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Ciliopathy in cholangiocytes of biliary atresia patients – association with ischemic cholangiopathy, patterns of ductular reaction and disease severity

Patrícia Alexandra Silveira Quelhas

Tese para obtenção do Grau de Doutor em
Biomedicina
(3º ciclo de estudos)

Orientador: Prof. Doutor Jorge Luiz dos Santos
Co-orientador: Profa. Doutora Sandra Maria Gonçalves Vieira
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Janeiro 2026

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11 Dezembro 2025

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Universidade da Beira Interior, Covilhã, 16 Janeiro 2026

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Identificação: BI14541453
Data: 2026-01-16 às 14:44:31

“Great works are performed not by strength but by perseverance.”

Samuel Johnson - The History of Rasselas, Prince of Abissinia

Para a minha avó, a luz eterna que me inspira no céu, e para os meus pais e irmão, os alicerces de amor e força que me sustentam na terra.

Agradecimentos

Chegar ao fim desta jornada foi um caminho de desafios, crescimento e superação, que não teria sido possível sem o apoio incondicional de pessoas muito especiais.

Ao meu orientador, Dr. Jorge Luiz dos Santos, agradeço profundamente pela orientação, paciência e por ter acreditado no meu trabalho e nas minhas capacidades em todas as etapas! Obrigada pelos conselhos, por toda a aprendizagem, pela exigência e por me ensinar que a ciência só avança quando se fazem as perguntas certas.

Aos meus co-orientadores, Dr. Ignácio Verde e Dra. Sandra Vieira, por todo o conhecimento partilhado, pela disponibilidade e por me guiarem sempre com entusiasmo e motivação. Obrigada por fazerem parte deste caminho.

À Fundação para a Ciência e a Tecnologia (FCT) pelo financiamento da minha bolsa (2020.06598.BD) que tornou esta investigação possível, permitindo dedicar-me inteiramente a este projeto e contribuir para o avanço do conhecimento na área.

Ao CICS-UBI, pelo acolhimento e por todas as condições proporcionadas para a realização desta investigação, bem como ao Serviço de Anatomia Patológica do CHU Coimbra, pelo apoio e disponibilidade na marcação das amostras de imunohistoquímica, etapa fundamental deste estudo. Às técnicas do CICS-UBI, pelo profissionalismo e suporte constante ao longo de todo o processo.

Ao Dr. Rui Caetano e Dr. Paulo Teixeira, pelo caloroso acolhimento no Serviço de Anatomia Patológica do CHUC e pelos valiosos conselhos que proporcionaram o avanço desta investigação.

À Dra. Michele Breton, que foi a minha companheira incansável de laboratório na fase inicial deste trabalho. Obrigada pela partilha de conhecimentos e pela amizade que tornaram o caminho mais leve nos primeiros passos desta jornada.

Às meninas do gabinete 46, Ana Oliveira, Ana Ribeiro e Mafalda Soares pela amizade e pelos momentos de partilha e gargalhadas que tornaram os dias de trabalho mais fáceis e mais divertidos.

À malta do basquete, o meu escape e anti-*stress* durante estes anos de doutoramento — por cada treino, aventura, jogo e gargalhada. À Vanessa, parceira de batalha no banco e

nas 4 linhas, por partilhar comigo a gratificante missão (e a dor de cabeça!) de treinar miúdas e provar que o basquete é mesmo o melhor remédio para a sanidade mental (ou para a falta dela!).

Às minhas amigas, que são casa, colo e força. À Margarida, a minha melhor amiga e irmã de coração, que me conhece nas vitórias e nas derrotas, que puxa por mim quando já não sei se consigo, e me faz rir até quando o mundo pesa. À Débora, a amiga furacão, que, mesmo longe, está sempre por perto. Porque cinco minutos de conversa contigo são suficientes para me arrancar um sorriso, aliviar o peso do dia e lembrar-me que a distância nunca foi barreira para a nossa amizade. À Emika, que me trouxe a paz quando a turbulência tomava conta, com a sua presença tranquila e palavras certas no momento certo. À Bernardino, pelo carinho silencioso, sempre presente, mesmo sem precisar de palavras. E à Nádia... a minha âncora em tantos momentos. A que acreditou em mim quando eu já não conseguia acreditar, que ouviu cada desabafo, cada dúvida, e nunca deixou que eu me perdesse no caminho. Levo cada uma de vós em cada linha desta tese, em cada conquista, e para sempre na vida.

Aos meus pais, Lucinda e Paulo, o meu pilar mais firme e constante. Obrigada por nunca terem deixado que eu duvidasse de que este caminho era possível, mesmo quando parecia demasiado longo, duro ou solitário. O vosso amor incondicional, o vosso sacrifício silencioso e a força com que sempre me ampararam estiveram presentes em cada passo que dei. Foram e são o meu maior exemplo — de coragem, de entrega e de carinho. Esta conquista é tanto vossa como minha. Obrigada por tudo, sempre.

Ao meu irmão, Renato, o meu melhor amigo e a outra metade do meu coração. Não há palavras que cheguem para agradecer tudo o que és para mim. Obrigada por seres o meu porto seguro, por me ouvires nos dias mais difíceis e por acreditares em mim com uma certeza que muitas vezes me faltou. És a prova de que, aconteça o que acontecer, nunca estarei sozinha.

À minha querida avó Conceição, que partiu sem ver este sonho terminado, mas que esteve comigo em cada passo do caminho. Pela força silenciosa, pelo amor incondicional e por me ensinar, sem precisar de palavras, que a verdadeira coragem está em nunca desistir. Queria muito que pudesses ler estas páginas... mas sei que, onde quer que estejas, estás orgulhosa.

A toda a minha restante família, que sempre teve uma palavra de incentivo e confiança. À minha tia Cecília, por estar sempre a puxar-me para cima, com aquela confiança inabalável de quem sabia que eu ia conseguir.

E por último, ao meu companheiro de todas as horas, Pedro. Obrigada por seres o meu equilíbrio e a minha luz em todos os dias cinzentos. Pela paciência infinita nos meus dias mais ansiosos, pelo apoio constante e por me relembrares, sempre, que sou capaz! A tua presença nesta caminhada foi essencial, e este capítulo não teria sido possível sem ti e sem o teu amor ao meu lado. Obrigada por seres o meu lar!

A todos os demais colegas e amigos que não referi acima (mas que sabem quem são), que de alguma forma, contribuíram para este trabalho – obrigada pela amizade, pelas palavras de incentivo e por tornarem este percurso mais leve e especial.

A todos vocês, o meu eterno bem-haja!

Resumo Alargado

A atresia biliar (AB) é uma colangiopatia neonatal rara caracterizada pela obstrução progressiva dos ductos biliares extra-hepáticos, levando a colestase, fibrose hepática e, frequentemente, à necessidade de transplante hepático. Embora os bebés afetados pareçam clinicamente saudáveis ao nascer, as evidências laboratoriais de colestase já são observáveis. Apesar dos avanços na compreensão dos mecanismos fisiopatológicos subjacentes, a etiologia da AB permanece incerta, com hipóteses causais que vão desde infeções virais e desregulação imunológica a predisposições genéticas e anomalias vasculares. Evidências recentes sugerem que a hipóxia e a disfunção dos cílios primários dos colangiócitos podem desempenhar um papel central na patogenicidade da AB, influenciando a progressão de colangiopatia e os desfechos clínicos.

Esta tese investiga a interligação entre a hipóxia, mais propriamente, a ativação da via do fator induzido por hipóxia 1-alfa (HIF-1α) no núcleo de colangiócitos (colangiopatia isquémica) e as alterações morfológicas e funcionais dos cílios primários em colangiócitos de pacientes com AB. A abordagem metodológica incluiu a análise imunohistoquímica, imunofluorescente e molecular de amostras hepáticas obtidas de pacientes submetidos a portoenterostomia, a caracterização detalhada da morfologia ciliar por técnicas avançadas de imagem digital, e a correlação desses resultados com parâmetros clínico-laboratoriais e desfechos pós-operatórios, incluindo a sobrevivência do fígado nativo.

Os resultados obtidos demonstraram um aumento da expressão nuclear de HIF-1α em colangiócitos de pacientes com AB, especialmente em áreas próximas ao plexo vascular peribiliar e em nichos de células progenitoras. Esta ativação não foi observada nas amostras de fígado utilizadas como controlo (pacientes com colestases neonatais, não AB), sugerindo um papel específico da hipóxia na patogénese da AB. Além disso, a análise de expressão génica revelou a sobre-regulação de vias moleculares relacionadas com a reação ductular, o stress oxidativo e a angiogénese, reforçando a hipótese de que a hipóxia desempenha um papel ativo na lesão biliar e na remodelação tecidual. Estes resultados justificam estudos adicionais focados nos mecanismos envolvidos na ativação da via HIF-1alfa e no papel e efeitos clínicos da hipoxia e/ou stress oxidativo que afetam os colangiócitos.

Em paralelo, a caracterização dos cílios primários em colangiócitos demonstrou alterações estruturais significativas em amostras de pacientes com AB. Observou-se uma redução no comprimento ciliar e um possível distúrbio no transporte da tubulina 4α-acetilada, um marcador chave da estabilidade ciliar, do citoplasma para o cílio. Estas alterações associaram-se ainda a piores desfechos clínicos, com uma redução significativa da sobrevivência do fígado nativo. Relativamente à associação da hipóxia com ciliopatia, a co-localização de HIF-1α e TUBA4A em amostras de AB, sugere uma associação inversa entre a positividade de HIF-1α e a presença de cílios primários na membrana luminal dos colangiócitos, tanto nas células biliares dos tratos portais como noutras áreas microanatómicas do fígado. A análise quantitativa das características ciliares mostrou que as células biliares sem características hipóxicas apresentaram preservação do cilio primário, sugerindo que a hipoxia pode ter um impacto negativo na formação ou manutenção do cilio primário. A correlação entre a ativação do HIF-1α e as disfunções ciliares sugere que a hipóxia não só promove a inflamação e a fibrose, como também compromete a integridade estrutural e funcional dos cílios primários, agravando a progressão da doença.

Estes resultados oferecem novas perspetivas para a compreensão dos mecanismos fisiopatológicos subjacentes à AB, sugerindo que a ativação do HIF-1α e as disfunções ciliares podem constituir biomarcadores relevantes para o prognóstico e potenciais alvos terapêuticos. A identificação dessas alterações moleculares permite não só antecipar a evolução clínica dos pacientes, como também explorar intervenções inovadoras que visem modular a resposta hipóxica e restaurar a função ciliar, com o objetivo de melhorar a sobrevivência hepática e a qualidade de vida dos doentes.

No conjunto, este trabalho contribui para o entendimento aprofundado da relação entre hipóxia, disfunção ciliar e colangiopatia na AB, abrindo novas linhas de investigação para o desenvolvimento de estratégias diagnósticas e terapêuticas mais eficazes, que possam mitigar os efeitos da hipóxia e preservar a função hepática em pacientes pediátricos com esta condição.

Palavras-chave

Atresia Biliar; Hipóxia; Colangiopatia Isquémica; HIF-1α; Ciliopatia; Cílios Primários; Tubulina

Abstract

Biliary atresia (BA) is a rare neonatal cholangiopathy characterized by the progressive obstruction of the extrahepatic bile ducts, leading to cholestasis, liver fibrosis, and often the need for liver transplantation. Although affected infants appear clinically healthy at birth, laboratory evidence of cholestasis is already detectable. Despite advances in understanding the underlying pathophysiological mechanisms, the etiology of BA remains unclear, with proposed causes ranging from viral infections and immune dysregulation to genetic predispositions and vascular anomalies. Recent evidence suggests that hypoxia and primary cilia dysfunction in cholangiocytes may play a central role in BA pathogenesis, influencing cholangiopathy progression and clinical outcomes.

This thesis investigates the interplay between hypoxia—specifically, the activation of the hypoxia-inducible factor 1-alpha (HIF-1α) pathway in cholangiocyte nuclei (ischemic cholangiopathy)—and the morphological and functional alterations of primary cilia in cholangiocytes of BA patients. The methodological approach included immunohistochemical, immunofluorescence, and molecular analyses of liver samples obtained from patients undergoing portoenterostomy, detailed characterization of ciliary morphology using advanced digital imaging techniques, and correlation of these findings with clinical and laboratory parameters and post-operative outcomes, including native liver survival.

The results demonstrated increased nuclear HIF-1α expression in cholangiocytes of BA patients, particularly in areas near the peribiliary vascular plexus and progenitor cell niches. This activation was absent in liver samples from control patients with other neonatal cholestases (non-BA), suggesting a specific role of hypoxia in BA pathogenesis. Furthermore, gene expression analysis revealed the upregulation of molecular pathways related to ductular reaction, oxidative stress, and angiogenesis, reinforcing the hypothesis that hypoxia actively contributes to bile duct injury and tissue remodeling. These findings justify further studies focused on the mechanisms involved in HIF-1α activation and the clinical effects of hypoxia and/or oxidative stress on cholangiocytes.

Additionally, the characterization of primary cilia in cholangiocytes revealed significant structural alterations in BA patient samples. A reduction in ciliary length and a possible

disruption in the transport of acetylated tubulin 4α—a key marker of ciliary stability—from the cytoplasm to the cilium was observed. These alterations were associated with worse clinical outcomes, including a significant reduction in native liver survival. Regarding the association between hypoxia and ciliopathy, the co-localization of HIF-1α and TUBA4A in BA samples suggests an inverse relationship between HIF-1α positivity and the presence of primary cilia on the luminal membrane of cholangiocytes, both in the portal tract and other microanatomical regions of the liver. Quantitative analysis of ciliary characteristics showed that biliary cells without hypoxic features maintained primary cilia integrity, suggesting that hypoxia may negatively impact ciliary formation or maintenance. The correlation between HIF-1α activation and ciliary dysfunction implies that hypoxia not only promotes inflammation and fibrosis but also compromises the structural and functional integrity of primary cilia, exacerbating disease progression.

These findings offer new insights into the pathophysiological mechanisms underlying BA, suggesting that HIF-1α activation and ciliary dysfunction may serve as relevant biomarkers for prognosis and potential therapeutic targets. Identifying these molecular alterations not only facilitates the prediction of clinical outcomes but also opens avenues for innovative interventions aimed at modulating the hypoxic response and restoring ciliary function to improve liver survival and patient quality of life.

Overall, this work advances the understanding of the relationship between hypoxia, ciliary dysfunction, and cholangiopathy in BA, paving the way for new research directions to develop more effective diagnostic and therapeutic strategies that mitigate the effects of hypoxia and preserve liver function in pediatric patients with this condition.

Keywords

Biliary Atresia; Hypoxia; Ischemic Cholangiopathy; HIF-1α; Ciliopathy; Primary Cilia; Tubulin

Funding

The experimental work presented in this thesis was carried out at the Health Sciences Research Center of the Faculty of Health Sciences of the University of Beira Interior (CICS-UBI) and at the Pathological Anatomy Laboratory of the University Hospital of Coimbra (CHUC). The collection of human samples was carried out and funded by the Hospital de *Clínicas de Porto Alegre*, Brazil. This work was supported by the project: "The role of ischemic cholangiopathy in situations of liver dysfunction" (POCI-01-0145-FEDER-028956) and by the doctorate fellowship granted to the author Patrícia Quelhas by the Foundation for Science and Technology (FCT), 2020.06598.BD.

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List of Abbreviations

A1ATd	Alpha-1 antitrypsin deficiency
ADD3	Adducin 3
ADPLD	Autosomal dominant polycystic liver disease
AFAP1	Actin filament associated protein 1
ARF6	ARF GTPase 6
ARPKD	Autosomal recessive polycystic kidney disease
ATX	Autotaxin
AURKA	Aurora kinase A
BA	Biliary Atresia
BARC	Biliary Atresia Research Consortium
BASM	BA Splenic Malformation syndrome
CABA	Cardiac-associated biliary atresia
CBA	Cystic BA
CD	Caroli Disease
CD62P	P-Selectin
CDG	Congenital Disorder of Glycosylation
CFC1	Cryptic Family 1
CHF	Congenital Hepatic Fibrosis
ChiLDREN	Childhood Liver Disease Research and Education Network
CICS-UBI	Health Sciences Research Centre – University of Beira Interior
CK19	Cytokeratin 19
CMV	Cytomegalovirus
CMV-BA	Cytomegalovirus-associated BA
CPE	Center of Experimental Research
CRs	Cilia ratio status
DB	Direct-reacting bilirrubin
DPM	Ductal plate malformation
DR	Ductular reaction
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
ExLap	Exploratory laparotomy with trans-operative cholangiography
FCT	Foundation for Science and Technology
FGF-2	Fibroblast growth factor-2

FOX2	Forkhead box protein P2
FRL-1	Frigida like 1
GEO	Gene Expression Omnibus
GPC1	Glypican-1
GSR	Glutathione-disulfide reductase
GSS	Glutathione synthetase
GWAS	Genome wide association study
HCPA	Hospital de Clínicas de Porto Alegre
HIF	Hypoxia-inducible factor
HIF-1α	Hypoxia inducible factor 1 alpha
HIF-3α	Hypoxia-inducible factor 3 alpha
HPC	Hepatic progenitor cell compartment
HRE	Hypoxia-responsive element
IBA	Isolated BA
IC	Ischemic cholangiopathy
ICAM	Intercellular adhesion molecule
IFN-γ	Interferon-gama
IFT	Intraflagellar transport
IFT-A	IFT complex A
IFT-B	IFT complex B
IHC	Intrahepatic cholestasis
IL-1	Interleukin 1
IL-10	Interleukin 10
IL-18	Interleukin 18
IL-6	Interleukin 6
INC	Idiopathic neonatal cholestasis
KIF3B	Kinesin-like protein KIF3B
LEFTY 2	Left-right determination factor 2
LEFTY1	Left-right determination factor 1
LTx	Liver transplantation
MAN1A2	Mannosidase alpha class 1A member 2
MDA	Methylene diphenyl diamine
MDI	Methylene diphenyl diisocyanate
MHC	Major histocompatibility complex
miRNAs	microRNAs
MSC	Mesenchymal stem cells
MT	Medial thickening

NLS	Native liver survival
NODAL	Nodal growth differentiation factor
PC	Primary cilia
PCNT	Pericentrin
PDGFA	Platelet-derived growth factor
PDGFA	Platelet-derived growth factor subunit A
PE	Portoenterostomy
PHIS	Pediatric health information system
PKD1L1	Polycystin 1 like 1
PLD	Polycystic Liver Disease
pVHL	Von Hippel-Lindau tumor suppressor protein
PVP	Peribiliary vascular plexus
REDOX	Reduction-oxidation
rhBMP7	Recombinant human bone morphogenesis protein-7
ROIs	Regions of interest
RVR	Rhesus Rotavirus
SAP-CHUC	Anatomo-Pathology Service at Coimbra Hospital and University Center
SPSS	Statistical Package for the Social Sciences
TB	Total bilirubin
TLR	Toll-like receptors
TLR3	Toll-like receptor 3
TNF-α	Tumour necrosis factor alpha
TPN	Total parenteral nutrition
TRPV4	Transient receptors potential cation subfamily V member 4
TTC17	Tetratricopeptide repeat domain 17
TUBA4A	Tubulin 4α-acetylated
TUSC3	Tumor suppressor candidate 3
UFRGS	Faculty of Medicine, Federal University of Rio Grande do Sul
USP8	Ubiquitin-specific protease 8
V2R	Vasopressin receptor
VCAM	Vascular cell adhesion molecule
VDAC1-ΔC	Voltage-gated anion channel
VEGFA	Vascular growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
VHL	Von Hippel-Lindau
ZIC3	Zic family member 3

List of Scientific Publications

Scientific articles, book chapters & conference papers included in the Thesis

Patricia Quelhas, Michele Breton, Rui Oliveira, Maria Augusta Cipriano, Pranav Shivakumar, Jorge Bezerra, Sandra Vieira, Carlos Kieling, Ignacio Verde and Jorge Luiz dos Santos. "Ischemic cholangiopathy in biliary atresia: immunohistochemical and molecular evidence", Journal of Hepatology V75(2):s191–s866, 2021, ISSN 0168-8278, https://doi.org/10.1016/S0168-8278(21)01843-2. (Conference abstract)

Patricia Quelhas, Carlos Cerski and Jorge dos Santos. "Update on Etiology and Pathogenesis of Biliary Atresia", Current Pediatric Reviews, Aug;19(1):48-67, 2022. doi: 10.2174/1573396318666220510130259.

Patricia Quelhas, Michelle Breton, Rui Oliveira, Maria Cipriano, Paulo Teixeira, Carlos Cerski, Pranav Shivakumar, Sandra Vieira, Carlos Kieling, Ignacio Verde, Jorge dos Santos. "HIF-1alpha-pathway activation in cholangiocytes of patients with biliary atresia: An immunohistochemical/molecular exploratory study", Journal of Pediatric Surgery, Mar;58(3):587-594, 2023. doi: 10.1016/j.jpedsurg.2022.08.020.

Patricia Quelhas, Rui Oliveira and Jorge Luiz dos Santos. "TOP-146 - Activation of the HIF-1alpha pathway in cholangiocytes of patients with biliary atresia and its association with cilia disruption", Journal of Hepatology, V80(1):S692,2024, ISSN 0168-8278. https://doi.org/10.1016/S0168-8278(24)01973-1. (Milan Conference abstract)

Patricia Quelhas, Rui Oliveira, Sandra Vieira, Carlos Kieling, Jorge dos Santos. "Structural Disruption of Cilia and Increased Cytoplasmic Tubulin in Biliary Atresia—An Exploratory Study Focusing on Early Postoperative Prognosis Following Portoenterostomy", Biomedicines, Jan; 13(1), 87, 2025. https://doi.org/10.3390/biomedicines13010087

Patricia Quelhas, Diogo Morgado, Jorge dos Santos. "Primary Cilia, Hypoxia, and Liver Dysfunction: A New Perspective on Biliary Atresia", *Cells*, 2025; 14(8):596. https://doi.org/10.3390/cells14080596

Scientific articles & book chapters not included in the Thesis

Patricia Quelhas, Joana Jacinto, Carlos Cerski, Rui Oliveira, Jorge Oliveira, Elisa Carvalho, Jorge Dos Santos. “Protocols of Investigation of Neonatal Cholestasis-A Critical Appraisal”, Healthcare, Oct;12;10(10), 2022. doi: 10.3390/healthcare10102012.

Patricia Quelhas and Jorge dos Santos. Encyclopedia entry: dos Santos, Jorge Luiz. "Protocols of Investigation of Neonatal Cholestasis". In, *Encyclopedia Journal*, 2022, https://encyclopedia.pub/entry/32315

List of Scientific Communications

Oral and Poster communications related to this doctoral thesis:

Oral Presentation: **Patricia Quelhas,** Michele Breton, Rui Oliveira, Maria Augusta Cipriano, Pranav Shivakumar, Jorge Bezerra, Sandra Vieira, Carlos Kieling, Ignacio Verde and Jorge Luiz dos Santos. “Ischemic cholangiopathy in biliary atresia: immunohistochemical and molecular evidence” at EASL International Liver Congress 2021, Online Conference

Poster Presentation: **Patrícia Quelhas,** Michele Breton, Rui Oliveira, Maria Cipriano, Carlos Kieling, Sandra Vieira, Ignacio Verde, Jorge Santos, "HIF-1alpha-pathway activation in cholangiocytes of patients with biliary atresia: An immunohistochemical/molecular exploratory study" at IV International Congress on Health Sciences Research: Towards Innovation and Entrepreneurship 2023, Covilhã, Portugal

Poster presentation: **Patrícia Quelhas**, Rui Oliveira, Sandra Vieira, Carlos Kieling, Jorge Santos, "Activation of the HIF-1alpha pathway in cholangiocytes of patients with biliary atresia and its association with cilia disruption" at EASL International Liver Congress 2024, Milan, Italy **(*Top Poster Award*)**

Oral and Poster communications unrelated to this doctoral thesis:

Oral Presentation: **Patrícia Quelhas, Jorge L. Santos**, “Ischemic cholangiopathy in the rat model of late ischemia-reperfusion injury” at 28th Meeting of European Society of Paediatric Clinical Research 2019, Paediatric Institute-Clinic of the University of Debrecen, Hungary.

Poster presentation: **Patrícia Quelhas**, Michele C. Breton, Tomaz Grezzana, Rui C. Oliveira, Maria A. G. Cipriano, Ignacio Verde, Jorge L. Santos, “Topical hypothermia as a protective method against ischemic cholangiopathy in rat liver" at XIV Annual CICS-UBI Symposium 2020, Health Sciences Faculty, University of Beira Interior, Covilhã, Portugal.

Thesis Overview

Biliary Atresia (BA) is a complex cholangiopathy that leads to progressive liver damage and often leads to the need for liver transplantation, being its most frequent indication during childhood and even infancy. The causes of BA remain largely unexplained, with ongoing research investigating genetic, environmental, and viral infectious/inflammatory contributions to the disease. Recent studies have pointed towards hypoxia as a significant factor in BA progression. Our work integrates molecular, cellular, and clinical research to explore the role of hypoxia and ciliary dysfunction in such an acquired cholangiopathy in BA, specifically focusing on the intracellular transport of tubulin proteins and the activation of the HIF-1α pathway.

The present thesis is the result of a collaborative effort between researchers from multiple institutions, including the Health Sciences Research Centre (CICS-UBI) and the Anatomo-Pathology Service at Coimbra Hospital and University Center (SAP-CHUC) in Portugal, as well as the Hospital de Clínicas de Porto Alegre, Faculty of Medicine, Federal University of Rio Grande do Sul (UFRGS) in Brazil. Together, they combined their expertise to analyze the molecular and histopathological alterations in BA. A focus was placed on understanding how the dysregulation of hypoxia-related pathways, particularly HIF-1α, correlate with cholangiopathy and changes in cholangiocyte primary cilia. We used advanced digital image analysis, gene analysis, and correlations with clinical prognosis to quantify changes in cilia structure and tubulin localization in liver samples from BA patients and to understand the role of hypoxia in the intrahepatic biliary tree in a subgroup of patients with BA and whether it is associated with ciliopathy.

The findings offer novel insights into how disrupted ciliary function might contribute to the cholangiopathy observed in BA. In particular, our work highlights potential new avenues for early diagnosis aimed at preserving native liver function in BA patients. Moreover, we discovered that a subset of BA patients exhibits ischemic cholangiopathy, suggesting that hypoxia and reduced blood flow may play a critical role in disease progression. Chapter 1 provides an overview of the current state of research on BA, offering a comprehensive review of its clinical subtypes, the pathogenic mechanisms underlying the disease, and the challenges involved in studying it. Chapter 2 delves into the role of ciliopathy in BA, exploring the interplay between primary cilia dysfunction and the effects of hypoxia on biliary structures, highlighting how these factors contribute to disease progression and impact therapeutic outcomes. Additionally, it explores the role of primary cilia and ciliogenesis in liver function, linking these

processes to hypoxia and its broader implications for disease progression. Chapter 3 presents the methodological approach used in this study and the outline of this thesis. The experimental findings, detailed in Chapter 4, provide histopathological and molecular evidence of HIF-1α activation in cholangiocytes, including its involvement with the peribiliary vascular plexus, in a group of patients with isolated biliary atresia. This was demonstrated through image analysis and gene quantification. In Chapter 5, our experimental findings suggest a disruption in the transport of tubulin 4α-acetylated (TUBA4A) from the cytoplasm to primary cilia, potentially due to hyper-stabilization of tubulin in the cytoskeleton. Given the complexity of the pathophysiological processes that may contribute to the early severity of BA, our findings suggest a plausible hypothesis: environmental factors, genetic variants, and/or embryological disturbances—such as hypoxia affecting the immature peribiliary matrix—may be associated with tubulin hyper-stabilization and disruption in the tubulin transport between cytoplasm and primary cilia, potentially leading to primary cilia disruption.

Finally, in Chapter 6, we discuss the main findings achieved in this study and their integration into understanding the molecular mechanisms underlying biliary atresia. Specifically, we focus on the development of novel methodologies for analyzing TUBA4A transport and cilia function, as well as the role of hypoxia in cholangiocyte dysfunction. These findings provide a foundation for future research aimed at unraveling the pathophysiology of biliary atresia and exploring potential therapeutic strategies to mitigate disease progression and improve patient outcomes.

Chapter 1

General Introduction – Part I
Update on Etiology and Pathogenesis of Biliary Atresia

This chapter was published in:

***Patricia Quelhas**, Carlos Cerski and Jorge dos Santos. "Update on Etiology and Pathogenesis of Biliary Atresia", Current Pediatric Reviews, 2022;19(1):48-67. doi: 10.2174/1573396318666220510130259. PMID: 35538816.*

Abstract

Biliary atresia is a rare inflammatory sclerosing obstructive cholangiopathy that initiates in infancy as complete choledochal blockage and progresses to the involvement of intrahepatic biliary epithelium. Growing evidence shows that biliary atresia is not a single entity with an unique etiology, but a phenotype resulting from multifactorial events whose common path is obliterative cholangiopathy. The etiology of biliary atresia has been explained as resulting from genetic variants, toxins, viral infection, chronic inflammation or bile duct lesions mediated by autoimmunity, abnormalities in the development of the bile ducts, defects in embryogenesis, abnormal fetal or prenatal circulation and susceptibility factors. It is increasingly evident that the genetic and epigenetic predisposition combined with the environmental factors to which the mother is exposed are potential triggers for biliary atresia. There is also indication that a progressive thickening of the arterial middle layer occurs in this disease, suggestive of vascular remodeling and disappearance of the interlobular bile ducts. It is suggested that hypoxia/ischemia process can affect portal structures in biliary atresia and is associated with both the extent of biliary proliferation and thickening of the medial layer.

Keywords: Biliary atresia, hypoxia, ischemia, etiology, genetics, epigenetics, gut microbiota, immunity

1.1. Introduction

Biliary atresia (BA) is an obstructive, inflammatory sclerosing cholangiopathy that initiates in infancy as complete choledochal blockage and progresses to the involvement of intrahepatic biliary epithelium [1, 2]. Although affected infants appear clinically healthy at birth, laboratory evidence of cholestasis is already observable [3]. In the following 8 postnatal weeks, the unsolved extrahepatic ductal obstruction associated with ongoing intrahepatic cholangiopathy and biliary fibrogenesis lead to cirrhosis [4]. Portoenterostomy, the surgical procedure developed by Dr. Morio Kasai [5] can restore biliary drainage and postpone or even deter the need for liver transplantation (LTx). The age at portoenterostomy, however, plays a key role in the outcome, since a late operation means progression to an early liver failure [6]. However, in most cases, irrespective to the timely performance of an adequate portoenterostomy, with initially successful results, the intrahepatic cholangiopathy progresses, leading to cirrhosis and chronic liver failure. The causes of such disappointing long-term results are not completely understood.

BA is a rare disease presenting a variable incidence from 4.2 to 32 per 100,000 live births [2], with the highest values reported in French Polynesian (3/10,000 live births) and East Asian countries (1.1/10,000 live births in Japan and 1.5/10,000 in Taiwan) [1, 7, 8]. Interestingly, in French Polynesian, as a result of a 30-year cohort of all BA cases (1979 – 2009), it was observed a noteworthy dynamic of seasonality, suggesting the role of environmental factors likely influenced by genetic background in that population [9]. Recent incidence estimates for BA in Western countries are in the range of 0.5 to 0.8/10,000 live births [10] and in Europe, it is approximately 1 in 12,000 live births [1]. BA is the most common cause of end-stage liver disease and LTx in children and it is urgent to unravel the etiology of biliary obstruction and why the pathophysiology of the progressive fibrogenic cholangiopathy.

It is known that BA involves both an extra- and intrahepatic progressive cholangiopathy that arises specifically in the neonatal period [11], sharing molecular pathways with other cholangiopathies such as primary sclerosing cholangitis and primary biliary cholangitis including immunity, ductular reaction and fibrosis [12]. An additional clinical association of BA, only recently unraveled [8] is cardiomyopathy in 70% of the patients with age around 8 months of life and in the waiting list for LTx. Cardiomyopathy is characterized by abnormal, distorted cardiomyocytes, and arterial medial layer thickening related to arterial hypertension, environmental factors such as

virus or toxins, specific gene variants, or with some primary systemic diseases. It is not defined whether the association between cardiomyopathy and BA is secondary to cirrhosis or there is a primary vascular abnormality in common between them. Our group observed medial layer thickening and distortion of the hepatic arterial walls in BA patients, apparently representing a primary event in the disease [13]. Uflacker and Pariente observed angiographic findings of peripheral vascular obstruction in hepatic artery branches at the time of portoenterostomy in all 45 patients of the sample they evaluated [14]. Image findings of medial layer thickening in the hepatic artery, and the presence of subcapsular telangiectasis (Figure 1.1) have been described specifically in BA patients, thus serving for the differential diagnosis between BA and cases of intrahepatic cholestasis [15-20].

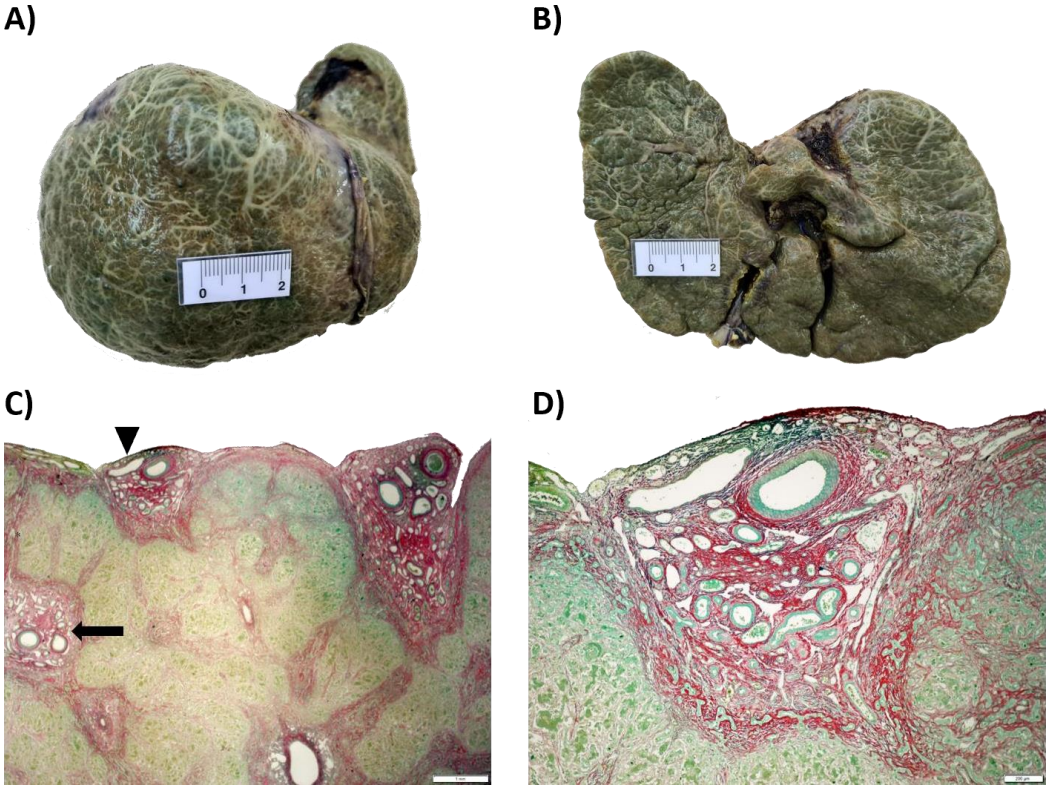

Figure 1.1. Subcapsular vascular agglomerates with features of telangiectasis in the explant of a patient with biliary atresia - macro anatomic images: (A) subcapsular telangiectasias in the diaphragmatic face of the liver explant; (B) subcapsular telangiectasias in the visceral face of the liver explant; micro anatomic images: (C) subcapsular vascular agglomerates with continuation through fibrovascular septa (asterisk) to a portal tract presenting (arrow) similar vascular features (magnification 100x); (D) detail of the subcapsular vascular agglomerate presented in Fig. C (arrowhead): note the increased number of arterial/arteriolar vessels with prominent medial layers and luminal dilatation accumulated within the subcapsular fibrous stroma. The images were acquired at the *Hospital de Clínicas de Porto Alegre* (HCPA) as part of a research study.

There are different clinical variants of BA that probably represent dissimilar etiopathogenic mechanisms and may imply distinct therapeutic response and prognosis (Table 1.1). An example of a different scenario is the cystic form of BA, which has a better prognosis compared to other types, since diagnosis is possible earlier through cyst seen in image exams. This is because it is typically diagnosed earlier, as the cysts are quickly visible on imaging exams [21]. So why the research and increasing knowledge around treatment and prognosis of the disease are still limited? Because the putative pathophysiologic processes involved in BA remain only partially solved [1, 11]. The fact that BA does not occur in humans as an outbreak suggests that there is no single etiological agent, but there may be a large group of etiological agents, including toxins and infectious agents [6] and this may be the explanation for the different associated types occurring in BA. Thus, identifying common injury and repair mechanisms in the context of BA may be a better approach to the development of therapeutic agents than looking for single etiological agents.

