



HIF-1alpha-pathway activation in cholangiocytes of patients with biliary atresia: An immunohistochemical/molecular exploratory study



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ABSTRACT

Background: Biliary atresia is a neonatal disease characterized by choledochal obstruction and progressive cholangiopathy requiring liver transplantation in most patients. Hypoxia-ischemia affecting the biliary epithelium may lead to biliary obstruction. We hypothesized that ischemic cholangiopathy involving disruption of the peribiliary vascular plexus could act as a triggering event in biliary atresia pathogenesis. **Methods:** Liver and porta hepatis paraffin-embedded samples of patients with biliary atresia or intrahepatic neonatal cholestasis (controls) were immunohistochemically evaluated for HIF-1alpha-nuclear signals. Frozen histological samples were analyzed for gene expression in molecular profiles associated with hypoxia-ischemia. Prospective clinical-laboratory and histopathological data of biliary atresia patients and controls were reviewed.

Results: Immunohistochemical HIF-1alpha signals localized to cholangiocytes were detected exclusively in liver specimens from biliary atresia patients. In 37.5% of liver specimens, HIF-1alpha signals were observed in biliary structures involving progenitor cell niches and peribiliary vascular plexus. HIF-1alpha signals were also detected in biliary remnants of 81.8% of porta hepatis specimens. Increased gene expression of molecules linked to REDOX status, biliary proliferation, and angiogenesis was identified in biliary atresia liver specimens. In addition, there was a trend towards decreased GSR expression levels in the HIF-1alpha-positive group compared to the HIF-1alpha-negative group.

Conclusion: Activation of the HIF-1alpha pathway may be associated with the pathogenesis of biliary atresia, and additional studies are necessary to confirm the significance of this finding. Ischemic cholangiopathy and REDOX status disturbance are putative explanations for HIF-1alpha activation. These findings may give rise to novel lines of clinical and therapeutic investigation in the BA field.

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Abbreviations: BA, biliary atresia; CK19, cytokeratin 19; DB, direct-reacting bilirubin; GSR, glutathione disulfide reductase; GSS, glutathione synthetase; HIF, 1alpha-hypoxia-inducible factor-1alpha; IHC, intrahepatic cholestasis; LTX, liver transplantation; PVP, peribiliary vascular plexus; REDOX, reduction-oxidation; TB,

total bilirubin; VCAM1, vascular cell adhesion molecule 1; VEGFA, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.

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1. Introduction

Biliary atresia (BA) is a neonatal disease involving choledochal obstruction and progressive cholangiopathy leading to the development of cirrhosis and necessitating liver transplantation (LTx) in most patients [1,2]. The occurrence of several clinical variants of BA suggests a variety of underlying pathogenic or etiological mechanisms. Deciphering the potential pathophysiology of BA may support the development of novel therapeutic approaches to improve the quality of life of affected patients.

The findings of progressive medial thickening of hepatic artery branches [3], peripheral arterial blockage with perivascular arterial tufts [4], and immunohistochemical expression of angiogenic factors in biliary structures suggest hypoxia and reactive angiogenesis in BA [5]. Liver specimens of patients with isolated BA show up-regulation of angiopoietins involved in pericyte recruitment to the vascular wall [6] as well as features of hypoxia-ischemia associated with disease aggravation [7]. Hypoxia-ischemia affecting the biliary epithelium may lead to biliary obstruction when the niches of progenitor cells are compromised [8–10].

We hypothesized that ischemic cholangiopathy involving injury of the peribiliary vascular plexus (PVP) could act as a triggering event in BA pathogenesis [11] and compromise the success of portoenterostomy. Aiming to address this research question, we performed an exploratory study of HIF-1 α pathway activation in the liver and porta hepatis of patients with BA.

2. Methods

2.1. Patients and samples

All the patients enrolled in this study underwent exploratory laparotomy between 2006 and 2015 as part of the diagnostic workup for neonatal cholestasis at Hospital de Clínicas de Porto Alegre, Brazil. Two groups were evaluated: the study group included 20 patients with BA in whom exploratory laparotomy preceded portoenterostomy; and the control group included five patients with intrahepatic cholestasis (IHC), in whom surgery was necessary to rule out BA. The clinical features of patients included in the control group were indistinguishable from BA, thus demanding the performance of a trans-operative cholangiogram for diagnostic differentiation. BA diagnosis was confirmed through both intraoperative cholangiogram and bile duct evaluation in porta hepatis. Morphological classification of BA following the Japanese Association of Pediatric Surgeons was possible in 13 out of the 20 cases for whom a surgical description was available, with 10 classified as type 3 (atresia of bile duct at the porta hepatis) and three as type 2 (atresia of hepatic duct). Isolated BA was characterized by the absence of biliary atresia splenic malformation (BASM), extrahepatic cysts or positive IgM serology for cytomegalovirus. Clinical-laboratory, molecular, and histological criteria defined the IHC group. The final diagnoses of IHC controls included idiopathic neonatal hepatitis ($n = 2$), alpha-1 antitrypsin deficiency ($n = 2$) and parenteral nutrition-associated cholestasis ($n = 1$). During the surgical procedures, tissue specimens were collected from the hepatic segment IV in cases and controls and from porta hepatis in patients with BA. In four patients with BA, porta hepatis specimens were available. For the remaining 16 BA patients, 11 had liver and porta hepatis specimens, and five cases only had liver samples. In addition, a piece of the liver samples from 11 BA patients was stored at $-80\text{ }^{\circ}\text{C}$ in RNA holder (BioAgency Biotecnologia, São Paulo, Brazil). Liver specimens from the control group were also collected and stored at $-80\text{ }^{\circ}\text{C}$, except for one patient with alpha-1 antitrypsin. The paraffin-embedded liver and porta hepatis samples were used for immunohistochemical analysis. Gene expression profiles were determined by RT-PCR in RNA isolated from the frozen liver sam-

