Published in final edited form as:
Hepatology. 2009 October ; 50(4): 1273–1281. doi:10.1002/hep.23134.

Advanced periductal fibrosis from infection with the carcinogenic human liver fluke *Opisthorchis viverrini* correlates with elevated levels of IL-6

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Abstract

More than 750 million people are at risk of infection with food-borne liver flukes. *Opisthorchis viverrini* is considered among the most important of these parasites, due to its strong association with cholangiocarcinoma (CCA). *O. viverrini* infection results in a chronic inflammatory challenge to the host, which can lead to advanced, pathogen-specific disease sequelae including obstructive jaundice, hepatomegaly, cholecystitis, as well as CCA. However, before disease sequelae are apparent, important inflammatory changes to the liver can be detected early during *O. viverrini* infection. In a case-control study involving 328 men and women with *O. viverrini* infection, we determined the presence of advanced periductal fibrosis in asymptomatic, *O. viverrini*-infected individuals and then measured cytokine responses to *O. viverrini* excretory/secretory products (ES). In the 200 participants with advanced periductal fibrosis (cases), levels of Interleukin (IL)-6 to *O. viverrini* ES were 8 times higher than levels of the 128 *O. viverrini*-infected individuals without advanced periductal fibrosis (controls). Moreover, elevated IL-6 to parasite ES was associated with increased risk of advanced periductal fibrosis by 63% in a model adjusted for sex and age. The risk of advanced periductal fibrosis was also found to increase with higher levels of IL-6: individuals in the third quartile of IL-6-ES production had a 127% higher risk of developing advanced periductal fibrosis than individuals in the first quartile of IL-6 production. *O. viverrini*-infected individuals with advanced periductal fibrosis showed other hepatobiliary abnormalities, including reduced gallbladder contractility and the presence of gallbladder sludge.

Conclusion—These data strongly implicate a role for parasite specific IL-6 in the pathogenesis of advanced periductal fibrosis in opisthorchiasis, with possible links to other hepatobiliary abnormalities, including cholangiocarcinoma.

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Keywords

chronic inflammation; opisthorchiasis; cytokine; periductal fibrosis; case-control study

Introduction

Food-borne trematodiases represent an important group of communicable diseases, and one of the most clinically significant infectious pathogens behind malaria, tuberculosis, and HIV (1, 2). At least 750 million people (10% of the world's population) are at risk of food-borne trematodiases, with more than 40 million people currently infected (1, 3). *Opisthorchis viverrini* is considered among the most important of the food-borne trematodes due to its strong association with liver disease (4) and cholangiocarcinoma (CCA) (5, 6). Humans become infected with *O. viverrini* by consuming raw or undercooked fish that contain the infective stage. The parasites migrate up the biliary tract, establish a chronic infection (on average 4–5 years) in the intrahepatic bile ducts, and produce eggs that are excreted in the feces. While the infection is effectively eliminated by the anthelminthic praziquantel, environmental and cultural factors of East Asia strongly favor the process of re-infection (7, 8).

O. viverrini infection represents a chronic inflammatory challenge to the host. The sustained production of growth factors and fibrogenic cytokines in response to local mechanical, toxic, and immune irritation results in a persistent inflammatory condition in the liver that progressively remodels and destroys the normal tissue architecture of the biliary epithelium (3, 9, 10). The cumulative damage caused by this chronic inflammation can lead to advanced, pathogen-specific disease sequelae including obstructive jaundice, hepatomegaly, cholecystitis, and CCA (4, 11). Whereas these are the acknowledged signs of morbidity in opisthorchiasis, important inflammatory changes to the liver can occur early in the course of the disease (12, 13), most of which will remain clinically silent unless actively detected by ultrasound or other imaging modalities (12, 14). Previous community-based ultrasound studies in *O. viverrini* endemic areas of Northeastern Thailand suggest that hepatobiliary abnormalities such as enlargement of the left hepatic lobe and the gallbladder, loss of gallbladder contractility, presence of sludge, and increased periportal fibrosis are common (14, 15). Due to the high prevalence of *O. viverrini* infection in East Asia, which can be as high 60–70% in populations resident in endemic areas (11), asymptomatic hepatobiliary abnormalities may represent the greatest part of the disease burden associated with opisthorchiasis. Multiple risk factors are likely to determine whether the host develops advanced periductal fibrosis from *O. viverrini* infection, including the duration of infection (15), the intensity of the infection (14, 15), and diet (nitrosamines) (16). However, the risk factors that induce periductal fibrosis are likely to pass through the pro-inflammatory cytokine network, which includes Transforming Growth Factor-β, Interleukin (IL)-1-α, and IL-6 (17).