Table 1.1. Types of biliary atresia proposed considering the anatomical and clinical aspects.

ANATOMICAL ASPECTS
Extra hepatic obstruction site a) Type 1 (5%) b) Type 2 (2%) c) Type 3 (90%) **Presence of bile cysts (regardless of the obstruction site)** a) Without cysts b) Extra Hepatic cystic form i. Type 1 associated ii. Type 2 associated **Presence of ductal plate malformations-like structures** **Correlation with histopathology / molecular analysis** a) Inflammatory type b) Fibrogenic type c) Hypoxic/Ischemia type d) Mixed form
CLINICAL ASPECTS **(association with congenital extra hepatic anomalies)**

Perinatal or isolated form (65%)

Embryonic form (35%)

a) Laterality anomaly associated
b) Non-syndromic pattern of congenital anomalies
c) Isolated intestinal malrotation-associated

Extrahepatic biliary cystic form (11%)

CMV-associated form

1.2. Clinical Types of Biliary Atresia

Currently, there is growing evidence that BA is not a single entity with a single etiology, but a phenotype resulting from multifactorial events whose common path is obliterative cholangiopathy [7, 22], thus hindering its classification.

There are many theories proposed to explain the etiology of BA, including the role of toxins, virus infection, genetic variants and autoimmune processes. Several studies have classified BA into different types that may involve variants of the disease in terms of pathogenesis and prognosis (Figure 1.2).

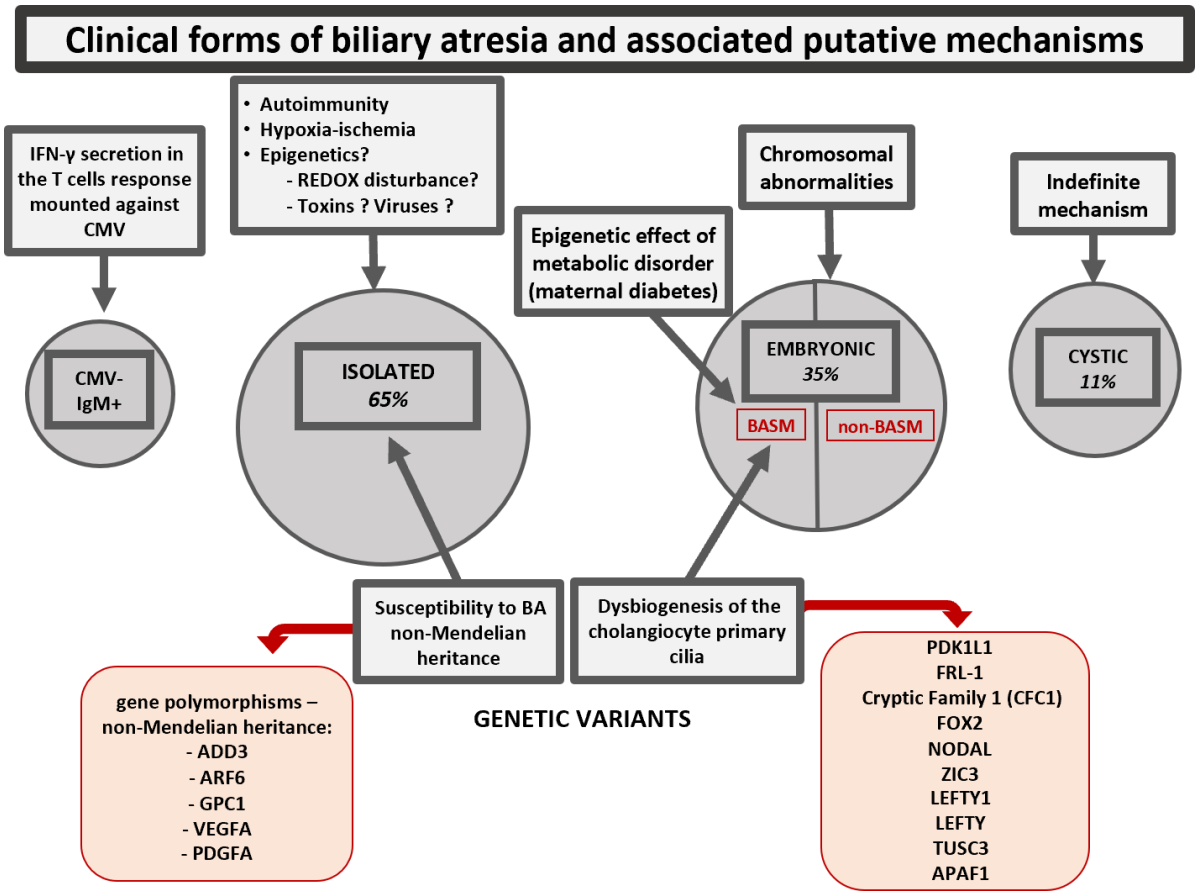

Figure 1.2. Diagram representing the putative mechanisms observed in BA etiology. This figure represents mechanisms probably involved in the development of the different BA clinical phenotypes, as discussed in the text.

The classification of patients with BA according to the anatomical location of the biliary obstruction defines the surgical approach to be performed since pervious segments of extrahepatic biliary structures are usable as an anastomosis site to the digestive tract in the Kasai procedure [23]. According to the Japanese Society of Paediatric Surgeons, BA can be classified into 3 different types according to the anatomical location of extrahepatic obstruction [23]. Type I is characterized by atresia in the common bile duct with permeability of the proximal biliary structures with a cystic biliary structure located upstream the obstruction [10] and accounts for approximately 5% of cases. Type II is characterized by atresia in the common hepatic duct and may be complemented by cysts in the porta hepatis, and accounts for only 2% of cases. Finally, type III is the most common anatomic variant, representing 90% of cases. In this type of BA all of the porta hepatis is solid, forming a fibrous cord, and a dense inflammatory proximal remnant is noted (Figure 1.3). At a clinical level, BA can be characterized into different types such as cystic BA (CBA), Cytomegalovirus-associated BA (CMV-associated BA), BA Splenic Malformation syndrome (BASM) [24] and Isolated BA (IBA) [1].

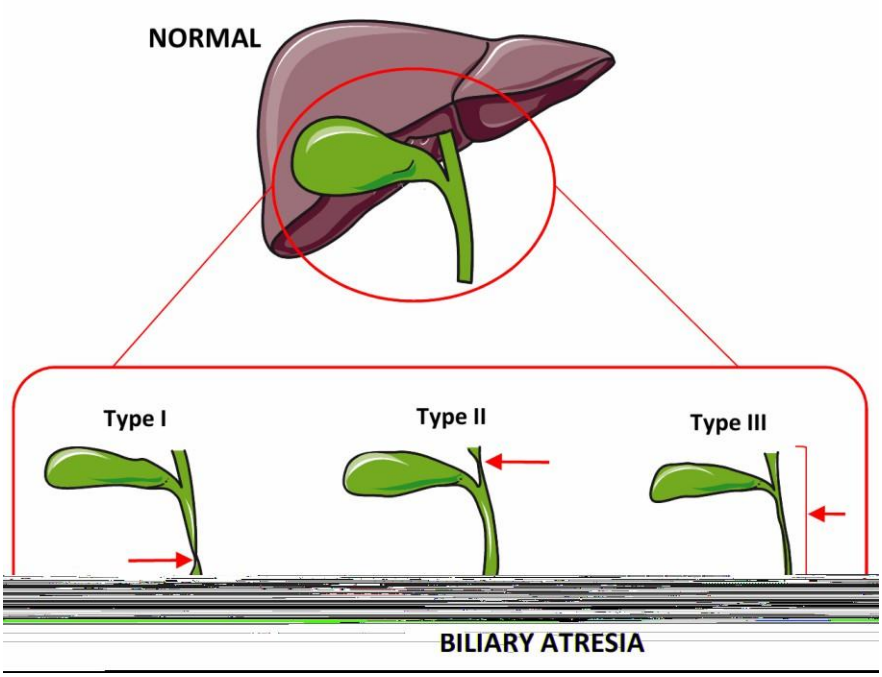

Figure 1.3. BA forms according to the anatomical location of extrahepatic mechanical obstruction, according to Japanese Society of Pediatric Surgeons. (Type I) Atresia limited to the common bile duct. (Type II) Atretic hepatic duct, keeping the proximal ducts pervious. (Type III) Found in 90% of cases, with the right and left hepatic duct and the porta hepatis atretic.

1.2.1. Cystic Biliary Atresia (CBA)

The cystic form of BA refers to the presence or absence of extrahepatic biliary cysts. CBA is the only type of BA that can be identified in the prenatal period (through ultrasound) and constitutes about 10% of all cases. It is characterized in the bile duct atresia of porta hepatis with a cystic structure at the common hepatic duct [1, 25]. Although cysts can be associated with any type of BA, they are placed upstream of an obstruction situated at the bile duct (Type I) or in a distal position to the porta hepatis obstruction (Type III). In CBA intrahepatic ducts are abnormal, irregular and hypoplastic with atretic gallbladder. In the postnatal period, the cystic structure neither increases nor decreases in size, as it is not connected to the biliary system [11].

Even if CBA can be detected in maternal uterus, the early diagnostic differentiation between CBA and choledochal cyst remains a hard task [26, 27]. Actually, there is no preoperative imaging diagnosis that can convincingly differentiate CBA from a congenital choledochal cyst disease, and the definitive diagnosis is only established at the time of surgery by surgical dissection and intraoperative cholangiography [25].

Some prenatal ultrasound characteristics have been proposed as differentiating between choledochal cyst and CBA, such as: small anechoic cysts in the hepatic hilum

would suggest BA, large echoic cysts, increasing in size, would suggest obstructed choledochal cyst; small and stable echoic cysts would point to an unobstructed choledochal cyst [28]. However, the small number of cases studied limits definitive assertions. Biliary cystic malformations, encompassing choledochal cyst and CBA, were considered an entity. In the presence of a choledochal cyst, however, the intrahepatic ducts are dilated, communicating widely with the cyst, and the gallbladder is distended [29]. In CBA, the intrahepatic ducts are abnormal, irregular and hypoplastic, and the gallbladder is atretic.

Irrespective to the differential diagnosis, CBA and choledochal cyst with complete luminal obstruction indicate the urgent need to remove the impediment to bile flow, given the risk of cirrhosis [30], which develops quickly in both disorders [31] .

1.2.2. Cytomegalovirus Biliary Atresia (CMV-associated BA)

Epidemiology studies have shown that the cause of BA can be environmental, and virus infections and toxins have also been studied as potential agents causing BA [6]. Several viruses, such as hepatitis B, reovirus and papillomavirus have been associated with BA, but no viruses are found in the liver or in the porta hepatis. Regarding the cytomegalovirus (CMV), a molecular signature suggestive of a specific response to the virus was observed in the livers of patients with BA [32]. CMV infection is a common perinatal infection that affects about 2% of infants at birth [33] and an association between CMV infection and neonatal cholestasis has been reported [34, 35] which is referred as CMV-BA and is characterized by immunological alterations [36]. However, CMV mechanisms that may lead to BA remain unproven [34], although CMV DNA has been demonstrated in 60% of liver biopsies from children with BA in China [6, 35].

Zani proposed that cholangiopathy can be explained by two suggested mechanisms: a primary disorder of bile duct development or viral exposure [37]. A study from Xu et al (2011) has demonstrated in human intrahepatic biliary epithelial cells that when there was CMV infection, the expression of cytokine IFN-γ was markedly downmodulated and cytokine IL-4 was significantly up-regulated, indicating that CMV infection can lead to a disequilibrium of cytokines' expression which is likely to suppress the functions of cellular immunity in the host [38] . Interestingly, this finding is in agreement with a study by Davenport et al (2016) who found a histological appearance in the liver of CMV-associated BA patients with an obvious mononuclear cell infiltrate consisting mainly of CD4+Th1+ T cells [1], i.e. the presence of altered immunologic features in CMV-BA patients seems to be related.

Previously, CMV-BA was thought to have the worst response to the Kasai procedure and to be the most susceptible to early death due to its immune-destructive pathogenesis following the viral process [39]. This may be partly because the CMV-BA variant is detected later than the others. However, more recent studies suggest that this view may not be entirely accurate. Current research indicates that the impact of CMV on BA outcomes is still being investigated, with some findings suggesting that prognosis may depend on multiple factors beyond the mere presence of the viral infection [40, 41].

An interesting study by Brindley et al (2013) has found that CMV infection was significantly associated with decreases in regulatory T cells in peripheral blood from BA patients which is important once deficits in the quantitative distribution of these cells could result in an inflammatory response leading to bile duct injury and autoimmunity. They also observed an immune reaction in liver tissue mounted against CMV in 38% of patients with BA [32].

1.2.3. Biliary Atresia Splenic Malformation Syndrome (BASM)

The presence of associated extrahepatic anomalies in a subset of BA patients led to the classification of the disease in perinatal (or isolated) BA and embryonic (or fetal) forms of the disease. Embryonic form occurs less frequently, the clinical evidence of cholestasis is early and there is absence of biliary remnants in the porta hepatis.

About 10% of BA cases have laterality sequence abnormalities and comprise the BASM type [42]. BASM syndrome is characterized by one or more congenital malformations within a wide spectrum of laterality defects along with abnormal biliary tract development thus suggesting a key role of genetic contributions to the etiology of BASM syndrome [42-44]. Infants with BASM may have intestinal malrotation, absent or interrupted inferior vena cava and preduodenal portal vein in addition to cardiovascular and gastrointestinal anomalies such as intestinal malrotation [45]. A study from Schwarz et al (2013) in which BA patients were classified in 3 subsets considering any anomalies in cardiovascular, pulmonary, gastrointestinal, genitourinary and splenic systems reported that 70% of the patients with laterality defects had splenic anomalies, suggesting the name alteration from BASM to “biliary atresia laterality sequence” [46].

A study from Berauer et al (2019) evaluating a large cohort of BASM patients through next-generation sequencing observed that Polycystin 1 like 1 (PKD1L1) gene may be

related to the occurrence of BASM [43]. This gene is involved in primary ciliary calcium signaling and thus its involvement with BASM may open a new field of investigation in the etiology of BA because the concept that the disordered function structure of cholangiocytes lead to the development of BASM seems plausible [43].

1.2.4. Isolated Biliary Atresia (IBA)

The isolated or perinatal form of BA is the largest clinical group, covering about 70% to 80% of all reported cases. In this group there are no distinctive clinical features, no congenital anomalies and, although laboratory evidence of cholestasis presents already at birth, the full disease picture is clearly evidenced in the second week of life. The etiology of IBA is not completely defined. It is not clear whether the atretic process in extrahepatic bile ducts results from an early primary teratogenic event, or if it is caused by a disruptive process occurring later in normally developed fetal structures [42]. Some gene polymorphisms show association with the development of BA, such as Adducin 3 (ADD3), ARF GTPase 6 (ARF6), Glypican-1 (GPC1), Vascular growth factor A (VEGFA) and Platelet-derived growth factors (PDGFA) [47, 48], but the role of these and other susceptibility genes probably follows a non-Mendelian inheritance.

There is strong evidence of the involvement of immune processes in the development of BA, mainly located in portal tracts. There is an inflammatory reaction in the liver of patients with IBA, located at the portal tract, with a significant increase in the expression of major histocompatibility complex (MHC) Class II antigens and the adhesion molecules intercellular (ICAM) and vascular (VCAM) in vascular and biliary epithelium [42]. There is also evidence of proliferating activated mononuclear cells, including Kupffer and mast cells [42].

In these immune responses, cholangiocytes are major players once they are presently considered the first line of defense in biliary tree against external substances and participate through various immunological pathways. There are already evidences of innate immune responses against viruses in intra- and extra-hepatic bile duct cholangiocytes that may lead to cholestasis and biliary obstruction additionally to increased cholangiocyte apoptosis suggested as a possible mechanism of BA development [49]. This also suggests the direct participation of cholangiocytes in the antimicrobial immune response with secondary apoptosis of the involved biliary cells.

1.2.4.1. Hypoxic-Ischemia Biliary Atresia: a new variant in the isolated group?

With the increase of studies in BA field, new developmental factors and variants for BA have been proposed. Our group observed that in BA there is a progressive thickening of the arterial medial layer, suggestive of vascular remodeling associated with the disappearance of interlobular bile ducts [13]. We also suggested that a process of hypoxia/ischemia can affect the portal structures in BA at portoenterostomy beginning at the porta hepatis and is associated both with the extent of biliary proliferation and medial layer thickening [50]. In normal situations, in the presence of a hypoxic stimulus, the hypoxia inducible factor 1 (HIF1alpha) induces the production of VEGFA. In the case of BA, it was observed that the increase in HIF1alpha gene expression was associated with decreased expression of VEGFA and vascular endothelial growth factor receptor 2 (VEGFR2). This is interesting, since when the initial stimulus is ischemia, HIF1alpha blocks the production of VEGF, which is what seems to happen in BA [51]. The HIF1alpha molecule is an identifier of hypoxia/ischemia and can be found positive in biliary cells of BA patients suggesting that an abnormality involving the peribiliary vascular plexus (PVP) can induce a hypoxic/ischemia on bile ducts, thus confirming that BA progression may involve ischemic cholangiopathy.

In addition to the medial thickening described by our group [13], other findings support the hypothesis of a hypoxic/ischemic clinical form of BA. These include immunohistochemical features of increased VEGF expression, suggestive of arterial/arteriolar and cholangiocyte hypoxia [50], overexpression of angiopoietins involved in pericyte recruitment to the arterial wall [52], and a gene expression profile consistent with liver hypoxia-ischemia [51]. These findings support our hypothesis that a hypoxic-ischemic mechanism may underlie a specific clinical form of BA. Supporting this idea, a group from Germany using a murine model of BA with Rhesus Rotavirus (RVR) infection observed a disturbance in the peribiliary vascular plexus (PVP) preceding luminal obstruction [53]. Thus, the hypothesis is that an injurious factor, such as virus, immune disorder, toxin or metabolites, affects the vascular endothelium of the PVP, leading to endothelial dysfunction and consequently inducing ischemic cholangiopathy (Figure 1.4). Recently, an innovative work from Min et al (2020) proposed a BA network that can be used for study both individual interactions among specific genes and the major cores of genes involved in fibrosis, immunity, inflammation, hypoxia, and BA development [54]. In this study, one of the pathways highlighted in the integrative network was hypoxia signaling. Perturbation with hypoxia inducible factor activator induced the biliary defects of BA in a zebrafish

model, which can be indicative of the involvement of hypoxia pathway in BA etiology [54]. Additionally, recent studies have explored genes implicated in ciliogenesis, uncovering potential links between defects in primary cilia function and the pathogenesis of BA, further expanding the understanding of its molecular mechanisms [55-62].

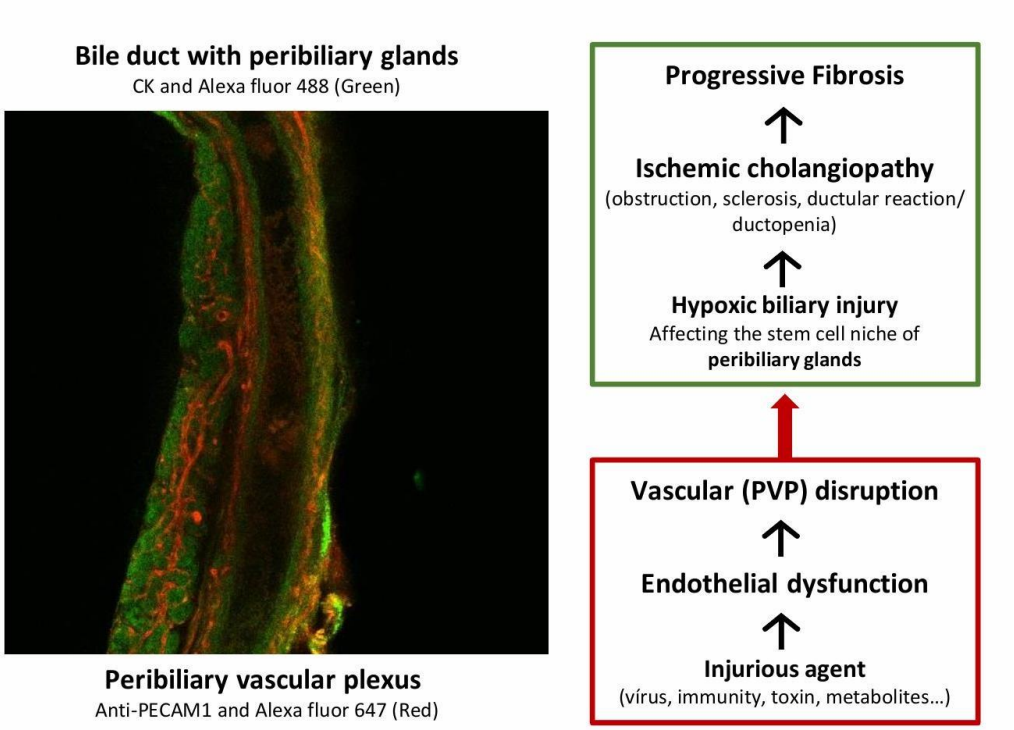

Figure 1.4. Putative role of ischemic cholangiopathy in the development of the hypoxic-ischemic clinical phenotype of BA. Left side figure shows the common bile duct of a normal neonatal rat stained both with anti-CK and anti-PECAM-1 evaluated by immunofluorescence. (Green channel) observe the bile duct lumen with the peribiliary glands; (Red channel) peribiliary vascular plexus (PVP), formed by small branches of the hepatic artery, which is the only source of irrigation with blood to the bile ducts in humans. Right side figure presents a scheme proposing that injurious agents, leading to endothelial dysfunction, can disrupt the PVP, decrease bile duct irrigation and induce ischemic cholangiopathy. The activation of molecular pathways related to hypoxia induces fibrogenesis and can aggravate the progression of fibrosis in these cases.

1.3. Biliary Atresia Pathogenesis

The pathogenesis of BA remains unclear and may involve a complex of stereotyped hepatobiliary tissue responses to different factors, and the host's immunogenetic constitution appears to act as a modulator of hepatobiliary damage. The disorder that leads to BA development may occur in the embryonic period, resulting from genetic or chromosomal alterations or a disruptive process in which the insult affects previously normal hepatobiliary structures after the organogenesis is complete.

The mechanisms proposed to explain the etiology of BA includes genetic variants, toxins [8], biliary viral infection, chronic inflammatory lesions or autoimmunity-mediated bile duct lesions, abnormalities in bile duct development [63], defects in embryogenesis, abnormal fetal or prenatal circulation and susceptibility factors (abnormal morphogenesis, environmental factors, among others) [64] (Table 1.2).

Table 1.2. Specification of the mechanisms putatively involved with BA pathogenesis.

GENETIC CHROMOSOMAL FACTORS
Numeric/structural chromosomal disorders • Trisomy 10q, 17, 18, 21, 22 • Deletion of chromosomal region portions- 18p, 2q73.3, 18q21, 17q12, 1p36, and 20p11.21 **Susceptibility to BA gene polymorphisms – non-Mendelian heritance:** • ADD3, ARF6, GPC1, VEGFA, PDGFA **Dysbiogenesis of the Cholangiocyte primary cilia (specifically in laterality determination defect)** • PKD1L1, Cripto, Frigida like 1 (FRL-1), Cryptic Family 1 (CFC1); Forkhead box protein P2 (FOX2), Nodal growth differentiation factor (NODAL), Zic family member 3 (ZIC3), Left-right determination factor 1 (LEFTY1), Left-right determination factor 2 (LEFTY 2), Actin filament associated protein 1 (AFAP1), Tumor suppressor candidate 3 (TUSC3)
EPIGENETIC FACTORS
Clinical evidence • Association of Biliary atresia splenic malformation (BASM) with diabetes **Molecular evidence** • **microRNAs (miRNAs) in BA:** miRNA-21; miRNA-29c (associated with fibrogenesis and affecting DNMT3B and DNMT3A); miRNA-19b; miRNA-29a/b, miRNA-30b/c, miRNA-195, miRNA-200, miRNA-320, miRNA-365, miRNA-140-3p, miRNA-200/429 and miRNA-222; miRNA-155 upregulation related to the immune response triggered via Interferon-gamma (IFN-γ). • **DNA methylation in BA:** Autotaxin (ATX) promoter methylation (associated with progression of fibrogenesis); CpG methylation in the Foxp3 promoter in Treg cells (associated with the biliary lesion); hypermethylation of promoter regulatory elements of T CD4+ cells and secondary decreased CD11a expression; methylation of repetitive elements Alu and LINE-1 in peripheral blood leukocytes; Platelet-derived growth factor subunit A (PDGFA) methylation (associated to biliary defects and fibrosis extent)

AUTOIMMUNE FACTORS
Clinical, histopathological, molecular evidence • Increased incidence of autoimmunity in relatives • Lymphocytic cell infiltrate in biliary epithelium (intra- and extrahepatic) • Oligoclonal expansion of T lymphocytes • Aberrant HLA II expression in biliary epithelium **Experimental evidence** • Th17 (IL-17) response both in human and in animal model
ENVIRONMENTAL FACTORS
Virus **Consistent laboratory evidence found for cytomegalovirus (CMV):** • Serum CMV-IgM-positivity in a subset of BA patients • IFN-γ secretion in the T cells response mounted against CMV peptides in 56% of BA patients, correlated with serum CMV-IgM positivity.
VASCULAR STRUCTURAL FACTORS
Histopathological evidence: • Progressive arterial and arteriolar medial layer thickening in the liver and porta hepatis of BA patients • Arteriolar vascular agglomerates in portal tracts, fibrous septa and subcapsular areas in BA patients **Ultrasound evidence:** • Subcapsular hypervascularization • Hepatic artery dilatation in the liver hilum **Arteriographic evidence:** • Peripheral occlusion of hepatic artery branches associated with formation of arteriolar tufts at the obstructed sites **Laparoscopic evidence:** • Presence of subcapsular spider telangiectasias in the liver of BA patients **Molecular evidence:** • Gene expression suggestive of hypoxia-ischemia profile in the liver of a subset of BA patients • Integration of High-Throughput Biological Data in BA patients: central role of Hypoxia signaling (perturbation with the HIF activator)

Although the etiology of BA is not fully understood, several evidence support that a perinatal viral infection can trigger a local autoimmune mediated acute episode by molecular mimicry on bile duct epithelia, where cellular and humoral immunity play important roles in the injury mechanism [35]. The potential contribution of

inflammatory and immune responses in the development of BA also leads to etiological hypotheses related to maternal risk factors (age, race-ethnicity, infection, seasonality and nutrient intake). A study by Carmichael et al. (2018) sought to find a relationship between nutrients that affect the pathways of inflammatory and immune responses (antioxidants, B-complex vitamins and minerals) with the development of the disease. The study, although limited by a small number of cases, reported a relationship between maternal intake of some nutrients with BA, suggesting that higher glycemic index was associated with increased risk [65]. Research and clinical management in biliary atresia has grown and many are the studies that have allowed a better understanding of its etiology in the field of immunity, genetics/epigenetics, toxic and developmental factors (Table 1.3).

Table 1.3. List of data sources that can be used to enhance Biliary Atresia basic research.

Biliary Atresia Data Repository
1. Genome wide association study (GWAS) public data set
2. Gene Expression Omnibus (GEO) public expression
3. Methylation data set
4. PHIS – Pediatric health information system
5. GeneMania network analysis
6. ChiLDREN - Childhood Liver Disease Research and Education Network (BASIC)
7. NIDKK central repository
8. Biliary Atresia Research Consortium (BARC)
9. OrphaNet
10. MedGen

1.3.1. Genetic Factors

There are evidence of the role of genetic factors in the development of BA. Several studies have unveiled putative genes associated with the pathogenesis of BA, since the existence of an embryonic form brings up a possible link between the disease and a genetic disorder. Regarding genetic studies and clinical analysis, there are already

several data sources that can be used to enhance biliary atresia basic research (Table 1.4).

Several investigators studied the relationship between BA and some genes associated with the development of extrahepatic biliary tree, because BA begins to affect the extrahepatic ducts first. Genes such as Pdx1, Hes1, Sox17 [66, 67], Invs, OneCut1, Hnf1b, Foxa1 and Lgr4 [68] have been investigated as possible modulating agents involved in the development of the biliary tree. Studies have reported that extrahepatic bile ducts originate from pancreatic embryonic tissue under pancreatic signaling, that is distinct from the molecular signaling involved in hepatic embryogenesis [69]. Sox17 is a protein involved in the formation of endodermal organs and controls the specification of the liver and bile ducts. This protein is necessary to induce a ductal fate in endodermal cells, with the separation in biliary or pancreatic lines being regulated by Hes1, a protein belonging to the Notch signaling pathway that can act in a feedback loop with Sox17 [70]. Additionally, Sox17 has been implicated in vascular development, particularly in the formation of the arterial system during embryonic angiogenesis. Notch signaling, which interacts with Sox17, plays a crucial role in regulating vascular morphogenesis and blood vessel maturation. This vascular development is essential for ensuring proper blood supply to the developing liver and biliary ducts, highlighting the interconnectedness between the biliary system and the vascular network [70]. Another study by Zhang et al (2018) demonstrated that the Hes1 gene is downregulated in patients with BA and may be causal to the cholangiopathy, since Hes1 contributes to the cellular maturation and duct-like structure formation [71]. Laochareonsuk et al (2018) and Zeng et al (2014) investigated in Thai population, the role of the Adducin 3 (ADD3) gene that is expressed in hepatocytes and biliary epithelia, and involved in the assembly of spectrin–actin membrane protein networks at sites of cell-to-cell contact [72, 73]). Mutations in this gene may result in excessive actin and myosin deposition, contributing to biliary fibrosis [8, 74-76]. The association between BA and single nucleotide polymorphisms in the ADD3 gene was demonstrated, suggesting a role of these variants in BA etiology. A study by Ye et al (2017) investigated the role of ADD3 overexpression regulated by micro-RNA-145 and concluded that it may be related with liver fibrosis in BA [77] .

Studies on cholangiocytes have also been done. Sira et al (2015) showed an aberrant immunostaining expression of P-selectin (CD62P) in the liver of patients with BA [78]. They found a significant expression of this molecule in the endothelium, platelets and biliary epithelial cells, suggesting an important role of this adhesion molecule in BA pathogenesis [78]. The authors suggest that platelets may play not only a central role in

the recruitment of inflammatory cells, but also in the secretion of fibrogenic mediators [78] . An interaction between NOTCH 2, a receptor from Notch pathway that plays a key role in the development of the biliary system, and CD14, a marker expressed on the surface of macrophage, neutrophils, and other myeloid-lineage cells was found in BA perhaps explaining the immaturity and malfunction of biliary epithelial cells in this disease [79].

Attributable to the remaining doubts regarding the pathogenesis of BA, the variety of genes proposed as causative factors of disease development is large. Genes involved in migration and adhesion of biliary epithelial cells such as GPC1, CFC1, ICAM1 and MIF [47, 80-82], in the fibrosis processes characteristic of cholestasis such as VCAM1, PLDL1 [83] and FGF21 [84], in biliary function such as CFTR, JAG1, A1AT [85] and ARF6 [86] and in immune responses such as VEGFA, EFEMP1 [47, 87, 88] and PDGFA [48] have been studied. However, the contribution of genetic factors to BA remains uncertain. It is becoming increasingly evident that genetic predisposition coupled with the environmental factors to which the individual is exposed are potential triggers of BA, thus explaining its distinct prevalence in the world population (higher prevalence in Asian countries). The clearest role of this genetic predisposition is the presence of ciliopathy gene mutations in the BASM form.

Table 1.4. Important research and clinical management that enriched the understanding of BA etiology.

Immune Factors	
Czech-Schmidt G, *et al.* J Surg Res 2001 [169]	Immune gap for infection
Carvalho E, *et al.* Gastroenterology 2005 [170]	TH1 response transcriptome
Mack C, *et al.* Clin Immunol 2005 [171]	Role of macrophages and $TCD4^+$ lymphocytes
Mack C, *et al.* Hepatology 2006	Cellular and Humoral immunity in BA
Leonhardt J, *et al.* Pediatr Surg Intern 2006 [172]	Additional participation of Th2 signals
Barnes B, *et al.* Liver Int 2009 [173]	Cholangiocytes as immunomodulators
Jafri M, *et al.* Am J Physiol Gastrointest Liver Physiol 2008 [174]	Role of integrins in cholangiocytes
Miethke A, *et al.* J Hepatol 2010 [175]	Decreased Tregs associated with disorders in NK activation
Lu B, *et al.* Gastroenterology 2010 [176]	Enolase as antigen of the humoral response
Saxena V, *et al.* Science Translational Medicine 2011 [177]	Dendritic cell role in BA
Li J, *et al.* J Clin Invest 2011 [125]	Blockage of the Th1 pathway leads to the occurrence of BA via the Th2 pathway
Lages Cs, *et al.* Hepatology 2012 [178]	Tregs modulate $CD8^+$ expansion induced by dendritic cells
Hill R, *et al.* J Pediatr Surg 2015 [118]	$CD4^+$ inflammatory infiltrates of Th1 and Th17 cells in BA livers
Shimada T, *et al.* Biomed Res 2017 [121]	CCL5 chemokine involved in immune response of TLR3 pathway

Wilasco M, *et al.* J Pediatr 2017 [1 1 4]	IL-10 associated with cirrhosis secondary to BA
Liang J, *et al.* Gene 2018 [113]	IL-18 identified as a disease susceptibility gene
Genetic/Epigenetic Factors	
Uemura M, *et al.* Development 2013 [179]	Sox 17 haploinsufficiency leads to BA (with associated subcapsular degeneration and vascular disorder)
Udomsinprasert, W, *et al.* Sci Rep 2016 [106]	Methylation of repetitive elements Alu and LINE-1 in BA
Cofer Z, *et al.* PLoS One 2016 [102]	PDGFA methylation mediates biliary defects and excessive fibrosis
Ye Y, *et al.* PLoS One 2017 [77]	*ADD3* overexpression regulated by microRNA-145 in liver fibrosis
Zhao D, *et al.* Dig Dis Sci 2017 [93]	Role of *miRNA-19b* in fibrogenesis
Zhao R, *et al.* Pediatr Res 2017 [101]	Contribution of *miRNA-155* to immune response in BA
Zhang R, *et al.* World J Gastroenterol 2018 [71]	*Hes1* gene is downregulated in BA and may be causal to the cholangiopathy
Lin Z, *et al.* Mol Ther Nucleic Acids 2018 [79]	Role of Notch 2 and CD14 in BA
Toxic Factors	
Lorent, at al. Sci Transl Med 2015 [151]	Induction of BA by injection of Biliatresone in ZF larvae
Waisbourd-Zinman O. Hepatology 2016 [180]	Biliatresone causes biliary damage by decreased Sox17 and GSH expression
Zhao X, et al. Hepatology 2016 [152]	Biliatresone leads to disturbed GSH homeostasis
Fried S, *et al.* Hepatology 2020 [181]	Biliatresone disruptive effect in cholangiocytes involves decreased GSH andaffects Wnt and Notch Signaling Pathways
Malik, A. Hepatology 2019 [148]	Polyurethane Chemicals (MDA and MDI) as new etiopathogenic agents in BA
Developmental Factors	
Amarachintha SP, *et al.* Hepatology 2021 [168]	Study of organoids using cholangiocytes of BA patients shows delayed epithelial development and barrier function in BA.

1.3.1.1. Epigenetic factors

The role of epigenetics in various pathologies has been increasingly investigated as it appears to have promising results, and the field of BA research is no exception. There have been many studies in this field aiming to understand the role of epigenetic processes such as DNA methylation, Micro-RNAs (miRNAs) and mRNAs circulation in the pathogenesis of BA.

The micro-RNAs are short, noncoding RNAs that regulate mRNA target genes and their cleavage through transcript degradation or translational repression [67, 89, 90]. Although miRNAs are known to be involved in various disorders in the gastrointestinal tract, information on their function in liver development remains poorly understood. Several studies have attempted to understand the role of these miRNAs in the characteristic fibrogenic process in the liver of BA patients and some have interesting results that may begin to open new lines of research in this disease. Makhmudi et al (2019) investigated the role of miRNA-21 in the liver fibrogenesis and found an

association of its expression with liver cirrhosis in BA patients [91]. Wang et al (2019) found that miRNA-29c levels are decreased in peripheral blood of BA patients and there is an association of fibrogenesis and the effects of this miRNA over two targets, DNMT3B and DNMT3A [92]. Zhao et al (2017) concluded that miRNA-19b is significantly less expressed in BA patients which can also be related to the fibrogenesis [93] . In addition, other miRNAs such as miRNA-29a/b [94], miRNA-30b/c, miRNA-195, miRNA-200, miRNA-320, miRNA-365 [95], miRNA-140-3p [96], miRNA-200/429 [89, 97] and miRNA-222 [98, 99] have also been investigated as potential contributors to the fibrogenic process of BA. The role of miRNAs in BA has also been investigated in immune processes [100]. Zhao et al (2017) reveal a contribution from miRNA-155 upregulation to the immune response triggered via IFN-γ in BA [101].