ples. Preoperative laboratory tests were performed in all patients, and surgery was performed if serum hemoglobin levels were adequate for a safe surgical procedure. The duration and type of anesthesia did not differ between patients and controls, and there was no evidence of intraoperative hypoxia in any of the infants studied.

2.1.1. Clinical-laboratory data collection

All patient-related clinical and laboratory data were prospectively collected and stored securely in a databank. Concerning the laboratory tests for comparison between groups, as described in the literature, serum bilirubin values were selected as the only indicator of native liver survival after portoenterostomy [12].

2.2. Immunohistochemical method

Paraffin-embedded samples were microtome-sectioned into $5\text{ }\mu\text{m}$ slices, deparaffinized with xylene, and rehydrated in decreasing ethanol concentrations and distilled water. Antigen retrievals were performed using EDTA/Tris buffer pH 8.0 in a water bath for 20 min at $95\text{ }^{\circ}\text{C}$. Immunohistochemical staining with recombinant HIF-1 α antibody (Abcam, Cambridge, UK, ab179483, 1:25) was performed using the avidin-biotin complex (ABC) detection system and a Ventana BenchMark ULTRA (Roche, CH) staining station. Two tissue specimens were used as on-slide controls: a human kidney specimen as positive control for HIF-1 α signal and a non-diseased liver control sample (See Fig. 1.1 and 1.2, Supplemental Digital Content (SDC) 1).

2.2.1. Immunohistochemical analysis of HIF-1 α positivity

HIF-1 α positivity was confirmed in the presence of brown granular nuclear staining in all microanatomic structures, both in liver and porta hepatis [13]. Detection and immunolocalization of HIF-1 α positivity were determined by consensus among three liver histopathology experts blinded to diagnosis.

2.2.2. Histopathologic variables associated with neonatal cholestasis

A histopathological study was performed to evaluate whether BA patients and controls were comparable concerning histopathological variables of interest, such as presence of ductular reaction, tissue disease severity, and vascular features (vascular agglomerates/hyperplasia in portal tracts, fibrous septa, or subcapsular area). Liver samples of 19 patients (14 BA cases and all five control patients) were stained with hematoxylin and eosin and picosirius red. A histopathologic protocol comprising 26 qualitative categorical variables was used (See Table 1, Supplemental Digital Content 2)

2.3. Gene expression analysis by qPCR

Total RNA was extracted from liver specimens using AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Carlsbad, CA) following the manufacturer's instructions. For cDNA synthesis, reverse transcription of $1\text{ }\mu\text{g}$ of RNA was performed using the NZY M-MuLV Reverse Transcriptase Kit (Nzytech, PT). Real-time PCR was performed using the iCycler IQTM real-time PCR detection system (Bio-Rad, CA) with the primers described in SDC Table 2 (Supplemental Digital Content 2). All primers were designed based on human mRNA sequences deposited in GenBank (NCBI), except for cytokeratin 19 (CK19), designed by Stathopoulou et al. [14]. mRNA expression was determined in comparison to controls using the $2^{-\Delta\Delta\text{CT}}$ method. CT values were normalized by the housekeeping gene ribosomal 18S.