IL-6 is a pleiotropic cytokine with a broad range of humoral and cellular immune effects relating to inflammation, host defense, and tissue injury (18). It is of particular importance in diseases where the pathology arises from chronic inflammation (see references 17 and 19 for review). Although crucial for the quick induction of an innate immune response (19), the persistent production of IL-6 may be central to the state of chronic inflammation induced during *O. viverrini* infection. Elevated levels of IL-6 have been reported in almost every chronic inflammatory disease of the liver (17), including alcoholic hepatitis (20) and hepatitis B (HBV) and hepatitis C (HCV) viruses (21–26). More importantly, IL-6 appears to be a pivotal cytokine for cholangiocarcinogenesis. In chronically inflamed biliary epithelium, (e.g., during *O. viverrini* infection), epithelial cells are constantly stimulated to

participate in the inflammation by continuously secreting chemokines and cytokines (3, 4), creating a cellular microenvironment beneficial to cancer growth. In this regard, IL-6 appears to be a pivotal cytokine for both inflammation and cholangiocarcinogenesis (17, 19).

On the basis of this background information, we sought to determine whether levels of pro-inflammatory cytokines are elevated among *O. viverrini*-infected individuals with advanced periductal fibrosis. We further sought to determine whether the association between markers of chronic inflammation and their association with advanced periductal fibrosis were modified by other factors including age, sex, and the intensity of *O. viverrini* infection.

Materials and Methods

Study Design and Setting

The current study represents an analysis of baseline data collected from a community–based, case-control study of the risk factors associated with the development of advanced periductal fibrosis in opisthorchiasis. Individuals from seven villages in the vicinity of the regional capital, Khon Kaen, Khon Kaen Province, Thailand were surveyed (27) (Supplemental Figure S1). For the current study, 1,032 individuals were screened for *O. viverrini* infection, with 554 positive (53.7 %) for *O. viverrini* infection. From this group of infected individuals, 200 males and females between the ages of 20 and 60 years old with advanced periductal fibrosis were enrolled as “cases” and asked to provide 30 ml of whole blood for baseline immunology and hematological parameters. Individuals who were positive for *O. viverrini* but negative for advanced fibrosis were defined as “controls” and matched with cases based on geographic location of residence (nearest-neighbor). As such, 128 individuals were identified as controls and were asked to provide 30 ml of blood for baseline immunology and hematological parameters. All individuals (cases and controls) positive for *O. viverrini* were referred to the local public health outpost for treatment with praziquantel, (standard of care). This study was approved by the Ethics Committee of Khon Kaen University School of Medicine, Khon Kaen, Thailand (reference number HE480528) and the Institutional Review Board of the George Washington University School of Medicine, Washington, D.C (GWUMC IRB# 020864).

Ultrasonography

A mobile, high resolution ultrasound (US) machine (GE model LOGIQ Book XP) was used. Hepatobiliary Abnormalities (HBA) including portal vein radical echoes, echoes in liver parenchyma, indistinct gallbladder wall, gallbladder size, sludge and suspected CCA were graded and recorded as previously described (28, 29). For the study of gallbladder function (contractility), the gallbladder was measured before and then 30 minutes after consumption of a fatty meal (28). Individuals were classified as periductal fibrosis grade 0 when no echoes were observed in any segment of the liver (Figure 1; Panel A); 1+ when echoes were observed in 1 segment of the liver (Figure 1; Panel B); 2+ when echoes were observed in 2 or 3 segments of the liver (Figure 1; Panel C); and, 3+ when echoes were observed in greater than 3 segments of the liver (Figure 1; Panel D). Individuals were then dichotomized into cases and possible controls as follows: “Non-Advanced Fibrosis” or “control” if the US grade was 0 or 1, and “Advanced Fibrosis” or “case” if the US grade was 2 or 3. Two radiologists performed US readings over the course of the study. A weighted kappa score was used to quantify level of agreement between the two readers (30). If a kappa fell below 0.6, a third ultrasonographer was consulted to read the image and determine the final outcome.

Fecal exams

Two grams of feces were weighed and preserved in 10 ml of 10% formalin in a 15 ml screw-cap centrifuge tube, labeled, mixed and kept at room temperature until processing for quantitative formalin/ethyl acetate concentration technique (15). The presence and intensity of *O. viverrini* infection was determined by quantitative formalin/ethyl acetate technique and expressed as eggs per gram of feces (epg). Eggs/parasite identification and egg counts were under light microscopy at 10× and 40× magnifications, with the results reported as the number of eggs per gram of stool.

Urine pregnancy testing

β-hCG testing was performed at the study site using urine pregnancy test kits approved by Thai and/or US regulatory agencies.

Parasite antigen preparation

Excretory/secretory antigen (ES) of cultured *O. viverrini* adult worms obtained from experimentally infected hamsters was prepared as described (31). In order to eliminate any possible stimulatory effects in tissue culture from bacterial lipopolysaccharide (LPS) in crude *O. viverrini* antigen preparations, LPS was removed from the crude antigen extract by phase separation using Triton X-114 (32). These studies were approved by the Animal Ethics Committee of Khon Kaen University (#0514.1.12.2/23) and the Institutional Animal Care and Use Committee, George Washington School of Medicine.

Blood collection by venipuncture

Approximately 2 ml of blood was collected in EDTA-coated tubes for hematology, and approximately 20 ml of whole peripheral blood was obtained in heparinized tubes for cellular immunology and cytokines assays.

Liver Functions Tests

Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), and Alkaline phosphatase (ALP) assays in serum samples from cases and controls were performed on an automated Synchron CX-4 system (Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions.