The state of DNA methylation has also been a focus of epigenetic research in BA etiopathogenesis, since changes in methylation status can be triggered by various agents such as viruses and other environmental factors [102]. Udomsinprasert et al (2017) demonstrated the possibility of using ATX promoter methylation status and its expression in peripheral blood as a biomarker that reflects the progression of postoperative hepatic fibrosis in BA [103]. Regulation of CpG methylation in the Foxp3 promoter in Treg cells also appears to play an important role in the development of the biliary lesion, which is prevented by decreased methylation [104]. Dong et al (2012) showed that hypermethylation of promoter regulatory elements of T CD4+ cells in infants with BA leads to decreased CD11a expression which may contribute to BA pathogenesis given that this CD11a deficiency leads to increased inflammatory response and increased susceptibility to virus infections [105]. Udomsinprasert et al (2016) investigated the effectiveness of methylation of repetitive elements Alu and LINE-1 in peripheral blood leukocytes as a cause of global methylation in BA patients after portoenterostomy and found that these elements are hypomethylated and this fact is associated with increased risk of BA compared to healthy controls. Additionally, LINE-1 was found to be associated with worst outcomes in BA patients [106]. The role of DNA hypomethylation in the pathogenesis of BA has also been investigated by Cofer et al (2016) who revealed the importance of PDGFA methylation in mediating biliary defects and excessive fibrosis [102]. PDGF receptor has been implicated in biliary and vascular development as well as in liver fibrogenesis and, since it is found in primary cilia which also appear to play an important role in BA and various types of congenital liver fibrosis, the study of this pathway may be of great importance [102]. Another line of investigation performed by Matthews et al (2011) observed that inhibition of DNA methylation in zebrafish larvae induced intrahepatic biliary defects and activation of

the IFNγ pathway genes. In the intrahepatic biliary epithelium of BA patients, DNA methylation was reduced as compared to patients with other neonatal cholestatic diseases [107]. Consequently, methylation analyses can provide an invaluable contribution to the investigation of specific epigenetic disturbances involved in the pathogenesis of biliary atresia. For instance, Low-pass genome-wide analysis of blood samples collected from patients with biliary atresia, compared with other cholangiopathies, could be used for epigenome-wide association studies to provide new insights into specific early life mechanisms of epigenomic dysregulation involved in biliary atresia [108, 109]. On the other hand, the methylation haplotypes observed in cell-free circulating DNA may be used to evaluate disease severity and identify distinct clinical subsets of BA [108, 110]. The study of epigenetic-mediated processes can unveil the underlying molecular network of BA, thus providing promising drug targets and subtypes identification helping the development of personalized treatment and precision medicine [111].

1.3.2. Immune Responses and Autoimmunity

Many investigations conducted in the field of BA focus on the immune system contribution to bile duct injury which plays a central role in the disease etiology (Figure 1.5). The innate immune system responds to any insult or infection by rapidly producing nonspecific inflammatory responses with the release of proinflammatory cytokines such as IL-1, IL-6 and Tumour necrosis factor alpha (TNF-α) [112].

The role of some inflammatory cytokines in BA pathogeny have been investigated. IL-18 has been associated with the production of IFN-γ which activates the inflammatory response of T and NK cells that are important cells in BA. In affected patients, IL-18 gene has been identified as a disease susceptibility gene [113]. Interleukin 10 (IL-10) has been associated with cirrhosis secondary to BA and has been identified as a possible early-stage biomarker of nutritional status deterioration in cirrhotic patients [114]. Increased expression of IL-2, IL-8 [115, 116] and IL-17 (Klemann et al., 2016) in patients with BA was related to increased liver fibrosis status and destruction of the biliary system. Interleukins involved in inflammasome-mediated epithelial injury were also investigated by Yang et al (2018) who suggested that interruption of the inflammasome could suppress the neonatal proinflammatory response, preventing experimental BA since the inflammasome genes are overexpressed in the BA models used. They concluded that targeted IL1R1 loss protected neonatal rats from bile duct obstruction in a rotavirus rhesus-induced model [35].

Regarding to immune mechanisms of biliary injury in BA the main studied mechanisms focus in Th1 and Th17 cell-mediated pathways. Th-17 (responsible for IL-17 secretion) and Th1 cells play a destructive role in autoimmune biliary diseases such as primary biliary cirrhosis and autoimmune hepatitis. A study by Davenport et al (2015) investigated the nature of CD4+ inflammatory infiltrate in BA livers and concluded that in different types of BA there are similar hepatic inflammatory infiltrates consisting of two subsets of Th1 and Th17 cells. They also observed that the inflammatory tissue composition in CMV-BA patients was different, consisting mainly of TH1 cells [118]. A study by Liu et al (2015) in a murine BA model provided evidence that overexpression of Th17 cells caused by excess IL-6 levels and suppression of Treg cells plays an important role in the pathogenesis of BA [119]. This can be the basis of a mechanism that if inhibited can lead to the prevention of autoimmune hepatobiliary lesion. Lages et al (2018), using the RVR murine model, proposed that dendritic cells produce IL-6 and promote differentiation of naive CD4+ cells into Th17 lymphocytes [120] leading to the production of chemokines by cholangiocytes that attract inflammatory macrophages which promote the destruction of bile duct epithelial cells. CCL5 chemokine is also involved in the pathogenesis of BA and it has been proven to be expressed in biliary epithelial cells. In the livers of BA patients and apparently being involved in the immune response of the Toll-like receptor 3 (TLR3) signaling pathway [121].

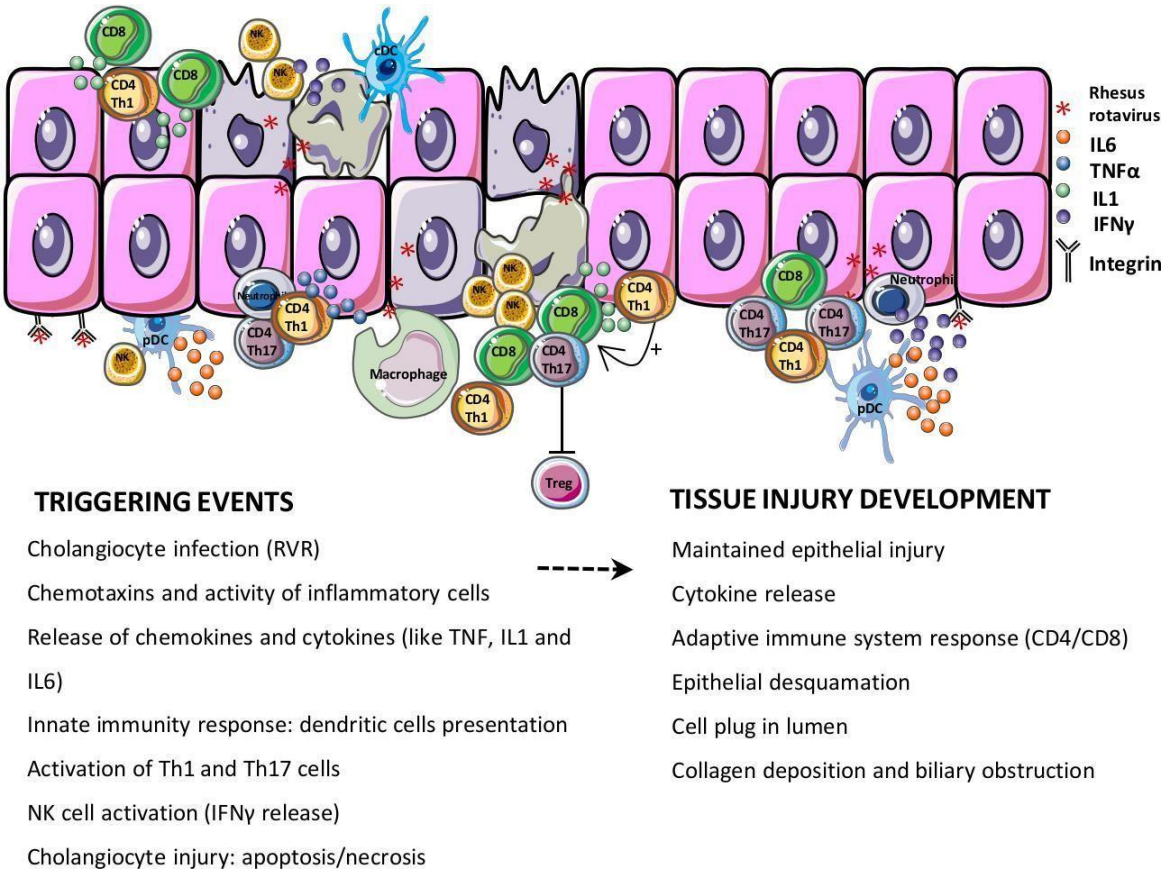

Figure 1.5. Epithelial damaging process involved in the Rota Virus Rhesus (RVR)-induced murine model of biliary atresia. Triggering events as cholangiocytes infection by RVR, chemotaxins and activity of inflammatory cells and natural killer (NK) cell activation lead to epithelial injury development with collagen deposition and biliary obstruction with an adaptive immune system response. Figure adapted from reference [95].

Biliary epithelial cells present innate immunity response associated with at least Toll-like receptors (TLR) from 1 to 6 and these receptors and adapter molecules are distributed throughout the cells lining of the biliary tree [122]. TLR 8, 3 and 7 present increased liver expression at the time of diagnosis of BA patients requiring transplantation, being related with bad outcomes [112]. Humoral immune responses are B cell-mediated responses, which begin by interacting the antigen with B-cell receptors and directly contacting cells with CD4+ T cells and TLR. B cells, in addition to those responsible for antibody production, also play a crucial role as antigen presenting cells for T cell activation. The contribution of these cells to the pathogenesis of BA is poorly studied, but recent facts suggest important roles in the development of the BA. Bednarek et al (2018) showed that neonatal B cells can contribute to an inflammatory disease like BA, through a cytokine-mediated mechanism, including upregulation of TLR4, 7 and 9 [123]. Feldman et al (2013) explored the role of B cells in bile duct injury and obstruction using the BA murine model with mb-1/CD79 knockout gene (Iga-/-) as the animals have loss of expression and function of B-cell receptors and are thus unable to present antigens or produce immunoglobulins. They concluded that these mice are

protected from biliary obstruction indicating a primary role of B cells in mediating disease pathology and the mechanism may involve lack of antigen presentation that will inhibit T cell activation and Th1 inflammation [124]. A study by Li et al (2011) using the murine model also proved that blocking the Th1 pathway leads to the occurrence of BA via the Th2 pathway with the participation of macrophages and B lymphocytes [125].

Treg cells also appear to play an important role in BA and are responsible for controlling T-cell-mediated immune responses and preventing activation of autoreactive T cells [8, 64]. In order to clarify the mediated changes that occur in the liver it is important to investigate the role of these cells in the hepatic hilar lymph nodes [64]. Sakamoto et al (2017) suggested the occurrence of a localized Treg cell-mediated immune reaction in the hepatic hilar lymph nodes and which is not detectable in peripheral blood [126]. However, a later study by Bove et al (2018) dismissed this hypothesis concluding that the absence of antigenic stimulation in lymph nodes parallel to the existence of biliary remnant supports the presence of another major alternative to infection in the etiology of BA [127].

NK cells are also important in BA pathogenesis as they have already been found in BA livers and induce cholangiocyte damage and rupture of the extrahepatic duct epithelium. They have been implicated as playing a crucial role in the development of inflammatory and fibrous liver disease and it has been shown that decreasing the activity of these cells at the onset of the biliary obstructive process can improve cholestasis, decrease tissue inflammation, prevent fibrosis development and improve survival [128]. However, it is important to refer that the accumulation of bile salts acids characteristic of cholestasis leads to immune changes including macrophages and inflammatory pathways [129], which must be taken into account when analyzing the results.

1.3.3. Structural Anomalies

Developmental and vascular factors have been another focus in the study of BA and within these, the structural anomalies observed in infants with this disease are key points in the understanding of BA etiology. A process proposed to contribute to the pathogenesis of BA involves abnormalities in the hepatic arteries and ductal plate malformation (DPM).

Anomalies of the vasculature can occur in 25% of cases of BA, related to the association of this condition with heterotaxy and left isomerism. They may include absence of the hepatic segment of the inferior vena cava with continuation of the azygos, pre-duodenal

portal vein, abdominal situs inversus with abnormal arborization of the hepatic arteries and portal veins, separate drainage of the hepatic veins for the right atrium, common arterial trunk of celiac and superior mesenteric arteries and anomalous anatomy of the superior vena cava. Angiography usually demonstrates abnormal vascularization in the porta hepatis region [130].

Regarding arteriopathies and their role in the development of BA, there are several histological as well as arteriographic and ultrasonographic evidence that show some alterations in the medial thickening (MT) of the branches of the hepatic artery. To better understand this disease aspect, knowledge of the behavior of angiogenic molecules becomes essential. Overexpression of angiopoietins (1 and 2) in the liver of BA patients have been studied, relating it to MT and concluding that it occurs regardless of the age at portoenterostomy and the variables associated with the severity of the disease (such as extent of fibrosis and ductular reaction) [52]. Masuya et al (2019) tried to analyze the behavior of two major vascular changes that occur in BA, the narrowing of the portal veins with an increase in the number of capillaries and endothelial proliferation and medial hypertrophy. They confirmed, in a study group of 25 patients that these two changes occur in the development of BA and considered them as essential vascular lesions in the pathology that may not occur secondary to liver fibrosis [131]. A study by Hong et al (2018) tried to relate the complications in the hepatic arteries that occur in liver transplants among patients with metabolic diseases and BA concluding that they are similar and should be approached with equal caution [132]. Microscopic studies have also brought news in the diagnosis of BA. Laparoscopic finding revealed the existence of small and dilated arteries in the hepatic subcapsular area in BA patients [133]. These vessels are branches of the hepatic arteries and, until now, this has not been mentioned in any other liver disease. In this study, hyperplasia or arterial hypertrophy was also documented in liver samples and the enlargement of the hepatic artery in BA patients [133]. Several imaging studies describe the dilation of the hepatic artery in the vicinity of the porta hepatis and the presence of subcapsular telangiectasia as indicative of BA [15-20, 134].

The presence of DPM in BA patients, or in a subset of affected patients with worse prognosis, is still a controversial matter that deserves additional investigation. The presence of DPM in histological studies represents a delay in the normal remodeling of the fetal biliary tract which results in an excess of embryonic structures of the bile duct. A study by Vuković et al. (2012) in a group of 28 children with uncorrectable BA—defined in the study as cases with advanced BA in which no effective biliary drainage could be achieved after portoenterostomy—assessed the prognostic impact of ductal

plate malformation (DPM) in long-term clinical outcomes [135]. These authors concluded that the presence of biliary structures with features similar to DPM can be considered as a subgroup of fetal BA and a marker of poor outcome, however DPM neither was associated with the early manifestation of clinical signs of BA nor with a worse native liver survival [135]. Desmet (1998) was the first pathologist to observe in BA liver samples biliary structures with features of DPM, and suggested that in the "precocious severe form" of BA, as he named, such a biliary malformative process was present [136]. Subsequently, Desmet informed that what is called DPM in BA is just a type of very intense ductular reaction [137] which aims at reconstituting bile flow and induce regeneration of the biliary tree. In this case, the greater the severity of cholestasis, the greater the production of structures similar to DPM. Ductal plate malformations are known to occur in autosomal recessive polycystic kidney disease (ARPKD), which is a ciliopathy. The study by Berauer et al (2019) in a large cohort of BASM patients showed that PKD1L1 variants associated with ciliopathy, and thus to renal and liver polycystic disease, are significantly prevalent in the BASM form. Studies correlating ciliopathy-related gene variants, cholangiocyte ciliary structure and the presence of DPM-like structures are warranted [43].

The epithelial-mesenchymal transition (EMT) (Figure 1.6) is another process that has been proposed in BA pathogenesis, where biliary structures start to present characteristics of collagen-producing mesenchymal cells, playing an active role in fibrogenesis [138]. Previous results by our group have already shown the relationship between the extent of biliary proliferation and the prognosis that follows portoenterostomy, with patients with the worst prognosis having the greatest extent of the ductular reaction [4]. Allam et al demonstrate an increase cytokeratin 7 expression in liver tissues as a discriminating factor between BA and non-BA, at initial diagnosis time [139]. There is increasing evidence of the cholangiocyte role in the fibrogenesis process, not only indirectly activating fibrogenic cells through cytokines, but directly transforming themselves through the EMT process into invading myofibroblasts [140]. The EMT process can thus be a future therapeutic target in the disease, once this process is blocked, there may be a decrease in the fibrotic process observed in BA. In fact, an interesting study from Zeisberg et al (2007) showed that TGF-β1 can activate fibroblasts and about 45% of activated fibroblasts derives from hepatocytes via EMT [141]. They also demonstrated in rat fibrotic livers induced by CCl_4, that the administration of recombinant human bone morphogenesis protein-7 (rhBMP7), a protein member of TGF-β1 family can inhibit the TGF-β1-induced EMT and decrease liver fibrosis [141]. Another hypothesis for portal tract fibrogenesis in BA is the

presence of biliary epithelial cells undergoing EMT that could serve as a source of myofibroblasts, surrounding bile ductules leading to periductular fibrosis, once these cells can rise the levels of type I collagen responsible for hepatic fibrosis [142]. A recent study from Wang et al (2019) showed in liver samples of infants with BA that TGF-β1-induced EMT significantly decreased the expression of miRNA-29c, maybe decreasing the fibrogenic process in BA [92]. However, there is no certainty of EMT in liver fibrosis, and there are serious arguments against it. Fabris et al reported that most studies supporting the EMT occurrence in cholangiocytes as a cause of biliary fibrosis are based essentially on a morphological approach, and are not confirmed in vivo by lineage-tracing experiments [143].

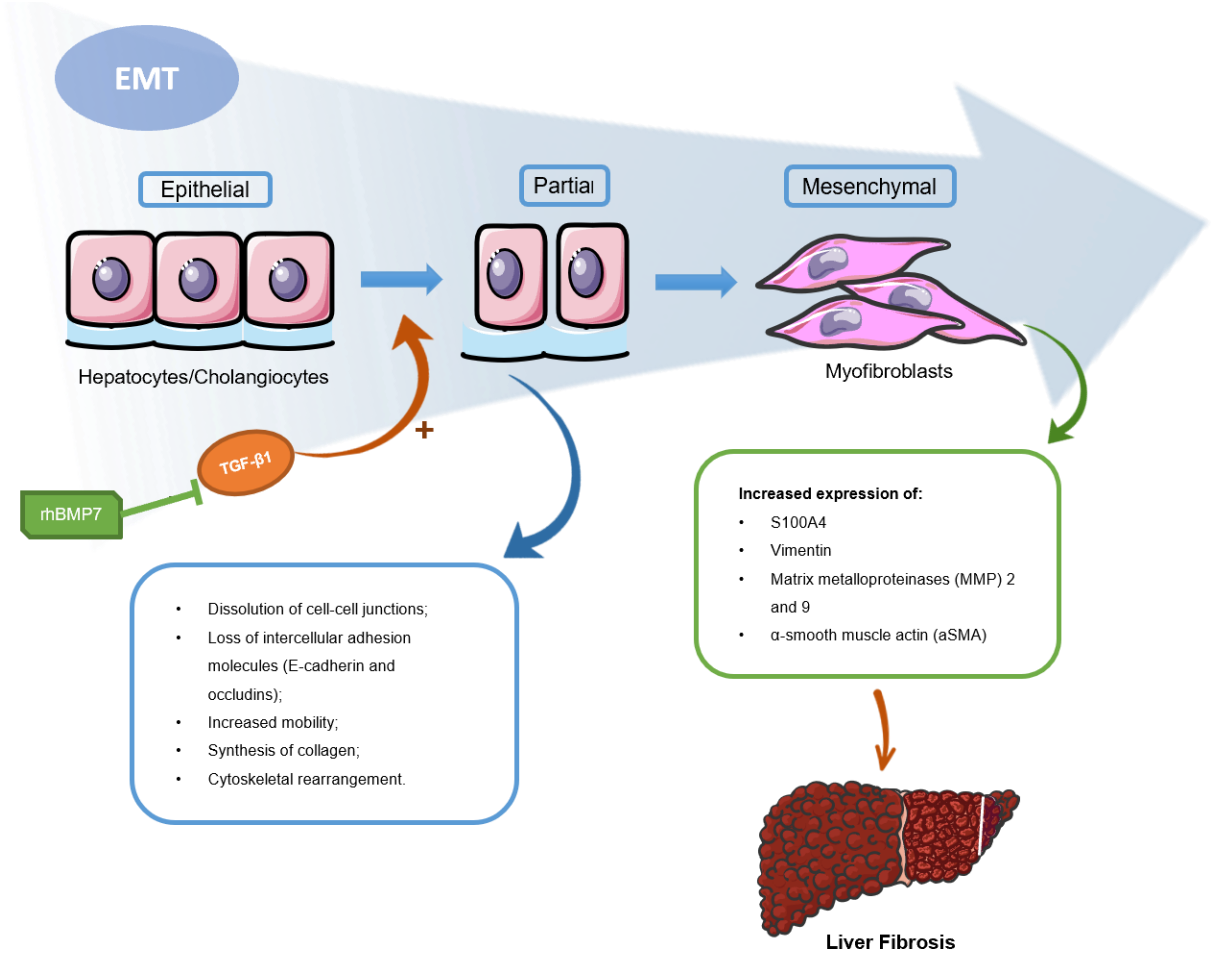

Figure 1.6. Epithelial-mesenchymal transition (EMT) in BA etiology. EMT is a process that has been proposed in BA pathogenesis, where biliary structures start to present characteristics of collagen-producing mesenchymal cells, playing an active role in liver fibrogenesis. TGF-β1 is an activator of this process, and the administration of recombinant human bone morphogenesis protein-7 (rhBMP7), a protein member of TGF-β1 family can inhibit this process and decrease fibrogenesis.

1.3.4. Environmental Factors

The environmental conditions to which the mother/newborn dyad is exposed during pregnancy also appear to play an important role in the development of BA. Certain studies have focused their line of research on the discovery of viruses and toxins that can cause the disease [144]. In the hepatic tissue of BA patients, a protein expression profile compatible with chronic inflammation is observed, secondary to the immune response like that occurring in a viral infection. In the case that viruses are responsible for BA, the direct evidence of their action seems to be diluted and can be identified with great difficulty, leaving the inflammatory reaction triggered by them. The role of viral infections in the development of BA is under investigation. The main proposed hypothesis focuses on the hepatotropic virus as a triggering agent of an autoimmune inflammatory process throughout the entire biliary tree [145]. One of the putative pathophysiology mechanisms involved in BA is the presence of an autoimmune process limited to the liver with features like other autoimmune liver diseases, such as autoimmune hepatitis and sclerosing cholangitis [146]. The main viruses investigated in the pathogenesis of BA include cytomegalovirus, human papillomavirus, human herpesvirus-5, Epstein-Barr virus and RVR [68] which has been used for the development of animal models that allow the study of this pathology. However, the identification of the different types of viruses has been inconsistent since there is a great variability in the methodologies used. The absence of viruses in the liver and porta hepatis, the methodological problems of virus research and the absence of an inflammation pattern specific to CMV are key points that hinder the development of this field of BA research. An interesting observation is that although the rotavirus-rhesus reovirus produces the animal model, the use of vaccines for this virus has not decreased the incidence of BA and the causality is not defined in relation to humans [147].

1.3.4.1. Natural Toxic Products

The study of chemical compounds found in environment and toxic products used in industry has been another important field in BA research. A recent study from Malik et al (2020) analyze the methylene diphenyl diisocyanate (MDI) and methylene diphenyl diamine (MDA) compounds and found a possible environmental factor associated with BA [148]. The isolation of an isoflavonoid called biliatresone from plants from gender *Dysphania* causes damage to the extrahepatic biliary system in zebrafish larvae, which develop a phenotype like human BA [149-151] and has been widely studied as a potentially toxin for the establishment of a novel BA animal model. Some studies focused to understand which toxicity mechanism has biliatresone to provoke BA. Zhao

et al (2016) performed a transcriptional profile of liver cells isolated from zebrafish larvae in the initial stage of bile duct injury. They concluded that oxidative stress resulting from disturbed glutathione metabolism is a critical factor in cholangiocyte injury induced by biliatresone, suggesting that variations in the intrinsic responses to stress depends on the susceptibility profile of liver cells [152]. The low antioxidant capacity of extrahepatic cholangiocytes may be a critical factor for the development of BA in humans. A study by Waisbourd-Zinman et al (2016) sought to understand the cellular changes caused by biliatresone in mammalian BA cells associated with fibrosis, through treatment with the toxin and with compounds that regulate glutathione. They found that biliatresone decreases glutathione and the expression of SOX17 (a gene associated with BA susceptibility), causing disruption of the apical polarity of cholangiocytes and loss of monolayer integrity, leading to increased permeability and rupture of the extrahepatic biliary tree and fibrosis [148].

1.3.4.2. Gut microbiota development: a new research path in BA.

The collection of gut microbiota community and their genes plays a critical role in health and disease once they perform an incredible array of essential functions to keep body functional. These includes breaking down and assimilating food, setting body metabolism, neutralizing drugs and carcinogens and synthesizing vitamins (like folate, vitamin K2 and choline) as well fatty acids and secondary bile acids, which are key signaling compounds in health [144, 153, 154]. Newborns do not have intestinal flora and, after birth, the neonatal intestine immediately faces vigorous alterations as it changes to a microbial-rich extra-uterine environment [155]. Since microbiome development is such an important process, researchers have tried to understand its influence on pediatric diseases and associations with immune responses have been made [154]. Some investigators have focused their research on the development of gut microbiome in BA and its influence in the progression of the disease. Wang et al (2019) conducted a cross-sectional analysis on 34 BA children and 34 healthy controls and analyzed changes in the intestinal microbiota after Kasai surgery in 16 of the BA patients. Their results concluded a decrease in microbial diversity and a remarkable structural change in the microbiome of BA patients as well as a marked decrease in intestinal bile acids, which may play a major role in microbial dysbiosis in BA [156]. This is an important result once BA microbial dysbiosis is strongly correlated with liver function, therefore being an important factor in the diagnosis of BA.

What has been shown is that after Kasai surgery bile flow correlates with native liver survival and that it is correlated with levels of total serum bilirubin and bile acids [157].

Microbes are capable of decoupling and transforming bile acids in the intestine, which will later affect the signaling of bile acids in the liver. This indicates a potential key role for the gut microbiome in the after Kasai outcome. A study from Tessier et al (2020) analyzed the fecal microbiome of 8 BA patients and concluded that stool microbiome differs between babies with BA and other forms of neonatal cholestasis and that this appears to be associated with differences in bile flow after portoenterostomy, once again highlighting the potential roles of bile flow and luminal bile in the development of the infant microbiome [157]. Jee et al (2017) also studied the microbiome development in BA. Using an animal model, they administered sulfamethoxazole and trimethoprim to the mother by vertically modifying the colonization of neonatal intestines by commensal bacteria. They observed microbiome changes associated with resistance to the experimental phenotype of BA, expressed as a decrease in cholestasis, biliary injury and improved long-term survival [155].

1.4. Animal Models: Challenges of Studying BA

The development of animal models that allow the study of BA has been a challenge for researchers in the area. Although there are already some models that can mimic the obstruction of the intra and extra hepatic bile ducts, the part of fibrosis in the disease has been difficult to mimic in vivo and the results obtained in animal models may not always be extrapolated to the human situation.

Some stipulated in vitro models fail to correlate the virus-induced cholangiopathy that is found in murine models with the development of BA. Models induced by RVR demonstrate the susceptibility of human cholangiocytes to infection by the virus, suggesting a potential role in the etiology of the disease [147], however fail regarding the survival rate of animals. An interesting model stipulated by Mohanty et al (2019) demonstrated that RVR infection results in an inflammatory cholangiopathy with a pathological phenotype like human BA with a clinical progression to obstructive jaundice and morphological and histological changes of the biliary tree [158]. Zhang et al (2018) using a mouse BA model inoculated with RVR combined with an antibody (anti-Ly6G) that reduces CD11b+ GR-1 + cells showed an improvement in BA syndrome and an increase in survival rates of the model, with a recovery of the fibrosis state, which may indicate a key role of GR-1+ cells in the etiology of BA [73]. Another study by Mohanty et al (2017) developed a mutant model of RVR with a change in the VP4 protein, where in vitro it reduced the binding and infectivity in cholangiocytes and in

vivo it produced less symptoms and less mortality in neonatal mice [159]. Using this same model, they tried to determine how the VP4 protein regulates susceptibility in cholangiocytes to infection both in vitro and in vivo and have identified a sequence of amino acids in the protein specific for the rotavirus strains that cause obstructive cholangiopathy [159]. Keyzer-Dekker et al (2015) tried to fill the gaps between BA murine models and the disease in humans, namely the fibrogenic process, once the presence of fibrosis is a key point of the disease [160]. Through the Biliary Atresia Research Consortium system, they described the morphological characteristics and the development of fibrosis in the RVR-induced animal model, having observed expansion of the portal tract in all animals and portal fibrosis with a portal-to-portal focal distribution of portal-portal bridges in just 2 mice after 14 days disease [160, 161]. Although some studies have been developed on the role of RVR in BA etiology, recent research shows that vaccination against this virus does not show any change in the prevalence of the disease [162, 163].

Another model that has been studied is the sea lamprey, since during its metamorphosis the lamprey liver loses the entire biliary system in a process that resembles human BA. This model has many characteristics similar to human BA, except liver failure and cirrhosis which are absent in lamprey. According to Chung-Davidson this spontaneous model could be useful in the study of the etiology of BA and other liver diseases to prevent biliary obstruction, fibrosis and eliminate cholestasis [164]. Biliatresone has also been studied as a potential agent in an animal model of BA. Studies by de Jong et al. (2023) demonstrated that fetal and neonatal bile ducts have high levels of hyaluronic acid, which plays a critical role in the development of the disease after injury [165, 166]. Damage to the fetal extrahepatic bile ducts leads to increased deposition of hyaluronic acid around the ducts, contributing to the progression of fibrosis in BA [165]. However, murine models have had limited success in demonstrating significant injury when exposed to low doses of biliatresone, as described in de Jong et al. (2024), where exposure resulted in altered bile metabolism but not significant fibrosis or duct damage [167]. Despite some progress in zebrafish models, the role of biliatresone in mammalian models remains inconclusive. Current research is still exploring whether higher or more sustained doses might yield a clinically relevant BA phenotype in mammals but concerns about toxicity and the models' limitations persist.

Another innovative model recently developed was organoids derived from liver biopsies from patients with biliary atresia, which generated lumen-containing spheroids with a layer of epithelial cells exhibiting a cholangiocyte-enriched gene expression signature

[168]. These organoids showed cellular, molecular, and functional characteristics that were unique to biliary atresia, such as fewer cholangiocyte-like cells expressing developmental and functional markers [168]. They also showed an abnormal pattern of expression of cellular polarity proteins, with cilia that pointed laterally or externally and with a formation of an epithelial layer with anatomical and functional evidence of increased permeability [168].

1.5. Conclusion And Future Perspectives

BA is progressively seen as a multifactorial disease that manifests itself in neonates and infants. BA has major consequences for infant health, with a quick progression to portal hypertension and end-stage liver disease. The window between diagnosis and surgical intervention with good results is tight and usually the outcome is poor. Increasingly, researchers agree that good clinical results of BA are difficult to achieve without a better understanding of the etiology underlying the disease. In future studies, the focus on identifying specific pathways of ductal epithelial injury and fibrogenesis, as well as viruses and toxins that can trigger these mechanisms, is of paramount importance. Also, the development of new human BA models through, for example, disease-specific pluripotent cells are important steps towards a better understanding of the genetic and immunologic basis of BA and as an opportunity for testing drugs that may have therapeutic effects on the disease. Investigating the role of hypoxia-ischemia in the development of BA also appears to be an important step, at least in the isolated variant of the disease. Abnormalities in PVP can cause ischemic cholangiopathy contributing to the progression of BA. Understanding why and which are the mechanisms of PVP injury can be a key point in understanding the pathogenicity and progression of BA.

1.6. References

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Chapter 2

General Introduction – Part II

Primary Cilia, Hypoxia, and Liver Dysfunction: A New Perspective on Biliary Atresia

This chapter was published in:

Patricia Quelhas, *Diogo Morgado, Jorge dos Santos, "Primary Cilia, Hypoxia, and Liver Dysfunction: A New Perspective on Biliary Atresia", Cells*, 2025; 14(8):596. https://doi.org/10.3390/cells14080596

Abstract

Ciliopathies are disorders that affect primary or secondary cellular cilia or structures associated with ciliary function. Primary cilia (PC) are essential for metabolic regulation and embryonic development, and pathogenic variants in cilia-related genes are linked to several pediatric conditions, including renal-hepatic diseases and congenital defects. Biliary atresia (BA) is a progressive infantile cholangiopathy and the leading cause of pediatric liver transplantation. Although the exact etiology of BA remains unclear, evidence suggests a multifactorial pathogenesis influenced by both genetic and environmental factors. Patients with BA and laterality defects exhibit genetic variants associated with ciliopathies. Interestingly, even isolated BA without extrahepatic anomalies presents morphological and functional ciliary abnormalities, suggesting that environmental triggers may disrupt the ciliary function. Among these factors, hypoxia has emerged as a potential modulator of this dysfunction. Hypoxia-inducible factor 1-alpha (HIF-1α) plays a central role in hepatic responses to oxygen deprivation, influencing bile duct remodeling and fibrosis, which are key processes in BA progression. This review explores the crosstalk between hypoxia and hepatic ciliopathies, with a focus on BA. It discusses the molecular mechanisms through which hypoxia may drive disease progression and examines the therapeutic potential of targeting hypoxia-related pathways. Understanding how oxygen deprivation influences ciliary function may open new avenues for treating biliary ciliopathies and improving patient outcomes.

Keywords: Ciliopathies; Ischemic Cholangiopathy; Therapeutic Interventions; HIF1alpha; Biliary Atresia; Liver

2.1. Hypoxia and Ciliopathy: Key Players in Biliary Atresia Pathogenesis?

Biliary atresia (BA) is a neonatal disease characterized by extrahepatic bile duct obstruction and progressive sclerosing intrahepatic cholangiopathy that frequently leads to chronic liver failure and necessitating liver transplantation (LTx) [1-3]. BA is a heterogeneous disease with distinct clinical subtypes, including isolated (non-syndromic) and syndromic forms. The latter is associated with congenital anomalies, such as biliary atresia splenic malformation (BASM) and cardiac-associated biliary atresia (CABA) [4-6]. Despite extensive research, the etiology of BA remains unclear, with causal hypotheses ranging from viral infections and immune dysregulation to genetic predisposition and vascular abnormalities [7-11]. One of the main histopathological features of BA is ductular reaction (DR), a reparative process characterized by biliary proliferation, activation of mesenchymal cells and fibrogenesis [12-14]. Recent studies have suggested that hypoxia may play a crucial role in modulating this process, and that HIF signaling contributes to both vascular remodeling and cholangiocyte dysfunction [15, 16]. Understanding these mechanisms is essential for identifying potential therapeutic targets beyond surgical interventions [17, 18].

Previous studies by our group have demonstrated, specifically in the isolated clinical form of BA, a medial layer thickening of hepatic arterial branches [19], immuno-histochemical features of vascular endothelial growth factor suggestive of arterial/arteriolar and cholangiocyte hypoxia [15, 19], overexpression of angiopoietins and their receptors that is involved in pericyte recruitment to the arterial wall [20] and a gene expression pattern of hypoxia-ischemia in the liver [10]. Based on these findings, a group in Germany developed a murine model of BA with RVR infection to further investigate the role of vascular disruption and hypoxia in disease progression. Their study revealed a disturbance in the peribiliary vascular plexus (PVP) preceding the biliary luminal obstruction [15, 21].

Hypoxia-inducible factors (HIF) 1, 2, and 3 play crucial roles in mediating cellular responses to hypoxia, and their stabilization is associated with primary cilium regression [22]. While HIF-1α and HIF-2α share structural and functional similarities, inducing distinct cellular responses upon binding to the hypoxia-responsive element (HRE) in target genes, HIF-3α acts as a negative regulator of HIF signaling under certain conditions [23]. This process occurs through the activation of HEF1/Cas-

L/NEDD9 and Aurora kinase A, which promote histone deacetylase-dependent tubulin depolymerization of the ciliary axoneme [22]. The degradation of HIF-α subunits depends on the von Hippel-Lindau tumor suppressor protein (pVHL), a negative regulator that drives the oxygen-dependent ubiquitin-mediated degradation of these subunits [23]. However, pVHL inactivation prevents HIF degradation, leading to its stabilization and consequent activation of the pathways responsible for ciliary regression. Although the α subunits of HIF-1 and HIF-2 share structural and functional similarities, they induce distinct cellular responses upon binding to the hypoxia-responsive element (HRE) in target genes [24]. These events highlight the intricate role of HIF stabilization in driving ciliopathy-associated processes under hypoxic conditions.