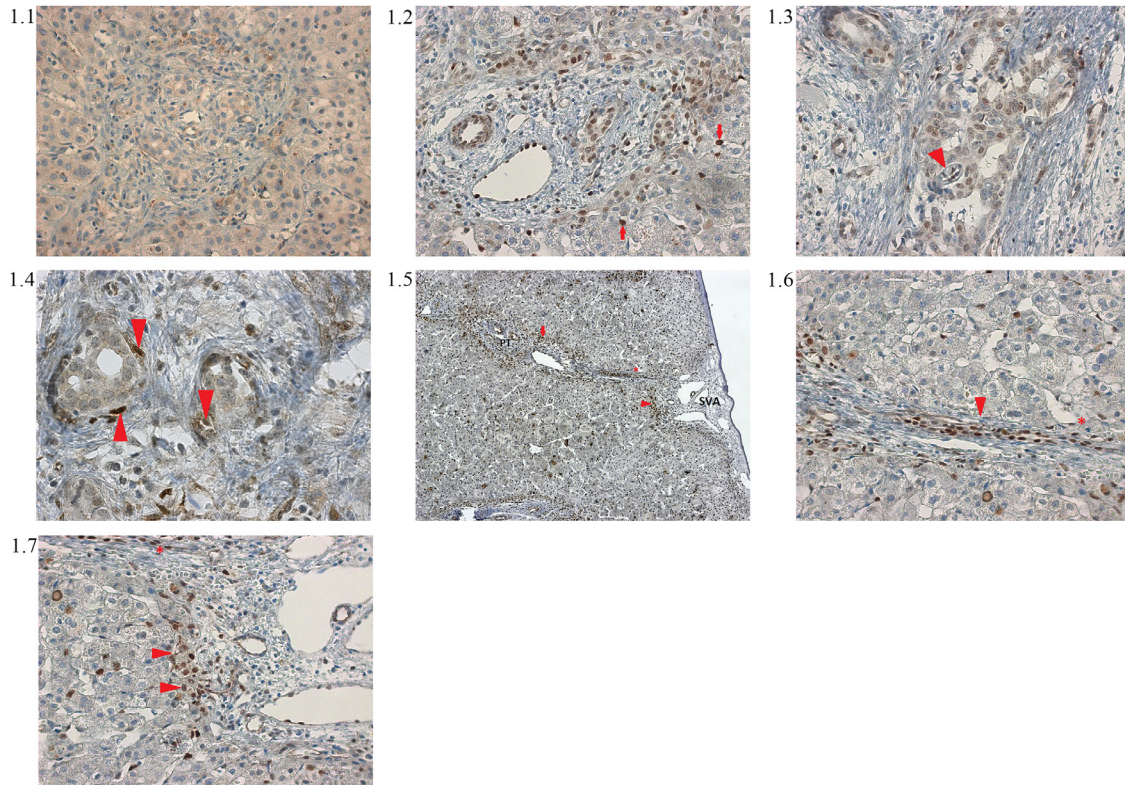


Fig. 1. HIF-1alpha positivity in the liver of biliary atresia patients and control patients with intrahepatic cholestasis. 1.1- 35-day old IHC control: idiopathic neonatal cholestasis with absent HIF-1alpha nuclear signals in hepatobiliary structures; 1.2- BA patient: portal tract with HIF-1alpha positivity in the interlobular bile duct, marginal ductular reaction, hepatic arteriolar branch including endothelium and medial layer muscle cells, portal venous endothelium. Note apparent HIF-1alpha positivity in sinusoidal membrane cells (arrows). 1.3- HIF-1alpha positivity in the ductular reaction area with features similar to ductal plate malformation. Also note endothelial HIF-1alpha positivity in the core of a structure with features of a mini ductal plate (arrowhead); 1.4- HIF-1alpha positivity in the endothelium of peribiliary vascular plexus; 1.5- Subcapsular vascular agglomerate (SVA, on the left) giving rise to a fibrovascular septum. Note the ductular reaction with positive HIF-1alpha nuclear signals at the external margin of the subcapsular fibrous stroma (arrowhead). The fibrous septum departs from the subcapsular area (asterisk), producing a marginal ductular reaction with HIF-1alpha positivity, which continues to the portal tract (PT) margin (arrow); 1.6 and 1.7- Fibrovascular septum (asterisk) and subcapsular vascular agglomerate showing associated HIF-1alpha positive ductular reaction at the interface between the fibrous stroma and parenchyma (arrowheads). Magnifications: 100x, 400x, 630x, 1000x.

2.4. Statistics

Quantitative variables were expressed as mean \pm SD or median (range), and categorical data were described as frequencies and percentages. Student's *t*-test, Mann–Whitney test or Kruskal–Wallis test were used for comparing groups depending on data symmetry. The Pearson Chi-square test was used for qualitative variables. Liver survival was compared in the study vs. control groups using a Kaplan–Meier test followed by the log-rank test. A two-tailed *p* value < 0.05 was accepted as significant. SPSS 27.0 (IBM, UK) was used for data processing and statistical analysis.

2.5. Ethics

Written informed consent for the use of histological specimens and clinical data was obtained from the patients' parents or guardians. The study was approved by the Ethics Committee at the Hospital de Clínicas de Porto Alegre, Brazil, and was performed in accordance with the ethical standards outlined in the Declaration of Helsinki.

3. Results

3.1. Clinical analysis of patient samples

Liver tissue samples of 25 patients were assessed: 20 with BA and five with IHC (control group). The demographic and clinical characteristics of patients and controls are presented in Ta-

ble 3 (Supplemental Digital Content 2). At the time of surgery, age ranged from 32 to 110 (mean 63 ± 19.8) days in BA patients, and from 35 to 81 (mean 59 ± 21) days in controls, and was not significantly different between the groups. Considering the total follow-up period (2005 until the end of the study in 2018), seven BA patients underwent LTx and eight died. Age of death ranged from 6 to 80 (median=9.5) months. Six patients (75% of the deceased patients) died in the first year of life, four (50%) without LTx. Age at LTx ranged from 6 to 84 (median = 26) months. Concerning bilirubin serum levels at portoenterostomy, total bilirubin (TB) ranged from 4.7 to 19.1 (mean 9.9 ± 3.8) mg/dL, and direct bilirubin (DB) from 3.5 to 14.3 (mean 7.3 ± 2.8) mg/dL. At 3 months post-portoenterostomy, TB values ranged from 0.3 to 25.7 (median = 5.4) mg/dL and DB values ranged from 0.1 to 18.8 (median = 4) mg/dL.