ELISA

O. viverrini-specific IgG, IgG1, IgG2, IgG3 and IgG4 were determined by indirect ELISA and parasite-specific IgE was examined by indirect avidin-biotin ELISA as described in Sripa and Kaewkes (31).

Cellular immune response and cytokine measurements

Cytokine production by PBMC in the presence of *O. viverrini* ES were examined at 0, 48 and 72 hours for levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, INFγ, TNFα, and TNFβ production. A whole blood culture technique was used for cytokine stimulation. Appropriately diluted (1:16) heparinized whole blood was cultured in RPMI 1640 medium in 24-well tissue culture plates in the presence of 35 μg/ml of *O viverrini* ES crude antigen extract or 2.5 μg/ml for phytohaemagglutinin (PHA)-L (Difco Laboratories, Detroit, MI, USA). Cells were cultured at 37°C in a humidified 5% CO_2 incubator. Supernatants were removed at predetermined times (when maximum secretion of each cytokine was obtained between 48–120 hours of culture) and stored in aliquots at −80°C. Twenty five to 50 μl of supernatant was used for all cytokine assays. The quantification of secreted cytokines was analyzed using commercial FlowCytomix bead-based multiplexing

assays kits (Beckman-Coulter). Values were quantified from standard curves using human recombinant cytokines.

Statistical analyses

Cases and controls were compared for dichotomous and categorical variables by percent and then differences tested with Fisher's Exact test. In the case of continuous variables, two methods were used to assess for statistical significance. When continuous variables were normally distributed, Student's T-test was used to determine differences between cases and controls. In the special case of gallbladder contractility, where the difference in gallbladder dimensions between pre and post fatty meal were the variable of interest, an Analysis of Covariance (ANCOVA) was used to test for differences between cases and controls. When the data were (a) highly skewed, (b) had a number of outlier values, or (c) were refractory to transformation (e.g. log-transformation), a quantile regression model was used to determine the median along with 95% Confidence Intervals (95% CI) to estimate the differences between cases and controls: e.g., cytokine level, liver function tests, and antibody levels. Where the data suggested a significant difference between cases and controls, such as in the levels of IL-6 to *O. viverrini* ES, the risk of this parameter on advanced periductal fibrosis was assessed using a logistic regression model. Odds Ratios (OR) were estimated on the risk of advanced periductal fibrosis in crude models and models adjusted for age and sex. In the case of IL-6 production to *O. viverrini* ES, the variable was treated in its original scale, a categorical scale (using the median value as a cutoff), and an ordinal scale determined by quartiles of IL-6 production to ES. Stata version 10 (StataCorp, College Park, TX) statistical software was used for all analyses. Level of significance in statistical tests was 0.05. All tests were two-tailed tests.

RESULTS

Study Design

The baseline characteristics of the enrolled participants are shown in Table 1, with 200 cases and 128 controls enrolled in the study. Overall, the sample included slightly more females (52%) than males (48%) ($P = 0.083$). The study sample had a mean age of 45.8 and an age range of 20 to 60 years, with 74% of the study sample over 40 years of age. The mean ages for cases (46.7 years of age) and controls (45.2 years of age) did not differ significantly ($P = 0.115$). As part of the inclusion criteria of the study, both cases and controls were positive for *O. viverrini* as determined by a single ovum in feces. No statistically significant difference ($P > 0.811$) was observed in the intensity of *O. viverrini* infection between cases (median = 36 epg) and controls (median = 38 epg). As shown in Table 1, in both case and control groups, there was a similar distribution of in the proportion of individuals when intensity of *O. viverrini* infection was stratified by tertiles of eggs per gram of feces, with the majority of cases (77%) and controls (77%) having less than 499 eggs per gram of feces.

Individuals with advanced periductal fibrosis show poor gallbladder function

Table 2 shows that the gallbladder did not contract as much after a fatty meal in individuals with advanced periductal fibrosis compared to individuals without advanced periductal fibrosis. In addition, individuals with advanced periductal fibrosis had a greater presentation of sludge than individuals without advanced periductal fibrosis for whom sludge was entirely absent. These data indicate that individuals with advanced periductal fibrosis from *O. viverrini* have reduced gallbladder function.

Advanced periductal fibrosis in opisthorchiasis does not appear to affect liver function

There was no difference in the levels of the liver function tests ALT, AST and ALP between cases and controls (Supplementary Table S1), indicating that pathology may be limited to advanced periductal fibrosis and did not affect liver function.

Intensity of infection was not a risk factor for advanced periductal fibrosis in *O. viverrini* infection

Table 1 shows the individuals by the intensity of *O. viverrini* infection: "≤ 499 epg" (77%), "500–999 epg" (10%), and "≥ 1,000 epg" (13%). No significant difference was found (P = 0.696) between the median epg for individuals with advanced periductal fibrosis (median, 141 epg) compared to controls (median, 121 epg). Table 2 shows that intensity of infection (expressed as epg) was not a risk factor for advanced periductal fibrosis. When epg was measured at an interval of 1 egg per gram of feces, the odds ratio was 0.99 (95% CI 0.99 to 1.00, P = 0.811) and when measured in the larger interval of 500 eggs per gram of feces the odds ratio remained unchanged 0.99, (95% CI 0.93 to 1.00, P = 0.811). The relationship between baseline levels of epg and advanced periductal fibrosis was not altered by analyses that adjusted for age, sex, or age and sex simultaneously.