Ciliary abnormalities are frequently observed in BA, although only patients with the syndromic type present with pathogenic variants associated with cholangiociliopathies [5]. In non-syndromic BA, ciliopathy likely represents an acquired disruptive condition, as has been observed in animal models of BA induced by both viruses and toxins [25-27]. Primary cilia (PC), membrane organelles present in cholangiocytes, regulate bile secretion in response to bile flow and composition and play a developmental role in biliary patterning, housing multiple signaling pathways [28, 29].

2.1.1. Molecular Mechanisms of Hypoxia-Inducible Factors in Ciliary Dysfunction

HIFs are mainly stabilized in response to decreased oxygen availability. Stabilized HIF induces the expression of target genes that maintain biological homeostasis. The levels of HIF-1α and HIF-2α are downregulated in normoxic cells but significantly increase during hypoxia [30], at which point they translocate to the nucleus to induce the transcription of hypoxia-inducible genes [31]. HIF-1α activates more than 70 genes with cell-type-specific responses that include angiogenesis (e.g., increased VEGFA expression), erythropoiesis, glycolysis, vasodilation, and anaerobic metabolism [32-34]. Under prolonged or severe hypoxia, HIF-1α can also initiate the transcription of genes involved in cell death pathways [35].

HIF-2α, unlike HIF-1α, is not hydroxylated under hypoxic conditions, preventing its binding to the tumor suppressor pVHL and the subsequent proteasomal degradation. This stabilization allows HIF-2α to promote the transcription of genes associated with erythropoietin synthesis, angiogenesis, cell proliferation and tumor growth [36]. The immunohistochemical detection of nuclear HIF positivity serves as an indicator of HIF

pathway activation. Recent findings have linked pVHL, which facilitates the nuclear translocation of HIF-1α, to the maintenance of ciliary integrity in cystic kidney disease [37]

In contrast to HIF-1α and HIF-2α, the role of hypoxia-inducible factor 3 alpha (HIF-3α) remains less understood, although evidence suggests that one of the alternative isoforms may bind to HIF-1α, thereby inhibiting its transcriptional activity [38]. This indicates a potential regulatory role for HIF-3α in modulating the broader hypoxic response, acting as a negative feedback mechanism that limits HIF-1α-driven gene expression under specific conditions.

HIF stabilization has been linked to the presence of a decreased number of cilia, as both processes share common regulatory pathways [39]. In BA, HIF-1α activation is evident in cholangiocytes; however, its precise role in ciliopathy affecting these cells remains unclear [15, 16]. While an association between HIF-1α and cilia absence was found, it did not correlate with prognosis [16]. However, cilia counting in BA may be affected by two putative biases: first, quantitative analyses of the presence of cilia are affected by the fact that all stem cells are ciliated, and BA pathophysiology involves the expansion of the progenitor cell niche; second, the proliferation of stem-like cells can be linked to HIF activation. Additionally, although hypoxia, ciliopathy, and the Hedgehog (Hh) signaling pathway have been correlated in other organs, their specific interaction in BA require further investigation [40-42]. Specifically, pharmacological inhibition, knockdown, or genetic ablation of HIF-1α abolishes Hh pathway activation [43], which is critical for ciliogenesis and cellular differentiation. Although these mechanisms have been extensively analyzed in other tissues, their implications in hepatic ciliopathy, particularly BA, remain unexplored and warrant further investigation.

2.1.2. Structural Basis of Ciliogenesis

Ciliogenesis is the process by which cilia are formed. It begins with the migration of the basal body to the apical surface of the cell during terminal differentiation [44]. PC develops when a cell exits the cell cycle and enters quiescence, a state in which the cell ceases to divide and becomes metabolically inactive, focusing instead on maintaining homeostasis and performing specialized functions [45].

PC has a diameter of 0.2–0.3 microns and lacks compartments such as the endoplasmic reticulum (ER) and free ribosomes. Assembly and maintenance involve the intraflagellar transport (IFT) system, consisting of IFT complex A (IFT-A) for retrograde transport, IFT complex B (IFT-B) for anterograde transport, and BBSome, a

protein complex that operates in PC biogenesis and homeostasis and is involved in transporting signaling molecules inside and outside PC [44] (Figure 2.1).

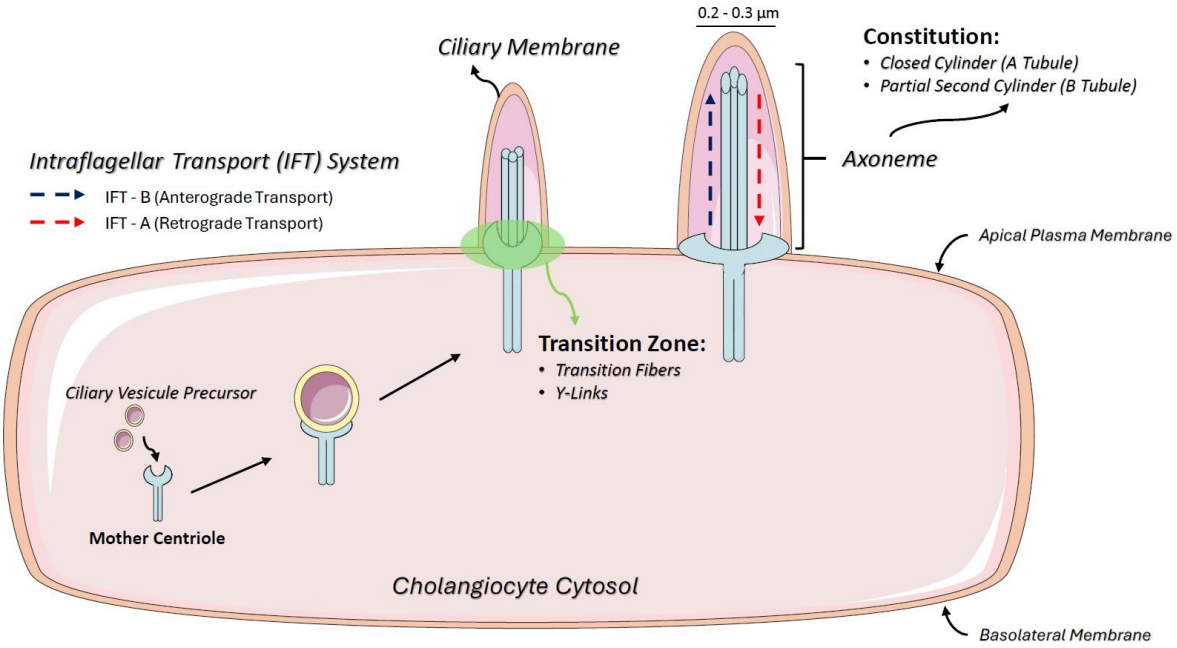

Figure 2.1. Ciliogenesis and Intraflagellar Transport Mechanisms in Cholangiocytes. The schematic illustrates the structure of primary cilia (PC) in cholangiocytes, highlighting their microtubule-based axoneme composed of an A-tubule (closed cylinder) and a B-tubule (partial cylinder). The figure also depicts the basal body derived from the mother centriole, which anchors the cilium to the cytoplasm. Additionally, intraflagellar transport (IFT) mechanisms are shown, including anterograde transport via IFT-B and retrograde transport via IFT-A, essential for ciliary assembly, maintenance, and signaling.

PC are structured by microtubules composed of alpha- and beta-tubulin dimers that form a closed cylinder (A-tubule) coupled with a partial second cylinder (B-tubule), collectively referred to as an axoneme. At the basal end, the doublet microtubules extend directly from the centriole microtubules, anchoring PC to the cytoplasm. The basal body, which is derived from the mother centriole, contains nine symmetrical microtubule triplets. Distal appendages extend outward from the basal body, docking it to the plasma membrane and facilitating ciliogenesis. The transition zone, located between the distal end of the proximal region of the axoneme and the basal body, includes transition fibers and Y-links that connect microtubule doublets to the ciliary membrane [46].

To concentrate and regulate signaling molecules within the ciliary membrane, membrane cargo is transported in vesicles from the cytoplasm to the cilium base. The lateral movement of these molecules into and out of the ciliary membrane compartment is restricted by a diffusion barrier at the basal portion of the cilium, primarily formed by the transition zone [47], which is critical for maintaining the

distinct composition of the ciliary membrane, an essential structure for signaling function [44].

2.1.3. Primary Cilia Play a Critical Role and Function in the Liver

PC are highly conserved organelles found on the surfaces of most growth-arrested or differentiated mammalian cells. They play crucial roles in regulating various cellular processes and maintaining cellular homeostasis [48] such as cell proliferation, differentiation, migration, cell polarity, signaling pathways, and other vital activities.

In the liver, PC are present in cholangiocytes and hepatic progenitor cells but are absent in mature hepatocytes, as progenitor cells lose their cilia upon differentiation [49]. Injured biliary epithelium regenerates from ciliated progenitor cells, and intrahepatic bile ducts rarely undergo hepatocyte metaplasia, although this remains debatable [13, 50]. In extrahepatic ducts, regeneration relies on biliary cell proliferation in the peribiliary glands [51]. Under normal conditions, cholangiocytes maintain homeostasis through self-replication, losing their PC during mitosis, and restoring them upon maturation [52]. This cyclical reconstruction of PC aligns with cholangiocyte differentiation. While liver repair is often termed “regeneration”, post-resection recovery in mammals is better described as “compensatory hyperplasia”, as it expands functional capacity rather than replacing lost lobes.

From a histopathological perspective, BA displays DR and precocious fibrogenesis [53, 54]. In BA, DR arises from the proliferation of progenitor cells located in the Hering canal within the Space of Mall [55] in the periportal region. These progenitor cells, including hepatoblasts, have the potential to differentiate into biliary and hepatocytic lineages, contributing to ductular reactions and liver regeneration. This niche also includes mesenchymal cells, inflammatory cells, and myofibroblasts, which contribute to the fibrogenesis associated with the ductular reaction (Figure 2.2) [56, 57]. Additionally, some biliary structures in BA resemble the “ductal plate malformation” (DPM) characteristic of cholangiociliopathies [13]. Although hepatocytic metaplasia at the portal interface has been suggested [58], its biological significance remains unclear.

Progenitor cells are involved in replacing lost liver parenchyma [59]. Specifically, the terminal segment of the biliary system, known as the canal of Hering, harbors progenitor cells capable of generating both hepatocytes and cholangiocytes under specific conditions. Lineage tracing experiments in rats and zebrafish have demonstrated hepatocyte regeneration from biliary stem cells [60]. However, in mice, the cre/lox

model did not show biliary-derived hepatocytes, although it confirmed the hepatocytic origin of biliary cells in cholangiocarcinomas [61]. Subsequent studies revealed that hepatic progenitor cells of biliary origin have liver repopulation capacity in mice when hepatocyte proliferation is blocked [62].

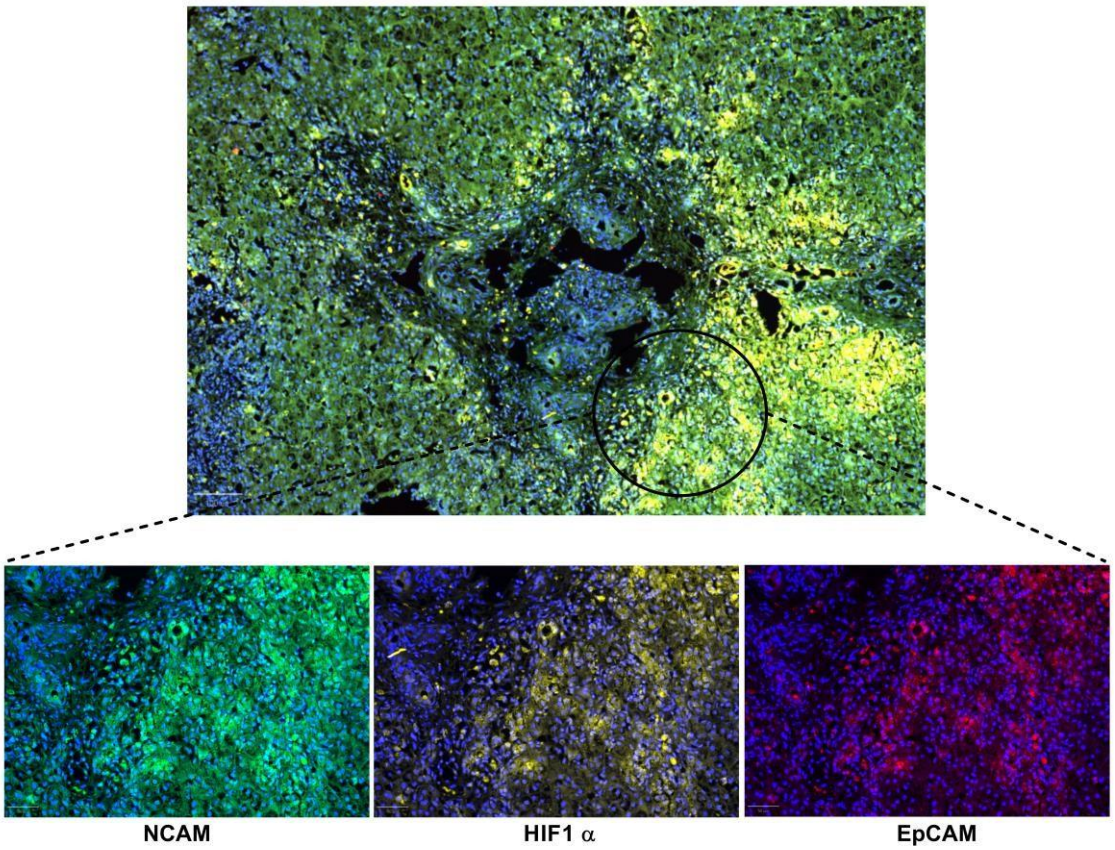

Figure 2.2. Representation of the cellular niche in biliary atresia, including mesenchymal cells, inflammatory cells, and myofibroblasts, which contribute to ductular reaction-associated fibrogenesis. Images were obtained from formalin-fixed paraffin-embedded liver tissues of children with Biliary Atresia. Staining highlights different markers: DAPI (nuclei, blue), HIF-1α (yellow, hypoxia marker), NCAM (Neural Cell Adhesion Molecule, associated with hepatic progenitor or mesenchymal cells, green), and EpCAM (Epithelial Cell Adhesion Molecule, marking epithelial cells, including cholangiocytes, red). Magnification: 20×.

2.2. Liver Function and Ciliopathies

In the liver, cholangiocytes, Kupffer cells, and endothelial cells each have cilia that play distinct and essential roles in maintaining liver health. Primary cilia in cholangiocytes act as mechanosensors and chemosensors, allowing them to detect changes in bile flow, composition, and osmolality [29, 63-65]. These cilia integrate various signaling pathways, including Hedgehog, Wnt, and Notch, which are crucial for biliary

development, repair, and homeostasis [66-69]. Dysfunction of these cilia can disrupt the regulation of bile flow and contribute to pathological conditions.

One important mechanism regulated by cholangiocyte cilia is calcium signaling [65]. Ciliary proteins, such as polycystin-1 and polycystin-2, form a complex within the primary cilium that facilitates the entry of extracellular calcium ions (Ca2+), thereby influencing intracellular signaling pathways [65]. Additionally, transient receptor potential cation subfamily V member 4 (TRPV4) channels in cholangiocyte cilia respond to changes in bile osmolality by activation when bile tonicity decreases and inhibition when it increases [70]. This mechanism regulates intracellular Ca2+ levels and subsequent cellular responses. Cholangiocyte cilia also possess chemosensory capabilities, with receptors such as P2Y12, which are activated by biliary nucleotides (ATP and ADP), thereby affecting cAMP signaling pathways [71].

Pathogenic variants in genes that encode structural or accessory proteins of PC can lead to disorders known as “ciliopathies” [48]. These conditions can manifest at different stages of life, including embryonic development, childhood, and adulthood. Ciliopathies often affect multiple organs and systems, leading to a wide range of overlapping symptoms.

Defects in PC structure and function lead to ductular reactions and changes in bile fluidity, thereby disrupting bile homeostasis [14]. Intact PC-based signaling is essential for the normal development of portal triads, including the branches of the biliary tree, and disruption of ciliary function can lead to a range of liver disorders.

Most hepatic fibrocystic diseases result from defective ciliary proteins, except autosomal dominant polycystic liver disease (ADPLD) and portal fibrosis associated with congenital disorders of glycosylation (CDG) type Ib [72]. The most common conditions include Congenital Hepatic Fibrosis (CHF), characterized by defective ductal plate remodeling, portal vein abnormalities, arterial hyperplasia, and progressive fibrosis [73, 74]; Caroli Disease (CD), marked by macroscopic dilations of medium and large intrahepatic ducts [73]; and Polycystic Liver Disease (PLD), involving isolated cysts derived from biliary microhamartomas (Von Meyenburg Complexes), which are typically unconnected to the intrahepatic biliary tree [75, 76].

Numerous studies have underscored the role of primary cilia (PC) in the pathophysiology of liver diseases (see Table 2.1). Pathogenic variants in genes such as PKD1 and PKD2 (related to Polycystic Liver Disease) [77, 78], PKHD1 (associated with

Congenital Hepatic Fibrosis and Caroli Syndrome) [79, 80], and DCDC2 (linked to Neonatal Sclerosing Cholangitis and Biliary Atresia) [81, 82] contribute to bile duct malformations. Pathogenic variants in IFT88, which are related to Bardet-Biedl Syndrome, can lead to liver fibrosis [72], while ALMS1 (linked to Alström Syndrome) [83-85] and NEK8 (associated with Nephronophthisis) [86-88] are involved in renal cysts and liver fibrosis. Pathogenic variants in ARL13B (related to Joubert Syndrome) and MKS1 (associated with Meckel-Gruber Syndrome) result in hepatic fibrosis and cysts, respectively [89-92]. Additionally, variants in TMEM67 are associated with liver disease in Joubert Syndrome [93]. Dysregulation of mTORC1 impacts nutrient sensing, autophagy, and stress responses, further exacerbating liver ciliopathies [45, 94].

Table 2.1. Genes Associated with Ciliopathy and Liver Dysfunction

Gene	Function	Disease	Impact of Pathogenic Variants	Ref.
PKD1 and PKD2	Encode polycystin-1 and polycystin-2, which are involved in calcium signaling and maintaining the structure of primary cilia	Polycystic Liver Disease (PLD) and Autosomal Dominant Polycystic Kidney Disease (ADPKD)	Cyst formation in the liver and kidneys, causing cystic enlargement and disruption of organ function	[65,73,74]
PKHD1	Encodes fibrocystin/polyductin, important for maintaining the architecture of the bile ducts and renal tubules	Autosomal Recessive Polycystic Kidney Disease (ARPKD), Caroli Syndrome and Congenital Hepatic Fibrosis (CHF)	Cyst formation in the liver and kidneys, causing cystic enlargement and disruption of organ function	[59,73,75]
DCDC2	Encodes doublecortin domain-containing protein 2, involved in microtubule organization and ciliary function.	Neonatal Sclerosing Cholangitis and Biliary Atresia	Disruption in bile duct development and function, leading to cholestasis and liver fibrosis	[77,78]
IFT88	Encodes a protein essential for intraflagellar transport, crucial for cilia formation and maintenance	Bardet-Biedl Syndrome (BBS) and Hepatic Fibrocystic Disease	Defective cilia lead to multi-organ fibrosis, including liver involvement, and other systemic manifestations	[73,74]
ALMS1	Encodes a protein involved in ciliary function and cellular signaling pathways	Alström Syndrome	Results in steatosis, with progressive liver fibrosis, along with retinal degeneration, cardiomyopathy, and other systemic features	[79,80]
NEK8	Encodes a serine/threonine kinase involved in ciliary function and cell cycle regulation	Nephronophthisis and Hepatic Fibrosis	Cystic kidney disease and liver fibrosis, indicating a shared pathogenesis involving ciliary dysfunction	[82–84]

ARL13B	Encodes a GTPase required for normal ciliary function and signaling	Joubert Syndrome and Hepatic Fibrosis	Ciliary signaling disruption, leading to cerebellar and hepatic fibrosis, and other systemic anomalies	[85]
MKS1	Encodes a protein involved in ciliogenesis and centrosome function	Meckel-Gruber Syndrome and Hepatic Fibrosis	Lethal multi-organ fibrosis, including hepatic and renal cysts, and other developmental anomalies	[86–88]
mTORC1	Influence the process of ciliogenesis by regulating the synthesis of proteins and lipids required for cilia assembly. It coordinates the cellular growth signals that are necessary for the formation of the ciliary membrane and axoneme.	Nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH)	Alterations in nutrient sensing, autophagy regulation and cellular stress response affecting ciliary function	[141]
MAN1A2	Involved in the processing of N-linked oligosaccharides during glycoprotein biosynthesis	Biliary Atresia	Knockdown of MAN1A2 results in poor biliary network formation and ciliary dysgenesis	[92]
KIF3B	Part of the kinesin-2 motor protein complex, which is essential for the anterograde transport of molecular cargos along microtubules in cilia		Variants in KIF3B can impair ciliary assembly and maintenance, potentially leading to defects in ciliary signaling pathways	[93]
TTC17	Involved in the organization of ciliary and centrosomal structures. It plays a role in the assembly and stability of ciliary axonemes		Defects in TTC17 can lead to ciliary dysfunction and impaired signaling	[93]
PCNT	Encodes a protein that is a key component of the centrosome and is involved in microtubule organization. It plays a critical role in the formation and function of primary cilia by anchoring and stabilizing the microtubules at the base of the cilium.		Variants in PCNT can cause structural and functional defects in cilia, leading to various ciliopathies	[93]
PKD1L1	Encodes a protein that forms part of a ciliary calcium channel complex with PKD2L1. This complex is involved in		Variants in PKD1L1 have been found in patients with Biliary Atresia Splenic Malformation (BASM) syndrome, indicating a link between	[91,96,97]

	mechanosensation and signal transduction within cilia		ciliary dysfunction and BA. The disruption of PKD1L1 can affect hepatobiliary development	
Planar polarity genes	Essential for positioning cells in 3D networks to establish the proper morphogenesis, structure, and function of organs during embryonic development		BA is closely linked to polygenic susceptibility involving 102 genes related to ciliogenesis and planar polarity effectors. Functional data point to issues in ciliary development, abnormal biliary epithelial cell formation, and disrupted vasculogenesis	[99]

2.2.1 Ciliary Dysfunction in Biliary Atresia: Insights and Implications

2.2.1.1. Genetic Variants in Ciliary Function and Their Impact on Biliary Atresia

The key role of primary cilia (PC) in maintaining biliary and hepatocellular health highlights the significance of ciliary function in preserving biliary homeostasis. This suggests that defects in cilia may contribute to the development and progression of biliary atresia (BA) (Figure 2.3). A study conducted by Hellen et al. (2023) utilized bile duct ligation in PKD1L1-deficient mice, successfully replicating the critical intrahepatic pathophysiological features of BA, including peribiliary fibroinflammation, hepatic arteriopathy, and ciliopathy [95]. This research provides a valuable opportunity to enhance our understanding of the disease and identify potential therapeutic targets [95].

Gene variants in MAN1A2 [96], kinesin-like protein KIF3B (KIF3B), tetratricopeptide repeat domain 17 (TTC17), pericentrin (PCNT) [97], and PKD1L1 [98-100] have been identified in patients with BA, contributing to this ciliary dysfunction. Lim et al. (2024) studied variants of the PKD1L1 gene, whose loss mimics syndromic BA in mice, and demonstrated that this loss causes ciliary dysfunction by disrupting ciliary signaling, thus classifying syndromic BA as a cholangiociliopathy [101]. These findings highlight the critical role of cilia and related gene mutations in the pathophysiology of liver diseases, including BA [102].

Glessner et al. (2023), through genome-wide association studies (GWAS) involving 811 biliary atresia patients, indicated that abnormal biliary development in BA is, in part, due to disruption of ciliogenesis and ciliary function through genes such as AFAP1 (actin filament integrity modulator) and TUSC3 (protein involved in cellular magnesium uptake) [103]. Additionally, they showed through an integrative analysis of hepatic BA transcription that downstream disruption of vascular and epithelial tube morphogenesis may explain portal vein atresia, which occurs in some cases of BA, indicating a possible association between BA and vascular development [103]. Karjoo et al. (2014) demonstrated, in a murine model of BA with rhesus rotavirus (RRV) infection, as well as in human tissue, that there is significant loss of extrahepatic cholangiocyte cilia, indicating the possibility that BA is an acquired ciliopathy [27].

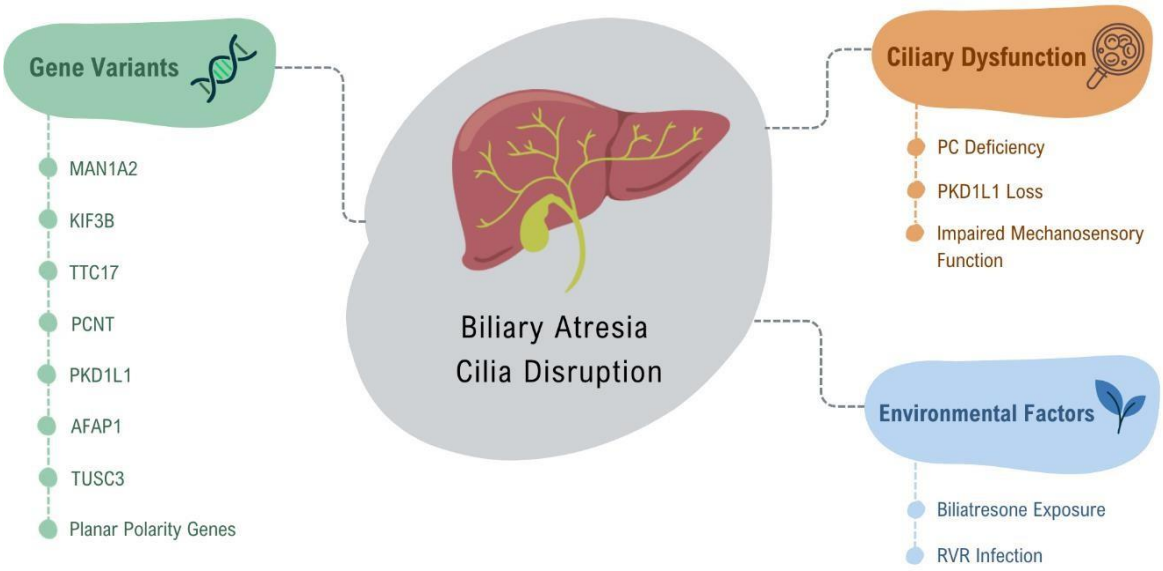

Figure 2.3. Factors contributing to ciliary disruption in Biliary Atresia. This figure illustrates the key genetic variants associated with ciliary dysfunction in biliary atresia, including MAN1A2, KIF3B, TTC17, PCNT, and PKD1L1, and environmental factors that contribute to BA pathogenesis, such as Bil-iatresone exposure and Rotavirus Rhesus infection. These factors disrupt ciliary structure and function, contributing to the pathogenesis of BA by impairing biliary flow and cellular signaling.

A recent study by Hai-Bing et al. (2024), which evaluated the effect of biliatresone on human liver organoids, confirmed that exposure to this toxin causes morphological and functional changes like those observed in BA [104]. The organoids exposed to biliatresone exhibited impaired ciliary function in cholangiocytes, characterized by a decreased number of cilia and compromised mechanosensory function [104]. In a previous study by Lorent et al. (2016), the effects of biliatresone on extrahepatic cholangiocytes were evaluated [105]. The researchers observed that cells treated with biliatresone experienced a reduction in primary cilia length, and staining for cellular

tubulin revealed a dose-dependent decrease in the number of visible microtubules. This suggested that biliatresone negatively affected microtubule stability [107]. Additionally, spheroids designed to mimic BA were utilized in that study, which led to the conclusion that biliatresone resulted in the loss of epithelial monolayer integrity, lumen closure, and disruption of apical-basal polarity [105].

Another line of investigation in ciliopathies in BA was highlighted in a recent study by our group, which showed that reduced PC length, PC bending, and increased cytoplasmic tubulin expression occur in the isolated clinical form of BA. Our findings suggest that a disruption in tubulin transport between the cytoplasm and PC negatively impacts early prognosis after portoenterostomy (Figure 2.4) [16].

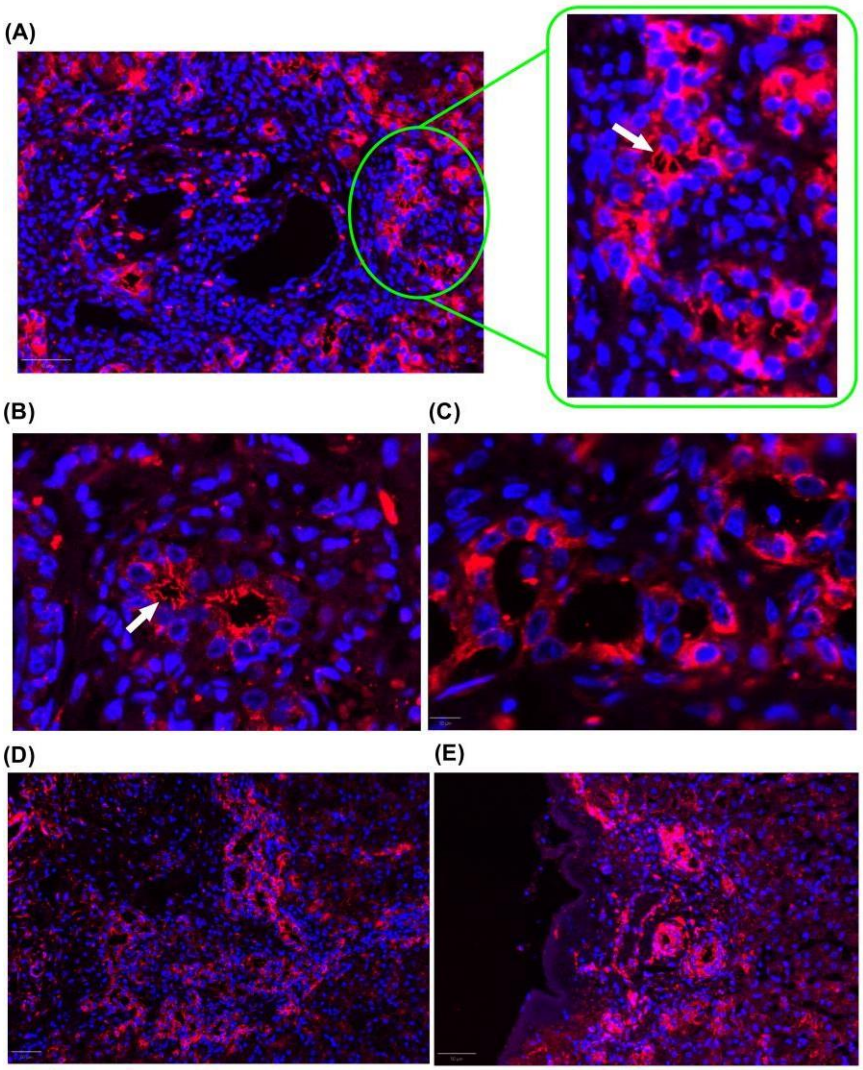

Figure 2.4. Representative image of cilia in cholangiocytes. Fluorescence microscopy images showing cilia labeled with TUBA4A (red) and nuclei stained with DAPI (blue). (A) Cytoplasmic expression and evident cilia in the portal space. White arrows indicate primary cilia. (B) Higher magnification (40×) image of the bile duct, highlighting prominent cilia in cholangiocytes. White arrow indicates primary cilia. (C) Control bile duct without primary cilia. (D) Cytoplasmic staining of cholangiocytes at the interface region. (E) Cilia and cytoplasmic staining in the biliary ducts in the subcapsular region. The images shown were obtained from formalin-fixed paraffin-embedded liver tissue from children with Biliary Atresia. Magnification: 20×.

2.2.1.2. The Role of Ciliary Genes in BASM

Approximately 20% of patients with BA exhibit a syndromic form known as embryonic BA, which includes conditions such as BASM [6, 106]. BASM is characterized by defects in laterality determination, which can lead to issues like malrotation, dextrocardia, and polysplenia [25]. These findings suggest that ciliary defects may play a significant role in the pathophysiology of these conditions [25].

During embryogenesis, abnormal function of primary cilia has been linked to laterality defects, highlighting the importance of proper ciliary structure in these developmental processes. Research by Chu et al. (2012) showed that cilia in liver specimens from patients with syndromic BA, regardless of laterality defects, were significantly shorter and less abundant than those in normal livers and other neonatal cholestatic diseases [25]. Similarly, Mitra et al. (2021) demonstrated notable abnormalities in primary cilia in patients with BA, with a significant reduction in their number compared to healthy controls [107].

Another study by So et al. (2020) indicated that the necessity for liver transplantation in BA cases may be influenced by sequence variants in the mannosidase alpha class 1A member 2 (MAN1A2) gene [96]. This gene interacts with the ARF6 and EGFR signaling pathways to regulate the development of the intrahepatic biliary network. Furthermore, it was observed that both MAN1A2 mRNA and protein expression levels were lower in the liver tissue of BA patients, potentially due to the gene's role in proper laterality determination and hepatobiliary morphogenesis through its influence on ciliogenesis [96, 108].

A novel hypothesis suggesting that primary cilia (PC) play a role in the pathogenesis of biliary atresia (BA) may lead to further investigations into the genes associated with ciliary development. This research could focus on patients presenting with laterality defects and other forms of BA linked to genetic susceptibility.

2.3. The Role of Hypoxia

2.3.1. Biliary Hypoxia: Mechanisms and Impacts

Investigating the role of hypoxia in hepatic and biliary tissues is becoming increasingly important. De Jong et al. (2021) showed that chronic biliary hypoxia resulting from microvascular disruption can lead to the development of non-anastomotic biliary structures [50]. This biliary hypoxia triggers the proliferation and differentiation of peribiliary gland stem cells into mature cholangiocytes, underscoring

the critical importance of oxygen supply to the biliary anatomy and the potential implications of hypoxia in biliary diseases [50].

In humans, both extrahepatic and intrahepatic biliary structures receive their blood supply exclusively from the arteriolar branches of the hepatic artery, which form the peribiliary vascular plexus (PVP). Recent studies on biliary atresia (BA) suggest that disruption of the PVP may initiate biliary hypoxia. In the livers of patients with BA, there is evidence of progressive thickening of the medial layer in hepatic artery branches [109], peripheral arterial blockages accompanied by the formation of perivascular arteriolar tufts [110], and immunohistochemical expression of angiogenic factors within the biliary structures [19]. These findings indicate the presence of hypoxia affecting the biliary tree and the occurrence of reactive angiogenesis.

Our research group demonstrated in 2014 that liver specimens from patients with the isolated form of BA showed upregulation of angiopoietins and their receptors, which are crucial for recruiting pericytes to the vascular wall [20], along with hypoxia-ischemia molecular features linked to disease progression [10]. Additionally, we found that activation of the HIF-1α pathway may play a role in the pathogenesis of BA, potentially involving ischemic cholangiopathy (IC) and/or disruption of the REDOX state (Figure 2.5) [15].

Ischemic cholangiopathy involves focal or extensive damage to the bile ducts due to reduced blood supply, as observed after LTx, hepatic arterial infusions of toxic agents, and certain vascular conditions [111-113] that compromise bile duct integrity through ischemia. PVP supplies oxygen to the intrahepatic bile ducts, and obstruction of its small vessels (<200 μm) leads to biliary duct ischemia and fibrotic strictures [10, 114]. During procedures such as LTx, cholangiocytes are highly sensitive to short ischemic episodes, resulting in structural alterations that impair ductal secretory function post-reperfusion [115-117]. This lack of adequate blood supply can also affect the peribiliary glands, which harbor stem cells essential for biliary epithelium regeneration [118] and large bile ducts, which consist of extrahepatic, segmental (400–800 μm), and area (300–400 μm) ducts [119]. Experimental models have indicated that hypoxia triggers periportal expression of factors such as VEGF and fibroblast growth factor-2 (FGF-2) [120], affecting cholangiocytes and hepatocytes and potentially promoting structural changes linked to cholestasis and biliary dysfunction [111, 115].

In addition to the lack of oxygen supply during LTx, the post-transplant reperfusion process is also a critical factor that can lead to LTx failure due to the occurrence of

large-scale REDOX stress [121, 122]. Ischemia-reperfusion injury affects not only hepatocytes and liver endothelial cells but also damages biliary structures. Biliary complications after LTx occur in 10% to 30% of operated patients and lead to increases in graft dysfunction, patient morbidity, graft loss and even death rates [115].