3.1.1. Detection and immunolocalization of HIF-1alpha positivity in the liver

HIF-1alpha positivity was not detected in any hepatobiliary structure in IHC controls (Fig. 1.1). Conversely, six of 16 (37.5%) patients with BA presented HIF-1alpha positivity in cholangiocytes, endothelial and medial layers of hepatic arterial branches, and less commonly portal venous endothelium (Fig. 1.2). HIF1-alpha positivity was also observed in sinusoidal endothelial cells; however, we were neither able to determine in which sinusoidal cell type, nor rule out a causal role of phagocytized bile pigments. HIF-1alpha-positive cholangiocytes were located in portal tracts including interlobular bile ducts (Fig. 1.2) and proliferative ductules along

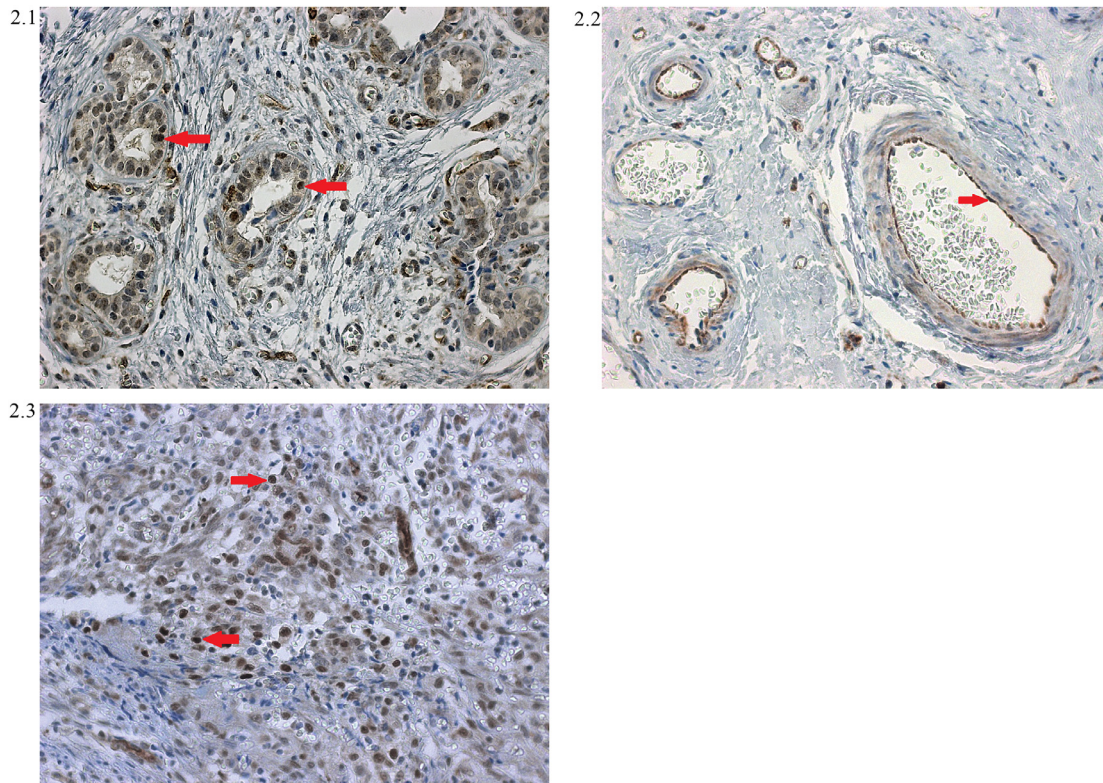


Fig. 2. HIF-1alpha positivity in the porta hepatis. 2.1- biliary remnants with HIF-1alpha positivity in cholangiocytes; 2.2- HIF-1alpha positivity in the endothelium of large and medium-sized hepatic artery branches; 2.3- HIF-1alpha positivity in cellular infiltrate. Magnification- 100x, 400x.

the portal margins. Cholangiocytes with HIF-1alpha positivity were also abundant in stroma of fibrous septa (Fig. 1.3), and in some instances presented a morphology reminiscent of ductal plate malformation containing “mini-ductal plates” with HIF-1alpha positivity in the endothelial cells of the vascular heart (arrow). Arteriolar endothelial HIF-1alpha positivity extended to the tiny vessels of PVP encircling biliary structures (Fig. 1.4). The fibrous septa emerging from both portal tracts and subcapsular vascular agglomerates (Fig. 1.5, left), particularly their margins (Fig. 1.5–1.7), constituted the preferential location of HIF-1alpha-positive cholangiocytes.

3.1.2. Detection and immunolocalization of HIF-1alpha positivity in porta hepatis

HIF-1alpha-positive cholangiocytes were observed in biliary remnants of nine out of the 11 (81.8%) porta hepatis specimens in this study (Fig. 2.1). HIF-1alpha positivity was also detected in the endothelium of large and medium-sized hepatic artery branches (Fig. 2.2), as well as in inflammatory infiltrates in regions of fibrosis (Fig. 2.3).