Levels of IL-6 to *O. viverrini* ES were significantly higher in individuals with advanced periductal fibrosis

Of the 11 cytokines tested (Supplementary Table S2), only levels of the inflammatory cytokine IL-6 were significantly elevated in individuals with advanced periductal fibrosis (Table 4). The frequency distributions of baseline IL-6 levels to *O. viverrini* ES are presented for cases and controls combined in Figure 2 Panel A, for cases only in Figure 2 Panel B, and for controls only in Figure 2 Panel C. IL-6 production from PBMC to *O. viverrini* ES among both cases and controls ranged between −2.7791 and 2.3082 pg/ml, with a range much greater in controls (−27,791 to 23,082 pg/ml) than in cases (−116.4 pg pg/ml to 14,221 pg/ml), indicating far less variation in the IL-6 response to *O. viverrini* ES among cases than controls. Median baseline levels of IL-6 in the presence of *O. viverrini* ES were 8 times higher in PBMC from cases than controls (3981 versus 507 pg/ml; P = 0.001). Individuals with "<499 epg" showed 12 times higher levels of IL-6 than controls (4305.9 versus 348.9 pg per ml, (95% CI 1, 500.9 to 5568.6, P = 0.001).

Elevated levels of IL-6 to *O. viverrini* ES significantly increase the risk of developing advanced periductal fibrosis

Table 5 shows that elevated levels of IL-6 to *O. viverrini* ES at baseline significantly increased the risk (OR = 1.63) of advanced periductal fibrosis in a model adjusted for age and sex (95% CI 1.01 to 2.54, P = 0.048). Moreover, as shown in Table 6, the risk of advanced periductal fibrosis increased with increasing quartiles of IL-6 production to *O. viverrini* ES: OR = 1.00 for Quartile 1 (reference quartile); OR = 1.39 for Quartile 2; OR = 2.27 for Quartile 3; and OR = 1.64 for Quartile 4. However, only for individuals in Quartile 3 of IL-6 production to *O. viverrini* ES was the risk (OR = 2.27) statistically significant for advanced periductal fibrosis (95%CI = 1.18 to 4.368, P = 0.014).

Antibody to *O. viverrini* ES did not associate with advanced periductal fibrosis

There was no significant increase between cases and controls in levels of IgG and its subclasses (IgG1, IgG2, IgG3, and IgG4) or IgE to *O. viverrini* ES (Supplementary Table S3). While important for *O. viverrini* infection, these data indicate that humoral immunity may not be a risk factor for advanced fibrosis.

Discussion

Liver fluke infection with *O. viverrini* is associated with a number of inflammation-induced hepatobiliary pathologies, the most common of which is periductal fibrosis (28). To our knowledge, this is the first report of an association between an inflammatory cytokine and periductal fibrosis in liver fluke infection. IL-6 levels to *O. viverrini* ES were on average 8 times higher in *O. viverrini*-infected individuals with advanced periductal fibrosis compared to *O. viverrini*-infected individuals without periductal fibrosis. Moreover, baseline PBMC production of IL-6 to *O. viverrini* ES significantly elevated the risk (63%) of advanced periductal fibrosis in a model adjusted for age and sex, with the risk of advanced periductal fibrosis increasing with increasing IL-6 levels to parasite antigen. The current data strongly support our hypothesis that opisthorchiasis represents a chronic inflammatory disease mediated by pro-inflammatory cytokines, specifically IL-6, which is significantly associated with the development of advanced periductal fibrosis.

Previous community-based ultrasound studies in *O. viverrini* endemic areas of Northeastern Thailand suggest that hepatobiliary abnormalities such as enlargement of the left hepatic lobe and the gallbladder, loss of gallbladder contractility, presence of sludge, and increased periductal fibrosis are common (15, 28, 34). In the current study, we also document deficits in gallbladder function, specifically reduced gallbladder contractility and the presence of sludge in *O. viverrini*-infected individuals with advanced periductal fibrosis. Recent studies in animals (34) and humans (4) have shown that opisthorchiasis is associated with chronic cholecystitis, which is characterized by inflammatory cell infiltration and prominent fibrosis of the gallbladder wall. The chronic inflammation and fibrosis of the gallbladder wall (14) can impair gallbladder contractility as observed in the current study among the individuals with advanced periductal fibrosis (14). The poor contractility of the gallbladder as well as the periductal fibrosis of the intrahepatic bile ducts may also cause functional bile stasis (or sludge). This can lead to precipitation of bile contents, which may even include *O. viverrini* eggs (35). Periportal and periductal fibrosis are among the most prominent histological features in chronic *O. viverrini* infection in humans (36) and in long term *O. viverrini* infection in hamsters (37). In a series of human autopsies conducted during the 1970s (38), no detectable changes were observed in the biliary epithelium and periductal areas of the liver in individuals with recent *O. viverrini* infection, while individuals with chronic *O. viverrini* infection had a proliferation of epithelial cells and the formation of acini and periductal fibrosis. Our observations support the hypothesis that, as with other chronic helminth infections (8), the asymptomatic abnormalities represent an important component of the disease burden in opisthorchiasis.

