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Neonatal cholestasis: new insights into pathophysiology and strategies to improve patients' diagnosis and prognosis

Ermelinda Santos Silva

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Ermelinda Ramalho Santos Silva

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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NEONATAL CHOLESTASIS: NEW INSIGHTS INTO PATHOPHYSIOLOGY AND STRATEGIES TO IMPROVE PATIENTS' DIAGNOSIS AND PROGNOSIS

Tese de candidatura ao grau de Doutor
em Ciências Médicas
Programa Doutoral da Universidade do Porto
(Instituto de Ciências Biomédicas Abel Salazar)

Orientadora – Professora Doutora Ana Isabel Lopes
Categoria – Professora Catedrática de Pediatria
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Co-Orientadora – Prof. Doutora Esmeralda Martins
Categoria – Professora Associada Convidada
Afiliação – Instituto de Ciências Biomédicas Abel Salazar

PREFACE

In the 21st century, neonatal cholestasis (NC) remains a broad clinical challenge involving several aspects. In many cases, the recognition of NC among jaundiced neonates continues to be delayed, often due to a lack of awareness of the corresponding red flags by healthcare professionals. Also, the great diversity of underlying entities makes the etiological diagnosis difficult as some of them have specific treatment that must be offered in time if prognosis is to be improved. Despite many improvements in the management of patients, morbidity and mortality remain high, and to survive, many patients rely on orthotopic liver transplantation (OLT).

Crucial advances in the field of pathophysiology have been achieved in the last two decades. However, we still lack the full translation of these achievements, mainly in improving treatment and prognosis.

At this time, a global vision that integrates all inputs, applies them for clinical purposes, and points out directions for future research is of paramount importance.

My particular interest in the theme of NC began very early in my medical career, during my residency in paediatrics. I have acquired about 30 years of experience during my clinical practice in a paediatric hepatology consultation in a tertiary hospital in Northern Portugal, for which I have been the head since 1997. My motivation for earning my PhD started when, in addition to my collaboration in the training of paediatric residents, I also began to collaborate in the teaching of medical students, from October 2011, but mainly after taking on the position of an Invited Professor of the Integrated Master's degree in Medicine (MIM) at the Instituto de Ciências Biomédicas Abel Salazar (ICBAS) in January 2015.

Here, I present my vision on the various aspects involving NC. Some currently accepted concepts will be revisited, and some old paradigms will be challenged. With the present work, I intend to contribute to a better clinical practice, with improvements in the diagnosis and prognosis of the patients. This contribution includes epidemiological, clinical and translational research. Additionally, some papers published prior to the period of this thesis will be cited.

In Portugal, there are no relevant studies performed in this area.

DEDICATION

To my patients,

Their suffering was the main motivation for this research.

For everything they taught me,

My endless gratitude,

Hoping this thesis might be useful to them in some way.

To my parents,

Benilde and Américo,

For faith, unconditional love and support,

My immense joy for being able to share this moment with them.

ACKNOWLEDGMENTS

Many thanks to everyone whose invaluable contributions made this work possible.

TO MY THESIS SUPERVISORS

To Professor Ana Isabel Lopes, who accepted the task of supervising this thesis, sharing with me enormous enthusiasm for the topic, and who, with her confidence in my abilities to carry out this project to the end, greatly honours and distinguishes me. I thank her very much for her clinical and scientific guidance, critical analysis and perspective and invaluable help in improving this manuscript.

To Professor Alice Santos Silva and her valuable team at the Faculty of Pharmacy of Porto. My deepest gratitude for the kindness and availability with which all received an unknown doctor who was looking for support on basic research for her doctoral thesis. To Professor Santos Silva for coordinating the translational study and for her critical appreciation and valuable suggestions improving the manuscript. I thank her very much for everything she has taught me, for her generosity and patience and for believing in the project and having done everything to make it successful.

To Doctors Susana Rocha and Cristina Catarino, the first for processing the blood samples, and both for the laboratory work (oxidative stress and inflammation), and for the teaching in the area of statistical analysis.

To Professor Esmeralda Martins, my long-time colleague who shared with me the diagnosis and treatment of so many patients. Many thanks for her trust, friendship, and support.

My sincere thanks also to Professor Dora Brites (extended to her team of the Faculty of Pharmacy of Lisbon). Although not being part of the supervision team, Professor Brites occupied a very special place in this thesis. Many thanks to her for having cherished the project with her invaluable participation in the translational study in the area of the bile acids. I am also very grateful for her warm encouragement.

TO MY MASTERS OF CHILDREN'S HEPATOLOGY

To Doctor Margarida Medina, my first Master in Children's Hepatology. The first patient I saw with NC was shown to me by her. It was also by her hand that I met the first Portuguese child with a liver transplant due to biliary atresia. I offer here my sincere acknowledgement for everything she taught me, for the opportunities she provided me and for her extraordinary example as a doctor, a relentless fighter at the bedside of her patients. Without her, my professional life would not have taken this course.

To Professor Etienne Sokal, my Master when I was a fellowship student in Belgium during my internship in pediatrics and with whom I maintain to this day a relationship of clinical and scientific collaboration. With him, I strengthened my work capacity and my resilience, and I learned to never give up. It was Professor Sokal who first introduced me to a basic science research laboratory where I observed the sacrifice of animals from experimental models. It was from him that I first heard how the clinic's success would be closely linked to the findings of basic research in a sort of pre-announcement of the translational research.

My eternal gratitude goes to my Masters.

TO MY COLLEAGUES

My appreciation and gratitude to all my colleagues who participated, contributed, helped and encouraged me along the way. And, very sincerely, thank you also to those who didn't make it easy, for the extra incentive that this provided for me in moments of relaxation or doubt.

Special thanks to Doctor Maria Luís Cardoso for her encouragement and Doctor Mafalda Barbosa for her precious proofreading of the original article presented in section 2.1 of Chapter IV.

TO THE INVOLVED INSTITUTIONS AND PEOPLE

No doctoral thesis can be carried out without the fundamental support of the institutions and the people who build them. Here I express my public acknowledgement of the institutions for which I work (and worked), including Hospital de Crianças Maria Pia, Centro Hospitalar Universitário do Porto (CHUP) and the Instituto de Ciências Biomédicas Abel Salazar (ICBAS).

Many thanks to all the professionals (nurses, operational assistants, administrative and telephone operators) for their institutional collaboration in the humanised care of all patients over three decades. Special thanks to the nurses of the birth block and outpatient clinic of Centro Materno-Infantil do Norte (CMIN's) for their invaluable collaboration in the blood collections carried out within the scope of the translational study.

Many thanks to my directors for having provided me, first, with the necessary training and, then, with the opportunity to treat the many patients for so many years. Many thanks to Professor Helena Jardim for having recommended me for the position of Invited Professor of Paediatrics of the Integrated MIM, for her trust in my teaching skills and for having challenged me to pursue a PhD.

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… goes to Professor Caldas Afonso, the current Director of Paediatrics department and of the Board Direction of CMIN, CHUP as well as Director of the Integrated MIM of ICBAS, University of Porto (UP) for all the institutional support given to the execution of the work of this thesis, fundamental and irreplaceable support without which this thesis would not have been possible.

And to, the Board of Directors of CHUP, which in this last year, and in these times of pandemic, authorised my request for a scholarship, allowing me a precious period of time which helped to initiate the writing of this manuscript

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- **# Articles - published, accepted or submitted**

Original Research article, 2017

Santos Silva E, Moreira Silva H, Lijnzaat LA, Melo C, Costa E, Martins E, Lopes AI. **Clinical practices among healthcare professionals concerning neonatal jaundice and pale stools.**

Eur J Pediatr 2017; 176 (3):361-369.

[IF 2017 = **2.242**; SJR = 0.992, Q1 in Paediatrics, Perinatology and Child Health]

Short Report, 2018

Santos Silva E, Klaudel-Dreszler M, Bakuła A, Oliva T, Sousa T, Fernandes PC, Tylki-Szymańska A, Kamenets E, Martins E, Socha P. **Early onset lysosomal acid lipase deficiency presenting as secondary hemophagocytic lymphohistiocytosis: two infants treated with sebelipase alfa.**

Clin Res Hepatol Gastroenterol 2018, 42: e77-e82.

[IF 2018 = **2.807**; SJR: 0.756, Q2 in Gastroenterology and Q2 in Hepatology]

Original Research article, 2019

Moreira-Silva H, Maio I, Bandeira A, Martins E, **Santos Silva E**. **Metabolic liver diseases presenting with neonatal cholestasis: at the crossroad between old and new paradigms.**

Eur J Pediatr 2019, 178: 515-523.

[IF 2019 = **2.305**; SJR = 0.913, Q1 in Paediatrics, Perinatology and Child Health]

Original Research article, 2020

Santos Silva E, Almeida A, Frutuoso S, Martins E, Valente MJ, Santos Silva A, Lopes AI. **Neonatal cholestasis over time: changes in epidemiology and outcome in a cohort of 154 patients from a Portuguese tertiary centre.**

Front Pediatr 2020, Jun 30;8:351.

[IF 2020: **3.418**; SJR = 0.960, Q1 in Paediatrics, Perinatology and Child Health]

Original Research article, 2021

Santos Silva E, Moreira Silva H, Catarino C, Dias CC, Santos-Silva A, Lopes AI. **Neonatal cholestasis: development of a diagnostic decision algorithm from multivariate predictive models.**

Eur J Pediatr 2021, 180 (5); 1477-1486.

[IF 2020 = **3.183,** SJR = 0.984, Q1 in Paediatrics, Perinatology and Child Health]

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Ashworth J, Tavares M, **Santos Silva E** & Lopes AI. **The stool color card as a screening tool for biliary atresia in the digital version of the Portuguese Child and Youth Health Booklet.**

Acta Med Port 2021 Sep;34(9):630-645.

[IF 2020 = **1.141**; SJR = 0.32, Q3 in Medicine (Miscellaneous)]

Original Research article, 2021.

Santos Silva E, Rocha S, Candeias Ramos R, Coutinho H, Catarino C, Lopes AI, Santos-Silva A & Brites D.

Bile acid profile and redox status in healthy infants.

Submitted to Pediatr Res, 28 August 2021.

Re-Submitted to Pediatr Res, 03 January 2022 (after revisions).

[IF 2020 = **3.756** SJR = 1.06, Q1 in Paediatrics, Perinatology and Child Health]

- **# Conference papers**

Moreira Silva H, Lijnzaat L, Melo C, Martins E, **Santos Silva E**. **Clinical practices among healthcare professionals concerning neonatal jaundice and pale stools.**
e-POSTER (with oral room presentation) in 49th Annual Meeting of European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) – Athens, 25-28 May 2016.
ABSTRACT published in *J Ped Gastroenterol Nutr* 2016, 62(1): 564. (IF: 2,779)

Moreira Silva H, Lavrador V, Bandeira A, Vilarinho L, Martins E, **Santos Silva E.** **Metabolic liver diseases presenting with neonatal cholestasis: a series of 13 cases.**
POSTER in 51st annual meeting of ESPGHAN, 11 May 2018, Geneva.
ABSTRACT published in *J Ped Gastroenterol Nutr* 2018, 66 (2): 776. (IF: 3,015)

Santos Silva E, Bandeira A, Martins E. **Liver failure detected by the newborn screening program – a case of DGUOK deficiency with high GGT.**
CLINICAL CASE in the Metabolic Liver Diseases Workshop. Hannover, 2-4 November 2016.

- **# Invited lectures**

Colestase neonatal

LECTURE in Escola de Inverno da Sociedade Portuguesa de Gastrenterologia, Hepatologia e Nutrição Pediátricas “Uma Viagem pelo Tubo Digestivo”, Monte Real, 2-4 de Março de 2017.

Colestase neonatal – a metodologia diagnóstica e o tratamento

LECTURE in Post-graduate Course of ICBAS and CMIN. Unidade de Formação Contínua em Neonatologia, Módulo VI: Nutrição, estado nutricional e patologia digestiva neonatal. Porto, 5 de Maio 2018.

Colestase neonatal – situação actual e perspectivas futuras

CONFERENCE in XXXII Reunião Anual da Sociedade Portuguesa de Gastrenterologia, Hepatologia e Nutrição Pediátricas, Curia, 22 Março 2019.

Fisiologia do Fígado – o fígado em todas as idades

LECTURE in Post-Graduate Course. Curso de Formação em Hepatologia Pediátrica “A Criança e o Fígado no Século XXI” (CMIN, sponsored by Sociedade Portuguesa de Pediatria and Sociedade Portuguesa de Gastrenterologia, Hepatologia e Nutrição Pediátricas), Porto, 18 Maio 2019.

Colestase Neonatal

LECTURE in Post-Graduate Course. Escola de Outono da Sociedade Portuguesa de Gastrenterologia, Hepatologia e Nutrição Pediátricas. Pedrogão Pequeno, 26 Outubro 2019.

THESIS RELATED PAPERS

Contemporaries of the thesis period --- In the setting of the participation in **GALA Study** (**G**lobal **AL**agille **A**lliance Study) (**https://www.galastudy.com/participating-centres**, accessed on 02-10-2021).

- **# Original Research article, 2021**

Vandriel SM, Li L-T, She H, Wang J-S, Gilbert MA, Jankowska I, Czubkowski P, Gliwicz D, Gonzales EM, Jacquemin E, Bouligand J, Spinner NB, Loomes KM, Piccoli DA, D'Antiga L, Nicastro E, Sokal E, Demaret T, Feinstein JA, Ebel NH, Fawaz R, Nastasio S, Lacaille F, Debray D, Arnell H, Fischler B, Siew S, Stormon M, Karpen SJ, Romero R, Kim KM, Baek WY, Hardikar W, Shankar S, Roberts AJ, Evans HM, Jensen MK, Kavan M, Sundaram SS, Chaidez A, Karthikeyan P, Davison S, Sanchez MC, Chazarreta M, Verkade HJ, Lee WS, Squires JE, Hajinicolaou C, Lertudomphonwanit C, Fischer RT, Larson-Nath C, Mozer-Glassberg, Arikan YC, Lin H, Bernabeu JQ, Alam S, Kelly D, Carvalho E, Ferreira CT, Indolfi G, Quiros-Tejeira RE, Bulut P, Calvo PL, Önal Z, Valentino PL, Desai DM, Eshun J, Rogalidou M, Dezsőfi A, Wiecek S, Nebbia G, Borges-Pinto R, Wolters VM, Tamara ML, Zizzo AN, Garcia J, Schwarz K, Beretta M, Sandahl TD, Jimenez-Rivera C, Kerkar N, Brecelj J, Mujawar Q, Rock N, Busoms CM, Karnsakul W, Lurz E, **Santos-Silva E**, Blondet N, Bujanda L, Shah U, Thompson RJ, Hansen BE, Kamath BM and The GALA Study Group**.** **Natural History of Liver Disease in an International Cohort of 1438 children with Alagille syndrome: Results from The GALA Study Group.**
Submitted to Gastroenterology, 23 September 2021
(IF 2020 = 22.682; SJR = 7.83, Q1 in Gastroenterology and in Hepatology)

- **# Conference papers, 2019-2021**

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Vandriel SM, Wang JS, Li L, Piccoli DA, Loomes KM, Sokal EM, Demaret T, Arnell H, Fischler B, Fawaz RL, Nastasio S, Hardikar W, Shankar S, Lee WS, Lertudomphonwanit C, Mogul D, Karpen, SJ, Fischer RT, Quiros RE, Targa Ferreira C, Bujanda L, Indolf G, Nicastro E, Calvo PL, Valentino PL, Schwarz KB, Papadopoulou A, Dezsőfi A, Mujawar Q, Pinto RB, Brecelj, J, Kerkar N, Sanchez MC, Karthikeyan P, **Santos-Silva E**, Jimenez-Rivera C, Mozer-Glassberg Y, Alam S, Hansen BE, Kamath BM and The GALA Study Group. **Clinical features and outcomes in an international cohort of 731 Alagille syndrome patients from 19 countries.**
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ABSTRACT published in Hepatology 2019, 70 (Suppl 1): 55A-56A. (IF: 14,679)

Vandriel SM, Li L, She H, Wang J-S, Czubkowski P, Jankowska I, Gliwicz D, Piccoli DA, Loomes KM, Spinner N, Sokal EM, Demaret T, D`Antiga L, Nicastro E, Fawaz R, Nastasio S, Siew S, Storman M, Arnell H, Fischler B, Kim K, Baek WY, Karpen S, Romero R, Hardikar W, Shankar S, Roberts AJ, Evans H, Sanchez MC, Cavaleri ML, Karthikeyan P, Davidson S, Jensen MK, Kavan M, Verkade H, Lee WS, Squires JE, Lertudomphonwanit C, Fisher R, Lin H, Karnsakul W, Kelly D, Quiros-Tejeira RE, Targa C, Carvalho E, Alam S, Banales JM, Indolfi G, Larson-Nath C, Bulut P, Calvo PL, Valentino PL, Ankan C, Schwarz K, Kanavaki I, Dezsofi A, Borges-Pinto R, Wiecek S, Mujawar Q, Brecelj J, Kerkar N, **Santos-Silva E**, Jimenez-Rivera C, Waisbourd-Zinman O, Nebbia G, Gonzalez E, Thompson R, Hansen B, Kamath B. **Clinical features and natural history of 1154 Alagille syndrome patients: results from the international multicenter GALA study group.**
POSTER in the Digital International Liver Congress (EASL), 27-29 August 2020.
ABSTRACT published in J Hepatology 2020, 73 (1): S1-S1001. (IF: 25,083)
EASL Young Investigator Bursary and Poster of Distinction (top 10% among all scored poster abstracts).

Vandriel SM, Li L, She H, Wang JS, Gilbert M, Spinner N, Loomes[4] K, Piccoli D, D'Antiga L, Nicastro E, Calvo PL, Hardikar W, Shankar S, Fawaz R, Nastasio S, Bulut P, Jankowska I, Czubkowski P, Gliwicz D, Sokal E, Demaret T, Siew S, Stormon M, Lacaille F, Debray D, Kim K, Baek W, Feinstein J, Ebel N, Karpen SJ, Romero R, Karthikeyan P, Davison S, Arnell H, Fischler B, Squires J, Verkade H, Jensen MK, Kavan M, Roberts A, Evans H, Lertudomphonwanit C, Lee WS, Sundaram SS, Chaidez A, Fischer R, Mozer-Glassberg Y, Larson-Nath C, Lin H, Bujanda L, Kelly D, Karnsakul W, Bernabeu JQ, Indolfi G, Mujawar Q, Valentino P, Nebbia G, Quiros-Tejeira R, Kerkar N, Schwarz K, Wolters V, Alam S, Jimenez-Rivera C, **Santos-Silva E**, Brecelj J, Sanchez MC, Cavalieri ML, Desai D, Önal Z, Tamara ML, Molera C, Arikan C, Wiecek S, Gonzales E, Thompson R, Hansen B, Kamath B, and The GALA Study Group. **Phenotypic divergence of JAGGED and NOTCH2-associated Alagille syndrome: Results from the international multicenter GALA study group.**

POSTER in The Liver Meeting Digital Experience (AASLD),13-16 November 2020.

ABSTRACT published in Hepatology 2020, 72 (Suppl. 1): 882A-884A. (IF: 17,425)

AASLD Presidential Poster of Distinction (top 10% among all scored poster abstracts).

Vandriel SM, Li L, She H, Wang JS, Gilbert MA, Jankowska I, Czubkowski P, Gliwicz D, Gonzales EM, Jacquemin E, Bouligand J, Spinner NB, Loomes KM, Piccoli DA, D'Antiga L, Nicastro E, Sokal E, Demaret T, Lacaille F, Debray D, Siew S, Stormon M, Fawaz R, Nastasio S, Kim K, Baek W, Feinstein JA, Ebel N, Karpen SJ, Romero R, Karthikeyan P, Davison S, Arnell H, Fischler B, Squires JE, Hardikar W, Shankar S, Jensen MK, Kavan M, Larson-Nath C, Roberts AJ, Evans HM, Verkade HJ, Lertudomphonwanit C, Waisbourd-Zinman O, Sundaram S, Chaidez A, Fischer RT, Lee WS, Lin H, Kelly D, Bulut P, Calvo PL, Bernabeu JQ, Indolfi G, Nebbia G, Valentino PL,Tamara ML, Desai DM, Zizzo A, Quiros-Tejeira RE, Kerkar N, Schwarz K, Wolters V, Mujawar Q, Molera C, Sandahl TD, Jimenez-Rivera C, **Santos Silva E**, Blondet N, Brecelj J, Banales JM, Sanchez MC, Cavalieri ML, Wiecek S, Karnsakul W, Lurz E, Alam S, Önal Z, Rogalidou M, Arikan C, Garcia J,Thompson RJ, Hansen BE, Kamath BM and The GALA Study Group. **Differential prevalence of extrahepatic clinical manifestations in patients with Jagged1 and NOTCH2-associated Alagille syndrome: Results from the International Multicenter GALA Study Group.**

ORAL COMMUNICATION in WCPGHAN (6th World Congress of Paediatric Gastroenterology Hepatology and Nutrition) 2-5 June 2021, Vienna-Austria.

ABSTRACT published in J Ped Gastroenterol Nutr 2021; 72 (Suppl. 1): 799-802. (IF: 2,839)

Nominated for The Annual Alex Mowat Prize for the best oral presentation in Hepatology.

Hansen BE, Vandriel SM, Vig P, Garner W, Li L-T, She H, Wang J-S, Gilbert MA, Jankowska I, Czubkowski P, Gliwicz-Miedzińska D, Gonzales EM, Jacquemin E, Bouligand J, Spinner NB, Loomes KM, Piccoli DA, D'Antiga L, Nicastro E, Sokal E, Demaret T, Ebel NH. Feinstein JA, Fawaz R, Nastasio S, Lacaille F, Debray D, Arnell H, Fischler B, Siew S, Stormon M, Karpen SJ, Romero R, Kyung, Kim KM, Baek WY, Hardikar W, Shankar S, Roberts AJ, Evans HM, Jensen MK, Kavan M, Sundaram SS, Chaidez A, Karthikeyan P, Davison S, Sanchez MC, Cavalieri ML, Verkade HJ, Lee WS, Squires JE, Hajinicolaou C, Lertudomphonwanit C, Fischer RT, Larson-Nath C, Mozer-Glassberg Y, Arikan C, Lin HC, Bernabeu JQ, Alam S, Kelly D, Carvalho E, Targa Ferreira C, Indolfi G, Quiros-Tejeira RE, Bulut P, Calvo PL, Önal Z, Valentino PL, Desai DM, Eshun J, Rogalidou M, Dezsőfi A, Wiecek S, Nebbia G, Borges Pinto R, Wolters VM, Legarda Tamara M, Zizzo AN, Garcia J, Schwarz K, Beretta M, Damgaard Sandahl T, Jimenez-Rivera C, Kerkar N, Brecelj J, Mujawar Q, Rock N, Molera Busoms C, Karnsakul W, Lurz E, **Santos-Silva E**, Blondet N, Luis Bujanda L, Shah U, Thompson RJ, Kamath BM and The Global ALagille Alliance (GALA) Study Group. **Application of real-world evidence analytics: A 6-year event free survival analysis in Alagille syndrome of the GALA clinical research database and maralixibat treated patients.**

Accepted for Oral Presentation in AASLD - The Liver Meeting, 12-15 November 2021, Anaheim, Califórnia, USA.

PAPERS PREVIOUS TO THE THESIS PERIOD

Santos Silva E, Moreira Silva H, Melo C, Rocha H, Medina M, Martins E. **Alpha-1-antitrypsin deficiency presenting as neonatal cholestasis: predictors of outcome and effect of ursodeoxycholic acid.** J Liver 2015, 4:4.

Santos Silva E, Cardoso ML, Vilarinho L, Medina M, Barbot C, Martins E. **Liver transplantation prevents progressive neurological impairement in argininemia.** JIMD Reports 2013; 11: 25-30.

Santos Silva E, Medina M, Rocha P, Bonet B, Ferreira de Sousa JA, Enes C. **Atrésia das vias biliares extrahepáticas: estudo clínico retrospectivo.** Nascer e Crescer 2011, 20 (1): 8-14. AWARD: best original article published in 2011.

Martins E, **Santos Silva E**, Vilarinho S, Saudubray JM, Vilarinho L. **Neonatal cholestasis: an uncommon presentation of hyperargininemia.** J Inherit Metab Dis, 2010;33 Suppl 3:S503-6.

Santos Silva E, Pó I, Gonçalves I. **Colestase neonatal – protocolo de abordagem diagnóstica (Sociedade Portuguesa de Pediatria, Consensos e Recomendações).** Acta Pediatr Port 2010. 0873-9781/10/41-3/141.

Santos Silva E, Martins E, Cardoso ML, Barbot C, Vilarinho L, Medina M. **Liver transplantation in a case of argininemia.** J Inherit Metab Dis 2001; 24: 885-887.

Santos Silva E, Lumbroso S, Medina M, Gillerot Y, Sultan C, Sokal EM. **Demonstration of McCune-Albright mutations in the liver of children with high γGT progressive cholestasis.** J Hepatol 2000; 32: 154-158.

Santos Silva E, Carvalho C, Martins E, Pereira F, Silvestre F, Medina M. **Colangite esclerosante neonatal afectando dois irmãos.** Acta Pediatr Port, 1998;29 (5): 471 – 474.

ABSTRACT

BACKGROUND & AIMS

Neonatal cholestasis (NC) is a syndrome with many underlying causes and entities. The outcome and prognosis of affected patients are determined by the underlying entities and by other factors, some of which are modifiable, such as the early recognition of cholestasis, timely diagnosis of the underlying cause and implementation of effective treatment.

Despite recent advances in the knowledge of the pathophysiology of cholestasis, and more specifically of some underlying entities, so far, it has not been possible to translate these achievements to therapeutic gains and significant outcome and survival improvements.

Data in the literature and our own experience on the subject have led us to the following considerations: 1) Healthcare professionals assume clinical practices that can delay the recognition of cholestasis. 2) Epidemiology, etiological diagnosis, outcome, and survival have changed significantly in recent decades, and some new therapies can change the prognosis of specific entities. 3) The current tools for diagnosis and prognosis need to be updated and can be based on the available evidence. 4) The immaturity of the neonatal and young infant liver includes a state of 'physiological cholestasis', where the bile acid profile, oxidative stress and inflammation are mutually associated, and, in this context, the type of diet may play a relevant role.

The aim of our work was to contribute to a better understanding on the pathophysiology of NC and to the improvement of clinical practices regarding early diagnosis and a prompt and adequate treatment to improve patients' prognosis and outcome.

MATERIAL & METHODS

A prospective cross-sectional, observational study was carried out involving healthcare professionals from the three levels of the National Healthcare Service in the northern region of Portugal to evaluate the adequacy of their clinical practices for the early diagnosis of NC.

A longitudinal, observational cohort study involving 154 patients with NC observed and treated in a tertiary centre (personal experience) was carried out for a better epidemiologic, etiologic and pathophysiologic characterisation of the disease in the northern region of Portugal. In this context, we present descriptions of two clinical cases with a rare metabolic disease (deficit in lysosomal acid lipase, early form) treated innovatively with enzyme replacement treatment.

Using the same sample population, we also performed a retrospective, observational study, based on clinical and biochemical variables upon patients' admission, we built predictive diagnostic and prognostic models. A study of 16 patients with metabolic diseases, with a presentation of NC, was also carried out.

A prospective cross-sectional, exploratory study in 32 two-month-old full-term healthy infants, born from healthy mothers by vaginal delivery and prospectively recruited, was carried out to research whether the bile acid profile, oxidative stress, inflammation and the type of diet might modulate 'physiological cholestasis'.

RESULTS

We found that almost one-third of the physicians had never ordered the evaluation of conjugated bilirubin concentration in jaundiced newborns exclusively breastfed; even in the presence of other signs or symptoms, about one-third of the physicians also did not require such evaluation. Using multiple linear regression models, we found that the specialisation of the healthcare professional was an independent variable significantly associated with the ability to recognize pale stools.

In the cohort study of 154 patients with NC diagnosed and treated over three decades, we observed that the age of recognition of NC significantly decreased over time and that some epidemiologic changes occurred, namely a significant decrease of cases with alpha-1-antitrypsin deficiency (A1ATD) and a significant increase of cases with transient cholestasis. The percentage of cases with idiopathic cholestasis remained unchanged. Over time, there were no significant changes in patients' outcomes, overall survival or survival with the native liver. The study of two patients with the early onset of lipase acid lysosomal deficiency showed that innovative enzyme replacement treatment, if not started early enough, cannot prevent a fatal outcome.

In the same 154 patients, multiple regression analysis identified lower gestational age as the only independent variable associated with an increased risk of transient cholestasis, and the presence of signs and symptoms of sepsis was associated to infectious and metabolic diseases. A gamma glutamyl transferase (GGT) value higher than 300 IU/L had a positive predictive value for the diagnoses of biliary atresia (BA) and A1ATD, as well as for an unfavourable prognosis. A model of diagnosis of A1ATD (n=34) showed an area under the ROC curve = 0.843 [confidence interval (CI: 0.773-0.912]. In the 16 cases of metabolic disease with the presentation of NC, we identified three main phenotypes of liver

disease (acute liver failure, chronic liver disease, and transient cholestasis). Based on the results obtained in this cohort of patients, personal experience and data from the literature, two diagnostic decision algorithms were built/proposed.

In the exploratory study carried out with two-month-old healthy infants, we observed that chenodeoxycholic acid (CDCA) percentage was significantly and negatively correlated with oxidative stress biomarkers [membrane bound hemoglobin (MBH) and oxidized gluthathione (GSSG), in erythrocytes]. MBH was a negative predictor of CDCA percentage and its predictive value was reinforced when the model was adjusted for type of diet. Exclusive breastfeeding was associated with a higher CDCA percentage.

DISCUSSION & CONCLUSIONS

Our studies showed that a significant percentage of healthcare professionals have engaged in clinical practices that prevent the timely recognition of cholestasis and pale stools and that their specialisation, rather than years of professional experience, was associated with better skills and practices. In this setting, we should highlight our initiative to obtain permission to use the stool colour card, validated by universal screening in Taiwan, for teaching and screening purposes in Portugal. An adapted version of the card was published in Acta Médica Portuguesa, a privileged mean for disclosure among Portuguese doctors, especially general and family medicine and paediatric medicine. We believe that it would be also important to include this tool in the digital Child and Youth Health Booklet and to use it in a 'yellow alert' style campaign or in a national screening programme.

The results of the cohort study suggest that transient cholestasis associated with perinatal risk factors has become a very important subgroup that requires a specific approach and that despite the advances observed in the earlier recognition of cholestasis and greater diagnostic diversity of underlying entities, improvement in the outcome and survival of patients is still not significant.

It was possible to build a diagnostic algorithm in which three of the five discriminators were chosen among the diagnostic and prognostic predictors identified in this sample of patients. Concerning the studied cases of metabolic disease with the presentation of NC, we must highlight transient cholestasis without associated risk factors as a clue to be followed for metabolic disease.

Data from the exploratory study suggested that under physiological conditions, in early postnatal life, bile acids profile may play a role in regulating the redox state (and/or vice versa) and that the type of diet appears to have impact on this process.

In summary, the results and conclusions from our clinical studies can help to improve the diagnosis and prognosis of patients with NC by leading to increased early detection and adequate clinical practices. The exploratory research study suggested a potential link between the bile acid profile and the redox state in healthy infants, strengthening a rationale for therapeutic and preventive strategies.

RESUMO

INTRODUÇÃO E OBJETIVOS

A colestase neonatal é uma síndrome com muitas causas e entidades subjacentes. O resultado e o prognóstico dos doentes afectados é determinado pelas entidades subjacentes e por outros factores, alguns deles modificáveis, tais como, o reconhecimento precoce da colestase, o diagnóstico atempado da causa subjacente, e a implementação de tratamento eficaz.

Apesar dos avanços recentes no conhecimento da fisiopatologia da colestase, e mais especificamente de algumas entidades subjacentes, até ao momento, ainda não foi possível transformar essas conquistas em ganhos terapêuticos e melhorias significativas dos resultados e da sobrevida dos doentes.

Os dados da literatura e a nossa experiência no assunto, conduziram-nos às seguintes considerações: 1) Os profissionais de saúde assumem práticas clínicas que podem atrasar o reconhecimento da colestase. 2) A epidemiologia, o diagnóstico etiológico, o resultado e a sobrevida mudaram significativamente nas últimas décadas; e algumas terapêuticas novas podem mudar o prognóstico de entidades específicas. 3) As actuais ferramentas de abordagem diagnóstica e preditivas do prognóstico precisam de ser actualizadas, e isso pode ser feito com base na evidência disponível. 4) A imaturidade do fígado neonatal e do pequeno lactente inclui um estado de “colestase fisiológica” onde o perfil dos ácidos biliares, o stress oxidativo e a inflamação estão mutuamente associados, e neste contexto o tipo de dieta pode desempenhar um papel relevante.

O objetivo da nossa investigação foi contribuir para um melhor conhecimento da fisiopatologia da colestase neonatal e para a melhoria das práticas clínicas relativamente ao diagnóstico precoce e a um tratamento rápido e adequado destes doentes, para conseguir melhorar os resultados e o seu prognóstico.

MATERIAL E MÉTODOS

Foi realizado um estudo prospectivo, transversal, observacional envolvendo profissionais de saúde dos três níveis do Serviço Nacional de Saúde na região Norte de Portugal, para avaliar a adequação das suas práticas clínicas a um diagnóstico precoce da colestase neonatal.

Foi realizado um estudo de coorte, longitudinal, observacional, envolvendo 154 doentes com colestase neonatal observados e tratados num centro terciário (experiência pessoal) para obter uma melhor caracterização epidemiológica, etiológica e fisiopatológica da síndrome no Norte de Portugal. Neste contexto apresentamos a descrição de dois casos clínicos com uma doença metabólica rara (défice em lipase ácida lisossomal, forma precoce) tratados de forma inovadora com tratamento de substituição enzimática.

Usando a mesma amostra da população, efectuamos também um estudo retrospectivo, observacional, tendo sido construídos modelos preditivos de diagnóstico e de prognóstico a partir de variáveis clínicas e bioquímicas registadas na admissão dos doentes. Adicionalmente, foi efectuado um estudo de uma série de 16 casos de doentes com doenças metabólicas com apresentação de colestase neonatal.

Foi efectuado um estudo transversal, exploratório, em 32 lactentes saudáveis com 2 meses de idade, nascidos de mães saudáveis, gestação de termo, e parto vaginal, e prospectivamente recrutados para estudar se o perfil dos ácidos biliares, o stress oxidativo, a inflamação e o tipo de dieta, podem modular a “colestase fisiológica".

RESULTADOS

No estudo realizado com os profissionais de saúde, quase um terço dos médicos assumiu que nunca requisitaria um doseamento de bilirrubina conjugada a recém-nascidos ictéricos amamentados ao seio materno; e mesmo na presença de outros sinais ou sintomas cerca de um terço dos médicos também não o faria. Além disso, usando modelos de regressão linear múltipla identificamos a especialização como uma variável independente significativamente associada com a capacidade de reconhecer as fezes despigmentadas.

No estudo de coorte de 154 doentes com colestase neonatal diagnosticados e tratados ao longo de três décadas observamos que a idade de reconhecimento da colestase neonatal diminuiu significativamente ao longo do tempo, e que as mudanças na epidemiologia foram relevantes, nomeadamente uma diminuição significativa dos casos de deficiência de alfa-1-antitripsina (A1ATD) e um aumento significativo dos casos de colestase transitória. A percentagem de casos com colestase idiopática não se alterou. Ao longo do tempo não houve mudanças significativas no resultado dos doentes nem na sobrevida global ou com fígado nativo.

A descrição dos dois doentes com défice de lipase ácida lisossomal, forma precoce, mostrou que o tratamento inovador de substituição enzimática quando não é iniciado precocemente não consegue impedir um desfecho fatal.

Nos mesmos 154 doentes, os modelos de regressão logística identificaram a prematuridade como a única variável independente associada a um risco aumentado de colestase transitória, e a presença de sinais e sintomas de sepsis associou-se a doenças infecciosas ou metabólicas. A gama glutamyl transferase > 300 UI/L na admissão teve um valor preditivo positivo para os diagnósticos de atresia das vias biliares e A1ATD, bem como para prognóstico desfavorável. Um modelo de diagnóstico de A1ATD (n=34) apresentou uma área abaixo da curva ROC = 0.843 [intervalo de confiança (CI): 0.773-0.912]. Na série de 16 casos de doença metabólica com apresentação de colestase neonatal identificamos três fenótipos principais de doença hepática (insuficiência hepática aguda, doença hepática crónica, e colestase transitória). Com base nos resultados obtidos nesta coorte de doentes, na experiência pessoal e na literatura foram construídos dois algoritmos de decisão diagnóstica.

No estudo exploratório realizado com os lactentes saudáveis observamos que a percentagem de ácido quenodesoxicólico (CDCA) se correlaciona significativa e negativamente com os marcadores de stress oxidativo [hemoglobina ligada à membrana (MBH), e glutatião oxidado nos eritrócitos (GSSG)]. A MBH foi um preditor negativo da percentagem de CDCA e o seu valor preditivo foi reforçado quando o modelo foi ajustado para o tipo de dieta. O aleitamento materno exclusivo associou-se a uma percentagem mais elevada de CDCA.

DISCUSSÃO E CONCLUSÕES

Os nossos estudos demonstraram que uma percentagem significativa de profissionais de saúde assumiu práticas clínicas que impedem o reconhecimento atempado da colestase e das fezes acólicas, e a sua especialização, e não os anos de experiência profissional, associou-se a melhores capacidades e práticas.

Neste contexto assume particular relevância a nossa iniciativa de obter a autorização para usar o cartão de cores de fezes validado pelo rastreio universal de Taiwan, para fins de ensino e rastreio em Portugal. Uma versão adaptada do cartão foi publicada na Acta Médica Portuguesa como meio privilegiado de divulgação entre os médicos portugueses, especialmente os Médicos de Medicina Geral e Familiar e os Pediatras. Esta ferramenta

poderá também ser incluída no Boletim de Saúde Infantil e Juvenil digital e utilizada numa campanha do estilo “alerta amarelo” ou num programa de rastreio nacional.

Os resultados do estudo de coorte sugerem que a colestase transitória associada a factores de risco perinatal se transformou num subgrupo muito importante e que requer uma abordagem específica, e que apesar dos avanços observados no reconhecimento mais precoce da colestase e na maior diversidade diagnóstica de entidades subjacentes, a melhoria do resultado e da sobrevida dos doentes ainda não é significativa.

Foi possível construir um algoritmo de diagnóstico em que 3 dos 5 discriminadores foram escolhidos entre os factores preditores de diagnóstico e de prognóstico identificados nesta amostra de doentes. Na série de casos de doença metabólica com apresentação de colestase neonatal destacamos a colestase transitória, mas sem factores de risco, como uma pista a ser seguida para doença metabólica.

Os resultados do estudo exploratório sugerem que, em condições fisiológicas, na vida pós-natal precoce, o perfil dos ácidos biliares pode desempenhar um papel na regulação do estado redox (e/ou vice-versa), e que o tipo de dieta pode ter algum impacto neste processo.

Em resumo, os resultados e as conclusões dos nossos estudos clínicos podem ajudar a melhorar o diagnóstico e o prognóstico dos doentes com colestase neonatal, conduzindo a maior precocidade no reconhecimento e a práticas clínicas mais adequadas. Os resultados do estudo de investigação exploratório sugeriram uma ligação potencial entre o perfil de ácidos biliares e o estado redox em lactentes saudáveis, fortalecendo um racional para estratégias terapêuticas e preventivas.

KEYWORDS

- Bile acids
- Breastfeeding
- Cholestasis
- Dark urine
- Diet
- Inflammation
- Infant
- Jaundice
- Liver physiology
- Neonatal cholestasis
- Newborn
- Oxidative stress
- Pale stools
- Strategies of Diagnosis and Prognosis

ABBREVIATIONS

A1AT – Alpha-1-antitrypsin
A1ATD – Alpha-1-antitrypsin deficiency
AA – aminoacids
AASLD – American Association for Study of liver disease
ABCG – ATP binding cassette
ALF – Acute liver failure
ALGS – Alagille syndrome
ALT – Alanine aminotransferase
AMP – Adenosine monophosphate
ASBT – Apical sodium dependent bile acid transport
AST – Asparate aminotransferase
ATP – Adenosine triphosphatase
BA – Biliary atresia
BD – Bile duct
BDL – Bile duct ligation
BPD – Bronchopulmonary dysplasia
BRIC – Benign recurrent intrahepatic cholestasis
BSEP – Bile salt export pump
CA – Cholic acid
CDCA – Chenodeoxycholic acid
CESD – Cholesterol ester storage disease
ChiLDRen – Childhood Liver Disease Research Network
Cho- Cholesterol synthesis
CHUP – Centro Hospitalar Universitário do Porto
CI – Confidence interval
CMIN – Centro Materno Infantil do Norte
CMV - Citomegalovirus
CV – Central vein
CYPs – Cytochrome P450 enzymes
DAMPs – Damage-associated molecular patterns
DBJ – Dubin-Johnson syndrome
DCA – Deoxycholic acid
EASL – European association for study liver disease
EC – Endothelial cells
ECMO – Extracorporeal circulation membrane oxygenation
ENR – European Reference Network
EO-LAL-D – Early-onset lipase acida lisosomal.
ERT – Enzyme replacement therapy
ESPGHAN – European Society of Gastroenterology, Hepatology and Nutrition
FCT – Fundação Ciência e Tecnologia
FDA – Food drug administration
FEDER - Fundo Europeu de Desenvolvimento Regional
FGF19 – Fibroblast growth factor
FGFR4 – Transmembrane receptor tyrosine kinase 4
FHL – Familial hemophagocytic syndrome
FR – Free radicals
FXR – Farnesoid X receptor
GALA – Global Alagille Alliance Study
GALD – Gestational alloimmune liver disease
GCDCA – Glicocheodeoxycholic acid
Ggn – Glycogen
GGT – Gamma glutamyl transferase
GLP axis – Glucagon like peptid
GNAS – G-protein alpha-subunit

GPBAR -1 – G-protein coupled bile acid receptor 1
GPs – General practioniers
GPx – Glutathione peroxidase
GS – Glutamin synthesis
GSH – Reduced glutathione
GSSG – Glutathione oxidized
GST – Glutathione transferase
HC - Hepatocytes
HIE – Hypoxic-ischemic encephalopathy
HLH – Hemophagocytic lymphohistiocytosis
ICBAS – Instituto de Ciências Biomédicas Abel Salazar
IDA – Imunodiacetic analogue
IEM – Inborn error of metabolism
IgG – Immunoglobulin G
IL – Interleukin
IQR – Interquartile range
IVH – Intraventicular hemorrhage
JPBAR – Japanese biliary atresia registry
KC – Kuppfer cells
Lac – Lactose
LAL-D – Lysosomal acid lipase deficiency
LCA – Lithocholic acid
LPAC – Low phospholipd associated cholelithiasis
LPO – Lipid peroxidation
LSD – Lysosomal storage disease
MAS – McCune-Albright syndrome
MBH – Membrane bound hemoglobin
MCT – Medium chain triglycerides
MCTES – Ministério da Ciência, e Tecnologia, e Ensino Superior
MDR – Multidrug resistance protein
MIM – Master`s degree in Medicine
mRNA – Messenger ribonucleic acid
MRP – Multidrug resistance-associated protein
NAPPED – Natural course and Prognosis of PFIC and Effect of biliary Diversion
NASPGHAN – North American Society for Pediatric, Gastroenterology, Hepatology and Nutrition
NBS – Newborn screening
NC – Neonatal cholestasis
NEC – Necrotizing enterocolitis
NGS – Next generation sequencing
NH – Neonatal hemochromatosis
NHS – National healthcare system
NICCD – Neonatal intrahepatic cholestasis caused by citrin deficiency
NTBC – [2-[2-nitro-4-trifluormetilbenzoil. 1.3 ciclohexanediol]
NTCP – Na+ taurocholate co-transporting
OATP – Organ anion transporting polypeptide
OATP – Organic anion transporting polypeptide family
OLT – Orthotopic liver transplantation
OR – Odds-ratio
OST alfa/beta – Anion transporting polypeptide family
PCr – Polymerase chain reaction
PDA- Patent ductus arteriosus
PE – Portoenterostomy
PFIC – Progressive familial intrahepatic cholestasis
PHH - Primary human hepatocytes
Pp – Periportal

PUFA – Poly unsatured fatty acids

Pv – Perivenosus

PV – Portal vein

RISC – RNA-induced silencing complex

ROC curve – Receiver operator characteristic curve

ROP – Retinopathy of prematurity

ROS – Reactive oxygen species

SCC – Stool color card

SD – Standard deviation

SERPIN – Serine protease inhibitor superfamily

SSBA – Sulfated bile acids

TAS – Total antioxidant status

TCA – Taurocholic acid

TGP- Target gene panels

TGR-5: Takeda-G-protein receptor5

THA – Terminal hepatic arteriole

TNF-alpha - Tumor necrosis factor

TPN – Total parenteral nutrition

TPV – Terminal portal vein

UCIBIO – Applied molecular biosciences unit

UDCA – Ursodeoxycholic acid

UDPGT1A1 – Uridine diphosphate glucuronosyltransferase

UK – United Kingdom

UPR – Unfolded protein response

USA – United Sates of America

VOUS - Variants of unknown significance

WCPGHAN – World Congress Pediatric Gastroenterology Hepatology and Nutrition

WES – Whole exome sequencing

WGS – Whole genome sequencing

CHAPTER I

INTRODUCTION

1. HISTORICAL PERSPECTIVE

Jaundice in newborns and infants has been known since antiquity. The Jews delayed circumcision when the male neonate had jaundice (1). The Chinese described neonatal jaundice in the year 610 (7th century) in the book 'On the Origins and Symptoms of Diseases' (2). Their reports reveal an awareness of congenital causes as they attributed the condition to 'heat and moisture' acquired through the womb or breast milk and environmental causes such as 'toxins and demons'. They also knew about biliary obstruction, referred to as 'blood flow stasis', and 'inherited diseases' (2).

During the Middle Ages, the border between physiological and pathological jaundice was not drawn since all serious neonatal diseases, regardless of their aetiology, were likely to be associated with jaundice, enhanced by starvation, dehydration, septicaemia and therapeutic attempts. Therefore, whatever therapeutic measures were taken, the general perception was that jaundice in newborns was most often a serious condition.

Later on, a landmark was a description of the haemolytic disease of a newborn in 1609 by a French midwife, Louise Bourgeois (3), in a set of twins: 'the first twin was markedly hydropic and was born nearly dead, while the second developed jaundice, rapidly worsening within a few hours and died three days after birth' [quoted by Reali 2007 (4)]. Nevertheless, this condition would only be fully characterised and its pathogenesis clarified in the 20th century.

In 1817, Prof John Burns, a surgeon at the University of Glasgow, gave a description of what seems to be biliary atresia (BA) in the textbook 'Principals of Midwifery, Including the Diseases of Women and Children': 'the jaundice of infants is a disease attendant with great danger, especially if it appears very soon after birth, and the stools evince, a deficiency of bile; for we have then reason to apprehend some incurable state of the biliary apparatus' [cited by Mowat (5)]. Later, by the end of the 19th century, a remarkable paper by John Thomson, a paediatrician from Edinburgh, was published with a review and discussion of data from 50 cases of BA, and his conclusions regarding anatomopathological findings were sustained for a century (6).

The first test for the measurement of bilirubin, including its separation into two chemically distinct compounds of direct- and indirect-reacting bilirubin, appeared in the 20th century [cited by Kramer et al. (7)]. Nevertheless, the discovery of an accurate and specific method of bilirubin assay with clinical practice application would not be achieved until after the commitment of many researchers, taking an additional 50 years. This achievement finally

allowed the characterisation of neonatal jaundice according to the amount of each type of bilirubin (7).

In the second half of the 20th century, patients with obstructive jaundice (pale stools and dark urine) started to receive more attention, and soon it was realised that not all had BA of the main biliary ducts. In 1952, Craig and Landing (8) described the characteristic histological appearance of the liver in cases with patent bile ducts, and the condition became known as 'neonatal giant cell hepatitis'. A decade later, Danks and Bodian (9) took the first steps into the search for genetic causes in this subset of patients by pursuing some clues non-existent in the BA group. By this time, the term 'neonatal hepatitis syndrome' had become popularised and encompassed both types of cholestatic neonatal jaundice (BA and neonatal hepatitis), and the diagnostic paradigm became the separation of patients with BA from patients with neonatal hepatitis.

In 1968, Morio Kasai, a Japanese surgeon, published his surgical procedure for patients with BA – the portoenterostomy (PE) – named after himself (the Kasai procedure) (10). The disease had much higher incidence in Asia than in Western countries, and the results of the Kasai procedure were very appealing. Thus, it became of paramount importance to differentiate among patients with 'neonatal hepatitis syndrome' those with BA, which could benefit from this surgery (11). Concomitantly, in 1963, Thomas Starzl, an American surgeon, tried the world's first orthotopic liver transplant (OLT) in a three-year old child with BA, but the child died during surgery (12). In 1967, Starzl had performed liver transplants in several patients with survival (13), but the procedure would only come to be long-term successful in the 1980s after the discovery of cyclosporine as an immunosuppressant that prevented graft rejection (14). Kasai's PE together with OLT radically changed the survival of patients with BA. Additionally, OLT also became crucial in improving the survival rates of other patients (15).

In the last quarter of the 20th century, there were important insights concerning bile acid metabolism, bile secretion and enterohepatic circulation (16). These discoveries enriched the understanding of hepatobiliary physiology in early human life, and in 1981, Suchy and Balistreri (17) introduced the concept of 'physiological cholestasis' caused by bile secretion immaturity and characterised by raised levels of serum bile acids. By the end of the 20th century, the interest in bile acid research was renewed by the discovery of signalling properties (18). By this time, the hypothesis that familial forms of neonatal cholestasis (NC) might be caused by inborn errors in hepatic metabolic or excretory function (altered membrane transport, bile acid biosynthesis or organelle dysfunction) experienced important developments. The first example of organelle dysfunction was homozygous alpha-1-

antitrypsin deficiency (A1ATD), which since 1969 has been known to be associated with liver cirrhosis and to manifest via NC (19). This disease became the prototype of an inborn error leading to liver disease, thus stimulating the search for other genetic/metabolic defects.

By the end of the 20th century and the beginning of the 21st century, basic research had enhanced our understanding of the molecular mechanisms concerning normal bile secretion and their disruptions in cholestasis. The identification of progressive familial intrahepatic cholestasis (PFIC) (20) and the characterisation of bile transporters led to a revolution in the pathophysiology of the now-named 'NC syndromes'. For the first time, it was possible to draw therapeutic strategies based on the known mechanisms of the disease (21). In parallel, a new subset of patients suffering from NC emerged from the reality of the neonatal intensive care units and brought about the new concept of 'transient NC' associated with risk factors (22), later regarded as related to immaturity, oxidative stress, infection/inflammation and starvation (23). Additionally, another syndrome from the 'non-BA group' emerged, 'neonatal hemochromatosis' (NH) (24). Once a highly lethal condition, it has become not only a treatable condition but a preventable one (25). Moreover, several studies have identified new forms of rare liver diseases underlying NC.

The discovery of so many underlying entities made the diagnosis approach to NC an enormous challenge, difficult to be accomplished in a promptly, non-invasive, and inexpensive way. The recent development of new-generation molecular sequencing tools (26, 27) is now challenging the diagnostic paradigm for the non-BA group of patients and is expected to contribute to reducing the category of patients with 'idiopathic NC'.

Currently, we are already living in a new era concerning NC. The 21st century brought the awareness that to obtain results in the investigation of rare diseases (NC is a syndrome with many underlying rare diseases), large cohorts have to be brought together to gain in scale. To fulfil this aim, patients' registries and multinational consortia were set up, such as the following: the Japanese Biliary Atresia Society Registry – JPBAR, the Childhood Liver Disease Research Network – ChiLDReN, the European Reference Network – ERN – RARE LIVER, the Natural course and Prognosis of PFIC and Effect of biliary Diversion (NAPPED) consortium and the Global Alagille Alliance Study – GALA. This strategy allied with the advances in basic and translational research has already brought significant results regarding BA and PFIC, and in the short term, the same is expected for Alagille syndrome (ALGS).

However, despite all the major advances in recent years, NC remains a remarkable clinical challenge. Specifically, early recognition fails in a considerable percentage of cases, the aetiological diagnosis is a complicated task due to the diversity of underlying entities and morbidity and mortality is high, with the survival of many patients dependent upon OLT. All of this suggests that further studies are still warranted to achieve the best outcome for the patients with NC.

2. CONCEPTS

- Jaundice

The word has an etymological origin in the French language in the word ‘jaune’, which means ‘yellow’. It refers to a morbid condition characterised by yellow eyes and skin, is caused by increased bile pigments in the serum and is a signal present in various diseases.

- Physiological neonatal jaundice

Here, jaundice appears after the first 24 hours of life and lasts for a maximum of seven days in full-term newborns and 14 days in preterm newborns. This type of jaundice is due to several adaptive processes and the immaturity of several enzymatic pathways in the neonate and is characterised by a predominant increase in the unconjugated fraction of bilirubin.

- Pathological neonatal jaundice

This refers to jaundice that may present in the first 24 hours of life and may be of great intensity requiring intensive/invasive therapy (double phototherapy or blood exchange-transfusion) or may be prolonged (> 14 days). This jaundice should raise suspicion of some underlying condition and is characterised by a predominant increase in either the unconjugated or the conjugated fraction of bilirubin (if conjugated, it is called cholestatic jaundice, or cholestasis).

- Cholestasis

Cholestasis is a clinical syndrome characterised by reduced bile formation or bile flow, resulting in the retention of bile compounds within the liver (28). Cholestasis is generally recognised by the serum elevation of conjugated bilirubin and bile acids, which are central and often coincident features of hepatobiliary dysfunction.

- Neonatal cholestasis

NC is defined as jaundice with conjugated bilirubin ≥ 1 mg/dl of the total bilirubin < 5.0 mg/dl, or > 20% of the total bilirubin > 5.0 mg/dl detected in a newborn or infant younger than four months old, according to the Guideline of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) and the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) of 2004 (29). In 2017, both societies extended the criterion by increasing the cut-off of conjugated bilirubin to > 1 mg/dl regardless the total bilirubin value (28). NC, defined in these terms, is always pathological and requires investigation in all affected newborns or infants.

NC is not a disease, but a syndrome, a clinical manifestation of several types of injury or diverse clinical underlying entities.

In addition to that mentioned above, it is also important to keep in mind the following concepts:

- Physiological neonatal cholestasis

Suchy et al. (17), in 1981, proposed the term 'physiologic cholestasis' to define the elevation of primary bile acid concentrations in healthy infants, reflecting the immaturity of the enterohepatic cycle in early postnatal life. However, in these physiological conditions, contrary to what is observed in pathological cholestasis, conjugated bilirubin is < 1 mg/dl.

- Transient neonatal cholestasis

By the end of the 20th century, a different subset of patients with NC has emerged from the neonatal intensive care units, requiring a redefinition of concepts. Jacquemin et al. (22), in 1998, proposed transient NC as the presence of cholestatic jaundice (as defined above), associated with known perinatal risk factors, detected in a newborn or infant younger than four months old and complete and spontaneous normalisation of liver function tests within the first six months of life.

3. EPIDEMIOLOGY

Dick and Mowat reported the overall incidence of NC as 1 per 2500 live births in a south east England population in a prospective study conducted between January 1971 and December 1973 (30). Previous studies had observed overall incidences between 1 per 5000 to 1 per 9000 live births in other populations (31, 32) although inclusion criteria differed among studies. Many scientific improvements were reported in the last 50 years, but no similar studies have been conducted to estimate the overall incidence of NC.