3.1.3. HIF-1alpha-positive biliary epithelium in the liver and demographic and clinical features of BA patients

BA patients with and without HIF-1alpha positivity in cholangiocytes did not differ with regards to age and serum bilirubin levels at the time of portoenterostomy and 3 months after portoenterostomy (See Table 4, Supplemental Digital Content 2). Five out of six (83%) patients with HIF-1alpha positivity were classified as type 3 BA according to the morphological classification proposed by the Japanese Association of Pediatric Surgeons, in comparison with five out of seven (71.4%) in the group of patients without HIF-1alpha positivity. The small number of cases precluded statistical comparison (See Table 3, Supplemental Digital Content 2). No significant difference was observed concerning the need for LTx or age at LTx. There was a statistical trend for correlation between

HIF-1alpha positivity in the liver and death before 1 year of age (Pearson chi-square, $p = 0.062$) (See Table 5, Supplemental Digital Content 2). Sixty-seven percent of the HIF-1alpha-positive patients died in the first postoperative year, compared with only 20% of HIF-1alpha-negative patients. At the end of the follow-up, only 16.7% of the HIF-1alpha-positive group survived with the native liver, whereas 50% of patients without HIF-1alpha positivity remained alive and non-transplanted.

3.2. Histopathological analysis of features associated with neonatal cholestasis in BA patients and controls

Given the small numbers of patients in each sample, comparative statistical analysis of histopathological features was not performed, and the variables of interest are described as frequencies and percentages. All patients in both the BA and IHC control groups presented ductular reaction in portal tracts, including the portal margins, and were thus comparable concerning HIF-1alpha activation in cholangiocytes. Parenchymal ductular reaction was noted in two IHC control patients. Cirrhotic nodules were present in three out of five (60%) patients with HIF-1alpha activation in cholangiocytes, in one out of nine (11%) HIF-1alpha-negative patients, and were absent in IHC control patients. Interestingly, vascular hyperplasia (agglomerates mostly of arterioles, some of which with a prominent medial layer) in portal tracts and fibrous septa only occurred in BA patients — including 100% of patients with and 78% of patients without HIF-1alpha positivity in cholangiocytes (See Figs. 2 and 3, Supplemental Digital Content 1).

3.3. mRNA expression of markers of cell function, cell death, and hypoxia

We investigated the gene expression of a representative set of molecules involved in metabolic and structural processes affected