Chronic inflammatory disorders such as those seen in opisthorchiasis are induced by persistent irritants that sustain the production of growth factors and fibrogenic cytokines, which in turn stimulate the deposition of connective tissue elements that progressively remodel and destroy normal tissue architecture, resulting in fibrotic elements (10). In opisthorchiasis, the persistent irritation can come from one or both of the following sources: (1) the feeding and migration of flukes (mechanical irritation), when oral and ventral suckers hook onto the biliary epithelium and damage tissue or (2) metabolic products that are released from the tegument and excretory openings of the parasite and enter into the bile duct epithelium of the human host (4). From the current data, we hypothesize that the persistent irritant derives from some component(s) of the crude ES antigen preparation that we employed to stimulate PBMC *in vitro*, which resulted in elevated IL-6 production. ES antigens have proven to be important immune modulators in a number of helminth infections (39), usually skewing the host cytokine response to create a microenvironment favorable to parasite survival. In the current study, we offer a novel role for helminth ES products: the idea that ES products from *O. viverrini* may be toxic or may interact with the

biliary epithelium as a mitogen (36) which results in chronic inflammation and subsequent periductal fibrosis and possibly even CCA. This hypothesis is consistent with much of the experimental literature on *O. viverrini* ES products in laboratory models. For example, when *O. viverrini* adults worms were co-cultured with human biliary cell lines in a non-contact transwell system, they induced a proliferative response (4), indicating that soluble products excreted or secreted from the parasite are indeed capable of stimulating cells to proliferate. A similar observation was also found when *O. viverrini* adult worms were co-cultured with mouse NIH-3H3 fibroblasts (40). There is also evidence that these immunological responses are, at least in part, specifically evoked by *O. viverrini* products: in this same non-contact transwell system, T-cell deprived hamsters did not show a proliferative response when co-cultured with adult worms (41). Our study extends these findings by indicating that ES products in *O. viverrini*-infected humans may cause a persistent irritation, resulting in a chronic inflammatory state, mediated by IL-6, which is strongly associated with advanced periductal fibrosis.

IL-6 is known to play a role in a number of chronic inflammatory conditions of the liver, e.g., alcoholic and viral hepatitis (42, 43) and other well known fibrotic lesions such as keloid pathogenesis (44). One route by which IL-6 may be responsible for periductal fibrosis in opisthorchiasis is the up-regulation of pro-fibrotic cytokines such as TGF–β1 (10), which has been observed in experimental *O. viverrini* infection in hamsters (45, 46). In addition, IL-6 is a pleiotropic cytokine involved in a variety of inflammation associated cancers (17), including CCA (47). In particular, in chronically inflamed biliary epithelium, epithelial cells are constantly stimulated to participate in the inflammation by continuously secreting chemokines and cytokines, which establishes a cellular microenvironment that is conducive to neoplasia (48). *Opisthorchis* may be the first helminth infection linking the development of fibrosis with parasite specific IL-6 production.

This study emphasizes the significant relationship between the inflammatory cytokine IL-6 and advanced periductal fibrosis in opisthorchiasis. Although less visible in clinical presentation, asymptomatic hepatobiliary abnormalities in opisthorchiasis may actually represent the greatest part of the chronic disease burden associated with this very prevalent but also neglected tropical disease (7, 8). Further studies are needed to enhance our knowledge of the relationship between pro-inflammatory cytokines, hepatobiliary pathology, and cholangiocarcinogenesis. Such studies will then allow us to identify additional risk factors and biomarkers for disease progression, highlighting those individuals with the highest risk of developing pathologic sequelae and CCA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial Support

This research was supported by award number UO1AI065871 from the National Institute of Allergy and Infectious Diseases. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIAID or the NIH.

We sincerely thank the volunteers who participated in this study. We also thank our regulatory affairs and data management colleagues including Preeyaporn Plaimee, Sangduan Wannachart, Sombat Thinkhamrop, Wilaiporn Thinkhamrop and Jittiawadee Pukdeepromma, and our laboratory and field technicians, including Arpa Surapitoon for assistance with the flow cytometric work. We thank Dr. Pyatat Tatsanavivat and Tarika Samor of the Clinical Research Collaboration Network, Thailand for guidance with compliance issues. This research was supported by award number UO1AI065871 from the National Institute of Allergy and Infectious Diseases (the content is solely the responsibility of the authors and does not necessarily represent the official views of the NIAID or the NIH).