After the year 2000, some studies reported the incidence of NC in neonatal intensive care units, a reflex of an emerging reality – the entry into the scene of 'transient cholestasis' associated with perinatal risk factors. In 2009, Tufano et al. (33) reported an incidence of 2% in a cohort of 1289 patients followed up for a period of 12 months after enrolment (4.5% incidence in newborns with gestational age ≤ 32 weeks). In 2012, Champion et al. (23) reported an incidence of 13.7% in a subset of neonates with gestational age < 34 weeks (n = 234) enrolled over a 12-month period. In 2021, Teng et al. (34) reported an incidence of 14.8% in 250 preterm infants with gestational age < 30 weeks and surviving for 28 days.

As previously mentioned, NC is a syndrome caused by different types of injury and with many underlying clinical entities. In a systematic review including 1692 patients (from 17 selected studies, 1963 to 2011), the most common entities were BA (25.89%), infection (11.47%), total parenteral nutrition (TPN)-associated cholestasis (6.44%), metabolic disorders (4.37%), A1ATD (4.14%), perinatal hypoxia/ischemia (3.66%) and idiopathic (26%) (35).

In older studies, such as that from Mieli-Vergani et al. (36), enrolling 1086 patients in a 20-year period (1970–1990), idiopathic neonatal hepatitis was the most common cause of NC, with an incidence of 31%. A more recent study in Germany (37) reports an incidence substantially lower (17%) in a consecutive series of 82 patients (8 preterm) in a 4-year period (2009–2013). However, an Australian series of 139 patients (99 preterm) in a 5-year period (2013–2017) reported by Ling et al. (38) classified 21 patients (15.1%) either with idiopathic neonatal hepatitis or unidentified cause, the majority of which were from the subset of full-term patients (13 patients, 32.5%).

In 2021, Hertel et al, in a prospective longitudinal study of ChiLDReN, involving 15 clinical sites in the Unites States of America (USA) and Canada over an 11-year period, reported 215 of 938 patients (23%) with idiopathic cholestasis; in this cohort, of participants with data

available, 22 of 90 (24%) were preterm (<37 weeks gestational age), and 25 of 89 (28%) had low birth weight (<2500g) (39).

We highlight the difficulties in interpreting the data from the different studies due to lack of uniformity in inclusion and exclusion criteria for the diagnosis of idiopathic cholestasis.

Table 1 displays a compilation of the most relevant studies carried out over the last decades in several countries reporting the main aetiologies underlying NC.

Authors, year of publication	Journal	Country	Period of time	N	BA	A1ATD	Transient	TPN	Others	Idiopathic
					n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
		Europe								
Dick & Mowat, 1985	Arch Dis Child	England	1971-1973	54						
Mieli-Vergani et al, 1991	Gut Supplement	England	1970-1990	1086	377 (35)	189 (17)			189 (17)	331 (31)
Fischler et al, 2001	Acta Paediatr	Sweden	1988-1997	85	30 (35)	11 (13)	0		52	22 (26)
Tiker et al, 2006	Indian J Pediatr	Turkey	2000-2003	42	1	0		3		6 (14)
Humphrey et al, 2007	Radiology	England	2002-2005	90	30 (33)	4 (4)	15 (17)			22 (24)
Ipek et al, 2013	Turk J Gastroenterol	Turkey	2006-2010	92	4	0	20	8		7 (8)
Hoerning et al, 2014	Frontiers Ped	Germany	2009-2013	82	34 (41)	1 (1)	8 (10)		25 (31)	11 (13)
		América								
Hitch et al, 1981	American J Surgery	USA	1978-1981	31	9	1				16 (52)
Spivak et al, 1987	J Pediatr	USA	1982-1985	28	7	0		5		6 (21)
Tolia et al, 1989	J Pediatr Gastroenterol Nutr	USA	NA	40	5	1				3 (8)
Mendoza et al, 2015	Rev Col Gastroenterol	Colômbia	2010-2013	21	4 ()	0				3 (14)
		Asia								
Yachha et al, 1996	Indian Pediatr	India	1992-1995	60	33 (55)	0			9 (15)	18 (30)
Aanpreung et al, 2005	J Med Assoc Thai	Thailand	1993-2004	252	56 (22)	0		46 (18)	92 (37)	58 (23)
Bazlul Karim & kamal, 2005	Indian J Gastroenterol	Bangladesh	1998-2003	62	16 (26)	1 (1,6)	0		22 (35)	15 (24)
Lee et al, 2008	J Pediatr Child Health	Malaysia	1996-2004	146	42 (29)	0	9 (7)		39 (27)	56 (38)
Rafeey et al 2008	Pak J Biol Sci	Iran	2002-2007	120	30 (25)	0				44 (36)
Jiang et al, 2013	Hepatobiliary Pancreat Dis Int	China	2009-2011	51	23	0				22 (43)
Chowdhury et al, 2014	J Banagladesh Coll Phys Surg	Bangladesh	2010-2011	40	17 (43)	0	0		13 (32)	10 (25)
Mathiyazhagan et al, 2017	Indian J Pediatr	India	2015-2016	64	16 (25)	0	10 (16)		32 (50)	6 (9)
		Oceania								
Danks et al, 1977	Arch Dis Child	Australia	1963-1974	162	55	6				59 (36)
Stormon et al, 2001	J Pediatr Child Health	Australia	1985-1996	205	34 (17)	12 (6)				52 (25)
Ling et al, 2020	J Ped Child Health	Australia	2013-2017	139	9			70		21
		África								
Johnson et al, 1980	Afr J Med Sci	Nigeria	?	101						
Motala et al, 1990	J Tropical Pediatr	South Africa	1975-1984	145	41	6				52 (36)
Bouyahia et al, 2008	La Tunisie Medicale	Tunisia	1995-2005	94	13 (14)	0	16 (17)		53 (56)	12 (13)
Gottesman et al, 2015	BMC Pediatrics	**Systematic Review**	1963-2011	1692	438 (26)	70 (4)		109 (6)		440 (26)

A1ATD (alpha-1-antitrypsin deficiency); BA (biliary atresia); NA (non-available); TPN (total parenteral nutrition)

Table 1 – Data from relevant epidemiologic studies on neonatal cholestasis

Morbidity and mortality rates are difficult to compare among studies due to the different follow-up periods in each study. In the study by Dick and Mowat (30), the overall mortality at 10 years follow-up was 19 out of 53 patients (35,8%). Persistent liver disease was observed in 4 out of 53 patients (7.5%), with one patient missed from follow-up. More recently, Hoerning et al. (37) found a higher mortality rate in BA among all patients of NC. The pre-transplant mortality rate for children with BA was ~12 *vs*. 4% in children with other disorders causing NC. Of the 82 children with NC, 10 died (~12%) in the longitudinal course either before or after liver transplantation during a four-year period. The majority of this subgroup with a poor outcome had BA (n = 8), of which four patients had a syndromic BA variant.

The outcomes and survival rates of NC patients vary with the underlying entities. Data are available for some subgroups such as BA (40-44), A1ATD (45-47) and ALGS (48-50) and will be further detailed in section 5.2.

On the contrary, regarding transient cholestasis, the long-term follow-up is scarcely reported. Teng et al. (34) recently described the outcome linked to NC in a cohort of preterm infants (gestational age < 30 weeks) surviving for at least 28 days in two tertiary centres (n = 250). Cases which developed cholestasis were matched on gestational week with two non-cholestatic controls. Cholestatic patients had more retinopathy of prematurity (ROP) and bronchopulmonary dysplasia (BPD). Multivariate models using logistic regression analysis found that ROP grade 3 and BPD were independent positive predictors of a poor outcome [odds-ratio (OR) 3.6 (95% CI 1.4–9.4) and 2.8 (95% CI 1.2–6.3), respectively]. The OR increased when the models were adjusted for gestational age, gender and mechanical ventilation > 2 weeks [OR 8.4 (95% CI 2.1–33.4) and 3.2 (95% CI 1.2–8.6)]. The mortality rate was 13.5% in cholestatic infants versus 2.7% in controls (p = 0.040). Nevertheless, all surviving infants with long-term follow-up (n = 101) were free of significant chronic liver disease by 10 years of age.

Also, the outcome and survival of patients with ‘idiopathic NC’, a definition which includes most of the unknown causes of intrahepatic cholestasis, are poorly established. The criteria for patient classification into this category are among other difficult-to-analyse data from published studies. This is a very heterogeneous subset suffering from variations over time and from one centre to another. Theoretically, outcomes and survival rates in this subset of patients should vary according to the underlying condition(s) and are expected to be different for sporadic and familial cases.

Very recently, the Hertel et al study (39) reported excellent outcomes for the idiopathic cholestasis subgroup (98% alive with native liver at 30 months of age), opposed to some old studies (30,51-54) reporting elevated rates of death and OLT (from 7-31%); this might be due to the current exclusion from this subgroup of patients of those with genetical or metabolic diseases, nowadays identifiable with the help of new diagnostic technologies, and with poor outcome; and to the inclusion of patients with transient cholestasis, with good outcome.

Despite all the advances, NC remains associated with high morbidity and mortality, in both the short and long term, for some of their underlying conditions.

4. PATHOPHYSIOLOGY – STATE OF THE ART

Understanding the pathophysiology of liver diseases, especially those underlying NC, requires a thorough understanding of the morphogenesis and differentiation of the liver, whose anatomy and function are immature at birth.

4.1 LIVER DEVELOPMENT AND ANATOMY

Human liver development begins during the third week of gestation from the ventral foregut endoderm cells by differentiation into hepatoblasts (the liver's progenitor cells) (Fig. 1) and is controlled by a network of transcription factors. The early stage of liver expansion depends on continuous interactions between hepatoblasts and adjacent mesodermal tissues and progresses with the invasion of the septum transversum (55).

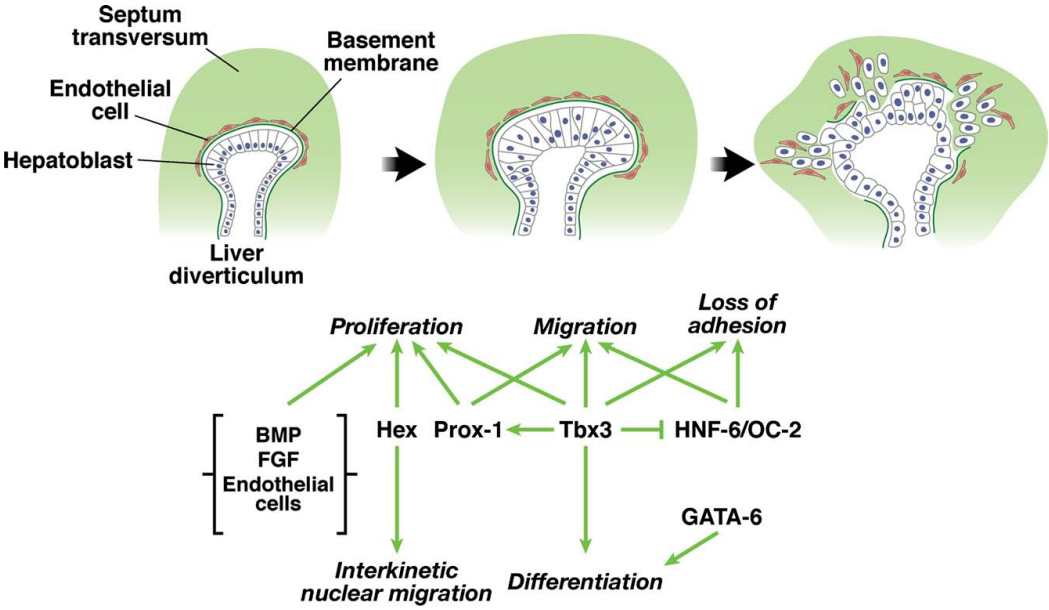

Figure 1 – Budding of the liver out of the endoderm.

Upper panel: Changes in morphology of the liver when it buds out of the endoderm. Lower panel: Control of key regulatory events by transcription factors and extracellular signalling.

Source: Reprinted with permission from Elsevier BV(55).

Hepatoblasts are bipotent and can differentiate into hepatocytes or cholangiocytes (55). The principles that guide the molecular mechanisms of liver specialisation are reasonably well known. The concept of cell fate decision applies, in theory, to hepatoblasts that have reached a stage of development in which they become committed to the hepatocyte or

cholangiocyte strains (Fig.2). The cells that differentiate themselves from the lineage of hepatocytes undergo a maturation process that consists of the progressive acquisition of morphology and physiological functions. Some factors enriched by the liver are important determinants of the morphology of hepatocytes in addition to their role in controlling metabolic functions.

The maturation of hepatocytes also requires the repression of a series of genes during the pre- and postnatal periods since at birth it is not finished. The neonatal period is a liver development phase often overlooked despite its importance being well illustrated by the three patterns of expression of the ontogenic genes of cytochrome P450 observed in mice (56), something very important for understanding the kinetics of xenobiotic metabolism during the neonatal period.

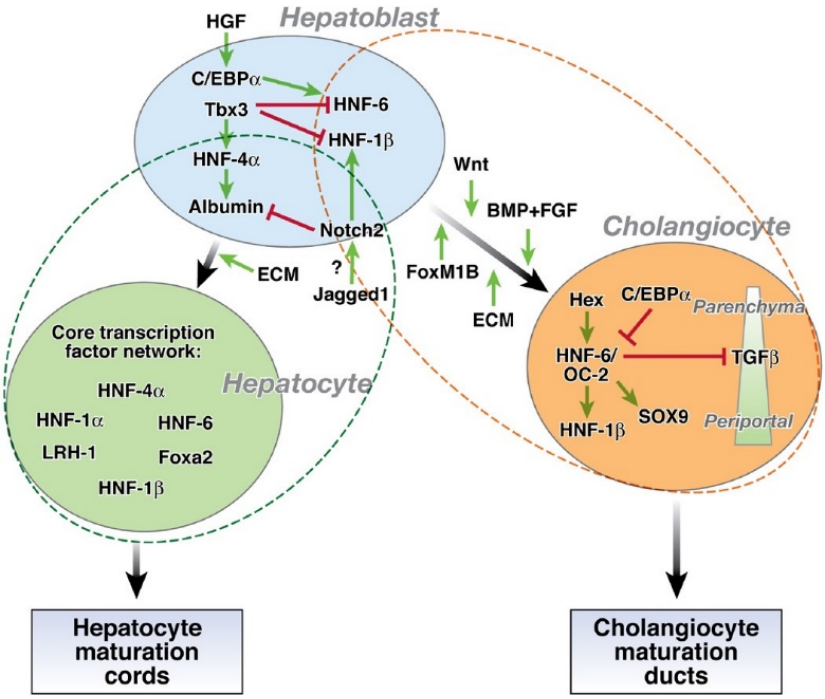

Figure 2 – Mechanisms of cell fate determination in the liver.

Source: Reprinted with permission from Elsevier BV (55).

Hepatocyte polarity (structural and functional). Hepatocytes form a layer of cells that separate sinusoidal blood from canalicular bile. To fulfil this purpose, they are polarised cells with a basal membrane facing the sinusoidal endothelial cells and an apical membrane that, together with other opposite hepatocytes, contribute to the formation of several bile ducts.

In addition, there is a polarised flow of several molecules through hepatocytes, from basolateral to canalicular membranes, and, whenever this flow is interrupted, by inherited or acquired diseases, structural depolarisation occurs.

In the human liver, hepatoblasts begin to differentiate into hepatocytes and cholangiocytes around seven weeks of gestation. The surface identity of embryonic hepatocytes is outlined at the beginning of development in their initial differentiation. However, the distribution of different surface proteins that mark the basolateral and canalicular domains varies for prenatal, neonatal and adult hepatocytes (57). The polarisation of hepatocytes and the formation of the canalicular network require the coordinated expression of several key elements conserved evolutionarily, each of which consists of many components, such as the extracellular matrix, narrow and adherent junctions and intracellular protein traffic machinery, including the endosome recycling, cytoskeleton and energy production.

Ng et al. (58) accidentally demonstrated that bile acids promote the polarisation of liver cells. They found that chenodeoxycholic acid (CDCA) potently induces polarity in Fao clones. In addition, Gissen and Arias (59) evaluated in their model the effect of taurocholic acid in the development of polarity. They found that this bile acid stimulates hepatocyte polarisation via a cAMP-Epac-MEK-LKB1-AMPK pathway. Bile salt export pump (BSEP) is positively regulated by stimulating taurocholate. The polarisation of hepatocytes is essential for many of their functions, and if it is altered, it can cause severe liver disease. Polarity defects are seen in rare inherited diseases of a single gene (Fig. 3) as well as in common infections, such as hepatitis C, and in multifactorial diseases, such as neoplasms (59).

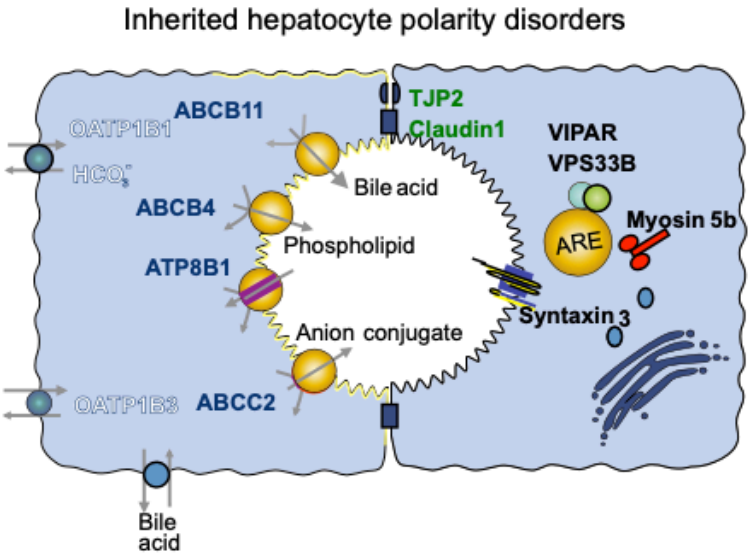

Figure 3 – Protein defects in inherited hepatocyte polarity disorders.

Source: Published by Elsevier Inc (59).

Bile duct morphogenesis. The morphological analysis of the developing liver suggests that the boundary between extrahepatic and intrahepatic bile ducts lies at the level of the hepatic ducts. The development of intrahepatic bile ducts is initiated near the hilum of the liver, before progressing towards the lobes (Fig. 4) (60).

The cholangiocytes that line the extrahepatic and intrahepatic bile ducts have different origins. Those of the extrahepatic biliary tract are derived from the endoderm and those of the intrahepatic bile ducts from hepatoblasts. The extrahepatic bile ducts develop before the intrahepatic bile ducts, and the mechanism by which they anastomose is unknown. In addition to tubulogenesis (formation of the tube) and the initiation of biliary transports, cholangiocyte epithelial maturation continues to strengthen the tight junction barrier function. The morphogenic importance of 'tight junctions' is emphasised by the role of claudins, which are the main constituents of 'tight junctions' and whose mutations in humans cause sclerosing cholangitis.

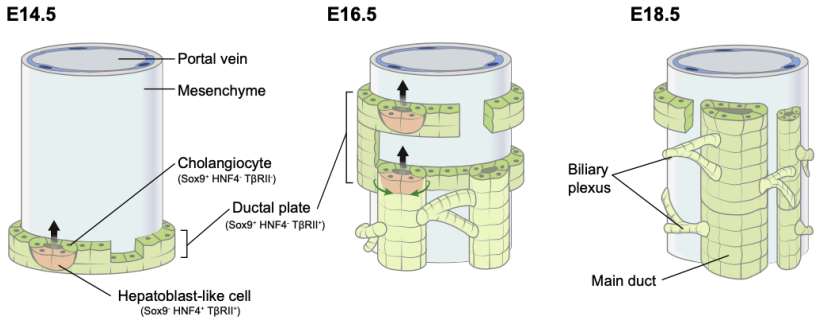

Figure 4 – Intrahepatic bile duct morphogenesis in embryonic mouse liver

Source: Published by Elsevier Inc (60)

Ductogenesis is not complete at 40 weeks' gestation and continues after birth. The number of bile ducts per portal tract progressively increases from birth until reaching the adult ratio of 1:1 by the age of 15 years, one bile duct to one arterial branch in each portal space.

Also, the cholangiocytes continue to acquire a significant degree of morphological and functional heterogeneity, depending on the position of cells in the branched duct network. Polarisation is a trademark of maturing cholangiocytes. Distinguishing the molecular mechanisms that regulate the onset of ductal plaque formation from those that control the differentiation of hepatoblasts into cholangiocytes remains difficult.

Biliary dysgenesis in humans has been associated with abnormal biliary differentiation. Congenital diseases of the extrahepatic ducts are rare, and their aetiology is, in most cases, unknown. The most important malformation of the extrahepatic bile ducts is BA, a syndrome of which the aetiology is still debated and for which several susceptibility genes have been proposed. As the biliary tree morphogenesis is not complete at birth, it is more susceptible to aggression. In addition, there may be genetic diseases that express themselves only after birth, in the final stages of biliary tree morphogenesis and in connection with exposure to various triggers or injuries. An abnormal bile flow can contribute or even cause abnormal ductal morphogenesis. After cholestatic injury, the bile duct morphology must adapt to deal with the toxic accumulation of bile. Ciliopathies can cause the aberrant differentiation of cholangiocytes, malformations of the ductal plaque, the absence of bile ducts and polycystic or dysplastic bile ducts (61). Blocking Notch signalling demonstrated that the Jagged1 and Notch2 mutations cause a scarcity of the bile ducts in patients affected by ALGS and that this pathway is necessary for the formation of the tube (62).

Morphogenesis of the hepatic vasculature. The original blood supply of the liver is the key engine of its development. However, during embryogenesis, it is not clear how the branching pattern of its vessels is established as to whether it results from angiogenesis or vasculogenesis. Also, very little is known about how the hepatocyte cords, bile ducts and vessels interact or connect in an organised manner to build a three-dimensional honeycomb-like structure.

The foetal liver receives highly oxygenated blood through the umbilical vein that connects to the venous duct. The latter acts as a shunt that transports this blood directly to the inferior vena cava. After birth, the umbilical vein is clamped, and the venous duct closes progressively, in most newborns in the first two weeks of life, but in premature infants, it may take longer. As a consequence, the portal circulation of the adult is established. The dual blood supply to the liver, by the hepatic artery and portal vein, is unique in the human body. The singularities of hepatic vascularisation and the changes that it undergoes in the postnatal period also influence the development and maturation of the liver in the initial postnatal period (60).

Regarding the vascularisation of the liver, we highlight the important role of the hepatic artery in the blood supply of the biliary tree. Any disturbance of this blood supply produces ischemia, with necrosis or stenosis, while the liver parenchyma can survive by the oxygen provided by the portal vein. This fact influences the transplantation techniques and may have impact on the survival of liver grafts (63, 64).

Macro-anatomy of the liver. The macro-anatomy is based on a segmental organisation (segments I to VIII), and each segment has its own independent vascular and biliary supply. Segment labelling begins with segment I (caudate lobe) in the left lobe and proceeds clockwise according to its position. Liver surgery, including liver transplantation, is strongly conditioned by this anatomical division (65).

Micro-anatomy and functional organisation of the liver. The micro-anatomy is intimately related to liver function and is best understood by linking individual cellular constituents and their local relationships with function. The liver parenchyma consists of a number of different cell types. About 80% are hepatocytes, 10% are sinusoidal endothelial cells, 5% are lymphocytes and 4% are Kupffer cells (hepatic macrophages), while biliary epithelial cells account for 1–3%.

The architecture is highly organised, with hepatocytes polarised at the interface between the endothelial sinusoids and the bile canaliculi. Between the endothelial cells and the basal surface of the hepatocytes lies the space of Disse. The hepatic stellate cells (previously known as Ito cells) are found in the space of Disse and produce extracellular matrix, cytokines and growth factors; store vitamin A and lipids; and have fine extensions surrounding the sinusoids, possibly related to the control of vascular tone.

The liver structural unit is the classical lobule, a hexagon where the proximal part of the efferent venous system (the central vein) is centrally located, while the portal tracts are at its periphery. In the bi-dimensional model described by Kiernan in 1833, the hepatocyte cords seem to radiate from the centre to the periphery. These hexagons are the base of a honeycomb-like architecture.

According to a description in 1954 by Rappaport (66), the acinus is the functional unit of the liver and develops in the third month of gestation (Fig. 5). The acinus is usually described as a parallelogram whose corners consist of two neighbouring central veins and the two portal triads in between.

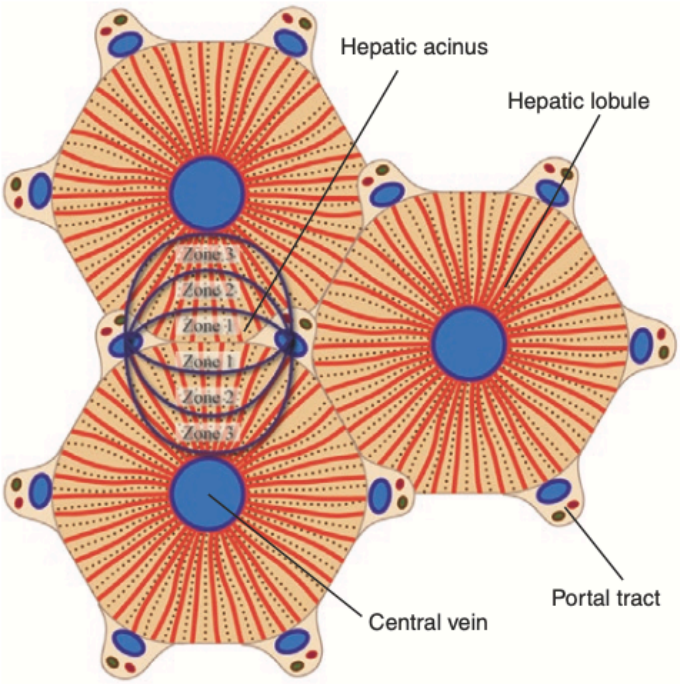

Figure 5 – A schematic representation of the basic lobule and the Rappaport acinus.
Source: Reprinted with permission from Springer, Cham.(66)

In this tri-dimensional structure, three acinar zones are defined according to the portal tract proximity and follow a different metabolic function and decreasing oxygenation – the periportal hepatocytes (zone 1) receive more oxygenated blood than the perivenular ones (zone 3). Hepatocytes of zone 2 receive intermediate levels of oxygen.

In this architecture, the hepatocytes are a heterogeneous population of cells expressing different sets of genes and exerting different metabolic functions depending on their location in the different zones of the liver acinus. This concept is known as metabolic zonation (67, 68) (Fig. 6), which develops only after birth (69) and is essential to determining the clinical expression of various liver diseases.

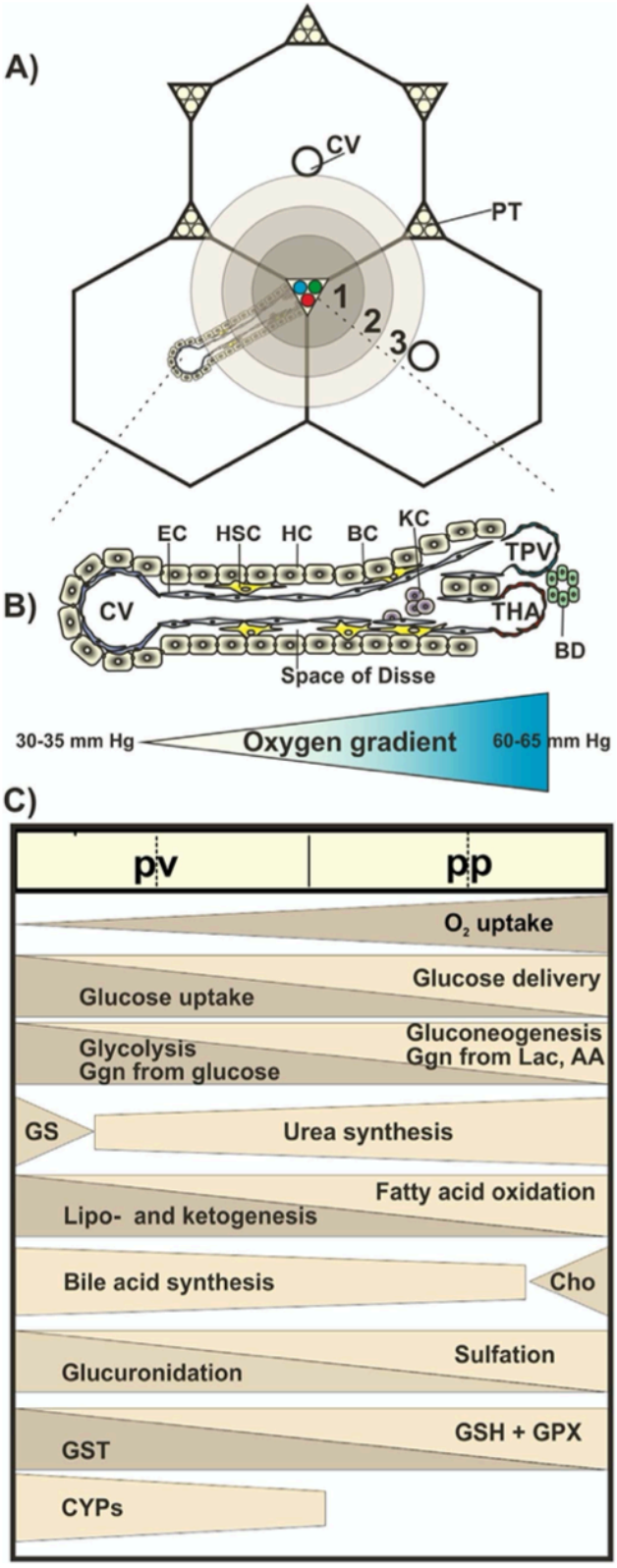

Figure 6 – Liver zonation.

Source: Published by Science Direct, Elsevier B.V.(62).

Legend: CV - central vein; PT – portal vein; TPV – terminal portal vein; THA – terminal hepatic artery; BD – bile duct; HC – hepatocytes; EC – endothelial cells; KC – Kupffer cells; pp – periportal; pv – perivenosus; AA – aminoacids; Cho – cholesterol; Ggn – glycogen; Lac – lactate; GS – glutamine synthetase; GST – glutathione transferase; GSH – reduced glutathione; GPX – glutathione peroxidase; CYPs – cytochrome P450 enzymes.

4.2 PHYSIOLOGY OF THE NEONATAL LIVER

The functional immaturity of the liver at birth lasts from days to weeks or even months depending on the enzyme systems involved; for example, liver enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are rapidly induced at birth, but the bilirubin conjugation will take weeks or months to reach adult levels. Healthy newborns do not suffer major consequences from this immaturity; however, sick or preterm newborns are at risk for developing cholestasis, hyperbilirubinemia, hypoglycaemia, bleeding and changes in drug metabolism (70).

Bile synthesis. The beginning of bile secretion occurs by the second trimester of gestation and is one of the immature processes at birth. Bile acids, one of its major compounds and driving forces, begin to be synthesised by the liver after the 14th week of intrauterine life. There are two pathways for bile acids synthesis, both departing from cholesterol. In the foetus, the alternative pathway is predominant, resulting in the synthesis of a comparatively higher percentage of CDCA over cholic acid (CA) as opposed to what occurs in children and adults (71). Moreover, there is also a predominance of forms conjugated to taurine over those conjugated to glycine (72).

At birth, the change from placental to enteral nutrition stimulates bile flow, bile acid secretion and enterohepatic recirculation. The pool of bile acids is small, and the enterohepatic recirculation is a highly immature process. During the first week of life, bile acids reach significantly higher serum concentrations than those of children and adults, a condition referred by Balistreri et al. (17) in 1981 as ‘physiological cholestasis’. The serum bile acid pattern does not reach maturity until the end of the first year of age.

Additionally, gallbladder maturation is incomplete at birth. The volume at fasting and contractility and the response to postprandial stimulation are decreased compared to older children (73).

Bilirubin metabolism. In the newborn, bilirubin is a by-product of the haemoglobin catabolism of senescent erythrocytes. Unconjugated bilirubin is highly lipophilic and insoluble in water, being difficult to remove from the body. The conjugation of bilirubin serves to make it soluble and, thereby, possible to be excreted through the bile ducts. This process requires a catalyst enzyme – uridine-diphosphate-glycosyltransferase-1A1 (UGT1A1) – whose expression at birth is residual (about 1% of the expression in adults). The expression of this enzyme gradually increases, but it takes several weeks or months to reach adult values (25% of the adult values at 3 months of age). Bilirubin reaches the intestine after being excreted by the bile, where it can be eliminated via stools or

unconjugated by bacteria or by the beta-glucuronidase enzyme and then reabsorbed into the blood for re-uptake by the liver.

Physiological jaundice is caused by the sum of three factors – increased production, decreased conjugation and increased resorption. In premature newborns, as immaturity of the bilirubin metabolism is greater, physiological jaundice starts earlier and lasts longer.

Metabolism of glucose and fatty acids. Under physiological conditions, the main source of glucose for the foetus is the placenta. The foetus can synthesise and degrade glycogen since from the eighth week the necessary enzymes are expressed. In contrast, gluconeogenesis does not occur during intrauterine life due to the rate-limiting enzyme phosphoenolpyruvate carboxykinase, which is only expressed after birth in the immediate newborn period (74). In newborns, glucose blood levels can vary significantly, and they are often exposed to hypoglycaemia, once maternal supply is interrupted at birth. The metabolism of the neonate needs to adapt quickly to enteral nutrition, since liver glycogen stores are scarce and are consumed in the first hours. Newborns have to metabolise lactose from milk and use the gluconeogenesis pathway. The other important source of energy is the metabolism of fatty acids, modulated by catecholamines. Premature newborns have even lower glycogen stores and reduced gluconeogenesis activity; in addition, they are not able to produce ketone bodies in response to hypoglycaemia. These combined factors cause premature babies to have an increased susceptibility to hypoglycaemia, which can last for several weeks (75).

Protein synthesis. The main protein synthesised by the foetal liver is alpha-fetoprotein. The serum levels of this protein are very high at birth, and they progressively decrease in the following months. Albumin is synthesised by the liver from the third trimester of foetal life and reaches adult serum levels by the end of gestation, which is why preterm newborns have low serum levels. Coagulation factors synthesised by the liver (all except for factor VIII) have low levels at birth, which, in addition to vitamin K deficiency that justifies universal prophylaxis, explains the susceptibility of newborns to haemorrhage. Serum levels similar to those of adults are reached only a few days after birth.

Biotransformation (detoxification). In utero, the placenta performs most of the metabolic and detoxifying functions that normally take place in the liver. Drug metabolism and liver transport mechanisms are immature in the neonatal period and mature during the first year of life. The degree of enzymatic expression and the pattern of development will depend on each pathway, on the existence of alternative routes and on inter-individual variability. For example, the expression of cytochrome P450, to which most of the biotransformation phase

I enzymes belong, at birth is 30% and will only reach the values of the adult at one year of age. Liver biotransformation involves the following two phases: I – oxidation, reduction and hydroxylation reactions; II – conjugation reactions to increase the solubility in water of endogenous and xenobiotic compounds, thus facilitating their renal or biliary excretion.

Liver enzymes. Liver enzyme expression varies significantly with age and gender (76, 77) and has been described as an inverted U-like relation curve (78). Kocylowski et al. reported a substantially reduced ALT in the blood cord of neonates with intrauterine growth retardation, suggesting a down-regulated hepatic activity at birth (79). In contrast, gamma-glutamyl transferase (GGT), located at the canalicular surface of the hepatocytes, is slightly elevated in the serum in the first few months of life (65). The significance of this finding is unclear, and most speculations revolve around the immaturity of the liver systems.

4.3 PATHOGENESIS OF CHOLESTASIS

The study of the mechanisms of cholestasis has been carried out mainly in animal models. The contribution of studies in humans (children and adults, with hereditary or acquired diseases) has been comparatively scarce, and these are mostly 'in vitro' and 'in situ' studies, which always have many limitations as they do not allow the study of the signalling and regulatory aspects of the various '*in vivo*' mechanisms.

'*In vivo*' animal models have led to numerous advances in clarifying the aetiology and pathogenesis of cholestatic liver diseases, but they are limited by inter- and intra-species differences, and each model has its specific constraints. The common bile duct ligation (BDL) model in rats and mice was the only one available model to study cholestatic diseases, for a long time (80). This model later underwent some modifications, namely the partial BDL and the selective BDL models. More recently, many other rodent models have been made available for different cholestatic diseases, addressing their specific underlying mechanisms. In these models, liver injury is induced by surgical intervention or viruses or chemicals; genetically modified mice are also currently used (81).

Translational studies in human cholestatic diseases have been hampered by several barriers, such as the rarity of the disorders, the difficulty in obtaining biliary tissue from across the spectrum of the disease stage and the difficulty in culturing and maintaining primary cholangiocytes, whose role is crucial in the pathophysiology of cholestasis. Their proliferation and death, their interaction with bile and their cross talk with the inflammatory milieu of disease play a key role in the evolution of cholangiopathies (82).

Organoids are groups of human cells that grow '*in vitro*', in a three-dimensional structure that is self-organised and differentiated into functional cell types, reproducing the structure and function of an organ '*in vivo*'. They are the bridge between in-line human cell culture and the '*in vivo*' model, and they promise to usher in a revolution in translational medicine as they are potentially able to overcome many of the constraints of research in animal models and clinical human studies (eliminating interspecies differences). This technology has many potential applications (e.g. human development, disease modelling, drug discovery and precision medicine, regenerative medicine, toxicology, gene editing) (83, 84) and is now available for studying the human liver (85). In hepatology the organoids' technology is already being used in personalised in vitro models (e.g. PFIC-2) and can be applied to clinical practice in autologous transplantation and gene editing (e.g. Wilson disease).

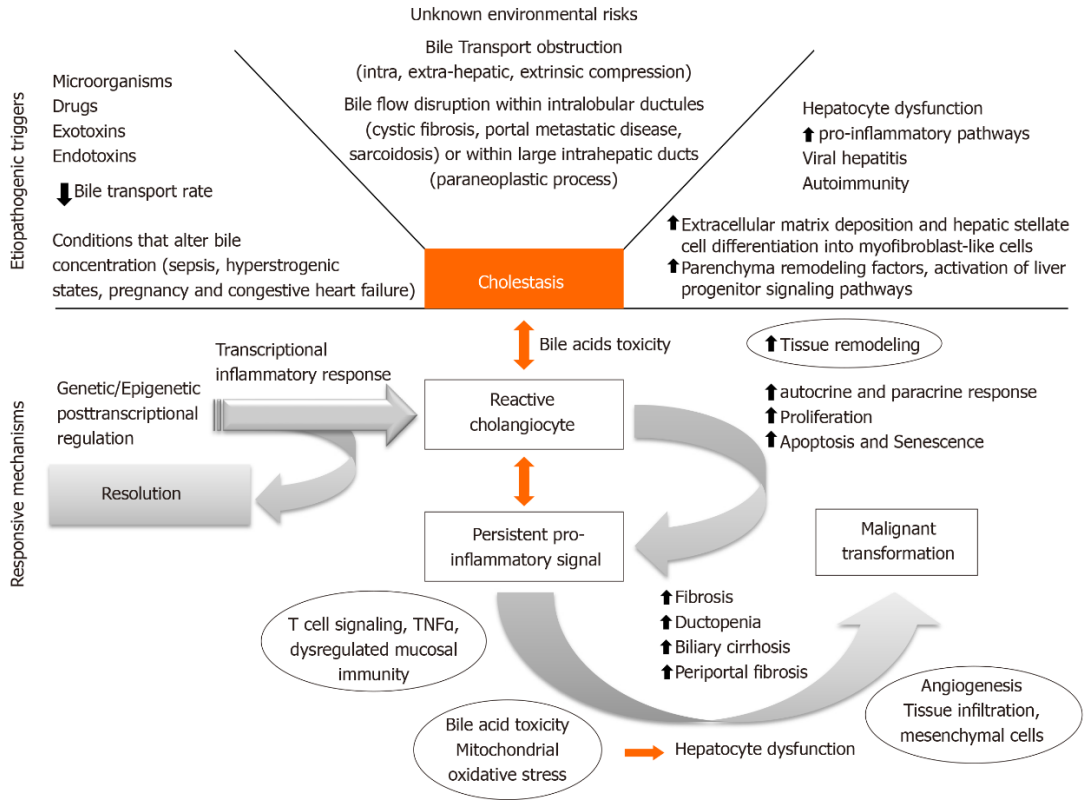

Figure 7 – Mechanisms of cholestatic liver diseases.

Source: Published by Baishideng Publishing Group (86).

The triggers. The pathogenesis of cholestasis depends on the action of a trigger followed by an initial reaction and individually managed adaptive responses (Fig. 7). The more known triggering factors are the obstruction of biliary transport (intra- or extrahepatic or by extrinsic compression) and the interruption of bile flow within the intrahepatic ducts and the

intralobular ductules. Other triggers are conditions that alter the bile concentration (sepsis, oestrogens, pregnancy, congestive heart failure), or cause hepatocyte dysfunction (e.g. viral hepatitis, autoimmune hepatitis); and, environmental factors although hypothesised (e.g. antigenic stimuli, microorganisms, xenobiotics, exotoxins and endotoxins), are mostly unknown (86).

The mechanisms of disease and the adaptive responses. Cholestasis is the manifestation of reduced bile formation or bile flow, resulting in the retention of biliary substances within the liver. Bile acids are probably the most important compounds through which metabolism is disturbed in this process.

Bile acids in the leading role – which came first, the chicken or the egg?

Bile acids are one of the two driving forces of bile flux. Cholestasis radically changes the bile acids' concentrations in the different compartments of the enterohepatic cycle, disrupting this driving force. Difficult to know, in many cases, is which came first: Is it the bile acids disturbing metabolism as the leading cause of cholestasis or merely a consequence?

Toxicity and mitochondrial dysfunction. Glicochenodeoxycholic acid (GCDCA), a hydrophobic molecule, is able to produce *in vitro* cell death by inducing apoptosis in rat hepatocytes or human hepatocellular carcinoma cells that overexpress Na^+-taurocholate co-transporting polypeptide (NTCP); however, studies performed in human primary hepatocytes showed that massively higher concentrations are required to induce any degree of cell death (87). Alternatively, the presence of the foci of liver necrosis has been demonstrated in the mouse liver, suggesting that the mechanism of hepatocyte death may be necrosis instead of apoptosis, as primarily conjectured. Additionally, *in vitro* studies (88) have demonstrated that GCDCA disrupted the mitochondrial membrane, leading to increased production of reactive oxygen species (ROS) and decreased mitochondrial mass and mitochondrial content. In spite of this, one must not ignore the interspecies differences, whose *in vitro* findings become difficult to translate to human disease; actually, the bile acid induced toxicity remains an open issue.

Neutrophil-mediated inflammatory response and oxidative stress. Experimental evidence supports the hypothesis that cholestatic liver injury is primarily a neutrophil-mediated inflammatory response and that neutrophil-induced oxidative stress is the main mechanism

of liver cell death. After the onset of cholestasis, bile acids stimulate hepatocytes to produce pro-inflammatory mediators that activate neutrophils.

Activated neutrophils produce and release reactive oxygen species (ROS) and other products that may induce lethal injuries to hepatocytes. Neutrophils respond to damage-associated molecular signs or "self" damage signals (DAMPs), driving an appropriate inflammatory response. DAMPs can amplify inflammation and liver damage by stimulating the release of cytokines by Kupffer cells or by directly activating neutrophils. DAMPs can also promote fibrosis through the activation of toll-like receptors on liver stellate cells. The release of ROS by activated neutrophils will cause moderate lipid peroxidation, whose products are chemotactic for neutrophils and activate hepatic stellate cells, stimulating them to produce collagen (89, 90). There are some studies showing oxidative stress involvement in cholestasis, either in animals (e.g. BDL rat model) (91), or in (blood and urine) human adults with primary biliary cirrhosis (90). However, it is also possible that in the human liver the activation of neutrophils, with development of oxidative stress, may stimulate adaptive responses that protect the liver from injury (e.g. oxidative stress may activate carrier proteins like multiple resistance protein2 (MRP2), which transports reduced glutathione (GSH) and all its conjugated forms, and BSEP, which is the bile acid excretory pump).

Signalling properties in an intricated network. Besides their fundamental role in lipid digestion, bile acids have remarkable signalling properties and activate a network of receptors in several organs and tissues of the human body (e.g. liver, intestine), including the farnesoid-X receptor (FXR), vitamin D3 receptor, membrane G protein-coupled bile acid receptor-1 (GPBAR-1) and Takeda-G-protein receptor5 (TGR5).

In this setting, the critical steps of the enterohepatic cycle are regulated by the bile acid receptor FXR, which limits bile acids' re-uptake and synthesis by enhancing biliary and basolateral bile acid export. Currently, a new hypothesis has come into the spotlight regarding the bile acid-induced proinflammatory signalling changes in a new pathway totally independent of FXR signalling, as observed in mouse hepatocytes and induced by taurocholic acid (92). This observation was not confirmed in primary human hepatocytes (PHH) exposed to taurocholic acid (TCA) (87); however, GCDCA did induce the expression of human cytokines (93).

Cholangiocytes in the leading role

The leading role of bile acids in the pathogenesis of cholestasis is being challenged lately by another hypothesis which put the cholangiocytes into the spot light. These cells regulate and modify the volume and the composition of the bile in a process that is the second driving

force of the bile flux. During cholestasis, the cholangiocytes may undergo a senescence-like reaction that promotes inflammation through a paracrine signalling pathway. Their reaction activates the hepatic progenitor cells, which may differentiate either in cholangiocytes or hepatocytes, and it may induce injury resolution or biliary fibrosis in the presence of perpetuating transcriptional inflammatory addiction. Currently, some authors consider this to be the core concept of the cholestatic liver injury mechanism (Fig. 7) (86).

To summarise, the primary rupture of the cholangiocytes would be damaging to hepatocytes. The infarction of the biliary tract results in hepatocyte damage and the release of bile acids and DAMPs. This releases cytokines and growth factors, which are signals recognised by neutrophils that adhere firmly to hepatocytes and induce cell death through potent ROS forms and other neutrophil activation byproducts.

Several lines of investigation converge on the idea that inflammation may be the main mechanism involved in cholestasis, especially after the rupture of the biliary tract. Animal studies have demonstrated that osteopontin released by cholangiocytes into the bile, and the disruption produced by the matrix metalloproteinases, generate a potent chemotactic stimulus responsible for neutrophil recruitment and for the initial inflammatory injury. Additionally, raised levels of biliary bile acids induce the formation of chemokins for hepatocytes (71). However, until now, this evidence has not been reproduced in humans with cholestasis. Other inflammatory cells may be involved during cholestasis (e.g. Kuppfer cells), and inflammation is, actually, a common feature in most types of liver injury. Within this concept, bile acids exert a major influence over cholangiocytes. In fact, cholangiocytes have receptors for bile acids and are the epithelial barrier to bile acids' toxicity. Taurocholic acid stimulates the cholangiocytes proliferation in order to hold on to the biggest load of bile possible and prevent biliary infarcts (94).

Finally, inflammation is directly linked to biliary pressure and the degree of cholestasis; therefore, the separation of inflammatory lesion from bile acid accumulation lesion is very difficult when it comes to defining the underlying mechanisms.

The pathway of no return. Whatever the initial mechanisms of cholestasis are, when the process that leads to dysfunctional matrix rearrangements and fibrogenesis is activated, we can consider that we are treading a path of no return to normality. This process is dynamic and has some variations depending on the specific disease. It seems to be involved with immunoinflammatory mechanisms, the secretion of tissue metalloproteinases, cytokine networks and derangements of mesenchymal cell infiltration, with a final loss of tissue maintenance homeostasis (86).

For example, in primary biliary cirrhosis, the pattern of extracellular matrix accumulation is characterised by the increased expression of mRNA encoding collagen types I, III and IV, which in mesenchymal cells promotes the expansion of the portal tracts, leading to excessive extracellular matrix deposition (95). Fibrogenic processes involve both damaged and undamaged bile ducts as well as the periportal sinusoidal system. While in primary sclerosing cholangitis, the fibrogenic process has been compared to the concentric recruitment of profibrogenic atherosclerotic cells, onion-like, and vascular damage with the ischemia of bile duct epithelial cells has been reported (86).

Genetic and epigenetic factors as modifiers of individual response. Patients with cholestatic liver diseases may carry genetic abnormalities responsible for the different characteristics of each disease. Furthermore, some genes may be directly related to the rate of disease progression, as occurs with a gene recently identified in patients with primary biliary cirrhosis (96). This gene or its transcriptional activators can become therapeutic targets. Several other immunogenetic genes have been associated with T cell and B cell function, and others have been described in association with loss of immunotolerance and epithelial permeability (86).

Genetic and epigenetic factors may also modify the cholestasis adaptive responses, thus explaining different clinical phenotypes.

Unravelling the pathogenesis of cholestasis has been critical to defining therapeutic targets. Currently, some pathways already have target drugs available.

4.4 BIOMARKERS OF CHOLESTASIS

Used in clinical practice, cholestasis biomarkers are some of the substances retained in blood when bile flow is interrupted, such as conjugated bilirubin and bile acids. In addition to these, GGT has also been described as a biomarker of diseases of the bile ducts.

Conjugated bilirubin. Bilirubin is the end product of heme degradation, is an important component of bile and is responsible for bile's colour. About 80% of bilirubin originates from the breakdown of erythrocyte haemoglobin, and the remaining 20% comes from ineffective erythropoiesis and other heme proteins (97). Unconjugated bilirubin is insoluble in water and binds to albumin so that it can be transported in plasma. When it passes through the liver (in the sinusoids), it is captured by the hepatocytes, where it will be conjugated so that it can be easily excreted in the bile. Conjugation is catalysed by the enzyme UGT1A1 in the

endoplasmic reticulum of hepatocytes. Excretion is mediated by ATP-dependent transporters (Fig. 8).

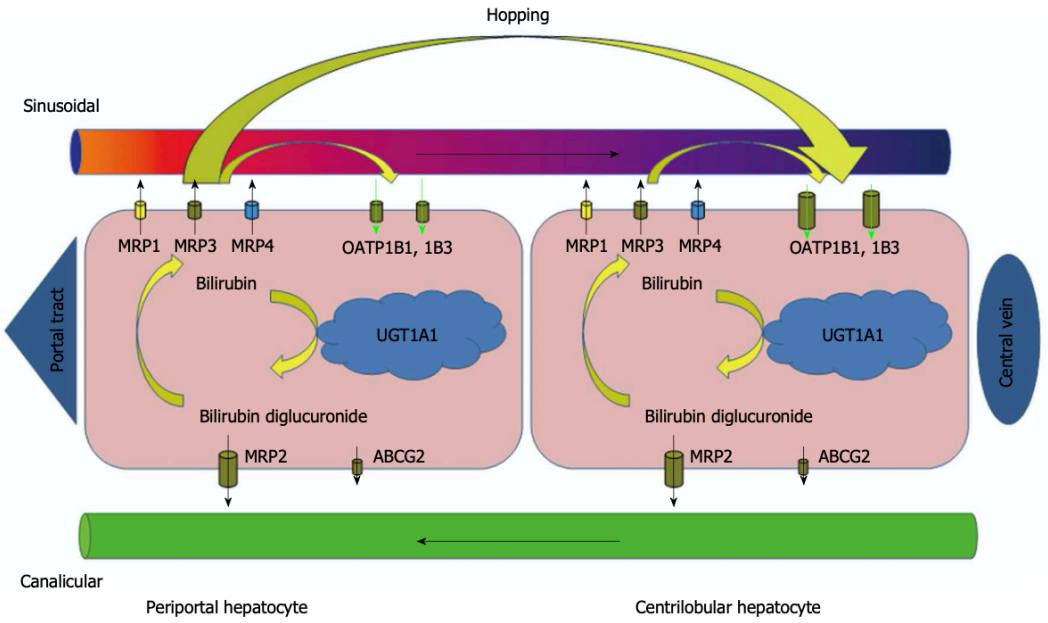

Figure 8 – Liver cycle of conjugated bilirubin.

Source: Published by Baishideng Publishing Group (97).

MRP – multidrug resistance-associated protein; OATP – organic anion transporting polypeptide; UGT – uridine diphosphate glucuronosyltransferase; ABCG – ATP binding cassette

Even under physiological conditions, a fraction of conjugated bilirubin is excreted by MRP3 into the blood through the sinusoid membrane, from where it can subsequently be taken up by the sinusoidal membrane transporters OATP1B1 (organic anion transporting polypeptide) and OATP1B3. The highest global expression of OATP1Bs was demonstrated in centrilobular hepatocytes. The shifting process (hopping) from the periportal to the centrizonal hepatocytes can act as protection of the periportal hepatocytes against high concentrations of various xenobiotics.

Some inherited diseases involving these metabolic pathways (e.g. Criggler-Najjar syndromes, Arias and Gilbert involving mutations in the *UGT1A1* gene, or Dubin-Johnson in the *ABCC2* gene, and Rotor in the *SLCO1B1* and *SLCO1B3* genes) have played an important role in the knowledge on all these processes. The deregulation of these pathways explains the existence of jaundice in many acquired diseases (just as an example, the reduced expression of MRP2 in sepsis and obstructive type cholestasis is followed by an upregulation of several MRP homologs in the basolateral membranes of the hepatocytes, leading bilirubin to overflow into the sinusoids and systemic circulation. Many of these adaptations occur to prevent the accumulation of potentially toxic components in bile and

other substrates in the liver). Conjugated bilirubin is the gold standard biomarker for the clinical diagnosis of NC.

Bile acids. Primary bile acids (CA and CDCA) are synthesised in hepatocytes from cholesterol and through two metabolic pathways, the classic (neutral) and the alternative (acidic) (98) (Fig. 9). The ratio of these two primary acids varies throughout human development. Foetal bile has a predominance of CDCA with a CA/CDCA ratio of ~ 0.85. Newborns and adults have a predominance of CA but with very different ratios (2.5 and 1.6, respectively) (99).

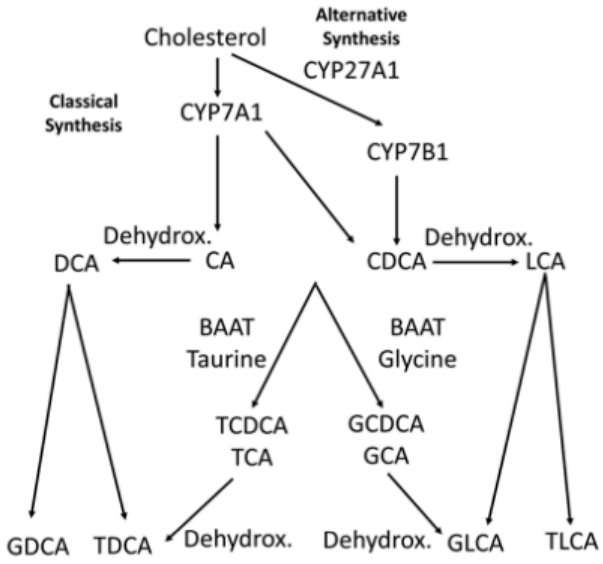

Figure 9 - Bile acids' synthesis

Source: Published by Ingenta (71).

Legend: BAAT – bile acid CoA:amino acid N-acyltransferase; CA – cholic acid; CDCA – chenodeoxycholic acid.; CYP7A1 – cholesterol 7-alpha-hydroxylase; CYP7B1 – cholesterol 7-beta-hydroxylase; CYP27A1 – cholesterol 27-alpha-hydroxylase DCA – deoxycholic acid; LCA – lithocholic acid; GCA – glycocholic acid; GLCA – glycolitocholic acid; GDCA – glycodeoxycholic acid; GCDCA –glycochenodeoxycholic acid; TCA – taurocholic acid; TDCA – taurodeoxycholic acid; TLCA – taurolithocholic acid.