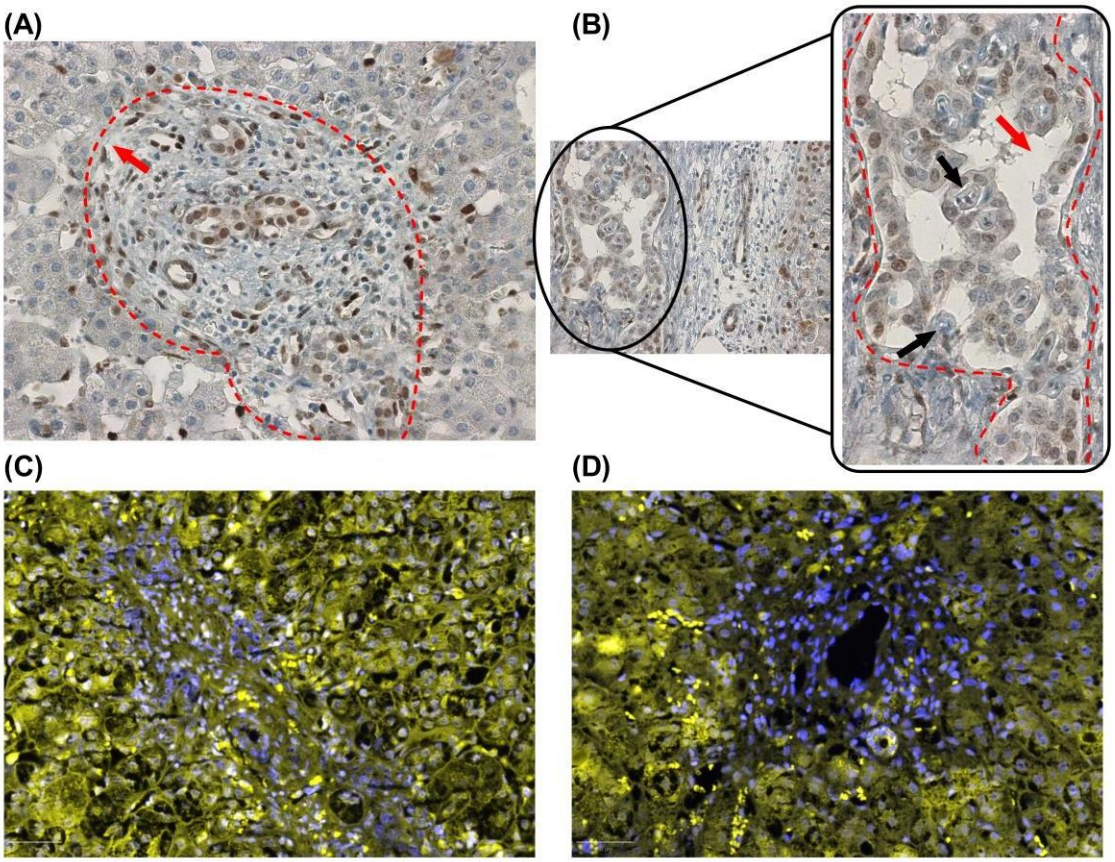

Figure 2.5. Representative images of nuclear positivity for HIF-1α. (A) Immunohistochemistry image of the portal area showing cholangiocyte nuclei strongly positive for HIF-1α (brown staining). HIF+ staining is observed in the portal area, interlobular bile ducts, peribiliary vascular plexus (PVP) endothelium, and interface (Mall's space). The dashed red line delimits the Portal Space, and the red arrow indicates the Mall Space. (B) Immunohistochemistry image with HIF+ staining in a structure resembling a Ductal Plate Malformation (DPM) (left) and at the interface of a fibrovascular septum (upper right). The dashed red line delimits the Space of Mall, with the red arrow indicating the same. Black arrows indicate PVP. (C) Immunofluorescence image showing intense nuclear HIF-1α staining associated with the development of a fibrovascular septum. (D) Immunofluorescence image of a portal area showing bile ducts with HIF+ cholangiocytes. HIF-1α is labeled yellow and DAPI blue. The images shown were obtained from formalin-fixed paraffin-embedded liver tissue from children with Biliary Atresia. Magnification: 20×.

2.3.2. Relationship Between Hypoxia and Ciliary Function

2.3.2.1. Hypoxia and Ciliopathy in Liver Diseases

Hypoxia plays an important role in the pathogenesis of different liver disorders [123], including lesions caused by hepatic ischemia/reperfusion [122, 124]. Cholestatic disorders can arise from liver ischemia through various mechanisms. In a rat model of arterial liver ischemia, hypoxia primarily affects cholangiocytes and hepatocytes, predominantly in the periportal area after complete arterial deprivation. In liver tissue, there is a marked reduction in the expression of hepatocyte membrane transporters, even before bile duct proliferation occurs [117]. In the clinical setting, only sustained postoperative hyperbilirubinemia is associated with high morbidity and mortality. However, experimental studies in rat liver ischemia models have demonstrated that liver resection procedures involving vascular clamping—and thus inducing hypoxia—can lead to transient elevations in bilirubin levels, suggesting a potential mechanistic link [125]. New studies are increasingly focusing on how hypoxia affects the liver PC, especially in relation to ischemia-reperfusion and its role in biliary injury, as hypoxia-related damage affects biliary structures in addition to hepatocytes [15].

A study by Esser et al. (2024) showed that liver pre-transplantation ischemic conditions shorten PC in biliary epithelial cells, and that stabilization of PC is an effective method for preventing biliary epithelial cell apoptosis, contributing to transplant success [126]. In that study, it was shown that damage to PC during ischemia triggers cellular senescence in biliary epithelial cells. Consequently, these cells lose their ability to proliferate, which leads to persistent biliary injury and impaired regeneration [126].

To our knowledge, the first description of HIF-1α nuclear positivity in cholangiocytes of the intrahepatic biliary tree was obtained by our group and may be associated with hypoxia, oxidative stress, and molecular pathways involved in ciliary disruption [15].

2.3.2.2. Ciliary Alterations Driven by Hypoxia beyond the Liver

HIF-1α: A Fundamental Player in Hypoxia and Cilia Dynamics

The impact of hypoxia on cilia has been a focus of research interest, not only in the liver but also in various other organs. Studies have shown that the HIF pathway and its associated genes, such as IFT52 and VHL, play critical roles in regulating ciliogenesis under different oxygen conditions, providing insights into how oxygen availability affects ciliary function in various tissues. Brown et al. (2003) observed in Tetrahymena

thermophila that the protein IFT52p (encoded by the IFT52 gene) is involved in signaling pathways that regulate cilia assembly, which may be particularly sensitive to hypoxic conditions [127]. The observed suppression of cilia assembly in IFT52 mutant cells suggests a direct link between ciliogenesis and hypoxia-mediated signaling, emphasizing the potential role of hypoxia in modulating ciliary dynamics at a fundamental level [127].

Research on the von Hippel-Lindau (VHL) gene has focused on its role in regulating hypoxia through the HIF-1α pathway. Lutz and Burk (2006) examined how the VHL gene contributes to the formation of primary cilia (PC) in renal-derived cells and its influence on hypoxia via the HIF-1α pathway [128]. VHL is essential for the ubiquitination process, as it facilitates the degradation of HIF-1α under normoxic conditions, thereby regulating the cellular response to hypoxia. Their study found that cells lacking functional VHL failed to develop cilia, leading to disrupted ciliogenesis, which is linked to renal cyst formation and the development of renal cell carcinoma. The significance of VHL in maintaining primary cilia has been emphasized, along with its role in mediating the HIF-1α pathway [128].

Further research by Troilo et al. (2014) highlighted the involvement of ubiquitin-specific protease 8 (USP8) in ciliogenesis, which works in conjunction with the VHL protein pVHL, as demonstrated through siRNA-based screening [129]. They found that USP8 acts as a deubiquitinating enzyme for HIF-1α, counteracting pVHL-mediated ubiquitination of HIF-1α. This interaction maintains basal levels of HIF-1α expression and HIF transcriptional activity under normoxic conditions, which is crucial for endosome trafficking and ciliogenesis [129].

Additional studies have shown interactions between HIF-1α and cellular components related to ciliogenesis in renal carcinoma and other hypoxia-sensitive tissues. For instance, mitotic aurora kinase A (AURKA) has been identified as a specific target of HIF-1α in the VHL syndrome. Dere et al. (2015) suggested that HIF-1α inhibits AURKA through β-catenin-directed transcription [130]. VHL protein (pVHL) regulates HIF subunits under normoxic conditions by promoting ubiquitination and subsequent proteasomal degradation. This process prevents the stabilization and nuclear translocation of HIF-1α and HIF-2α, thereby maintaining cellular homeostasis.

Recent findings also underscore the role of pVHL in maintaining the integrity of primary cilia, particularly in the context of cystic kidney disease. Dysfunction of pVHL

disrupts ciliogenesis and endosome trafficking, leading to the stabilization of both HIF-1α and HIF-2α, resulting in the upregulation of AURKA (Figure 2.6) [131].

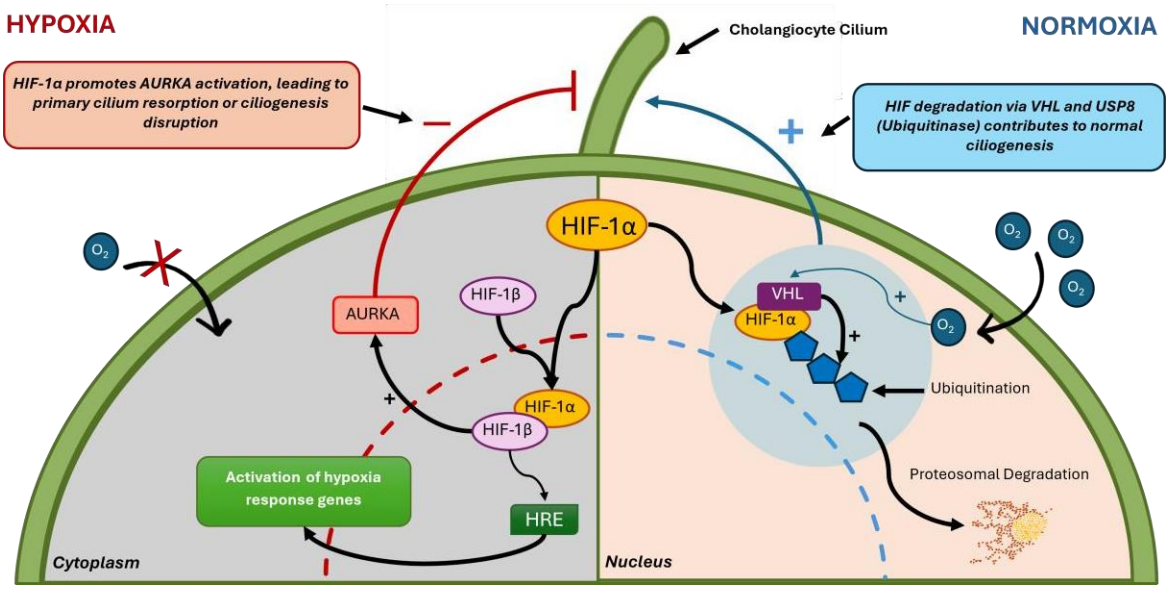

Figure 2.6. Schematic representation of the relationship between HIF-1α, VHL, AURKA, and primary cilia in hypoxic and normoxic conditions. Normoxia (right side): Under normal oxygen levels, HIF-1α is ubiquitinated by the von Hippel-Lindau protein (VHL) and degraded via the proteasomal pathway. USP8, a deubiquitinating enzyme, counteracts VHL-mediated HIF-1α ubiquitination to maintain the basal HIF transcriptional activity. This regulation contributes to normal ciliogenesis, as VHL plays a crucial role in maintaining primary cilia integrity. Hypoxia (left side): Under low-oxygen conditions, HIF-1α is stabilized and translocates to the nucleus, where it dimerizes with HIF-1β and binds to hypoxia-responsive elements (HREs) to activate the transcription of hypoxia-related genes. Hypoxia also promotes the activation of AURKA, leading to primary cilium resorption or disrupted ciliogenesis.

Research has also explored the molecular mechanisms by which hypoxia influences ciliogenesis. Fabbri et al. (2020) examined the regulation of ciliogenesis in renal carcinoma cells, with a focus on the hypoxic form of the voltage-gated anion channel (VDAC1-ΔC) [132]. Their findings indicate that PC loss in these cells is dependent on the pVHL/HIF/hypoxia pathway [132], supporting the idea that changes in cellular structure and function due to hypoxia are associated with VHL/HIF signaling.

Furthermore, HIF-1α appears to affect ciliary architecture and motility in tissues other than the kidneys. Resnick (2016) discovered that cilia in tissues stabilized by HIF were more flexible than those in non-stabilized tissues [39]. This flexibility may suggest that cells adjust cilium length and flexibility in response to fluctuating oxygen levels [39]. Their study indicated that longer cilia are associated with increased flexibility, implying that cells regulate cilium length to maintain consistent sensitivity and functionality under varying oxygen conditions [39].

In primary human nasal epithelial cells, HIF-1α has been linked to cilia loss and increased proliferation of goblet cells, potentially through the phosphorylation of NLRP3, an essential component of the inflammasome [133]. Similarly, HIF-1α regulates primary cilia formation in HeLa cells via ROS-induced NPHP3 expression and ERK activation during serum deprivation, highlighting its role in adapting to stress [134]. In mesenchymal stem cells (MSC), silencing HIF-1α prevents cilia loss under hypoxic conditions, while constitutively active HIF-1α reduces primary cilia formation, underscoring its critical role in maintaining ciliary stability under low-oxygen conditions [37]. A recent study from our group examined the effects of hypoxia and HIF-1α pathway activation on primary cilia in cholangiocytes and found an inverse relationship between HIF-1α expression and the presence of cilia. This suggests that hypoxia disrupts ciliary formation and maintenance, contributing to biliary dysfunction [16].

Impact of HIF-2α on Primary Cilia

Recent findings have suggested that HIF-2α plays a role in PC signaling beyond its transcriptional activity. Specifically, HIF-2α appears to contribute to the pathology of osteoarthritis by promoting PC loss through the HIF-2α/AURKA/NEDD9 pathway, whereas HIF-1α does not exhibit this effect [42]. Leu et al. (2023) further identified the accumulation of HIF-2α in the ciliary axoneme, where it interacts with IFT88, influencing MEK/ERK signaling, and enhancing cell survival under hypoxic conditions [135]. Similarly, Johnston et al. (2024) demonstrated that increased HIF-2α activity upregulates PC-related genes and promotes longer cilia in hypoxic murine neuronal cells through interactions with an IFT88 homolog, potentially aiding cellular adaptation to low-oxygen levels [136].

To examine the relationship between primary cilia and HIFs in inflammatory signaling, Wann et al. (2013) conducted a study using primary articular chondrocytes from both bovine and human sources [137]. They concluded that the sequestration of HIF-2α in cilia negatively regulates its expression and may influence HIF-2α activity, illustrating for the first time that primary cilia play a regulatory role in HIF signaling during inflammation [137]. Qiao et al. (2021) investigated the roles of HIF-1α and HIF-2α in the PC of human retinal epithelial cells and found that hypoxia elongated cilia in a dose-dependent manner when using CoCl2 (a model for hypoxia), with this increase being reversible and dependent on exposure time [138]. This finding contrasts with observations in the liver, where hypoxia appears to impact ciliary structure and function differently [126]. This suggests that hypoxia-induced ciliary elongation serves

as a cellular adaptation mechanism, albeit with varying modulation across different tissues.

Hypoxia and mTOR: Effects on Ciliary Function and Autophagy

Another molecular pathway affected by hypoxia is the mTOR pathway, which is a member of the PI3K-related kinase family. This pathway consists of two distinct complexes: mTORC1 and mTORC2, both sensitive to rapamycin [139, 140]. Each complex has unique functions and associated proteins that account for its different roles. mTORC1 primarily regulates cell growth and metabolism, while mTORC2 is involved in cell survival and cytoskeletal organization. Hypoxia can disrupt both pathways, leading to various cellular responses [141]. Wang et al. (2015) demonstrated that cilia and autophagy influence each other reciprocally, suggesting that the mTOR pathway is integral to this regulation [142]. Specifically, suppression of mTOR signaling enhances autophagy, promoting ciliary elongation, while increased mTOR activity shortens cilia by inhibiting autophagic processes (Figure 2.7). These findings emphasize the complex relationship between cellular pathways and ciliary dynamics, indicating that mTOR plays a key role in the interaction between autophagy and ciliogenesis [142]. Recent research supports this complex relationship. Morleo et al. (2023) proposed that primary cilia serve as specialized sites for the control of autophagy, with selective autophagic degradation impacting both cilia formation and elongation [143]. However, the impact of autophagy on ciliogenesis appears to be context-dependent, as contradictory findings have been reported [143]. The complexity of this interaction is further underscored by emerging evidence implicating mitochondria and lysosomes in ciliary regulation, although their precise roles remain unclear [144-146].

The increasing number of genes associated with both ciliopathies and autophagy regulation highlights the broader implications of this interaction in diseases [147]. As suggested by Morleo et al., targeting autophagy could offer therapeutic potential for ciliopathies, and ciliogenesis itself might serve as a useful biomarker for therapies aimed at modulating autophagy in diseases that extend beyond ciliary dysfunction [143].

Evidence indicates a connection between the mTOR and HIF-1α pathways. Sakamoto et al. (2014) demonstrated that crosstalk between MT1-MMP and mTOR inhibits FHI-1, which leads to the activation of HIF-1α [148]. The activation of the PI3K/mTOR pathway increases the levels of the HIF-1α protein by enhancing its translation, rather than affecting mRNA expression [149]. Furthermore, overexpression of Rheb activates

mTOR, which boosts HIF transcriptional activity under hypoxic conditions; this effect can be reversed by rapamycin [150-152]. Additionally, mTOR regulates HIF-1α through CAP-dependent translation mechanisms, which influence its stabilization and synthesis [150].

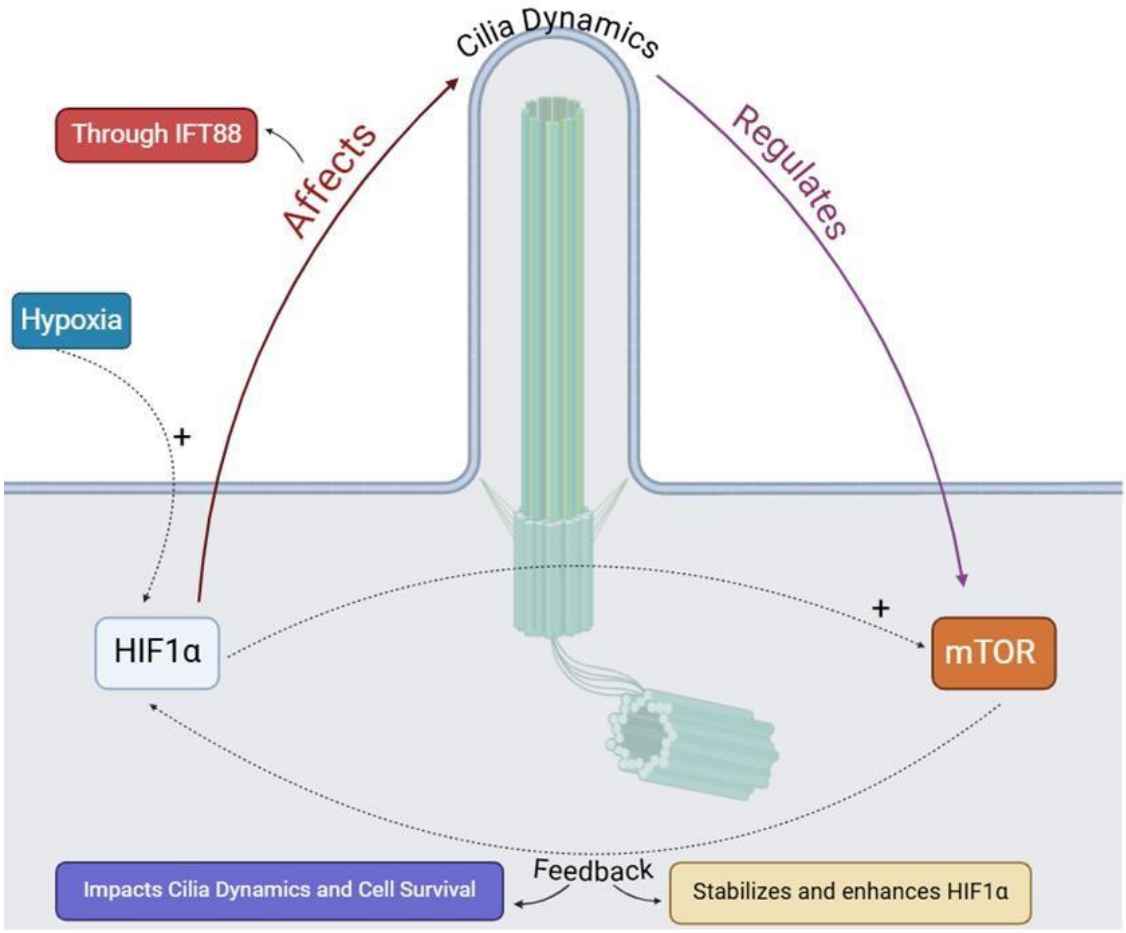

Figure 2.7. Interaction between hypoxia, mTOR pathway, and cilia dynamics. Hypoxia activates HIF1α, which influences ciliary function and cell survival. The mTOR pathway plays a stabilizing role by enhancing HIF1α activity in response to cellular stressors. Arrows indicate regulatory interactions, with feedback mechanisms modulating the overall dynamics of the cilia and cellular adaptation.

2.4. Potential Therapeutic Interventions

With increasing evidence highlighting the role of ciliopathies in liver diseases, therapeutic strategies focused on preserving primary cilium integrity, especially regarding biliary health, could greatly improve native liver survival and enhance outcomes of liver transplantation. This area presents excellent opportunities for further research. Potential strategies include the use of senolytics and treatments that stabilize cilia to maintain their structure and function during and after liver transplantation [126, 153].

Defects in ciliary structure or function led to decreased intracellular calcium and increased cAMP levels, making these pathways promising therapeutic targets [65]. In a PCK rat model of polycystic kidney and liver disease, Masuyk et al. (2007) demonstrated that octreotide, a somatostatin analog, reduced cAMP levels in cholangiocytes, which suppressed liver cyst growth and fibrosis [154, 155]. In polycystic kidney disease, targeting ciliary dysfunction through cAMP modulation, growth factor inhibition, microRNA-17 inhibition, and mTOR inhibition has demonstrated therapeutic potential [156]. Increased cAMP levels and abnormal vasopressin receptor (V2R) signaling lead to cyst expansion. Tolvaptan, a V2R antagonist, effectively reduces cAMP levels and slows disease progression, although it has hepatotoxic limitations [157, 158].

Therapeutic strategies targeting the mTOR pathway in polycystic kidney disease (PKD) have produced inconsistent results. Deletion of the PKD1 or PKD2 gene increases cilium length and activates the mTOR pathway [159]. However, mTOR inhibitors like sirolimus and everolimus have demonstrated limited clinical success. While these treatments resulted in modest reductions in kidney volume, they had minimal effects on renal function and were associated with significant adverse effects [160].

PC function as key sensory organelles, housing G protein-coupled receptors, receptor tyrosine kinases, and ion channels, and their localization is controlled by a diffusion barrier in the transition zone, making them potential therapeutic targets [161, 162]. Their structure dynamically regulates cell proliferation and differentiation, and ciliogenesis plays a crucial role in these processes. Modulating ciliogenesis in non-tumor cells may help regulate differentiation under pathological conditions, thereby reducing susceptibility to disease [163].

AURKA inhibits ciliogenesis by phosphorylating histone deacetylase 6 and is overexpressed in various cancers. Targeting AURKA may suppress tumor proliferation by promoting ciliogenesis, with compounds such as iCRT14, bexarotene and alisertib identified as potential inhibitors [161, 164]. Additionally, PC is crucial for Hh signaling, affecting liver fibrosis and regeneration through smoothened translocation [165, 166]. Promising therapeutic targets include USP8, the USP8-Trichoplein-AurA pathway, and USP54, which may enhance ciliogenesis and inhibit tumor progression, particularly in colorectal cancer [165, 167]. Advanced tools, such as transcriptomics, mass spectrometry, and genome editing, are crucial for elucidating these pathways and expanding therapeutic possibilities.

PC has become a significant therapeutic target in cancer due to its role in critical processes, such as adaptation to hypoxia, resistance to apoptosis, autophagy, angiogenesis, metabolic reprogramming, and migration, all of which contribute to metastasis [153, 168]. However, the role of PC in cancer progression is complex and varies across different tumor types. This variation is likely influenced by tissue-specific ciliation patterns and the effects of the tumor microenvironment on cilia dynamics. Several anticancer compounds have been developed to target cilia dynamics. For instance, alisertib inhibits AURKA to prevent ciliary disassembly, while vinblastine disrupts microtubules and reduces ciliation [168]. Additionally, as signaling hubs, primary cilia are targeted by drugs such as sonidegib [169] and vismodegib [170], which inhibit Hh signaling pathways.

PC interacts with HIFs and the tumor microenvironment. HIF inhibitors, such as belzutifan, which specifically target HIF-2α, have demonstrated promising results in cancers related to von Hippel-Lindau syndrome. These inhibitors work by preventing the accumulation of non-hydroxylated HIF-2α under hypoxic conditions. By inhibiting HIF-2α, they reduce angiogenesis, cell proliferation, and tumor growth, highlighting the potential of targeting pathways associated with low-oxygen levels [36]. In addition, primary cilia (PC) play a crucial role in regulating immune responses and vascular permeability, which influences both metastasis and the outcomes of cancer therapies. When primary cilia are absent, the expression levels of ZO-1, a protein essential for tight junctions, are decreased. This reduction results in disorganization of intercellular junctions and an increase in endothelial permeability. Furthermore, many anti-cancer drugs unintentionally affect the structure and function of cilia. This highlights the importance of evaluating the impact of these drugs on ciliary dynamics to optimize cancer treatments and minimize resistance mechanisms [171].

2.5. Conclusion

In summary, these findings enhance our understanding of the effects of hypoxia on cilia at the molecular level, providing new avenues for research and valuable insights into ciliary dysfunction associated with various diseases. Although few studies have focused specifically on the liver, we believe that this area has significant potential for exploration. While existing research suggests a connection between hypoxia, the HIF pathway, and other molecular pathways that regulate cilia, the evidence is fragmented and occasionally contradictory. This highlights the need for further investigation to clarify these complex interactions.

The potential of primary cilia as a key focus for research on liver pathologies and complications following transplantation is promising, particularly because hypoxia and cilia-associated pathways, such as AURKA, HIFs, and mTOR, play critical roles. Improving our understanding of the relationship between cilia dynamics and hypoxic signaling could lead to innovative therapeutic strategies to alleviate liver dysfunction and improve transplant outcomes.

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Chapter 3

Aims of the Thesis

Aims of the Thesis

Biliary atresia (BA) is a progressive neonatal hepatobiliary disorder characterized by extrahepatic bile duct obstruction and intrahepatic cholangiopathy. While timely surgical intervention with portoenterostomy can improve outcomes, the disease often advances to cirrhosis, thus making BA the main indication of paediatric liver transplantation. Deciphering the still unclear nature of BA may enable the development of novel therapeutic options.

This work is part of the research line about the role of arterial disturbance in BA aetiology. Previous studies of our group observed in the liver of BA patients, medial layer thickening of hepatic arterial branches; immunohistochemical features of vascular endothelial growth factor suggestive of arterial/arteriolar and cholangiocyte hypoxia; overexpression of angiopoietins, involved in pericyte recruitment to arterial wall and presence of a gene expression pattern of hypoxia-ischemia in the liver, suggesting that BA aetiology may include an ischemic cholangiopathy.

From a histopathological perspective, BA displays ductular reaction (DR) and precocious fibrogenesis. In BA, DR arises from the proliferation of progenitor cells located in the Hering canal within the Space of Mall in the periportal region. These progenitor cells, including hepatoblasts, can differentiate into biliary and hepatocytic lineages, contributing to both ductular reaction and liver regeneration. This cellular niche also includes mesenchymal cells, inflammatory cells, and myofibroblasts, which play a key role in the fibrogenesis associated with DR. Although some biliary structures in BA may resemble the 'ductal plate malformation' (DPM) characteristic of cholangiociliopathies, current evidence suggests that DR is primarily driven by progenitor cell activation rather than mature hepatocyte transdifferentiation. Primary cilia are cholangiocyte membrane organelles that regulate bile secretion according to bile flow/composition. Only patients with the syndromic BA variant present mutations associated with cholangiociliopathy. However, ciliopathy happens frequently in BA, being probably acquired given that it occurs in animal models of BA induced by both virus and toxin. Stabilization of HIF deranges cilia, since ciliogenesis and HIF metabolism are regulated by common pathways. Studies confirm the relation between hypoxia and ciliopathy in several organs but still lack regarding the liver.

Since hypoxia leads to ciliopathy, our hypothesis is that in BA it is correlated with and acquired cholangiociliopathy, prompting continuance of investigation by deepening

histologic evaluation, and undertaking molecular studies in ultra-frozen liver samples of the same patients and additional infants.

Thus, the primary goal of this Thesis is to study the presence of an ischemic cholangiopathy in the hepatobiliary structures of BA patients through analysis of the HIFs' nuclear positivity. Furthermore, this work aims to define the association of the presence of cholangiocyte cilia abnormalities with ischemic cholangiopathy in BA (HIF1alpha nucleus positivity in cholangiocytes).

Therefore, to successfully achieve these purposes, the main objectives of this work were to:

1. Assess the percentage of nuclear HIF1a positivity stained by immunohistochemistry in relation to the total amount of cells present in each specific hepatobiliary structure in the BA patients;
2. Correlate the values found with clinical and histologic variables that affect the postoperative prognosis after portoenterostomy;
3. Analyze the association between histologic findings and native liver survival (defined as survival without death or liver transplantation) at one and two years following portoenterostomy.
4. Analyze the genetic expression of molecules involved in HIF1a pathway (VEGFA and receptor, VCAM, GSS, GSR, CK19, Caspase 3 and Fibrocystin).

The work carried out to achieve these goals is fully described in Chapter 4. Regarding the association between cilia abnormalities and a possible ischemic cholangiopathy the objectives were:

1. Evaluate the primary cilia of cholangiocytes in liver samples of patients with BA and intrahepatic cholestasis;
2. Assess by digital image analysis the ciliary features in terms of their presence, shape and length;
3. Compare the features of cilia in BA patients with and without ischemic cholangiopathy, defined by the presence or absence of HIF-1α nuclear positivity in cholangiocytes;
4. Correlate the presence of the previously mentioned variables with clinical-histological data associated with the post-operative prognosis in BA patients.

The data regarding this work is presented in Chapter 5. Overall, the accomplishment of these goals will represent a novelty in BA etiology, given the absence of similar studies and the originality of the hypothesis of ischemic cholangiopathy related to a ciliopathy in BA, the results will be an important contribution to this field of knowledge.

Chapter 4

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

This chapter was published in:

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Abstract

Background: Biliary atresia is a neonatal disease characterized by extrahepatic ducts 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.

Keywords: Ischemic cholangiopathy, Biliary atresia, Neonatal cholestasis, Oxidative stress.

4.1. Introduction

Biliary atresia (BA) is a neonatal disease involving extrahepatic biliary 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 upregulation 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-1alpha pathway activation in the liver and porta hepatis of patients with BA.

4.2. Methods

4.2.1 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 °C in RNA holder (*BioAgency Biotecnologia*, São Paulo, Brazil). Liver specimens from the control group were also collected and stored at −80°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 samples. 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.

4.2.1.1. 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].

4.2.2. Immunohistochemical method

Paraffin-embedded samples were microtome-sectioned into 5 μm 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˚C. Immunohistochemical staining with recombinant HIF-1alpha 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-1alpha signal and a non-diseased liver control sample (Figure 4.1 A and B).

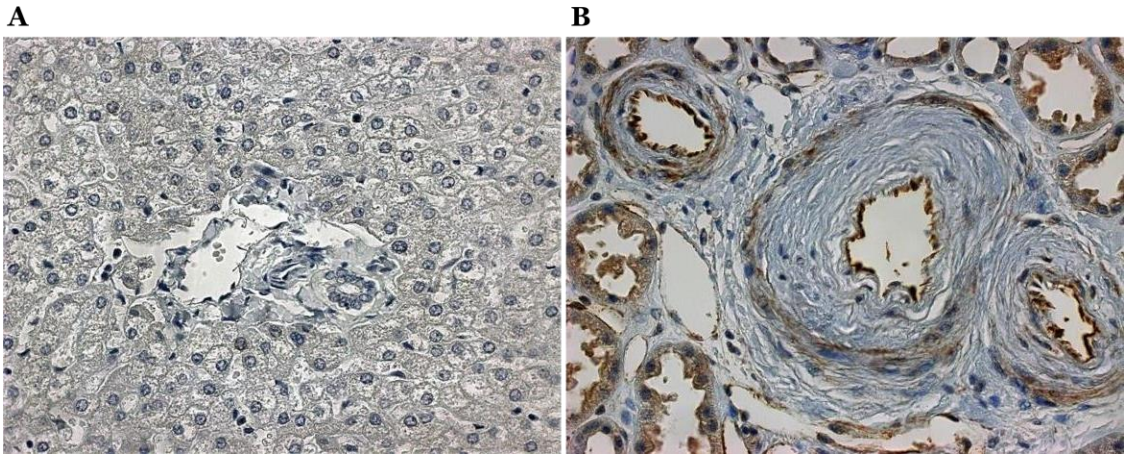

Figure 4.1. Liver samples used in this study as "on-slide" external controls for HIF-1alpha staining. A- liver specimen of a patient without hepatopathy; B- positive control: kidney showing HIFn-positivity in arterial vascular endothelium. Magnification 200x, 400x.

4.2.2.1. Immunohistochemical analysis of HIF-1alpha positivity

HIF-1alpha 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-1alpha positivity were determined by consensus among three liver histopathology experts blinded to diagnosis.

4.2.2.2. 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 picrosirius red. A histopathologic protocol comprising 26 qualitative categorical variables was used (Table 4.1).

Table 4.1. Histopathologic variables evaluated in the liver of studied patients

Variables (categories: yes/no)
1. Portal tract expansion
2. Ductular reaction
3. Ductular reaction in portal tracts
4. Ductular reaction in portal interface
5. Ductular reaction (focal) in parenchyma
6. Bile duct paucity
7. Porto-portal bridging fibrosis
8. Portal-central bridging fibrosis
9. Cirrhotic nodules
10. Biliary structures resembling ductal plate malformation
11. Mini ductal plates
12. Vascular agglomerates (hyperplasia) in portal tracts and fibrous septa
13. Vascular agglomerates (hyperplasia) in subcapsular area
14. Bile plugs in bile ducts
15. Bile plugs in canaliculi
16. Bile pigment in hepatocytes
17. Bile pigments in Kupffer cells
18. Prominent arterial medial layers (qualitative assessment)
19. Presence of pseudo-acini
20. Giant cell transformation
21. Feathery degeneration
22. Biliary infarcts
23. Macrovesicular steatosis
24. Microvesicular steatosis
25. Hepatocellular necrosis
26. Intracellular (cytoplasmic/nuclear) inclusions

4.2.3. Gene expression analysis by qPCR

Total RNA was extracted from liver specimens using AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Carlsad, CA) following the manufacturer's instructions. For cDNA synthesis, reverse transcription of 1 μg 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 Table 4.2. 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-ΔΔCT method. CT values were normalized by the housekeeping gene ribosomal 18S.

Table 4.2. Primer sets used for qPCR gene expression analysis

Application	Primer	Forward	Reverse
Housekeeping	***Ribosomal 18S***	AAACGGCTACCACATCCAAG	CCTCCAATGGATCCTCGTTA
Hypoxia	***HIF-1alpha***	CATCCATGTGACCATGAGGA	GAATGTGGCCTGTGCAGTG
Apoptosis	***Caspase 3***	TTTTTCAGAGGGGATCGTTG	CGGCCTCCACTGGTATTTTA
Angiogenesis	***VEGFA***	GGGCAGAATCATCACGAAGT	ATCTGCATGGTGATGTTGGA
	VEGFR2	GCTTGGACAGCATCACCA	CCAAGCCAAAGTCACAGATTT
Ductular reaction	***CK19****	GGGCAACGAGAAGCTAACC	GGTACCAGTCGCGGATCTT
Inflammation	***VCAM1***	GGACCACATCTACGCTGACA	TCCAGAGGGCCACTCAAAT
Ciliopathy	***Fibrocystin***	AACAGCAGAGAGGACCAGGA	CTCCCATCCAGATCCTTGAA
REDOX status	***GSS***	GCCTCCTACATCCTCATGGA	ACGTGCTTGTTCATCACGAG
	GSR	CAGTGGGACTCACGGAAGAT	AAACCCTGCAGCATTTCATC

Abbreviations: HIF-1alpha – hypoxia-inducible factor-1alpha; VEGFA – vascular endothelial growth factor A; VEGFR2 – vascular endothelial factor receptor 2; CK19 – cytokeratin 19; VCAM1 – vascular cell adhesion molecule 1; REDOX – reduction-oxidation; GSS – glutathione synthetase; GSR – glutathione-disulfide reductase. *Designed by Stathopoulou et al, 2006 [14]

4.2.4. Statistics

Quantitative variables were expressed as mean ± 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 < .05 was accepted as significant. SPSS 27.0 (IBM, UK) was used for data processing and statistical analysis.