Abbreviations

CCA	Cholangiocarcinoma
PBMC	Peripheral Blood Mononuclear Cells
ES	Excretory/Secretory
IL	Interleukin
CI	Confidence Interval
INF	Interferon
TNF	Timor Necrosis Factor
ml	Milliliter
US	Ultrasonography
HBA	Hepatobiliary Abnormalities
LPS	Lipopolysaccharide
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ALP	Alkaline Phosphatase
Ig	Immunoglobulin
ELISA	Enzyme Linked Immunosorbent Assay
M	Molar
PBS	Phosphate Buffered Saline
HRP	Horseradish Peroxidase
Hr	Hour
OD	Optical Density
OPD	*Ortho*-Phenylenediamine
ANCOVA	Analysis of Covariance
OR	Odds Ratio
Epg	Eggs per Gram of Feces

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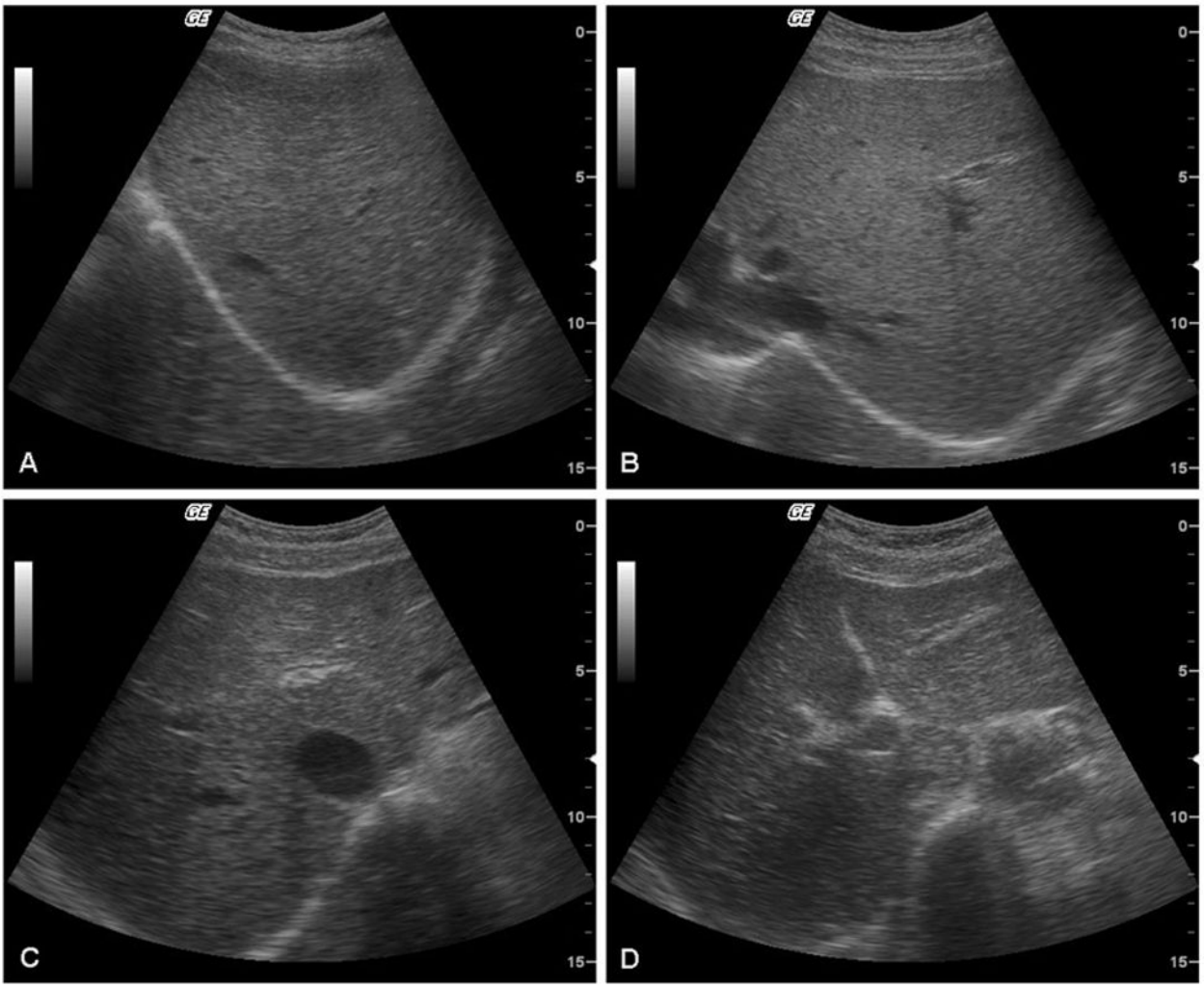

Figure 1.
Representative ultrasonographs, revealing increasing periportal echoes and markedly enlarged gallbladder, in individuals categorized as grade 0 (panel A), 1+ (B), 2+ (C) and 3+ (D) periportal fibrosis.). Individuals were classified as periductal fibrosis grade 0 when no echoes were observed (A); 1+ when echoes were observed in 1 segment of the liver (B); 2+ when echoes were observed in 2 or 3 segments of the liver (C); and, 3+ when echoes were observed in greater than 3 segments of the liver (D). Individuals were then dichotomized into Non-advanced fibrosis" or "control" if the ultrasonography (US) grade was 0 or 1 and "Advanced Fibrosis" or "case" if the US grade was 2 or 3.