After synthesis, bile acids are conjugated to taurine or glycine mainly in peroxisomes. Newborns conjugate bile acids predominantly with taurine while older children and adults predominantly with glycine. Once conjugated, bile acids are excreted into the bile ducts and form a fundamental component of bile. Bile acids are excreted through the canalicular membrane by the BSEP encoded by the *ABCB11* gene. Mutations in this gene have been described in association with PFIC-2 disease.

When bile acids are excreted in the bile, they will reach the intestine, where they will be metabolised by bacterial enzymes to become secondary bile acids. Primary bile acids are

deconjugated and dehydroxylated to deoxycholic and lithocholic acids, respectively, which make up the majority of bile acids excreted in stools. Ursodeoxycholic acid (UDCA) can be produced by the epimerisation of the CDCA but is generally found in low concentrations in humans.

Only 3–5% of primary and secondary bile acids are excreted in stools. The rest are reabsorbed in the terminal ileum and returned to the liver through the portal vein, where they are excreted again. This circuit is called bile acid enterohepatic circulation (Fig. 10).

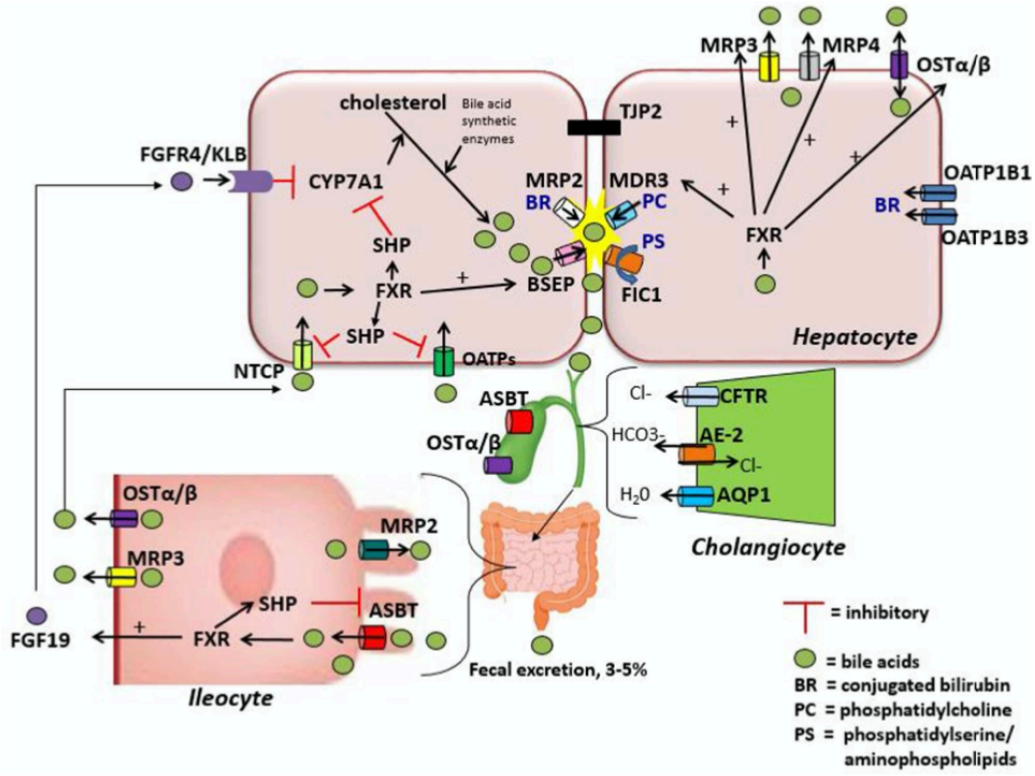

Figure 10 - Enterohepatic recirculation of bile compounds.

Source: Published by Frontiers Media S.A.(98)

Legend: AE – anion exchanger; AQP1 – aquaporin 1; ASBT – apical sodium dependent bile acid transporter; CYP7A1 – cholesterol 7-alpha-hydroxylase; CFTR – cystic fibrosis transmembrane conductance regulator; FIC1 – family intrahepatic cholestasis; FXR – farnesoid X receptor; FGF19 – fibroblast growth factor-19; FGFR4/KLB – transmembrane receptor tyrosine kinase 4/ beta Klotho; MRP – multidrug resistance associated protein; NTCP – Na+ sodium taurocholate co-transporting polypeptide OATPs – organic anion transporting polypeptides; OATPB – organic anion-transporting polypeptide B; OST a/b – organic anion transporting polypeptide; SHP – small heterodimer partner; TJP – tight junction protein.

The uptake of bile acids by enterocytes occurs through the action of the apical sodium-dependent bile acid transporter (ASBT) located on the apical membrane, also encoded by the *SLC10A2* gene. Then they are exported to the blood of the portal circulation through the basolateral membrane through the alpha and beta transporters (OSTalfa-OSTbeta). These transporters also exist in the basolateral membranes of hepatocytes and

cholangiocytes, as well as other tissues, and can transport bile acids back to the sinusoides if necessary.

Bile acids are taken up by hepatocytes, through their basolateral membranes, mainly by the sodium taurocholate co-transporter polypeptide (NTCP). Then they are excreted again in the bile, completing the enterohepatic cycle.

Many innate errors of metabolism have been described in the metabolic pathways of bile acid synthesis and diseases of peroxisomes (e.g. Zellweger syndrome), which can lead to the accumulation of toxic bile acid intermediates and which may present as cholestasis in the neonatal period.

During cholestatic processes, the basolateral membranes of hepatocytes also have pumps capable of transporting bile acids in sinusoids, such as MRP3 (*ABCC3* gene) and MRP4 (*ABCC4* gene) in addition to OSTalpha-OSTbeta.

Several hereditary diseases have been described in which changes in the expression of the enzyme or in the regulation of the transporter of various components of bile affect the enterohepatic circulation of bile acids. Alternatively, several acquired cholestatic conditions can also interfere in the enterohepatic circulation of bile acids.

The synthesis and enterohepatic recirculation of bile acids is strongly regulated. FXR encoded by the *NR1H4* gene (nuclear receptor subfamily 1, group H, member 4) is a nuclear hormone receptor that strongly influences, in many ways, the synthesis and transport of bile acids, and mutations in this gene can lead to PFIC-5. The activation of FXR promotes excretion and reduces the uptake of bile acids, helping to maintain homeostasis within the hepatocyte. In situations of cholestasis, it leads to the increased expression of OSTalpha/beta, MRP3 and MRP4, which causes bile acids to reflux through the hepatocyte basolateral membrane to the sinusoids to reduce their intracellular concentration and mitigate their toxic effects in the cell.

Another alternative family of receptors known as the G-protein-coupled receptor family, of which the best known is GPBAR1, also known as TGR5, is also capable of binding bile acids, especially secondary ones. GPBAR1 has signalling effects, immunoregulatory effects in liver diseases due to its presence in Kupffer cells, and through its expression in sensory nerves it can play a role in the regulation of pruritus.

Other regulatory pathways for bile acid synthesis have been discovered more recently, such as the unfolded protein response (UPR). This is an adaptive cellular response to the stress

of the endoplasmic reticulum that works to regulate homeostasis but can also trigger apoptosis. The activation of UPR has been observed in several liver diseases (e.g. fatty liver, viral hepatitis and cholestasis).

To summarising, for all that has been exposed, bile acids are an important cholestasis biomarker.

Gamma glutamyl transferase (GGT). This sialoprotein is found in the cell membranes of many tissues, catalyses the transfer of gamma-glutamyl from gamma-glutamyl peptides to other peptides or L-amino acids and plays an important role in the metabolism of GSH (100). It exists in large quantities in the kidneys (in the proximal tubule and in the loop of Henle) and in other organs with functions of absorption and secretion, including the liver (bile ducts and gallbladder), pancreas, intestine and prostate as well as the heart, lungs, brain and spleen. It also exists in small amounts in erythrocytes, granulocytes and lymphocytes. However, its activity in skeletal muscle is residual, and GGT is not elevated in muscle diseases. Although GGT activity in the kidneys is approximately 10 times greater than that in the liver, serum GGT is considered to be mainly of hepatobiliary origin and therefore has been used as a 'liver test' for decades.

The rise in GGT appears earlier and lasts longer than other enzymes. It is the most sensitive of liver enzymes although it is one of the least specific. The elevation of GGT has been described in relation to several hepatobiliary diseases, including in paediatric age (101-103). There are several isoenzymes, but no relationship has been found between any of them and specific hepatobiliary diseases. Its elevation has also been described in healthy people, with an emphasis on newborns and small infants in what is thought to be attributable to the immaturity of the liver in this age group (100).

The gamma-glutamyl cycle proposed by Meister in 1970 incorporated GGT, giving it a prominent role. However, we now find that while the description of GSH biosynthesis was correctly represented, the part regarding GSH degradation and the role of GGT has changed radically. In reality, GGT does not play a role in the transport of amino acids as initially thought, and the translocation of gamma-glutamyl amino acids (or GSH) by GGT does not exist (104).

It is currently agreed that the main function of GGT is to save the GSH exported by the cytosol (the conjugates and the oxidised GSH, and even the reduced GSH). In the last decade, we found that serum GGT concentrations are increased not only in an aetiologically diverse spectrum of hepatobiliary disorders but also in an increasing number of extrahepatic conditions, such as cardiovascular conditions; chronic kidney disease; cancer and

neurological, pulmonary and bone diseases (105). GGT has also been described as a predictor of prognosis (106).

Thus, GGT is currently a sensitive biomarker and, although nonspecific, predictive of diagnosis and prognosis, but its interpretation must be made by specialists and in an integrated clinical and laboratory context. To interpret the results, the evolutionary phase of human development, inter-individual differences and some environmental factors (e.g. alcohol consumption) must also be taken in to account.

New biomarkers. Nowadays, we need new biomarkers applicable to clinical practice, such as biomarkers of oxidative stress, inflammation and apoptosis. With the current level of knowledge concerning the pathogenesis of cholestasis involving inflammation, development of oxidative stress and changes leading to cell death, this ambition seems increasingly possible to materialise.

Some studies have shown promising results with biomarkers of ROS and some secondary and sulfated bile acids. In 2016, Masubuchi et al. (107), in a study involving 304 blood samples from 150 adult patients with liver diseases (hepatitis B and C, alcohol liver disease, non-alcoholic fatty liver disease and drug-induced liver injury) and 46 blood samples from the same number of healthy volunteers, found a significant association between the following biomarkers with a cholestasis type injury: lithocholic acid (LCA) and deoxycholic acid (DCA) levels and elevation of the serum sulfated bile acid (SSBA), liver fibrosis marker IV collagen (type IV collagen) and hyaluronic acid (HA) and ROS levels. The largest area under the ROC curve was found for ROS. In contrast, elevations in the LCA and type IV collagen levels and a reduction in the UDCA level were significantly associated with hepatocellular injury.

4.5 OXIDATIVE STRESS AND DISEASE IN THE NEONATAL PERIOD

Oxidative stress occurs when the balance between free radical (FR) production and intracellular antioxidant defences are disrupted. The newborn, mainly the preterm, is at greater risk for oxidative stress for several reasons. First is the transition from a poorly oxygenated foetal environment to an oxygen-rich atmosphere. Second, sick newborns may need supplemental oxygen. Also, asphyxia and elevated free serum iron (common in the newborn) increase the risk of oxidative stress. Furthermore, preterm newborns have less of their own and mother-borne antioxidant defences.

Maternal obesity and tobacco consumption enhances the foetus's exposure to oxidative stress, and the effects continue in the neonate following birth (108). The former is currently an epidemic mainly in the industrialised countries.

In 1988, Saugstad introduced the concept that during reoxygenation, oxygen radicals are formed in excess and may simultaneously attack different organs, leading to a condition referred to as oxygen radical disease of the newborn (109) (Fig. 11).

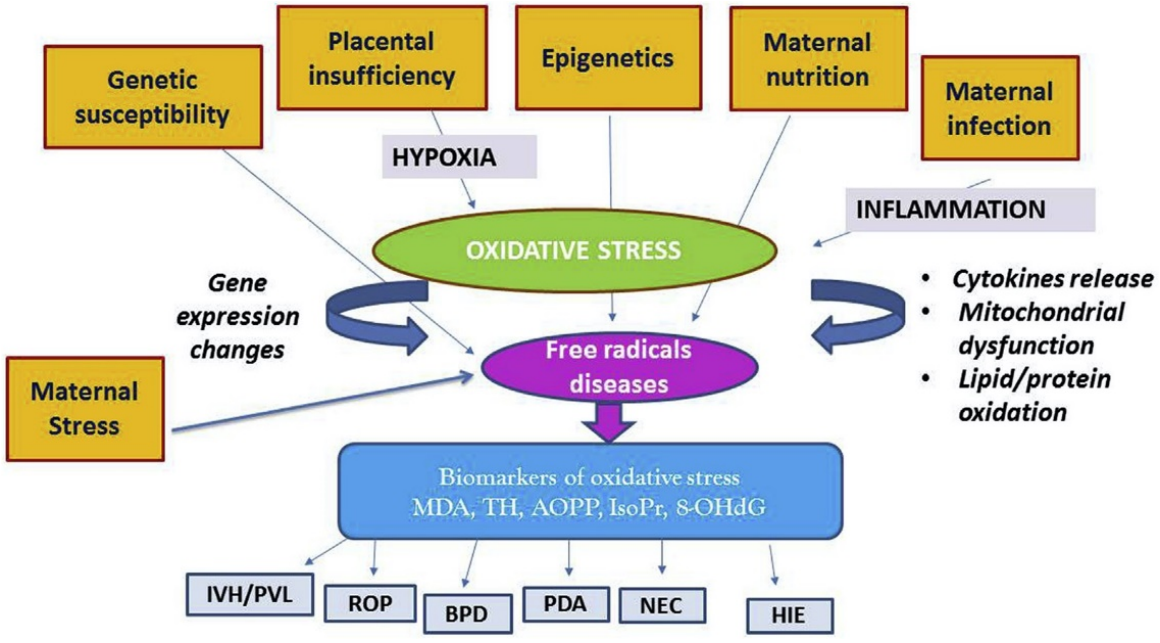

Figure 11 - Central role of oxidative stress in the pathogenesis of neonatal diseases

Source: Reprinted with permission from Elsevier (112)

Legend: BPD – bronchopulmonary dysplasia; IVH/PVL – intraventricular haemorrhage / periventricular leukomalacia.; HIE – hypoxic ischemic encephalopathy; MDA – malondialdehyde; NEC – necrotizing enterocholitis; TH – total hydroperoxides; AOPP – advanced oxidative protein products; IsoPr – Isoprostane; 8–OHdG – 8-hydroxi-deoxyguanosine; PDA – patent ductus arteriousus; ROP – retinopathy of prematurity.

Erythrocytes were the first cells to reveal neonatal susceptibility to oxidative stress, manifested by the oxidation of haemoglobin and the damage of the erythrocyte membranes (110).

Nowadays, evidence is strongly supporting the notion that oxidative stress is the cause of disease in the neonate with manifestations in several organs, such as the lung (BDP), brain (HIE – hypoxic-ischemic encephalopathy; IVH – intraventricular haemorrhage; PVL – periventricular leukomalacia), eye (ROP), intestine (NEC – necrotising enterocolitis) and heart (PDA – patent ductus arteriosus). So, instead of different diseases, we might be dealing with different aspects of the same disease sharing common pathogenic mechanisms. Oxidative stress acts directly on gene expression and also results in epigenetic changes.

So far, the liver has not been included on the list of these disorders despite the clues associated to transient cholestasis and some sporadic forms of NC (109, 111).

Oxidative stress is not easily characterised *in vivo*, and none of the existing biomarkers can do it on their own. The routine use of oxidative stress biomarkers in clinical practice may be useful in the early identification of children at higher risk for tissue damage. Additionally, those biomarkers may be applied to evaluate the results of therapeutic and preventive strategies.

Current biomarkers of oxidative stress are, for example, compounds from plasma [total antioxidant status (TAS), lipid peroxidation (LPO)] or erythrocytes [membrane bound haemoglobin (MBH), glutathione peroxidase (GPx), GSH, and glutathione oxidised (GSSG)].

Among the new biomarkers are the microRNAs, which are promising candidates since they have a role in the cell responses of redox imbalance (112).

5. CLINICAL MANIFESTATIONS

5.1 CLINICAL FEATURES

The clinical presentation of NC may vary according to the underlying entity. However, the cardinal findings in a neonate/infant with cholestasis are jaundice, dark urine and pale stools.

Concerning jaundice, it is important to characterise the moment it starts and how it evolves. Jaundice can start in the first days (or even in the first 24 hours) of life and last more than 14 days; it can also start in the first days of life followed by a free interval and recur later (> 7 days) and can start only later on (> 7 days). In the first case, cholestasis can set in at any time during the evolution of jaundice. It is important to bear in mind that jaundice may decrease during the first weeks of life as the percentage of indirect bilirubin decreases, thus giving a false impression that it is disappearing. In cases where jaundice occurs later, after a free interval, the suspicion of cholestasis arises more easily.

Dark urine is frequent as an indicator of conjugated hyperbilirubinemia and can be easily confirmed by a urine test strip although a negative test does not exclude it. On the other hand, the presence of pale stools is suggestive of extrahepatic biliary obstruction or severe intrahepatic cholestasis. On the contrary, the presence of coloured stools suggests the permeability of the extrahepatic biliary tree and generally makes BA unlikely. However, in the early stages of BA, stools can be normal or intermittently coloured, and, therefore, it is very important that stool colour be evaluated in a serial manner in all jaundiced newborns/infants. The stool colour card is a validated method for this purpose (43).

When exploring clinical data, it is also important to emphasise the presence or absence of other signs/symptoms of disease, such as eating disorders (anorexia/vomiting), weight gain, fever/hypothermia, seizures and bleeding manifestations. It is essential to explore the family history and specific features of personal history. The existence of parental consanguinity, or affected siblings, points to inherited diseases. It is also important to observe any physical stigma in the parents (e.g. ALGS). The obstetric history may suggest maternal infection (TORCHS infection, hepatitis B) or pregnancy cholestasis (e.g. PFIC). It is also particularly important to explore some perinatal risk factors for the development of transient cholestasis.

In the clinical observation, it is important to give relevance to the nutritional status (low birth weight or intrauterine growth restriction, failure to grow or malnutrition), vital signs (hemodynamic instability, fever/hypothermia), jaundice intensity and extension and

bleeding manifestations (petechiae, suffusions, bleeding from the umbilical stump or venous punctures). Microcephaly or dysmorphia (e.g. peculiar facies) may be present as well as cardiac murmurs (e.g. peripheral pulmonary strictures or structural cardiac anomalies). Examination of the abdomen may show the presence of organomegalies (liver and spleen) or palpable masses (choledochal cyst) and ascites. Ocular anomalies (cataracts, chorioretinitis, cherry red-spot, posterior embriotoxon, ocular coloboma, hypoplasia of the optic nerve) and neurological features (irritability/lethargy, hypotonia, convulsions or nystagmus) should be investigated. Direct observation of the stools is part of the patients' clinical observation.

5.2 MAJOR CLINICAL ENTITIES

The diversity of the entities underlying NC has been growing significantly in recent decades. Advances in the knowledge of pathophysiology and the development of new diagnostic techniques (e.g. next-generation molecular techniques) have enabled the identification of new underlying entities. Consequently, the classification tables of the underlying entities have undergone many changes over time. The older ones classify the various entities using as classifiers the supposed anatomical location of the pathologies (extrahepatic or obstructive, intrahepatic or hepatitis, miscellaneous and idiopathic).

In 2003, Balistreri et al. (113) emphasised the importance of an adequate nomenclature and, quoting a Chinese proverb – 'the principle of wisdom is to call things by their true names' – proposed a new classification with a division between genetic and sporadic forms. In this scenario, and at that time, it was hard to classify an iconic underlying entity such as BA. Afterward, as more metabolic diseases were progressively identified, it was tempting to classify them as an important subgroup (114). Time has come to prove that Balistreri et al. were right. Nowadays, with the advances in the knowledge of pathophysiology, the consensus of this type of classification is increasingly evident, and increasingly more entities can fit into it.

Table 2 shows a list of underlying entities that are not intended to be exhaustive.

	Underlying entities	Genes
Familial (Genetic) forms	**Genetic and metabolic disorders**	
	Alfa-1-antitrypsin deficiency	*SERPINA1*
	Alagille syndrome	*JAG1, NOTCH2*
	Arginimemia	*ARG*
	Arthrogryposis-renal dysfunction-cholestasis syndrome	*VPS33B*
	Caroli disease and congenital hepatic fibrosis	*PKHD1*
	Citrin deficiency	*SLC25A13*
	Cystic fibrosis	*CFTR*
	Congenital disorders of glycosylation	*PMM2*
	Disorders of bile acids synthesis	*AKR1D1, AMACR, CYP7A1, CYP7B1, CYP27A1 HSD3B7, …*
	Disorders of bile acids conjugation	*BAAT, SLC27A5*
	Fatty acid oxidation defects	*ACADS, ACADL*
	Galactosemia	*GALT*
	Glycogen storage disease type IV	*GBE1*
	Lymphedema cholestasis (Aagenaes syndrome)	*LCS1*
	Lipid storages diseases	*LIPA,* GBA
	Hepatic-pancreatic-renal syndrome	*NPHP3*
	Hereditary fructose intolerance	*ALDOB*
	Hemophagocytic lymphohistiocytosis (FLH) (familial)	*PRF1, STX11, STXBP-2, UNC13D*
	Mitochondrial respiratory chain disorders	*DGUOK, MPV17, POLG, …*
	Neonatal cholestasis and type 5 MODY	*HNF1B*
	Neonatal ichthyosis-sclerosing cholangitis syndrome (Claudin 1, NISCH syndrome)	*CLDN1*
	Neonatal sclerosing cholangitis	*DCDC2*
	Niemann-Pick disease type C	*NPC1, NPC2*
	North American indian childhood cirrhosis	*CIRH1A*
	Peroxisomal disorders (Zellweger syndrome, Refsum syndrome)	*PEX2, PEX3, PEX5, PEX6, PEX7, PEX10, PEX12, PEX13, PEX14, PEX16, PEX19, PEX26, PEX11B*
	Progressive familial intrahepatic cholestasis (PFICs)	*ATP8B1, ABCB11, ABCB4, TJP2, NR1H4, MYO5B,*
	Tyrosinemia	*FAH*
	Chromosomal disorders	
	Trissomy 21	
	Turner syndrome	
	Endocrine	
	Hypothyroidism	
	Panhypopituitarism	
Sporadic forms		
	Infectious	
	Viruses (CMV, Herpes types 1, 2, ...)	
	Bacteria (E.coli, Streptococus haemoliticus group B,...)	
	Spirochaetes (Syphilis)	
	Toxins	
	Drugs	
	Endotoxins	
	Total parenteral nutrition-associated cholestasis	
	Herbal products	
	Immune	
	Gestational alloimune liver disease	
	Congenital lupus	

Obstruction of biliary tree	
Biliary atresia	*ADD3, FOXA2, GPC1, EFEMP1, STIP1, PKD1L1 (polymorphysms)*
Choledochal cyst	
Cholelithiasis	
Biliary sludge	
Inspissated bile	
Spontaneous perforation of common bile duct	
Malignacy	
Others	
Cardiovascular and circulatory disorders	
Haemophagocytic lymphohistiocytosis	
MacCune-Albright syndrome	*GNAS (postzygotic mosaicism)*
Transient	*ABCB11, ABCB4*
Idiopathic	

Table 2 – Neonatal cholestasis underlying entities and coding genes.

Below, we briefly describe some of the most relevant entities underlying NC and important contributions made to the understanding of its pathophysiology.

5.2.1 Familial forms

A1ATD. A1ATD is an autosomal recessive disease with the co-dominant expression of both alleles characterised by low serum levels of this glycoprotein. Alpha-1-antitrypsin (A1AT) belongs to the SERine Protease INhibitor superfamily (SERPIN), and is synthesised mainly in the liver, with small contributions from leukocytes, the lungs and the intestines. Among its various functions, such as acute phase protein with immunomodulatory and anti-inflammatory properties, A1AT is an important inhibitor of the neutrophils elastase in the lower respiratory tract, protecting the lung parenchyma from excessive destruction that ultimately leads to emphysema (115). Serum A1AT levels are defined by mutations in the *SERPINA1* gene, and the normal alleles are called M. The most common deficient alleles are called Z and S. Homozygosity ZZ can result in very low A1AT serum levels and abnormal protein deposition in the endoplasmic reticulum of the hepatocytes.

A1ATD is most common in Northern Europe (mainly Scandinavia, Denmark), the Baltic Republics and the Iberian Peninsula and is extremely unusual in Asia. In the Swedish population, the PiZZ phenotype has a prevalence of 1/1500 and the PiSZ of 1/750 (116). In the Portuguese population, the PiZZ estimated prevalence is 1/5249 and for PiSZ is 1/281 (117). In Portugal, multiple rare alleles have been identified, but their frequency in the population is unknown (115). The PiZZ phenotype can lead to the development of severe and early emphysema and also liver cirrhosis (19).

Lung and liver disease have origins in different mechanisms, and, therefore, the protein replacement therapy used for lung disease has no effect on the liver. Liver disease results from the intrahepatic accumulation of the A1AT mutant Z protein, which triggers a cell injury cascade of hepatocellular death, a low level of compensatory liver regeneration and, ultimately, fibrosis, cirrhosis and hepatocellular carcinoma (118). Allelic variations in the A1AT gene itself, or in the genes involved in the proteolytic systems, may alter the susceptibility to liver injury by changing the efficiency of the degradation of the accumulated abnormal protein. Environmental or genetic modifiers of protein secretion, degradation, apoptosis or regeneration may have influence on the progression of liver disease in an individual patient (118).

The first manifestation of liver disease may be NC, which occurs in only 10% of PiZZ patients (45). Recently, a retrospective and prospective study performed on the French DEFI-ALPHA cohort (153 patients from 28 centres born from 1989 to 2016) identified NC as an independent risk factor for severe liver disease even when adjusted for age and gender (OR = 4.5, $p < 0.01$) and that long-term survival without severe liver disease was significantly lower when NC had been the clinical form of presentation ($p < 0.05$). In this study, PiZZ was the most frequent genotype associated with liver disease; however, other genotypes were also found to be involved, especially when associated to other predisposing factors (119).

Nebbia et al. (1983) described a 13,3% mortality rate in a cohort of 45 patients with A1ATD presented by NC (47). Sveger et al. (1988) reported the results of a cohort of 127 PiZZ patients identified in a screening at birth from a sample of 200 000 Swedish infants. These patients were followed from infancy to 12 years of age. Five patients died (mortality rate ~3,94%), from which two died of liver cirrhosis, a third died of aplastic anaemia but had liver cirrhosis upon autopsy and two from unrelated causes (anaphylactic shock, accident) (45). Recently, Teckman et al. (2020) reported a 12% rate of progressing to transplantation or death and a 28% rate of experiencing portal hypertension during a period of follow-up of 10 years (46).

The current management of liver disease focuses on supportive care. Some old reports claimed the benefits of UDCA on A1ATD patients (120). Nowadays, experimental data give promising perspectives on novel medicines (121, 122).

PFIC. Cholestasis results from the impairment of bile flow and can be caused by defects of the hepatocytes (involving the complex process of bile formation and secretion) and/or of the cholangiocytes (secretory machinery). The PFIC are a group of rare diseases caused by autosomal recessive mutations in the genes that encode proteins, expressed mainly in the apical membrane of the hepatocytes, which play important roles in the process of bile formation and flow (123).

In 2019, a systematic review recorded an incidence of intrahepatic cholestasis, including but not limited to PFIC, of 1/18 000 live births in one study that did not used genetic testing. In two studies of infants and children (2–18 years) with cholestasis, 12–13% had genetically diagnosed PFIC (20).

Historically, three types of PFIC were described, types 1–3, and recently, new types have been added (currently 9 types). We briefly describe here only the so far best characterized types 1–6 (Table 3 and Fig.12).

Disease	Gene	Molecular mechanism
PFIC-1	*ATP8B1*	Encodes the familial intrahepatic cholestasis 1 (FIC 1) protein, involved in translocating phospholipids in membranes and in the organisation of the apical membranes of polarised cells.
PFIC-2	*ABCB11*	Downregulation or absence of functional bile salt export pump (BSEP), which is a crucial mechanism to bile flow.
PFIC-3	*ABCB4*	Lowers the expression of MRP3, a phosphatidylcholine flippase localised in the canalicular membrane of hepatocytes, which facilitates the transport of this but no other phospholipids.
PFIC-4	*TJP2*	Loss of function mutation in the tight junction protein 2. The tight junction protein claudin-1 (CLDN1) fails to localise, especially in the parenchyma of the hepatic lobule.
PFIC-5	*NR1H4*	Encodes farnesoid X receptor (FXR; a transcription factor for bile formation, activated by bile acids and directly involved in the expression of BSEP and MDR3).
PFIC-6	*MYO5B*	Encodes an actin-associated molecular motor known as MYO5B. Diminished activity of the MYO5B/RAB11A is related to the disruption of the BSEP localisation and of the polarisation of epithelial cells. Mutations are also associated with microvillus inclusion disease.

Table 1 – Overview of PFIC.

Clinically, PFICs 1, 2, 4 and 5 may present with severe NC, with high serum bile acids and bilirubin levels. In all types, GGT is low except for PFIC-3, in which it is high, and PFIC-5, in which it is normal.

Patients with PFIC-1 will typically present itching (sometimes uncontrollable) and extrahepatic manifestations (diarrhoea, growth failure, exocrine pancreatic insufficiency and progressive sensorineural hearing loss). PFIC-2 patients will also present itching and may progress to cirrhosis and hepatocellular carcinoma or cholangiocarcinoma. In PFIC-3, symptoms often appear later in life, in childhood or in adulthood; mutations in the same gene will also cause low phospholipid-associated cholelithiasis (LPAC), parenteral nutrition-associated liver disease, transient NC and intrahepatic cholestasis of pregnancy. PFIC-4 presents with severe NC, and extrahepatic symptoms may occur mainly in the neurological and respiratory systems; some patients may develop hepatocellular carcinoma. PFIC-5

patients may present with NC and the undetectable expression of BSEP in the bile canaliculi. PFIC-6 presents with symptoms of microvillus inclusion disease, which affects the enterocytes and leads to diarrhoea and malabsorption. Cholestatic liver disease possibly occurs as a consequence of TPN, which is required for life.

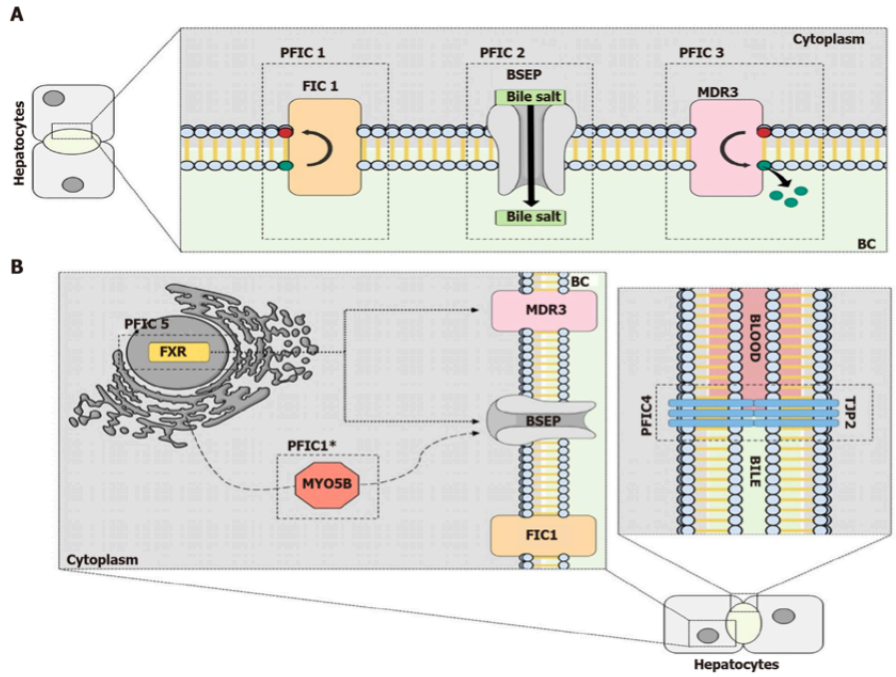

Figure 12 - Molecular mechanisms behind PFIC.

Source: Published by Baishideng Publishing Group Inc (123).

Legend: BSEP – bile salt export pump; FIC – familial intrahepatic cholestasis; FXR – farnesoid X receptor; MDR – multidrug resistance-related protein; PFIC – progressive familial intrahepatic cholestasis

Treatment will involve support measures such as itching control (119) and nutritional support. UDCA may be effective in PFIC-3. The ASBT inhibitors and the FXR agonists (e.g. obeticholic acid) may be useful in PFIC-5. TPN is required for life in PFIC-6. Sometimes surgery (partial internal or external biliary diversion surgery, or ileal exclusion) may be an option.

All PFIC may progress to end-stage liver disease, and the final option is liver transplantation. In PFIC-1 the extrahepatic manifestations will persist or even worsen (124). In PFIC-2, the development of allo-reactive antibodies specific to BSEP in the allograft may cause BSEP deficiency in the transplanted liver (124). In PFIC-5, steatosis has been reported in the transplanted liver. In PFIC-6, a combined bowel-liver transplant should be considered.

The mortality rate of PFIC was 0–87% across 10 studies, with a median age at death of four years in one study (20).

Alagille syndrome. ALGS is an autosomal dominant, multisystem disorder with highly variable clinical features, including NC. It is a rare condition, occurring in approximately one in 70 000 live births but is the most common cause of cholestasis in children. Mutations were found in two genes, *JAG1* (90% of the clinically diagnosed patients) and *NOTCH2* (in a small percentage); expression of the mutated genes in the liver is associated with defects in the embryogenesis of the intrahepatic ducts (canalicular paucity), which manifests itself in cholestasis (including NC).

Other clinical features beyond the liver include peculiar facies, cardiac malformations (peripheral pulmonary artery stenosis, tetralogy of Fallot), other vascular malformations (renal artery stenosis, aneurysms or cerebral arteriovenous malformations), bone malformations (wing butterfly vertebrae) and ocular features (posterior embriotoxon). They may also include other minor characteristics such as a high-pitched voice, delayed growth and nephron-urological malformations.

The characterisation of the natural history of these patients may promote better management and the settlement of endpoints for future clinical trials. Currently, this knowledge is based on retrospective studies in which many patients do not have molecular characterisation. Recently, a multicentre observational study involving 293 patients with ALGS and NC sought to assess the frequency of liver complications and the survival rate with native liver as well as to explore the determinants of growth (49). This study shows the previously underestimated weight of liver disease associated with early cholestasis and a second wave of liver disease impact later in childhood associated with portal hypertension, with less than 25% of patients reaching adulthood with their native liver. Higher bilirubin values were associated with a decrease in weight and height z-scores.

In ALGS, overall survival may be influenced beyond the liver by other organs' severity of disease, namely cardiovascular diseases or some other vascular anomalies. Lykavieris et al. (2001) reported a cohort of 163 patients (1960–2000) from which 132 presented with NC; of these, 48 patients died, 17 related to complications of liver disease (~10,4%). In the whole series, actuarial survival rates with native liver were 51% and 38% at 10 and 20 years, respectively, and overall survival rates were 68% and 62%, respectively. Neonatal cholestatic jaundice was associated with poorer survival with native liver ($p = 0.0004$) (48). Kamath et al. (2020) reported the largest multicentre natural history study of cholestasis in ALGS ($n = 293$), demonstrating a previously underappreciated burden of liver disease

since, by 20 years of age, 40% of participants had developed portal hypertension. The estimated liver transplant-free survival at the age of 18,5 years was 24% (49). Previously, Kamath et al. (2004) had reported the results of a cohort of 268 patients with ALGS in which 25 patients (9%) had non-cardiac vascular anomalies or events, and vascular accidents accounted for 34% of mortality (50).

To summarise, morbidity and mortality are high, and, as previously mentioned, ALGS is generally associated with the evolution of liver disease (cirrhosis and portal hypertension), cardiac malformations (severe Fallot) or complications of vascular malformations (e.g. rupture of cerebral aneurysm). Among the morbidities associated with liver disease, we lack studies to assess the impact of intractable itching and refractory hypercholesterolemia, conditions that can put pressure on liver transplantation, the former due to the poor quality of life and the second due to future cardiovascular risks.

Metabolic diseases, and other genetic diseases. The number of these diseases described in association with NC has increased in recent years (see Table 2) with the availability of new-generation molecular genetic techniques. We highlight the deficit of lysosomal acid lipase (Wolman's disease), which will be part of the central articles of this thesis, and others, neonatal sclerosing cholangitis, argininemia, and McCune-Albright syndrome (MAS) that are part of the works published before the thesis period.

Lysosomal acid lipase deficiency (LAL-D) is an autosomal recessive lysosomal storage disease caused by mutations in *LIPA* gene. The clinical spectrum of the disease varies from the very rare (1/ 500 000) and most severe early onset form (EO-LAL-D) (125), to the less rare (1/40 000) form known as cholesterol esters storage disease (CESD) affecting children and adults (126). The EO-LAL-D presents in the first weeks of life with failure to thrive, progressive hepatosplenomegaly, dyslipidemia, liver dysfunction, and adrenal calcifications; it leads to death within the first year of life (125). The diagnosis of the EO-LAL-D should be suspected in the presence of lymphocytes with cytoplasmic vacuolation in peripheral blood smear and/or calcification of the adrenal glands. LAL-D, in its both forms, was associated to NC (113, 114, 127).

Neonatal sclerosing cholangitis, first described by Ameedee-Manesme et al in 1987 (128), is a rare and severe cholangiopathy of neonates, presenting by NC and pale stools with progression to liver cirrhosis and need for OLT. The high parenteral consanguinity and the recurrence of the disease among siblings suggested an autosomal recessive pattern of inheritance (129). Recently, a genetic basis was found, pointing to its possible

pathophysiology – a liver based non-motile ciliopathy sometimes associated to nephronophthisis (130,131).

Argininemia is a rare inborn error of metabolism, due to hepatic arginase deficiency, which is the last enzyme of the urea cycle. The onset of the disease is usually in childhood, and clinical manifestations include progressive spastic paraparesis and mental retardation (132). Liver involvement is less frequent and usually not as severe as observed in other urea cycle disorders. NC has been rarely associated to argininemia as the first clinical manifestation of the disease (133,134). The mechanism by which arginase deficiency may be associated to NC is unknown.

MAS is characterized by a clinical triad of café-au-lait spots, polyostotic fibrous dysplasia and sexual precocity (135,136); the first two signs and/or a hyperfunctional endocrinopathy are enough to confirm the diagnosis, but other tissues may be affected (137) including liver tissue (138,139). Liver involvement is not frequent but NC may be the first clinical manifestation of the syndrome (140). Intrahepatic bile duct paucity has been reported requiring a differential diagnosis with ALGS (141). Although some authors have reported a spontaneous resolution of cholestasis with persistent elevation of liver enzymes, mainly GGT, recently, others described progressive liver diseases with development of liver tumors (142), or severe cholestasis with early progression to end-stage liver diseases requiring OLT (143). An activating mutation in the G-protein alpha-subunit (GNAS) gene (an oncogene) has been identified in the affected tissues with a transmission pattern consistent with a post-zygotic mosaic distribution, early embryogenesis (144).

5.2.2 Sporadic forms

Biliary atresia. BA is a severe neonatal liver disease caused by fibro-inflammatory obliteration of the intra and extrahepatic biliary tree progressing to terminal liver disease in the first two years of life (145). The ‘poster child’ of this disease is cholestatic jaundice and pale stools in an otherwise healthy full-term neonate/infant.

The disease has been known since the late 19th century (5) and has much higher incidence rates in Asia [~1 per 10 000 in Japan (146) and ~1 per 2700 in Taiwan (147) than in Western countries (1 in 12 000–18 000 infants) (148).

Morbidity and mortality rates in the long term are a high burden to patients, families and societies. The two major advances achieved in the 20th century with an impact on outcomes

and survival were Kasai's PE (10) and liver transplantation (12). More recently, in some countries the centralisation of diagnosis and treatment (40, 41) and in others universal newborn screening (NBS) programmes (43) have produced advances with an impact on overall survival and survival with native liver. In France, in a cohort study (1986–2015) enrolling 1428 patients, 22% reached the age of 30 years without transplantation, and the five-year overall survival is nowadays 87%. The improvement in BA prognosis was due to both, lowering the mortality before OLT and improving outcomes after OLT (42). Also, the Japanese Biliary Atresia Society Registry, where each patient is followed-up for 30 years, enrolled 3160 patients between 1989 and 2015, and OLT was performed in 1236 patients. The 20-year overall survival and native liver survival rates were 89% and 49%, respectively (44).

The current clinical challenge in BA is to be able to perform an early diagnosis to increase the success of PE, to prevent the progression of liver disease after successful PE and to treat the long-term complications from liver transplantation and immunosuppression. However, the outstanding challenges are to reverse BA in its earliest stages and to prevent BA, both requiring increasing knowledge of the underlying causes and pathogenesis (145).

Currently, it is consensual that BA is a multifactorial clinical entity that requires the existence of exposure and susceptibility factors, and evidence points to its beginning in prenatal life (145) (Fig. 13).

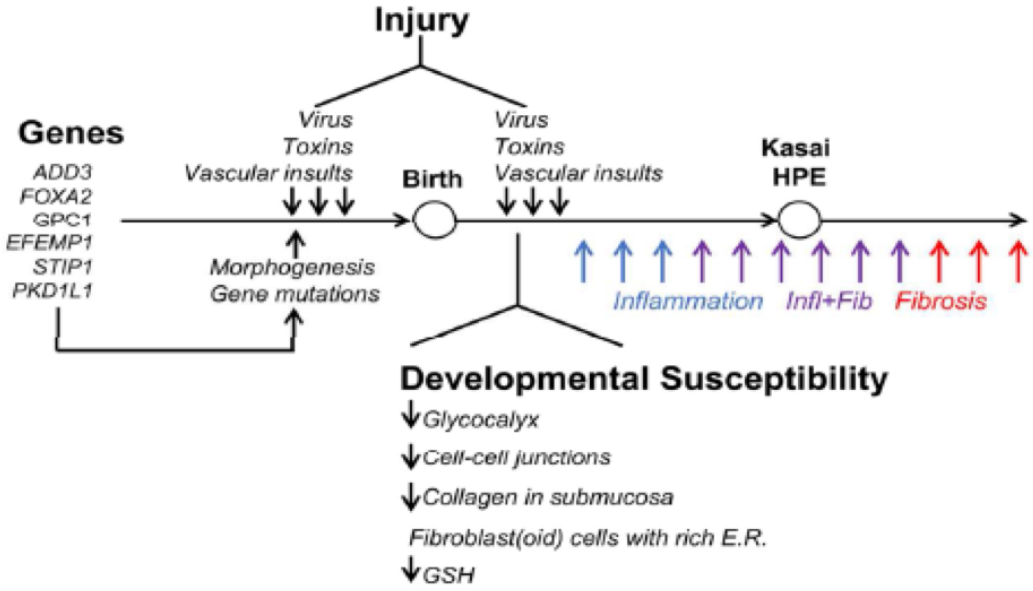

Figure 13 - Factors implicated in aetiology and pathogenesis of BA.

Source: Reprinted with permission from John Wiley and Sons (145).

Legend: GSH – reduced glutathione; ER – endoplasmic reticulum; HPE – hepato-portoenterostomy

Exposure. Several viruses have been proposed as triggers to BA, but cytomegalovirus (CMV) infection is the one with the largest body of evidence for this association. CMV DNA was identified in 60% of BA patients at diagnosis in one study (149), and immunological tests reported a liver memory T-cell response to CMV of 56% (150). Detailed modern molecular viral analyses may help to clarify the role of CMV in BA patients.

Data from studies in human subjects (151) and animal models (152) have linked an environmental toxin (biliatresone) to biliary damage. Human exposure to this toxin (a compound from a plant) is unlikely, but further studies may come to identify other toxins to which humans may be more likely exposed.

Susceptibility factors. BA affects only neonates or small infants, which suggests that the developmental immaturity of the bile ducts may play a role in the disease pathogenesis. Also, interestingly, the junction between the intrahepatic and the extrahepatic bile ducts occurs at the hilum level (bile ducts morphogenesis), the same site as the anastomosis of Kasai's PE.

In fact, studies in neonatal mice have confirmed very relevant data, such as a marked lack of glycocalyx in cholangiocytes (a sort of bicarbonate 'umbrella' in the apical membrane to protect cholangiocytes from bile salt toxicity) (153), poorly developed cholangiocyte junctions and a lack of submucous collagen bundles in extrahepatic bile ducts (145). These anatomic and functional immaturity features enhance the potential for fibrotic response.

Also, gene sequence variants related to biliary morphogenesis (e.g. ALGS, ciliopathies) make some patients uniquely prone to cellular injury and to a prominence in the expression of the extracellular matrix. Additionally, there is growing evidence that BA patients have a genetic susceptibility to potentiate the biliary injury initiated by some triggers and to develop a pathological repair. Some candidate genes were identified mainly in animal models; for example, studies with an environmental toxin (biliatresone) demonstrated that some genetic variants affecting the human glutathione (GSH) metabolism are potential risk factors for biliary injury (154).

The beginning. An important issue to be clarified is whether BA arises in foetal life or only postnatally. Against foetal onset are the absence of BA in stillbirths and the absence of an association with prematurity and low birth weight. In favour of prenatal onset are the forms with cystic anomalies observed in prenatal ultrasounds and especially a recent study that demonstrated an elevation of conjugated bilirubin in the first 24–72 hours of life in 34 of 34 patients with BA (155). This result was confirmed by a large-scale prospective study and points to conjugated bilirubin as a screening method (156) potentially more precocious than

the stool colour card (SCC) (43). Combined data indicate that BA is a disease with prenatal onset with clinical manifestations after birth, after the loss of maternal and placental physiology protection.

The progression. After the initial damage produced by a virus, toxin or other insult, damage to the cholangiocytes will arouse the newborn's immune system, which has an innate pro-inflammatory bias. In BA, there is indirect evidence of autoimmunity (animal model) as well as circumstantial evidence of immune dysfunction. Interestingly, immunosuppression does not prevent the progression of the disease after Kasai's PE, which shows that its nature is similar to that of other autoimmune cholangiopathies (autoimmune cholangitis and primary biliary cirrhosis). The implication of innate immunity in disease pathogenesis highlights their suitability as a therapeutic target (157).

Type 2 cytokines (IL-13, IL-33, IL-4) are elevated in BA patients (158). These interleukins are associated with the activation of fibrogenesis. Several non-immune factors can initiate and amplify fibrogenic signals in cholangiopathies, but they are scarcely studied in BA. Also, some drugs when administrated in the neonatal period may facilitate bile leaks that in turn stimulate fibrogenesis.

All these advances in the knowledge of the pathogenesis need to be translated into new medical therapies, fulfilling the aims of reversing or preventing BA.

Neonatal hemochromatosis. NH is a clinical condition in neonates encompassing severe liver disease and extrahepatic siderosis with a distribution similar to that of hereditary hemochromatosis (159).

NH was initially seen as an inherited disease of iron metabolism (160). The existence of cirrhosis at the time of diagnosis caused suspicion of the onset of liver damage even during intrauterine life. The existence of older siblings affected caused suspicion of a genetic disease although the pattern of recurrence defied all the rules of heredity. A woman could have several healthy children before having one affected, and after having the first affected child, the probability of the following being affected was > 90%. Maternal half-siblings were affected but not paternal half-siblings, and surviving girls had unaffected offspring. NH appeared to be congenital and familial but not hereditary. This recurrence pattern pointed to an alloimmune maternal disease.

In 2010, Pan et al. reported that liver damage was mediated by complement in a process initiated by anti-IgG antibodies binding to foetal hepatocytes (161). This discovery led to the deduction that gestational alloimmune liver disease (GALD) is the main cause of NH.

Furthermore, NH is not a disease but merely a phenotypic expression of severe liver disease in the perinatal period. GALD can have other phenotypic expressions, and more rarely (in about 2%), NH can also have other causes besides GALD (e.g. perinatal infection, trisomy 21, *DGUOK* gene mutations, bile acids synthesis deficiencies and GRACILE syndrome).

GALD occurs when a woman is exposed to liver antigens that her body does not recognise as being of the 'self'. These antigens are expressed only during foetal development, and the woman may have lost tolerance to these antigens over time. They cross the placenta, and this exposure results in the sensitisation and production of immunoglobulin G (IgG) antibodies against foetal hepatocytes that are also able to cross the placenta in reverse, initiating an innate immune reaction. The complement's terminal cascade is activated by the classical pathway, resulting in the formation of the C5b-9 complex (161).

Liver tissue displays severe injury with a marked loss of hepatocytes, while the remaining ones show siderosis, giant-cell transformation and canalicular bile plugs. There may be observed pan-lobular parenchymal fibrosis and regenerative nodules, and 50% of the patients may have cirrhosis. In some cases, GALD may induce a hyperacute foetal liver injury without siderosis, which ends in stillbirth and neonatal demise and also may induce neonatal liver cirrhosis without iron overload.

Extrahepatic siderosis in NH is most frequently seen in the exocrine pancreas, thyroid, salivary glands, oronasopharynx and respiratory tree, among other tissues. Foetal liver damage leads to decreased hepcidin production, and this has a negative effect on ferroportin; consequently, the transport of iron through the placenta to the foetal liver is excessive. In addition, there is a decrease in the expression of the transferrin gene, giving rise to a decrease in the iron-binding capacity. Together, this results in a foetal iron overload.

There have been descriptions of renal hypoplasia in GALD, with the dysgenesis of the proximal tubules and paucity of the peripheral glomeruli. It is thought that these abnormalities in kidney development may be due to a decreased expression of the angiotensin gene that is synthesised exclusively in the liver.

GALD can present from 18 weeks of gestation to three months postpartum. Presentation is usually as liver failure in the neonatal period, typically with changes in the functions of hepatic synthesis and glucose homeostasis, in the absence of liver cytolysis markers (almost normal transaminases). Intrauterine growth retardation, oligohydramnios and prematurity are frequent. The diagnosis of NH depends on the evidence of severe liver

disease in association with evidence of extrahepatic siderosis (e.g. histology of the oral mucosa plus pancreas MRI has 80% sensitivity). The presence of increased iron deposits in the liver is common to many other liver diseases and does not contribute to the diagnosis, and its absence does not exclude it (e.g. hyperacute GALD); however, liver histology can be useful to demonstrate C5b-9 complexes. Serum ferritin levels are a sensitive but non-specific indicator.

Several questions remain unanswered, namely those concerning the mother's genetic and environmental susceptibility factors.

In the past, treatment consisted of a cocktail of antioxidants and iron chelators based on the hypothesis that liver damage would be secondary to oxidative stress caused by iron overload. With this treatment, survival was very low (10–20%). Liver transplantation improved survival to 35% (162). After the discovery of GALD, the proposed treatment switched to exchange transfusion with a two-volume exchange (to remove circulating antibodies) followed immediately by the administration of a high dose of IgG immunoglobulins (1g/kg) to block antibody-induced complement activation. With this new treatment, survival improved significantly (>80%) compared to the historical group at > 80% survival without liver transplantation (162, 163). Complete liver recovery can take two to four years.

Transient neonatal cholestasis. Transient cholestasis, as previously defined (22, 164), has been described in the last two decades, mainly in the setting of neonatal intensive care units. The preterm born and critically ill neonates have a high incidence of cholestasis of multifactorial aetiology.

Some retrospective studies have identified gestational age (33, 165, 166) and neonatal surgery (167) as risk factors for the development of NC, while others have also pointed to an association with ischemia/asphyxia (33, 168) and with sepsis (33, 169). In 2012, another prospective study, conducted by Champion et al. (23), involved 460 newborns admitted to a neonatal intensive care unit over a 12-month period. This study identified the following risk factors: prematurity (< 34 weeks), small for gestational age, parenteral feeding > 7 days and abdominal-pelvic or thoracic surgery. The incidence rate was 13.7% in patients with those risk factors, at a mean age (SD) of 14.7 (12.9) days. All patients who developed cholestasis had received parenteral nutrition. None of the patients without risk factors developed cholestasis. Gestational age and neonatal surgery were strong predictors of cholestasis with OR = 4.4 (95% CI 1.6–12.5) and 4.6 (95% CI 1.7–12.3), respectively.

In 2021, in another retrospective study, involving 250 patients, Teng et al. (34) reported an incidence rate of cholestasis of 14.8%, with increasing rates in lower gestational ages. In this study, the risk factors were pre-eclampsia and born small for gestational age.

Pathogenesis. Given the identified risk factors, some authors (21, 23) hypothesised that oxidative stress could play an important role in the pathogenesis of transient NC, but, as far as we know, no quality research studies have tested this hypothesis. Currently, oxidative stress biomarkers are not of common use in clinical practice.

Other authors argue in favour of a pro-inflammatory state as the main mechanism by which these patients develop transient cholestasis. They claim that certain evidence may support their hypothesis, such as, the reports that show C-reactive protein and interleukin-1b as predictors of cholestasis (170) and those showing higher liver stiffness in infants who developed cholestasis (171). Clinical and basic research has well demonstrated that inflammation can induce or contribute to cholestasis in several liver conditions (172), but data on transient cholestasis there is lacking. The gut microbiome may also be a player in this scenario, and we will come to this issue later.

Clinical challenge. In this subset of patients, the presence of risk factors that can justify cholestasis by themselves makes it difficult to decide on a diagnostic approach for underlying entities, which is a very relevant problem in clinical practice. Some studies showed that the incidence rates of underlying entities are significantly lower in preterm infants with cholestasis than in full-term ones. (38). In fact, some important underlying entities, such as BA, do not associate with prematurity. So, the existing diagnosis algorithms for NC may need to be modified for application to the preterm cohort in an efficient and costly manner.

TPN-associated cholestasis. Among all risk factors for the development of NC, the most consistently reported is TPN (38, 165-167, 173). Liver steatosis and fibrosis are observed in these infants, and prolonged cholestasis can lead to cirrhosis and liver transplantation. Since most infants develop TPN-associated cholestasis following intestinal failure requiring prolonged TPN, the terminology ‘intestinal failure associated liver disease’ is sometimes used to classify the patients with short-gut syndrome and cholestasis (38). The development of TPN-associated cholestasis in infants is multifactorial (174), likely by the following mechanisms:

Gut-liver cross talk. TPN-associated cholestasis is reversible upon restoration of enteral feeding; actually, it can progress to cirrhosis and end-stage liver disease if early enteral feeding is not started. Recent research into gut-systemic cross talk via the FXR-FGF19 axis

and via the TGR5-GLP axis has greatly broadened our understanding of TPN-associated injury (175) (Fig. 14). For example, the potent natural FXR agonist (CDCA) prevented cholestasis and TPN-associated liver disease in an animal model (176), and both animal and human studies have demonstrated the immaturity of the enterohepatic cycle, including a decreased bile acid reservoir and gallbladder contraction in neonates, particularly in the preterm neonates, increasing their susceptibility (177).