4.2.5. Ethics

Written informed consent for the use of histological specimens and clinical data was obtained from the patient´s 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.

4.3. Results

4.3.1 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 Table 4.3. 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.

Table 4.3. Clinical and Laboratory data of patients included in study group

Patient features			Clinical variables			Laboratory variables			
Patient number and diagnosis	Sex	Type of BA*	Age at ExLap (days)	Age at LTx (months)	Age at death (months)	TB (PE)	DB (PE)	TB (3 months post-PE)	DB (3 months post-PE)
BA1	F	3	58	45	80	14.4	9.5	14.4	9
BA2	F	3	110	7	NA	9.5	7.5	Miss	Miss
BA3	M	3	56	NA	8	7.0	5.4	6.4	4.9
BA4	F	3	48	NA	NA	6.3	4.4	4.9	4.4
BA5	M	2	54	NA	11	6.7	5.3	Miss	Miss
BA6	F	3	59	NA	11	5.8	4.2	Miss	Miss
BA7	F	3	93	NA	8	10.0	6.7	25.7	18.8
BA8	M	3	68	NA	NA	12.0	8.8	0.8	0.4
BA9	M	3	69	NA	NA	7.5	6.0	Miss	Miss
BA10	F	2	66	NA	NA	12.9	9.5	0.5	0.1

BA11	F	NA	89	NA	NA	12.4	9.0	Miss	Miss
BA12	M	NA	75	26	26	9.8	7.1	10	7.8
BA13	M	NA	44	NA	6	9.5	6.6	14.7	8.4
BA14	M	NA	45	NA	NA	5.7	4.4	4.1	2.4
BA15	F	3	65	6	6	19.1	14.3	Miss	Miss
BA16	F	NA	52	8	NA	11.3	7.3	10.1	7.2
BA17	F	3	56	48	NA	16.6	11.8	5.9	3.6
BA18	M	NA	92	84	NA	8.6	6.6	4.6	3.4
BA19	F	2	32	NA	NA	4.7	3.5	0.3	0.2
BA20	M	NA	43	NA	NA	9.4	5.8	1.4	1.1
Control group									
IHC1 (A1ATd)	M	*NA*	81	*NA*		*NA*			
IHC2 (INC)	F		35						
IHC3 (INC)	M		78						
IHC4 (INC + TPN)	F		62						
IHC5 (A1ATd)	M		40						

Abbreviations: BA – biliary atresia; ExLap – exploratory laparotomy with trans-operative cholangiography; IHC – intrahepatic cholestasis; A1ATd – alpha-1 antitrypsin deficiency; INC – idiopathic neonatal cholestasis; TPN – total parenteral nutrition; PE – portoenterostomy; LTx – liver transplantation; TB – total bilirubin; DB – direct bilirubin; NA – not applicable; Miss – missing data. Observation – in BA patients. *According to the Japanese Association of Pediatric Surgeons.

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

HIF-1alpha positivity was not detected in any hepatobiliary structure in IHC controls (Figure 4.2A). 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 (Figure 4.2B). 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 in portal tracts including interlobular bile ducts (Figure 4.2B) and proliferative ductules along the portal margins. Cholangiocytes with HIF-1alpha positivity were also abundant in stroma of fibrous septa (Figure 4.2C), 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 (Figure 4.2D).

The fibrous septa emerging from both portal tracts and subcapsular vascular agglomerates (Figure 4.2E), particularly their margins (Figure 4.2E-G), constituted the preferential location of HIF-1alpha-positive cholangiocytes.

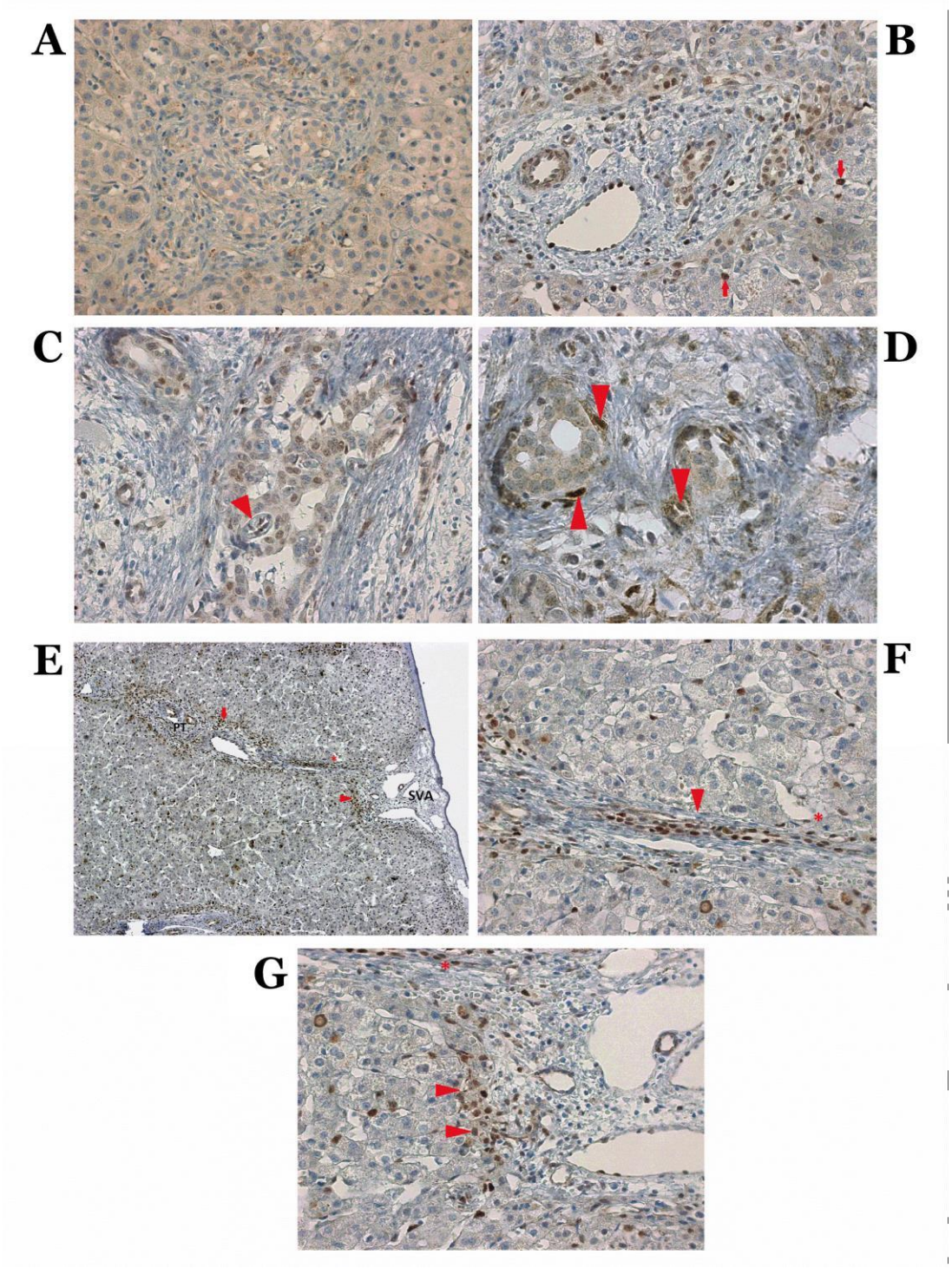

Figure 4.2. HIF-1alpha positivity in the liver of biliary atresia patients and control patients with intrahepatic cholestasis. A- 35-day old IHC control: idiopathic neonatal cholestasis with absent HIF-1alpha nuclear signals in hepatobiliary structures; B- BA patient: portal tract with HIF-1alpha positivity 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). C- 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); D- HIF-1alpha positivity in the endothelium of peribiliary vascular plexus; E- 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); F and G- 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.

4.3.1.2. 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 (Figure 4.3A). HIF-1alpha positivity was also detected in the endothelium of large and medium-sized hepatic artery branches (Figure 4.3B), as well as in inflammatory infiltrates in regions of fibrosis (Figure 4.3C).

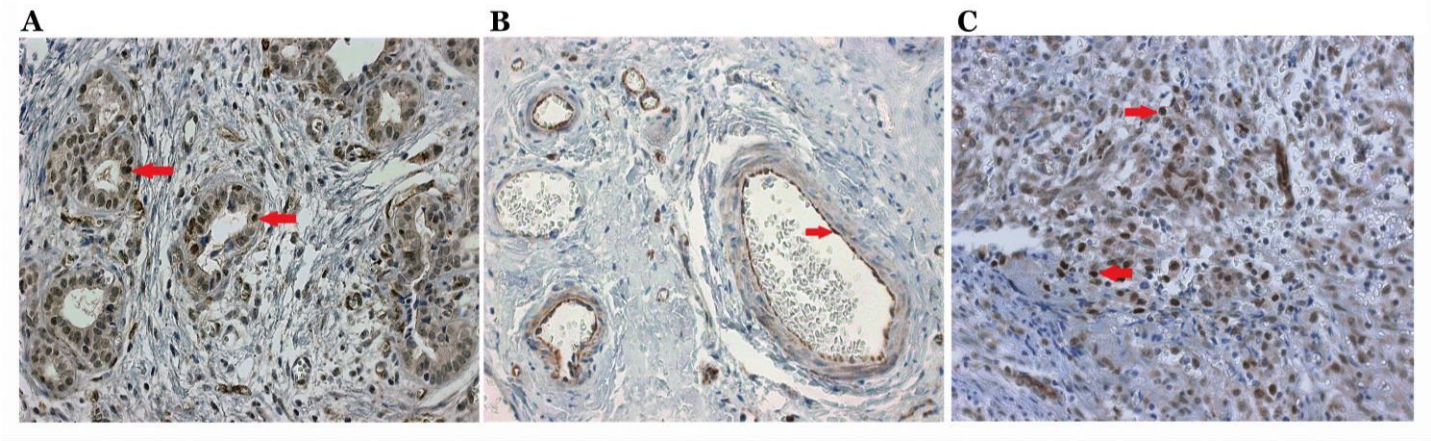

Figure 4.3. HIF-1alpha positivity in the porta hepatis. A- biliary remnants with HIF-1alpha positivity in cholangiocytes; B- HIF-1alpha positivity in the endothelium of large and medium-sized hepatic artery branches; C- HIF-1alpha positivity in cellular infiltrate. Magnification- 100x, 400x.

4.3.1.3. 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 (Table 4.4).

Table 4.4. Correlation between HIF-1alpha nuclear signal status in intrahepatic biliary epithelium and clinical variables age and bilirubin serum levels at portoenterostomy and 3 months post-portoenterostomy

Clinical factors	HIF-1α nuclear status		p-value
	Positive (n=6)	Negative (n=10)	
Age at portoenterostomy (days)	64.17 ± 9.3	61.6 ± 6.3	.875
TB at portoenterostomy	8.2 ± 1.3	10.4 ± 1.4	.368
DB at portoenterostomy	6.1 ± 0.8	7.5 ± 1	.368
TB 3 months post-Kasai	8.5 ± 2.9	6.6 ± 2.9	.392
DB 3 months post-Kasai	6.1 ± 1.4	4.6 ± 2.1	.243

Abbreviations: TB – total bilirubin; DB – direct bilirubin.
Statistics – Mann-Whitney U Test.

Five out of six (83%) patients with HIF1-alpha 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 (Table 4.3). 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) (Table 4.5). 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.

Table 4.5. Death and native liver survival in patients with positive HIF-1alpha nuclear signal vs. negative HIF-1alpha nuclear signal in cholangiocytes

	Death after portoenterostomy		Native liver survival	
Group	% (Group total)	Pearson Chi-Square	% (Group total)	Pearson Chi-Square
HIFn-P *(n=6)*	66.7%	P = .062	16.7%	p = .182
HIFn-N *(n=10)*	20%		50%	

Abbreviations: HIFn-P – positive HIF1A nuclear signal; HIFn-N – negative HIF1A nuclear signal. Statistics – Pearson Chi-Square test.

4.3.2. 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 HIC 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 (Figure 4.4 and Figure 4.5).

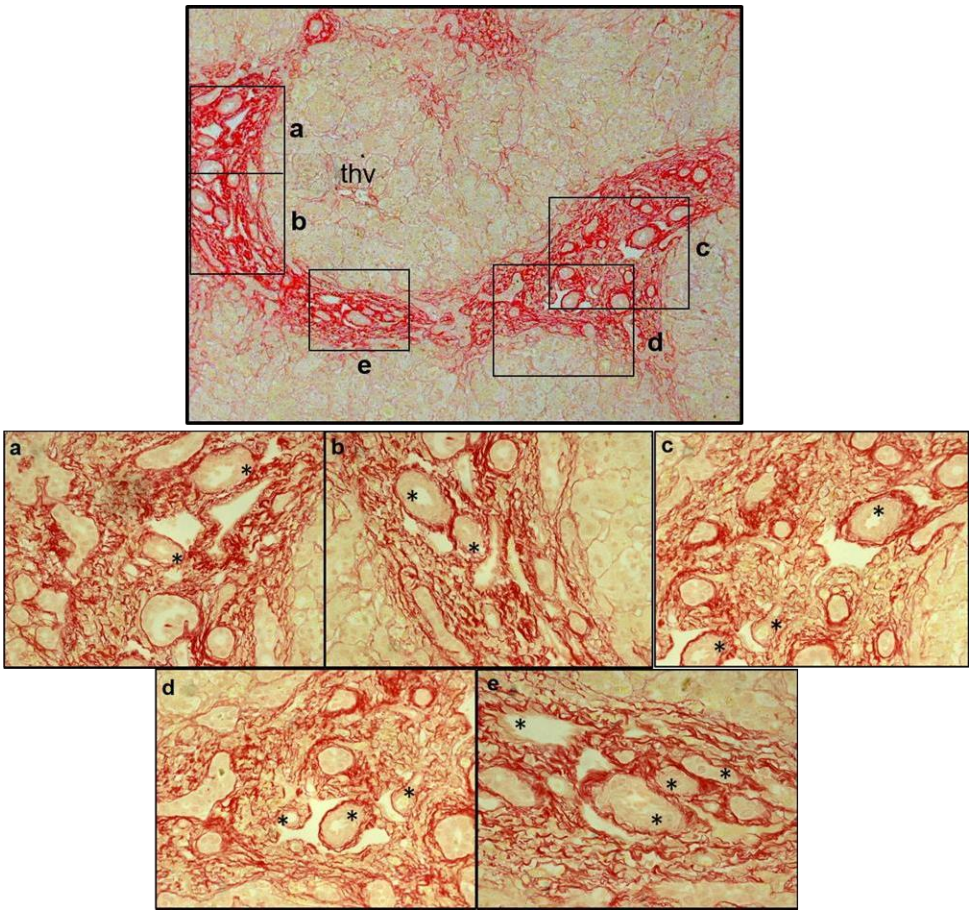

Figure 4.4. Histopathological features of ductular reaction in biliary atresia. Histopathological features in the liver of a patient with biliary atresia (59 days old) showing fibrous septa, presenting a pattern of arteriolar hyperplasia with prominent medial layers. Images a-e showing the arterioles in higher magnification (asterisks). Picrosirius red (magnifications 100x and 400x). Abbreviation- thv- terminal hepatic vein branch.

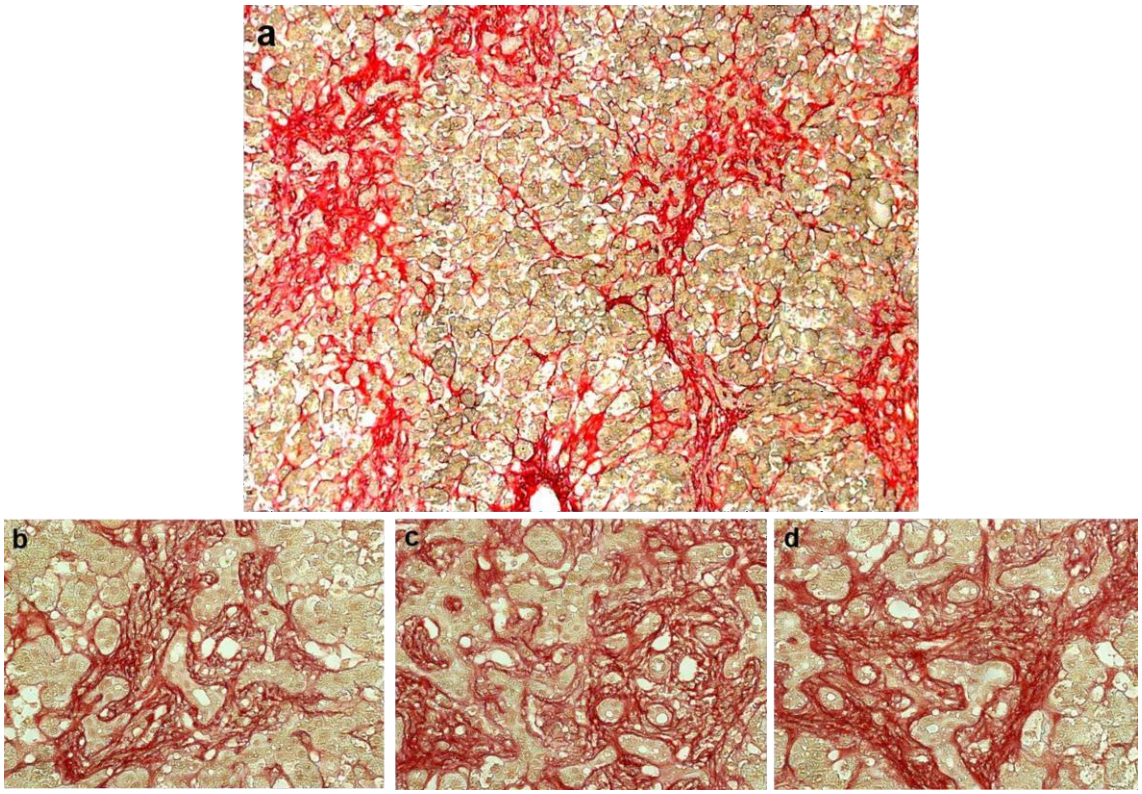

Figure 4.5. Histopathological of ductular reaction in diseased control. Histopathological features of the ductular reaction, including fibrous septa, with absent vascular arteriolar hyperplasia in a diseased control (idiopathic neonatal hepatitis, 35 days old). Picrosirius red (a- magnification 10x; b-d 20x).

4.3.3. 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 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) (Figure 4.6).

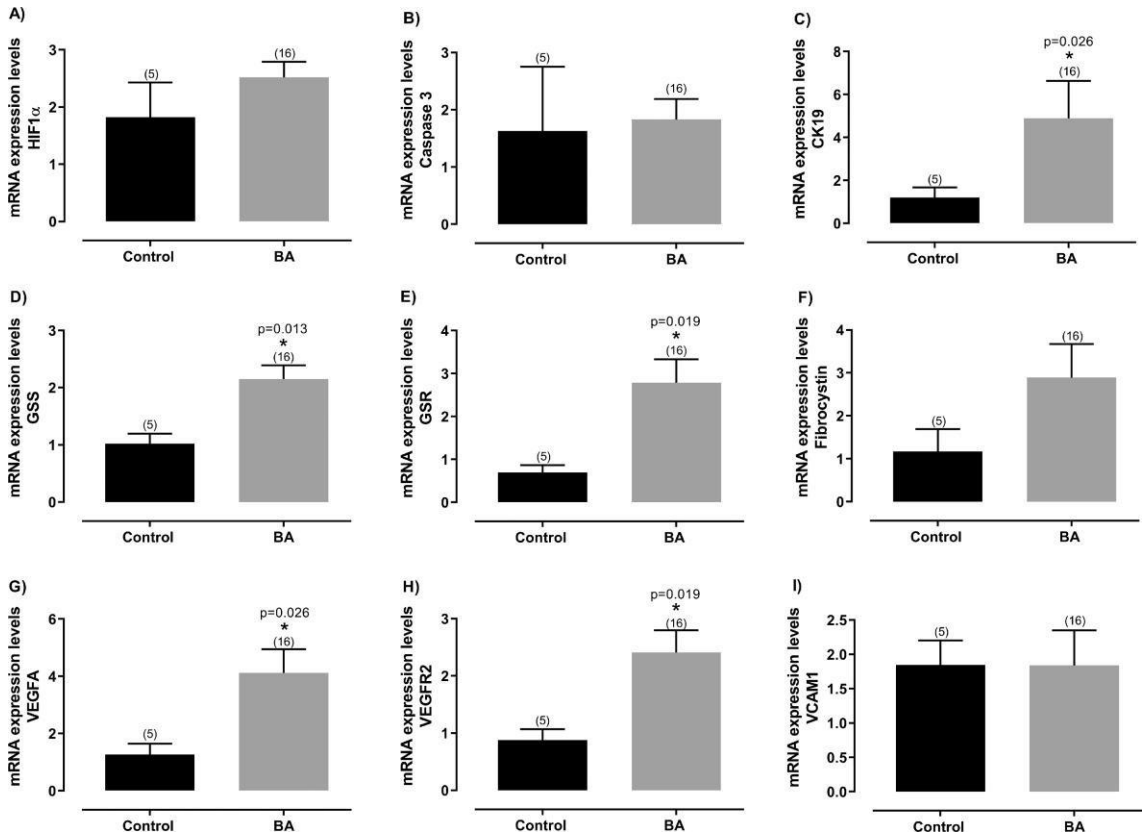

Figure 4.6. Gene expression of molecules involved in metabolic and structural processes affected by the hypoxia-ischemia in the liver. Abbreviations: HIF-1alpha- hypoxia-inducible factor-1alpha; GSS- glutathione synthetase; GSR- glutathione-disulfide reductase; VEGFA vascular endothelial growth factor 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).

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) (Figure 4.7).

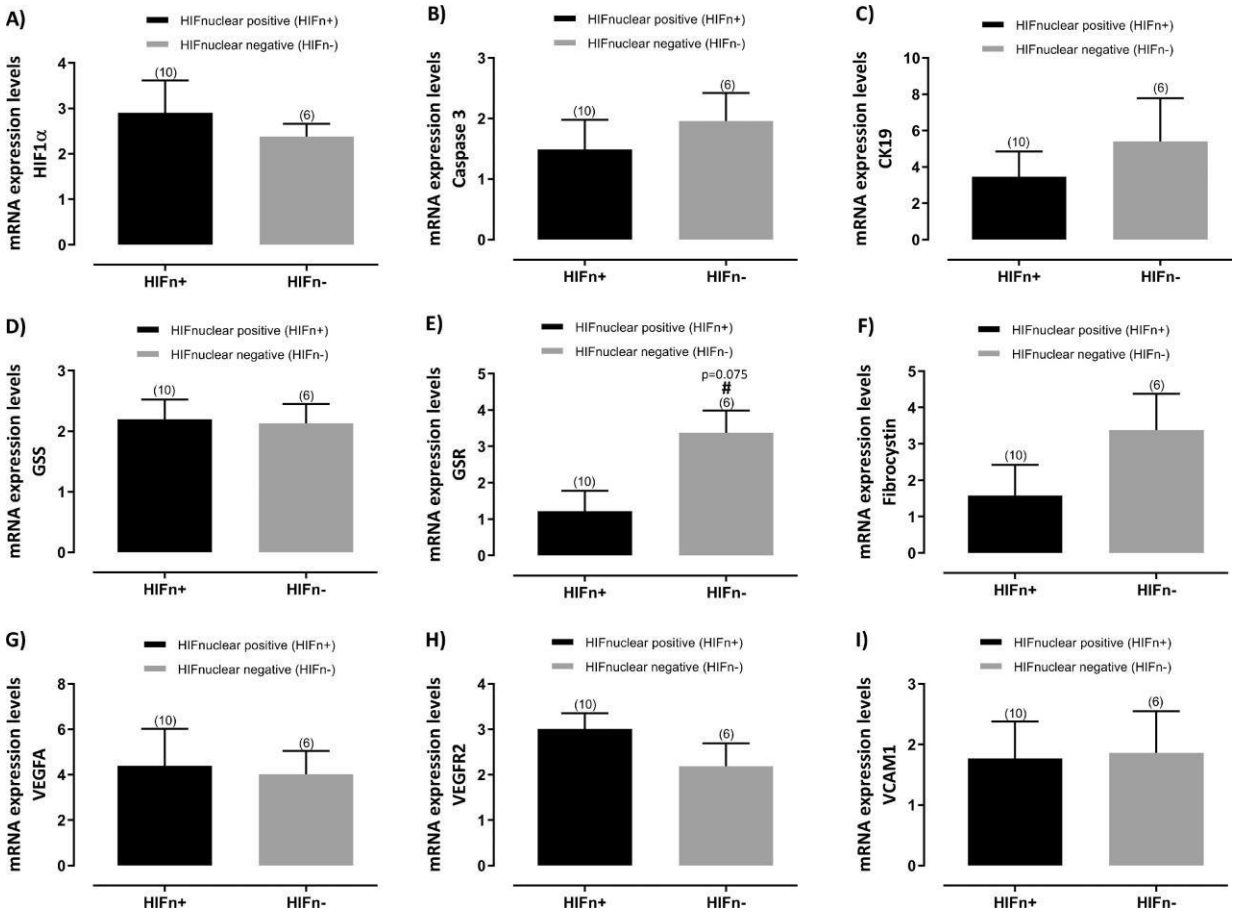

Figure 4.7. Gene expression of molecules involved in metabolic and structural processes affected by the hypoxia-ischemia in the liver of BA patients according to HIF-1alpha nuclear signal status. Abbreviations: HIF-1alpha- hypoxia-inducible factor-1 alpha; GSS- glutathione synthetase; GSR- glutathione-disulfide 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).

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 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 (Figure 4.2).

Endothelial cells of arterioles encircling the bile ducts and representing the branches of PVP were also positive for HIF-1alpha (Figure 4.2). HIF-1alpha activation was also observed in biliary structures displaying features of ductal plate malformations and mini ductal plates (Figure 4.2) [21]. In addition to these findings in liver, 81.8% of the porta hepatis specimens investigated (Figure 4.3) showed HIF-1alpha activation in cholangiocytes of biliary remnants as well as in endothelial cells of hepatic artery branches and inflammatory infiltrate (Figure 4.3).

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 (Figure 4.2) 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 subunits become stabilized, translocate to the nucleus, heterodimerize with the beta subunits, and regulate 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 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 (Figure 4.3), 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 (Figure 4.3E and F). 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, Figure 4.3G) [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 (Figure 4.6G and H). VEGFA secretion affects and is affected by cholangiocyte proliferation, playing a crucial part in the crosstalk 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 Figure 4.6C),

demonstrates the extensive ductular reaction that characterizes BA and supports the presence of ongoing liver fibrogenesis in patients with BA [55]**.** Additionally, we found increased GSS and GSR gene expression in BA patients compared to controls (Figure 4.6E). 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, like the effects caused by toxins biliatresone 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 Figure 4.8 describes the results associated with gene expression in BA patients with or without HIF-1alpha activation and in IHC controls.

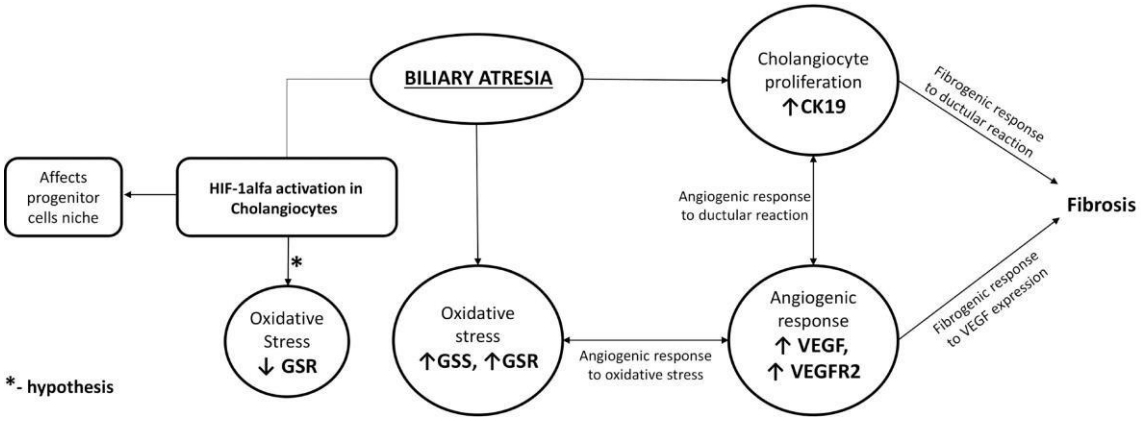

Figure 4.8. Diagram representing the putative relationships between study data. The liver samples of biliary atresia patients showed overexpression of genes associated with ductular reaction and angiogenesis, two interrelated processes that are responsible for fibrogenesis. Molecules involved in REDOX 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.

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 (Table 4.5 and Figure 4.7E).

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.

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Chapter 5

Structural Disruption of Cilia and Increased Cytoplasmic Tubulin in Biliary Atresia – an exploratory study focusing on early postoperative prognosis following portoenterostomy

This chapter was published in:

***Patricia Quelhas**, Rui Oliveira, Sandra Vieira, Carlos Kieling, Jorge dos Santos." Structural Disruption of Cilia and Increased Cytoplasmic Tubulin in Biliary Atresia—An Exploratory Study Focusing on Early Postoperative Prognosis Following Portoenterostomy", Biomedicines, Jan, 13(1), 87, 2025. https://doi.org/10.3390/biomedicines13010087*

Abstract

Introduction: Biliary atresia (BA) is a progressive hepatobiliary disease in infants, leading to liver failure and the need for transplantation. While its etiopathogenesis remains unclear, recent studies suggest primary cilia (PC) disruption plays a role. This study investigates correlations between PC and cytoplasmic tubulin (TUBA4A) alterations with hypoxia in patients with the isolated form of BA, focusing on native liver survival.

Methods: Using qualitative and quantitative digital image analysis of immunofluorescence-stained liver samples, we assessed PC and TUBA4A features correlating these findings with HIF-1α nuclear positivity, clinical–laboratory data, and early native liver survival. Liver samples from fourteen BA patients and six controls with another liver disease were analyzed by digital image analysis, with data evaluated using Spearman's correlation and independent *t*-tests.

Results: HIF-1α positivity in cholangiocytes was observed in 42.8% of BA patients. While the PC ratio per biliary structure (cilia ratio status, CRs) was similar between BA patients and controls, PC length was decreased in BA patients. Cytoplasmic TUBA4A levels were elevated in BA patients. CRs positively correlated with lower cytoplasmic TUBA4A expression and was higher in patients without HIF-1α nuclear positivity. Reduced cilia length correlated with higher bilirubin levels at portoenterostomy. Predictors of early poor prognosis (death or need for transplantation until 1 year of life) included HIF-1α positivity, elevated direct bilirubin levels, decreased cilia length, PC bending, and increased TUBA4A expression.

Conclusions: Reduced PC length, PC bending, and increased intensity of cytoplasmic TUBA4A expression occur in the isolated BA clinical type and negatively impact the early prognosis after post-portoenterostomy. These findings suggest the existence of a disruption in the tubulin transport between cytoplasm and PC. The detrimental effect of HIF-1alpha pathway activation over early native liver survival was confirmed, although independently from PC or cytoplasmic tubulin features.

Keywords: Biliary Atresia; Primary Cilia; Cytoplasmic Tubulin; Hypoxia; Native Liver Survival

5.1. Introduction

Biliary atresia (BA) is a rare, life-threatening disease that starts in infancy and comprises a progressive obstructive cholangiopathy expanding from the extrahepatic biliary tract to the intrahepatic biliary tree [1, 2]. In BA, there is an early extensive ductular reaction and associated fibrosis, which unleashes cirrhosis and chronic liver failure, leading to the need for liver transplantation (LTx), or death, until the second year of life. Presently, the only treatment for BA is a portoenterostomy developed by Morio Kasai. If timely performed, Kasai portoenterostomy enables 5- and 10-year survival rates of around 75% and 67%, respectively [3, 4]. Whenever portoenterostomy is ineffective, post-operative native liver survival (NLS) does not surpass 2 years of life. Around 22% of BA patients reach 30 years of life without LTx given the increased NLS rates after portoenterostomy presently obtained [5]. The occurrence of different clinical forms of BA, including the most frequent variant, called isolated BA, suggests the existence of diverse etiopathogenetic mechanisms for the development of the disease [6,7]. The etiology of BA seems to result from viral infection, toxins, immunogenetic factors, and defects in embryogenesis. Genetic and epigenetic predispositions, associated with environmental factors affecting the mother, are potential triggers for BA [8, 9]. Concerning the effects of environmental factors on the mother, a recent large case-control study indicates an association between prenatal maternal intestinal or genitourinary tract infection and the occurrence of BA in the offspring [10].

Our group, after confirming a progressive medial layer thickening of hepatic arterial branches suggestive of vascular remodeling in patients with isolated BA [11, 12], investigates the role of hypoxia-ischemia affecting the biliary structures as a putative cause of the cholangiopathy. We detected histopathologic alterations and specific gene expression profiles in VEGF, Angiopoietins, their receptors, and HIF-1alpha (HIF-1α) pathways [13-15]. Later we uncovered through immunohistochemistry, a strong HIF-1α positivity in intrahepatic biliary structures of at least 35.7% of patients with isolated BA, involving the portal tracts, portal-parenchymal interfaces, fibrovascular septal and subcapsular areas [16]. Since HIF-1α pathway was found to be activated in cholangiocytes within the portal-parenchymal interface, where is located the hepatobiliary progenitor cells niche, it is conceivable that hypoxia and other processes involved with HIF-1α activation affect liver regeneration and fibrogenesis [17]. These consecutive studies suggest that at least a subset of BA patients present a cholangiopathy attributable to hypoxia [6, 18]. A relevant investigation involving systems analysis integrating high-throughput biological data confirmed a central role of

hypoxia and the HIF-1α pathway activation in the development of BA [19]. One recent research confirmed the presence of hepatic artery medial layer thickening in BA and its detrimental effects on the liver, attributable to hypoxia, relating the vascular disruption with a disorder of the Notch3/Hey1 pathway [12]. Another study, using hypoxic injury over fetal/neonatal extrahepatic bile ducts in sheep, revealed that hypoxia causes luminal narrowing and formation of biliary mucous plugs, a process which, after completion of the fetal/neonatal period, transitions into the adult scarring/fibrosing process, characteristic of BA [20]. Thus, hypoxic injury represents a putative mechanism for the pathophysiology of BA.

Primary Cilia (PC) are solitary luminal projections present in cholangiocyte apical membrane, that function as tunable sensing organelles, and whose permanent loss leads to disease. The structural base of PC, as that of cytoplasmic microtubules, consists of doublets of alpha- and beta-tubulin dimers assembled as closed cylinders that collectively are referred to as axoneme [21, 22]. Sensory and signaling functions of PC are essential for embryonic development, and gene variants that cause loss of PC result in ciliopathies. Primary cilia act as chemoreceptors and mechanoreceptors in the biliary system, likely involved in cell proliferation, senescence, activation of progenitor cell compartment, regeneration, and embryonic developing mechanisms [22, 23]. Concerning BA, PC abnormalities seem to occur in BA patients with or without associated extrahepatic anomalies [24, 25]. A reduced amount of PC in intrahepatic biliary structures in the livers of patients with BA seems to worsen the post-operative prognosis after portoenterostomy [26]. However, as far as we know, alterations in the behavior of the cytoplasmic microtubule Tubulin-alpha 4A (TUBA4A), which are known to be associated with PC disorders [27, 28] have not yet been analyzed in BA.

Given the described alterations of cholangiocyte PC in biliary atresia patients and their effects on the disease prognosis in the scarce references from the literature [26-29] , we performed this study using adequate quantitative image analysis of fluorescent stained samples to 1) confirm specific cholangiocyte PC characteristics, including deciliation, PC length and bending in patients with the isolated form of BA; 2) investigate the effects of ciliary alterations over the 1-year native liver survival after portoenterostomy. And, considering the recognized effects of hypoxia over PC dysfunction and the previous evidence of HIF-1alpha activation in the biliary epithelium of a subset of BA patients, we aimed to correlate the HIF-1α pathway activation in cholangiocytes of BA patients with the primary cilia features, and the 1-year native liver survival. Finally, since the intensity of cytoplasmic TUBA4A expression has not been evaluated in BA

patients, we intended to correlate this variable with PC features and the 1-year native liver survival.

5.2. Materials and Methods

This histopathological study analyzed selected microanatomic structures in the liver of each patient included in a convenience sample of the isolated BA group, compared to controls with another disease, to identify specific histopathological (immunofluorescence and morphometric) behavior patterns involving the variables of interest (Table 5.1 and Table 5.3).

Table 5.1. Compared variables in the present study.