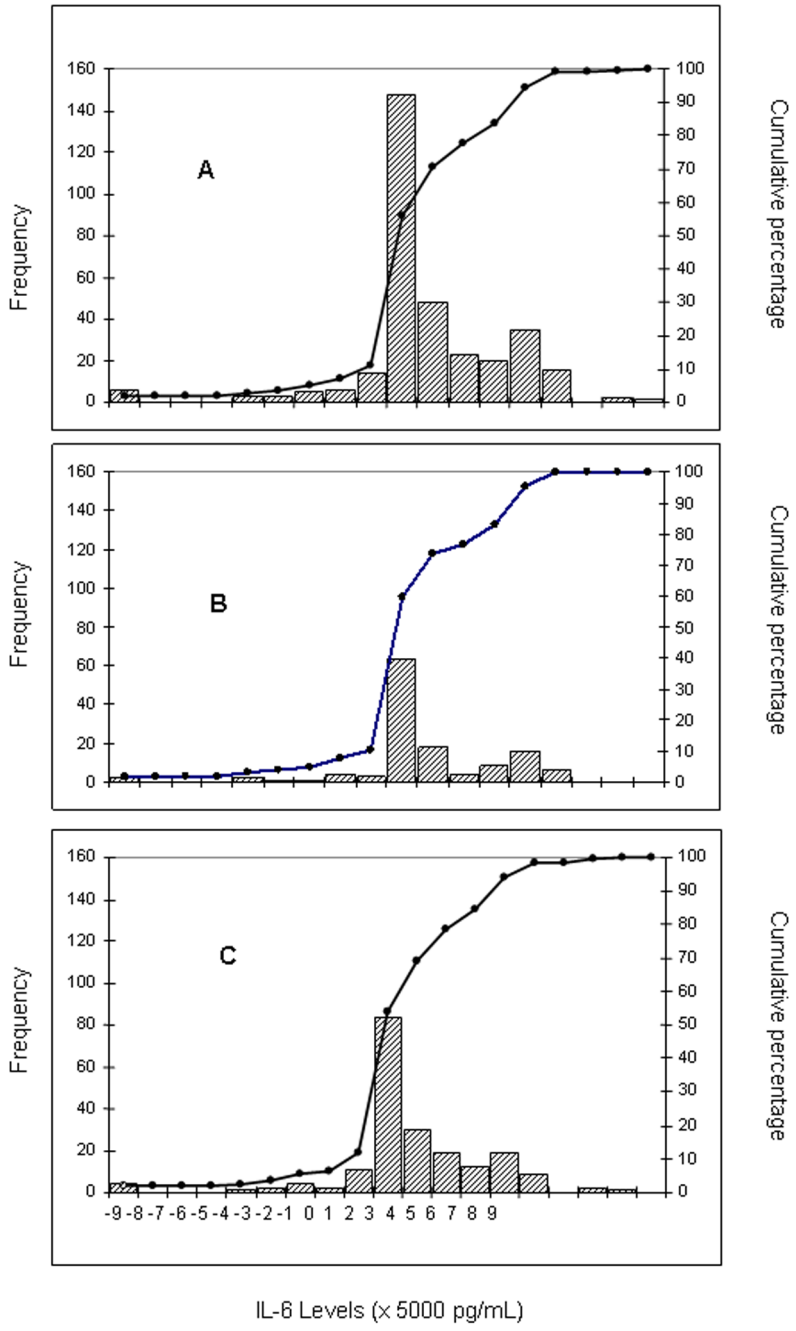

Figure 2.

The frequency and cumulative percentage of production of Interleukin (IL)-6 to *Opisthorchis viverrini* excretory/secretory (ES) products among cases and controls in a community-based case control study. The bars refer to frequency of production of IL-6 and the line refers to cumulative frequency of IL-6. Panel A refers to all individuals in the study (n = 328); Panel B refers only to cases (n = 200), and Panel C refers only to controls (n = 128)

Table 1

The relationship between advanced periductal fibrosis status as determined by ultrasonography in *Opisthorchis viverrini*-infected individuals by age, sex and level of infection.

	Advanced Periductal Fibrosis	
	Negative (0 and 1)	**Positive (2 and 3)**
Total	128	200
Sex		
Male	54 (42.2%)	104 (52.0%)
Female	74 (57.8%)	96 (48.0%)
Age (years)		
20–29	4 (3.1%)	17 (8.5%)
30–39	21 (16.4%)	42 (21.0%)
40–49	56 (43.8%)	75 (37.5%)
50+	47 (36.7%)	66 (33.0%)
Intensity of infection		
1–499 epg	98 (76.6%)	154 (77.0%)
500–999 epg	16 (12.5%)	18 (9.0%)
>1000 epg	14 (11.0%)	28 (14.0%)

epg refers to eggs per gram of feces.