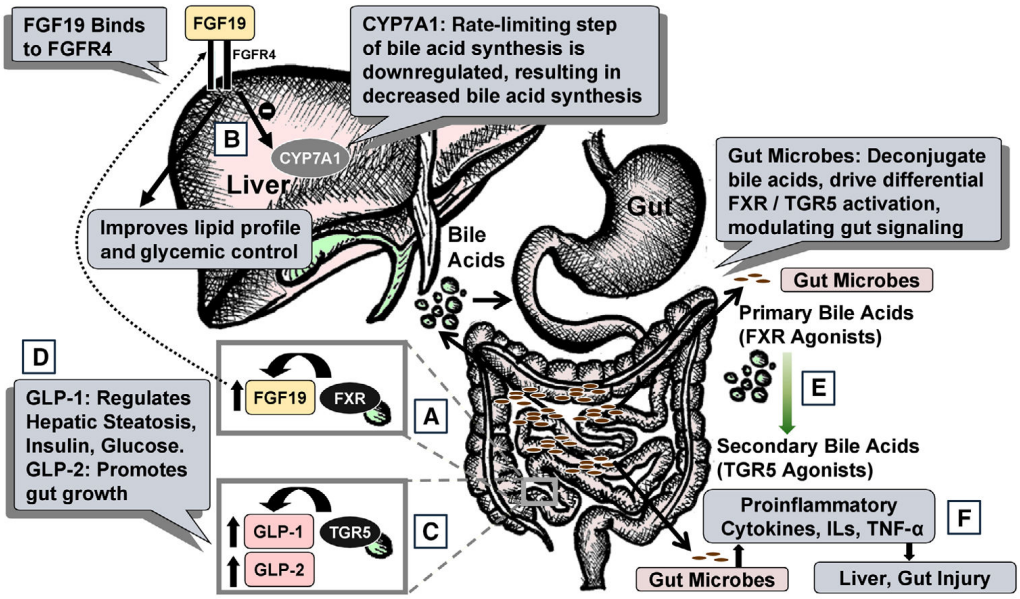

Figure 14 - Parenteral nutrition-associated injury.

Source: Reprinted with permission of Wiley and Sons (175).

Legend: CYP7A1 – cholesterol 7-alpha-hydroxylase; FGF19 – fibroblast growth factor 19; FGFR4 – transmembrane receptor tyrosine kinase 4; FXR – farnesoid X receptor; GLP-1 e 2 – glucagon like peptide; ILs – interleukins; TGR5 – takeda-G-protein-receptor-5; TNF – tumor necrosis factor.

Gut mucosal atrophy, bacterial translocation and central line infection-driven liver injury. Lack of enteral feedings leads to gut mucosal atrophy, decreased motility, bacterial overgrowth, increased permeability, bacterial translocation and sepsis. Also, intestinal resection leads to inflammation and increased permeability. Patients under TPN have higher incidence rates of central line infections compared with patients under enteral feeding, which may be due to bacterial translocation.

Bile acids and gut microbiota. Bile acids promoted gut mucosal regeneration via the activation of TGR5 in intestinal stem cells of mice and in intestinal organoids (178); additionally, a neonatal pig model has shown that bile acid receptor agonists prevented gut

atrophic changes (179). Primary bile acids are transformed into secondary bile acids by the gut microbiota, and primary bile acids are preferential ligands for FXR while secondary bile acids are for TGR5, and this explains the mechanism by which gut microbes can regulate bile acids' signalling properties. Gut microbiota comprises a key player not only in gut proliferation and inflammation but also in the gut's absorption of different nutrients and vitamins.

In many patients, these are non-avoidable and non-modifying risk factors for developing NC.

The role of lipids. In this scenario, the search for modifiable factors directed the investigation over the composition of the lipid emulsion of TPN. Conventionally used soybean oil-based lipid emulsions have high polyunsaturated fatty acid (PUFA) content and phytosterols, which may contribute to adverse effects, including TPN-associated liver disease. Nevertheless, a systematic review from the Cochrane Database (2019) resulted in insufficient data from randomised studies to determine the potential benefit of fish oil-containing lipid emulsion over other types of lipid emulsions (180). In fact, it seems there is still more to learn about the molecular effects of the lipid components have on protecting and promoting injury to the liver. Further studies are needed to separate the role of phytosterols from that of omega-6 fatty acid lipid emulsions and to examine other ingredients that can be added to lipid emulsions to promote better outcomes (174). Meanwhile, some recent studies have shown that cyclic TPN was an independent factor that significantly decreased TPN-related cholestasis incidence without increasing the risk of hypoglycaemia (181).

Survival in patients receiving long-term TPN has significantly increased over time, and this raises new challenges to improve patient outcomes.

CMV infection. CMV infection can be congenital or acquired. The first diverges from the second by the positive viral load in the blood in the first seven days of life. In Portugal, the blood sample collected for the Guthrie card under the national neonatal screening programme can be used for this purpose, requiring authorisation from patients' legal guardians.

CMV infection is a multisystem infection and may include cholestatic hepatitis or coexist in a condition of NC determined by another underlying entity, becoming a major confounding factor (182). Although diagnosis seems simple, indeed it is not. Strictly speaking, only the demonstration of the presence of the virus in the liver tissue can unequivocally affirm the

diagnosis of CMV hepatitis. It should be noted that the presence of viral load in the blood is not always accompanied by the presence of viruses in the liver tissue (183). However, in a compatible clinical context, the presence of positive viral load in the blood or the excretion of virus in the urine will be sufficient to suggest the diagnosis but will continue requiring the exclusion of other underlying causes (182).

In patients with BA and concomitant CMV infection, some authors have reported worse outcomes, lower survival with native liver, and higher mortality (184). The suggestion that CMV infection may be one of the triggers for BA is controversial (185). Very recently, Chatterjee et al. (186) demonstrated that the strains that infect intrahepatic bile ducts are phylogenetically different from those infecting extrahepatic bile ducts.

It is very important to exclude or confirm the diagnosis of CMV infection in the context of NC since a specific and effective antiviral treatment (ganciclovir and valganciclovir) is available.

6. DIAGNOSIS ADVANCES – EARLY RECOGNITION

The early recognition of NC is essential to ensure timely treatment and to improve prognosis. The main symptom of NC is jaundice. Other cardinal symptoms are dark urine and pale stools.

Jaundice is a very common symptom in this age group and usually related to benign clinical conditions (physiological jaundice, breastfeeding- or breast milk-associated jaundice); the latter is associated to prolonged jaundice (> 14 days), sometimes up to two to three months of age. Prolonged jaundice is a relatively common problem, affecting up to 15% of newborns. Because it is common and generally associated with benign conditions, jaundice is a clinical manifestation that tends to be devalued by healthcare professionals and caregivers.

Dark urine and pale stools are not easy to recognise by those who are less aware. Dark urine should be suspected when urine stains the diapers. Healthcare professionals will only identify the warning sign if they actively seek it, such as by performing macroscopic observation of urine and/or an examination with a test strip. Pale stools are not a mandatory sign in all cases of NC and their presence is usually associated with obstruction of the bile ducts or severe intrahepatic cholestasis with impairment of the excretory function of hepatocytes.

Recognising pale stools is also a major task. They may appear coloured when mixed with urine in patients' diapers. In addition, there is the inexplicable myth that inhabits the minds of many caregivers, and some healthcare professionals, that pale stools are due to the fact that newborns and infants are fed with milk. Instead, pale stools are due to a lack of bile in the intestine when the bile flux is severely impaired and not because they are stained by any white pigment from milk.

6.1 CLINICAL PRACTICES

Over the past two decades, several scientific paediatric societies have published standardised recommendations concerning the management of the jaundiced newborn and newborn/infant with NC (28, 29, 187). Nevertheless, some surveys have demonstrated that many healthcare professionals do not know or do not apply these guidelines.

Excellence of clinical practices requires both the elaboration of standardised recommendations as well as their disclosure among all healthcare professionals at all levels of care.

Currently, it is consensually recommended that all newborns who remain jaundiced after 14 days of life should be seen by a doctor, and if they show any of the other cardinal signs of cholestasis, or signs and symptoms of disease, they should be tested for serum conjugated bilirubin; some authors argue that the assay should be done regardless of the presence/absence of other cardinal signs of cholestasis. Obviously, any newborn with other signs or symptoms of alarm and with less than 14 days old should also be tested for conjugated bilirubin immediately.

6.2 UNIVERSAL SCREENING PROGRAMMES

The eligibility criteria for universal screening are related to disease characteristics, screening test and treatment effectiveness. Despite the rare incidence (1:2500 live births) the high impact due to morbidity and mortality may justify the costs of a NC screening programme. Additionally, the prognosis depends on the early-onset of effective treatment for some of the underlying entities.

Nevertheless, NC as a clinical condition candidate for universal screening presents some issues hard to solve. The existence of many underlying entities (sporadic and genetic forms) with a very large amplitude in the timing of clinical presentation makes it very difficult to effectively apply any screening method, whichever is chosen. Second, the screening test must be sensitive and specific, reproducible and reliable and economical and non-invasive as well as be accepted. Such method has so far not been found although some attempts have been made.

Some studies have tried to use serum cholestasis biomarkers as a screening method for NC. Mushtaq et al. measured the conjugated bile acids in dried blood spots obtained from newborn infants at seven to 10 days of age for the Guthrie test using tandem mass spectrometry. The results were not encouraging due to the low sensitivity of the method (62% for all cholestatic diseases and 79% for BA), thus making it a non-feasible option for mass screening (188).

Twelve years later, the same idea but using a more accessible biomarker – the gold-standard diagnosis, the conjugated bilirubin assay – was tested by Harpavat et al. (155, 156) with promising results. A measurement of serum direct or conjugated bilirubin was

performed in the newborns before hospital discharge (< 60 hours) (Test 1). A second test was performed only in the Test 1 positives by two to three weeks of life. For the diagnosis of BA, the sensibility was 100% (95% CI, 19.8–100.0), and the specificity was 99.9% (95% CI, 99.8–99.9), with a positive predictive value of 18.2% (95% CI, 3.2-52.2). This study may be useful to track all forms of cholestasis beginning in intrauterine life, which may include entities such as BA, GALD and genetic forms of NC. Anyway, there will be some sporadic cases beginning in the post-natal period escaping screening at this time and for which prevention and early recognition will be most suitable.

Meanwhile, some screening methods have been developed for tracking only the following certain specific underlying entities:

BA universal screening. In Taiwan, Hsiao et al. (2008) developed a SCC to screen for BA which proved to be effective in identifying pale stools with 95.2% sensitivity (43). This method consists of a SCC with which physicians must compare the colour of the infant's stool in the surveillance consultation carried out at one month of age. The card has a scale with three colours for 'normal' and six colours for 'abnormal' (Fig. 15).

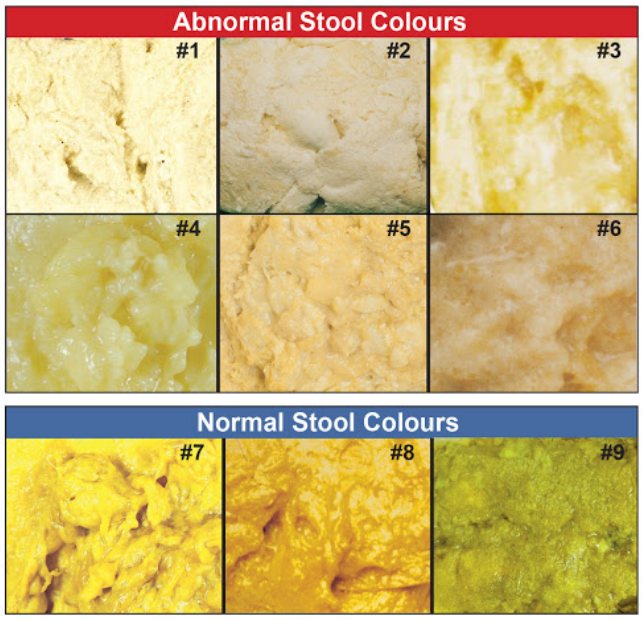

Figure 15 - Stool colour card for screening BA in the UK.

(courtesy from the Health Promotion Administration, Ministry of Health and Welfare, Taiwan, and Prof. Mei-Hwei Chang)

Source: http://www.perinatalservicesbc.ca/our-services/screening-programs/biliary-atresia-home-screening-program (accessed 17-05-2021)

All ‘abnormal’ cards must be notified to a centralised facility that contacts the parents to report immediately to a specialised medical service for a full assessment of the patient. The universal screening allowed an early diagnosis and the performance of Kasai surgery, with an important impact on the surgery’s success and predictably on global survival and long-term survival with native liver. The incidence of BA in Asian countries (e.g. Taiwan 1/2700 live births) makes this screening cost-effective in those countries (189, 190). In European (191) and American countries (192), where BA is rarer, this screening has not yet widely been recognised as cost-effective despite the attempts of several authors and institutions.

This SCC alone is not a suitable method for NC universal screening since not all patients with cholestasis have pale stools. In addition, choosing the right time to apply the screening test is hampered by the wide spectrum of time available for the clinical expression of NC.

Metabolic diseases screening. Metabolic diseases, whose prognosis may depend on the promptness of medical intervention, are at risk of not being diagnosed in a timely manner. NBS programmes using tandem mass spectrometry are used in neonatal screening for several inborn errors of metabolism (IEM) and have been widely adopted in Europe. In Portugal, an expanded NBS programme was implemented in 2005 for 24 treatable disorders (193).

Some IEM presenting with NC are covered in NBS, namely tyrosinemia type I, citrullinemia type II and argininemia.

6.3 THE ROLE OF CAREGIVERS

The contribution of caregivers to the early recognition of cholestasis in jaundiced newborns is essential. This role has been recognised, and some instruments have already been designed to integrate this contribution.

Home-based screening programmes for BA using stool colour cards performed in Canada proved to be cost-effective (194, 195). In Asia, a mobile phone application was developed with the stool colour card accessible to everyone, including caregivers (196). Other countries (e.g. Brazil, Switzerland, Japan) have included a page with information on the cardinal signs of cholestasis and a stool colour card in the Child and Youth Health booklet of those countries as a reminder for healthcare professionals and caregivers.

Yellow alert campaigns. Some countries (e.g. United Kingdom, France) have promoted the 'yellow alert campaign' for the early recognition of infant liver disease through publicity of materials such as the ones presented below (Figs. 16 and 17).

While the UK poster from the Children's Liver Disease Foundation presents clear information and an emergency contact line (phone/e-mail), the French poster may lead to the misconception that in a jaundiced newborn, the suspicion of cholestasis will only arise in the presence of pale stools.

We have not seen, yet, the published results of these initiatives.

In Portugal, as far as we know, there have been no initiatives at the level of the central healthcare system on this matter. On a local level, a university hospital in Porto uses a rudimentary print of a stool colour scale stapled to a page of the Child and Youth Health booklet.

Figure 16 - Alerte Jaune, Association Maladies du Foie Enfants, France.

Source: http://www.alertejaune.com (accessed 22-06-2021)

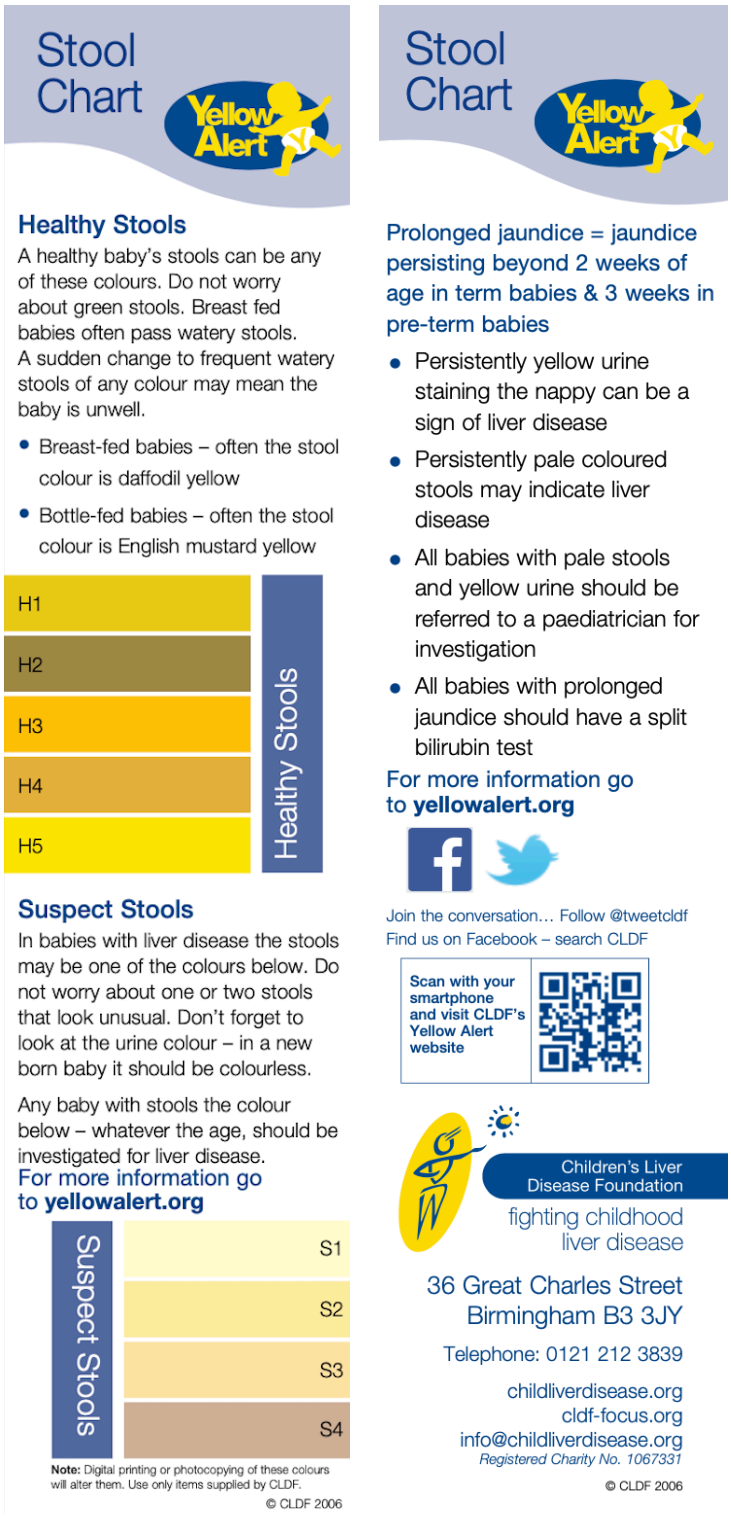

Figure 17 - Yellow alert, Children's Liver Disease Foundation, UK.

Source: https://www.childliverdisease.org/wp-content/uploads/2018/01/Yellow-Alert-Stool-Chart-Bookmark.pdf (accessed 17-05-2021)

6.4 DIAGNOSIS ASSESSMENT AND WORK-UP

The first approach to neonatal jaundice. The first approach to a jaundiced newborn or infant must aim to classify jaundice as cholestatic or of free bilirubin. The diagnosis of cholestasis should be suspected immediately in the presence of dark urine, pale stools or other signs/symptoms of illness and whenever jaundice lasts beyond 14 days of life. The confirmation is made by the serum measurement of bilirubin, total and conjugated. Secondly, it is critical to identify the presence of red flags, such as signs or symptoms of liver failure and pale stools, which call for urgency in the approach.

Liver failure is screened through an evaluation of liver functions, such as synthesis function (e.g. coagulation tests, serum albumin), glucose homeostasis (serum glycaemia and lactate) and detox (serum ammonia). Other biochemical markers may be increased in neonates or infants with cholestasis, but they have no specific diagnostic or prognostic value – raised transaminases (ALT, AST) indicates a nonspecific hepatocellular injury and GGT is an enzyme in the biliary epithelium whose increase is generally associated with cholestatic diseases involving the intra or extrahepatic bile ducts (e.g. BA, A1ATD, ALGS, etc.).

The diagnosis of underlying entities is complex and to avoid inadequate, invasive and expensive investigation, the experience of the teams is assumed to be essential and the performance of complementary exams should be guided by clinical expertise. For example, the presence of risk factors, especially in preterm newborns, may in itself explain cholestasis, and, for this reason, selective criteria are recommended in the investigation of these cases.

Guidelines and algorithms. The physician's expertise does not dismiss the development of tools to support the clinical decision, involving the following two levels: first, early recognition of cholestasis, which has already been addressed above; secondly, diagnosis of the underlying entities, which we will deal with here, highlighting the documents produced mainly by scientific societies.

The most recent guidelines were produced in 2004 by Moyer et al. (29) and in 2017 by Fawaz et al. (28), both on behalf of NASPGHAN and ESPGHAN. The first guideline presents a complex decision algorithm. The most recent guideline reviews the cholestasis diagnostic criterion for a conjugated bilirubin value greater than 1 mg/dl, regardless of the total bilirubin value. No decision algorithm was produced in this guideline. Also, some national guidelines have been produced by some countries, such as that of the Italian Society of Paediatric Gastroenterology (187).

In Portugal, a consensus with an algorithm was published in 2010 on behalf of the Portuguese Society of Paediatric Gastroenterology (197). A consensus guideline was also produced by the Portuguese Society of Neonatology, which is available on their website at https://www.spneonatologia.pt/wp-content/uploads/2016/11/2013-Colestase_Neonatal.pdf (accessed 22-06-2021).

Following, we highlight some **complementary exams** that are particularly important and/or that have made great progress recently:

Viral PCr. The remarkable progress in the diagnostic techniques of virology and their accessibility in the last decade has greatly benefited patients with NC by allowing easy confirmation of acute infections by viral agents, especially for some for whom effective treatment has been developed. The paradigm is CMV infection, in which it is possible to differentiate a congenital infection from an acquired infection in the postnatal period, with implications for the outcome and the prognosis of patients.

Imaging evaluation. The abdominal ultrasound is an important tool in the initial diagnostic approach and is the most useful imaging study. It can assess the size, echostructure and appearance of the liver, the number and size of spleen (as they can be abnormal in biliary splenic malformation syndrome) and the presence of ascitis. It can establish the diagnosis of choledochal cysts or to identify causes of extrahepatic obstruction (e.g. choledochal cysts, gallstones or biliary sludge). It can also demonstrate a small or absent gallbladder in fasting, which may suggest BA. The discovery of the triangular cord signal, an echogenic area of the 'porta hepatis' due to a cone of fibrous tissue, is specific to BA, but its visualisation requires an experienced radiologist. Dilation of the common bile duct is not seen in BA and suggests a distal obstruction or a broken form of choledochal cyst.

Hepatobiliary scintigraphy with technetium-99 labelled with immunodiacetic acid analogue (IDA) has high sensitivity (80–100%) but low specificity (33–80%) for BA. When pale stools are present, this exam adds little information.

In some centres with specific magnetic resonance, cholangiopancreatography has been used to define bile duct patency; however, these techniques require general anaesthesia in infants and have not yet replaced intraoperative cholangiography as the gold-standard diagnostic test for BA in the large majority of centres.

Non-invasive liver stiffness evaluation. Non-invasive methods to assess liver stiffness as an estimate of fibrosis are now available in many tertiary hospitals although with limitations in human resource skills and suitability of devices for very young children. These methods, such as transient elastography or acoustic radiation force impulse (ARFI), were found to be able to evaluate fibrosis in correlation with the degree found in histology and to be predictive of BA among patients with NC (198-200). Theoretically these techniques may also be useful in the long-term for monitoring the progression of fibrosis in BA (after Kasai's PE) and in other patients, avoiding liver biopsy.

Histopathological evaluation. Percutaneous liver biopsy remains an essential diagnostic tool in evaluating NC, but it should only be carried out after other tests have enabled a first diagnostic guidance. In BA, some features are highly predictive of the diagnosis, such as bile plugs in portal bile ducts or ductules, portal stromal oedema, absence of bile duct paucity, absent to rare giant cell transformation and absent to rare extramedullary haematopoiesis (201). However, it should be emphasised that other causes of cholestasis can mimic the histological appearance of BA, so the perioperative cholangiography is the diagnosis gold-standard.

Liver biopsy can also help to identify other causes of cholestasis, including A1ATD deficiency (periodic acid Schiff-positive, diastase-resistant hepatocyte globules), ALGS (bile duct paucity), neonatal sclerosing cholangitis (necro-inflammatory duct lesions), metabolic liver disease (steatosis and pseudo-acinar formation of hepatocytes), PFIC and storage diseases (electron microscopy findings), transient NC (multinucleated giant cells, extramedullary hematopoiesis and hepatocellular cholestasis without portal tract involvement) and viral infection (Herpes and CMV inclusions on immunohistochemistry). Even so, liver biopsy is increasingly less used for diagnostic purposes in these situations since other non-invasive methods including genetic tests have taken its place. It is also important to notice that several underlying entities leading to NC are dynamic (e.g. BA, A1ATD) and that characteristic liver biopsy sample findings may not all be present at initial biopsy in infants within the first weeks of age and might evolve over time.

Role for molecular genetics. Careful analysis of clinical features and biochemical markers can narrow the diagnostic possibilities in NC, but this traditional assessment paradigm can be time-consuming and expensive. Moreover, there have been important advances in molecular diagnostic techniques that make them feasible in the near future as well as more affordable and, therefore, can change the paradigm of the diagnostic assessment of patients with NC.

Next-generation sequencing (NGS) is performed by several high-throughput platforms using massively parallel processing of spatially separated amplified DNA templates. Targeted gene panels (TGPs), whole-exome sequencing (WES), whole genomic sequencing (WGS) and bioinformatics pipelines are now clinically available tools in many centres and countries which can identify all known gene variants that have been associated with cholestatic diseases (114). NGS can be particularly useful to identify infants that might be eligible for new and emerging therapies early in their course, to identify those children whose diseases might be contraindications for liver transplantation (for example, in mitochondrial disease and Niemann–Pick disease type C) and to enable families to receive specific genetic counselling and risk estimation for future offspring. In addition, NGS and bioinformatics analyses provide the capacity to discover new genetic causes of NC. For example, in a Japanese study of 109 patients with NC, a molecular genetic diagnosis was made for 28 patients (26%) using an NGS panel with 18 genes (26). In 22 out of 31 patients, the molecular genetic diagnosis was consistent with the clinical diagnosis of 'genetical cholestasis'. For the remaining six subjects, four out of 46 were of the subset 'unknown diagnosis without complications', and two out of 32 patients were of the subset 'unknown diagnosis with complications'. The NGS panel confirmed the diagnosis of ALGS, n = 1; neonatal Dubin Johnson syndrome (DJS), n = 3; neonatal intrahepatic cholestasis by citrin deficiency (NICCD), n = 1; and PFIC-2 / benign recurrent intrahepatic cholestasis (BRIC)-2, n = 1.

The use of TGPs can be useful to quickly and reliably confirm a clinical suspicion, while WES with the classification of variants has been adopted to aid in diagnostic investigation in unclear clinical settings (27). WES is powerful but problematic because it can detect a large number of variants of unknown significance (VOUS) that can be difficult to interpret and lead to misunderstandings.

As the cost and turnaround time of TGPs and WES continue to decrease, after BA is excluded and as other treatable disorders are evaluated, we may be tempted to proceed in the patient evaluation with an NGS panel. However, caution is advised in over-interpreting VOUS or single allele variants or pathologic variants that have not undergone functional genomics verification. Consultation for the interpretation of these types of variants with expertise at the genetics laboratories performing these tests is strongly recommended.

Prospective multicentre studies that collect and review the VOUS identified on multi-gene panels might lead to the identification of new diseases or elucidate the pathogenesis of certain causes of NC.

7. THERAPEUTIC OPTIONS

Treatment should be adapted to the patient's stage of disease. Early treatment includes specific medical or surgical interventions directed to a confirmed diagnosis as it is essential to improve the patient's outcome and survival. Supportive therapy is important to mitigate the consequences of cholestasis whatever the aetiology and must be initiated immediately; moreover, it is essential as a bridge to OLT. Liver transplantation is required for patients with end-stage liver disease. The management of portal hypertension is beyond the scope of this Introduction.

7.1 SUPPORTIVE TREATMENT

Cholestasis, regardless of aetiology, is responsible for several disturbances in the short or long term which require appropriate supportive treatment (Fig.18).

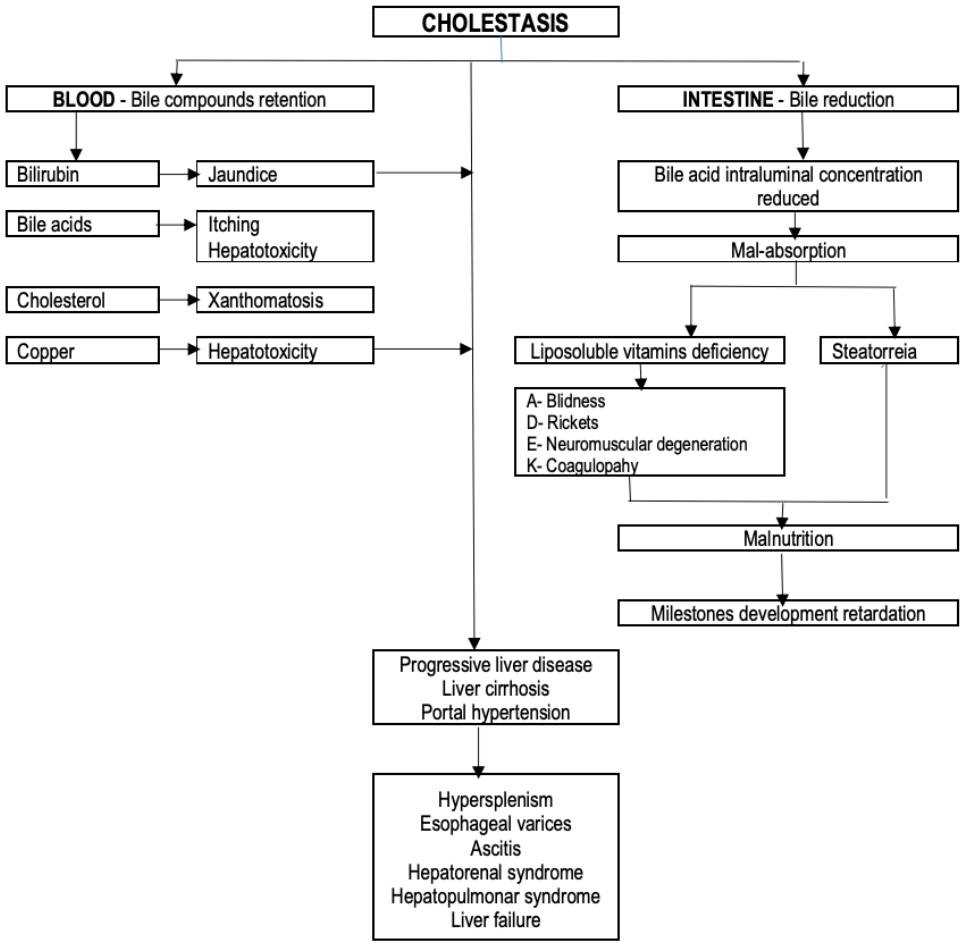

Figure 18 - Cholestasis disturbances in the short and long-term

Source: Adapted from de Carvalho *et al* (202), published by Sociedade Brasileira de Pediatria.

Bile flow reduction (or interruption), as already referred, has consequences – on one hand, the retention of bile compounds with leakage into the blood, and on the other hand, the decrease of bile in the intestinal lumen. The first emphasises the retention of bilirubin (jaundice), bile acids (itching and hepatotoxicity), cholesterol (xanthomatosis) and copper (hepatotoxicity). Regarding the second, there is a reduction in bile acids in the intestine and, consequently, malabsorption (steatorrhea, malnutrition and a deficit of fat-soluble vitamins). Regarding fat-soluble vitamins, the consequences include (vitamin) A – blindness, D – rickets, E – neuromuscular degeneration and K – coagulopathy (Fig. 18), due to deficiencies in the liposoluble vitamins A,D,E and K, respectively . Malnutrition and vitamin deficiency lead to psychomotor development delay.

Even patients with underlying entities without specific treatment can benefit from supportive therapy, which is also essential as a bridge to liver transplantation. In both cases the benefit is the greater the sooner treatment is instituted.

Nutrition. Cholestasis patients often have steatorrhea (a decrease in bile acids in the intestine leading to the malabsorption of lipids) and increased energy expenditure. Therefore, caloric intake should be approximately 125% of the recommended daily intake based on the ideal body weight (203). Medium chain triglycerides (MCT) are more easily absorbed than long chain fatty acids and are the best source of lipid calories. In fact, MCTs are relatively soluble in water, do not require the solubilisation of bile acid micelles and can be directly absorbed by the portal circulation.

Breastfeeding should be encouraged, and if infants are unable to suckle according to their needs, they may be fed by spoon. Sometimes, neonates and infants will require a nasogastric tube for nocturnal or continuous enteral feeding (203). Enteral feeding (even if only trophic feeding) should be initiated as soon as possible as it increases bile flow, gallbladder contraction and intestinal motility. Parenteral feeding should be kept only for the strictly required time (175).

Vitamins. The intestinal absorption of liposoluble vitamins (A, D, E and K) is reduced in patients with cholestasis due to bile acid scarcity in the intestinal lumen. To prevent deficiencies in these vitamins, supplements should be administered, ideally from water-soluble formulations that have been developed as orphan drugs. If this is not possible, the fat-soluble formulations should be administered in boosted doses. Monitoring can be done by measuring serum levels. The first deficit to evaluate is that of vitamin K, which appears within a few days, so supplementation should start immediately after the diagnosis of

cholestasis. Supplementation of other vitamins should continue for at least three months after jaundice resolves as there is a delay before normal bile flow is restored (203).

Itching control. UDCA has been associated with different beneficial effects in cholestasis, and, thus can be used as first-line therapy for pruritus and also in parenteral nutrition related-cholestasis, in BA post-Kasai procedure and in A1ATD. UDCA has several interesting mechanisms of action from which we highlight the following two: (a) replacement of the bile acid pool by other hydrophilic acids less hepatotoxic ones and (b) stimulation of the bile flow. The therapeutic dosage is 15 to 20 mg/kg/day in two to three doses/day. The only frequent side effect is diarrhoea, which usually responds to a dose reduction.

Rifampicin inhibits the absorption of bile acids by hepatocytes and induces microsomal liver enzymes. It is recommended for the treatment of refractory itching (10 mg/kg/day), but liver function should be monitored due to its potential for hepatotoxicity (see Table 4) (98).

New drugs are being studied for the control of chronic itching, a particularly worrying problem in patients with ALGS, and may have great impact on the quality of life of the patients (204).

7.2 SPECIFIC TREATMENT

7.2.1 Medical treatment

Special diets. The prognosis of some IEM also depends on early diagnosis and specific medical treatment, particularly those who present with signs of liver failure, and which may include the treatment of metabolic imbalances and the establishment of special diets (e.g. lactose-free diet for galactosemia, low-protein diet with specific amino acids composition for tyrosinemia).

Specific pharmacological intervention. Some infectious diseases have specific and effective treatments, such as bacterial sepsis to streptococcus agalactiae or gram-negative bacilli, E. coli urinary infection, congenital syphilis or CMV infection. Also, herpes virus types I and II infections are treatable although less effectively in preventing fatalities or sequelae.

For some metabolic diseases, specific drugs are also available, such as NTBC [2-(2-nitro-4- trifluorometilbenzoil) -1.3-ciclohexanediona] for tyrosinemia (205,206); however, treatment success depends on patient adherence to both NTBC and a diet low in tyrosine and phenylalanine, which can be difficult to achieve in a significant percentage of individuals

(207). Therefore, the overall survival of these patients additionally depends on the availability of OLT for those with progression to end-stage liver disease or at high risk of hepatocellular carcinoma.

GALD has specific and effective treatment directed against the mechanism of the disease (immunoglobulin), as mentioned in section 3.2.1.

Currently, three decades after the empirical introduction of UDCA into clinical practice, a new paradigm in the treatment of cholestatic liver diseases is underway (208). The progress in the understanding of the pathophysiological mechanisms of hepatocellular and cholangiocellular cholestasis has identified many potential targets and led to the development of a variety of novel therapeutic options which are under evaluation, some already in clinical trials. These new drugs are specific modifiers of hepatobiliary secretion and form cell protection mechanisms against bile acids-mediated cytotoxicity, aiming to minimise cholestasis and prevent progression to liver fibrosis (Table 4).

For example, Tang et al. demonstrated that nor-ursodesoxycholic acid (norUDCA) administration to PiZZ transgenic mice was associated with increased autophagy, reduced A1AT protein accumulation and reduced liver injury (121). The same authors unravelled a novel cellular mechanism by which nor-UDCA modulates autophagy (122). Nor-UDCA is currently being administrated to patients with primary sclerosing cholangitis in a phase 3 double-blind randomised clinical trial (NCT03872921) and has already proven to be safe and well tolerated.

Enzymatic replacement therapy (ERT). Patients with lysosomal storages diseases (LSD) with some residual enzyme activity were found to have less severe phenotypes than patients with null enzyme activity. ERT rationale was supported by this evidence (209). However, it is no cure since it does not fix the cause of the lack of enzyme activity.

ERT was first approved as a treatment for the LSD Gaucher disease in 1991 and is currently approved for eight LSDs by the FDA. ERT may help to improve clinical symptoms and slow down disease progression except for neurological conditions due to its inability to cross the blood-brain barrier. Intra-thecal administration throughout placed catheters was applied in animal models and is being considered. Other limitations are the high cost, the possibility of patient non-compliance in the long term since it requires a life-long commitment and the development of humoral immune responses which may lead to loss of efficacy over time.

The first LSD treated with macrophage-targeted ERT was Gaucher disease. Long-term results from large cohort studies demonstrated that ERT (imiglucerase) partially or completely ameliorated the clinical manifestations of Gaucher disease type 1 (210).

The other LSD which may manifest as NC is the early-onset of lipase acid lysosomal deficiency (EO-LAL-D), also known as Wolman disease, which accounts for approved ERT (sebelipase alpha, weekly, intravenously). This is an extremely rare condition (1/500 000 live births), and the results of two open label studies containing 19 patients were reported in 2021 (211). The findings of these studies demonstrated prolonged survival (79% at 12 months and 68% at five years of age, estimated by the Kaplan-Meier method), with normal psychomotor development; improved growth, hematologic and liver parameters; and an acceptable safety profile.

Therapy	**Proposed mechanisms of action**	**Paediatric disease with a favourable outcome reported in animal models (*or human subjects)**
UDCA	More favourable bile composition, increased expression of BA transporters, reduced apoptosis	*PSC at standard doses, *PFIC3
Nor-UDCA	Increased choleresis, cholehepatic shunting, and bicarbonate secretion	PSC and PFIC3 ($mdr2^{-/-}$ mouse), CFRLD, A1ATD
CA	Decrease synthesis of toxic bile acid intermediates	*BASD (approved for use in BASD 2015)
Rifampicin	FXR agonist, altered gut flora	
Bile acid sequestrants	Increased faecal BA secretion; increased hydrophilic BA, decreased inflammation and fibrosis; increased biliary proliferation	PSC and PFIC3 ($mdr2^{-/-}$ mouse)
Chemical chaperones	Improved trafficking of transport proteins to membrane surface	*PFIC1, *PFIC2, A1ATD, CFRLD
ASBT inhibitors	Increased faecal BA secretion	PSC and PFIC3 ($mdr2^{-/-}$ mouse, *PSC, *PBC, *ALGS
FXR and TGR5 agonists	Suppressed BA synthesis, increased BA secretion across canalicular membrane	PSC and PFIC3 (MDR2-/- mouse), *PBC
FGF19 analogues	Suppressed BA synthesis	*PSC
Anti-inflammatory/anti-fibrotic therapies, hepatocyte or stem-cell transplants	CCR2/CCR5 inhibition, multiple anti-inflammatory/anti-fibrotic pathways	Rat fibrosis model, PFIC3 ($mdr2^{-/-}$ mouse), BA (RRV/mouse model)

Table 2 – Current and potential therapies in paediatric cholestatic liver diseases.

Source: Published by Frontiers Media S.A.(98)

Legend: A1ATD – alpha-1-antitrypsin deficiency; ALGS – Alagille syndrome; ASBT – apical sodium-dependent bile acid transporter; BA – biliary atresia; BASD – bile acids synthesis disorders; CFRLD – cystic fibrosis-related liver disease; FGF – fibroblast growth factor; FXR – farnesoid X receptor; PBC – primary biliary cirrhosis; PFIC – progressive familial intrahepatic cholestasis; PSC – progressive sclerosing cholangitis; TGR5 – G-protein-coupled bile acid receptor; UDCA – ursodeoxycholic acid.

7.2.2 Surgical interventions

Kasai's PE. In 1968, the Japanese surgeon Morio Kasai first described a surgical procedure which aimed to treat BA (10). Kasai's procedure consists of the removal of the fibrosclerotic extrahepatic bile ducts (including the gallbladder) and the anastomosis of a small intestine loop to the hepatic hilum (after extensive removal of fibrous tissue) (Fig. 19). This procedure aims to restore bile flow.

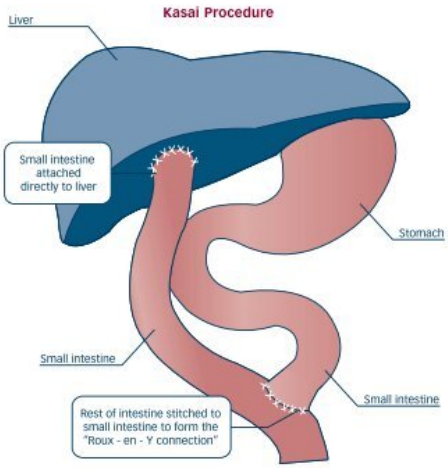

Figure 19 - Kasai's PE.

Source: https://gikids.org/wp-content/uploads/2019/12/Biliary-Atresia.pdf (accessed 17-05-2021)

If successful, the first clinical sign will be the normalisation of stool colour, followed by a decrease in or normalisation of the bilirubin levels. Short- and medium-term complications include bacterial cholangitis and the onset of fibrosis, portal hypertension and liver cirrhosis. If unsuccessful, progression to terminal liver disease occurs in the first two years of life, and patient survival depends on liver transplantation.

The timing of Kasai's PE in BA is decisive for better outcome and long-term survival. Several studies show that the earlier the surgical intervention is carried out, the greater the probability of success and the greater the probability of long-term survival with native liver (40-42, 44). In 2009, Serinet et al. (41) estimated that if every patient with BA underwent the Kasai surgery before 46 days of age, 5.7% of all liver transplantations performed annually in France in patients younger than 16 years could be spared. Recently, Fanna et al. (42), in a cohort of 1340 patients, demonstrated a 25-year survival with native liver of

37.9%, 27.4%, 22.2% and 18.6% in patients operated on in the first, second and third month of life or later, respectively; $p < 0.0001$).

Other surgical interventions on the biliary tree. Also, choledochal cysts must be treated by surgical intervention, a bile flow restoration surgery which in many cases will be Kasai's PE. Spontaneous perforation of the main bile duct or obstruction by stones may also require surgical intervention.

Liver transplantation. The main indications for liver transplantation in the context of NC are as follows: BA, metabolic diseases and acute liver failure (ALF). Worldwide, BA accounts for more than half of transplants performed in paediatric age (212-214), although there may be significant variability between different age subgroups (212). In the context of cholestatic disease, transplantation is indicated for patients with cirrhosis and portal hypertension, malnutrition and growth retardation that does not respond to nutritional support and intractable itching that does not respond to medical therapy or biliary bypass.

Contraindications for liver transplantation are severe sepsis (especially fungal), but infection located in the liver is not a contraindication; severe extrahepatic disease that is not reversible after transplantation; and liver tumours with extrahepatic metastasis.

Pre-transplant assessment and preparation should include optimisation of nutritional status, treatment of infections (e.g. tooth caries), immunisations and psychological preparation of the child and family. Nutritional status is a key element for post-transplant success. The recipient of a cadaver organ is chosen based on blood group compatibility, size matching, medical urgency and time on the waiting list. Unlike kidney transplantation, there is no benefit in HLA matching, and hyperacute rejection is rare.

OLT involves three procedures of donor hepatectomy, graft preparation and graft implantation in the donor. The graft may be a whole liver (rare in children, especially under 2 years old), a reduced liver (usually segments II and III of an adult implanted in children under 2 years of age) or a 'split-liver' (the right lobe implanted in an adult recipient and the left lobe in a child). The donor may be a brain-dead patient or a living related donor (mother or father) who undergoes a left lobe hepatectomy.

Post-transplant complications include, among others, in the short/medium term, vascular thrombosis of the hepatic vessels, biliary stenosis/leakage in the biliary tree, infection and graft rejection and, in the long term, side effects of immunosuppressive medication and malignancies. After successful liver transplant, children are usually able to catch-up growth, and they may attend school and practice sports without limitations.

Liver transplantation has radically changed the prognosis of children dying of end-stage liver failure. The European Liver Transplant and the American Liver Transplant Registries reported 1-year, 5-year and 10-year survival rates of over 90%, 85% and 60%, respectively (215, 216), while a recent study reported long-term (up to 20 years) survival rate of 79% (217). Children transplanted before one year of age may have worse outcome (218) although very recent reports challenge this traditional assumption (219).

Liver transplantation has major limitations; besides a still-significant short-term mortality and morbidity risk, it requires lifelong immunosuppression and is often complicated by severe long-term hepatic and extrahepatic problems (220).

7.3 ADVANCED TECHNIQUES

Cell therapy. Cell therapy aims to treat children and adults with liver failure or with metabolic liver diseases by transplanting cells that perform specific liver functions. This technique has been developing for the past two decades and, although considered experimental, has already shown potential not only as a bridge to but as an alternative to OLT. The success of this technique is mainly determined by the functionality of the employed cells (221).

Currently, the preferred cells are primary human hepatocytes (PHH) isolated from donors' livers. Alternatively, hepatocytes derived from the differentiation of several types of stem cells have been employed in experiments, with promising results. PHH freshly isolated from adult livers are the gold standard for metabolic functions, but they also have some disadvantages, such as scarce availability due to organ shortage, variability between donors, being unsuited for urgent application or repeated infusions and poor engraftment and rejection. In fact, livers made available for digestion are mostly organs rejected for OLT due to reduced quality [patients on extracorporeal membrane oxygenation (ECMO)], non-heart beating donors, long cold ischemia time) or remnant tissues from unused split livers or liver reductions; also, marginal donors (severe steatosis, older donors, ischemia time > 14hours) are a possibility, but obtained hepatocytes are often suboptimal. Organ shortage is the major limitation to the use of PHH for liver cell transplantation. Cryopreservation of PHH allows consideration of liver cell transplantation in fulminant or acute-on-chronic liver failure or acute decompensations of metabolic diseases, allowing multiple infusions over days or weeks. Nevertheless, cryopreservation affects the viability and metabolic functions, which significantly reduce the efficacy and often make them unsuitable for clinical use.

Stem cells may be used as an alternative. They are characterised by easier availability, high expansion potential and resistance to cryopreservation and thus are promising candidates for overcoming part of the limitations of PHH but present other challenges and limitations.

The best candidates for this procedure are patients with ALF since they need prompt replacement of liver function and may not have a suitable donor within a timely manner for liver transplantation. So, theoretically, they are good candidates; and, if their livers regenerate, they may avoid OTL. Patients with acute-on-chronic liver failure, which consists of acute decompensation of underlying cirrhosis, may benefit from liver cell therapy as a bridge to OLT. Also, some with IEM may be good candidates if the long-term efficacy of this procedure is to be achieved. Finally, liver cell therapy may also be indicated in neonates and infants with liver failure who are too young for OTL.

Cells are administrated by perfusion though the portal system, and the procedure protocol includes anticoagulation and immunosuppression.

Over the last 20 years, liver cell therapy has evolved into a safe procedure routinely performed in several centres around the world but is still hampered by significant unsolved problems that preclude its widespread use. Major concerns are the difficulties in obtaining a sufficient amount of cell engraftment within the target organ and unknown long-term safety with the use of stem cells due to hypothetical tumour transformation.

Gene therapy. Gene therapy consists of delivering a nucleic acid to specifically diseased cells to achieve a long-term therapeutic effect. Monogenic liver diseases are ideal candidates for this type of therapy. They represent approximately 50% of paediatric chronic liver diseases and 20% of paediatric liver transplants. Several of these diseases can manifest themselves as NC (e.g. A1ATD, ALGS, tyrosinemia, argininemia, cystic fibrosis, Wolman disease, PFIC1). So far, several techniques have been developed, namely gene therapy by adding or replacing the gene, by RNA interference (RNAi) or by editing the genome.

Gene replacement therapy consists of adding new genes to a patient's cells to replace others that are missing or malfunctioning and depends on vectors that can transport them into the cells. There are two types of vectors – viral (especially adeno-associated viral vectors) and non-viral (nanoparticles created by engineering). The viral vector technique has already shown safety and efficacy in animal models (222). Gene therapy via adeno-associated virus has shown promise in restoring phospholipid excretion and preventing liver injury in murine models of PFIC3 (223).

However, there are some immunological problems that need to be resolved (e.g. pre-existing antibodies and cellular responses directed to the AAV capsid). Fewer immunogenic vectors are available than needed that can be efficiently (re) administered at a large scale. In addition, in diseases where there is damage to the liver structure and hepatocyte function, permanent modification of the genome may be necessary to prevent the loss of therapeutic DNA. The alternatives are naked DNA techniques and non-viral vectors (e.g. nanoparticles produced by engineering, namely liposomes and polymers). Non-viral vectors are less immunogenic and less costly (224).

RNA interference is an endogenous cellular mechanism for controlling gene expression. It is mediated by small RNAs (siRNAs), which promote the disruption of the target mRNA with complementary sequences through the RNA-induced silencing complex (RISC). Synthetic siRNAs can be introduced into cells and used to silence specific genes (two examples in which this technique proved effective were hypercholesterolemia and A1ATD). The siRNA can be transported by lipid nanoparticles.

Genome editing is defined as the specific and permanent modification of the genome in a living organism. This is achieved by inserting, deleting or replacing DNA at a precise location in the genome. Then, the edited/corrected genome is transmitted to the daughter cells after proliferation, without loss of information or therapeutic efficacy. Various techniques have been developed to repair DNA, such as site-specific synthetic endonucleases (225). However, these techniques still require improvements in their overall efficiency, especially in post-mitotic cells such as those in the adult liver. Highly efficient and more specific endonucleases with transient activity and few off-target effects are needed.

Many gene-based treatments for inherited liver disease are in development and will become available in the next decade. Currently, several phase III clinical trials in humans are underway (e.g. NCT04370054, NCT03861273). At this time, within the scope of NC, PFIC-3 and PFIC-4 are the diseases that are best positioned to benefit from long-term gene therapy (223).

8. PREVENTION TRENDS

The possibility that NC may become a preventable condition arises when the existence of sporadic forms will be recognised, possibly triggered, or enhanced by exposure to several modifiable risk factors. Genetic forms may also be prevented by molecular genetics intervention in the prenatal diagnosis with selective abortions in subsequent pregnancies.

Although as of yet no global preventive strategies have been implemented, some interesting contributions are coming from the management of some underlying entities, such as the following:

– NH is a very serious phenotype of NC and is the 'poster child' for the prevention goal. Most cases are caused by GALD, which has a 90% recurrence rate in future pregnancies. GALD's recognition and understanding of its pathogenesis allowed not only the effective treatment of patients but also the prevention of disease recurrence in younger siblings by administering intravenous immunoglobulin to the mother during pregnancy (24). This entity represents the paradigm of a serious and fatal disease whose successful outcome and patient survival are due to the unravelling of the mechanisms of its pathogenesis. It is an example to be followed for other sporadic forms of NC with phenotypes of severe disease and in utero onsets, such as BA. It is also a model of maternal-foetal disease, specifically with its intrauterine onset and clinical manifestation and progression in the immediate postnatal period.

– Early diagnosis and treatment of perinatal infections, such as bacterial sepsis, syphilis or CMV infection, for which there is specific and effective antibiotic, antiparasitic or antiviral treatments, may also be able to prevent some sporadic forms of NC with its progression to damage of the biliary tree or more severe phenotypes of liver disease such as liver failure, chronic hepatitis or cirrhosis.

– The maintenance of a trophic diet, whenever possible, and the administration of cycled TPN have been able to reduce the incidence rate of cholestasis associated with TPN. The lipid composition of TPN has also been adjusted to reduce the risk of lipoperoxidation although the effectiveness of this intervention is still not agreed upon. Furthermore, transient cholestasis associated with modifiable risk factors can be prevented if these are reduced or mitigated.

– Finally, the existence of some drugs that can intervene in the mechanisms of the disease and adaptive reactions are dawning and may soon make a difference if administered early, preventing the development of some severe phenotypes of NC.

CHAPTER II

STUDY RATIONALE AND AIMS

HYPOTHESIS

The early recognition of NC is essential to ensure timely treatment and optimal prognosis. Currently, there are few studies evaluating skills and clinical practices of healthcare professionals to recognize NC, and, in Portugal, none.

Hypothesis 1: The current clinical practices of healthcare professionals (doctors and nurses) delay the recognition of NC.

Knowledge of the epidemiology of NC is essential to address the needs for clinical care and research in this area. The identification of the underlying causes or entities determines a targeted treatment and impacts the patient's outcome and prognosis. Currently, few well-characterized cohorts allow assessing the impact of the last advances in etiological diagnosis and treatment, and, in Portugal, there is none.

Hypothesis 2: NC epidemiology, etiological diagnosis, outcome and survival have changed in the last decades: a) The spectrum of underlying entities, mortality and survival have changed significantly; b)New therapies are changing the outcome of some specific underlying entities.

A proper diagnostic methodology helps to identify the underlying entities more promptly and in a less costly and invasive manner. Current diagnostic algorithms have been built based on experts' opinions and do not comply with potential epidemiological changes or the availability of new molecular genetic techniques. Concomitantly, predictive prognostic tools on admission can help establish priorities for patient care. However, so far, a few predictive models have been developed but only for biliary atresia.

Hypothesis 3: The current tools for diagnostic approach and prognostic prediction need to be updated and may be improved based on available clinical evidence.

Despite all the advances made in recent decades, the overall morbidity and mortality rates of NC remain high. New health gains for these patients will only be achieved through research on the underlying pathophysiological mechanisms and the respective translation of knowledge for diagnosis and treatment.

Hypothesis 4: Considering that the mechanisms underlying neonatal 'physiological cholestasis' are still poorly understood, we hypothesise that in healthy infants: a) Bile acid profile, oxidative stress and inflammation may be mutually associated; b) Type of diet may play a role in this setting.

OBJECTIVES

GENERAL OBJECTIVE

To contribute to a better clinical practice with improvement in the diagnosis and prognosis of patients with NC.

SPECIFIC OBJECTIVES

Objective 1

To evaluate the clinical practices and educational needs of Portuguese healthcare professionals (doctors and nurses) concerning NC, at several levels of the National Healthcare System (NHS).

Objective 2

To perform a detailed clinical characterization of a group of patients with NC from a Portuguese tertiary center with representative experience (personal experience) including:

- A retrospective analysis of a cohort over a period of 30 years.
- Descriptions of rare underlying entities and related innovative therapeutic interventions.

Objective 3

Contribute to the development of new diagnostic and prognostic tools, including an evidence-based diagnostic algorithm (personal and peer' s experience).

Objective 4

To evaluate some specific features concerning the liver physiology in early post-natal life, namely:

- A potential association of bile acid profile, oxidative stress and inflammation biomarkers.
- The potential influence of diet in this setting.

CHAPTER III

MATERIAL AND METHODS

This chapter presents an overall description of the material and methods used within this thesis. Detailed methodologies are described in the corresponding published articles.

STUDY DESIGNS AND SUBJECTS

Objective 1

Study 1.1 – Cross-sectional study including the application of a locally validated questionnaire administered to a sample of 266 participants (166 nurses and 100 physicians) recruited from the three levels of health institutions. The questionnaire had the following two sections: eight multiple-choice questions, different for doctors and nurses, and a panel with eight photographs of stools to classify as normal or suspicious (see appendices).