- **Diagnostic (groups)**
  Isolated biliary atresia
  Intrahepatic neonatal cholestasis
- **Clinical–laboratory**
  Age at portoenterostomy (days of life)
  Total and direct-reacting bilirubin serum levels at exploratory laparotomy
- **Prognostic outcome evaluation**
  Age at liver transplantation (days of life)
  Age at death (days of life)
  Cause of liver transplantation
  Cause of death
  1-year native liver survival
- **Immunofluorescence features**
  Subjective (qualitative) method
  HIF-1alpha nuclear positivity in intrahepatic cholangiocytes
  Objective (quantitative, morphometric) methods
  (*portal biliary structures; cells of the portal, or septal/parenchymal interface; and areas of ductular reaction distributed externally to parenchymal zone 1*)
  - HIF-1alpha nuclear positivity (according to the positivity threshold of the control specimen)
  - TUBA4A positivity in primary cilia (according to the positivity threshold of the control specimen):
    - Cilia ratio status (ratio between cilia count/number of bile ducts and ductules analyzed per patient)
    - Cilia length
    - Cilia bending
  - Cytoplasmic tubulin TUBA4A positivity (according to the positivity threshold of the control specimen)
  - DAPI-positive nuclei

5.2.1 Patients and samples

All the patients enrolled in this study underwent exploratory laparotomy between 2005 and 2018 as part of the diagnostic workup for neonatal cholestasis at *Hospital de Clínicas de Porto Alegr*e, Brazil. The inclusion criteria for patients in this study

included infants with isolated BA and diseased controls with intrahepatic neonatal cholestasis of comparable age—submitted to exploratory laparotomy with trans-operative cholangiogram in the process of diagnostic investigation—and patients with BA that maintained postoperative follow-up until the end of the study or died. Exclusion criteria comprised infants with clinical types of BA different from the isolated form, absence of histopathological confirmation of BA through the analysis of biliary remnants in the porta hepatis, and BA patients that did not fulfill the follow-up until the completion of the study.

The study group comprised fourteen patients with BA in whom exploratory laparotomy preceded portoenterostomy, and the diseased control group included six patients with intrahepatic cholestasis (IHC), in whom this invasive diagnostic procedure was necessary to rule out BA (Table 5.3). The diagnosis of BA was confirmed by both exploratory laparotomy with intraoperative cholangiogram and postoperative bile duct evaluation in porta hepatis. Isolated BA was characterized by the absence of biliary atresia splenic malformation (BASM), extrahepatic bile duct 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 cholestasis (n=3), alpha-1 antitrypsin deficiency (n=1), cytomegalovirus hepatitis (n=1), and parenteral nutrition-associated cholestasis (n=1). During the exploratory laparotomy, a wedge liver biopsy was excised from the hepatic segment IV in each patient. In those patients with image evidence of choledochal anatomical blockade, the porta hepatis was extracted. Liver samples were paraffin-embedded and used for immunofluorescence analysis. Preoperative laboratory tests were collected on all the patients, and the surgery was performed if serum hemoglobin levels were adequate for a safe 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.

5.2.2 Clinical-laboratory data collection

Clinical and laboratory data were prospectively collected and stored in a secure databank (Table 5.3). Concerning the laboratory tests for comparisons between groups, the serum bilirubin level was selected as an indicator of NLS after portoenterostomy.

5.2.3 Immunofluorescence staining

Liver paraffin-embedded samples were microtome-sectioned into 5 μm slices, deparaffinized with xylene, and rehydrated in decreasing ethanol concentrations (100%, 95%, and 70%) and distilled water. Since the samples were fixed in formalin, and formaldehyde residues are autofluorescent in the green spectrum, a treatment with 0.02% methanol-peroxidase was performed for 30 minutes at room temperature under constant agitation. Antigen retrievals were executed using Sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) in a pressure cooker for 6 min after boiling.

Samples were permeabilized with PBS 1% Tween for 15 minutes at room temperature and blocked with PBS 10% fetal bovine serum for 1 hour at room temperature. Immunostaining with recombinant HIF-1α (Novus Biological, AF1935; 1:100) and TUBA4A (Santa Cruz Biotechnologies, Sc-23950; 1:100) antibodies was carried out at room temperature for 3 hours. Secondary antibodies (Alexa-568 – A175474, Abcam and Alexa-647 – Ab150115, Abcam, 1:20 000) were stained for 1 hour at room temperature, and nuclei were stained using DAPI, 10 mins at room temperature. A human gallbladder specimen was used as a positive control for the HIF-1α signal, and a human testicle for the TUBA4A signal (Figure 5.1).

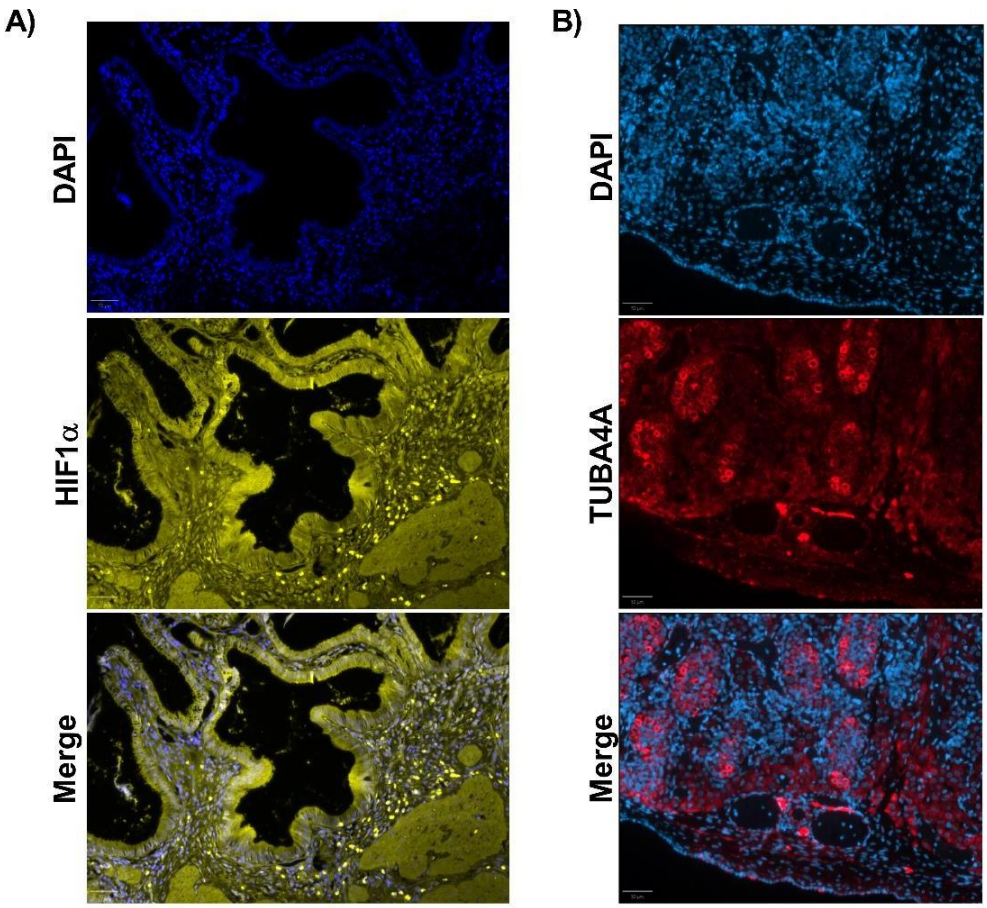

Figure 5.1. Positive controls for primary antibodies. A) Human gallbladder stained for HIF-1αalpha (yellow). B) Human testicle stained for TUBA4A (red). Blue: Dapi ; Red: TUBA4A ; Yellow: HIF-1α . Magnification: 40x.

5.2.4 Image analysis

5.2.4.1. Qualitative analysis

An initial qualitative analysis of TUBA4A and HIF-1α labeling was performed by the authors RCO and JLS, with expertise in liver histopathology, separately and after under consensus. Concerning both HIF-1α and TUBA4A, the distribution of their staining was observed in the cholangiocytes according to our previous study [16]; this included within the portal tracts, at the portal–parenchymal interface, in fibrovascular septa and subcapsular areas, as well as in the ductular reaction spreading to the parenchyma. HIF-1α positivity in cholangiocytes was defined as the strong nuclear-positivity exclusively in biliary structures. Although HIF-1α positivity occurred in hepatocytes with varying parenchymal zonal locations and intensities, we did not analyze these data for the present study. In the case of TUBA4A, the location of positivity was classified as cytoplasmic, apical, and ciliary.

5.2.4.2. Quantitative analysis

HIF-1α nuclear positivity analysis

The analysis of fluorescence images was conducted using QuPath 0.4.4 software [29], following a standardized protocol to ensure reproducibility. To define a positivity threshold for HIF-1α staining, a positive control (human gallbladder, large cholangiocytes) was used (Figure 5.1A). Nuclei were identified and isolated using the DAPI channel, and their intensities in the HIF-1α channel were quantified using the 'Positive Cell Detection' tool in QuPath ('Intensity Threshold Parameters: Nucleus DAPI mean'). Subsequently, quantifications (mean intensity per nucleus) were exported (QuPath: 'Measure – show detection results'), and a histogram of the average intensity per nucleus was generated. This histogram revealed two distinct peaks corresponding to positive and negative populations, with the threshold set at the midpoint between these peaks to differentiate the two groups.

Portal tracts, portal-parenchymal or septal-parenchymal interfaces and areas of ductular reaction extending externally to parenchymal zone 1 were selected as regions of interest (ROIs). These areas were manually delineated using QuPath's annotation tools. The 'Positive Cell Detection' tool in QuPath was then applied to count HIF-1α – positive nuclei. Cells were categorized into two groups – negative or positive, based on the predefined threshold.

TUBA4A Analysis – Cytoplasmic Expression

Cytoplasmic expression for TUBA4A was conducted using QuPath 0.4.4 software. To define the positivity threshold, a positive control for TUBA4A staining (human testicle) was used (Figure 5.1B).

Bile ducts, ductules and interface structures were selected and, through 'Positive Cell Detection' tool in QuPath ('Intensity Threshold Parameters: Cytoplasm TUBA4A mean'), positive cells in cytoplasm were counted and divided into 2 categories: low expression and high expression for TUBA4A. Cells were isolated using DAPI, and their intensities in the TUBA4A channel were quantified. Subsequently, quantifications (mean intensity per cell) were exported (QuPath: 'Measure – Show detection results'), and the histogram of the average intensity per cell was observed, revealing two peaks (positive and negative). The value between these peaks was set as the positivity threshold and subsequently divided into the groups low and high expression.

TUBA4A Analysis – Cilia Quantification

For cilia analysis, at least 5 images per sample were acquired with a 40x magnification (Axio Imager Z2 Microscope, Zeiss, Germany). Subsequently, the CiliaQ v0.1.4 plugin [30] from the image analysis program ImageJ was used to quantify and calculate the length and bending of cilia in cholangiocytes per image. The plugin presents three steps for image segmentation and processing. In the first step of the workflow, using the CiliaQ v0.1.2 Preparator, the Hysteresis Threshold was applied to segment the fluorescence channel labelling TUBA4A (cilia), and in the second step (CiliaQ v0.0.3 Editor), everything within the threshold that was not cilia (labeling debris) was discarded. Finally, in the CiliaQ v1.4 step, cilia were quantified, and the length and bending of each cilium was measured. Given that in BA samples there is a larger number of biliary structures, mainly in portal-parenchymal interface, ductular reaction and fibrovascular septa, to calculate the total number of cilia per sample, a ratio of the total number of cilia to the total number of ducts/ductules analyzed, called cilia ratio status (CRs), was computed (Figure 5.2).

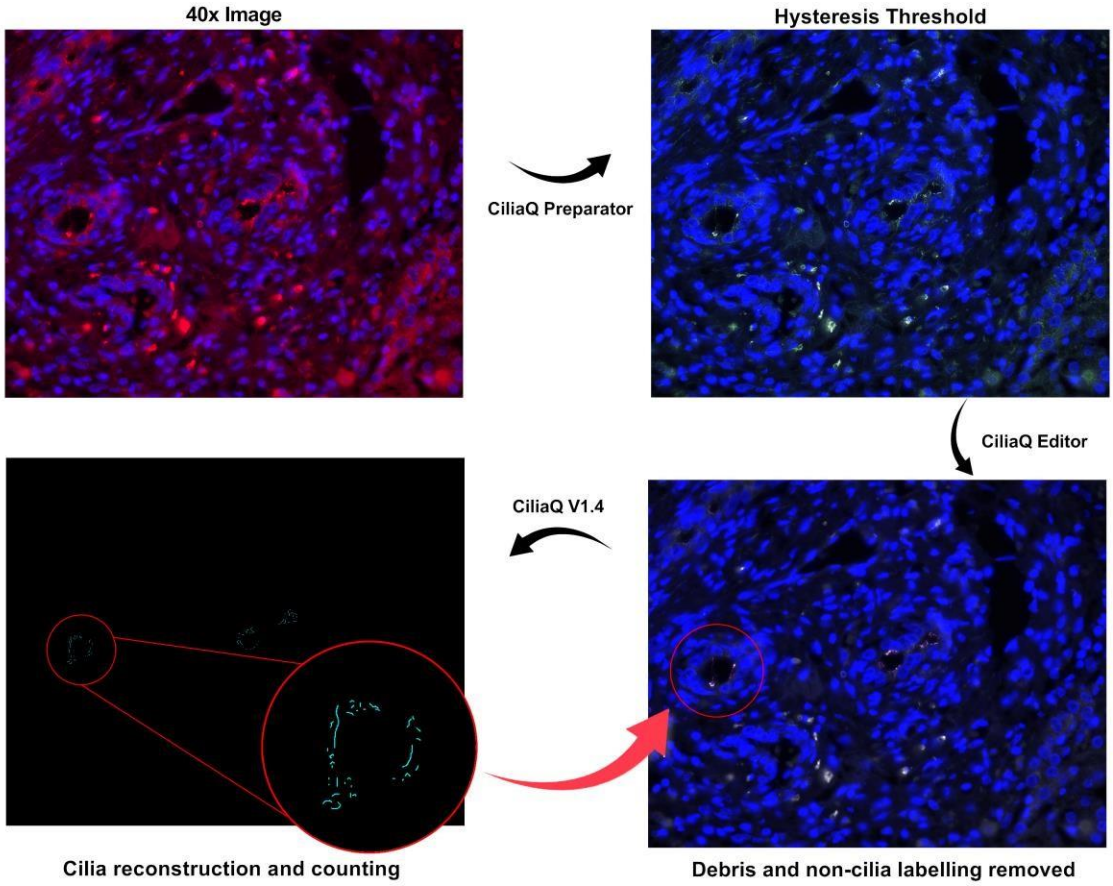

Figure 5.2. Schematic resume of CiliaQ plugin workflow. Blue – Dapi; Red – TUBA4A; Green – Processed Cilia by the program; Purple: Final cilia, after debris removed. Magnification: 40x.

5.2.5 Correlation of Image Analysis Data with Postoperative Outcomes: Death, LTx, and Native Liver Survival after Portoenterostomy

To evaluate the impact of cholangiocyte cilia characteristics (presence/absence, length, and bending) and cytoplasmic TUBA4A expression on the outcomes following portoenterostomy (Figure 5.3), we focused on the 1-year native liver survival rate as the primary outcome measure. Spearman correlation analyses were conducted to explore associations between clinical data and image analysis results to provide insights into how these factors may influence the early postoperative prognosis.

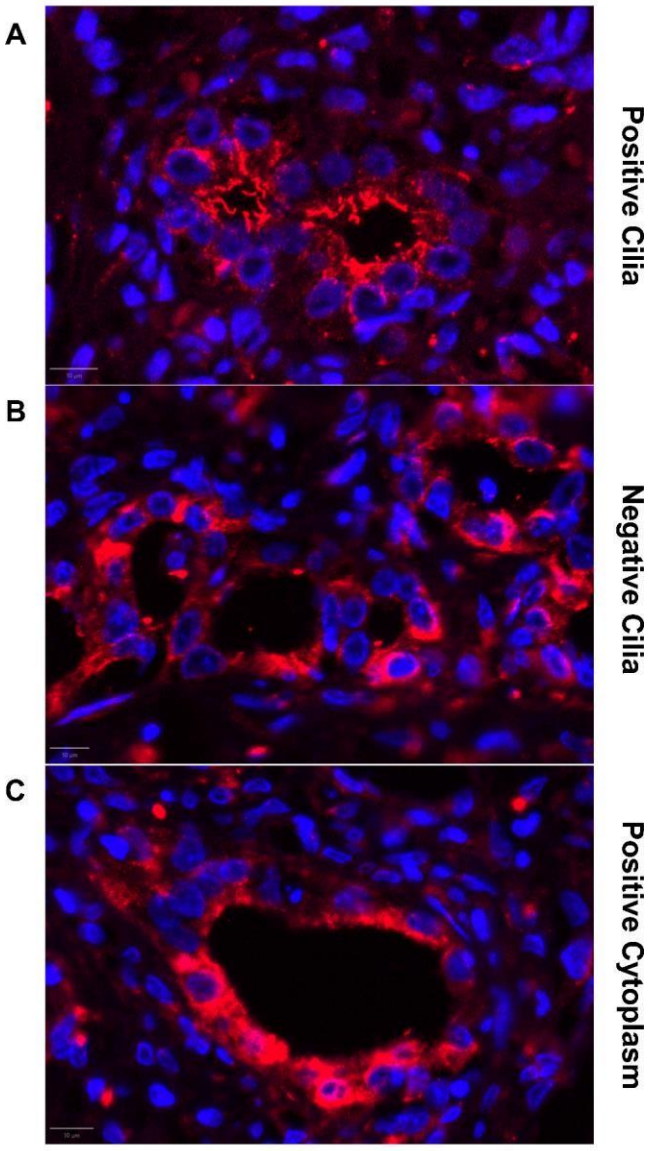

Figure 5.3. Representative images illustrating the microanatomical structures and staining patterns analyzed in this study. These images highlight key features relevant to the investigation, showcasing the structural organization and specific staining used for detailed analysis. A) Representative biliary duct presenting cilia in cholangiocytes; B) Representative biliary duct without cilia in cholangiocytes; C) Representative biliary duct with cholangiocytes positive for Tubulin in cytoplasm (Blue – DAPI; Red – TUBA4A, Magnification: 63x).

5.2.6 Statistics

Quantitative variables were expressed as individual data points and mean ± SD and categorical data were described as frequencies and percentages. The Shapiro-Wilk and Levene tests were performed to assess the normality of the quantitative variables, and all were found to follow a normal distribution. Therefore, an independent student's t-test was used to compare the groups. The Spearman correlation was applied to correlate quantitative and qualitative variables. The statistical power of significant results was calculated using Cohen's d, with data interpretation according to the Table 5.2. A two-tailed p-value < 0.05 was accepted as significant. Statistical Package for the Social Sciences (SPSS, version 27.0, IBM Corp., Armonk, NY, USA) was used for data processing and statistical analysis, and GraphPad Prism 8 for graph images and processing.

Table 5.2. ***Cohen's effect size.*** Interpretation of the effect size statistics according to Hopkins (2002)

Magnitude of the difference	Effect size (d Cohen)
Trivial	0.001|—
Small	0,2 |—
Moderate	0,6 |—
Large	1,2 |—
Very large	2,0 |—
Nearly perfect	4,0 |—
Perfect	∞ |—

Reference: [47]

5.2.7 Ethics

This study was approved by the Research Ethics Committee at the Hospital de Clínicas de Porto Alegre (HCPA), Brazil (project: Arteriopathy in Biliary atresia and its relation with the postoperative prognosis after portoenterostomy, number 130030) and performed by the ethical standards outlined in the Declaration of Helsinki. Informed consent for using biological specimens and clinical data of the infants was obtained from parents or legal guardians. Information from the Data Bank used in this study was pseudonymized to protect the privacy of patients and families. The liver samples were stored in the "Biorepository of Biological Specimens in Pediatric Hepatology" of the Center of Experimental Research (CPE) of HCPA, respecting ethical and methodological aspects. Eventually, this study was approved by the board of the Foundation for Science and Technology (FCT) under the European/Portuguese

collaboration project Portugal 2020 (Title: The role of Ischemic cholangiopathy in conditions of hepatic dysfunction, Project number 02/SAICT/2017 - IsChoHep FCT FEDER).

5.3. Results

5.3.1 Clinical and laboratory data of the BA patients

Liver tissue samples of 20 patients were assessed: 14 with BA and 6 with IHC (control group). The demographic and clinical characteristics of patients and controls are presented in Table 5.3. At the time of surgery, age ranged from 32 to 110 days (mean 62.7 ± 21.4) in BA patients and from 35 to 78 days (mean 52.7 ± 17.1) in controls, with no significant difference between the groups. During the total follow-up period (2005 to 2018), five patients underwent LTx, and five patients died, four of them without having undergone LTx. Age at LTx ranged from 3.8 to 85.7 months (median 46.8 months). Age at death with the native liver ranged from 5.5 to 9.5 months (median 8.3 months), reflecting a native liver survival of less than one year. Nine BA patients survived until the end of the study, 5 of them with their native liver. Regarding bilirubin serum levels at portoenterostomy, total bilirubin (TB) ranged from 4.6 to 19.1 mg/dL (mean 9.6 ± 4.1), and direct bilirubin (DB) ranged from 3.5 to 14.3 mg/dL (mean 7.1 ± 3.1).

Table 5.3. Demographic and clinical characteristics of patients and controls.

PATIENT ID	DIAGNOSIS	AGE PE (days)	AGE LTX (days)	AGE DEATH (days)	CAUSE OF LTX	CAUSE OF DEATH	TOTAL BILIRUBIN (mg/dl)	DIRECT BILIRUBIN (mg/dl)
BA1	Isolated Biliary Atresia	110	118	*Alive*	Liver Failure	*Alive*	9.5	7.5
BA2		56	*No LTx*	208	*No LTx*	Cirrhosis; liver failure; hemorrhage	7	5.4
BA3		48	*No LTx*	*Alive*	*No LTx*	*Alive*	7.8	3.5
BA4		54	*No LTx*	285	*No LTx*	Cirrhosis; liver failure	6.7	5.3
BA5		59	*No LTx*	283	*No LTx*	Cirrhosis; liver failure	5.8	4.2
BA6		93	*No LTx*	165	*No LTx*	Cirrhosis; liver failure	10	6.7

BA7		59	*No LTx*	*Alive*	*No LTx*	*Alive*	12	8.8
BA8		92	2559	*Alive*	Cirrhosis; liver failure	*Alive*	12.9	9.6
BA9		69	*No LTx*	*Alive*	*No LTx*	*Alive*	7.5	6
BA10		65	114	122	Liver failure	Cirrhosis; liver failure	19.1	14.3
BA11		52	212	*Alive*	Cirrhosis; liver failure	*Alive*	11.6	8.6
BA12		56	1409	*Alive*	Recurrent cholangitis; cirrhosis; liver failure	*Alive*	16.6	11.8
BA13		32	*No LTx*	*Alive*	*No LTx*	*Alive*	4.7	3.5
BA14		43	*No LTx*	*Alive*	*No LTx*	*Alive*	4.6	4
C1	Idiopathic Neonatal Cholestasis	35	*No LTx*	*Alive*	*No LTx*	*Alive*	14.6	10
C2		78	*No LTx*	*Alive*	*No LTx*	*Alive*	19.2	14.9
C3		62	*No LTx*	*Alive*	*No LTx*	*Alive*	12.1	8.8
C6	Parenteral Nutrition-Associated Cholestasis	62	*No LTx*	*Alive*	*No LTx*	*Alive*	14.6	10
C4	A1ATD	40	*No LTx*	*Alive*	*No LTx*	*Alive*	9.2	6.4
C5	CMV Hepatitis	35	*No LTx*	*Alive*	*No LTx*	*Alive*	*Not Available*	*Not Available*

5.3.2 Detection and immunolocalization of HIF-1α and TUBA4A by qualitative method

HIF-1α nuclear positivity was not detected in any biliary structures in IHC controls (Figure 5.4A). In contrast, five out of 14 (35.7%) patients with BA showed HIF-1α nuclear positivity in cholangiocytes of the portal tracts, portal-parenchyma interface, ductular reaction expanding outwards to parenchymal zone 1, fibrovascular septa, and subcapsular area (Figure 5.4A).

In BA samples, TUBA4A positivity was observed in the cytoplasm and/or apical membrane of cholangiocytes, in the last case, with or without associated ciliary structures (Figure 5.4B). Cytoplasmic TUBA4A positivity was noted in cholangiocytes located in the portal tracts, portal-parenchymal interface, ductular reaction expanding outwards to parenchymal zone 1, fibrovascular septa, and subcapsular area (Figure 5.4B).

5.3.2.1. Qualitative vs Quantitative analysis

Qualitative and quantitative methods were used to characterize HIF-1α nuclear positivity. According to the quantitative method, a higher number of cases (six out of 14; 42.8% of cases) presented HIF-1α nuclear positivity in cholangiocytes than through the qualitative method (five out of 14; 35.7% of cases), showing a small variability between the two evaluation processes.

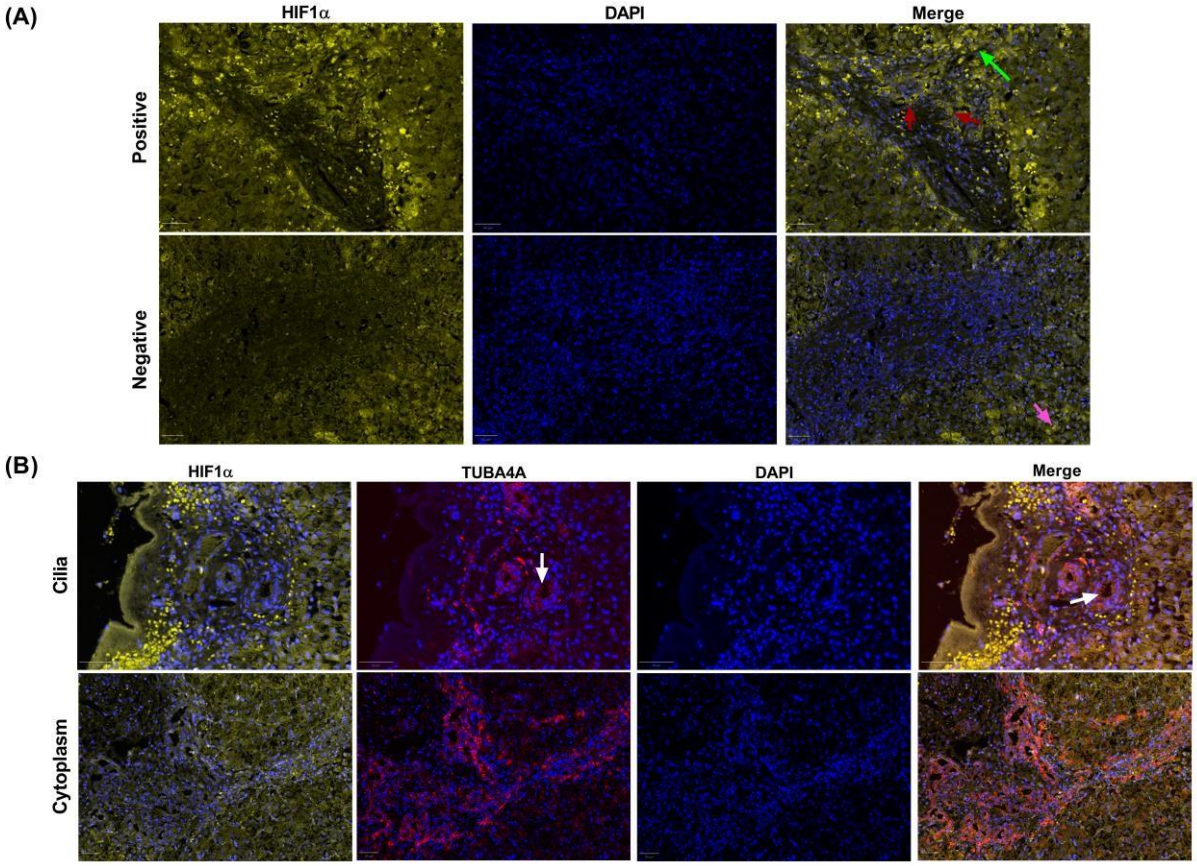

Figure 5.4. HIF-1α nuclear activation status in cholangiocytes. (A) Above (positive): HIF-1α nuclear positivity in the liver of a BA patient. HIF-1α positivity was observed in cholangiocytes of the portal tracts, including interlobular bile ducts (red arrows); the portal–parenchymal interface (green arrow); ductular reaction expanding outwards to parenchymal zone 1; and fibrovascular septa, including intra-septal and septal interface ductular reactions. Below (negative): a patient with idiopathic neonatal cholestasis. Hepatocytes also exhibited HIF-1α nuclear positivity (pink arrow) with variable intensity according to the zonal distribution (pericentral or periportal), in both BA and control samples, independent of the biliary HIF-1α positivity status. Images magnification: 20x. Blue: DAPI staining; Yellow: HIF-1α staining. (B) Above (cilia): representative image from a BA sample with TUBA4A staining in cholangiocytes of the portal tracts with ciliary configuration (white arrows). Below (cytoplasm): representative image from BA sample with TUBA4A staining in cytoplasm of cholangiocytes in the portal tract and portal–parenchymal interface. Images magnification: 20x. Blue: DAPI staining; Yellow: HIF-1α staining; Red: TUBA4A staining.

5.3.3 Quantitative analysis of ciliary characteristics in cholangiocytes of biliary atresia patients

When evaluating the CRs across the different assessed areas, no difference was observed between the CRs of BA patients versus controls (Figure 5.5B). However, regarding primary cilia length, the cilia in the biliary structures of BA patients were notably shorter (Figure 5.5C).

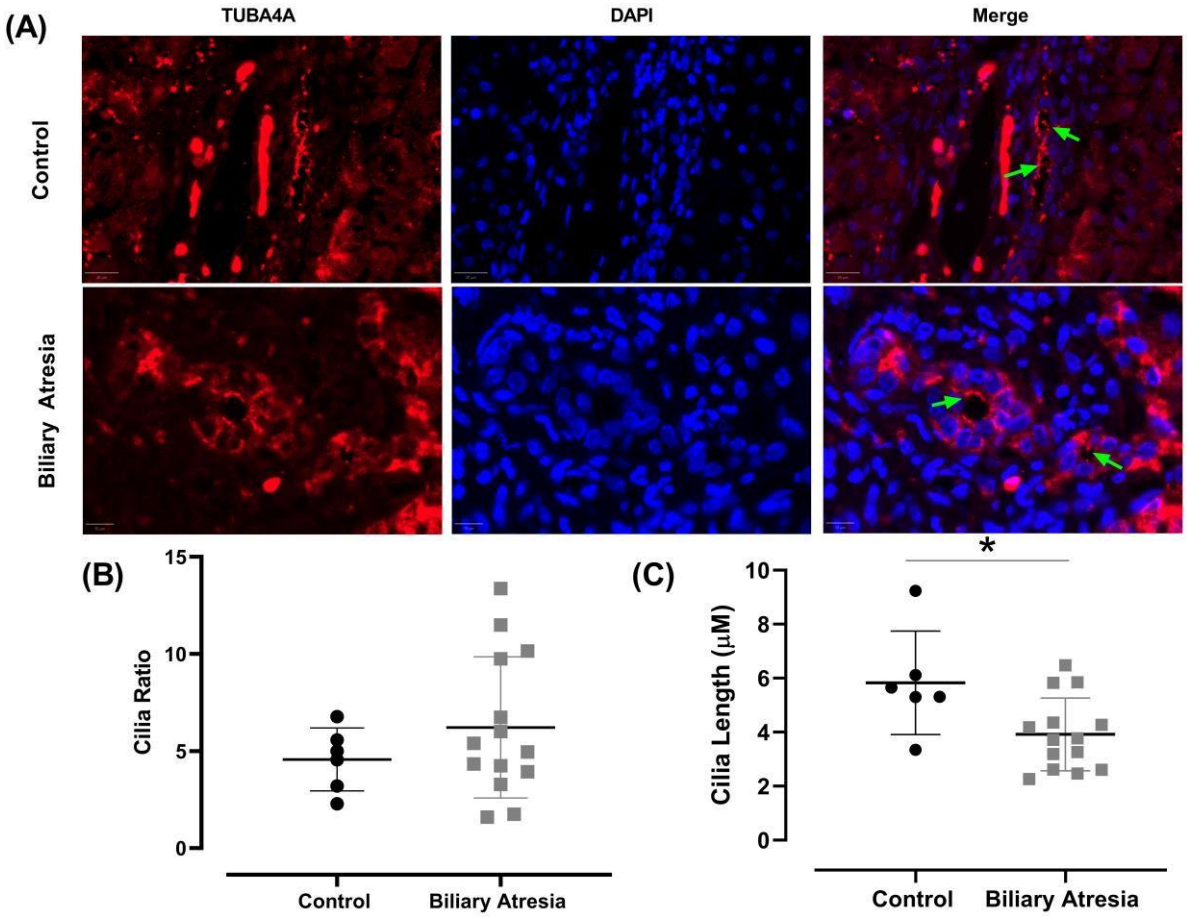

Figure 5.5. Comparison of cholangiocyte primary cilia features in biliary atresia and controls. (A) Representative images showing the differences between controls and BA. Green arrows indicate the cilia of cholangiocytes. Images Magnification: 40x. Blue: DAPI staining; Red: TUBA4A staining. (B) Comparison of cilia ratio status between controls and BA. (C) Comparison of cilia length between controls and BA (Student's t-test, * p = 0.010, d = 1.249). Dots represent individual data points. Horizontal lines indicate the mean, and vertical lines represent the SD.

A significant increase in cytoplasmic TUBA4A expression was observed in BA patients compared to diseased controls (Figure 5.6). TUBA4A expression intensity was higher in biliary structures within the portal tracts and along the portal-parenchymal interfaces (Figure 5.6A and B). The cilia ratio status presented a strong positive correlation with a lower cytoplasmic TUBA4A expression (Table 5.4).

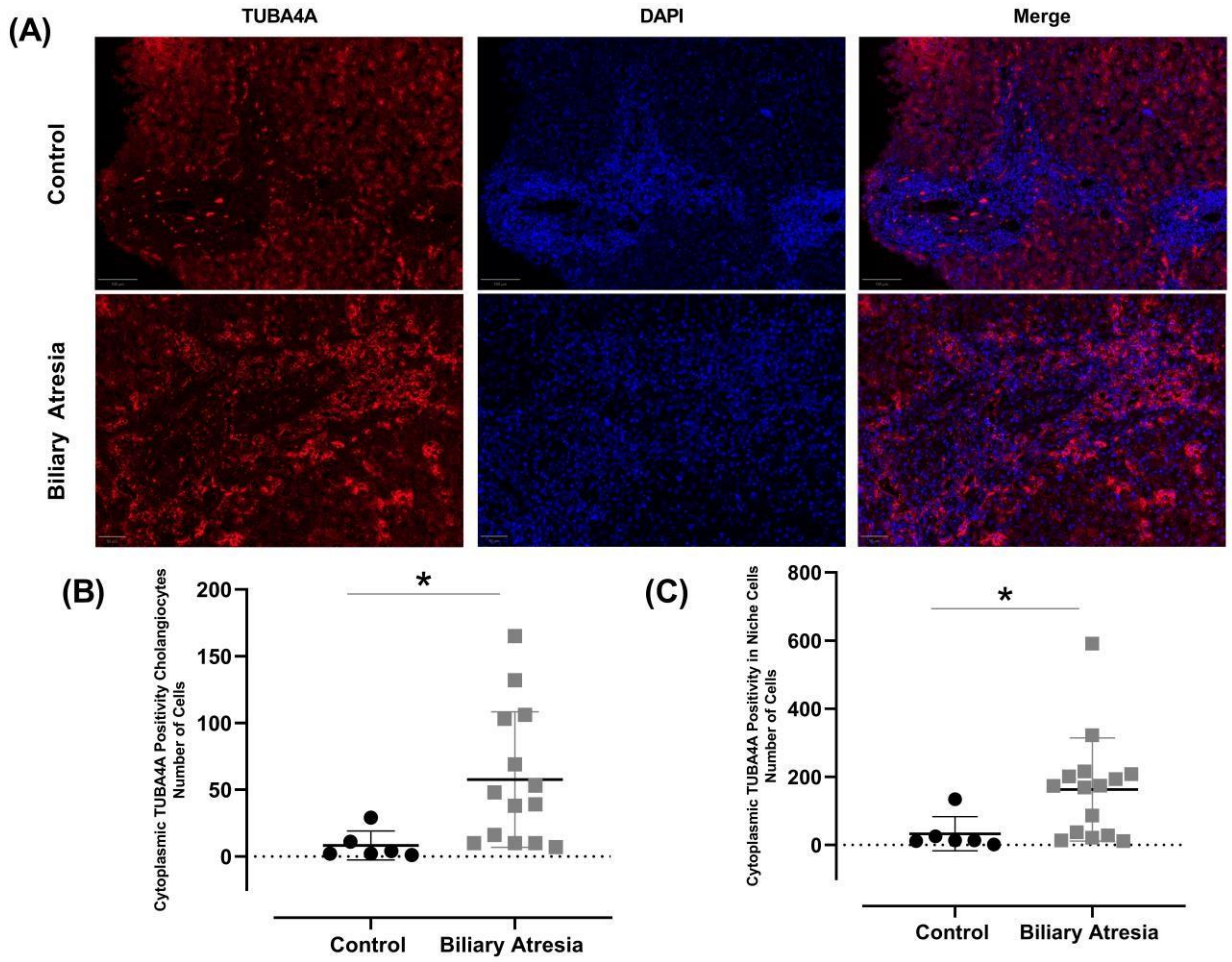

Figure 5.6. Comparison of the cytoplasmic TUBA4A positivity in cholangiocytes and niche cells. (A) Representative images showing the differences between controls and BA. Images Magnification: 40x. Blue: DAPI staining; Red: TUBA4A staining. (B) Comparison of cytoplasmic TUBA4A cholangiocyte positivity between controls and BA (t-test, * p = 0.005, d = 0.953). (C) Comparison of cytoplasmic TUBA4A positivity in niche cells between controls and BA (t-test, * p = 0.045, d = 0.924). Dots represent individual data points. Horizontal lines indicate the mean, and vertical lines represent the SD.