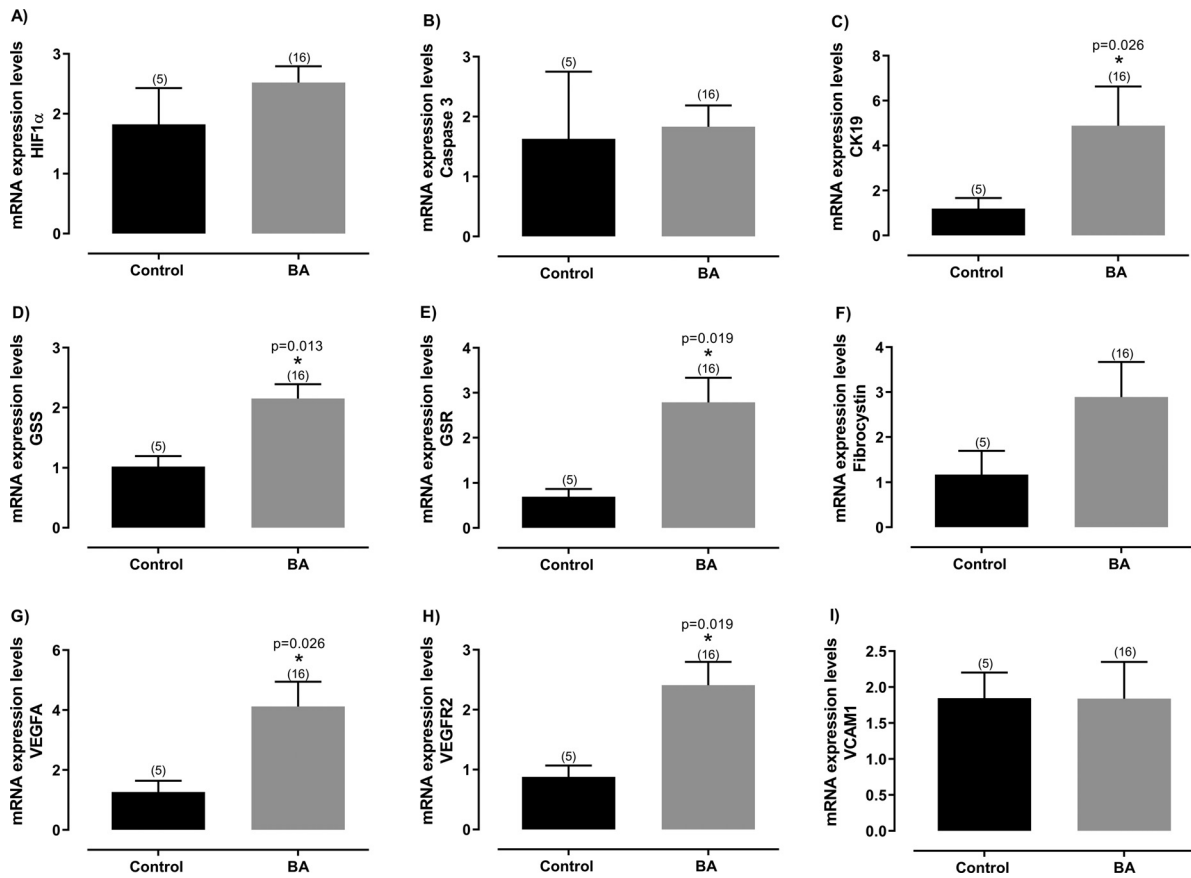


Fig. 3. Gene expression of molecules involved in metabolic and structural processes affected by the hypoxia-ischemia in the liver. Abbreviations: HIF-1alpha- hypoxia-inducible factor-1alpha; GSS- glutathione synthetase; GSR- glutathione-disulfide reductase; VEGFA vascular endothelial growth factor A; VEGFR2- vascular endothelial growth factor receptor 2; VCAM 1- vascular cell adhesion molecule 1; CK19 - Cytokeratin 19. Bars represent the mean and vertical lines the SEM. (Mann-Whitney test).

by the hypoxia-ischemia process in the liver. In comparison to IHC controls, BA patients presented overexpression of genes associated with REDOX status (glutathione synthetase [GSS] $p = 0.013$; glutathione-disulfide reductase [GSR], $p = 0.019$), cholangiocyte proliferation (CK19, $p = 0.026$), and angiogenic response (VEGFA, $p = 0.026$; VEGFR2, $p = 0.019$) (Fig. 3). No statistically significant differences were observed between BA patients with and without HIF-1alpha positivity regarding relative levels of mRNA expression of these molecules, despite a trend toward decreased gene expression of GSR in HIF-1alpha-positive patients ($p = 0.075$, two-sided) (Fig. 4).

4. Discussion

In animal models, regions of hypoxia develop in the liver after injuries caused by toxins and bile duct ligation [15,16], resulting from coagulation system activation, production of vasoactive mediators, or anatomical vascular block. Instead, biliary epithelial hypoxia results from PVP disruption leading to ischemic cholangiopathy [17]. Activation of HIF-1alpha is a cardinal feature of hypoxia, although some cytokines, growth factors, and oxidative stress can also activate the HIF-1alpha pathway [18]. In the current animal models of cholestatic diseases used to study HIF-1alpha pathway activation, positive signals have been detected strictly in parenchyma, but not in cholangiocytes [19,20]. In this study, liver HIF-1alpha signals were located in the biliary epithelium in 37.5% of BA patients, including interlobular bile ducts and ductular reaction at the margins of portal tracts and in fibrovascular septa (Fig. 1). Endothelial cells of arterioles encircling the bile ducts and representing the branches of PVP were also positive for HIF-1alpha

(Fig. 14). HIF-1alpha activation was also observed in biliary structures displaying features of ductal plate malformations and mini ductal plates (Fig. 13) [21]. In addition to these findings in liver, 81.8% of the porta hepatis specimens investigated (Fig. 2.1) showed HIF-1alpha activation in cholangiocytes of biliary remnants as well as in endothelial cells of hepatic artery branches and inflammatory infiltrate (Fig. 2.2).

Activation of the HIF-1alpha pathway in both cholangiocytes located to the liver and porta hepatis in a subset of BA patients supports the hypothesis that ischemic cholangiopathy plays a role in the pathogenesis of BA. The presence of HIF-1alpha-positive inflammatory cellular infiltrates (Fig. 2.3) in the porta hepatis suggests the existence of an integrated network of processes involving hypoxia, inflammation, fibrosis, and biliary obstruction [11]. In patients with BA, previous studies have shown peripheral arterial blockage with perivascular arterial tufts [4], with a VEGFA immuno-localization pattern suggestive of hypoxia affecting the biliary epithelium with reactive angiogenesis [5]. In the liver of patients with the isolated variant of BA, there are molecular features of hypoxia-ischemia associated with disease aggravation [7]. VEGFA is secreted in response to hypoxia through stabilization of hypoxia-inducible factors which are the primary mediators of hypoxia. Under hypoxic conditions, the HIF protein alpha subunit becomes stabilized, translocates to the nucleus, heterodimerizes with the beta subunit, and regulates the expression of genes responsible for cellular adaptation to hypoxia [22]. In arterial vessels, HIF-1alpha is a major mediator of reactional angiogenesis [23]. The observed HIF-1alpha positivity in this study potentially correlates with the biological behavior of VEGFA within the biliary structures of patients with BA [5]. We hypothesized that an injurious agent affecting the