Study 1.2 – Proposal for the use of the stool colour card (SCC), adapted from the original SCC of Taiwan, as a screening (and teaching) tool for biliary atresia, to be integrated into the organisation of the Portuguese healthcare system at the central level and nationwide.

Objective 2

Study 2.1 – Retrospective cohort study of 154 NC patients diagnosed and treated at the hepatology outpatient clinic of a single tertiary hospital from 1 January 1985 to 31 October 2019.

Study 2.2 – Clinical case report concerning a new therapy for a rare entity underlying NC in two patients identified in two tertiary centres (Centro Hospitalar Universitário do Porto and Children's Memorial Health Institute, Warsaw, Poland).

Objective 3

Study 3.1 – Development of diagnostic and prognostic models, and creation of a diagnostic algorithm, based on data from a retrospective study enrolling 154 patients with NC diagnosed and treated at a single tertiary centre from 1 January 1 1985 to 31 October 2019 and from the published evidence.

Study 3.2 – Retrospective study of 16 patients with inborn errors of metabolism presenting as NC, selected from two independent large cohorts (n = 126; n = 582) during a 30-years period (1987– 2017) at a single tertiary hospital.

Objective 4

Study 4.1 – Cross-sectional, exploratory study conducted over a period of two years, in a Portuguese maternal and children's tertiary hospital, enrolling 32 two-month- old full-term healthy infants born by vaginal delivery and prospectively recruited.

INSTITUTIONAL AND ETHICAL APPROVAL

The studies were reviewed and authorised by the Institutional Review Board of CHUPorto.

Additionally, the study 1.1 was also reviewed and authorised by the Board Direction of Centro Hospitalar da Póvoa de Varzim/Vila do Conde, the Board Direction of ACeS Grande Porto IV - Póvoa de Varzim/Vila do Conde and the Scientific Board of ACeS Grande Porto VI - Porto Oriental.

Study 1.2 required the permission to use the original SCC and its validated images from both, the author (Professor Mei-Hwei Chang) and the Taiwan Ministry of Health and Health Promotion Administration. Written permission was required and obtained.

The principles of the Declaration of Helsinki and the internal rules of the CHUPorto were observed. The confidentiality of participants and data collection was guaranteed in all studies. All databases were constructed and analysed anonymously.

DATA COLLECTION

Study 1.1– Data were collected from the questionnaires completed by the participants.

Studies 2.1, 2.2, 3.1 and 3.2 – These were based on the analysis of clinical charts and institutional database records.

Study 4.1 – Before blood sample collection, infants had an appointment with a paediatrician, a complete clinical examination was performed and clinical data were recorded.

LABORATORY ASSESSMENTS (STUDY 4.1)

Whole-blood samples (in EDTA) were collected by peripheral vein puncture, before a meal (2–4 hours after the previous meal). Samples were sent to CoreLab, CHUPorto, and to the Faculty of Pharmacy, University of Porto.

The samples sent to CoreLab were processed immediately to evaluate the following parameters: liver function tests [total and conjugated bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), alkaline phosphatase, albumin, uric acid] and serum alpha-1-antitrypsin (A1AT).

The samples sent to the Faculty of Pharmacy, University of Porto, were processed within two 2 hours of collection to obtain plasma, washed RBCs and erythrocyte membrane suspensions by sequential centrifugation and hypotonic lysis.

Samples were stored at -80 ºC until assayed. Afterward, the following parameters were evaluated: inflammatory parameters [tumour necrosis factor (TNF)-alpha, C-reactive protein] and oxidative stress parameters [(membrane bound haemoglobin (MBH) and lipoperoxidation (LPO) in the membranes) and catalase activity, total antioxidant status (TAS), glutathione peroxidase (GPX) activity, glutathione reduced (GSH), glutathione oxidised (GSSG), GSH/GSSG ratio in erythrocytes].

Some stored samples were sent to the Faculty of Pharmacy, University of Lisbon, and the following parameters were evaluated: total and conjugated bile acid profiles (plasma).

Detailed descriptive analytical methods are described in the original article on study 4.1.

STATISTICAL ANALYSIS

Categorical data were expressed as numbers (and percentages), and continuous data were described using mean ± standard deviation (SD) or median and interquartile range (IQR).

Categorical data were compared using Pearson's qui^2 test or Fisher`s exact test. Continuous variables were compared with Student's t-test or an analysis of variance (ANOVA) test if their distribution was parametric, and with the Mann-Whitney U test or Kruskal-Wallis test if their distribution was non-parametric.

Survival curves were constructed using the Kaplan-Meier method, with comparison between groups done by log-rank test.

A two-sided P-value of <0.05 was considered statistically significant.

Statistical calculations were performed using SPSS version 26.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 9.1.0 (216) (GraphPad Software, La Jolla, CA).

The distinct statistical analyses used in some studies will be described in the respective Results chapter.

CHAPTER IV

RESULTS

This chapter presents the results of the studies performed within the scope of the thesis and which were subsequently published (6 papers) or submitted for publication (1 paper).

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1.1 CLINICAL PRACTICES AMONG HEALTHCARE PROFESSIONALS CONCERNING NEONATAL JAUNDICE AND PALE STOOLS.

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ORIGINAL ARTICLE

Clinical practices among healthcare professionals concerning neonatal jaundice and pale stools

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Abstract Jaundice and pale stools are major indicators of neonatal liver disease. Prognosis depends on timely diagnosis and management. We evaluated the clinical practices among healthcare professionals concerning jaundiced newborns and their ability to recognize pale stools. We supplied a questionnaire and a panel with eight photographs of stools, both locally validated, to physicians and nurses of the National Healthcare Service. Analysis was conducted according to professional status, specialization and years of experience of professionals and level of healthcare. Questionnaires were administered to 266 participants (100 physicians, 166 nurses). The decision to send patients to medical observation depended on the intensity of jaundice for a significant percentage of nurses. Concerning jaundiced newborns breastfed and otherwise healthy, 28.9% of physicians would never request a conjugated bilirubin assay, and only 43.3% would request it after 14 days old; for those with other signs/symptoms of disease, only 69.1% of physicians would request it immediately. Multiple linear regression

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analysis identified specialization as an independent variable significantly associated with the ability to recognize pale stools.

Conclusion: A significant percentage of healthcare professionals assumed clinical practices that preclude the timely recognition of cholestasis/pale stools, reinforcing the idea of educational needs. Specialization, rather than years of experience of professionals, was associated with better skills and practices.

What is Known:

- *Neonatal cholestasis is a condition with some rare underlying entities having high mortality and morbidity. Early diagnosis is crucial to improve prognosis. Yet, many cases remain late recognized and referred.*
- *Studies evaluating the ability of healthcare professionals to recognize neonatal cholestasis are scarce.*

What is New:

- *In this study, a significant percentage of professionals assumed clinical practices that preclude timely recognition of neonatal cholestasis and pale stools, reinforcing the idea of educational needs.*
- *Specialization of professionals was associated with better skills and practices.*

Keywords Newborn jaundice · Neonatal cholestasis · Pale stools · Dark urine · Biliary atresia

Abbreviations

BA	Biliary atresia
cBt	Conjugated bilirubin
GP	General practice
IQ	Interquartile
NHS	National Health Service
PHCUs	Primary Health Care Units
UK	United Kingdom
USA	United States of America

Introduction

Neonatal cholestasis is always pathological and presents with persistent jaundice as part of a clinical constellation that may include dark urine and/or pale stools, as well as other symptoms based on the underlying entity. The differential diagnosis is broad and can be categorized into obstructive and intrinsic processes, most of which are individually rare (Table 1) [23]. Biliary atresia (BA) remains one of the most common causes, while many intrahepatic familial causes have also been associated. The sub-group of "transient neonatal cholestasis" has become common, especially among preterm infants (multifactorial transient cholestasis), while the number of cases of "idiopathic neonatal hepatitis" is decreasing. Prognosis in some of these entities depends on early recognition (e.g. BA, some inborn errors of metabolism, endocrine and infectious diseases) since they can benefit from specific, successful treatment [10, 11]. But even when specific treatment is neither available nor curative, early recognition is always important because medical management and the optimization of nutrition are beneficial while waiting for liver transplantation.

However, in clinical practice, there are several obstacles to the early diagnosis of neonatal cholestasis (conjugated hyperbilirubinemia). Jaundice, the main clinical manifestation, is extremely common, affecting up to 60% of term newborns [28]. It is mostly related to benign and self-limited conditions, such as physiologic and breastfeeding jaundice (unconjugated hyperbilirubinemia) [9]. The latter can be responsible for prolonged jaundice, affecting up to 15% of newborns [12] and extending beyond 14 days (sometimes up to 3 months) [35]. Perhaps for this reason, healthcare professionals tend to underestimate the symptoms, delaying diagnosis, especially in cases in which jaundice is not accompanied by other signs or symptoms of disease. On the other hand, many healthcare professionals (physicians and nurses), especially at the primary healthcare units (PHCUs), tend to be unfamiliar with the other cardinal signs of cholestasis (dark urine and pale stools) [2, 27].

According to national and international recommendations [1, 3, 6, 19, 25, 29], any newborn who remains jaundiced beyond 14 days of life requires a medical evaluation. Those who present with other signs or symptoms of disease, including dark urine and/or pale stools, should be immediately referred to a specialized centre and subjected to further investigation [3, 6, 10, 11, 25, 29].

The aims of our study were (1) to evaluate clinical practices among healthcare professionals concerning jaundiced newborns and (2) to evaluate their ability to recognize pale stools.

Methods

This was a cross-sectional study conducted between January and April 2014 among physicians (general practice (GP) physicians, general paediatricians and neonatologists) and nurses (with and without paediatrics specialization), from primary healthcare systems and *A*- and *B*-level hospitals from NHS (*A*-level = tertiary level of care, university hospital; *B*-level = secondary level of care, regional hospital), in the north region of Portugal.

The study instrument was a questionnaire consisting of two main sections. The first section included eight multiple-choice questions, differing for physicians and nurses (Appendices 1 and 2), to evaluate clinical practices concerning jaundiced newborns. The second section was a panel with eight stool photographs to classify as "normal" or "suspicious" (Fig. 1); four were normal and four suspicious (two totally pale, one uniformly and one unevenly discoloured). Responses were registered according to the number of photographs correctly identified (minimum 0, maximum 8). Stool photographs were obtained from jaundiced newborns using a digital camera and computer colour calibration for ambient light.

Table 1 Neonatal cholestasis underlying entities

Extrahepatic causes	
	Biliary atresia
	Choledocal cyst
	Bile duct stricture
	Neonatal sclerosing cholangitis
	Congenital perforation of the common bile duct
	Inspissated bile syndrome (in severe ABO incompatibility)
	Choledocolithiasis
	Mass (intraductular: rhabdomyosarcoma; extraductular: hepatoblastoma, neuroblastoma)
Intrahepatic (familial) causes	
Disorders of bile acid biosynthesis	Primary: oxysterol 7α- hydroxylase, Δ4–3-oxosteroid-5β- reductase deficiency, 3β-hydroxy-Δ5- C27- steroid dehydrogenase deficiency
	Secondary: Zellweger spectrum disease, and Smith-Lemli-Opitz syndrome
Disorders of membrane transport	Disorders of "canalicular" secretion
	Ion transport (CFTR deficiency, cystic fibrosis)
	Bile acid transport (BSEP deficiency, PFIC2, BRIC2)
	Phospholipid transport (MDR3 deficiency, PFIC3)
	Complex/multiorgan disorders
	Defective aminophospholipid flippase (FIC1 deficiency, PFIC1, BRIC1)
	Lymphedema cholestasis (Aagenaes syndrome)
	Defective tight junction (Claudin1, NISCH syndrome)
	Defective conjugation of bile acids (TJP2 and BAAT, familial hypercholanaemia)
Disorders of embryogenesis	Impaired Jagged-Notch signalling (Alagille syndrome)
	Congenital fibrosis or infantile polycystic disease, Caroli disease
	Hepatic-pancreatico-renal syndrome (Ivemark, Jeune, Bardet-Biedl syndrome)
	Repression of HNF-1 beta activity (neonatal cholestasis and type 5 MODY)
Metabolic disorders	Amino acid metabolism: tyrosinemia
	Carbohydrate metabolism: galactosemia, fructosemia, glycogen storage disease IV
	Lipid metabolism: lipase acid lysosomal deficiency (Wolman disease), Niemman-Pick Type C disease, cerebrotendinous xanthomatosis, Gaucher disease
	Urea cycle disorders: argininemia, citrulinemia type II (citrin deficiency)
	Mitochondrial disorders
	Congenital disorders of glycosylation (CDG)
Endocrinopathies	Pan-hypopituitarism (septo-optic dysplasia)
	Hypothyroidism
Unclassified	Alpha-1-antitripsin deficiency
	MacCune-Albright syndrome
	North American Indian childhood cirrhosis (CIRH1A-mutation)
	Neonatal iron storage disease
	Hemophagocytic lymphohistiocytosis (HLH)
Sporadic neonatal cholestasis	
Cholestasis associated with infection	Bacterial
	Sepsis with endotoxemia (urinary tract infection, gastroenteritis), Syphilis, listeriosis, tuberculosis
	Parasites
	Toxoplasmosis, malaria
	Viral
	Common: CMV, HIV
	Rare: hepatitis B or C
	Associated with neonatal liver failure: HSV 1–2, HHV 6, paramyxovirus
Cholestasis associated with toxins	Parenteral nutrition associated cholestasis
	Drugs: faetal alcohol syndrome, maternal amphetamine abuse
Perinatal hypoxia/hypoperfusion	Cardiac insufficiency (severe)
	Vascular disorders (Budd-Chiari syndrome, multiple haemangiomata)

Table 1 (continued)

Miscellaneous	Haematological disorders: Langerhans cell histiocytosis, Inspissated bile Neoplastic: neonatal leukaemia Autoimmune: neonatal lupus, autoimmune haemolytic anaemia with giant cell hepatitis Intestinal obstruction
Idiopathic "neonatal hepatitis"	
Transient neonatal cholestasis	
Multifactorial	Mainly in preterm newborns/infants
Disorders of metabolic transport	FIC1 polymorphisms, MDR3 polymorphisms
Idiopathic	

Both questionnaires were previously validated by five experts (two paediatric gastroenterologists, three neonatologists) and pretested in 20 subjects (10 paediatricians and 10 nurses specialized in paediatrics, of an *A*-level hospital). Participation was voluntary and anonymous. The application occurred after the weekly staff meeting (physicians) or at a daily turn-switch meeting (nurses). It took less than 10 min to complete. After the questionnaires were collected, the first author (of this paper) provided a lecture in the interested units. The study was approved by Executive Councils and by the local ethics committees of all participating healthcare institutions.

Fig. 1 Panel with the eight stool photographs shown to participants

Statistical analysis All variables were reported as medians (interquartile range) or proportions. The normality of data was tested using the Kolmogorov-Smirnov test. Differences between groups were analysed using Student's *t* test or the Mann-Whitney test, according to the results obtained in the Kolmogorov-Smirnov test. Multiple comparisons between groups were performed by one-way ANOVA supplemented with Tukey's HSD post hoc test. The association between categorical variables was analysed using the chi-squared test or Fisher's exact test. Multiple linear regression analysis, using the forward stepwise introduction method, was used to determine independent factors affecting the number of right identified stool pictures. For both models, physicians and nurses, we include three variables: specialization and years of experience of professionals, and the institution level of healthcare. Data were analysed using the program SPSS 23.0 for Windows (SPSS, Inc., Chicago, IL). $p < 0.05$ was accepted as statistically significant.

Results

A total of 266 participants responded to the survey (physicians $n = 100$ (37.6%), nurses $n = 166$ (62.4%)), with a response rate higher than 80% in all groups. Sample characterization is depicted in Table 2.

Sending patients for medical observation

The question, asked only to nurses, was "*When do you send a jaundiced newborn for medical observation?*" The answers are represented in Table 3.

Regarding newborns aged less than 7 days, 34.6% of the nurses said it would "*depend on jaundice degree of intensity*". Only 37.7% said they would send patients "*immediately*" for medical observation. There were no significant differences

Table 2 Sample characterization

	Physicians		Nurses	
Participation rate	Primary care	80.3% (49/61)	Primary care	86.4% (38/44)
	B-level hospital	100% (14/14)	*B*-level hospital	100% (47/47)
	A-level hospital	100% (25/25)	*A*-level hospital	82.7% (62/75)
Workplace	Primary care	61.0% (61/100)	Primary care	26.5% (44/166)
	B-level hospital	14.0% (14/100)	*B*-level hospital	28.3% (47/166)
	A-level hospital	25% (25/100)	*A*-level hospital	45.2% (75/166)
Years of professional experience	<5 years	20.2% (20/99)	<5 years	8.4% (14/166)
	5–10 years	17.2% (17/99)	5–10 years	23.5% (39/166)
	11–20 years	10.1%(10/99)	11–20 years	42.8% (71/166)
	>20 years	52.5% (52/99)	>20 years	25.3% (42/166)
Specialization	General practice	61.0% (61/100)	Paediatrics	26.5% (44/166)
	Paediatrics	22.0% (22/100)	Other specialization	73.5% (122/166)
	Neonatology	17.0% (17/100)		

Table 3 Results

When should nurses send patients for medical observation?					
Newborn <7 days			Newborn >7 days (infant breastfed, apparently in good health)		
Immediately		37.7%	Immediately		26.7%
Depends on the jaundice intensity		34.6%	Depends on the jaundice intensity		58.0%
			(If jaundice persists >14 days)		(81.3%)
			(If jaundice persists >28 days)		(18.7%)
Depends on the presence of other signs and symptoms		4.3%	Jaundice persists >14 days		11.3%
Depends on the jaundice intensity and the presence of other signs and symptoms		23.4%	Jaundice persists >28 days		4.0%
When should physicians request a conjugated bilirubin assay?					
Newborn *WITH* other signs and symptoms of disease			Newborn breastfed and *NO* other signs and symptoms of disease		
At any age, as it is probably a pathological situation		69.1%	I do not request one, as jaundice is probably related to breast-feeding		28.9%
Jaundice persists >14 days		24.7%	Jaundice persists >14 days		43.3%
Jaundice persists >28 days		6.2%	Jaundice persists >28 days		23.7%
Jaundice persists >2 months		0%	Jaundice persists >2 months		4.1%
Panel of stools photographs (total number of right responses)					
Physicians	*p = 0.258*	Median (IQ)	Nurses		Median (IQ)
		7 (6–8)			7 (6–8)
Healthcare level	*p = 0.005*		Healthcare level	*p = 0.175*	
PHCUs		7 (6–8)	PHCUs		7 (6–8)
B-level hospital		7 (5–7)	*B*-level hospital		7 (6–7)
A-level hospital		8 (7–8)	*A*-level hospital		7 (6–8)
Specialization	*p = 0.062*		Specialization in paediatrics	*p = 0.012*	
General practice		7 (6–8)			
Paediatrics		7 (6–8)	Yes		7 (7–8)
Neonatology		8 (7–8)	No		7 (6–8)
Years of professional experience			Years of professional experience		
	p = 0.425	7 (7–8)		*p = 0.573*	
<5 years		7 (6–7)	<5 years		7 (4–7)
5–10 years		8 (6–8)	5–10 years		7 (6–8)
11–20 years		7 (6–8)	11–20 years		7 (6–8)
>20 years			>20 years		7 (6–8)

when comparing PHCU nurses with hospital nurses (*B*-level + *A*-level hospital) ($p = 0.653$).

Regarding newborns aged more than 7 days, breastfed and apparently in good health, 58% of nurses said it would "*depend on jaundice degree of intensity*". The proportion of hospital nurses who chose this response was significantly higher to PHCU nurses (64.2 vs 41.5%; $p < 0.05$). On the other hand, the proportion of PHCU nurses who said "*beyond 14 days*" was significantly higher than of hospital nurses (24.4 vs 6.4%; $p < 0.05$).

Request for conjugated bilirubin

The question, asked only to physicians, was "*When do you request a conjugated bilirubin assay in a jaundiced newborn?*" The answers are represented in Table 3.

Regarding newborns with other signs/symptoms of disease, only 69.8% of the physicians said they would request immediately a conjugated bilirubin assay (cBt). There were no significant differences according to specialization, years of experience of professionals or the level of the healthcare system. Further, there was no significant difference when comparing PHCUs + the *B*-level hospital with the *A*-level hospital.

Regarding the breastfed newborns in apparently good health, most GP physicians (34.5%) stated that they "*would not request a conjugated bilirubin assay because jaundice is probably related to breast-feeding*". However, there were no significant differences when accounting for specialization, years of experience of professionals or healthcare level. Concerning the same issue, there were neither significant differences when comparing GP physicians with hospital specialists (paediatricians + neonatologists) nor when comparing PHCUs with hospital levels (*B*-level + *A*-level hospitals).

Observation of stools and urine colour

Eighty-one percent of all professionals reported that in their current practice, they usually observe stool colour and 78% observe urine colour. All paediatricians and neonatologists reported that they routinely observe stool colour; 90.9% of paediatricians and all neonatologists routinely observe urine colour. However, only 68 and 63% of GP physicians observe stool and urine colours, respectively.

Regarding nurses, 97.7% of the specialists versus 75.2% of non-specialists in paediatrics observe stool colour, and 100% of the specialists versus 71.9% of non-specialists observe the urine colour.

Panel of stool photographs

There were no significant differences in the number of correct answers to the panel of stool photographs when comparing physicians and nurses (7 (6–8) vs 7 (6–8) correct responses; $p = 0.258$). The answers are represented in Table 3.

When analysing physicians and nurses separately, we found that there was a significant difference in the physicians' number of correct answers to the panel of stool photographs when comparing the level of healthcare ($p = 0.005$). On the other hand, when the comparison involved the physicians' specialization or their years of professional experience, there were no significant differences. Yet, multiple linear regression analysis identified the specialization of physicians as an independent variable significantly associated with the capacity to recognize pale stools, with neonatologists having superior performance (b = 0.200, $p = 0.048$) (Fig. 2a).

Concerning nurses, there was a significant difference in their number of correct answers to the panel of stool photographs when comparing their specialization ($p = 0.012$). No significant difference was found when comparing their years of professional experience or level of healthcare. Multiple linear regression analysis confirmed the specialization of nurses as an independent variable significantly associated with the capacity to recognize pale stools, with nurses specialized in paediatrics having superior performance (b = − 0.168, $p = 0.031$) (Fig. 2b).

There were no significant differences in the number of correct answers to the panel of stool photographs when comparing professionals who declared to have, or not to have, the practice of observing stool colour (physicians 7 (6–8) vs 7 (7–8), correct responses, $p = 0.790$; nurses 7 (6–8) vs 7 (6–7) correct responses, $p = 0.510$) or observing urine colour (physicians 7 (6–8) vs 7 (7–8) correct responses, $p = 0.860$; nurses 7 (6–8) vs 7 (6–7) correct responses, $p = 0.398$).

Discussion

Neonatal cholestasis is a condition with some rare underlying entities having high mortality and morbidity, for which early diagnosis is crucial to improve prognosis. The management of these patients at reference centres shows many cases for which recognition and referral should have been done earlier [14, 18]. Despite this, few studies have been done evaluating the ability of healthcare professionals to recognize neonatal cholestasis in jaundiced newborns [2, 27]. Our study is the first of this kind performed in our country.

One prior study [27] involved general paediatricians affiliated with a children's hospital and in the other [2] physicians and nurses from three university hospitals. Our study, although of regional scope, included physicians and nurses, with several degrees of specialization and practice time, of the three levels of care of our NHS.

Palermo et al.'s study [27] evaluated the primary care management of early and prolonged jaundice through a questionnaire in which approximately 70% of paediatricians reported being unfamiliar with the guidelines. Few paediatricians said to order confirmatory blood tests when the transcutaneous bilirubin is low. Most of them would order a fractionated bilirubin

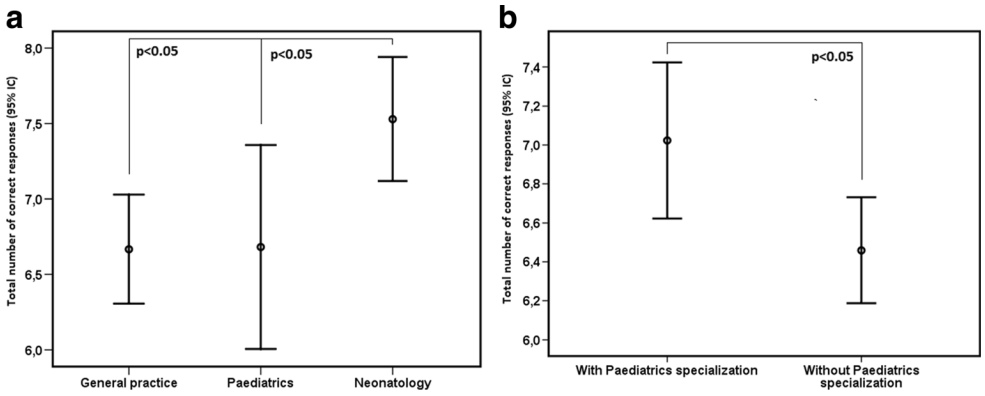

Fig. 2 Differences in the detection of pale stools by specialization. **a** Physicians. **b** Nurses

to neonates with jaundice persisting at 4 weeks of age, but one in four do not routinely see the infants between 1 and 8 weeks of age. In our study, a significant percentage of nurses (especially hospital nurses) request medical observation depending on the intensity of jaundice. This matter is relevant because jaundice intensity is neither revealing of cholestasis nor indicative of underlying disease or its severity [4]. In addition, almost 30% of physicians do not immediately request a cBt for a newborn with other signs and/or symptoms of disease. In the otherwise well, breastfed, jaundiced newborns/infants, 28.9% physicians do not request a cBt, regardless of the infant's age, and 4.2% only request it after they're 2 months old. Additionally, one third of GP physicians do not observe the stool and urine colours. These practices preclude the timely intervention and discrimination between benign unconjugated hyperbilirubinemia and cholestasis. According to national and international recommendations [1, 3, 6, 19, 25, 29], any jaundiced newborn/infant with other signs and/or symptoms, or aged more than 14 days, should be immediately observed and have a cBt determination.

In their study, Bakshi et al. [2] report that more than one third of participants did not identify suspicious pale stools, and the results seemed to be independent of the institution and professional background of healthcare professionals. In our study, physicians and nurses revealed a similar capacity to recognize pale stools. The independent variable associated with this skill was the specialization, both in physicians and nurses. The more specialized they were, the better they performed. The years of professional experience did not have an influence. Physicians from the *A*-level hospital revealed a greater capacity than did those of the *B*-level hospital and PHCUs.

Palermo et al. [27] proposed changes in the system of care [13] including mandatory routine care schedules at 3–4 weeks of age. For Bakshi et al. [2], if healthcare professionals are not able to recognize pale stools, even with a visit at 4 weeks old instead of the Well Baby Review at 6 weeks, early recognition rates may not improve. In our country, the Child and Youth Healthcare Surveillance Plan, with visits at 2, 4 and 8 weeks, has a high rate of success [22], yet our study led us to agree with these authors: it will be difficult to achieve better results without specific training programs.

Newborns/infants with BA may be at significant risk of not being diagnosed in a timely manner. They are otherwise well, jaundice is of a low intensity and pale stools may not be identified. The timely diagnosis of BA is particularly important since successful surgery able to re-establish the bile flux (porto-enterostomy of Kasai [17]), as well as long-term survival with native liver [31], both depend on early performance of the first one. The success of Kasai porto-enterostomy is significantly higher (~55 to 74%) when performed before 60 days old [7, 8, 20, 21] and is much lower (39.5%) when the median age at surgery is beyond this age [34]. Yet, some studies have shown that other factors, such as the team's surgical skills, may have a significant influence on the success rates [26, 30]. On the other hand, survival with a native liver decreases when age at a Kasai operation increases. A comparison of the 15-year survival rate with a native liver between patients with BA having undergone a Kasai operation before and after 45 days of life showed a 12.1% difference, with a better outcome in the first group [31].

In Taiwan, a stool colour card proved to be effective for screening pale stools with 95.2% sensitivity [5]. The incidence of BA makes this screening cost effective in Asian countries [15, 32]. In the UK, the Children's Liver Disease Foundation produced a similar stool colour card, and Bakshi et al. [2] proposed its mandatory use. They argue that checking a baby's stool against the chart does not require substantial training and can be done very quickly. In European countries, where BA is rare, this screening was not yet recognized as cost effective despite the attempts of several authors [16, 24, 33].

However, not all patients with cholestasis have pale stools, and among them, patients with other serious underlying diseases (metabolic or infectious), and whose prognosis may depend on a promptness medical intervention, may be at risk of not being diagnosed in a timely manner. This reinforces the need for having trained professionals for early diagnosis and/or promoting universal screening through other instruments.

The universal screening of cholestasis is justified by the incidence of 1:2500 live births, the several underlying entities with effective treatment and the prognosis depending on the earliness on which it is started [10, 23]. However, due to the

various underlying diseases, it is difficult to find the most suitable method and timing for screening.

The results of the present study point to the existence of clinical practices in our setting that significantly prevent the early recognition of neonatal cholestasis, despite an adequately programmed and accomplished schedule of visits; the independent variable associated with the skill of recognizing pale stools was the specialization. Ideally, the Child Health Surveillance Plan should be in charge of paediatricians and nurses with a specialization in paediatrics. From a more cost-effective perspective, GP physicians and non-specialized nurses should have proper training for early recognition of neonatal cholestasis and pale stools, but general paediatricians and hospital nurses also need to reinforce their skills in this area; the investigation of the underlying cause of neonatal cholestasis must be the responsibility of more specialized physicians at reference centres. For a better and overall knowledge of the clinical practices in our country, a national survey should be performed.

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Authors' contributions Ermelinda Santos Silva: Dr. Santos Silva conceptualized and designed the study, coordinated and supervised data collection, analysed and interpreted data, drafted the initial manuscript and approved the final manuscript as submitted.

Helena Moreira Silva: Dr. Moreira Silva carried out acquisition, analysis and interpretation of data; critically reviewed the manuscript and approved the final manuscript as submitted.

Lia Azevedo Lijnzaat: Dr. Lijnzaat carried out acquisition, analysis and interpretation of data; critically reviewed the manuscript and approved the final manuscript as submitted.

Cláudia Melo: Dr. Melo carried out analysis and interpretation of data, critically reviewed the manuscript and approved the final manuscript as submitted.

Elísio Costa: Prof. Costa carried out analysis and interpretation of data, critically reviewed the manuscript and approved the final manuscript as submitted.

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Ana Isabel Lopes: Prof. Lopes critically carried out analysis and interpretation of data, reviewed the manuscript and approved the final manuscript as submitted.

All the authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

Compliance with ethical standards

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Ethical approval This study involved human participants and all procedures performed were in accordance with the ethical standards of the participating healthcare institutions committees and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Clinical trial registration Not applicable.

Informed consent Informed consent was obtained from all individual participants included in the study.

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1.2 THE STOOL COLOR CARD AS A SCREENING TOOL FOR BILIARY ATRESIA IN THE DIGITAL VERSION OF THE PORTUGUESE CHILD AND YOUTH HEALTH BOOKLET.

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The Stool Color Card as a Screening Tool for Biliary Atresia in the Digital Version of the Portuguese Child and Youth Health Booklet

O Cartão de Cores das Fezes como Instrumento de Rastreio da Atrésia Biliar na Versão Digital do Boletim de Saúde Infantil e Juvenil Português

Keywords: Biliary Atresia/diagnosis; Feces; Jaundice, Neonatal; Neonatal Screening
Palavras-chave: Atresia das Vias Biliares/diagnóstico; Fezes; Icterícia Neonatal; Rastreio Neonatal

Dear Editor

Biliary atresia (BA) is a rare entity (incidence in Europe of 1/18 000 live births) leading to great morbidity and mortality and represents the first indication for pediatric liver transplantation.[1] The long-term prognosis depends on the timing of bile flow restoration (Kasai portoenterostomy). One of the major determining factors of prognosis and survival of the native liver is surgical intervention before 45 days of life.[2]

Taiwan has one of the highest incidence rates of BA in the world. Therefore, a universal screening program was started there in 2002, using a validated stool color card (SCC). Subsequently, in 2011, it was observed that the median age of patients undergoing Kasai portoenterostomy decreased substantially, thus improving prognosis.[3] Meanwhile, other countries have demonstrated the cost-effectiveness of this tool,[4] and some have already started using it as a teaching and surveillance tool, by enrolling parents and caregivers. In Portugal, and as far as we know, there have been no initiatives at the level of the central healthcare system on this matter.

The SCC was originally created by Prof. Mei-Hwei Chang and her team, from the National University of Taiwan Medical College. It includes nine stool-colored images: three with normal colors and six with abnormal colors, which are considered red flags requiring urgent observation of the newborn/infant by Pediatricians.

A study performed in Northern Portugal has identified a significant percentage of healthcare professionals with clinical practices that may delay recognition of BA.[5] Recently, we have obtained written permission from both Prof. Mei-Hwei Chang and the Taiwan Ministry of Health and Health Promotion Administration to use the original SCC and its validated images for the purposes of a screening program in Portugal.

We propose the use of SCC as a teaching tool for parents at the time of discharge from the maternity hospital, and the inclusion of an early ambulatory screening in the first two visits after discharge by inserting the SCC into the Child and Youth Health Booklet. If the Portuguese Directorate-General of Health will convert the current paper booklet into a digital format, as it has been preliminarily admitted, we propose that a SCC should be part of this update (Fig. 1) and that a digital version should be accessible to both parents and caregivers, for example, through a web link or an app. In this scenario, the outcome of the implementation of this practice must be validated and a referral network should be implemented.

AUTHORS CONTRIBUTION

JA: Requested for permission to use the original SCC and its validated images, drafted the manuscript and approved its final version.

MT: Contributed with intellectual insights to the work, critically reviewed the manuscript and approved its final version.

ESS: Designed the work, supervised the request for

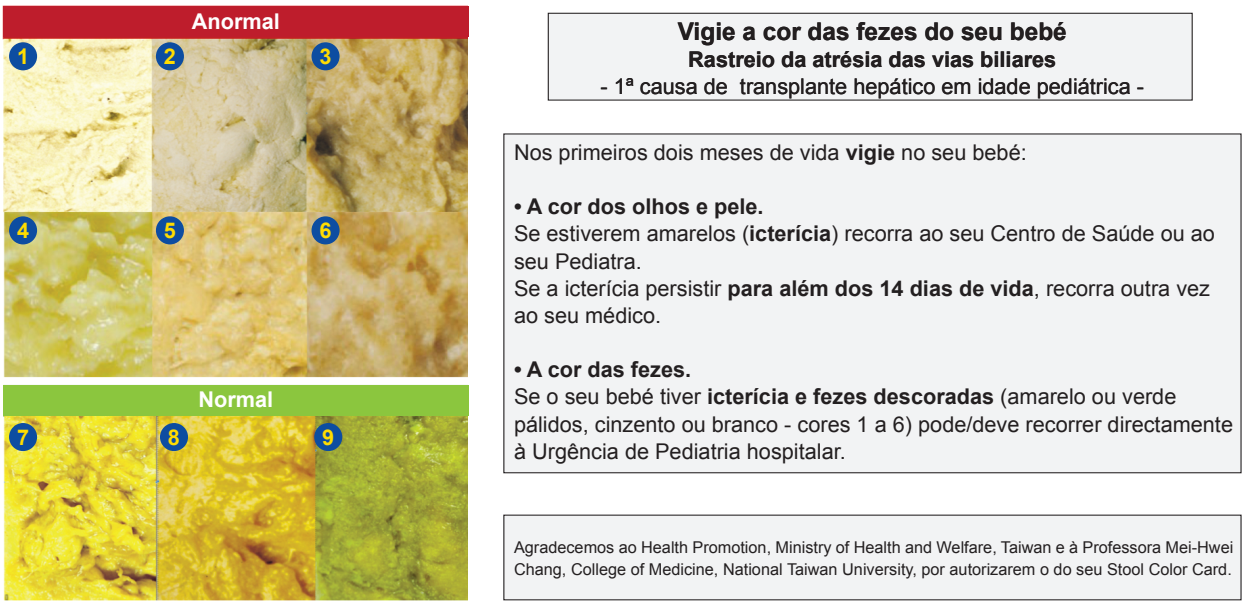

Figure 1 – Portuguese stool color card for screening biliary atresia in newborns and infants

permission to use the original SCC and its validated images, critically reviewed the manuscript and approved its final version.

AIL: Designed the work, critically reviewed the manuscript and approved its final version.

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2.1. NEONATAL CHOLESTASIS OVER TIME: CHANGES IN EPIDEMIOLOGY AND OUTCOME IN A COHORT OF 154 PATIENTS FROM A PORTUGUESE TERTIARY CENTER.

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Neonatal Cholestasis Over Time: Changes in Epidemiology and Outcome in a Cohort of 154 Patients From a Portuguese Tertiary Center

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Introduction: In the last two decades there have been advances in the diagnosis and management of neonatal cholestasis, which may have changed its epidemiology, diagnostic accuracy, outcomes, and survival. Our goal was to characterize these changes over time in our setting.

Methods: Retrospective cohort study in a tertiary center, enrolling patients born between January 1985 and October 2019. The cohort was divided into two periods, before (A; $n = 67$) and after (B; $n = 87$) the year 2000; and in two groups, according to patient's outcome (favorable, unfavorable). Overall survival and survival with and without orthotopic liver transplant (OLT) were evaluated in the two periods (A and B) and in different subgroups of underlying entities.

Results: We found that the age of cholestasis recognition decreased significantly from period A to period B [median 43 days and 22 days, respectively, ($p < 0.001$)]; the changes in epidemiology were relevant, with a significant decrease in alpha-1-antitrypsin deficiency ($p < 0.001$) and an increase in transient cholestasis ($p = 0.004$). A next-generation sequencing (NGS) panel available since mid-2017 was applied to 13 patients with contributory results in 7, but, so far, only in 2 patients led to conclusive diagnosis of underlying entities. The number of cases of idiopathic cholestasis did not vary significantly. Over time there was no significant change in the outcome ($p = 0.116$). Overall survival and survival without OLT had no significant improvement during the period of observation (in periods A and B, 86 vs. 88%, and 85 vs. 87%, respectively). However, in period B, with OLT we achieved the goal of 100% of survival rate.

Conclusions: Our data suggest that transient cholestasis became a very important subset of neonatal cholestasis, requiring specific guidance. The NGS panels can provide

important inputs on disease diagnosis but, if applied without strict criteria and expertise, they can open a Pandora's box due to misinterpretation. Despite all the advances in accurate diagnosis and timely management—including early recognition of cholestasis—the improvement in patient outcomes and survival were still not significant.

Keywords: neonatal cholestasis, transient cholestasis, neonatal cholestasis epidemiology, cholestasis risk factors, neonatal cholestasis survival, next generation sequencing panel

INTRODUCTION

In the twenty-first century, neonatal cholestasis (NC) remains a major clinical challenge for several reasons. Recognition of NC among jaundiced neonates is delayed in a significant number of cases, often due to the lack awareness of healthcare professionals (1–3). Furthermore, the diagnosis is difficult due to the great diversity of underlying entities, some of them with specific treatment that should be offered in a timely manner to improve prognosis. Finally, morbidity and mortality are still high and many patients survive at the expense of orthotopic liver transplantation (OLT) (4).

Nevertheless, in the last two decades, diagnosis and management has improved, which may have had a significant impact in epidemiology and outcome of NC (5–7). New underlying entities have been added to the long list of etiological causes of NC (3) requiring specialized diagnostic tools and clinical expertise (8). Next-generation sequencing (NGS) technology is a new and appealing diagnostic tool in this field, yet to be incorporated in clinical practice (9). Additionally, the recent advances in understanding the pathophysiology of NC has not yet fully translated into new treatments or prevention strategies (10).

A few studies (11–14) have focused on NC epidemiology and how outcomes have changed over time. A systematic review of 17 studies including a total of 1,692 patients recruited ascertained from 12 countries and 5 continents from 1963 to 2011, focused only in the etiology of NC and was flawed by the inconsistency of diagnostic approach (15).

Moreover, the variations in the terminology used over time and in different centers make it even more difficult to analyse the underlying etiologies. For example, sometimes the term "neonatal hepatitis" is used regardless of existence of a known underlying entity; similar patients may be classified as "transient cholestasis" or as "total parenteral nutrition (TPN) associated cholestasis;" and finally, there is barely no consensus on which cases should be under the broad label of "idiopathic cholestasis." In addition, the selected studies do not allow us to draw conclusions about the epidemiological trends over time. Outcomes and survival were not systematically assessed.

The aim of this study—the first one in Portugal—was to characterize NC evolution over time, epidemiology, diagnostic accuracy, outcomes, and survival.

PATIENTS AND METHODS

Patients and Study Design

Retrospective cohort study of NC patients diagnosed and treated at the hepatology clinic of a single tertiary hospital, from 1 January 1985 to 31 October 2019. The study was based on the analysis of clinical charts and institutional database records. Patients with incomplete clinical records were excluded.

This study was compliant with the ethical standards of the participating healthcare institution committee [Studies N/REF.[a] 2016. 081 (069-DEFI/066-CES) and N/REF.[a] 2016. 084 (072-DEFI/069-CES)], and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Patients were referred from institutional departments of Emergency, Neonatology and Pediatrics as well as external institutions (primary and secondary healthcare providers).

We analyzed the cohort according to the period of diagnosis using the year 2000 as a symbolic landmark: period (A) from 1985 to 1999, and period (B) from 2000 to 2019. The cohort was also analyzed according to the outcome (I), unfavorable [death, OLT, advanced chronic liver disease], or (II) favorable (mild chronic liver disease, cure of liver disease). Furthermore, we studied the 7 subgroups of underlying entities of our cohort [biliary diseases, alpha-1-antitrypsin deficiency (A1ATD), infectious diseases, metabolic diseases, transient cholestasis, other diseases, idiopathic cholestasis].

Clinical Data, Diagnostic Approach and Treatment

Patients Were Managed by the Same Nuclear Team Throughout the Years

NC was defined as jaundice with conjugated bilirubin $\geq$1 mg/dl (and $>$ 20% of total bilirubin, if total bilirubin $>$5.0 mg/dl), detected in a newborn or infant younger than 4 months old, according to the Guideline of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) and European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) (16). For the retrospective analysis, transient cholestasis was defined as the presence of cholestatic jaundice, with known risk factors, in the same age group, and complete and spontaneous normalization of liver function tests within the first 6 months of life (17, 18).

The risk factors for NC included those described by Champion et al. (19), as well as sepsis and asphyxia, among others (20, 21). Signs and symptoms of sepsis were moaning, lethargy, hypotonia, fever, and/or hypothermia. Signs and symptoms of liver failure were coagulopathy not correctable by vitamin K administration, ascites, and hypoglycaemia, in accordance to the Pediatric Acute Liver Failure Study Group (22).

Diagnostic approaches evolved over the years reflecting the evolution of international guidelines and accumulated personal experience (8, 16). Diagnosis of underlying entities was based

TABLE 1 | List of genes included in the next generation sequence (NGS) panel.

	NGS panel evolution	
	Mid-2017 (54 genes)	**Since the end of 2019 (95 genes)**
Genes		
	ABCB11, ABCB4, ACAD9, AKR1D1, ASAH1 ATP8B1, BAAT, BCS1L CC2D2A, CFTR, CLDN1 CYP27A1, CYP7A1, CYP7B1 DCDC2, DGUOK GBA, GBE1, HSD3B7 INVS, JAG1, LIPA MKS1, MPV17, MYO5B NOTCH2, NPC1, NPC2, NPH3, NR1H4 PEX1, PEX10, PEX11B, PEX12, PEX13, PEX14, PEX16, PEX19, PEX2, PEX26, PEX3, PEX5, PEX6, PEX7 PKHD1, POLG, POLG2 RRM2B SERPINA1, SLC25A13 TJP2, TRMU VIPAS39, VPS33B	ABCB11, ABCB4, ABCC2, ABCD3, ABCG5, ABCG8, ADK, AKR1D1, ALDOB, AMACR, ATP7B, ATP8B1, BAAT, BCS1L, CC2D2A, CFTR, CLDN1, COG7, CYP27A1, CYP7A1, CYP7B1 DCDC2, DGUOK, DHCR7, EHHADH, EPHX1, FAH, GALT, GBA, GBE1, GNAS, GPBAR1 HADHA, HAMP, HFE, HFE2, HNF1B, HSD17B4, HSD3B7, IARS, INVS, JAG1, LIPA, MKS1, MPI, MPV17, MVK, MYO5B, NOTCH2, NPC1, NPC2, NPHP1, NPHP3, NPHP4, NR1H4, PEX1, PEX10, PEX11B, PEX12, PEX13, PEX14, PEX16, PEX19, PEX2, PEX26, PEX3, PEX5, PEX6, PEX7, PKHD1, POLG, POLG2, RPGRIP1L, SCP2, SERAC1, SERPINA1, SLC10A1, SLC10A2, SLC25A13, SLC27A5, SLC30A10, SLC40A1, SMPD1, TALDO1, TFR2, TJP2, TMEM216, TMEM67, TRMU, TTC37, UGT1A1, USP53, UTP4, VIPAS39, VPS33B

on biochemical, imaging, histological, and enzymatic tests; molecular studies were also performed when appropriate and available. Diagnosis of biliary atresia was confirmed by per-operatory cholangiography (with simultaneous liver biopsy). Diagnosis of liver disease for A1ATD was based on the serum level of the protein and its phenotype, and on liver histology.

Since 2005 the national newborn screening program (performed between the 3rd and 6th day of life) includes the screening of 24 treatable disorders, namely hypothyroidism, tyrosinemia, argininemia, citrullinemia type II, some organic acidurias and beta-oxidation fatty acids disorders, and cystic fibrosis (23).

In the mid-2017 a customized NGS panel targeting inborn errors of metabolism and genetic cholestatic disorders became available—initially comprised of 54 genes, and currently 95 genes (**Table 1**). This panel was offered to some patients fulfilling the following criteria: no known underlying entity (liver failure survivors, and/or with evolving chronic liver disease), suspicion of a second underlying entity, and cholestasis evolving transiently without risk factors.

Patients with biliary atresia were submitted to Kasai porto-enterostomy (PE), performed by a stable and skilled surgical team after January 2000 (24). Patients with A1ATD born after 1994 were treated by ursodeoxycholic acid (UDCA), 15–20 mg/kg/day, bid (25). Metabolic diseases were treated with special diets and specific drugs, according to the state of art, which included OLT in some patients (26, 27). Pediatric OLT was available in our country since January 1994 (one center); before was only available through foreign centers. All patients with chronic cholestasis were managed with supportive therapy in accordance to international guidelines (28).

Statistical Analysis

Clinical, biochemical, and genetic data were compared by using the following tests, as appropriate: chi-square, Fisher's and Mann-Whitney. Overall survival and survival without OLT were compared between groups using the Kaplan-Meier method and the Log Rank. The significance level used was $P < 0.05$. Statistical analysis was performed using the software Statistical Package for the Social Sciences v. 24.0.

RESULTS

We analyzed 154 clinical charts of NC patients (6 were excluded due to insufficient data). The descriptive analysis of the cohort is available on **Table 2**.

The male gender showed a higher prevalence ($n = 92$; 60%). Parental consanguinity was present in 9 patients (6%) and 11 (7%) had siblings affected by related clinical conditions.

A comparison between period A (before year 2000) and period B (after year 2000) is also available in **Table 2**. We analyzed 67 patients in period A and 87 patients in period B. The median follow-up was, as expectable, significantly longer in period A ($p < 0.001$].

Cholestasis Recognition Over Time

The median age of onset of jaundice, cholestasis recognition, and admission to the tertiary center, before and after year 2000 are described and compared in **Table 2**.

We verified that the age of cholestasis recognition decreased significantly after the year 2000 [median = 43 days (IQ: 21–72) vs. 22 days (IQ: 6–42), $p < 0.001$], while the time interval between the cholestasis recognition and admission to the tertiary center increased significantly [median 6 days (IQ: 0–14) vs. 12 days (IQ: 1–49), $p = 0.017$]; however, the age of admission to the tertiary center decreased in period B, but non significantly.

Role of Risk Factors

Risk factors for developing neonatal cholestasis were present in 45% ($n = 69/85$) of the cohort. Gestational age ($p < 0.001$) and birth weight ($p = 0.002$) were both significantly lower in period B.

All patients from the subgroup of transient cholestasis had at least one risk factor, and the same was observed in 35% ($n = 45/85$) of all other patients ($p < 0.001$) (**Table 3**).

The presence of one or more risk factors was significantly less frequent in patients with biliary atresia in comparison to other patients, even when patients with transient cholestasis were excluded from analysis ($p = 0.008$), which is in contrast to what was observed with A1ATD patients ($p = 0.541$).

TABLE 2 | Descriptive analysis of the cohort and comparisons between periods A (before year 2000) and B (after year 2000).

	Cohort (*N* = 154)			Before year 2000 (A)			After year 2000 (B)			
	N	**Median/IQ**	**Range**	***n***	**Median/IQ**	**Range**	***n***	**Median/IQ**	**Range**	***p*-value***
1. Age of onset of jaundice (days)	154	2 (2–4)	1–118	67	3 (2–7)	1–80	87	2 (2–3)	1–118	**0.015**
2. Age of cholestasis recognition (days)	153	30 (8–54)	1–165	66	43 (21–72)	2–165	87	22 (6–42)	1–124	**<0.001**
3. Age of admission to the tertiary center (days)	153	54 (30–75)	3–192	66	61 (33–79)	3–165	87	45 (30–69)	7–192	0.072
4. Age of diagnosis of underlying entity (days)	154	65 (42–97)	4–2,520	67	75 (45–97)	4–2,520	87	61 (42–100)	10–270	0.371
Interval between 1 and 2 (days)	153	22 (6–41)	0–163	66	31 (15–58)	0–163	87	16 (3–33)	0–75	**<0.001**
Interval between 2 and 3 (days)	153	8 (1–27)	0–143	66	6 (0–14)	0–99	87	12 (1–49)	0–143	**0.017**
Interval between 3 and 4 (days)	152	7 (4–14)	0–2,458	65	6 (5–11)	0–2,458	87	7 (4–17)	0–221	0.355
Gestational age (weeks)	144	39 (36–40)	26–42	63	40 (39–40)	30–42	81	37 (34–39)	26–41	**<0.001**
Birth weight (grams)	148	2,858 (2,200–3,280)	570–4,400	66	3,045 (2,400–3,425)	1,000–4,400	82	2,615 (1,626–3,070)	570–4,000	**0.002**
Age at Kasai surgery (days)	24	62 (42–72)	31–128	9	84 (64–110)	42–128	15	47 (39–63)	31–69	**0.002**
Age at OLT (months)	28	24 (13–69)	8–132	18	30 (18–90)	8–132	10	16 (12–46)	8–81	0.088
Age of death (months)	18	9 (4–23)	2–32	9	14 (5–27)	2–32	9	9 (2–19)	2–25	0.248
Follow-up (months), maximum 216 (18 years)	154	118 (21–216)	2–216	67	216 (72–216)	2–216	87	60 (18–126)	2–216	**<0.001**

**Mann-Whitney test; IQ, interquartile; OLT, orthotopic liver transplantation. The bold values correspond to results with significative differences.*

Diagnosis of Underlying Entities Over Time

A1ATD ($n = 34$, 22%) and biliary atresia ($n = 24$, 16%) were the most prevalent underlying entities. Transient cholestasis was one of the major sub-groups of patients ($n = 24$, 16%). Over the years, epidemiological trends underwent significant changes in our cohort (**Figure 1**).

Biliary Atresia and A1ATD—The Old Players

While the frequency of biliary atresia remained stable, A1ATD has significantly lost prominence over the years [period A: $n = 25$ (37%); period B: $n = 9$ (10%), $p < 0.001$]. The A1AT phenotypes were ZZ ($n = 29$), SZ ($n = 3$), M1Z ($n = 1$), and FZ ($n = 1$). One of the SZ patients had concomitantly biliary atresia.

Transient Cholestasis—The New Kid on the Block

From 33 patients referred with transient cholestasis, only 24 remained with this diagnosis after investigation; in 9 of these patients we identified post-natal Cytomegalovirus (CMV) infection, A1ATD, choledochal cyst, and chromosomal disorders. Transient cholestasis emerged as the major subgroup of patients in period B [$n = 4$ (6%) vs. $n = 20$ (23%), $p = 0.004$].

Idiopathic Neonatal Cholestasis—What's Been Left in the Bag

Idiopathic neonatal cholestasis accounted for 8% ($n = 12$) of all patients, and the difference over time was not significant.

Contribution of Molecular Studies

Molecular studies were more commonly used in period B ($n = 21$; 24%) than in period A ($n = 6$; 9%) ($p = 0.014$). Time lag between admission and etiological diagnosis was significantly longer than in patients who did not undergo genetic testing [23 days (IQ: 3–91) vs. 6 days (IQ: 4–11), $p = 0.011$].

In period A, molecular studies were performed in three patients with galactosemia, one with argininemia (29), and one with MacCune-Albright syndrome (30), previously reported. In period B, molecular studies were performed in patients with Alagille syndrome and some metabolic diseases (27).

Additionally, since the NGS panel became available, we applied it, so far, to 13 patients of this series (see details in **Table 4**) (31–36). We highlight a pair of siblings (brother and sister), diagnosed with neonatal sclerosing cholangitis in period A, based on clinical, biochemical, imaging, and histological criteria (26). NGS panel in the sister (the brother declined testing) allowed us to identify the genetic basis of their disease (case 1).

Treatment

Kasai's surgery was performed at a significantly younger age over time ($p = 0.002$) (**Table 2**), but the success of this surgery did not increase significantly after the year 2000 (22 vs. 60%), $p = 0.072$).

Treatment with UDCA, administered to patients with A1ATD was not associated with a significantly better outcome (55 vs. 83%, $p = 0.111$). Four out of the 23 treated patients had unfavorable outcome, in comparison with 5 out of 11 untreated patients; no side effects were observed.

OLTs were significantly more frequent in period A ($p = 0.014$). Differences in relation to the median age at which they were performed were not significant. In both periods, OLT performance varied significantly according to the underlying entity. In period A, OLT was performed in 6 out of 9 patients with biliary atresia, 6 out of 25 with A1ATD, 2 out of 2 with neonatal sclerosing cholangitis, 2 out of 2 with tyrosinemia, 1 out of 1 with

TABLE 3 | Risk factors for neonatal cholestasis: comparison between transient cholestasis and other patients.

	Cohort (*N* = 154)		Transient cholestasis		Other patients		
	N	**(%)**	***N***	**(%)**	***n***	**(%)**	***p*-value***
RISK FACTORS							
Gestational age							**<0.001**
Term	114	(74)	4	(17)	110	(85)	
Preterm ≥34 weeks	16	(10)	4	(17)	12	(9)	
Preterm <34 weeks	24	(16)	16	(17)	8	(6)	
Birth weight							**<0.001**
Normal	93	(62)	4	(17)	89	(69)	
Low	34	(23)	8	(33)	26	(20)	
Very low	8	(5)	3	(13)	5	(4)	
Extremely low	10	(7)	9	(38)	1	(0.8)	
Big	6	(4)	0	0	6	(5)	
Missings					3		
Birth weight vs. gestational age							0.125
Adequate	110	(71)	13	(54)	97	(75)	
Small	40	(26)	10	(42)	30	(23)	
Big	4	(3)	1	(4)	3	(2)	
TPN >7 days					130		**<0.001**
Yes	38	(25)	21	(88)	17	(13)	
Infection/sepsis							**<0.001**
Yes	56	(36)	21	(88)	35	(27)	
Surgery							**0.045**
Yes	7	(4)	3	(13)	4	(3)	
Thoracic	1	(0.6)	1	(4)	0	0	
Abdominal	5	(3)	2	(8)	3	(2)	
Pelvic	0	(0)	0	0	0	0	
Brain	1	(0.6)	0	0	1	(0.8)	
Invasive ventilation							**<0.001**
Yes	25	(16)	16	(67)	9	(7)	
Hemodynamic instability							**<0.001**
Yes	16	(10)	8	(33)	8	(6)	
Number of RF (19)							**<0.001**
0	85	(55)	0	0	85	(65)	
1	40	(26)	6	(25)	34	(26)	
2	20	(13)	10	(42)	10	(8)	
3	9	(6)	8	(33)	1	(0.8)	
4	0	(0)	0	0	0	0	
One OR more RF							**<0.001**
Yes	69	(45)	24	(100)	45	(35)	

**Chi-square test; RF, Risk factors; TPN, total parenteral nutrition. The bold values correspond to results with significative differences.*

argininemia, and 1 out of 3 with Alagille syndrome ($p = 0.035$). In period B, OLT was performed in 8 out of 15 patients with biliary atresia, and in 2 out of 9 with A1ATD ($p = 0{,}020$).