5.3.4 Relationship between HIF-1α expression and ciliary characteristics in BA

Regarding the colocalization of HIF-1α and TUBA4A in BA samples, the presence of HIF-1α nuclear positivity in cholangiocytes often correlated with a cytoplasmic pattern of TUBA4A positivity without identifiable PC. Conversely, HIF-1α negativity was frequently associated with identifiable PC structures in the apical membrane of cholangiocytes. The staining of HIF-1α and TUBA4A was analyzed to understand the behavior of cilia under hypoxic conditions. Cholangiocytes with lower levels of HIF-1α expression exhibited increased TUBA4A staining, indicating a higher rate of PC presence (Figure 5.7A). Due to the high variability in CRs, the data were divided into

two groups based on the median value: normal CRs and decreased CRs (Figure 5.7B). This division allowed for a clearer understanding of the relationship between HIF-1α expression and cilia presence. Considering the quantitative evaluation of the extent of HIF-1α nuclear expression and the cilia ratio status in the sample of BA, a significant increase in cilia ratio status was observed when the amount of HIF-1α negative cells increased.

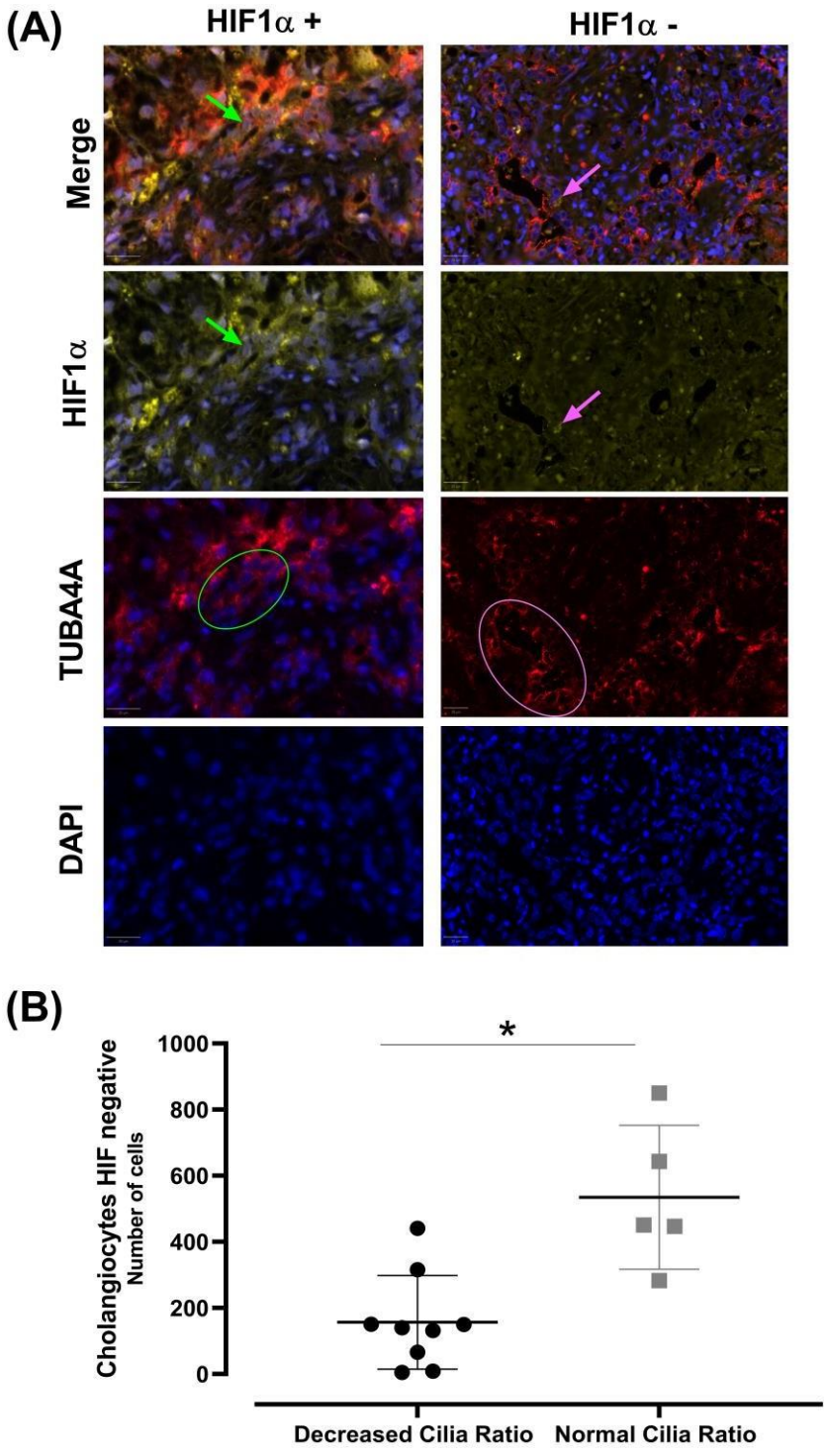

Figure 5.7. HIF-1α influence over cholangiocyte cilia. (A) Representative images demonstrating the influence of HIF-1α positivity on cholangiocyte cilia. The images in the left column display a biliary duct positive for HIF-1α (indicated by green arrows) with no visible cilia in the duct (highlighted by the green circle). In contrast, the right column shows a biliary atresia sample with HIF-1α-negative cholangiocytes (indicated by pink arrows) where the cilia in the ducts are clearly evident (highlighted by the pink circle). Images magnification: 40x. Blue: DAPI staining; Yellow: HIF-1α staining; Red: TUBA4A staining. (B) Considering the quantitative evaluation of the extent of HIF-1α nuclear expression and the cilia ratio status in the sample of BA, a significant increase in cilia ratio status when the amount of HIF-1α negative cells is higher is observed (Student's t-test, * $p < 0.001$, $d = 2.216$). Dots represent individual data points. Horizontal lines indicate the mean, and vertical lines represent the SD.

5.3.5 Effects of HIF-1α as well as Ciliary and Cytoplasmic TUBA4A Morphometric Features on the Clinical–Laboratory Status and Native Liver Survival

Table 5.4 presents the results of the correlations between quantitative and qualitative clinical variables, and native liver survival.

Table 5.4. Spearman's correlation between quantitative and qualitative clinical variables.

Variable Pair	Spearman's Correlation (ρ)	*p*-Value	Significance
Clinical–laboratory status			
Total Bilirubin vs. Cilia Length	−0.665	0.013	*
Direct Bilirubin vs. Cilia Length	−0.758	0.003	**
Morphometric variables			
Lower TUBA4A vs. CRs	0.890	<0.001	**
Outcomes			
LTx vs. Direct Bilirubin	0.624	0.023	*
LTx vs. Cilia Length	−0.802	0.001	**
Death vs. HIF-1α Positive Nuclei	0.692	0.009	**
Death vs. Higher TUBA4A	0.626	0.022	*

Abbreviations: CRs—cilia ratio status; HIF-1α: hypoxia-inducible factor-1alpha in cholangiocytes; LTx—liver transplantation; TUBA4A—cytoplasmic alpha-tubulin 4 acetylated; PE—portoenterostomy; NLS—native liver survival. **Death and Liver transplantation:** represents the outcome until 1 year of life. **Characterization of the type of variables under study:** Qualitative variables: death; liver transplantation. Quantitative variables: bilirubin serum levels; cilia length; age; levels of TUBA4A and HIF-1α. In the table, * indicates p-values between 0.05 and >0.01, while ** indicates p-values ≤0.01, representing higher statistical significance.

5.3.5.1. Correlations between clinical-laboratory, image morphometric and outcome variables

Concerning BA patients, some relevant correlations occurred between clinical laboratory data and quantitative morphometric results. Age at portoenterostomy did not present correlations neither with HIF-1α positivity nor with TUBA4A expression in cholangiocytes. However, cilia length presented negative correlations with total bilirubin (ρ =-0.665; P=0.013) and direct reacting bilirubin (ρ=-0.758; P=0.003).

Concerning the statistical relationships among the different image morphometric variables, lower TUBA4A cytoplasmic expression presented a strong positive

correlation with Cilia Ratio status (ρ=0.890; P<0.001). Regarding the outcome variables, early LTx (until 1 year of life) was positively correlated with direct-reacting bilirubin serum levels at portoenterostomy (ρ=0.624; P=0.023) and negatively correlated with Cilia Length (ρ=-0.802; P=0.001). On the other hand, early death (until one 1 year of life) showed moderate positive correlations both with HIF-1α nuclear positivity (ρ=0.692; P=0.009) and higher cytoplasmic TUBA4A expression (ρ=0.626; P=0.022) in cholangiocytes.

Confirming our previous immunohistochemical findings [16], HIF-1α nuclear positivity correlated with early death rates (ρ=0.692; P=0.009).

5.3.5.2. Native liver survival

Concerning the correlations between the 1-year NLS after portoenterostomy and the PC features in BA, patients presenting decreased PC length and decreased PC bending either died or needed liver transplantation within 1 year after portoenterostomy (Figure 5.8). These results suggest that specific alterations in PC morphology are associated with poorer prognosis in BA patients after portoenterostomy.

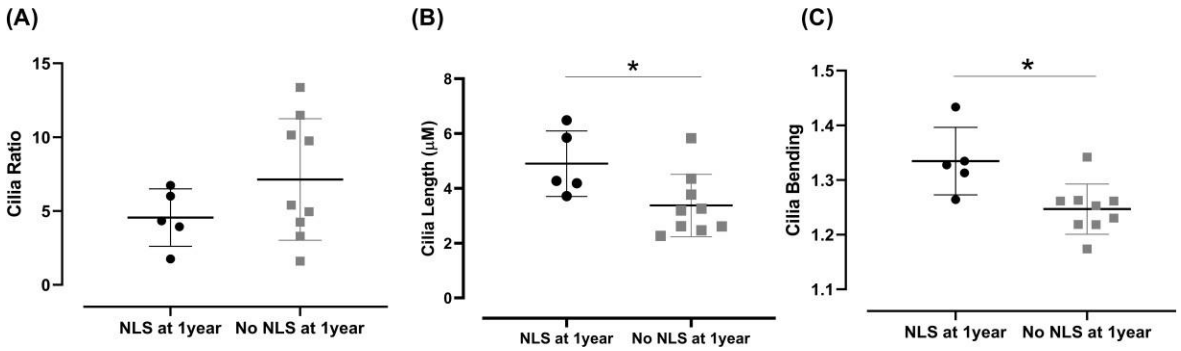

Figure 5.8. Correlations between native liver survival within 1 year after portoenterostomy and morphometric primary cilia features in the sample of BA patients. (A) Comparison of cilia ratio status between BA patients with and without NLS. (B) Comparison of cilia length between BA patients with or without NLS (t-test, * p = 0.018, d = 1.317). (C) Comparison of cilia bending between BA patients with or without NLS (t-test, * p = 0.005, d = 1.694). Dots represent individual data points. Horizontal lines indicate the mean, and vertical lines represent the SD.

A significant difference concerning the cytoplasmic TUBA4A expression in cholangiocytes occurred between BA patients with and without NLS until 1 year of life (Figure 5.9A). Increased cytoplasmic TUBA4A expression in cholangiocytes was associated with non-achievement of a 1-year NLS (Figure 5.9A). These findings suggest

that higher levels of cytoplasmic TUBA4A in cholangiocytes correlate with poorer outcomes in BA patients.

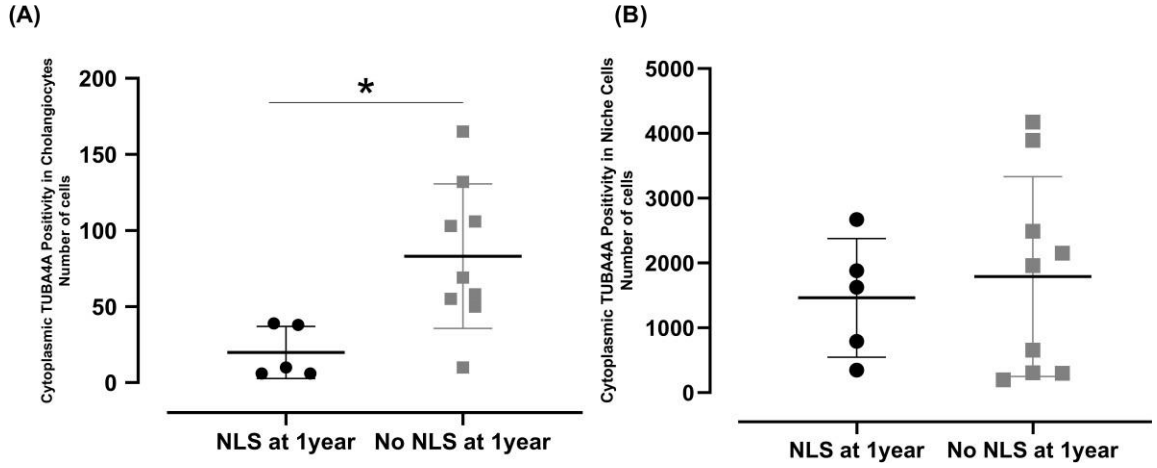

Figure 5.9. Effects of TUBA4A expression in the cytoplasm of cholangiocytes within the portal tracts and along the portal–parenchymal interface over 1-year rates of native liver survival after portoenterostomy. (A) Increased cytoplasmic TUBA4A expression in cholangiocytes was associated with non-achievement of 1-year native liver survival (t-test, * p = 0.020, d = 1.005). (B) Difference between TUBA4A cytoplasmic positivity in niche cells regarding native liver survival. Dots represent individual data points. Horizontal lines indicate the mean, and vertical lines represent the SD.

5.4. Discussion

5.4.1 Clinical data of the BA patients for a prognostic evaluation

Regarding the clinical data of BA patients under study, given the small sample available we were not able to evaluate the 6-month NLS after portoenterostomy. However, during the full follow-up period (2005 to 2018), 7 out of 14 (50%) BA patients died and/or were transplanted in the first year of life, which we consider an early bad prognosis. One of these patients (BA10) with an early bad prognosis underwent LTx at 114 days of life and died 8 days later due to systemic complications of liver cirrhosis and chronic liver failure. Four of the patients with early bad prognosis died with their native liver. Three other patients were transplanted and/or died after two years of life.

5.4.2 Detection and immunolocalization of HIF-1α and TUBA4A

In this study, according to the qualitative evaluation, 35.7% of BA patients showed HIF-1α nuclear positivity in cholangiocytes of portal tracts, portal-parenchymal interfaces, ductular reaction expanding outwards to parenchymal zone 1, fibrovascular septa, and subcapsular area, whereas HIF-1α positivity was absent in the biliary epithelium of all

the diseased controls (Figure 5.4A). However, the quantitative analyses of HIF-1α positivity in cholangiocytes showed a higher extent of HIF-1α positivity by using the QuPath 0.4.4 software. Six out of fourteen BA cases (42.8%), and not only 35.7%, presented HIF-1α activation in the intrahepatic biliary epithelium, suggesting that the quantitative method has higher sensitivity rates for HIF-1α detection in immunofluorescence-stained samples.

These findings agree with previous studies published by our team [11, 13, 14, 16, 17, 31, 32] in that, at least in a subset of patients, hypoxia-ischemia seems to have a pathophysiological role in BA, corresponding to an ischemic cholangiopathy [18]. Recently, it was demonstrated that HIF-1α pathway plays a central molecular role in the development of BA [19]. Chang et al. (2024) confirmed that a precocious hepatic arterial medial layer thickening in BA, apparently associated with the Notch3/Hey1 pathway, leads to peribiliary hypoxia [12]. Another group, using a model of hypoxic injury over fetal extrahepatic bile ducts in sheep [20], endorsed that hypoxic injury over fetal/neonatal extrahepatic bile ducts activates a fetal wound healing program and leads to extrahepatic biliary obstruction. A recent epidemiological genetic publication evaluated 811 BA cases in a genome-wide association study and observed a strong correlation of BA with genes involved in planar polarity, which integrates ciliary dysgenesis and abnormal vascular and biliary epithelial cell development [33].

Concerning tubulin, TUBA4A positivity was observed in the cytoplasm and/or apical membrane of cholangiocytes, occasionally with a ciliary configuration (Figure 5.4B), both in BA and diseased controls. In BA, cytoplasmic TUBA4A positivity was noticed in cholangiocytes located in the portal tracts, portal-parenchymal interface, ductular reaction expanding outwards to parenchymal zone 1, fibrovascular septa, and subcapsular area (Figure 5.4B).

5.4.3 Quantitative analysis of ciliary characteristics in cholangiocytes of biliary atresia patients

Concerning the CRs, unlike previous studies [24, 26], our morphometric evaluation did not find difference between BA patients and diseased controls (Figure 5.5B). However, a remarkable difference was observed concerning cilia length, notably shorter in BA (Figure 5.5C). Cilia length showed significant correlations with clinical and laboratory indicators of cholestasis severity, including total and direct bilirubin serum levels. The presence of PC and their correct structure and functioning indicate the ability of

cholangiocytes to detect and respond to extracellular stimuli [34] aiming to regulate fundamental signaling pathways [35].

In this context, PC length is an important variable concerning ciliary function [22]. Cilia length is maintained by the addition or removal of tubulin at the tip of PC. Protein synthesis does not occur in PC, and cilia growth involves the import of tubulin monomers from the cytoplasm into the cilium through tightly regulated processes. Changes in cilia length represent a significant alteration in ciliary volume, and altered molecular concentrations within the cilium are associated with processes that affect PC growth [22]. Genetic or environmental factors can lead to reduced cilia length and the development of ciliopathies [36-38].

Given that axoneme length depends on the transit of molecules, including tubulin, between cytoplasm and cilium, alterations in the microtubule network within the cytoplasm can lead to alterations in the levels of soluble tubulin within PC [27]. Microtubule cytoplasmic stabilization reduces the pool of soluble tubulin and, therefore, may erode the distal tip of the axoneme [38], leading to decreased cilia length or even deciliation.

Additional information obtained in this study was evidenced by the significant increase in cytoplasmic TUBA4A expression in BA patients compared to the diseased controls (Figure 5.6). Increased TUBA4A expression was particularly evident within the portal tracts and along the portal-parenchymal interfaces. Table 5.4 shows that higher CRs presented a very strong positive correlation with low or negative cytoplasmic TUBA4A expression ($\rho = 0.890$; $P < 0.001$).

Importantly, elevated cytoplasmic TUBA4A expression was correlated with increased mortality in BA patients ($\rho = 0.626$; $P = 0.022$), Table 5.4. Concerning deciliation, which can be identified in the present study through the variable CRs, it is caused by environmental stresses, including hypoxia [37].

5.4.4 Relationship between HIF-1α expression and ciliary characteristics in BA

Regarding the colocalization of HIF-1α and TUBA4A in BA samples, in the qualitative analysis, we observed an inverse association between HIF-1α positivity and the presence of PC at the luminal membrane of cholangiocytes, both in biliary cells of portal tracts and at other microanatomic liver areas (Figure 5.7B). The quantitative

analysis of the ciliary characteristics showed that biliary cells without hypoxic characteristics presented preservation of PC, suggesting that hypoxia, indicated by HIF-1α positivity, may negatively impact the formation or maintenance of PC. As previously described, hypoxia constitutes an environmental stress that causes deciliation [37,39].

A correlation between HIF-1alpha pathway activation and decreased early death was confirmed in the present study (Table 5.4). HIF-1α pathway activation occurs within the portal-parenchymal interface, location of the progenitor cell niche, per se can stimulate liver fibrogenesis, with detrimental effects on prognosis [17]. In this study, CRs correlated with HIF-1α positivity but not with the postoperative prognosis after portoenterostomy.

5.4.5 Effects of Ciliary and Cytoplasmic TUBA4A Alterations on the Clinical–Laboratory Status and Native Liver Survival

5.4.5.1. Effects over clinical-laboratory status

Correlations were observed between the need for early LTx, decreased cilia length, and bilirubin serum levels (Table 5.4). Bilirubin serum levels at the time of portoenterostomy seemed to be affected by decreased cilia length, which can disturb the bile acids signaling pathways [35, 40] . Additionally, increased direct-reacting bilirubin serum levels, a marker of cholestasis severity, and decreased cilia length correlated with the need for early LTx.

5.4.5.2. Effects over native liver survival

This study found correlations between ciliary and cytoplasmic tubulin features and the early postoperative prognosis following portoenterostomy. Decreased cilia length, ciliary bending, and increased cytoplasmic TUBA4A expression in the biliary cells reduced the span of NLS (Figure 5.8). The detrimental effects of the PC disruptions observed in the present study are understandable since PC regulate several signaling pathways crucial for liver function [35, 40].

A unique finding of this study was an increased expression in BA patients of the cytoplasmic TUBA4A in cholangiocytes in comparison to diseased controls (Figure 5.9A), suggesting that increased cytoplasmic levels of TUBA4A in cholangiocytes correlate with poorer outcomes in BA patients. Tubulin, a fundamental constituent of the cellular cytoskeleton, is affected by post-translational modifications by diverse

molecular factors [41], altering microtubule structures and their associated functions. Toxic compounds, such as alcohol, harm liver cells through tubulin cytoplasmic hyper-stabilization [42-46].

5.4.6 Limitations of the study

The limitations of the present study include the small sample size of both BA and diseased controls. Biliary atresia and intrahepatic neonatal cholestasis are rare hepatobiliary diseases, and the study design of investigations concerning details of pathophysiology and clinical correlations may be affected by the sample size, thus requiring the performance of multicenter clinical studies or the use of experimental models. An additional aggravating factor, specifically concerning BA, is the existence of different clinical variants, which may affect the accuracy of results. Given these concerns, we have chosen to limit our BA sample to the isolated type and to bypass the sample size constraints by performing exploratory histopathological analyses. Histopathological studies involving digital image analyses enable the evaluation of a large series of microanatomic structures in each patient, making it possible to identify specific histopathological and morphometric patterns related to our subjects of interest. Thus, we used a convenience sample of patients who were cared for and followed in the clinical practice and conducted this exploratory histopathological analysis, being able to obtain statistically significant results with reasonable internal validity. Aiming to confirm the clinical effect of the statistically significant data, we determined the magnitude of the effect size developed by Cohen [47]. Our future next steps will include confirmatory experimental methods. Additionally, studies with larger sample sizes are necessary to extrapolate our findings to the population of BA patients, i.e., to ensure their external validity, not only for the isolated BA variant but also for the remaining clinical types.

5.5. Conclusion

This study evaluated the presence and clinical-laboratory outcomes attributable to alterations in the primary cilia and cytoplasmic tubulin expression in the intrahepatic biliary epithelium, observing that reduced PC length, decreased PC bending and increased intensity of cytoplasmic TUBA4A occur in the isolated BA type, and negatively impact the postoperative prognosis after portoenterostomy (Figure 5.10). These findings suggest the existence of a disruption in the tubulin transport between cytoplasm and PC. The detrimental effect of the HIF-1alpha pathway activation on

native liver survival was confirmed but unassociated with PC or cytoplasmic tubulin features.

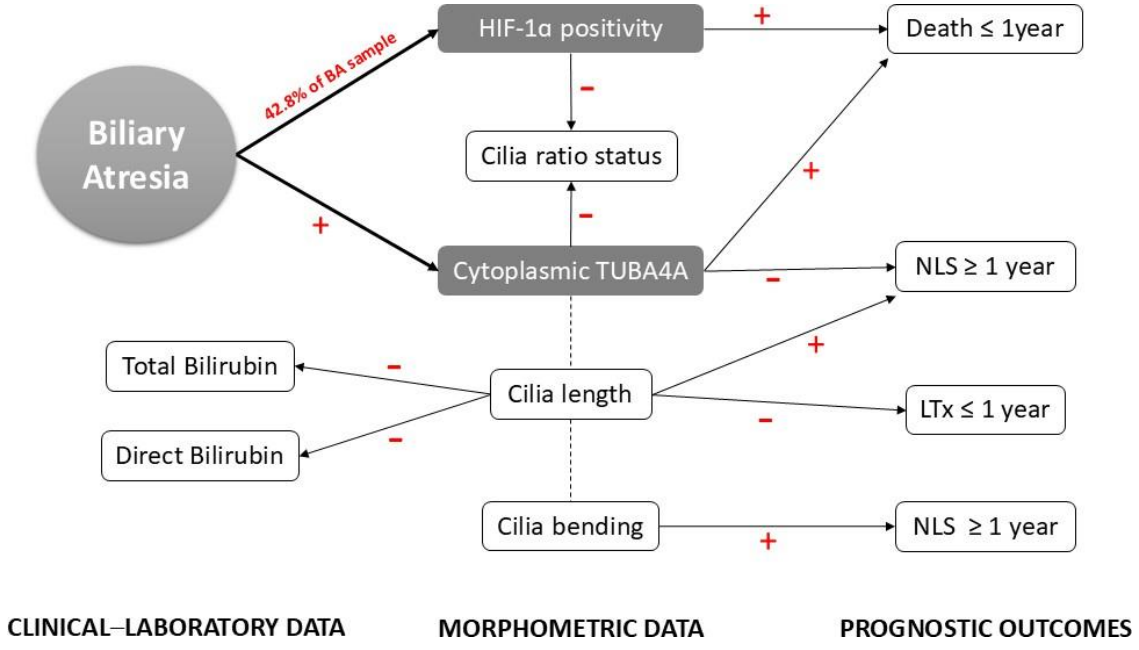

Figure 5.10. Overview of study findings. The flowchart represents the relationship between BA clinical–laboratory parameters, morphometric data, and prognostic outcomes analyzed in the present study, highlighting the role of PC dysfunction and HIF-1α activation in BA prognostic (+: directly proportional; –: inversely proportional).

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Chapter 6

General Conclusions and Future Perspectives

6.1. Study Goals and Main Findings

Biliary atresia, although a rare disease, remains an urgent pediatric health concern as it is the leading indication for liver transplantation during infancy and childhood. Despite advances in medical research, BA etiology remains not completely deciphered, with genetic, environmental, and immunological factors implicated in its pathogenesis. Recent evidence suggests that hypoxia and disruption of cholangiocyte cilia play critical roles in disease progression, highlighting the need for an understanding of their molecular and cellular effects. Furthermore, BA presents unique challenges in diagnosis and treatment, mainly due to the small-time window for post-diagnosis success and the limited understanding of the mechanisms driving its progression and lack of targeted therapeutic strategies.

In this context, this thesis aimed to unravel the role of hypoxia and its associated molecular pathways in BA, focusing on their impact on ciliary function in cholangiocytes. The processes that are being investigated will not only shed light on critical aspects of BA pathophysiology but also open new research paths for the development of diagnostic tools and therapeutic interventions that could mitigate disease progression and improve patient outcomes.

Herein, we first provided evidence for ischemic cholangiopathy in a subset of patients with isolated BA by demonstrating histopathological nuclear HIF-1α positivity in cholangiocytes (**Chapter 4**). This observation represents a groundbreaking step in understanding the molecular basis of hypoxia-driven biliary injury that is essential to the pathophysiology of BA. Remarkably, this is the first study to establish a direct link between the activation of HIF-1α and involvement of the peribiliary vascular plexus, suggesting that hypoxia-induced ischemic damage may significantly contribute to the disease's progression.

Based on this, **Chapter 5** extended the analysis to evaluate the clinical and molecular implications of PC dysfunction and cytoplasmic tubulin alterations in BA. We found that intrahepatic biliary epithelium in isolated BA is characterized by reduced PC length and bending, along with increased cytoplasmic TUBA4A expression. The aforementioned changes then negatively influenced postoperative outcomes after portoenterostomy, showing evidence of an impairment in tubulin transport between cytoplasm and PC. Furthermore, we established that HIF-1α activation indeed has a detrimental impact on native liver survival; however, this effect was independent of

primary cilia or cytoplasmic tubulin alterations. In conclusion, our studies have supported the pathophysiology of BA as multifactorial and complex. Together, these studies underscore the intricate relationship between hypoxia, cellular damage, and clinical endpoints in BA, providing insights for further studies and possible therapeutic interventions.

6.2. Study Limitations

The work developed in this study represents a significant step forward in understanding the molecular and cellular mechanisms underlying BA, particularly the roles of hypoxia, primary cilia dysfunction, and tubulin transport disruption. However, as with any exploratory research, there are several technical and logistical challenges, which contributed to certain limitations that should be acknowledged. These limitations affected both the scope of our analysis and the optimization of our methodologies, yet they also highlight important lessons for future studies in this field:

1. Limitations related to protein analysis in frozen liver samples: Initially, our study design included the analysis of total protein levels using western blot. However, the frozen liver samples stored in RNA Later, while effective at preserving RNA integrity, did not adequately protect the proteins due to the age of the samples and the limitations of RNA Later for protein preservation. This issue rendered the samples unsuitable for western blot analysis and significantly limited our ability to perform protein quantification and further characterization.

2. Immunofluorescence challenges with frozen samples: Another planned objective was to conduct immunofluorescence analyses on the frozen liver samples, which were more numerous than the paraffin-embedded samples. However, the frozen samples had not been fixed prior to storage in RNA Later, and despite testing numerous protocols, this lack of fixation resulted in poorly preserved tissue structure and non-specific staining, preventing us from obtaining defined cellular structures or specific marker localization. Consequently, we were unable to perform the immunofluorescence analysis as intended.

3. Switch to paraffin-embedded samples with a smaller sample size: Due to the limitations of the frozen samples, we shifted our analysis to paraffin-embedded samples. While this allowed for successful immunofluorescence and

histopathological analyses, the number of paraffin-embedded samples available for study was smaller than initially anticipated. Moreover, the paraffin itself posed additional challenges, particularly its autofluorescence in the green spectrum, which required extensive protocol optimization to achieve clear visualization of results. Testing and refining these protocols consumed significant time and resources yet ultimately allowed us to obtain interpretable results. While we were able to generate significant findings with the paraffin-embedded samples, these limitations highlight the importance of ensuring optimal sample preservation and securing collaborations in future studies to enhance the scope and impact of research.

4. Clinical variants of BA: The heterogeneity of BA, which includes different clinical variants, poses a challenge to the accuracy of pathophysiological and clinical correlation studies. To minimize variability, we limited our analysis to the isolated BA type, which may have excluded insights relevant to other variants. Consequently, the findings presented in this thesis are specific to the isolated form of BA and may not apply to syndromic or other clinical subtypes of the disease. Expanding future studies to include all clinical variants will be important for a comprehensive understanding of BA pathophysiology.

5. Lack of experimental validation of findings: Although this study provided compelling evidence for the involvement of HIF-1α activation, primary cilia abnormalities, and tubulin transport disruption in BA, experimental validation using in vitro or in vivo models was not conducted. For instance, the specific role of HIF-1α activation in ischemic cholangiopathy and its potential interaction with oxidative stress remains to be experimentally confirmed. Similarly, the mechanisms underlying the observed disruption in tubulin transport and its impact on primary cilia function require further investigation.

6. Limited generalizability and external validity: As the study was conducted using a convenience sample of patients treated and followed at specific centers, there may be limitations in extrapolating these findings to the broader BA population. Multicenter clinical studies and experimental models are necessary to ensure the external validity of these findings and to explore potential geographic, genetic, or environmental variations in BA pathophysiology.

7. Lack of longitudinal data: While we evaluated postoperative outcomes following portoenterostomy, a detailed longitudinal analysis of how hypoxia, primary cilia dysfunction, and tubulin transport abnormalities evolve over time in BA patients was not performed. Such data could provide valuable insights into the progression of the disease and the impact of these molecular and cellular changes on long-term clinical outcomes.

Despite these challenges, we successfully optimized the protocols for paraffin-embedded samples, enabling us to obtain high-quality data and draw meaningful conclusions about the role of hypoxia, primary cilia dysfunction, and tubulin transport in biliary atresia. These experiences underscore the importance of methodological flexibility, collaboration, and meticulous planning in addressing the inherent challenges of working with rare and difficult-to-preserve tissue samples. Future studies should prioritize securing well-preserved and diverse sample cohorts, including prospective sample collection, to overcome such limitations and build on the insights generated in this work.

6.3. Future Perspectives

The results obtained in this thesis mark a significant advancement in our understanding of BA and the intricate molecular processes involved in its pathogenesis. While important progress has been made, further investigations are necessary to deepen our knowledge and address unanswered questions. To build upon the findings presented in this study, the following future directions are proposed to advance research in this area:

1. Analysis of primary cilia in organoids: Organoid models derived from cholangiocytes or liver progenitor cells could provide a controlled and reproducible system to study primary cilia structure and function in BA. By using organoids, it would be possible to directly observe cilia-related abnormalities, such as changes in cilia length, bending, or protein transport, in a more physiologically relevant three-dimensional environment. Additionally, organoids could serve as a platform to test the effects of hypoxia, oxidative stress, and pharmacological interventions on ciliary dynamics and cholangiocyte function.

2. In Vivo studies of hypoxia and ischemic cholangiopathy: The role of hypoxia and HIF-1α activation in BA should be further explored using in vivo models.

Experimental models of BA, such as Biliatresone mouse model could allow a better understanding of how hypoxia and ischemia contribute to biliary injury and disease progression. Specifically, these studies could help elucidate the mechanisms by which HIF-1α activation impacts cholangiocyte function, the peribiliary vascular plexus, and primary cilia. Additionally, in vivo studies could help clarify the interplay between hypoxia, oxidative stress, and other molecular pathways in the BA pathogenesis.

3. Expansion of sample size and multicenter studies: A larger and more diverse sample cohort is essential to validate the findings of this study and ensure their external validity. Future research should prioritize multicenter collaborations to obtain a greater number of well-preserved liver samples, involving different clinical variants of BA (e.g., syndromic and non-syndromic types). These efforts would not only improve statistical power but also allow for the investigation of possible genetic, environmental, or geographic factors that may influence BA pathophysiology.

4. Molecular analysis of tubulin transport mechanisms: Further studies are needed to elucidate the mechanisms underlying the disruption of tubulin transport in cholangiocytes. For example, experiments using cell culture systems, organoids, or in vivo models could focus on the molecular regulators of tubulin transport, such as motor proteins and microtubule dynamics, and how these are impacted by hypoxia and other factors. Understanding these pathways may uncover new therapeutic targets for restoring cilia function in BA.

5. Longitudinal studies to assess disease progression: Long-term follow-up studies in BA patients are necessary to evaluate how hypoxia, primary cilia dysfunction, and tubulin transport abnormalities influence disease progression and clinical outcomes over time. Such studies could provide valuable insights into the natural history of BA and help identify early biomarkers or predictors of disease severity, which could inform prognosis and guide clinical management.

6. Development of new diagnostic and therapeutic approaches: The findings of this study suggest potential avenues for developing targeted therapies aimed at mitigating hypoxia-induced biliary injury and restoring ciliary function. Future research should focus on identifying small molecules or other interventions that can modulate the HIF-1α pathway, improve tubulin transport, or protect primary cilia. Additionally, molecular and imaging biomarkers related to

hypoxia, cilia dysfunction, or tubulin transport could be developed to facilitate earlier diagnosis and more personalized treatment strategies.

7. Exploration of the peribiliary vascular plexus: Given the observed involvement of the peribiliary vascular plexus in ischemic cholangiopathy, future studies should focus on understanding its role in BA pathogenesis. Investigating the relationship between vascular alterations, hypoxia, and biliary injury may uncover novel insights into the mechanisms driving disease progression and identify potential therapeutic targets for preserving biliary and vascular integrity.

In conclusion, the perspectives outlined above provide a roadmap for advancing research on BA. By integrating advanced in vitro models, in vivo studies, expanded patient cohorts, and innovative diagnostic and therapeutic approaches, future investigations have the potential to unravel the complex pathophysiology of BA and ultimately improve outcomes for affected patients.