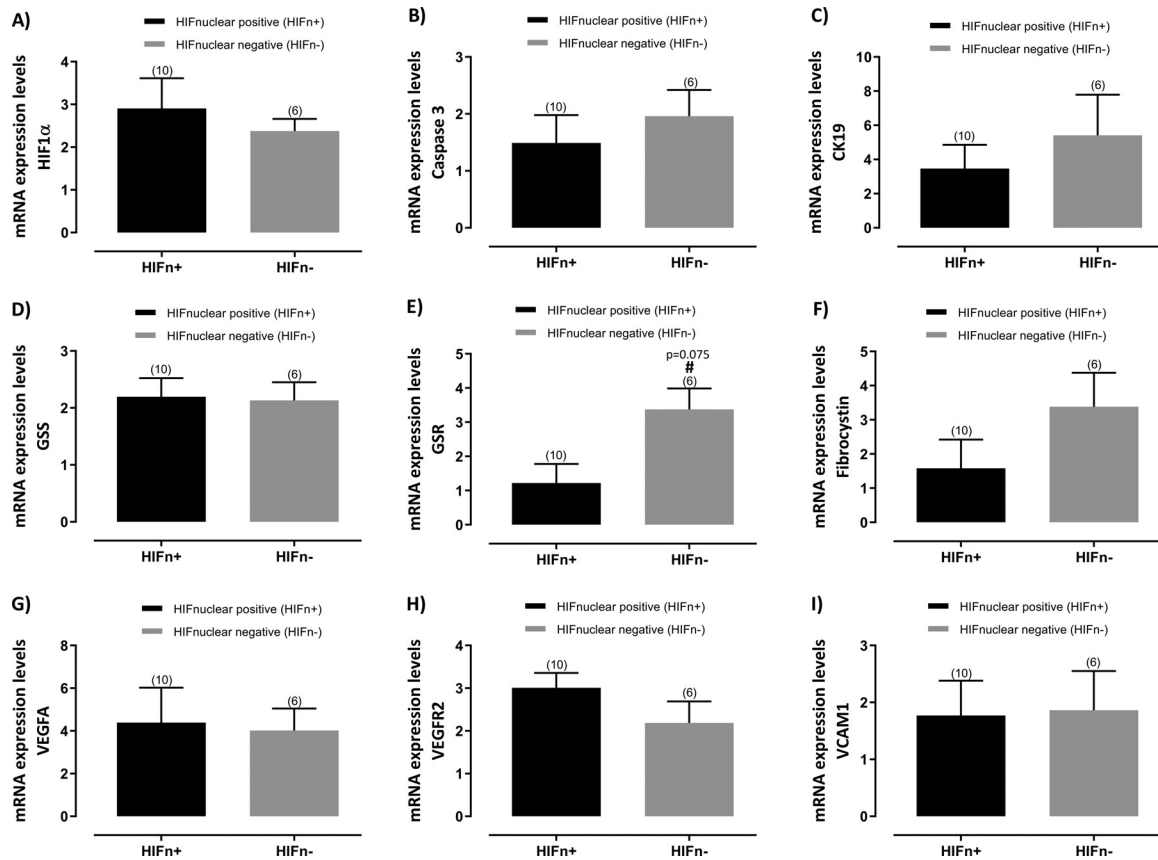


Fig. 4. Gene expression of molecules involved in metabolic and structural processes affected by the hypoxia-ischemia in the liver of BA patients according to HIF-1alpha nuclear signal status. Abbreviations: HIF-1alpha- hypoxia-inducible factor-1 alpha; GSS- glutathione synthetase; GSR- glutathione-disulfide reductase; VEGFA- vascular endothelial growth factor A; VEGFR2- vascular endothelial growth factor receptor 2; VCAM 1- vascular cell adhesion molecule 1; CK19- Cytokeratin 19. Bars represent the mean and vertical lines the SEM. (Mann-Whitney test).

vascular endothelium of PVP would lead to endothelial dysfunction and secondary ischemic cholangiopathy. The identification in the Rhesus-rotavirus induced BA of a derangement in PVP immediately before luminal obstruction [24], and the HIF-1alpha positivity patterns detected in our study strengthen this hypothesis.

The specific immunolocalization of HIF-1alpha positivity to biliary structures in BA, involving the PVP (Fig. 1), rather than to the parenchymal location described in other cholestatic diseases [20,25], suggests simultaneous vascular and biliary disruption, since human bile ducts are supplied exclusively with arterial blood via PVP [26,27].

Additionally, since imaging studies from several other groups have described increased subcapsular blood flow specifically in BA patients, and representing spider telangiectasias [28–35], we evaluated vascular agglomerates in the subcapsular region (Fig. 1.5 and 1.6). Like bile ducts, the subcapsular region is irrigated exclusively by blood from hepatic artery branches [26,27]. Extensive HIF-1alpha positivity in the ductular reaction was evident in the limiting plates in areas of subcapsular vascular agglomerates, as well as in septa departing from these areas and subsequently merging with portal tract margins. Therefore, HIF-1alpha-positive structures involve the hepatic progenitor cell compartment (HPC, Fig. 1.7) [36]. The HPC represents the histologic recess that is critically involved in the control of proliferation, differentiation, and pluripotency of progenitor cells [20], influencing the development of ductular reaction and secondary fibrogenesis. According to Desmet [21,37], ductular reaction consists of a regenerative process triggered by hypoxic stimuli that leads to an angiogenic response with beneficial effects on the hepatobiliary system. In contrast,

several studies have shown that instead of a beneficial regenerative response to improve bile flow, ductular reaction may produce detrimental outcomes resulting in progressive liver fibrogenesis [38–43], including in BA. Ductular reaction refers not only to the epithelial components, but also to expanding precursor niches for cells that are responsible for fibrogenesis [36,44–49], so that the development of ductular reaction leads to concomitant production of fibrillary collagens [36,49]. HIF-1alpha itself controls secretion of profibrotic mediators during the development of liver fibrosis [19], including VEGF [23,50]. The HIF-1alpha activation observed in the HPC compartment [36] may thus produce profound effects on hepatobiliary pathophysiology by playing a direct role in stem cell regulation [20,38,51]. The mechanisms involved in the observed HIF-1alpha activation may result from the effects of hypoxia or oxidative stress, or even both, on the biliary epithelium and HPC [52]. Oxidative stress and hypoxia are intricately linked, and both lead to endothelial dysfunction [25,53,54].