Outcome, Morbidity, and Mortality

Outcome was unfavorable in 47 patients (31%), without significative difference over time. Co-morbidities were identified in 26% ($n = 40$) and death occurred in 12% ($n = 18$), both not significantly different over time (**Table 5**). Early death rate (<12 months-old) occurred in 7% ($n = 10$; 55% of all mortality), from which 7 patients had metabolic or infectious diseases or idiopathic cholestasis—*the fast killers*. Mortality was not significantly different between genders.

Mortality details were as follows: 9 patients died in period A (biliary atresia $n = 3$, Caroli disease $n = 1$, A1ATD $n = 1$, Alagille syndrome $n = 1$, Herpes infection $n = 1$, idiopathic cholestasis $n = 2$); from those, 5 deaths were potentially avoidable: 3 died while waiting for OLT (2 with biliary atresia and 1 with neonatal idiopathic hepatitis), 1 with Caroli disease died from sepsis, and one with A1ATD died from CMV infection; additionally, 2 died

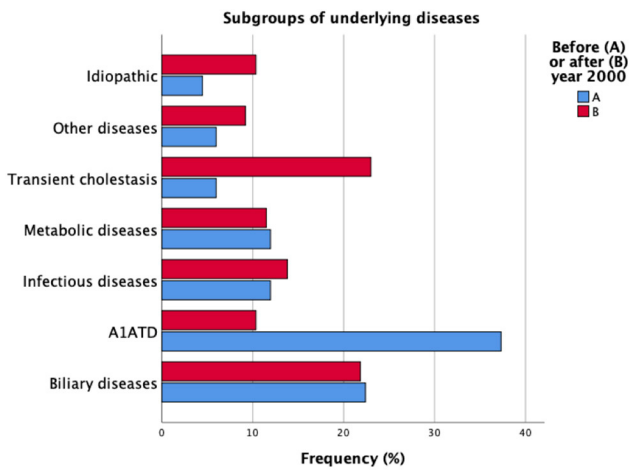

FIGURE 1 | Underlying entities in the cohort of patients with neonatal cholestasis ($N = 154$). Comparison between periods of time A and B.

after OLT complications. The same number of patients died in period B (mitochondrial disorders $n = 3$, Zellweger syndrome $n = 1$, Menkes disease $n = 1$, Short-bowel syndrome $n = 1$, idiopathic cholestasis $n = 2$), but these were unavoidable deaths and none died after OLT.

Overall survival and survival without OLT had no significant improvement during the period of observation (in periods A and B, 86 vs. 88%, and 85 vs. 87%, respectively). However, in period B, with OLT we achieved the goal of 100% of survival rate. Survival rates of patients with and without OLT are disclosed in **Figure 2**.

Survival rates were different according to subgroups of underlying entities. The idiopathic cholestasis subgroup exhibited significantly lower overall survival rates than other patients (63 vs. 89%) as well as the metabolic diseases subgroup (72 vs. 89%) (**Figure 3**). The survival rate in patients with biliary atresia, with and without OLT, was not significantly different (93 vs. 75%); the same happened with patients with A1ATD (100 vs. 96%).

DISCUSSION

NC is the form of expression of many types of liver injury with a myriad of underlying entities (15). Geographic variability is explained by genetic and environmental factors, and also depends on the expertise of the healthcare services and their technical resources. Over time, advances in diagnostic tools has changed the epidemiological landscape of this entity.

Biliary atresia is the most common cause of NC (15). A1ATD was the first genetic disease to be reported presenting with NC and progressing to liver cirrhosis (37) and played an important role in some European populations, namely in England (38) and Sweden (39). However, this may no longer be the case in England taking into account the different results obtained by Humphrey et al. (13) vs. Mieli-Vergani et al. (38). In our series, A1ATD was overall more frequent than biliary atresia, with a slightly higher rate than what had been observed in English and Swedish studies, and much higher than the rate of 4% described in the systematic review (15). The prominence of this specific condition in our cohort may be explained by the high prevalence of the disease in Northern Portugal (40), a region known for its Celtic genetic heritage. Noteworthy, we have seen a prevalence decrease over the past two decades. Several reasons can explain the lower number of cases: decrease in birth rate, greater diagnostic capacity in secondary healthcare facilities, better outcome due to early diagnosis and better supportive treatment, and our availability for external consultancy, all together avoiding the referral. In parallel with the loss of predominance of A1ATD after the year 2000, we observed the arrival and rise of the "new kid on the block"—the transient cholestasis—surpassing the "old players."

After the year 2000, several studies reported an emergent subset of patients, denominated as "transient," "prematurity-associated," "TPN-associated," or "sepsis-associated" (15). This new subset of patients shares a higher level of exposure to risk factors (19, 41). Their role as determinants or co-factors of NC is far from being totally clear. In our study, one third of the other patients also had risk factors. Interestingly, the biliary atresia patients had significantly less risk factors. This is in contrast with the A1ATD patients in which the pathophysiology of the liver disease (42), may be affected by the risk factors, and so the prevention or treatment of those may also be contributing to the downtrend in prevalence. On the other hand, the increase in transient cholestasis may be explained by the increased rate of prematurity and survival of premature and sick newborns, and an increase in referrals of these to tertiary centers. Previous studies (17, 18) did not address underlying entities as possible determinants or cofactors of cholestasis in patients with transient cholestasis, but in our study we have verified that in out of 9 patients referred with this diagnosis we could identify both sporadic (e.g., CMV infection, choledochal cyst) and genetic etiologies (e.g., A1ATD). Finally, identification of etiological diagnosis might have been limited due to insufficient diagnostic tools. The assumption of the diagnosis of transient cholestasis is one of exclusion and the current guidelines for diagnostic approach do not fully address this problem (8). In clinical practice the current main question is: "*how far should we investigate these patients for underlying entities?*"

The prevalence of idiopathic cases has decreased significantly according to a recent European study (11). In our center, the prevalence rate has not decreased over time—as a matter of fact, we obtained similar figures to the ones reported by Hoerning et al. Improved detection rate is likely to be attained with the availability of new molecular technologies. Recently, Nicastro et al. (43) developed a prospective study that included 125 patients, of which 50 subjects underwent a through diagnostic protocol that included genetic testing—and they obtained a detection rate of 60% which is much higher than reported in previous studies (44). Our preliminary data supports the perspective that patients that remain undiagnosed despite standard of care diagnostic tools may benefit from NGS panels for further clarification. Neonatal sclerosing cholangitis is the poster child of this paradigm as it allowed to establish

TABLE 4 | Results from NGS panel in 13 patients.

ID	Gender	Birth year	Liver clinical phenotype/diagnosis	Outcome	Genes	Variants	Interpretation
1	F	1986	CLD *Neonatal sclerosing cholangitis*	Alive OLT	86	***DCDC2*** (NM_001195610.1)—c.942del [p. (Gly315Glufs*32)]—in Intron 9 and Exon 19, in HOMOZYGOSITY	**A variant not previously described, leading to a premature STOP codon, most likely pathogenic**. In this gene were previously described other pathogenic variants in association with **neonatal sclerosing cholangitis** (AR), with deafness type 66 and **nephronophthisis type 19**.
2	F	1992	CLD *Argininemia + a second disease?* (pruritus + dislipidemia)	Alive OLT	86	***ARG1*** (NM_001244438.1)—c.61C>T [p.(Arg21*)]—Exon 2, in HOMOZYGOSITY	A variant in homozygosity, previously associated with argininemia (31). **No second disease confirmed.**
3	M	1998	Liver failure	Alive Cured	54	No variants potentially pathogenic	No underlying disease confirmed.
4	F	2002	Liver failure	Alive Cured	54	No variants potentially pathogenic	No underlying disease confirmed.
5	M	2009	*Transient cholestasis* (without risk factors)	Alive Cured	54	***DCDC2*** (NM_001195610.1)—c.1283A>T [p. (Asp428Val)]—Exon 10, in HETEROZIGOSITY ***DGUOK*** (NM_080916.2)—c.4G>T [p. (Ala2Ser)]—Exon 1, in HETEROZIGOSITY ***VP5338*** (NM_018668.4)—c.1148T>A [p.(Ile383Asn)]—Exon 15, in HETEROZIGOSITY	**Three variants in heterozygosity, all of unknown clinical significance**. The bioinformatic analysis indicates that the first two will be benign, and the third (not previously described) will be pathogenic.
6	M	2011	CLD *Idiopathic cholestasis* (with raised total bile acids; no improvement with UDCA)	Alive CLD	54	***CYP781*** (NM_004820.3)—c.928C>T [p. (Arg310Trp)]—Exon 4, in HETEROZIGOSITY	**A variant of unknown clinical significance**, whose bioinformatic analysis indicates that it will be pathogenic. Both progenitors have normal liver tests; the father has raised total bile acids; genetic tests of both parents are in course.
7	F	2015	CLD *Biliary atresia + a second disease?* (microcephaly + cognitive delay + enteropathy)	Alive OLT	54	***ATP8B1*** (NM_005603.4)—c.607A>G [p. (Lys203Glu)]—Exon 7 in HETEROZYGOSITY	A rare variant (rs56355310) of **unknown clinical significance.** There are pathogenic variants reported in this gene in patients with PFIC-1 (AR). Heterozygosity was described in association with transient cholestasis (32).
8	M	2016	*Transient cholestasis* (without risk factors) *Alagille Syndrome?* (facies + embriotoxon)	Alive ALT elevated	95	***NOTCH2*** (NM_024408.3)—c.7223T>A [p. (Leu2408His)]—Exon 34 in HETEROZYGOSITY	**A variant** formerly identified by Sanger sequencing of the gene, classified as of unknown clinical significance and considered as potentially pathogenic, is **currently seen as benign.** The parents refused to do the genetic test.
9	M	2016	*Transient cholestasis* (without risk factors)	Alive Cured	54	***ABCB11*** (NM_003742.2)—c.1460G>A [p. (Arg487His)]—Exon 14, in HETEROZIGOSITY	A variant in heterozygosity, **previously associated with PFIC-2** (33).
10	M	2016	*Transient cholestasis* (without risk factors)	Alive Cured	54	No variants potentially pathogenic	No underlying disease confirmed.
11	F	2018	CLD *Deficit alfa-1-AT (ZZ) + a second disease?* (pale stools)	Alive CLD	54	***SERPINA1*** (NM_001127701.1)—c.1096G>A [p. [Glu366Lys)]—Exon 7, in HOMOZIGOSITY	A variant in homozygosity, previously associated with deficit of alpha-1-antitrypsin (34). **No second disease confirmed.**
12	M	2018	*Transient cholestasis* (with UTI *E. coli*)	Alive Cured	86	No variants potentially pathogenic	No underlying disease confirmed.
13	M	2019	*Transient cholestasis)* (with starvation)	Alive Cured	86	***CFTR*** (NM_000492.3)—c.1210-11T>G (r.?)—Intron 9, in HETEROZIGOSITY ***CFTR*** (NM_000492.3)—c.2991G>C [p.(Leuc997Phe)]—Exon 19, in HETEROZIGOSITY	Both variants are currently classified as variants of **unknown clinical significance.** The first was previously described as responsible for primary ciliary dyskinesia (AR) (35). The second was previously described as responsible for congenital bilateral absence of the vas deferens (AR) (36).

CLD, chronic liver disease; OLT, orthotopic liver transplant; UTI, urinary tract infection; AD, autosomal dominant; AR, autosomal recessive.
Here the classification of "Transient NC" is applied by the authors only in accordance with the evolution of liver clinical phenotype.

TABLE 5 | Outcome, morbidity and mortality: comparison between periods A and B.

	Cohort (*N* = 154)		Before year 2000 (A)		After year 2000 (B)		*p*-value*
	N	**(%)**	***N***	**(%)**	***N***	**(%)**	
OUTCOME							0.108
Favorable	107	(70)	42	(63)	65	(75)	
Biliary diseases (n = 34)							0.171
Favorable	14	(41)	4	(27)	10	(53)	
Biliary atresia (n = 24)							0.191
Favorable	7	(2)	1	(11)	6	(40)	
A1ATD (n = 34)							>0.999
Favorable	25	(4)	18	(72)	7	(78)	
Infectious diseases (n = 20)							0.400
Favorable	19	(95)	7	(88)	12	(100)	
Metabolic diseases (n = 18)							0,664
Favorable	10	(56)	5	(63)	5	(50)	
Transient cholestasis (n = 24)							-
Favorable	24	(100)	4	(100)	20	(100)	
Other diseases (n = 12)							>0.999
Favorable	8	(67)	3	(75)	5	(63)	
Idiopathic (n = 12)							0.523
Favorable	7	(58)	1	(33)	6	(67)	
Status of liver disease at present							0.125
Cure	86	(56)	35	(52)	51	(59)	
CLD without cirrhosis/PTH	21	(14)	7	(10)	14	(16)	
CLD with cirrhosis/PTH	3	(2)	0	(0)	3	(3)	
OLT	26	(17)	16	(24)	10	(12)	
Death	18	(12)	9	(13)	9	(10)	
CO-MORBIDITIES							0.881
Yes	40	(26)	17	(25)	23	(26)	
Brain	17	(11)	7	(10)	10	(12)	
Eyes	1	(.6)	1	(2)	0	(0)	
Ears	1	(.6)	1	(2)	0	(0)	
Others	11	(7)	6	(9)	5	(6)	
Brain and others	8	(5)	0	(0)	8	(9)	
Brain and eyes	2	(1)	2	(3)	0	(0)	
MORTALITY							0.554
Yes	18	(12)	9	(13)	9	(10)	
Early death (<12 months old)	10	(7)	4	(6)	6	(7)	0.817
Cause of death							0.959
Liver disease	8	(44)	4	(44)	4	(44)	
Other organs related to UE	7	(39)	3	(33)	4	(44)	
Other disease	3	(17)	2	(22)	1	(11)	

**Chi-square test; A1ATD, alpha-1-antitrypsin deficiency; CLD, chronic liver disease; PTH, portal hypertension; OLT, orthotopic liver transplantation; UE, underlying entity.*

a genetic basis and point to the possible pathophysiology—a novel liver-based non-motile ciliopathy, sometimes associated to nephronophthisis (45, 46); which our study also supports since we describe a novel variant. Still, despite the advances that new molecular technologies brought to this field, a word of caution is warranted as genetic studies can be misleading (e.g., case 8, Alagille syndrome, later unconfirmed), of difficult interpretation (e.g., case 5, co-existence of 3 variants in heterozygosity) or raise more questions instead of simply giving us answers (e.g., when parental testing is required for re-classification of variants which comes at increased costs). We did not offer NGS panel testing to patients with transient cholestasis but we did offer it to patients with transiently evolving cholestasis without risk factors, mainly searching for some treatable diseases such as Niemman-Pick type C (47) or citrin deficiency (48), and we had some surprising results requiring further clarification.

According to Feldman and Sokol (7), genetic evaluation will necessarily change the diagnostic paradigm and give rise to

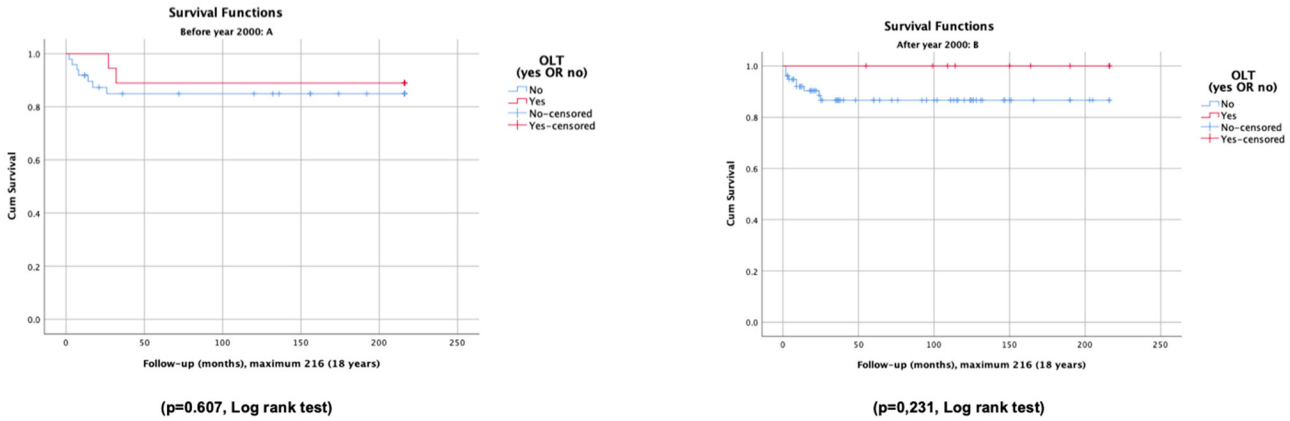

FIGURE 2 | Overall survival and survival without OLT of patients with neonatal cholestasis, before (A) and after (B) the year 2000.

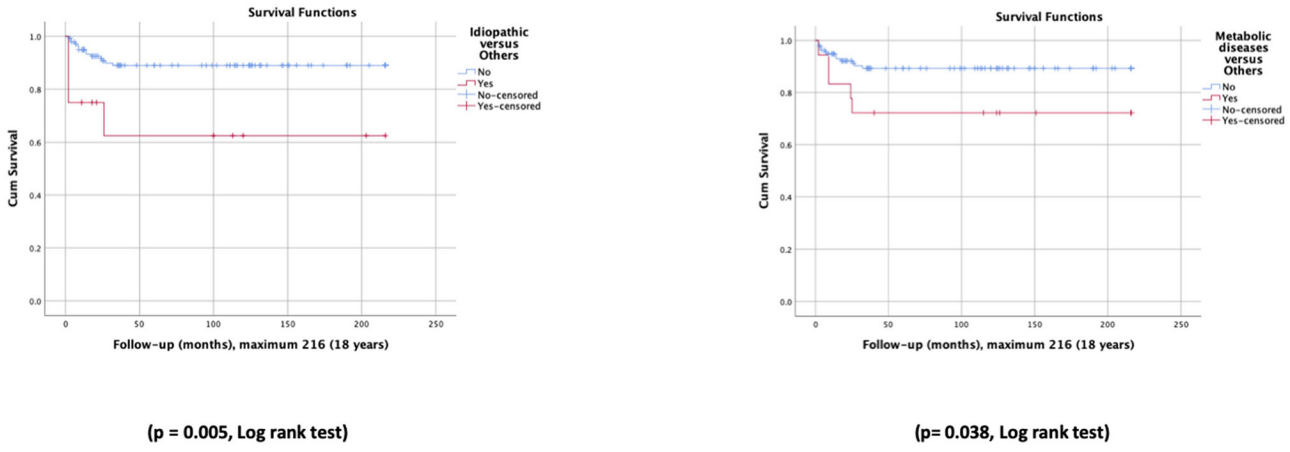

FIGURE 3 | Survival curves for patients with idiopathic cholestasis vs. other patients, and metabolic diseases vs. other patients.

current and emerging diagnosis algorithms that will certainly include this tool. From our experience, NGS panel should be used with caution and in selected cohorts. As we recently stated elsewhere (27), we believe that this tool must not be used as first-line, and should definitely not be used in the absence of skilled clinical guidance. Therefore, it does not make sense to talk about a paradigm shift, since any decision algorithm must always have clinical and biochemical markers. Additional work is needed to formally assess the cost-effectiveness of these studies and to understand what is their ideal place in the algorithm tree. In Portugal, we still need to improve on NGS panel availability and turn-around times that allow for diagnosis in a timely manner. We propose its use as previously described by Moreira da Silva et al. (27). In clinical practice we do not recommend transient cholestasis patients to be offered NGS panels. However, including these patients in research studies will be useful as they might contribute to a better understanding of the pathophysiology of NC. We highlight the fact that no positive genetic test will ever subside one of our greatest sources of anxiety—the exclusion of biliary atresia—since it can coexist with other entities.

Despite all the advances, NC remains globally associated with high mortality and morbidity (14). Our data is in line with this observation, as one third of the patients had an unfavorable outcome. In addition, a significant number of patients with "favorable outcome" presented mild chronic liver disease and their long-term outcome (adulthood) is uncertain. Mortality and morbidity rates are difficult to compare, due to the different follow-up periods in each study. However, the comparison of our findings with the study by Hoerning et al. (11) showed a similar mortality rate, but, contrary to this study, which found a higher mortality rate in biliary atresia (before and after OLT), in our study, metabolic diseases and idiopathic cases were the main responsible for mortality.

As far as we know, there are no publications with long-term survival studies, except in the subgroup of biliary atresia that has been well-studied. In biliary atresia, first the Kasai surgery (49, 50), then OLT (51, 52), and, more recently, in some countries the centralization of diagnosis and treatment (5, 6), and in others, universal newborn screening programs (53), produced advances with impact on overall survival and survival with native liver

(5, 54). Our study presents the survival curves of a cohort of 154 patients with NC for a period of nearly 35 years, namely overall survival and survival without OLT, as well as the comparison of these curves before and after the year 2000. We concluded that outcomes and survival did not improve significantly over time, and the number of patients who survived due to the benefits of OLT was not significant. Outcomes and survival varied with the underlying entities. In our study, patients with biliary atresia had long-term overall survival similar to other patients, and survival rates without OLT were not significantly lower. Metabolic diseases and idiopathic cholestasis showed significantly shorter survival curves than those of other patients.

Early recognition of cholestasis is fundamental to attain a better outcome (55). In our cohort, early recognition of cholestasis improved significantly over time. This was due to a continuous investment in the postgraduate training of pediatricians and pediatric nurses—in a pilot study developed locally that entailed a survey and photographs of normal and pale stools, these healthcare professionals obtained the best results (3). However, there is still room for improvement, especially at the level of primary healthcare services. Taking in to account the high morbidity and mortality, a universal screening program for neonatal cholestasis would be very useful; however, there are numerous underlying entities expressing at variable timings, which makes screening a very complex task. So far, only one screening method has been developed successfully: the stool color card that screens for biliary atresia. This screening tool as not been adopted in our country yet—but could easily be integrated into our Health Surveillance program of the Child and Adolescent. There are some promising studies using serum levels of conjugated bilirubin in the first days of life (56, 57) as a screening tool; these studies, if validated in larger populations, could point toward a screening method for other underlying entities in addition to biliary atresia. In our country, this parameter can also be easily added to the existing endocrine-metabolic screening program (23).

Other factors may interfere with the outcome of patients, such as the accuracy in the diagnosis of the underlying entities and the availability of specific medical and surgical treatments (10). In our center, patients had access to modern diagnostic techniques, as well as medical and surgical therapies according to international guidelines and to our institutional resources over time. After the year 2000, patients benefited from a qualified and stable surgical team, but the improvement in the success rate of Kasai surgery was not significant. Given the low number of cases/year, and according to data from other countries (6), since April 2019, the patients managed in our center were referred to perform Kasai surgery at the only pediatric liver transplant center in Portugal.

In summary, our data suggest that transient cholestasis became a very important etiology of NC, it can hide underlying entities, and requires specific guidance. The challenge on to what extent pursuing an etiological diagnosis still remains. The role played by risk factors in NC is far from clear. NGS panels can provide important inputs on disease diagnosis, but if applied without strict criteria and expertise they can open a Pandora's box due to misinterpretation. Despite all the advances in accurate diagnosis and timely management—including early recognition of cholestasis—the improvement in patient outcomes and survival were still not significant.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: The data are recorded in the patients' clinical files and in the hospital databases. In order to have access, it is necessary to ask for authorization from the Ethics Committee and the Board of Directors of the hospital. This authorization was requested and obtained to carry out this study. Requests to access these datasets should be directed to Ethics Committee, secretariado.etica@chporto.min-saude.pt, and Departement of Education, Training and Research (DEFI), secretariado.cg.defi@chporto.min-saude.pt.

ETHICS STATEMENT

This study, involving human participants, was analyzed and approved by the Ethics Committee (Comissão de Ética para a Saúde, CES) and by the Research Coordinating Office of the Department of Education, Training, and Research (Gabinete Coordenador da Investigação do Departamento de Educação, Formação, e Investigação, DEFI) and afterwards reviewed and approved by the board of administration (Conselho de Administração) of our hospital (Centro Hospitalar e Universitário do Porto, Portugal).

AUTHOR CONTRIBUTIONS

ES diagnosed and followed patients, designed the study, collected and analyzed data, and elaborated the draft of the manuscript. AA and SF diagnosed and followed patients, created databases, and collected and analyzed data. EM diagnosed and followed patients and collected data. MV analyzed data and critically reviewed the manuscript. AS-S and AL critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.2. EARLY ONSET LYSOSOMAL ACID LIPASE DEFICIENCY PRESENTING AS SECONDARY HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS: TWO INFANTS TREATED WITH SEBELIPASE ALFA.

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CASE REPORT

Early onset lysosomal acid lipase deficiency presenting as secondary hemophagocytic lymphohistiocytosis: Two infants treated with sebelipase alfa

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Abbreviations: ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; CESD, Cholesteryl ester storage disease; EO-LALD, Early onset lysosomal acid lipase deficiency; FHL, Familial hemophagocytic lymphohistiocytosis; HLH, Hemophagocytic lymphohistiocytosis; HSCT, Hematopoietic stem cell transplantation; rhLAL, Recombinant human lysosomal acid lipase; LDL, Lactic dehydrogenase; LAL-D, Lysosomal acid lipase deficiency; GGT, Gamma glutamyl transferase.

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KEYWORDS
Hemophagocytic lymphohistiocytosis;
Early onset lysosomal acid lipase deficiency;
Wolman disease;
Hepatosplenomegaly;
Neonatal cholestasis;
Sebelipase alfa

Summary Two unrelated infants were diagnosed with and initially treated for hemophagocytic lymphohistiocytosis (HLH), but progressed to cholestasis and liver failure. Early onset lysosomal acid lipase deficiency (EO-LAL-D) was suspected due to lymphocytes with cytoplasmic vacuolation and/or adrenal calcifications and confirmed by enzymatic and genetic analysis. Enzyme replacement therapy with sebelipase alfa was implemented, but both children died, despite initial improvement. Since this inborn error of metabolism progresses rapidly in infants, early diagnosis is crucial, and appropriate treatment should be started as soon as possible. The authors suggest that the diagnosis of EO-LAL-D should be considered in infants with symptoms of HLH.

Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a rare life-threatening disease, either congenital or acquired, that affects children of any age. It is characterized by hyperinflammatory features (prolonged fever, pancytopenia), progressive splenomegaly, biochemical features of liver injury, hypertriglyceridemia and hemophagocytosis found in bone marrow and other tissues [1]. Primary (congenital) HLH — familial hemophagocytic lymphohistiocytosis (FHL) — is caused by mutations in a few genes (*PRF1*, *MUNC 13-4*, *STX 11*) and affects mainly infants, while the secondary forms of HLH may be associated with malignancy, infections, rheumatologic disorders and some metabolic diseases [1,2], including early onset lysosomal acid lipase deficiency (EO-LAL-D) [3—7].

EO-LAL-D, also referred to as Wolman disease, is a rare (1/500,000), autosomal recessive, lysosomal storage disease caused by mutations in the *LIPA* gene and leading to nearly null lysosomal acid lipase activity [8]. This most severe form of LAL-D presents in the first weeks of life with failure to thrive, progressive hepatosplenomegaly, dyslipidemia, liver dysfunction, and adrenal calcifications; it leads to death within the first year of life [9]. Another form of LAL-D, known as cholesteryl ester storage disease (CESD), affects both children and adults (1/40,000). CESD is less severe due to only partial deficiency of lysosomal acid lipase activity [10,11].

Historically, the only treatment for Wolman disease was allogeneic hematopoietic stem cell transplantation (HSCT), which has seen variable success [9,12—14]. Sebelipase alfa (Kanuma®, Alexion Pharmaceuticals, New Haven, CT, USA), a recently developed recombinant human lysosomal acid lipase (rhLAL), has demonstrated safety and efficacy in several clinical trials performed in children and adults [15—18] and more recently in infants [19].

We describe two infants treated with sebelipase alfa after a delayed diagnosis of EO-LAL-D, presenting as secondary HLH that was initially misdiagnosed as FHL.

Case report — 1

A Portuguese male infant, the second son of first cousins of Roma ethnicity, was born by normal delivery after a 38-week gestation (weight: 3340 g, percentile: 53, score z: +0.07; length: 51 cm, percentile: 48, score z: −0.04; head circumference: 34.5 cm, percentile: 39, score z: −0.29). He was exclusively breastfed, with good tolerance and normal growth. He did not have neonatal jaundice.

At two months, he was diagnosed with bronchiolitis to syncytial respiratory virus. At day seven of symptoms, he was hospitalized for hypoxemia. Fever recurred and he had hepatosplenomegaly, anaemia and elevated serum concentration of ferritin and triglycerides. Levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactic dehydrogenase (LDH), C-reactive protein and procalcitonin were elevated, while haemostasis and fibrinogen were normal (Table 1). He was transferred to a paediatric oncology division. A bone marrow smear showed hemophagocytosis. Due to suspected HLH (six of eight diagnostic criteria fulfilled), he received treatment according to the HLH 2004 protocol [19], including cyclosporine and dexamethasone IV twice daily, etoposide IV once weekly, and gamma globulin IV once monthly.

The infant showed intermittent improvement of HLH parameters, including disappearance of fever. He was exclusively breastfed, without vomiting or diarrhoea, and then began to fail to thrive. Breast milk was also provided by a nasogastric tube. In addition, supplements of maltodextrin, medium chain triglycerides and fat-soluble vitamins were administered. After six weeks, he developed cholestatic jaundice; one month later, he had a voluminous hepatosplenomegaly and coagulopathy. Meanwhile, molecular analysis excluded FHL caused by the known gene mutations.

The peripheral blood smear showed lymphocytes with cytoplasmic vacuolation (Fig. 1). An abdominal X-ray showed adrenal calcifications. LAL activity tested from a dried blood spot sample by an international reference laboratory (Biochemistry Department, Queen Elizabeth University Hospital, Glasgow) was 0.05 nmol/punch/h, (normal range: 0.37—2.30). A diagnosis of EO-LAL-D was suggested, and the patient was transferred to our Gastroenterology Unit; HLH protocol treatment was discontinued, except for steroids. Since this enzyme activity level was considered high compared to other EO-LAL-D previously diagnosed by the same laboratory, LAL activity in fibroblasts was also tested by a local laboratory and was 1.0 nmol/mg/h protein (normal range: 459—2050). Molecular PCR analysis by direct sequencing revealed a variant, not reported at the time [*LIPA*] c.966 + 2T > G-intron 9 (in homozygosity), that affects a splice site and consequently mRNA processing. This

Table 1 Clinical, haematological and biochemical parameters of both patients at presentation.

	Patient 1	Reference values	Patient 2	Reference values
Signs and symptoms of Wolman disease				
Age of onset: early infancy	2 months		4 months	
Splenomegaly	Yes		Yes	
Hepatomegaly	Yes		Yes	
Failure to thrive	Yes		Yes	
Clinical findings				
Anemia	**6,3**	9,5—13,5 (g/dL)	**8,4**	10,0—13,0 (g/dL)
Hypersplenism	No		Yes	
Hypertriglyceridemia	**785**	44—150 (mg/dL)	**539**	< 150 (mg/dL)
Lymphocytes with vacuoles	Yes		No	
Hypoalbuminemia	**29,0**	38—53 (g/L)	**19,0**	38,0—54,0 (g/L)
Additional examinations				
Adrenal calcifications (US/X-ray)	Yes		Yes	
Bone marrow biopsy: haemophagocytosis described in a few patients in publications [16,17]	Yes		Yes	
Extremely decreased activity of LAL in DBS test	Yes		Yes	
Diagnostic criteria of HLH				
Fever lasting > 7 days	Yes		No	
Splenomegaly	Yes		Yes	
Cytopenias				
Hb < 9 g/dL[a]	**6,3**	9,5—13,5 (g/dL)	**8,4**	10,0—13,0 (g/dL)
Platelets < 100×10^9	318×10^9	$150—400 \times 10^9$	**117** $\times 10^9$	$150—250 \times 10^9$
Neutrophiles < 1000×10^9	6120×10^9	$1000—10000 \times 10^9$	8500×10^9	$1000—10000 \times 10^9$
Fasting TG >265 mg/dL or low fibrinogen < 1,5 g/L	**785**	44—150 (mg/dL)	**539**	< 150 (mg/dL)
Low fibrinogen < 1,5 g/L	2,5	2,0—4,0 g/L	3,18	1,90—3,60 g/L
Hemophagocytosis found in bone marrow, lymphnode or spleen	Yes		Yes	
High ferritin ≥ 500 μg/L	**19867**	6—320 μg/L	**21577**	4,63—204 mg/dL
Soluble CD25 ≥ 2400 U/mL	**2879**	158—623 UI/L	**2538**	< 500 UI/mL
Low or absent activity of NK cells	Not tested		No	
Other laboratory abnormalities commonly found in HLH				
Hypoalbuminemia (g/L)	**29,0**	38—53 (g/L)	**19,0**	38,0—54,0 (g/L)
High LDH (IU/L)	**3140**	67—248 UI/L	**3300**	< 451 UI/L
Elevated AST and ALT (IU/L)	**299/68**	39/42 UI/L	**540/130**	84/60
Age at				
Diagnosis of HLH	2 months		4 months	
Diagnosis of LAL-D	4 months		5 months	
Death	5 months		6 months	

US: ultrasound; LAL: lipase acid lisosomal; DBS: dried blood spot; HLH: hemophagocytic lymphohistiocytosis; TG: triglycerides; LDH: lactic dehydrogenase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; the bold was used to highlight the abnormal values.

[a] In newborns < 10 g/dL.

prediction was made using in silico tools and was thus presumed to be the cause of LAL-D in this infant. More recently, this mutation was also described by Ruiz-Andres et al. [20].

A single dose of sebelipase alfa (Kanuma) 1 mg/kg IV, was administered at five months and eight days of age, with no side effects. A slight improvement in the intensity of cholestasis and coagulopathy was noted, but over the next few days the infant's condition deteriorated. He died seven days later of multi-organ failure.

Case report — 2

A Polish female infant, born after a normal 40-week pregnancy (birth weight: 2600 g, percentile: 2, score z: −2.07), developed hepatosplenomegaly, vomiting and failure to thrive by four months (weight: 5300 g, percentile: 16, score z: −0.09). Early symptoms of the disease, such as large belly and progressive umbilical hernia, had appeared at two months.

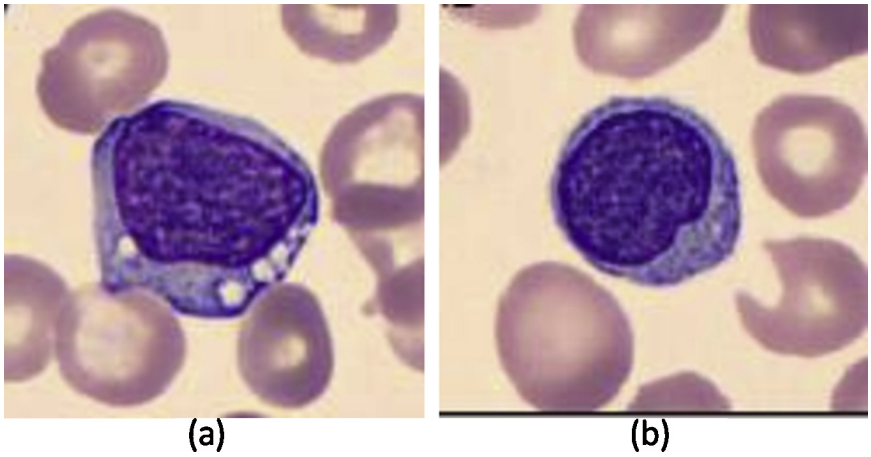

Figure 1 Peripheral blood film; a: lymphocyte with cytoplasmic vacuolation in patient 1; b: normal lymphocyte. Cellavision® DM96 analysis. Blood film, MGG stain.

She presented at the regional university hospital with anaemia, conjugated hyperbilirubinemia, elevated activity of aminotransferases, GGT and LDH, severe coagulopathy, hyperferritinemia, hypertriglicerydemia and hypercholesterolemia (Table 1). Hemophagocytosis was found in the bone marrow smear, and elevated sCD25 was noted. At that point, she met five of eight diagnostic criteria for HLH, although fever was missing. The child received intravenous dexamethasone and cyclosporine A, according to the HLH 2004 treatment protocol [21], but showed no improvement. Because of progressive liver disease and profound coagulopathy, with the suspicion of acute liver failure due to HLH, she was admitted to our Department of Paediatric Gastroenterology.

Immunologic investigation revealed normal cytotoxic activity of NK cells and perforin expression. FHL and other primary immunodeficiencies presenting as HLH were excluded. Abdominal ultrasound showed calcifications of adrenal glands. LAL activity tested from a dried blood spot sample by a local laboratory, was very low: 0.3 nmol/punch/h (normal range: 8.7—34.4 nmol/punch/20 h). Based on suspicion of HLH secondary to EO-LAL-D, cyclosporine A was stopped, although steroids were maintained to treat symptoms of hyper-inflammation, partial parenteral nutrition and 20% albumin were given to treat malnutrition and sebelipase alfa was started as bridging treatment for HSCT, since the patient had a perfectly matched sibling donor.

After initial improvement achieved after two doses of sebelipase alfa (1 mg/kg/dose once weekly), coagulopathy and cholestasis progressed, and the child's general condition continued to worsen. We considered this a contra-indication for HSCT. Despite six doses of sebelipase alfa once weekly, as following: 1 mg/kg IV (two weeks), 3 mg/kg IV (two weeks), and 5 mg/kg IV (two weeks), the patient died at the age of 6 months of age due to cardiopulmonary insufficiency in the course of chronic liver failure. Molecular analysis of the *LIPA* gene identified two mutations in compound heterozygous state: c.509C > A(p.S103R)/c.796G > T(p.G266X), both known to be pathogenic.

Discussion

Both reported patients presented with at least five diagnostic criteria of HLH [21]; FHL seemed the most likely diagnosis because they were infants. Interestingly, patient 2 had no fever, which is a cardinal symptom of FHL [1,21].

Secondary HLH has been described in association with EO-LAL-D in infants [3—7]. All had adrenal calcifications, a highly suggestive feature of EO-LAL-D [8], and some had lymphocytes with cytoplasmic vacuolation in peripheral blood smear, a suggestive feature of metabolic disease [22]. All were treated with support therapies and/or chemotherapy, and all developed cholestasis and/or liver failure and died before six months of age. Adrenal calcifications were detected in both our patients. Additionally, in patient 1, the peripheral blood smear showed lymphocytes with cytoplasmic vacuolation. These findings, if actively searched for, could have led to an earlier correct diagnosis.

The pathophysiology of the various forms of secondary HLH is not fully understood. Multiple factors, including genetic predisposition and infectious triggers, are considered important contributors to final macrophage activation. Taurisano et al. [6] suggested that in EO-LAL-D the tendency to cholesteryl esters form crystals and their ability to stimulate activation of macrophage-mediated inflammation could be a trigger of sustained acquired HLH, which could then be perpetuated by cytokines and ferritin macrophage production.

Diagnosis was confirmed in both patients by enzymatic and genetic analysis. The mutation found in patient 1 (of Roma ethnicity) was recently reported as frequent in the Spanish population [20]. Ruiz-Andres et al. reported 23 patients (10 of Roma ethnicity), of which 13 had EO-LAL-D. The novel splicing mutation c.966 + 2T > G in intron 9 was detected in 75% (20/26) of the Wolman patient alleles, always in homozygosity. Using in silico tools, Ruiz-Andres et al. predicted the disruption of the correct slicing process and presumably an insertion or a deletion of some amino acids in the protein, but studies on cDNA need to be performed to know the exact effect it causes. From the clinical

point of view, patients presenting this mutation in homozygosity did not show differential trait than other EO-LAL-D patients with other mutations. Haplotype analysis showed that the novel c.966 + 2T > G mutation also co-segregated with a unique haplotype and was presumed to be transmitted by a common ancestor [20]. Both mutations found in patient 2 are known to be pathogenic; one of them causes a very severe course of the disease [23].

HSCT seems to be the only cure for FHL [24]; it has occasionally been attempted to treat EO-LAL-D, with variable success and complications like veno-occlusive disease [9,12—14]. Recently, enzyme replacement therapy (ERT) became available through the development of a recombinant human lysosomal acid lipase (rhLAL) that catalyses the hydrolysis of esters of cholesterol and triglycerides to free cholesterol, free fatty acids and glycerol [15]. Its safety and eficacy have been demonstrated in clinical trials involving children and adults affected with CESD [15—18] and very recently in infants with EO-LAL-D [19]. Jones et al. performed an open-label single-arm study with nine infants (aged 1—6 months) in comparison to a historical control group ($n = 21$) with similar clinical presentation. Six sebelipase alfa-treated infants survived to 12 months of age, compared with none in the historical control group. Survivors exhibited improvements in weight-for-age, reductions in markers of liver dysfunction and hepatosplenomegaly and a decrease in anaemia and gastrointestinal symptoms. Three deaths occurred early (in the first few months of life), two because of advanced disease. A fourth death occurred at 15 months of age and was related to other clinical conditions. Five patients have survived to age ≥ 24 months with continued sebelipase alfa treatment; all have displayed marked improvement in growth parameters and liver function.

Both of our patients were initially treated for HLH; the disease was very advanced when ERT was started. We did not observe significant infectious complications that could be attributed to the HLA treatment and thus be responsible for the deterioration of the patients' clinical condition. On the other hand, dexamethasone alone may be considered appropriate for treating inflammatory hyperactivity in secondary HLH. However, the clear message we want to send with these case descriptions is that HLH secondary to EO-LAL-D cannot be treated as HLH alone. If the EO-LAL-D diagnosis had been made sooner, ERT could have been started earlier, and perhaps our patients would have had a better outcome.

At the time, the Polish patient was treated with ERT as a bridge for HSCT, since she had a perfectly matched sibling donor [25]; ERT was seen as able to improve liver function and nutritional status in the pre-transplant stage and to keep the concentration of LAL stable, optimizing patient outcome post-transplant. Currently, with the evidence on the efficacy and safety of ERT demonstrated by the study of Jones et al. [19], it is our opinion that HSCT can no longer be considered as a first-line therapy. It should be considered as a second-line option with a less favourable risk—benefit ratio, although when successful it can achieve remission of the disease in the long term [13,14].

Although EO-LAL-D is a rare disease, its true prevalence is unknown, and evidence suggests that it may be considerably under- or misdiagnosed [26]. It can be quickly and accurately diagnosed using an inexpensive, recently developed enzymatic blood test to determine LAL activity [27], and, since it is a rapidly progressing disease, early diagnosis is crucial. The inclusion of EO-LAL-D in the neonatal screening program would likely not be cost-effective due to the extreme rarity of the condition and because diagnosis cannot be made by adding a parameter to the tandem mass spectrometry work-up.

We propose that EO-LAL-D be considered in any infant presenting with marked failure to thrive, massive hepatosplenomegaly, abnormalities in lipoproteins and neonatal cholestasis with rapidly progressive liver disease, including those that fulfil the HLH criteria. We recommend that searching for lymphocytes with cytoplasmic vacuolation in peripheral blood smear and imaging of the adrenal glands be part of the work-up of infants with HLH. The possibility of an EO-LAL-D diagnosis should be considered in these patients.

Disclosure of interest

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3.1. NEONATAL CHOLESTASIS: DEVELOPMENT OF A DIAGNOSTIC DECISION ALGORITHM FROM MULTIVARIATE PREDICTIVE MODELS

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ORIGINAL ARTICLE

Neonatal cholestasis: development of a diagnostic decision algorithm from multivariate predictive models

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Abstract

Despite the recent advances involving molecular studies, the neonatal cholestasis (NC) diagnosis still relays on the expertise of medical teams. Our aim was to develop models of etiological diagnosis and unfavourable prognosis which may support a rationale diagnostic approach. We retrospectively analysed 154 patients born between January 1985 and October 2019. The cohort was divided into two main groups: (A) transient cholestasis and (B) other diagnosis (with subgroups) and also in two groups of outcomes: (I) unfavourable and (II) favourable. Multivariate logistic regression analysis identified the lower gestational age as the only variable independently associated with an increased risk of transient cholestasis and signs and/or symptoms of sepsis with infectious or metabolic diseases. Gamma-glutamyl transferase serum levels > 300 IU/L had a positive predictive value for both diagnosis of biliary atresia and for alpha-1-antitrypsin deficiency (A1ATD) and for unfavourable prognosis. A model of diagnosis for A1ATD (n = 34) showed an area under the ROC curve = 0.843 [confidence interval (CI): 0.773–0.912].

Conclusion: This study identified some predictors of diagnosis and prognosis which helped to build a diagnostic decision algorithm. The unusually large subgroup of patients with A1ATD in this cohort emphasizes its predictive diagnostic model.

Keywords Alpha-1-antitrypsin deficiency · Biliary atresia · Diagnostic decision algorithm · Multivariate prediction models · Neonatal cholestasis · Transient neonatal cholestasis

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What Is Known
- *The etiological diagnosis of neonatal cholestasis (NC) requires a step-by-step guided approach, and diagnostic models have been developed only for biliary atresia.*
- *Current algorithms neither address the epidemiology changes nor the application of the new molecular diagnostic tools.*

What Is New
- *This study provides diagnostic predictive models for patients with A1ATD, metabolic/infectious diseases, and transient cholestasis, and two models of unfavourable prognosis for NC.*
- *A diagnostic decision algorithm is proposed based on this study, authors expertise and the literature.*

Introduction

Neonatal cholestasis (NC) is the form of expression of many types of liver damage and many underlying entities, which makes etiologic diagnosis a very complex issue. Although it is recognised that no guideline can replace the skills of a specialised clinical team, over time there have been several attempts to respond to this challenge with the construction of diagnostic tools, the most recent being the recommendations of Fawaz et al. [1].

In the last few decades, there is some evidence that epidemiology has changed, with the loss of relevance of some entities and the emergence of others [2, 3]. Transient cholestasis, among the emerging ones, is a diagnosis of exclusion [4], whose patients can sometimes suffer from underlying entities bringing additional challenges, such as when and how far to investigate. In addition, some new diagnostic tools are available that need integration into clinical practice (e.g. molecular next generation sequencing (NGS) technologies) [5]. NGS panels, if used judiciously, can be an important diagnostic asset, but they can also be misleading and must not be in the first line of approach [3, 6, 7].

Our hypothesis is that clinical and biochemical markers are far from exhausted and can be explored in innovative ways. So far there have been developed some predictive models of diagnosis only for biliary atresia [8–10]. The aim of this study was to develop models of diagnosis and prognosis, by finding clinical and biochemical predictors at admission to support the design of a diagnostic algorithm for patients with NC.

Methods

Participants and study design

Retrospective study of one hundred and sixty patients with NC diagnosed and treated at a tertiary Portuguese centre over ~ 35-year period (January 1, 1985, to October 31, 2019). The study was based on the analysis of clinical charts and institutional database records. We excluded patients with incomplete clinical records.

Patients were referred from institutional departments of neonatology, emergency, and general paediatrics, but also from other institutions (primary and secondary healthcare providers). Our centre is one of the two tertiary paediatric centres within the city of Oporto, serving a population of approximately 2 million people. After January 1, 2000, the department of neonatology reported 45,211 live-births (3901 preterm; 341 preterm ≤28-week gestation) and 5243 neonatal intensive care unit admissions.

We divided our sample into two main groups: (A) patients with transient cholestasis and (B) other diagnosis. The latter was further divided according to the underlying entities: biliary diseases, alpha-1-antitrypsin deficiency (A1ATD), infectious diseases, metabolic diseases, other diseases, and idiopathic cholestasis.

In addition, we also divided the sample into two groups of outcomes: (I) unfavourable (death, orthotopic liver transplantation and/or portal hypertension) or (II) favourable (cured or mild liver disease). The classification of "mild liver disease" required a minimum follow-up of 5 years (regardless of the diagnostic subgroup), and an age-related criterion was established as follows: 5 years and 10 years of age. native liver, normal liver tests, and no clinical, ultrasound or endoscopic signs of portal hypertension; 18 years of age, native liver and no short-term transplant forecast (see Online Resource 1 which shows a participant flow diagram).

Clinical data, diagnostic approach, and treatment

We collected demographic data (age, sex, and ethnic background), past medical history, and clinical and laboratory data. Clinical and biochemical variables at admission were studied for the purpose of diagnostic and prognostic value (see also Online Resource 2 which shows the list of those variables).

NC was defined as jaundice with conjugated bilirubin ≥ 1.0 mg/dL (and > 20% if total bilirubin > 5.0 mg/dL) detected in a newborn or infant up to 4 months old [11]. Transient cholestasis was the presence of cholestatic jaundice with known risk factors and complete and spontaneous normalisation of liver function tests within the first 6 months of life [4]. Risk factors were those described by Champion et al., but also sepsis and asphyxia among others [12, 13]. Signs and symptoms of sepsis were moaning, lethargy, hypotonia, fever, and/or hypothermia. Signs and symptoms of liver failure were coagulopathy not correctable by vitamin K administration, ascites, and hypoglycaemia in accordance to the Paediatric Acute Liver Failure study group [14]. Diagnostic approach

methodology was carried out by the same team over the years, progressed according to international guidelines [1, 11] and the team's experience, and was previously detailed by our group [3]. NGS panel targeting inborn errors of metabolism and genetic cholestatic disorders was available in mid-2017, initially comprising fifty-four genes and currently ninety-five genes, and was applied with the following criteria: idiopathic cases, suspicion of a second underlying entity, and cholestasis evolving transiently without risk factors.

Statistical analysis

Categorical variables were described as absolute frequencies (n) and relative frequencies (%). Median and percentiles were used for continuous variables. When testing a hypothesis about continuous variables, nonparametric tests were used as appropriate, taking in to account normality assumptions and the number of groups compared. When testing a hypothesis about categorical variables, a chi-square test and Fisher's exact test were used, as appropriate. The time elapsed from diagnosis to the last time of follow-up was evaluated using survival analysis: the cumulative probabilities of event-free survival were estimated using the Kaplan-Meier method and the logrank. Logistic regression was applied to determine the relationship between some clinical and demographical factors and some subgroups of diagnosis or unfavourable outcome using Enter approach. For goodness of models fit, a Hosmer-Lemeshow test was computed. The significance level used was 0.05. Statistical analysis was performed using the software Statistical Package for the Social Sciences v. 24.0.

Results

We analysed one hundred and fifty-four patients (six were excluded due to insufficient data) with a median age at admission of 54 days (Interquartile (IQ): 30–75 days) and a male preponderance (n = 92, 59.7%).

We identified twenty-four patients with transient cholestasis (group A) and the other one-hundred and thirty (group B) were subdivided according to underlying entities: biliary diseases (n = 34), A1ATD (n = 34), infectious diseases (n = 20), metabolic diseases (n = 18), other diseases (n = 12), and idiopathic cholestasis (n = 12) (see Online Resource 3 which shows a detailed description of the underlying entities). The A1ATD phenotypes were the following: ZZ (n = 29), SZ (n = 3), FZ (n = 1), and M1Z (n = 1).

The majority of patients with transient cholestasis were referred after the year 2000, as follows: before the year 2000 (n = 4; 16.7%) and after the year 2000 (n = 20; 83.3%) (see Online Resource 4 which shows the comparison of clinical and biochemical features at admission between transient cholestasis and "other diagnosis", and Online Resource 5 which shows the comparison of overall survivals; see also Online Resource 6 which shows the comparison between some subgroups of "other diagnosis").

NGS panel testing was applied to thirteen patients. The results were enlightening in a case of neonatal sclerosing cholangitis. In the remaining cases, NGS testing was unrevealing as it found genetic variants of unknown/uncertain significance. Further details concerning the results of the NGS panel were recently published by our group [3].

Subgroups of outcomes were as follows: unfavourable (n = 47) and favourable (n = 103). We have excluded from outcome analysis four patients (biliary atresia n = 2, A1ATD n = 2) with early predictors of favourable outcome for their diseases, but without a minimum follow-up of 5 years (see Online Resource 7 which shows a comparison of clinical and biochemical features at admission between favourable and unfavourable outcome).

Variables at admission with diagnostic predictive value

Univariate and bivariate analysis

Gestational age and birth weight were significantly lower in transient cholestasis, whilst other risk factors such as parenteral nutrition > 7 days, sepsis, hypoxia (invasive ventilation), and haemodynamic instability were also significantly more associated with transient cholestasis.

Signs and/or symptoms of liver failure were only significantly more associated with infectious or metabolic diseases (42.1% versus 8.6%; p = < 0.001).

Pale stools were significantly more frequent in biliary diseases (79.4% versus 12.5%; p = < 0.001), but not in other subgroups of underlying entities, namely, the A1ATD subgroup (30.6% versus 29.2%; p = 0.391).

Aspartate aminotransferase (AST) was significantly lower in transient cholestasis (76 IU/L versus 138 IU/L;p = 0.005) but not alanine aminotransferase (ALT) or AST/ALT ratio.

Gamma glutamyl transferase (GGT) serum levels were significantly higher in the subgroups of biliary diseases (median: 769 IU/L; IQ: 400-1219 IU/L;p = <0.001) and A1ATD (median: 591 IU/L; IQ: 351–1075 IU/L;p = <0.001). In contrast, infectious or metabolic diseases were associated with significantly lower GGT serum levels (median: GGT 171 IU/L; IQ: 95-320 IU/L;p = 0.004). GGT serum levels were also significantly lower in transient cholestasis.