Table 2

The relationship between gall bladder dimensions “pre” and “post” fatty mean and sludge with the presence of advanced periductal fibrosis by case and control status

	Advanced Periductal Fibrosis		
Gall bladder abnormalities	**Negative Mean (SD)**	**Positive Mean (SD)**	***P***
Post Fatty Meal Reduction[†]			
Length	1.88 (1.35)	1.59 (1.20)	0.007
Width	0.71 (0.55)	0.68 (0.56)	0.154
Cross-sectional	0.61 (0.47)	0.55 (0.57)	0.261
	n (%)	*n* (%)	
Presence of sludge	0	13 (6.5)	0.002

[†]Refers to the difference in gall bladder dimensions (measured in centimeters) “pre” and “post” fatty meal.

Table 3

Crude and Adjusted Odds Ratios for intensity of infection, expressed as egg per gram of feces, for *Opisthorchis viverrini* infection and advanced periductal fibrosis

	Odds Ratio	95% CI	*P* value
Intervals of 1 epg			
Crude	0.99	0.99, 1.00	0.811
Adjusted			
Age	0.99	0.99, 1.00	0.987
Sex	0.99	0.99, 1.00	0.646
Age and sex	0.99	0.99, 1.00	0.818
Interval of 500 epg			
Crude	0.99	0.93, 1.00	0.811
Adjusted			
Age	0.99	0.94, 1.06	0.987
Sex	0.99	0.93, 1.05	0.646
Age and sex	0.99	0.93, 1.06	0.818

† epg refers to eggs per gram of feces. CI refers to Confidence Interval.

Table 4

Baseline concentrations (median) of IL-6 in supernatants from peripheral blood mononuclear cells (PBMC) stimulated with *Opisthorchis viverrini* excretory/secretory products after 48 hours according to Case (Advanced Periportal Fibrosis Positive) and Control (Advanced Periportal Fibrosis Negative) Status and in relationship to sex, age, and intensity of infection

	Unstimulated PBMC			Stimulated PBMC			IL-6 Production by PBMC		
N	**Control 128**	**Case 200**	***P***	**Control 128**	**Case 200**	***P***	**Control 128**	**Case 200**	***P***
Total	999.2	1408.9	0.478	4478.6	8415.3	0.145	507.1	3981.2	0.001
Sex									
Male	1842.3	1288.0	0.501	908.3	7436.4	0.016	4.1	2772.6	0.039
Female	739.0	1506.4	0.138	8032.1	10440.6	0.680	1404.7	4966.2	0.082
Age (years)									
20–29	2452.4	421.6	0.036	19.7	21.5	0.999	−154.7	2.5	0.963
30–39	404.4	2540.5	0.018	8262.3	3729.6	0.530	495.9	1298.6	0.344
40–49	1872.4	104.6	0.143	636.0	8790.8	0.005	3.2	3648.2	0.014
50–59	820.9	1920.4	0.030	8604.9	9217.9	0.873	1687.8	6030.0	0.116
Intensity of infection (epg)									
<499	959.5	1532.0	0.318	4286.7	8603.0	0.113	348.9	4305.9	<0.001
500–999	3645.7	280.5	<0.001	3817.5	3918.1	0.939	2135.9	3473.4	0.333
1000–2000	2877.2	2572.2	0.984	12694.4	9077.3	0.991	9017.3	2870.9	0.524

Production refers to the median of the differences between Stimulated PBMC and Unstimulated PBMC (control wells) as expressed by the equation: Production = Median (Stimulated PBMC – Unstimulated PBMC).

Table 5

Crude and Adjusted Odds Ratios for levels of IL-6 by Peripheral Blood Mononuclear Cells (PBMC) stimulated by *Opisthorchis viverrini* excretory/secretory products (ES) for advanced periductal fibrosis[†].

	Odds Ratio	95% CI	*P*-value
Crude	1.51	0.94, 2.41	0.089
Adjusted			
Age	1.56	0.97, 2.51	0.066
Sex	1.55	0.96, 2.50	0.070
Age and Sex	1.63	1.01, 2.54	0.048

[†]Refers to" *in vitro* cultures of PBMC with *O. viverrini* ES after 48 hours. CI refers to Confidence Interval.

Table 6

Crude and Adjusted Odds Ratios by quartiles of the difference between IL-6 produced by PBMC that were unstimulated or stimulated by *Opisthorchis viverrini* excretory/secretory products (ES)for advanced periductal fibrosis among *O. viverrini* infected individuals.

	Quartile IL-6 in production to *O. viverrini* ES (range, pg/ml)				
	1 (−47073.0 to −2.44)	**2 (−2.45 to 2695.8)**	**3 (2695.9 to 12519.1)**	**4 (12519.2 to 44055.3)**	***P* for Trend**
Crude					
OR	1.00	1.35	2.07	1.49	
95% CI	—	0.73 – 2.50	1.09 – 3.92	0.80 – 2.78	*P* = 0.106
P	—	0.346	0.026	0.207	
Adjusted					
OR	1.00	1.38	2.27	1.64	
95% CI	—	0.74 – 2.59	1.18 – 4.38	0.87 – 3.08	NA
P		0.312	0.014	0.127	

OR indicates Odds Ratio. CI refers to Confidence Interval. Adjusted models include age and sex simultaneously. NA refers to not applicable to adjusted models.