The gene expression analysis of molecular pathways affected by hypoxia-ischemia in our samples showed overexpression of VEGFA and VEGFR2 in BA patients as compared to controls, which suggests triggering of the angiogenic pathway (Fig. 3G and 3H). VEGFA secretion affects and is affected by cholangiocyte proliferation, playing a crucial part in the cross-talk between cholangiocytes and PVP [23,50]. In BA, VEGFA is strongly expressed in portal structures and may be involved in the mechanistic regulation of progressive cholangiopathy [5]. Another finding, the increased gene expression of CK19 in BA patients (described in Fig. 3C), demonstrates the extensive ductular reaction that characterizes BA and supports the presence of ongoing liver fibrogenesis in patients with BA [55]. Ad-

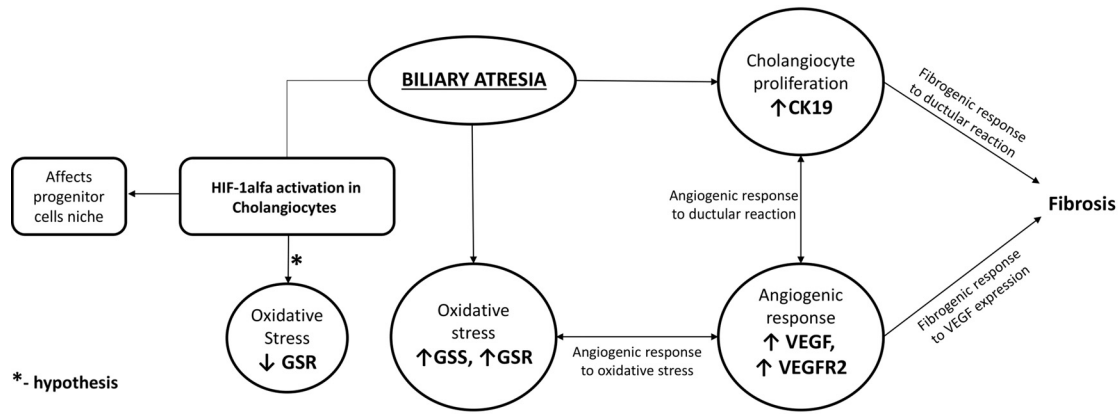


Fig. 5. Diagram representing the putative relationships between study data. The liver samples of biliary atresia patients showed overexpression of genes associated with ductular reaction and angiogenesis, two interrelated processes that are responsible for fibrogenesis. Molecules involved in REDOX status equilibrium were also overexpressed in BA, evidencing oxidative stress, which may be correlated with angiogenesis. A subset of BA patients presented HIF-1alpha activation in cholangiocytes, and PVP, involving the progenitor cell niche.

ditionally, we found increased GSS and GSR gene expression in BA patients compared to controls (Fig. 4E). GSS and GSR are important modulators of the glutathione pathway and critically interrelated with the fibro-inflammation, progression, and survival of BA [56]. Our findings confirm that livers of patients with BA are under a continuum of oxidative stress associated with altered glutathione metabolism, similar to the effects caused by toxins biliary atresia and methylenedianiline [57,58]. In a murine model of bile duct ligation, the hepatic expression of glutathione synthetic enzymes increased early in an adaptive response to oxidative stress (which entails a protective role), but decreased markedly during later stages characterized by advanced fibrosis [59]. The increased expression of GSS and GSR observed in BA liver specimens in this study represents an adaptive hepatic response against oxidative stress. The diagram in Fig. 5 describes the results associated with gene expression in BA patients with or without HIF-1alpha activation and in IHC controls.

A limitation of this study was the small sample size, which precluded confirmative statistical evidence for two of our hypotheses: first, the existence of a correlation between HIF-nuclear positivity in cholangiocytes and decreased early native liver survival; and second, that reduced native liver survival might have resulted from the loss of the protective role of GSR against oxidative stress, as observed in an experimental model of BA [60]. However, given the small size of the sample, for both these correlations we were only able to detect a trend for statistical significance (SDC2 Table 5 and Fig. 4E).

In conclusion, to the best of our knowledge, our study is the first to provide histopathological evidence of HIF-1alpha activation in cholangiocytes, also involving the peribiliary vascular plexus, in a group of patients with isolated biliary atresia. These findings warrant further studies focused on the mechanisms involved in HIF-1alpha pathway activation and on the role and clinical effects of hypoxia and/or oxidative stress affecting cholangiocytes, especially in the hepatic progenitor cell compartment.

Level of evidence

III

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jpedsurg.2022.08.020.

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