GGT had positive predictive value for the diagnosis of biliary diseases (area under the ROC (receiver operator characteristic) curve (AUC) = 0.773 (confidence interval (CI): 0.688–0.858)); sensibility: 85.3%; specificity: 60.5%; cut-off value = 325 IU/L), biliary atresia (AUC = 0.793 (CI: 0.715–0.870); sensibility: 91.7%; specificity: 64.3%; cut-off value = 381 IU/L), and for A1ATD (AUC = 0.709 (CI: 0.621–0.797);

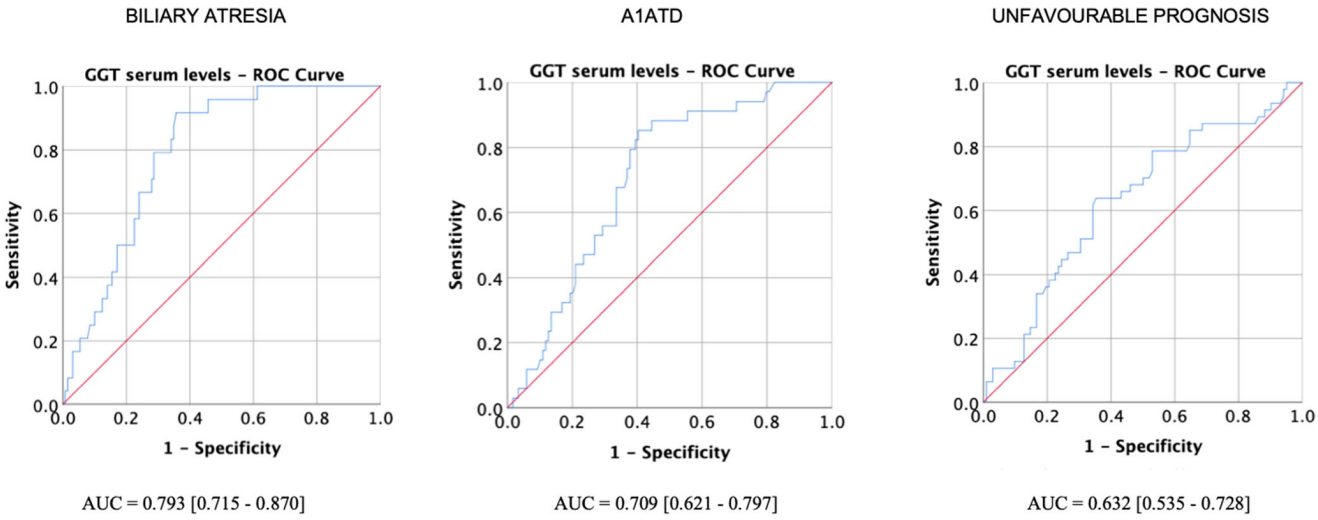

Fig. 1 GGT serum levels predictive value (ROC curves) *GGT* gamma glutamyl transferase, *A1ATD* alpha-1-antitrypsin deficiency, *ROC* receiver operator characteristic, *AUC* area under the roc curve

sensibility: 85.3%; specificity: 59.7%; cut-off value = 321 IU/L) (see Fig. 1, which demonstrates the ROC curves of GGT serum levels for biliary atresia and for A1ATD).

Multivariate analysis

A multivariate logistic regression model was performed for each of the various subgroups of diagnosis of underlying entities of NC. In addition, among the biliary diseases, we made a separate model for patients with biliary atresia.

We observed that a low birth weight, pale stools and GGT serum levels > 300 IU/L were associated with the diagnosis of biliary diseases. The same variables were even significantly more associated with the diagnosis of biliary atresia. The presence of prematurity, pale stools, signs and/or symptoms of liver failure, and GGT serum levels > 300 IU/L were also associated with the diagnosis of A1ATD. Signs and symptoms of sepsis were independently associated with a greater risk of infectious and metabolic diseases. The gestational age was the only variable independently associated with the risk of the diagnosis of transient cholestasis (see Table 1, and Figs. 2a and b).

Variables at admission with prognostic predictive value

Univariate and bivariate analysis

Gestational age, but not birth weight, was significantly associated with the outcome with term infants having a significantly more unfavourable outcome (p = <0.001). The absence of parenteral nutrition for >7 days was associated with an unfavourable outcome (p = 0.002). Signs and symptoms of sepsis and/or liver failure were not significantly associated with the outcome. Pale stools were significantly associated with an unfavourable outcome (p = 0.003).

Higher total and conjugated bilirubin serum levels were significantly associated with an unfavourable outcome. Unconjugated bilirubin serum levels and the AST/ALT ratio were not significantly associated with the outcome (see Online Resource 7).

The GGT serum levels were significantly different between the two groups of outcomes: unfavourable (median = 528 IU/L (IQ: 200–960)) and favourable (median = 255 IU/L (IQ: 99–644)); p = 0.010). GGT had a positive predictive value for an unfavourable outcome (AUC: 0.632 (CI: 0.535–0.728); sensibility: 51.1%; specificity: 69.6%; cut-off value = 525 IU/L; see Fig. 1, which demonstrates the ROC curve of GGT serum levels for unfavourable prognosis).

Multivariate analysis

Two multivariate logistic regression models, differentiated by the GGT serum levels, were performed for the unfavourable outcomes. In model A, total bilirubin, unconjugated bilirubin, and GGT serum levels < 100 IU/L were associated with an unfavourable outcome. In model B, total bilirubin, unconjugated bilirubin, and GGT serum levels > 300 IU/L were associated with an unfavourable outcome (see Table 2 and Fig. 3).

Discussion

NC represents a complex diagnostic challenge, mainly because it may not be readily identified, and the etiological diagnosis is broad. The build of a diagnostic guidance algorithm plays an important role on the first approach, although it is recognised that it will not replace specialised teams. Over

Table 1 Multivariate regression logistic models for underlying entities diagnosis

	Biliary diseases (n = 34)				Biliary atresia (n = 24)				A1ATD (n = 34)			
	p value	OR	95% CI for OR		p value	OR	95% CI for OR		p value	OR	95% CI for OR	
Gestational age												
Term	Ref				Ref				Ref			
Preterm	0.168	0.330	0.068	1.598	0.087	0.127	0.012	1.347	0.047	0.244	0.061	0.979
Birth weight												
Adequate	Ref				Ref				Ref			
Small	0.015	0.154	0.034	0.695	0.048	0.163	0.027	0.988	0.498	1.424	0.512	3.957
Big	-	-	-	-	-	-	-	-	0.678	1.996	0.076	52.217
Failure to thrive[1]	0.232	0.423	0.103	1.735	0.137	0.279	0.052	1.498	0.510	1.474	0.465	4.670
Pale stools[1]	< 0.001	14.627	4.981	42.952	< 0.001	34.978	6.834	179.035	0.001	0.164	0.055	0.485
Symptoms and/or signs of liver failure[1]	0.659	0.618	0.073	5.237	0.619	2.080	0.116	37.388	0.030	0.081	0.008	0.784
GGT serum levels (> 300 IU/L)	0.045	3.688	1.029	13.219	0.038	6.685	1.108	40.354	< 0.001	10.882	3.315	35.721

	Infectious/metabolic diseases (n = 38)				Transient cholestasis (n = 24)			
	p value	OR	95% CI for OR		p value	OR	95% CI for OR	
Parental consanguinity[1]	0.986	0.983	0.149	6.474				
Affected siblings[1]	0.610	0.610	0.091	4.076	Gestational age (weeks)	0.017	0.680	0.496	0.933
Failure to thrive[1]	0.051	2.594	0.995	6.764	Birth weight (grams)	0.945	1.000	0.999	1.002
Signs and/or symptoms of sepsis[1]	0.002	7.126	2.095	24.236	Signs and/or symptoms of sepsis	0.266	0.271	0.027	2.709
Signs and/or symptoms of liver failure[1]	0.966	0.960	0.152	6.086	and/or liver failure[1]				
Signs and/or symptoms of neurological involvement[1]	0.080	3.247	0.869	12.133					
Ratio AST/ALT	0.856	1.049	0.623	1.767	Organomegaly[1]	0.163	0.385	0.101	1.472
GGT serum levels (≤ 100 IU/L)	0.867	1.096	0.740	3.210	GGT serum levels (≤ 300 IU/L)	0.152	2.758	0.689	11.040
Prothrombin time[2]	0.681	0.696	0.124	3.907					

A1ATD, alpha-1-antitrypsin deficiency; *OR*, odds ratio; 95% CI, 95% confidence interval; *GGT*, gamma glutamyl transferase; [1] the reference category is "NO"; [2] the reference category is "ABNORMAL". Hosmer-Lemeshow test: biliary diseases (p = 0.989); biliary atresia (p = 0.871); A1ATD (p = 0.605); infectious/metabolic diseases (p = 0.541); and transient cholestasis (p = 0.470)

time, several attempts have been made; however, some algorithms are complex, whilst others do not detail the approach of all emerging and urgent entities or do not address transient cholestasis.

The most recent guideline by the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) and North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) [1] does not include a diagnostic algorithm and the role of new technologies is not addressed, while recent proposals by some authors are not always sufficiently comprehensive [7, 15, 16]. Traditionally, the choice of discriminators was based on the experience/opinion of experts (levels of evidence 4 and 5) instead of studies establishing their predictive diagnostic value. More recently, the application of advanced statistical tools made it possible to establish some predictive models of diagnosis, but only for biliary atresia [8–10]. We applied those tools, and based on our data and expertise, we give our contribution to a diagnostic decision algorithm (Fig. 4 [17]).

Biliary atresia is the most frequent cause of NC [18] and of liver transplant in pediatric age [19]. Shneider et al. [9] identified thirteen features that were significantly different (GGT, pale stools, and birth weight as the best predictors), and using a stepwise logistic regression model, they identified seven factors in a predictive model that yielded an 81% true positive rate for biliary atresia, and 0.2 (n = 120) yielded an 11% false negative rate. In their study, GGT serum levels were not stratified and bilirubin serum levels were not significantly different. In our study, we identified some variables significantly associated with biliary diseases and biliary atresia. After applying univariate and bivariate analysis, we identified two predictors, pale stools and GGT serum levels, and a multivariate logistic regression model added the variable, birth weight (small for gestational age with significantly lower OR). As

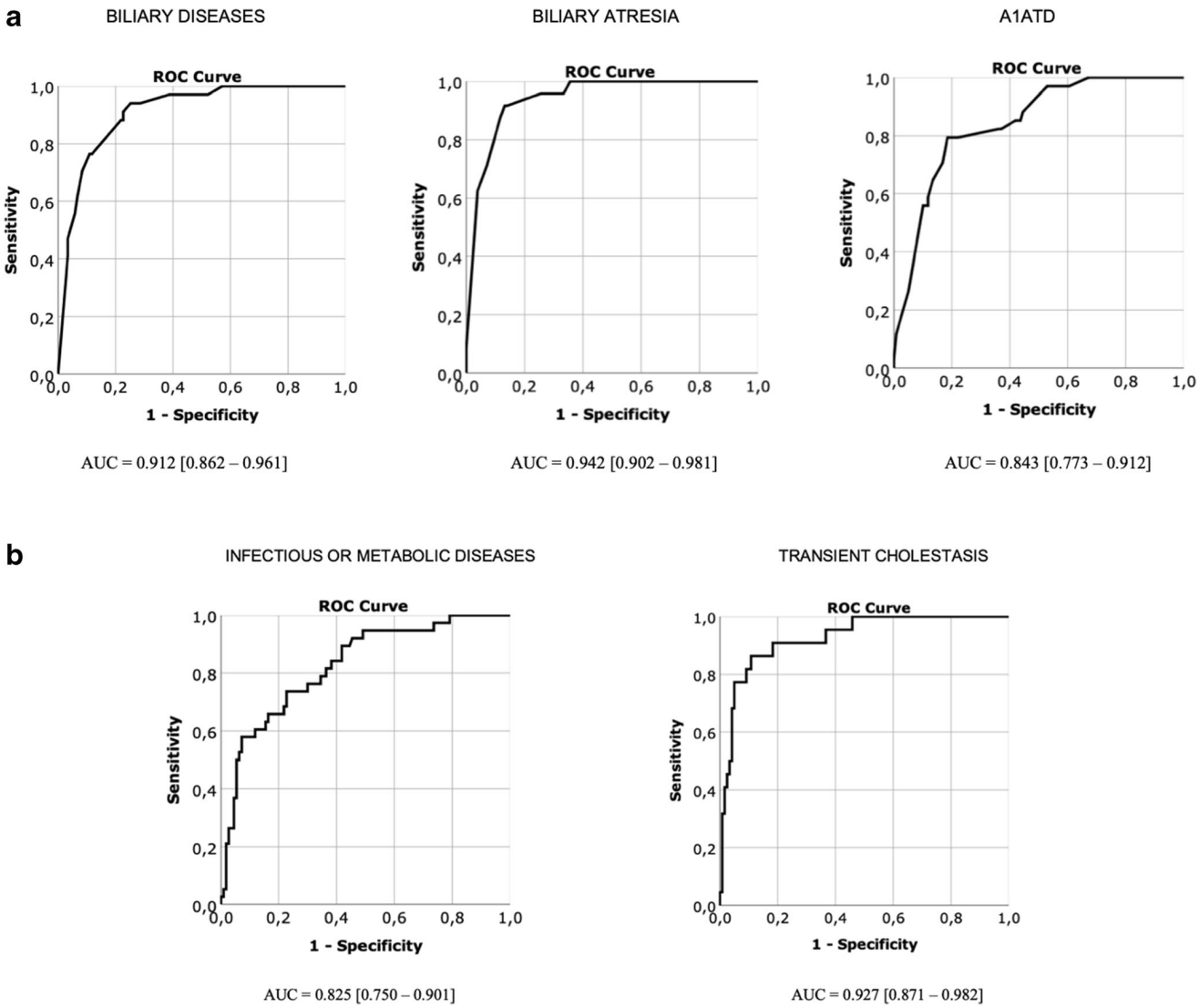

Fig.2 **a** and **b** Models of diagnosis (ROC curves) *A1ATD* alpha-1-antitrypsin deficiency, *ROC* receiver operator characteristic, *AUC* area under the roc curve

Table 2 Multivariate regression logistic models for unfavourable prognosis ($n = 47$)

Model A					Model B				
	p value	OR	95% CI for OR			*p* value	OR	95% CI for OR	
Signs and/or symptoms of sepsis[1]	0.153	0.362	0.090	1.458	Signs and/or symptoms of sepsis[1]	0.180	0.382	0.093	1.561
Signs and/or symptoms of liver failure[1]	0.183	3.310	0.569	19.246	Signs and/or symptoms of liver failure[1]	0.177	3.532	0.565	22.063
Comorbidities[3]	0.461	0.715	0.293	1.744	Comorbidities[1]	0.595	0.780	0.312	1.950
Serum total bilirubin	0.009	1.113	1.028	1.206	Serum total bilirubin	0.013	10.107	1.021	1.200
Serum unconjugated bilirubin	0.008	0.771	0.637	0.933	Serum unconjugated bilirubin	0.005	0.760	0.627	0.921
Ratio AST/ALT	0.468	0.822	0.484	1.396	Ratio AST/ALT	0.680	0.889	0.510	1.551
GGT serum levels (≤ 100 IU/L)	0.016	4.566	1.323	15.750	GGT serum levels (> 300 UI/L)	0.002	3.772	1.602	8.880
Prothrombin time[2]	0.066	4.861	0.899	26.282	Prothrombin time[4]	0.106	4.266	0.735	24.777

OR, odds ratio; 95% CI, 95% confidence interval; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma glutamyl transferase; [1] the reference category is "NO"; [2] the reference category is "ABNORMAL"; [3] the reference category is "YES"; [4] the reference category is "NORMAL". Hosmer-Lemeshow test: model A ($p = 0.879$); model B ($p = 0.616$)

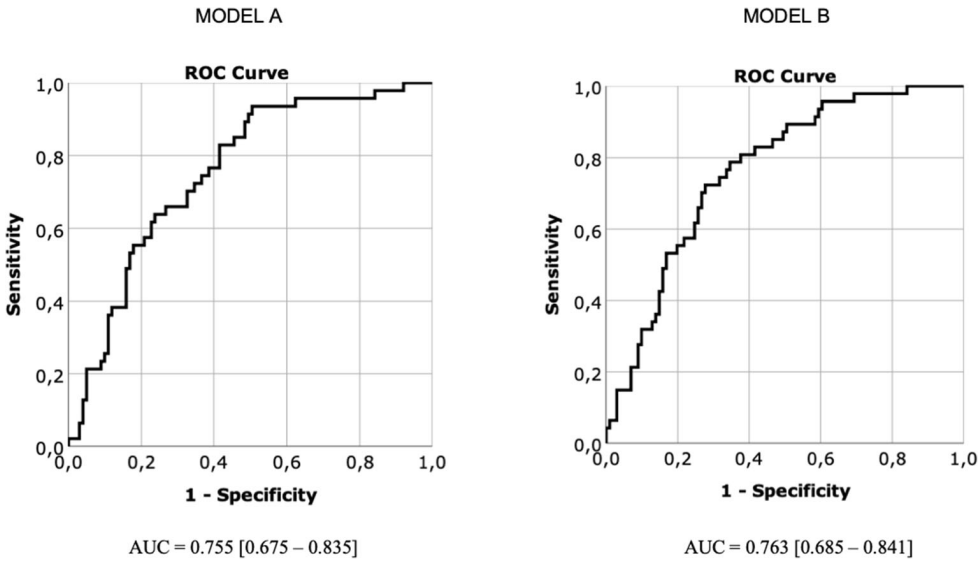

Fig. 3 Models of prognosis (ROC curves) *ROC* receiver operator characteristic, *AUC* area under the roc curve

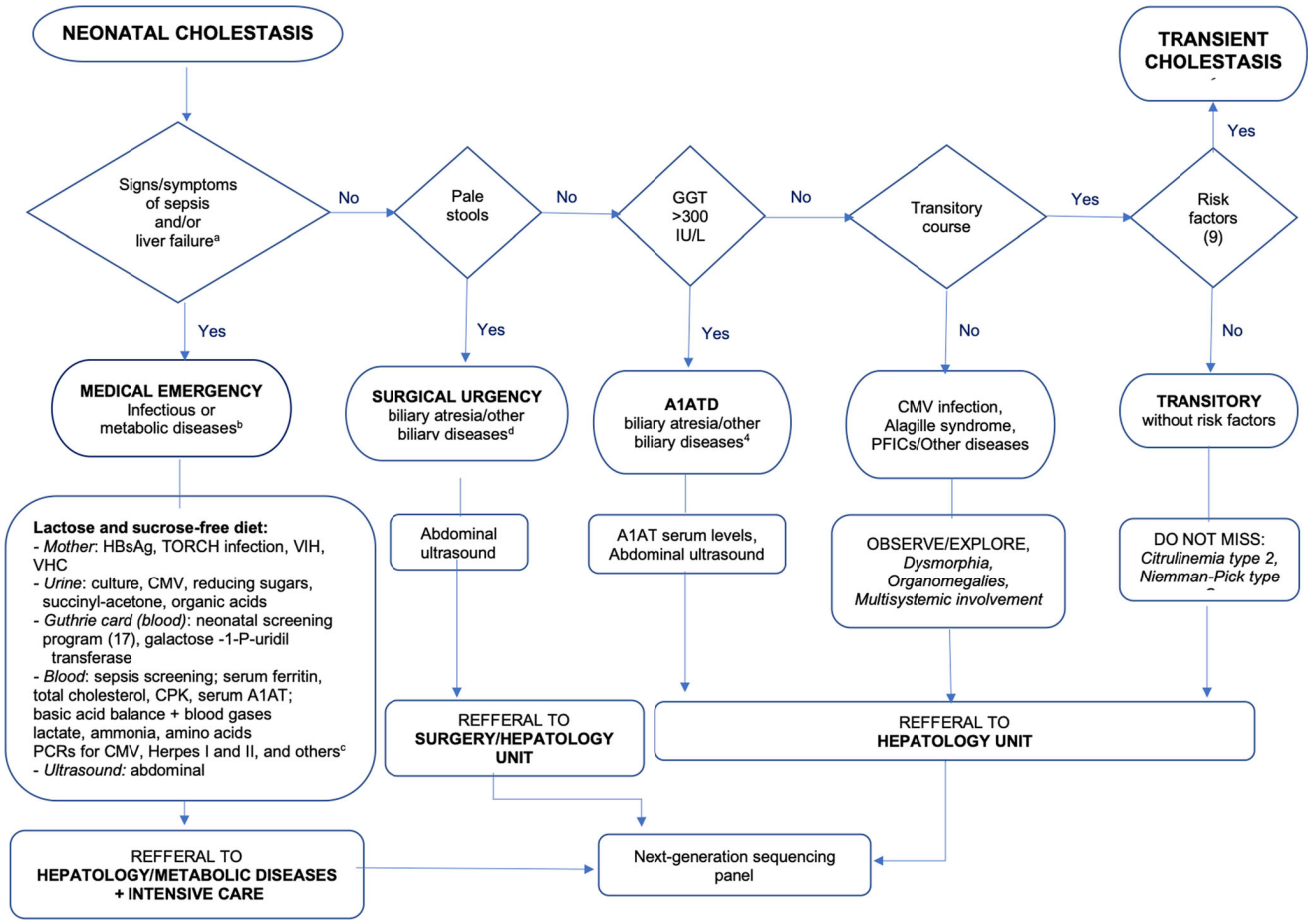

Fig. 4 Diagnosis decision algorithm *GGT*, gamma glutamyl transferase; *A1ATD*, alpha-1-antitrypsin deficiency; *CMV*, Cytomegalovirus; *PFICs*, progressive familial intra-hepatic cholestasis; *HBsAg*, surface antigen of hepatitis B virus; *TORCH*, (T)oxoplasmosis, (O)ther Agents, (R)ubella, (C)ytomegalovirus, and (H)erpes Simplex; *HIV*, human immunodeficiency virus; *HCV*, hepatitis C virus; *CPK*, creatine phosphokinase; *A1AT*, alpha-1-antitrypsin; *PCR*, polimerase chain reaction [a]Moaning, hypotonia, lethargy/irritability, seizures, vomiting, fever. Ascites, bleeding, hypoglycaemia, hypoalbuminaemia, coagulation abnormalities despite vitamin K administration [b]Bacterial septicaemia/meningitis, CMV, herpes I or II, HIV, hepatitis B, syphilis. Galactosaemia, tyrosinaemia type I, fructosemia, mitochondrial depletion syndromes [c]Adenovirus, parvovirus B19, echovirus, etc [d]Biliary sludge/stones, choledochal cyst, Caroli syndrome/disease, neonatal sclerosing cholangitis

previously described in the literature, GGT serum levels above 300 IU/L had a positive predictive value, while conjugated bilirubin serum levels did not [8, 9].

Shneider et al [9] concluded that the high precision required to differentiate biliary atresia from non-biliary atresia was not achieved in their model and speculate that the accuracy of the model could increase if A1ATD and Alagille syndrome were carefully assessed. Based on our data, we were able to establish a predictive diagnostic model for A1ATD, in which we found a common predictor with biliary atresia (GGT serum levels above 300 IU/L (OR higher than for biliary atresia)). On the contrary, pale stools were associated with a reduced risk of A1ATD, as well as prematurity and signs and/or symptoms of liver failure. Yet, in our opinion, the main problem is that the diagnosis of A1ATD will never exclude biliary atresia since both entities can coincide in one patient.

Concerning the subset of infectious and metabolic diseases, it is known that they can rapidly progress to liver failure, turning this condition into an emergency. We performed multivariate logistic regression analysis on the thirty-eight patients in our sample, and the signs and symptoms of sepsis at admission were independently associated with the highest risk for these entities.

Transient cholestasis became progressively more relevant in the past two decades [20]. It is a transitory condition associated with risk factors whose role needs to be further clarified. As an exclusion diagnosis, it currently poses a question not answered by the guidelines, namely, when and to what extent underlying entities should be investigated. In our sample, multivariate analysis only confirmed gestational age as an independent variable associated with this diagnosis. Further studies with a larger sample are required to better characterise this subgroup and, above all, to identify and explore the predictors that should motivate the investigation of underlying entities. This may be one of the subgroups of patients who will benefit most from the discovery of new biomarkers of the disease. Based on our data, we propose that patients with NC, regardless of whether they have risk factors or not, should undergo further investigation when they have one of the known positive predictors of biliary disease, A1ATD, metabolic/infectious diseases, and/or other signs/symptoms of disease.

The combination of univariate, bivariate, and multivariate analysis strengthened the evidence of some principles already recognized and helped us to find three of the five discriminators we used to build the algorithm: signs and/or symptoms of sepsis and/or liver failure; pale stools; serum GGT levels; transient course; and risk factors (Fig. 4). Although pale stools are the discriminative marker with the highest OR (which justifies the choice of this screening method for biliary atresia through the coloured stool card [21], we chose the signs and symptoms of sepsis (and/or liver failure) as the first discriminator because it identifies emergencies and pale stools as the second because it identifies surgical urgency. The third discriminator was serum GGT levels above 300 IU/L. This choice was based on the predictive capacity for diagnosis, in addition to the predictive capacity for prognosis, as we believe that patients with a worse prognosis should have priority in the approach. In accordance to our results, the study by Lu et al. linked serum GGT levels to prognosis and, interestingly, for very similar cut-off values [22].

Concerning the NGS technologies, they can be used to look for specific conditions or as part of a standard panel. These panels are designed to identify genetic variants irrespective of the clinical phenotype. Thus, it is our opinion that they should be judiciously managed in tertiary centres by specialised and multidisciplinary teams. As we argued elsewhere [3, 15], we think NGS panels should not be offered as the first approach but incorporated into diagnostic algorithms of NC and when the standard workup is unsuccessful.

The main limitation of this study is its retrospective design and the relatively small sample size. However, we accounted with the same nuclear team over the years and databases and clinical charts of good quality that yielded a sufficient ability to rigorously apply the statistical analysis. The major advantage was that it encompasses a large cohort of paediatric patients with A1ATD, providing a first diagnostic predictive model for this condition.

In conclusion, we think that it is premature to state a paradigm shift in the diagnostic approach of NC. Biochemical markers [23–25] and others [26, 27] are far from being fully explored, and genetics poses new questions while answering to some old ones. Our study intend to be a preliminary contribution for larger prospective studies that may constitute (or not) an external validation for the results and the proposal we present here.

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Authors' contributions ESS diagnosed and followed patients, designed the study, collected and analysed data, and elaborated the draft of the manuscript.

HMS collected and analysed data, contributed to the design of the study, and critically reviewed the manuscript.

CC analysed data and critically reviewed the manuscript.

CCD built and helped to interpret the predictive models of diagnosis and prognosis, revised all statistical analysis, and critically reviewed the manuscript.

ASS and AIL contributed to the conception of the study and the interpretation of data and critically reviewed the manuscript.

All authors approved the final version of the manuscript, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Data Availability The data analysed in this study is subject to the following licenses/restrictions: The data are recorded in the patients' clinical files and in the hospital databases. In order to have access, it is necessary to ask for authorization from the Ethics Committee and the Board of Directors of the hospital. This authorization was requested and obtained to carry out this study. Requests to access these datasets should be directed to Ethics Committee, secretariado.etica@chporto.min- saude.pt, and Departement of Education, Training and Research (DEFI), secretariado.cg.defi@chporto.min-saude.pt.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was in accordance with the ethical standards of the participating healthcare institution committee (Studies N/REF.ª 2016. 081 (069-DEFI/066-CES) and N/REF.ª 2016. 084 (072-DEFI/069-CES)), and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent to participate Informed consent was obtained from all individuals included in the study.

Consent for publication Informed consent was obtained from all individuals included in the study.

Code availability Software Statistical Package for the Social Sciences v. 24.0.

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3.2. METABOLIC LIVER DISEASES PRESENTING WITH NEONATAL CHOLESTASIS: AT THE CROSSROAD BETWEEN OLD AND NEW PARADIGMS.

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ORIGINAL ARTICLE

Metabolic liver diseases presenting with neonatal cholestasis: *at the crossroad between old and new paradigms*

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Abstract

Metabolic liver diseases (MLD) are an important group of disorders presenting with neonatal cholestasis (NC). The spectrum of liver involvement is wide and the presumptive diagnosis is traditionally based on clinical and laboratory findings. Recently, next-generation sequencing (NGS) panels have emerged as an appealing tool to diagnose neonatal/infantile cholestatic disorders. The aim of this study was to identify clinical phenotypes of liver injury and contribute to find a diagnostic methodology that integrates new molecular diagnostic tools. We retrospectively analyzed the clinical and biochemical features of 16 patients with MLD and NC. Patients were categorized into three groups: A—*NC with liver failure* ($N = 8$): tyrosinemia type I ($n = 2$), classic galactosemia ($n = 5$), mitochondrial DNA depletion syndrome ($n = 1$); B—*NC evolving with chronic liver disease* ($N = 5$): argininemia ($n = 2$); mitochondrial cytopathy ($n = 1$); congenital disorders of glycosylation type Ia ($n = 1$); Zellweger syndrome ($n = 1$); and C—*transient NC* ($N = 3$): Niemann-Pick type C ($n = 2$), citrullinemia type II ($n = 1$).

Conclusion: MLD presenting with NC can be categorized into three main clinical phenotypes of liver injury. We highlight transient NC as a clue for MLD that must be pursued. New molecular diagnostic tools can play a key role, but application criteria must be established to make them cost-effective.

What is Known:
- *Metabolic liver diseases are an important group of disorders presenting with neonatal cholestasis.*
- *The diagnostic approach is challenging and traditionally based on clinical and laboratory findings. Next-generation sequencing is a recent and rapidly developing tool in pediatric hepatology.*

What is New:
- *We provide a liver-targeted characterization of metabolic liver diseases presenting with neonatal cholestasis, categorizing them into three clinical phenotypes that may narrow the diagnostic possibilities.*
- *A clinical decision-making algorithm is proposed, in which the NGS technology is integrated.*

Keywords Neonatal cholestasis · Transient neonatal cholestasis · Liver failure · Metabolic liver diseases · Next-generation sequencing panels

Abbreviations

cB	Conjugated bilirubin
CDG	Congenital disorder of glycosylation
GGT	Gamma-glutamyltransferase
HE	Hepatic encephalopathy
IEM	Inborn errors of metabolism
INR	International normalized ratio
LF	Liver failure
MLD	Metabolic liver diseases
NBS	Newborn screening
NC	Neonatal cholestasis
NGS	Next-generation sequencing
NP-C	Niemann-Pick type C
OLT	Orthotopic liver transplant
PFIC	Progressive familiar intrahepatic cholestasis

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Introduction

Neonatal cholestasis (NC) is an infrequent pathological condition, affecting approximately 1 in 2500 live births [1, 22]. The diagnostic approach is challenging since the differential diagnosis is broad and can be divided into two main categories: biliary (e.g., biliary atresia) and hepatocellular (e.g., genetic and/or metabolic disorders) [26].

As the liver is a key metabolic organ, metabolic liver diseases (MLD) often involve disarrangements in enzymes and/or metabolic pathways highly expressed in the liver. Additionally, many multisystemic diseases such as mitochondrial disorders can primarily manifest as a cholestatic syndrome in infancy.

Newborn screening (NBS) programs using tandem mass spectrometry are used in neonatal screening for several inborn errors of metabolism (IEM) and have been widely adopted in Europe [3]. In Portugal, an expanded NBS program was implemented in 2005 for 24 treatable disorders [32]. However, most IEM presenting with NC are not covered in NBS. In addition, there are multiple rare types of genetic cholestasis presenting with a similar phenotype. Therefore, genetic testing may contribute to a precise diagnosis in this setting. The single gene approach (Sanger technique) is reliable for detecting single mutations, but it is expensive and time consuming. In contrast, next-generation sequencing (NGS) is a recent and rapidly developing tool in pediatric hepatology that may contribute to a prompt identification of patients with MLD [15, 25]. However, NGS should not be interpreted irrespective of the clinical phenotype.

Herein, we report the data (clinical presentation, laboratory profile, and outcome) of a cohort of 16 children with MLD presenting with NC, providing a workup approach and a clinically oriented overview of the differential diagnosis. We will also discuss the driving shift from the classical scenario, in which diagnostic hypotheses arise from clinical and biochemical data to multi-gene panel testing and its inclusion in diagnostic algorithms.

Methods

We retrospectively analyzed a cohort of 126 patients presenting with NC, referred to a tertiary university hospital in the northern region of Portugal, during a 30-year period (1987–2017). We identified a group of 13 patients with MLD. Additionally, three out of 582 patients with MLD followed at our institution in the same period were included as they were retrospectively identified as having NC. Patients with alpha-1-antitrypsin deficiency and cystic fibrosis were excluded. A final cohort of 16 patients was enrolled and analyzed.

NC was defined as prolonged jaundice with conjugated bilirubin (cB) ≥ 1 mg/dL (in combination with a total bilirubin of < 5.0 mg/dL) or a cB fraction of > 20% of the total, detected either in a newborn or an infant up to 4 months old [24]. Transient cholestasis was retrospectively defined as the presence of cholestatic jaundice resolving in the first 6 months of life, in the absence of known risk factors for NC [7] and with complete and spontaneous normalization of liver function tests.

Liver failure (LF) was defined according to the Pediatric Acute Liver Failure Study Group [30] by the following: biochemical evidence of liver injury and coagulopathy [international normalized ratio (INR) ≥ 1.5], not correctable by vitamin K administration, in the presence of hepatic encephalopathy (HE) or an INR ≥ 2 regardless of presence or absence of HE.

Demographic data, family background, presenting symptoms, age at diagnosis, and laboratory investigations at admission were analyzed [full blood cell count, blood chemistry including direct and indirect bilirubin, serum aminotransferases (AST, ALT), gamma-glutamyltransferase (GGT), albumin, creatinine, urea]. Baseline metabolic workup included serum ammonia and lactate, plasma and urinary amino acids, urinary organic acids profiles, acyl-carnitine profile, plasma carbohydrate-deficient transferrin, and urinary reducing substances. Amino acids profile was determined by liquid ion-exchange chromatography. Organic acids were measured by gas chromatography-mass spectrometry. Other laboratory/metabolic investigations were carried out in a case-by-case approach. Diagnosis were confirmed by biochemical, enzymatic, and/or molecular testing, when available.

Results

During the study period, we analyzed 16 infants with NC and MLD with a mean age at presentation of 3.5 weeks (1–8 weeks) and a male preponderance (56.2%). Regarding the outcome, four patients died and three received orthotopic liver transplant (OLT)—Table 1. Retrospective analysis of clinical and laboratorial features allowed the categorization into three different groups: A - *NC with liver failure* ($N = 8$): tyrosinemia type I ($n = 2$), classic galactosemia ($n = 5$), mitochondrial DNA depletion syndrome ($n = 1$); B - *NC evolving with chronic liver disease* ($N = 5$): argininemia ($n = 2$); mitochondrial cytopathy ($n = 1$); congenital disorders of glycosylation type Ia ($n = 1$); Zellweger syndrome ($n = 1$); and C - *transient NC* ($N = 3$): Niemann-Pick type C ($n = 2$), citrullinemia type II ($n = 1$).

Group A—neonatal cholestasis with liver failure

Cases #1 and 2 These patients presented before the expanded NBS program.

Table 1 Clinical, biochemical, and molecular data

	Patient no.	Sex	Year of birth	Parental consanguinity	Week of cholestasis	TB/CB (mg/dl)	AST/ALT GGT (UI/L)	Hepatomegaly	Failure to thrive	Hypotonia/ development delay	Diagnosis	Molecular study	Cholestasis resolution	Outcome (liver disease/other)
Group A	1	M	1990	No	8	NA	NA	Yes	Yes	No	TYR I	ND	No	OLT/none
	2	M	1993	No	8	3.1/1.9	230/157 256	Yes	Yes	No	TYR I	ND	8 weeks	3rd OLT/none
	3	M	1989	Yes (first cousins)	2	26.0/3.8	254/258 150	Yes	Yes	No	GAL	ND	NA	None/none (lost from follow-up after 18 years old)
	4	F	1991	NA	4	8.7/7.4	119/103 45	Yes	Yes	No	GAL	ND	NA	None/ovarian failure, osteopenia
	5	M	1993	NA	2	17.0/7.0	245/NA 299	Yes	Yes	No	GAL	Homozygous Q188R mutation (GALT gene)	10 weeks	None/cognitive impairment, osteopenia
	6	M	1997	No	1	9.5/8.4	211/NA 103	Yes	Yes	No	GAL	Homozygous Q188R mutation (GALT gene)	4 weeks	None/osteopenia
	7	F	1998	No	1	16.5/10	134/124 NA	Yes	Yes	No	GAL	Homozygous Q188R mutation (GALT gene)	5 weeks	None/ovarian failure, osteopenia
	8	M	2015	No	4	3.3/1.34	101/51 1109	No	Yes	Yes	DGUOK	Compound heterozygous mutation (DGUOK gene)	No	Died at 8 months old
Group B	9	F	1992	No	8	10.8/9.4	800/530 80	Yes	No	No	ARG	Homozygous R21X mutation (ARG1 gene)	No	OLT/none
	10	F	2009	Yes (first cousins)	3	5.7/1.6	51/38 1295	No	No	No	ARG	Homozygous R21X mutation (ARG1 gene)	5 weeks	None/none
	11	M	2005	No	1	6.5/3.2	150/200 89	Yes	No	Yes	CDG Ia	ND	8 weeks	Transaminitis/severe neurological impairment
	12	M	2010	No	8	3.9/2.3	99/52 547	Yes	Yes	Yes	M CYT	Homozygous mutation (EARS2 gene)	6 months	Transaminitis/ neurological impairment
	13	F	2015	No	3	9.27/8.64	616/204 206	Yes	Yes	Yes	ZEL	Homozygous mutation (PEX12 gene)	No	Died at 9 months old
Group C	14	F	1983	No	1	7.2/3.6	NA	Yes	No	No	NP-C	ND	12 weeks	Died at 9 years old
	15	F	1983	No	1	8.3/3.8	NA	Yes	No	No	NP-C	ND	12 weeks	Died at 9 years old
	16	M	2007	No	1	12.7/1.8	80/93 131	Yes	Yes	No	CIT II	Compound heterozygous mutation (SLC25A13 gene)	5 months	None/none

Tyr I, tyrosinemia type 1; *Gal*, galactosemia; *ARG*, argininemia; *CDG Ia*, congenital disorder of glycosylation type Ia; *M CYT*, mitochondrial cytopathy; *ZEL*, Zellweger syndrome; *NP-C*, Niemann-Pick disease type C; *CIT II*, citrullinemia type II; *OLT*, orthotopic liver transplantation; *NS*, neurosensorial; *NA*, non-available; *ND*, not done

Male patients with NC and liver dysfunction during the second month of life, in addition to craneotabes and renal tubulopathy. High urinary succinyl acetone suggested the diagnosis of *tyrosinemia type 1*, confirmed by enzymatic assay on fibroblasts. Patient 1 underwent OLT at the age of 11 months due to progression to liver failure, with no major complications. Patient 2, despite treatment with nitisinone since 3 months of age, received a first OLT at the age of 3 years due to a dysplastic nodule with suspected malignancy (later non-confirmed); complications related to OLT led to a second and then a third OLT.

Cases #3–7 Newborn infants with similar presentation of acute sepsis-like syndrome on the first 2 weeks of life, characterized by vomiting and hemodynamic instability (3 patients had *E. coli* sepsis). Laboratory workup revealed cholestatic liver dysfunction with coagulopathy and hypoalbuminemia. Physical examination showed hepatomegaly and congenital cataracts in patient #7. The diagnosis of *galactosemia* was confirmed by enzymatic assay in blood cells. Three patients had diagnostic confirmation by molecular study of GALT gene (Table 1). The liver disease resolved under galactose restriction.

Case #8 Male neonate with secondary biomarkers of liver disease (raised tyrosine and methionine) identified by the extended NBS program. On day 12, blood tests showed acidosis (pH 7.29), hyperlactacidemia (lactate 4.16 mmol/L), hypoglycemia, and coagulopathy. By the age of 2 months, he developed cholestatic liver injury (Table 1), severe hypotonia, rotational nystagmus, and cardiomyopathy. Genetic study confirmed the diagnosis of *mtDNA depletion syndrome* (compound heterozygous mutation c.677A>G (p.H226R) and c.749T>C (p.L250S) in DGUOK gene). He died at 8 months old due to an infection leading to acute-on-chronic liver failure.

Group B—neonatal cholestasis evolving with chronic liver disease

Cases #9 and 10 were previously described by our group [5, 14, 29].

Case #9 Two-month-old female with new onset of jaundice and hepatosplenomegaly. The elevation in plasmatic arginine to 1756 μmol/L (normal range 22–88) and ammonia led to the suspicion of *argininemia*, confirmed by the absence of arginase A1 activity in blood red cells and the molecular analysis of ARG1 gene (homozygous for R21X mutation). The patient had progressive biliary cirrhosis complicated with portal hypertension in the absence of neurological impairment. At the age of 7 years, she underwent successful OLT.

Case #10 Asymptomatic neonate, second child of first-degree consanguineous parents, diagnosed with *argininemia* through NBS (arginine level of 360 μM on day 5). At 21 days of age, she was found to have cholestatic jaundice and hyperammonemia. Plasmatic arginine was high (1600 μmol/L, $N < 140$) so as urinary orotic acid (5.3 μmol/mmol creat, $N = 0.1$). Homozygous R21X mutation on the ARG1 gene confirmed the diagnosis. Under proper diet and medical treatment, cholestasis resolved before 3 months old.

Case #11 Male newborn with cholestatic jaundice detected in the first week of life associated with severe feeding difficulties and failure to thrive. Coagulopathy was also predominant due to low prothrombin and antithrombin III. Physical examination revealed hepatomegaly and dysmorphic features (abnormal distribution of fat, inverted nipples, hypogonadism) that led to the suspicion of a congenital disorder of glycosylation (CDG), confirmed by isoelectric transferrin focusing (*CDG type 1a*).

Case #12 Male infant who had physiologic jaundice in the first week of life after which he developed a cholestatic pattern detected in the eighth week, in addition to episodes of hypoglycemia, metabolic acidosis, and hyperlactacidemia. He also had feeding difficulties and failure to thrive. On the physical exam, he had hepatomegaly in association to global hypotonia and hyperreflexia. Magnetic resonance imaging showed features of leukoencephalopathy involving the thalamus and brainstem. The genetic study revealed a homozygous mutation on the gene EARS2, confirming a *nuclear mitochondrial disorder*.

Case #13 Female patient presenting with NC during the first month of life. She had severe hypotonia and dysmorphic features (dolichocephaly, high forehead, large fontanelles). The very long-chain fatty acids in plasma were greatly increased. The PEX1 gene had no mutations. A diagnosis of *Zellweger syndrome* was confirmed by the identification of a homozygous mutation in the PEX12 gene from a NGS panel of genes associated with peroxisomal disorders. She died at 9 months of age.

Group C—transient neonatal cholestasis

Cases #14 and 15 Twin females who presented at a neurology consultation at the age of 5 years because of marked cognitive impairment and ataxia. Upon physical examination, hepatosplenomegaly was noted. Retrospectively, they were found to have had cholestatic liver disease in the first week of life. Filipin staining of skin fibroblasts was positive, confirming a diagnosis of *Niemann-Pick type C* (NP-C).

Case #16 Male newborn presenting with cholestasis in the first week of life associated with poor weight gain. Developmental milestones and neurological exam were normal. The cholestasis resolved spontaneously by 5 months of age. A diagnosis of *citrullinemia type II* was suggested by hyperammonemia and raised citrulline and methionine. It was later confirmed by molecular study (a compound heterozygous mutation c.1056-1060 del A/ c.1231-1G>A in gene SLC25A13 gene).

Discussion

Recent advances in molecular genetics have led to NGS technology, resulting in a dramatic reduction in the time and cost required to perform DNA mutation analyses [2]. In contrast to Sanger sequencing, where genes are sequenced one at a time, in NGS, the entire sequence or a significant portion of the sequence of DNA is sequenced in a single procedure, improving the diagnostic efficiency particularly in entities with broad differential diagnoses, such as MLD. In clinical practice, NGS can be used to look for specific conditions (like Sanger sequencing) or as part of a standard panel [13, 15]. These panels are increasingly becoming an important procedure when the standard workup is unsuccessful, but no one has yet established guidelines for their use. Some centers have built NGS panels for NC, comprising a variable number of genes, designed to include not only IEM but also genetic cholestatic disorders [13, 31], irrespective of the clinical phenotype [25].

At first glance, the idea of NGS panels as a diagnostic tool for suspected MLD allowing the decentralization of the diagnosis of these patients is undoubtedly appealing. However, we believe that albeit exciting to have access to such a powerful instrument, NGS should be applied after a pre-test counseling to avoid a burden of interpretative challenges. In fact, NGS can detect variants of uncertain significance, not definitively linked to a disorder [16]. It is important to stress that the meaning of a gene mutation or polymorphism should be matched within the clinical context [9, 11], and in monogenic disorders where there is a predictable genotype-phenotype match [25], NGS panels have no advantage over the Sanger technique. In addition, it is critically important to state that it takes weeks to report the results of NGS; therefore, it is advisable not to depend on this instrument to diagnose urgent and treatable conditions. Finally, the cost-effectiveness of NGS needs to be assessed as it is still far more expensive than clinically oriented biochemical tests.

At our institution, infants with NC undergo a stepwise evaluation in which detailed clinical and analytical assessments are the main crossroads, fundamental to pinpointing the diagnosis [9, 34]. Additional and more specific tests are tailored according to the presenting features and suspected diagnosis (Table 2). Concerning the NGS technology, until the compilation of the last patient in this case series in 2015, we only had sub-panels for MLD subgroups, such as peroxisomal disorders (patient #13). It was only since mid-2017 that we had available a customized NGS panel comprising 54 genes related not only to IEM presenting with NC but also to genetic cholestatic disorders.

Our data provide a liver-targeted characterization of MLD presenting with NC, categorizing them into three clinical phenotypes that may narrow the diagnostic possibilities: *NC with liver failure*, *NC evolving with chronic liver disease*, and *transient NC*. Nine out of 16 cases had molecular studies (eight by Sanger sequencing), which was crucial to confirm the diagnosis in two (#8 and #12); only one patient was diagnosed by targeted NGS panel (#13). Among the remaining seven patients, five underwent invasive procedures that could have been avoided if genetic studies had been available in due time. Overall, NGS panel would have advantage over Sanger sequencing in 4 patients (#8, #12, #14, and #15).

It is true that in some instances, the inclusion of targeted NGS or a panel-based approach in diagnostic processes may prove useful. However, due to time and other logistic restraints, it is difficult to apply the NGS approach to individual patients with MLD irrespective of their clinical phenotype. The paradigmatic example is the category of patients with *NC with liver failure*, which represents a medical emergency. The differential diagnosis includes, among others, classic galactosemia [4], tyrosinemia type I [21], and mitochondrial disorders. In both galactosemia and tyrosinemia type I, the clinical assessment in combination with specific biochemical markers (Table 2) can guide towards the specific diagnosis. In these settings, establishing a timely and accurate diagnosis is fundamental to promptly institute a specific therapy. Later on, the Sanger technique may confirm diagnosis. Thus, a panel-based approach in such cases appears counter-wise. Nevertheless, in some other entities, the metabolite profile can be abnormal but not characteristic or, if present, the gold-standard investigations can be invasive. An example is the mitochondrial DNA depletion syndromes (e.g., DGUOK gene mutations) that have a severe course characterized by the onset in infancy of progressive LF and neurological abnormalities [12, 17, 18] (Table 1), as observed in patient #8. Respiratory chain disorders can also present with LF, but they often manifest with cholestatic jaundice in association with minor liver disease (e.g., transaminitis), as exemplified by patient #12. In both mitochondriopathies, multisystemic involvement (mainly neurological involvement) along with metabolic acidosis and hyperlactacidemia are important clues. However, the specific diagnosis is highly dependent on invasive diagnostic procedures (liver and/or muscle biopsies), and gene expression can be heterogeneous limiting the application of the traditional Sanger technique. In these cases, we believe that a targeted NGS or an NGS panel could be a valuable tool to overcome these diagnostic difficulties and at the same time determine the specific mutation, avoiding futile OLT and

Table 2 Suggested workup during evaluation of cholestatic infant with suspected MLD

	Clinical clues	Laboratory investigation	Molecular analysis (gene)
NC with liver failure			
Galactosemia (OMIM 230400)	Congenital cataracts Gram-negative septicemia	Urinary reducing substances Galactose-1-phosphatase uridyl transferase activity in red blood cells (↑)	GALT
Tyrosinemia type 1 (OMIM 276700)	Rickets Renal tubular dysfunction	Plasma and urine amino acids Plasma alpha-fetoprotein Urine organic acids—succinylacetone (↑)	FAH
Mitochondrial DNA depletion syndrome (OMIM 203700, 251880, 256810)	Multisystemic involvement (neurological—hypotonia, rotational nystagmus; cardiac—cardiomyopathy)	Metabolic acidosis Lactate, piruvate Biopsy (muscle/liver)	POLG DGUOK MPV17
Wolman disease (OMIM 278000)	Hepatosplenomegaly Adrenal calcification Diarrhea and malabsorption Failure to thrive	Lipid profile (type IIb dysliproteinemia) Peripheral blood smear (lymphocytes with cytoplasmic vacuolation) Lysosomal lipase acid (↓) in peripheral blood monuclear cell	LIPA
NC with transient cholestasis			
Neonatal intrahepatic cholestasis caused by citrin deficiency (OMIM 605814)	Feeding difficulties Failure to thrive Fatty liver	Plasma ammonia (↑) Urine organic acids Plasma amino acids (↑citrulline and methionine)	SLC25A13
Niemann-Pick type C (OMIM 257220)	Hepatosplenomegaly Vertical ophtalmoplegia and ataxia (later).	Plasma LDL and HDL (↓), triglycerides (↑) Plasma chitotriosidase (↑) Skin biopsy (filipin staining of skin fibroblasts)	NPC1 (>95%) NPC2 (4%) Other genes (1%)
Peroxisomal disorders Infantile Refsum disease (OMIM 601539) Zellweger syndrome (OMIM 214100)	Craniofacial dysmorphism (dolichocephaly, esotropia, epicanthic folds, broad nasal bridge, high-arched palate, low-set ears, and anteverted nostrils) Severe axial hypotonia Pigmentar retinitis Neurosensorial hearing loss	Plasma very long-chain fatty acids (↑) Pattern of plasmalogens Urine organic acids (↑phytanic acid, pristanic acid)	PEX1 or PEX 6 (majority); PEX12, PEX26, PEX10, PEX2, PEX5, PEX13, PEX16, PEX3, PEX19, PEX14, PEX11β
NC with chronic liver disease			
Argininemia (OMIM 207800)	Feeding difficulties Vomiting	Plasma ammonia (↑) Amino acids profile Arginase A1 activity in blood red cells	ARG1
Congenital disorders of glycosylation type 1a (OMIM 212065)	Dysmorphic features (abnormal distribution of fat, inverted nipples, hypogonadism) Failure to thrive Neurological involvement	Plasma transferrin isoforms Triglycerides ↑ Coagulopathy (ATIII ↓, factor XI ↓, protein C and S ↓)	PMM2
Progressive familial intrahepatic cholestasis syndromes (PFIC) type 1 and 2 (OMIM 211600; 601847)	Pruritus	GGT (normal or ↓)	ATP8B1 (PFIC 1) ABCB11 (PFIC 2)
Defects in bile acid metabolism - Δ(4)-3-oxosteroid 5-β-reductase deficiency (OMIM 235555) - 3β-hydroxy-Δ5-C27-steroid dehydrogenase deficiency (OMIM 607765) - Oxysterol 7α-hydroxylase deficiency (OMIM 613812)	No pruritus	Normal serum bile acid levels Urinary bile acid analysis	AKR1D1 HSD3B7 CYP7B1

LDL, low-density lipoprotein; *HDL*, high-density lipoprotein

allowing genetic counseling. Figure 1 illustrates a proposed clinical-based diagnostic algorithm for MLD presenting with NC followed at our institution, and it additionally incorporates NGS.

In the other two clinical phenotypes with normal liver synthetic function, the differential diagnosis is broad and includes, among others, argininemia, CDG, and mitochondrial disorders—*NC evolving with chronic liver disease*—and citrin deficiency, peroxisomal biogenesis disorders (Refsum disease), and NP-C—*transient NC*. Argininemia is the second most common urea cycle disease in Portugal [14, 32]. Although moderate transaminitis may be observed in all urea cycle defects, NC as the first presentation of hyperargininemia is rare, but well-established by our group [14]. It is now diagnosed through the expanded NBS program, thus overcoming the initial diagnostic difficulties. CDG, similar to mitochondrial hepatopathies and peroxisomal biogenesis disorders, presents with multisystemic manifestations early in life [10, 19, 20] and can also have a wide spectrum of liver involvement, from mild disease (most common), as observed in patient #12, to liver failure (rarely). Chronic liver disease occurs in a minority of the reported CDG types (22%) [19] and the prognosis is poor, often precluding liver transplantation. Additional clinical and biochemical clues for the diagnosis are depicted in Table 2. However, the diagnosis may not be straightforward and these MLD may be candidates for an NGS-based approach due to extreme clinical and genotypic heterogeneity, complexity, and/or the invasiveness of traditional diagnostic approaches [13].

MLD causing transient cholestasis (i.e., citrin deficiency, Refsum disease, and NP-C) pose a diagnostic challenge as they can be misinterpreted as prolonged physiologic jaundice and the diagnosis can be missed early in the disease course. NP-C is the paradigmatic example of this heterogeneous group of disorders, with manifestations occurring along a continuum in the disease course. The neonatal-onset NP-C has a more aggressive clinical course [33] and remains the most challenging. Neonatal cholestasis, the first phase of the disease (and sometimes the only presenting symptom), is usually self-limiting, with resolution at 2–4 months of age [23]. Additionally, other NP-C manifestations are not specific and only appear later in the disease course, e.g., hepatosplenomegaly and central nervous system involvement as occurred in our twin patients. In these cases, when there are no clear pointers to the diagnosis, NGS panels or even whole exome sequencing can be considered to improve the diagnostic yield (Fig. 1). However, it should be ascertained whether it is cost-effective to routinely apply an NGS panel to all transient NC.

Finally, we highlight other MLD causing cholestatic liver disease in infancy not present in our cohort: Wolman disease [6, 27], progressive familiar intrahepatic cholestasis (PFIC) [28], and bile acid synthesis defects [8] (Table 2). These disorders are being increasingly recognized thanks to the application of NGS panels reducing the percentage of idiopathic cases [25]. In our cohort of patients with NC, some patients deceased without a definitive diagnosis. Therefore, we believe that this may be a subgroup of patients in which NGS

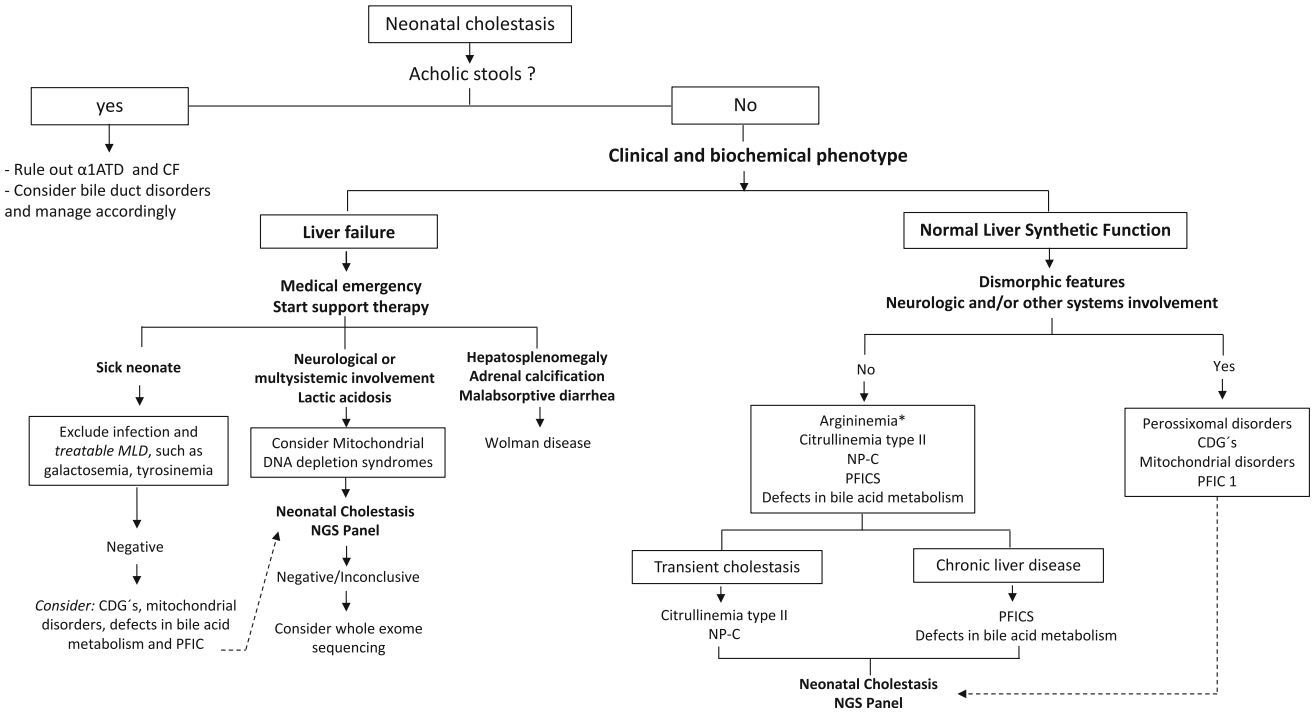

Fig. 1 Diagnostic algorithm to identify MLD in neonatal cholestasis. An asterisk indicates that it is identified through the NBS program

technology could have an added value, allowing to increase the diagnostic efficiency, provided that there are stored samples.

In summary, our study highlights the critical role of clinical and biochemical evaluations for decision-making in the setting of MLD. The proposed "phenotypic" categorization can be achieved in a tertiary center with high level of competence. NGS is an emerging and appealing tool that in our opinion should be judiciously applied and not used as a first-line approach nor in a decentralized model of care. Although we believe that NGS will never replace clinical and biochemical assessments in the management of MLD, incorporating NGS into the diagnostic algorithm of MLD may improve the accuracy of diagnosis. However, it may be advisable to centralize NGS technology in a few hospital centers (tertiary centers) with clinical and laboratorial expertise to overcome some of its restraints. Further work is required to formally assess the cost-effectiveness of NGS and explore the optimal approach to the timing of NGS in the diagnosis of MLD.

Authors' Contributions Helena Moreira Silva: study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript.

Inês Maio: acquisition of data, analysis and interpretation of data.

Anabela Bandeira and Esmeralda Martins: patient diagnosis and follow-up, analysis and interpretation of data; critical revision of the manuscript.

Ermelinda Santos Silva: patient diagnosis and follow-up, study concept and design; study supervision; critical revision of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was approved by our institutional scientific and ethics committee [Study N/REF.ª 2016. 081 (069-DEFI/066-CES)].

Clinical trial registration Not applicable.

Informed consent Informed consent was obtained from all individual participants included in the study.

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4.1. BILE ACIDS PROFILE AND REDOX STATUS IN HEALTHY INFANTS

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ABSTRACT

Introduction: At birth, human neonates are more likely to develop cholestasis and oxidative stress as a consequence of immaturity and/or other causes. We aimed to search for any potential association among bile acids profile, redox status and type of diet in healthy infants. Methods: A cross-sectional, exploratory study was conducted on 32 two-month-old full-term infants. We measured the plasma bile acids (total and conjugated) and the erythrocyte oxidative stress biomarkers. The type of diet was tested as an independent variable. Results: The percentage of conjugated chenodeoxycholic acid (CDCA) correlated significantly and negatively with the percentage of membrane-bound hemoglobin (MBH) (r = -0.635, $p < 0.01$) and with oxidized glutathione (r = -0.403, $p < 0.05$) levels in erythrocytes. Oxidative stress biomarkers (especially MBH) were predictors of conjugated CDCA percentage, and this predictive value was enhanced when adjusted for the type of diet (e.g. MBH, R = 0.452, $p < 0.001$). Exclusive breastfeeding was associated with higher percentages of CDCA. Conclusion: Our data suggest that the bile acids profile might play a role in the regulation of redox status (and/or vice-versa) in early postnatal life, and the type of diet may have some impact on this process.

KEYWORDS

Bile acids; Biology Development; Cholestasis; Diet; Infant; Oxidative stress

RUNNING TITLE

Bile acids, oxidative stress and infants

IMPACT STATEMENT

- The percentage of conjugated CDCA in plasma is negatively correlated with biomarkers of erythrocyte oxidative stress in healthy infants.
- Specific biomarkers of erythrocyte oxidative stress (e.g. MBH, GSH, GSSG) may be promising predictors of conjugated CDCA representation in plasma.
- The type of diet may influence the predictive capacity of hit erythrocyte oxidative stress biomarkers (e.g. MBH, GSH, GSSG).
- Our findings suggest a link between 'physiological cholestasis' and the redox status in healthy infants potentially modulated by diet type.
- The recognition of this link may contribute to the development of preventive and therapeutic strategies for neonatal cholestasis.

INTRODUCTION

Neonates and young infants may develop cholestasis due to genetic conditions or in response to several insults in the setting of an immature liver [1].

Liver immaturity at birth, particularly involving the mechanisms of bile formation and secretion [2], predisposes human neonates to a condition defined by Suchy et al [3] as 'physiological cholestasis'. In this condition, serum total bile acid concentrations in healthy full-term infants are more elevated in the first months [4-6] and then gradually decrease to the adult level [7], despite normal conjugated bilirubin serum levels. Additionally, glycocholic acid (GCA) is the major bile acid in the early neonatal period, but between 1-3 months glycochenodeoxycholic (GCDCA) acid predominates [7]. Thus, percentage of chenodeoxycholic acid (CDCA) conjugated with glycine and taurine become prevalent at these ages [3,7], in contrast to healthy children and adults [6,8,9]. Also, the taurine conjugated bile acids that prevail in the first days of life, turn also conjugated with glycine [4,10].

Newborns are more prone to oxidative stress due to labour stress and adaptation to a more oxygenated environment immediately after birth (among other acute or chronic perinatal stress), and because they have less antioxidant defences [11]. Interestingly, oxidative stress seems to be a common denominator among different types of insult or risk factors associated with neonatal cholestasis [12-15].

Oxidative stress was described in association with some adult cholestatic liver diseases, such as primary biliary cirrhosis [16]. More recently, Tan *et al* [17] clearly demonstrated, in liver tissue of adult patients with obstructive cholestasis, that GCDCA is more cytotoxic, induces mitochondrial injury and increases the production of reactive oxygen species (ROS). This bile acid proved to be toxic to primary human hepatocytes by inducing apoptosis, however, the tested concentrations are far from those found *in vivo* raising the hypothesis that inflammation may also have a key role in cholestasis [18]. Inflammation and oxidative stress are considered to be interrelated [19], yet, it was not found an association between the pro-inflammatory liver status, the decrease in the antioxidant capacity and augmentation of oxidative stress markers, at least in the liver cirrhosis [20]. So far, it is not clear if oxidative stress may be the cause instead of a consequence of cholestasis. And previous studies suggest that ROS are drivers of pathologic changes during cholestasis [21].

On the other hand, it is admitted that physiological jaundice may be part of the antioxidant protective shield of term and preterm neonates since the antioxidant properties of bilirubin

have been definitively shown *in vitro* and in animal model [22]. However, the significance of the immature bile acid profile and all its implications remain unexplained, mainly because it extends far beyond the first 1-2 weeks of physiological jaundice.

Breastfeeding in animals (guinea pigs and baboons), in contrast to formula, led to elevations in total bile acids [23] and increased concentrations of conjugated CDCA [23,24]. Moreover, breastfed infants were shown to have lower concentrations of cholic acid than the formula diet up to 5 months old, and the last type of diet caused lithocholic acid in the stools at an earlier age than the first type of diet [25]. Thus, it has been suggested that progressive maturation of the bile acid profile may occur during the first year of life [26], due to several determinants, including the impact of breastfeeding on the intestinal microbiota [27]. There is also evidence that human milk may reduce oxidative stress in preterm infants [28] and infants with necrotizing enterocolitis [29].

In this study, we aimed to evaluate the association (if any) between bile acid signature, oxidative stress biomarkers, and inflammation indicators in healthy infants, and the potential impact of the type of diet.

We chose erythrocytes as a minimally invasive model of cumulative oxidative stress damage for use in healthy infants. Erythrocytes are fit models because they have deficient repairing mechanisms and biosynthetic capacity, considering that they are anucleated and without organelles [30,31]. Among others, we used the membrane-bound hemoglobin (MBH), oxidized glutathione (GSSG) and reduced glutathione (GSH) as erythrocyte oxidative stress markers. The MBH results from hemoglobin oxidation and irreversible binding to the erythrocytes membrane, and leads to a less flexible membrane, and consequently, to an increased susceptibility to hemolysis [31]. Likewise, GSSG and GSH levels are overall markers of intracellular redox homeostasis, with a shift towards the oxidized species whenever increased oxidative stress is present [32]. Additionally, it has been shown that bilirubin [33] and bile acids, namely GCDCA and TCDCA [34] may cause eryptosis in healthy individuals.

MATERIALS AND METHODS

Study design

We conducted a cross-sectional and exploratory study in a Portuguese tertiary mother-child hospital. Patients' recruitment was performed prospectively over a two-year period (between April 2017 and March 2019).

Inclusion criteria: The participants were two-month-old healthy infants, born from healthy non-smoking mothers, after an uneventful full-term pregnancy, by vaginal delivery, without evidence of perinatal asphyxia. They were conveniently enrolled at birth before discharge from the hospital and requested to comeback for an appointment at two-month old.

Exclusion criteria: evidence of illness in the two weeks before blood collection, C-reactive protein serum levels out of normal range (reference value < 300 ng/ml), alpha-1-antitrypsin deficiency, and insufficient blood sample.

We divided the subjects according to the type of diet, into the following groups: A – exclusively breastfed; B – mix-fed (breast milk and formula); and C – exclusively formula-fed. The standard infant formula contained long chain-polyunsaturated fatty acids (LC-PUFAS) according to ESPGHAN recommendations [35] as well as to National and European legislations [36,37]. We also analyzed data according to gender.

Study population

Enrollment of 45 participants (22 males) from which two were excluded according to the previously defined exclusion criteria. Additionally, eleven were excluded due to: withdrawal of consent (*n* = 8), impossibility of collecting blood on the scheduled date (*n* = 2), and loss of bile acid sample during processing (*n* = 1).

Thirty-two healthy infants (17 males) were analyzed. Their mothers had a mean age of 29.9 ± 5.4 years and a body mass index (BMI) of 29.8 ± 4.5. For 17 of those mothers, this was their first child. Neonates were born at a median gestational age of 39 weeks (IQR: 38-40), with a mean birth weight of 3239 ± 322 grams (males) and 3381 ± 336 grams (females). Twelve developed neonatal jaundice in their first week of life, and seven required phototherapy. None of the subjects had obvious jaundice at two-month old (total bilirubin = 0,71 mg/dL, IQR: 0.47-1.11).

At the time of sample collection, infants had a mean age of 61.2 ± 2.6 days. Their mean weight was 5268 ± 285 grams (males) and 5023 ± 590 grams (females). Seventeen were exclusively breastfed, six were fed with formula milk and nine with both types of milk.

Clinical data, blood collection, and processing

At two months-old, infants had an appointment with a pediatrician where clinical data were registered and a full clinical examination was performed, before blood collection.

Whole-blood samples (in EDTA) were collected by peripheral vein puncture, before a meal (2-4 hours after the previous meal). The samples were processed in less than 2 hours after collection to obtain plasma, washed erythrocytes and erythrocyte membrane suspensions by sequential centrifugation and hypotonic lyses, as previously described [38]. Samples were stored at -80 ºC until assayed.

Analytical methods

Descriptive analytical methodology, according to previously established methods, is indicated in Table 1. Samples were used to evaluate the parameters presented in Tables 2 and 3.

Bile acids in the plasma were assessed using the Bile Acids Kit from Randox Laboratories for total bile acids and high-performance liquid chromatography for the most common conjugated bile acids [9,39,40], together with other previously established methods [38].

A more detailed description is given for oxidative stress biomarkers since some techniques were adapted:

Membrane-bound hemoglobin (MBH) was measured by spectrophotometry in erythrocytes membrane suspensions, as previously described [38]. Erythrocyte membrane lipid peroxidation was evaluated using the thiobarbituric acid test method [38] adapted from Mihara and Uchiyama [41].The ferric reducing ability of plasma (FRAP) assay [42] was adjusted to assess the total antioxidant status of whole erythrocytes and plasma samples, as previously described [38].

Enzyme catalase (CAT) activity of whole erythrocytes was determined using a method based on the peroxidase activity of catalase, in which low molecular weight alcohols can serve as electron donors, as reported by Johansson and Borg [43]. Briefly, erythrocytes samples (20 µL) were incubated for 20 min at room temperature with methanol (30 µL) and H_2O_2 (20 µL) in 100 mM, pH 7.0 phosphate buffer (100 µL), after which 10 M KOH (30 µL) was added to stop the reaction. Finally, 30 µL of the chromogen (Purpald 70 mM, Sigma-Aldrich) was added to react with the formaldehyde byproduct, which was measured spectrophotometrically at 540 nm. A formaldehyde standard curve was performed for each assay (0–75 µM).

Glutathione peroxidase (GPx) activity was assessed in whole erythrocytes through the adaptation of Weydert and Cullen's essay [44]. This assay is based on the activity of glutathione reductase (GR) and the accompanying depletion of reduced nicotinamide adenine dinucleotide phosphate (NADPH), which is monitored by spectroscopy at 340 nm. The assay was carried out by adding glutathione (GSH)/GR (1 mM/1 U/mL, Sigma-Aldrich) solution and NADPH (0.2 mM, Sigma-Aldrich) to erythrocytes samples (20 µL) in 50 mM, pH 7.0 phosphate buffer. After a 10 min incubation at 25 °C, cumene hydroperoxide (15 mM, Sigma-Aldrich) was added as a GPx substrate and the rate of decrease in absorbance at 340 nm was recorded for 10 min at 25 °C). A standard curve was performed for each assay (GPx 0–100 mU/mL, Sigma-Aldrich).

Total glutathione (GSH) and oxidized glutathione (GSSH) were measured by a spectrophotometric method adapted from Griffith, [45] and Shaik and Mehvar [46]. Briefly, 15 µL of washed erythrocytes were added to 720 µL of perchloric acid, centrifuged (10,000 g, 15 min, 4 °C, Gyrozen 1730R Frilabo), and the supernatants stored at -80 °C until assayed. On the day of the assay, the supernatants were neutralized with an equal volume of 0.76 M $KHCO_3$, centrifuged (10,000 g, 10 min, 4 °C), mixed, and incubated with 240 µM of NADPH and 1.3 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB, Sigma-Aldrich) in 70 mM, pH 7.5 phosphate buffer for 15 min at 30 °C. Afterward, 2.0 U/mL of GR was added and the rate of 2-nitro-5-thiobenzoic acid (TNB) formation was monitored at 405 nm (3 min at 30 °C). GSSG was quantified using the same method, but with a pre-incubation of the supernatants with 2-vinylpiridine (GSH masking reagent, Sigma-Aldrich) for 1 h, at 4 °C. Standard curves for GSH (0–15 µM, Sigma-Aldrich) or GSSG (0–8 µM, Sigma-Aldrich) were performed for all assays. The reduced GSH was estimated using the difference between total GSH and GSSG, and the GSH balance, by calculating the ratio between reduced GSH and GSSG.

All erythrocytes and membrane biomarkers were normalized using the sample's total protein concentration, to standardize the data. The total protein concentration of washed erythrocytes and erythrocytes membrane suspensions was determined according to Bradford's method [47].

Statistical analysis

Data distribution normality was accessed by the Shapiro-Wilk test. As most our data had a non-Gaussian distribution, all analytical parameters are presented as the median and interquartile range (IQR). Data were compared using the following statistical tests as appropriate: Chi-squared test, Fisher`s exact test, Kruskal-Wallis, and Mann-Whitney U test. Correlations were calculated using Spearman`s correlation. A linear regression model

was applied to determine the relationship between CDCA and some oxidative stress parameters (Enter approach) and adjusted for the type of diet. The significance level accepted was $p < 0.05$. Statistical analysis was performed using Statistical Package for the Social Sciences (version 26.0) software.

Ethical approval

This study was following the ethical standards of the participating healthcare institution committee [Study N/REF.ª 2016.085(073-DEFI/070-CES)] and with the 1964 Helsinki declaration and its later amendments, or comparable ethical standards.

RESULTS

The median value of total bile acids (14.80 µmol/L, IQR:9.25 – 18.00), found in the plasma of infants at two-month old, was increased when compared to values in older children reported in the literature (3.61 µmol/L, 95% confidence interval 3.09 – 4.12) [8], with a predominance of taurocholic (TCA), taurochenodeoxycholic (TCDCA), GCA and GCDCA acids. The percentage of cholic acid (CA) was slightly increased over that of CDCA conjugates. Secondary bile acids accounted for nearly 15% of total bile acids. Taurine and glycine conjugates were found in similar proportions (Table 2). We also observed a small elevation in the median serum conjugated bilirubin levels (0.36 mg/dL, IQR: 0.22 – 0.46) in comparison with the normal values of older children [48] (Table 2). The overall results of other liver function tests, and oxidative stress and inflammation parameters are also displayed in Table 2.

We find no gender-related differences in the bile acids profile, but male infants exhibited lower GSH erythrocyte levels ($p = 0.012$), as well as lower alanine aminotransferase (ALT) serum levels ($p = 0.003$) (Table 2).

We also find no early postnatal clinical jaundice-related differences (n = 12, data not shown) in bile acid profile, bilirubin levels and other oxidative stress biomarkers, at two-month old. On the contrary, infants with a history of early postnatal clinical jaundice showed significantly lower albumin serum levels as follows, median 3.96 g/dL (IQR: 3.86 - 4.10) *vs.* 4.12 g/dL (IQR: 3.96 - 4.32), $p = 0.026$.

Analysis by type of diet

There were 17 participants exclusively breastfed (group A), 9 with breastfeeding and milk formula (diet mix) (group B), and 6 exclusively fed with milk formula (group C).

Among the 3 groups of diet no significant differences were found in gender (p = 0.354), gestational age (p = 0.989), birthweight (p = 0.706), mother`s age (p = 0.304), mother´s BMI (p = 0.317), age (p = 0.503) and weight (p = 0.226).

Data concerning bile acids, liver function tests, inflammation and erythrocytes oxidative stress parameters, according to type of diet, are shown in Table 3.

We notice that the group exclusively breastfed shows a trend towards higher percentage of CDCA conjugates, (Fig.1) and though not significant, in this group we observed increased levels of GCDCA, decreased ratios of CA to CDCA and increased ratios of primary to secondary bile acids, compared to those fed with a mix or formula diet. However, we did not find significative differences of TCA and GCA among different feeding modes.

The exclusive breastfeeding group showed a trend towards lower oxidative stress and inflammation (Table 3). In liver function tests, there were no significant differences, including in serum albumin levels (p = 0.517).

Bile acid profile *vs*. oxidative stress

TCA showed to be positively correlated with bilirubin plasma levels as follows: TCA with total bilirubin (TB) (r = +0.463, p = 0.008), TCA with unconjugated bilirubin (B) (r = +0.454, p = 0.009), and TCA with conjugated bilirubin (r = +0.392, p = 0.027).

CDCA percentage, referring to the sum of the glycoconjugate (GCDCA) and tauroconjugated (TCDCA) species, showed to be negatively correlated with the erythrocyte's membrane-bound hemoglobin (MBH) (r = -0.635, p < 0.001), and with the oxidized glutathione (GSSG) (r = -0.403, p = 0.022), as well as positively correlated with reduced glutathione (GSH) (r = +0.420, p = 0.017) (Fig.2).

The GCDCA was more inversely correlated to MBH than the TCDCA (r = -0.447, p = 0.010 *vs*. r = -0.351, p = 0.049). In addition, GCDCA did not correlate significantly with the GSSG, in contrast to the TCDCA (r = -0.200, p = 0.273 *vs*. r = -0.491, p = 0.004). On the other hand, GCDCA was more positively correlated with GSH than the TCDCA (r = +0.503, p = 0.003 *vs*. r = +0.360, p = 0.043).

Linear regression models demonstrated that MBH, GSSG, and GSH were predictors of the percentage of plasma's CDCA conjugates; for all three, the predictive ability improved when the unadjusted model was adjusted for the type of diet. The strongest association was found for CDCA and MBH, which further improved in the adjusted model (R = 0.452, p < 0.001) (Table 4).

DISCUSSION

Bile acids are molecules with signaling properties, regulating the activity of many genes [49, 50]. They are critical in the control of hepatocellular function and their enterohepatic circulation is tightly regulated [51]. The disruption of bile acid homeostasis is implicated in the pathogenesis of chronic liver diseases [6,52,53]. Our study intended to explore the homeostatic imbalance due to liver immaturity and propensity to oxidative stress and cholestasis that characterizes the 'physiological cholestasis' in early postnatal life.

In terms of bile acids, and as previously described in animal models [24,25] and human infants [3], we found an increase in the plasma level of total bile acids with a medium of 14.8 µmol/L (IQR = 9.25-18.0), as well as a higher percentage of CDCA conjugates. These findings are in accordance with other studies that indicate 11.0 ± 8.7 µmol/L for the total bile acid levels in the first month of life [7], or also to values of 19.6 ± 5.2 µM/L in newborns *vs.* 5.1 ± 2.9 µM/L in adults [5]. The immaturity of many enzyme pathways particularly the regulation of the enterohepatic cycle [51] has been pointed to be involved. Actually, the sodium taurocholate cotransporter polypeptide (NTCP), one of the major transporters responsible for the liver bile acid re-uptake, requires approximately one year to achieve complete NTCP glycosylation in human hepatocytes [54]. The mean ratio of CA (TCA+GCA) to CDCA (TCDCA+GCDCA) was slightly over 1.0 and is in line with the literature in that it decreases from values >1.5 to levels <0.5 at 3 months of age [7]. The same occurs with glycine conjugated bile acids (glycoconjugates) to taurine conjugated ones that also decreases from birth (3.0 ± 3.1) until 1 month of life, a period where the values are lower and close to those in children and adult ones [4,7], which are usually around 2.0 [10, 55] or lower (1.4 ± 0.1) [56], close to the values we found in our infants, as depicted in Table 2. Moreover, the elevation of primary to secondary bile acids is also characteristic of infants with less than 1 year of age [7]. These Authors also describe that though GCA predominates in the neonatal period, after 1 to 3 months GCDCA starts to predominate, thus justifying our ratio of 1.05. As other Authors, we also did not find gender-related differences [5] and neither to early postnatal jaundice, despite these latter-ones having significantly lower levels of serum albumin, an indicator of more liver immaturity.

The most impressive finding in our study was, however, the correlation between the percentage of CDCA and more favorable redox status in erythrocytes, somehow opposite to what is described in the scenario of cholestatic liver diseases [21, 57]. Considering the CDCA hydrophobicity, its major representation may somewhat increase the toxicity to the liver. CDCA is a detergent-like molecule and is capable to damage cell membranes [51], and to induce eryptosis, which may accelerate the clearance of erythrocytes [34]. Will this

mechanism contribute to a faster replacement by adult erythrocytes? Anyhow, its association with biomarkers that reflect a more favorable redox status seems paradoxical, such as the strong negative correlation with MBH, reinforced by the correlations with GSSG (negative correlation) and with GSH (positive correlation) in erythrocytes.

In our opinion, the explanation may lie in some physiological mechanisms responsible for preserving homeostasis. We speculate that CDCA may cause a concentration-dependent Darwinian phenomenon, in which it penetrates cell membranes destabilizing only the most structurally fragile erythrocytes (i.e., with more oxidative stress-induced alterations) which, therefore, would be destroyed more quickly, sparing the more resistant (with better redox status). Thus, simply stated, our results point to that, the lower the oxidative injury/status presented by erythrocytes the higher the levels of circulating CDCA can co-exist. Other mechanism that can be associated include the activation of G protein-coupled bile acid receptor-1 (GPBAR1) by CDCA, which is a strong agonist of the FXR [58]. The GPBAR1 functions as a cell surface bile acid receptor and is encoded by GPBAR1 gene, present in rodent and humans [49]. Besides glucose regulation, GPBAR gene also modulates inflammation by reducing phagocytosis and inhibiting pro-inflammatory cytokine production, while FXR, expressed in the liver and intestine, is the most important nuclear receptor for maintaining bile acid homeostasis [59]. Moreover, Wang et al [60] reported that bile acids strongly inhibit the cysteine dioxygenase type-1-mediated (CDO1-mediated) cysteine catabolic pathway via an FXR-dependent mechanism, and attenuation of this bile acid repressive effect leads to the depletion of the free cysteine pool and a decrease in GSH concentration in the mouse liver. Among the bile acids, CDCA is a strong agonist of FXR associated to a defective plasma clearance of low-density lipoprotein (LDL) [61], what may enhance the thiol status [62].

We also highlight, in our study, the linear regression models between CDCA and oxidative stress biomarkers showing significant results, especially for MBH, and in which the type of diet was a clear influence since in the models adjusted for this factor the predictor capacity of each oxidative stress parameter invariably increased. In fact, the positive correlation between the percentage of conjugated CDCA and the most favorable redox status in erythrocytes seems to be improved by breastfeeding. This may occur either because human milk may promote an increase in CDCA percentage and/or may improve the erythrocytes redox status.

Breastfeeding was previously indicated as delaying the maturation of bile acids profile, at least in animal models [24, 25]. In sum, breastfeeding trends to promote a more immature bile acid pattern, with a higher percentage of CDCA and a lower percentage of secondary

bile acids; although, conversely, a trend towards higher glycine conjugates than in formula-fed ones, and in this respect more similar to the adult profile. Motta et al [63] described that hepatic CYP7A1 activity was low, but sevenfold higher in formula-fed *vs*. breastfed infant baboons, and that may explain the higher CDCA percentage in the latter. Our results, despite the small sample size, are in accordance with the higher concentration of total bile acids, and mainly CDCA percentage, in breastfeeding infants more than in those receiving mix or formula diet. Our study sample encompasses a small number of cases that most likely hindered the statistical analysis power of the results, which have no statistical significance.

Diet has also been shown to affect the bile acid metabolism in human infants [64, 65], and breastfeeding molds the developing neonatal gut microbiota in early life [27]. Moreover, the lipid composition of cell membranes varies according to the diet [66], as infants fed with breast milk have a better balance of LC-PUFAS, and maybe more protected from lipid peroxidation [67]. Exclusive breastfeeding may have contributed to a lower MBH through this mechanism. In fact, in the exclusively breastfed group we observed a trend towards median lower values in the inflammatory and oxidative stress parameters.

Finally, human milk provides many bioactive components (e.g. microRNAs), whose functions have been the subject of extensive research [68]. One of these functions could be the regulation of bile acid metabolism, for example, through GPBAR1 gene expression. There is recent evidence suggesting that environmental factors may influence the expression of this gene [58]. To test this hypothesis further studies are needed, namely the exploitation of miRNA in human milk, matched with infant blood samples.

Strengths and limitations - As far as we know this is the first report associating bile acids profile with oxidative stress according to the type of diet in healthy infants. This study is performed with human samples which nullifies the interspecies differences in the bile acid physiology of other studies. We emphasize the difficulty of obtaining samples from healthy infants, which led to the main limitation of the study – a smaller sample size than initially planned.

Conclusions - Our data suggest that the maturation of the bile acid profile may play a role in the regulation of erythrocytes redox status (and/or vice-versa), in early postnatal life. Exclusive breastfeeding slightly delayed bile acid profile maturation by the liver, and adaptive/compensatory mechanisms may involve a better redox status in healthy full-term infants to activate compensatory circuits and improve bile acid homeostasis.

Future implications - Our findings suggest a link between 'physiological cholestasis' and the redox status in healthy infants. Future studies should confirm such association that may be useful for preventive and therapeutic strategies mainly in transient cholestasis and in other non-familial forms of neonatal cholestasis.

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AUTHOR CONTRIBUTIONS

ESS, designed the study, collected and analyzed data, and elaborated the draft of the manuscript.

SR, processed the blood samples, evaluated the oxidative stress biomarkers and analyzed data.

RCR and HC, extracted, analyzed and quantified the serum bile acids.

CC, analyzed the inflammatory biomarkers.

FT and GH, analyzed the liver function tests and alpha-1-antitrypsin serum levels

AIL, ASS and DB, designed the study, analyzed data, and critically reviewed the manuscript.

All authors approved the final version of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

All data relevant to the study are included in this article. Additional data may be shared on a reasonable request.

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Tests	Equipment	Method description
Liver function tests		
Total and conjugated bilirubin	Roche, c701	Total and conjugated bilirubin – Diazo, colorimetric
AST and ALT		AST and ALT– IFCC, optimized
GGT		GGT – Colorimetric, enzymatic
Albumin		Albumin – Colorimetric
Serum alpha-1-antitrypsin	Roche, e702	Alpha-1-antitrypsin – Immunoturbidimetric
Inflammatory parameters		
TNF-alpha	ELISA assays	Human TNF-α Quantikine High Sensitivity ELISA Kit & Human C-Reactive Protein Quantikine ELISA Kit R&D Systems, Minneapolis, Minnesota, USA
C-reactive protein		
Oxidative stress biomarkers		
Erythrocyte's membrane	Multi-mode microplate reader Bio-tek Synergy HTX	A detailed description of these methods is given in the text of the manuscript. Some of these methods were described by Rocha S, et al [38].
MBH		
LPO		
Erythrocytes:		
TAS		
Catalase activity		
GPX activity		
GSSG, GSH reduced		
GSH/GSSG ratio		
Total bile acids (plasma)	Clinical Chemistry Analyzer Horiba ABX-Pentra C200	Total bile acids were evaluated by the 5th Generation Bile Acids Kit from Randox Laboratories (Ref. BI3863), using the control chemistry calibrator serum level 3 (Ref, CAL2351 from Randox) and the quality control human assayed multi-sera - level 2 (HUM ASY Control 2, Ref. HS2611 from Randox).
Conjugated bile acid profile (plasma)	High Performance Liquid Chromatography (HPLC) Shimadzu (Ref. 641228-45102-38 LC-2010 AHT)	Bile acids were extracted from plasma using Sep-Pak C18 cartridges as published [39]. Individual amidated bile acids were determined by HPLC based on a previously described method [40] with further modifications [6,9].

Legend: ALT – alanine aminotransferase, AST – aspartate aminotransferase, DIFF – differential blood count, ELISA – enzyme-linked immunosorbent assay, GGT – gamma-glutamyl transferase, GPX – glutathione peroxidase, GSSG – oxidized glutathione, GSH - glutathione, IFCC – International Federation of Clinical Chemistry, HGB - hemoglobin, HPLC – high-performance liquid chromatography, LPO – lipid peroxidation, MBH – membrane-bound hemoglobin, TNF - tumor necrosis factor, TAS – total antioxidant status.

Table 1 – Description of analytical methods

	Total (n = 32)	Male (n = 17)	Female (n = 15)	p value*
	Median (IQR)	**Median (IQR)**	**Median (IQR)**	
Liver function tests				
TBb (mg/dL)	0.71 (0.47 - 1.11)	0.85 (0.48 – 1.36)	0.54 (0.42 – 0.97)	0.896
UnconjBb (mg/dL)	0.40 (0.24 - 0.68)	0.47 (0.24 - 0.80)	0.31 (0.25 - 0.52)	0.450
ConjBb (mg/dL)	0.32 (0.22-0.46)	0.40 (0.26 - 0.55)	0.28 (0.18 - 0.43)	0.151
AST (UI/L)	37.0 (31.0 - 42.0)	34.0 (30.5 - 40.0)	40.0 (34.0 - 59.0)	0.061
ALT (UI/L)	**27.5 (24.0 - 35.5)**	**25.0 (20.0 - 30.0)**	**31.0 (27.0 - 66.0)**	**0.003**
GGT (UI/L)	37.5 (28.5 - 61.5)	35.0 (26.5 - 58.5)	42.0 (32.0 - 71.0)	0.157
Alkaline phosphatase (UI/L)	321.5 (280.3 – 425.0)	313.5 (284.8 – 440.5)	343.0 (264.0 – 394.0)	0.633
Albumine (g/dL)	4.08 (3.93 - 4.22)	3.96 (3.83 - 4.15)	4.11 (3.96 - 4.28)	0.082
Inflammation				
C-reactive protein (ng/mL)	99.94 (68.51 - 184.46)	75.65 (48.81 - 242.39)	133.62 (83.15 - 181.64)	0.558
TNF-alfa (pg/mL)	0.766 (0.693 – 0.970)	0.735 (0.610 - 0.826)	0.849 (0.708 - 1.007)	0.136
Oxidative stress				
Erythrocytes				
TAS (mM)	4.51 (3.72 – 6.22)	4.51 (4.00 – 6.51)	4.43 (3.50 – 5.79)	0.461
Catalase activity (nmol/min/mL)	10619 (8545 - 12391)	10799 (9020 - 12436)	10141 (8465 - 12443)	0.637
GPx activity (mU/mL)	2001 (1876 - 2305)	2056 (1915 - 2353)	1975 (1793 - 2241)	0.308
GSH (µM)	**25.15 (18.40 – 31.63)**	**20.30 (17.35 - 26.35)**	**29.70 (23.80 - 33.9)**	**0.012**
GSSG (µM)	2.61 (1.46 - 5.24)	2.450 (1.010 - 4.200)	2.63 (1.82 - 6.03)	0.664
GSH / GSSG	9.35 (4.39 - 20.78)	9.10 (3.29 - 24.60)	9.59 (4.69 - 16.20)	0.835
Erythrocyte's membrane				
MBH (%)	0.78 (0.52 - 1.13)	0.72 (0.51 - 1.07)	0.83 (0.51 - 1.39)	0.484
LPO (p.d.u)	0.07 (0.04 - 0.11)	0.07 (0.02 - 0.09)	0.09 (0.06 - 0.11)	0.290
Bile acids (total, species, percentages and ratios)				
Bile acids, total (µmol/L)	14.80 (9.25 - 18.00)	14.80 (9.75 - 20.85)	14.80 (8.70 - 18.10)	0.762
Primary				
TCA (µmol/L)	2.65 (1.20 - 5.15)	3.40 (1.30 - 5.30)	2.20 (0.90 - 5.00)	0.385
TCDCA (µmol/L)	1.85 (0.93 - 4.43)	1.30 (0.80 - 4.50)	2.20 (1.00 - 4.20)	0.484
GCA (µmol/L)	2.55 (1.53 - 4.73)	2.60 (1.55 - 5.30)	2.50 (1.50 - 4.50)	0.496
GCDCA (µmol/L)	2.70 (1.40 - 4.75)	2.80 (1.10 - 5.70)	2.60 (1.50 - 4.80)	0.955
Secondary				
TDCA (µmol/L)	0.40 (0.23 - 0.50)	0.40 (0.25 - 0.50)	0.40 (0.20 - 0.50)	0.923
TLCA (µmol/L)	0.30 (0.20 - 0.48)	0.30 (0.20 - 0.45)	0.30 (0.20 - 0.50)	0.578
TUDCA (µmol/L)	0.25 (0.10 - 0.40)	0.30 (0.10 - 0.40)	0.20 (0.10 - 0.40)	0.816
GDCA (µmol/L)	0.60 (0.20 - 0.80)	0.60 (0.20 - 0.80)	0.70 (0.20 - 0.90)	0.818
GLCA (µmol/L)	0.25 (0.13 - 0.40)	0.30 (0.20 - 0.45)	0.20 (0.10 - 0.40)	0.539
GUDCA (µmol/L)	0.20 (0.10 - 0.30)	0.20 (0.10 - 0.30)	0.20 (0.10 - 0.30)	0.875
Percentages				
Cholic acid (TCA + GCA)	44.40 (36.13 - 54.15)	44.80 (41.60 – 54.80)	39.70 (31.50 – 54.70)	0.317
Chenodeoxycholic acid (TCDCA + GCDCA)	39.70 (26.10 - 50.00)	29.10 (25.20 – 45.80)	42.2 (28.7 – 51.70)	0.385
Deoxycholic acid (TDCA + GDCA)	7.90 (4.78 - 12.78)	7.20 (4.10 – 13.40)	9.80 (5.00 – 11.70)	0.821
Litocholic acid (TLCA + GLCA)	4.15 (2.53 - 6.80)	3.60 (2.30 - 6.65)	5.90 (2.90 - 6.90)	0.597
Ursodeoxycholic acid (TUDCA + GUDCA)	3.70 (2.25 - 5.03)	3.40 (1.90 – 5.45)	4.00 (2.40 – 5.10)	0.584
Molar ratios				
Cholic to Chenodeoxycholic	1.25 (0.70 - 2.00)	1.50 (0.95 - 2.00)	1.00 (0,60 - 2.10)	0.384
Glyco- to Tauroconjugates	1.05 (0.73 - 1.50)	1.30 (0.60 - 1.55)	1.00 (0.80 - 1.30)	0.609
Primary to Secondary	6.40 (3.98 - 12.00)	7.00 (3.90 - 12.40)	6.10 (3.90 - 11.10)	0.720
Trihydroxy- to Dihydroxylated	0.95 (0.63 - 1.58)	1.00 (0.90 - 1.45)	0.80 (0.50 - 1.60)	0.306

Legend: IQR, interquartile range; *Mann-Whitney U test, $p<0.05$ was considered statistically significant

TBb, total bilirubin; UnconjBb, unconjugated bilirubin; ConjBb, conjugated bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; TNF, tumor necrosis factor; TAS, total antioxidant status; GPx, glutathione peroxidase; GSH, glutathione reduced; GSSG, glutathione oxidized; MBH, membrane-bound hemoglobin; LPO, lipid peroxidation; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TLCA, taurolithocholic acid; TUDCA, tauroursodeoxycholic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GLCA, glycolithocholic acid; GUDCA, glycoursodeoxycholic acid.

Table 2 – Outcome measurements in two-month old full-term healthy infants (n = 32) and comparison by gender

	Breastfeeding (*n* = 17)	Mix (*n* = 9)	Formula (*n* = 6)	*p* value*
	Median (IQR)	Median (IQR)	Median (IQR)	
Liver function tests				
TBb (mg/dL)	0.70 (0.47 - 1.11)	0.84 (0.50 – 1.07)	0.57 (0.25 – 2.90)	0.896
UnconjBb (mg/dL)	0.41 (0.25 - 0.70)	0.41 (0.23 - 0.58)	0.29 (0.17 – 2.11)	0.868
ConjBb (mg/dL)	0.30 (0.23 - 0.45)	0.41 (0.27 - 0.49)	0.29 (0.13 - 0.77)	0.715
AST (UI/L)	40.0 (31.5 - 55.0)	35.0 (32.5 - 41.0)	33.0 (25.3 – 57.5)	0.393
ALT (UI/L)	31.0 (22.5 - 53.0)	27.0 (25.5 - 31.5)	25.0 (17.5 – 41.0)	0.503
GGT (UI/L)	41.0 (26.5 - 67.5)	35.0 (28.5 - 59.5)	44.0 (31.0 – 105.5)	0,648
Alkaline phosphatase (UI/L)	326 (290 - 432)	354 (286 - 413)	282 (246 – 383)	0.472
Albumine (g/dL)	4.12 (3.95 - 4.26)	3.96 (3.71 - 4.09)	4.10 (3.87 – 4.22)	0.157
Inflammation				
C-reactive protein (ng/mL)	91.25 (59.09 - 155.99)	149.42 (56.89 – 242.39)	151.62 (79.06 – 316.29)	0.346
TNF-alfa (pg/mL)	0.74 (0.69 - 0.85)	0.802 (0.681 – 1.073)	0.897 (0.698 – 1.063)	0.257
Oxidative stress				
Erythrocytes				
TAS (mM)	4.43 (3.48 – 6.28)	4.63 (4.05 – 6.97)	5.10 (3.71 – 6.33)	0.778
Catalase activity (nmol/min/mL)	9665 (8315 - 12386)	10439 (9476 – 11333)	12128 (10527 – 13112)	0.325
GPx activity (mU/mL)	1975 (1898 - 2584)	2010 (1705 – 2240)	2106 (1883 – 2442)	0.613
GSH (µM)	26.40 (17.25 - 32.65)	24.70 (19.05 – 31.65)	24.35 (18.30 – 27.08)	0.843
GSSG (µM)	2.76 (1.01 - 6.63)	2.58 (1.91 – 6.79)	2.14 (0.61 – 3.06)	0.409
GSH / GSSG	9.06 (3.29 - 24.90)	9.10 (3.61 – 13.50)	11.35 (6.87 – 39.45)	0.594
Erythrocyte's membrane				
MBH (%)	0.83 (0.57 - 1.17)	0.71 (0.41 – 0.91)	0.98 (0.58 – 1.88)	0.223
LPO (p.d.u)	0.067 (0.03 - 0.11)	0.073 (0.033 – 0.109)	0.072 (0.049 – 0.109)	0.978
Bile acids (total, species, percentages and ratios)				
Bile acids, total (µmol/L)	14.80 (11.35 - 18.25)	9.90 (7.30 – 16.90)	14.00 (8.50 – 25.43)	0.484
Primary				
TCA (µmol/L)	2.90 (0.85 - 5.20)	1.70 (1.15 – 4.65)	3.00 (2.08 – 8.03)	0.564
TCDCA (µmol/L)	2.00 (0.90 - 4.60)	1.10 (0.80 – 3.55)	1.80 (0.98 – 3.08)	0.757
GCA (µmol/L)	2.60 (1.60 - 4.65)	1.80 (1.20 – 5.45)	2.65 (1.58 – 6.03)	0.817
GCDCA (µmol/L)	4.00 (1.90 - 4.95)	1.60 (1.10 – 2.90)	1.40 (1.15 – 7.05)	0.213
Secondary				
TDCA (µmol/L)	0.30 (0.20 - 0.50	0.50 (0.30 – 0.80)	0.40 (0.30 – 0.58)	0.361
TLCA (µmol/L)	0.30 (0.15 - 0.45)	0.30 (0.25 – 0.45)	0.40 (0.18 – 0.53)	0.562
TUDCA (µmol/L)	0.20 (0.10 - 0.35)	0.30 (0.15 – 0.40)	0.30 (0.18 – 0.43)	0.520
GDCA (µmol/L)	0.60 (0.20 - 0.95)	0.60 (0.20 – 0.80)	0.75 (0.58 – 1.35)	0.370
GLCA (µmol/L)	0.20 (0.15 - 0.40)	0.30 (0.10 – 0.35)	0.40 (0.18 – 0.90)	0.504
GUDCA (µmol/L)	0.20 (0.10 - 0.30)	0.20 (0.10 – 0.20)	0.30 (0.10 – 0.40)	0.452
Percentages				
Cholic acid (TCA + GCA)	39.70 (31.25 – 52.60)	44.40 (42.65 – 58.20)	48.45 (39.88 - 54.78)	0.335
Chenodeoxycholic acid (TCDCA + GCDCA)	**42.60** (28.75 – 54.15)	**30.40 (23.80 – 46.75)**	**31.55 (21.95 - 41.35)**	0.104
Deoxycholic acid (TDCA + GDCA)	5.70 (4.25 – 10.55)	9.80 (6.10 – 14.15)	10.15 (5.20 – 13.45)	0.334
Litocholic acid (TLCA + GLCA)	3.60 (2.35 – 6.35)	6.50 (2.60 – 7.35)	6.10 (2.88 – 8.25)	0.424
Ursodeoxycholic acid (TUDCA + GUDCA)	3.40 (2.10 – 4.35)	4.30 (2.80 – 5.00)	4.25 (2.43 – 6.50)	0.594
Molar ratios				
Cholic to Chenodeoxycholic	1.00 (0.55 - 2.00)	1.50 (0.95 – 2.35)	1.55 (1.05 – 2.38)	0.219
Glyco- to Tauroconjugates	1.00 (0.70 - 1.45)	1.10 (0.65 – 1.70)	1.10 (0.80 – 1.58)	0.893
Primary to Secondary	9.80 (4.40 - 12.55)	4.90 (3.35 – 10.10)	5.50 (3.68 – 9.60)	0.337
Trihydroxy- to Dihydroxylated	0.80 (0.50 - 1.45)	1.00 (0.90 – 1.80)	1.10 (0.85 – 1.70)	0.272

Legend: IQR, interquartile range; * Kruskal - Wallis test; $p<0.05$ was considered statistically significant.

TBb, total bilirubin; UnconjBb, unconjugated bilirubin; ConjBb, conjugated bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; TNF, tumor necrosis factor; TAS, total antioxidant status; GPx, glutathione peroxidase; GSH, glutathione reduced; GSSG, glutathione oxidized; MBH, membrane-bound hemoglobin; LPO, lipid peroxidation; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TLCA, taurolithocholic acid; TUDCA, tauroursodeoxycholic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GLCA, glycolithocholic acid; GUDCA, glycoursodeoxycholic acid.

Table 3 – Outcome measurements in two-month old full-term healthy infants according to the type of diet

Dependent variable	Model adjusted *R* square; Sig.	CDCA_N	Unstandardized Coefficients		Standardized Coefficients Beta	t	Sig.
			B	Std. error			
Independent variables	1 0.285; *0.001*	Constant	54.556	4.878		11.184	***<0.001***
		MBH_N	**-18.198**	5.132	-0.557	-3.546	***0.001***
	2 **0.452**; *<0.001*	Constant	60.784	4.898		12.652	***<0.001***
		MBH_N	**-21.229**	4.598	-0.649	-4.617	***<0.001***
		Type of diet	-6.232	2.018	-0.434	-3.089	***0.005***
Independent variables	1 0.132; *0.025*	Constant	21.487	7.382		2.911	***0.007***
		GSH	**0.664**	.281	0.402	2.362	***0.025***
	2 0.189; *0.020*	Constant	25.136	7.443		3.377	***0.002***
		GSH	**0.649**	0.272	0.393	2.387	***0.024***
		Type of diet	-4.242	2.445	-0.285	-1.735	*0.094*
Independent variables	1 0.117; *0.036*	Constant	45.104	3.845		11.729	***<0.001***
		GSSG_N	**-1.881**	0.855	-0.384	-2.199	***0.036***
	2 0.167; *0.032*	Constant	47.978	4.124		11.633	***<0.001***
		GSSG_N	**-1.747**	0.835	-0.356	-2.093	***0.046***
		Type of diet	-4.199	2.558	-0.279	-1.641	*0.112*

Type of diet was divided in 3 groups: exclusive breastfeed (n = 17), exclusive formula milk (n = 6), and mixed milk diet (n = 9)

Legend: CDCA_N, chenodeoxycholic acid normalized; MHB_N, membrane-bound hemoglobin normalized; GSH, reduced; glutathione; GSSG_N, oxidized glutathione normalized.

Table 4.– Predictor parameters of CDCA (%) assessed by linear regression models, 1) unadjusted and 2) adjusted for type of diet

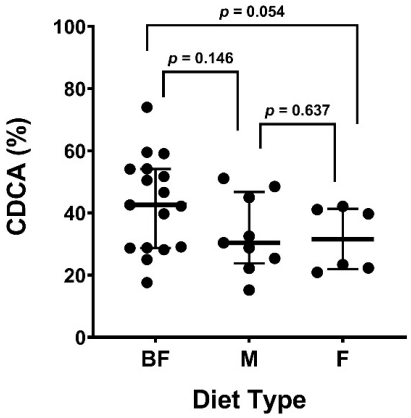

Figure. 1 – CDCA (%) according to the type of diet of two-month-old full-term healthy infants.

Data presented as median (IQR); p < 0.05 was considered statistically significant (Kruskal-Wallis test)

Legend: CDCA – chenodeoxycholic acid; BF – exclusively breastfed (n = 17); M – mix diet (human milk and formula; n = 9); F – exclusively formula-fed (n = 6).

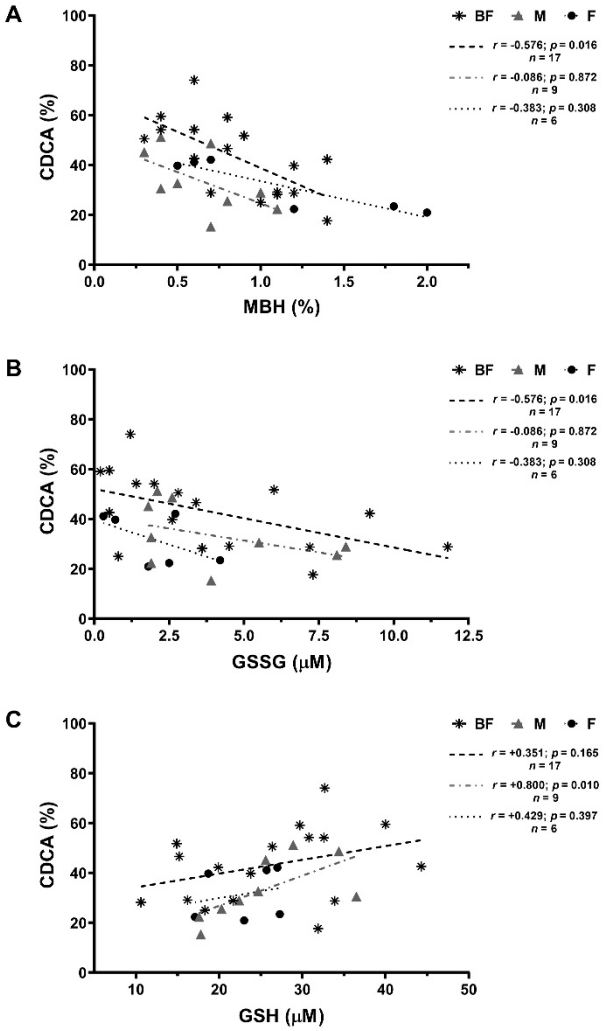

Figure. 2 – Correlations between CDCA (%) and MBH (A), and GSSG (B) and GSH (C), according to the type of diet of two-month-old full-term healthy infants.

p < 0.05 was considered statistically significant (Spearman`s rank correlation test)

Legend: CDCA – chenodeoxycholic acid; MBH – membrane-bound hemoglobin; GSSG – oxidized glutathione; GSH – reduced glutathione; BF – exclusively breastfed; M – mix diet (human milk and formula); F – exclusively formula-fed.

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CHAPTER V

GLOBAL DISCUSSION AND CONCLUSIONS

Neonatal cholestasis (NC) is a heterogeneous syndrome that remains a significant clinical challenge in the 21st century. Current strategies to improve the diagnosis and prognosis of this condition need to be strengthened while new strategies are developed. The strategies for improving diagnosis involve, first, early recognition of cholestasis and, second, prompt and accurate diagnosis of the underlying causes or entities. The strategies for improving prognosis include early establishment of support and specific treatment after accurate diagnosis.

However, current evidence indicates that further gains will only be achieved by improving knowledge of the pathophysiology of NC. This would facilitate improvements in both diagnosis (e.g., the development of new biomarkers) and treatment (e.g., novel targeted therapies), with translational research playing a major role. As with any other clinical condition, advances in the outcome and prognosis of NC rely on a circle of knowledge in which pathophysiology, diagnosis, treatment, and prevention are the main players (Fig. 20). This circle had important inputs in the last 40 years and mostly over the last decade; however, prevention has played an almost non-existent role.

Since the beginning of the 1980s, research in several scientific areas has enriched our knowledge of the physiology of the neonatal liver (17, 226), but the main contribution to unravelling the pathophysiology of some of the entities underlying NC was made in the field of molecular genetics at the end of the 20th century (21).

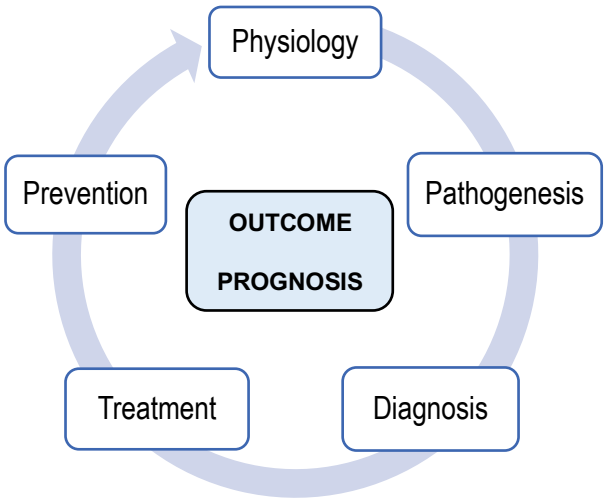

Figure 20 - Circle of knowledge

Source: Original

In some countries, organisational changes in healthcare services have improved outcomes for the subgroup of NC patients with biliary atresia (BA) (40), while in other countries a similar effect has been achieved through universal screening programmes (43,189). On the treatment front, the success of orthotopic liver transplantation (OLT) merits mention (227), although certain other medical interventions have also contributed (228,229); however, the full benefits of translational research have not yet been realised, and there are currently no preventive strategies.

In this chapter, we discuss the contributions of this thesis to this circle of knowledge and focus on their potential applications in the global and Portuguese settings. We present this discussion in two parts: first, we discuss the clinical contributions with a focus on diagnosis, treatment, and outcome/prognosis; second, we discuss the contribution of our translational research focusing on physiology and pathophysiology.

1. THE CLINICAL CHALLENGES AND STRATEGIES FOR IMPROVING PATIENTS DIAGNOSIS AND PROGNOSIS.

1.1. NEONATAL CHOLESTASIS EARLY RECOGNITION - TRAINING OF HEALTHCARE PROFESSIONALS AND SCREENING STRATEGIES

Delayed recognition of NC remains one of the main causes affecting the outcome and prognosis of patients. Two factors can contribute to this delay. One is the tendency to underestimate jaundice, the main clinical manifestation of NC, because it is common in neonates and is mainly associated with benign and self-limited conditions (e.g, physiological jaundice or breast milk-related jaundice). The other is that too many healthcare professionals are still unaware of the other possible cardinal signs of NC (dark urine and pale stools).

So far, few studies have evaluated the ability of healthcare professionals to recognise NC. Two studies were reported in 2012, the Palermo et al. study, performed in the USA, involving general paediatricians affiliated with a paediatric hospital (230), and the Bakshi et al. study, carried out in England and involving physicians and nurses from three university hospitals (231). Both studies confirmed that a significant percentage of healthcare professionals were unaware of the guidelines and adopted clinical practices that prevented prompt recognition of NC and/or pale stools. Within this research field, our work, published in 2017 (232), was the first to be performed in Portugal. In line with these studies, we also found that the clinical practices of a significant percentage of healthcare professionals prevented the early recognition of NC. In addition, we found that specialisation rather than length of practice was associated with better skills in recognising pale stools.

These studies are crucial for providing evidence to support decisions on healthcare policies. The authors of the American study highlighted the lack of a surveillance protocol, and proposed the reorganisation of their surveillance scheme. The authors of the English study concluded that it was not enough to have an adequate surveillance scheme without professionals equipped with the knowledge and skills to recognise NC and proposed an investment in the education and training of healthcare professionals.

Our study, conducted in Portugal, showed that the most substantial flaw was at the level of primary care. Despite the existence of a dedicated scheme, the Child and Youth Healthcare Surveillance programme, which includes medical appointments at postnatal weeks 2 and 4, healthcare professionals are not specialized in paediatrics. Additionally, education and continuous training in the relevant clinical practices and the dissemination of the national

guidelines (197) have taken place almost exclusively through paediatric channels, and have not been included in general practicioners (GPs) training programs.

In our view, the education and training of healthcare professionals (physicians and nurses) needs to be extended and strengthened. However, this will not be enough. In the Portuguese context, we believe that the best strategy would be to reorganise the National Healthcare System (NHS), assigning the provision of primary healthcare to newborns and infants to paediatricians and nurses specialised in paediatrics. Nevertheless, we understand that this approach requires significant human and financial resources and that it may not be cost effective to implement to address NC alone. Similar studies in other areas of healthcare provision for this age group may be helpful in strengthening the rationale and support for this strategy. From a cost-effectiveness perspective, the more generalised improvement in health of the population of this age-group that this strategy could bring should be considered.

The other possible strategy for achieving early recognition is universal screening for NC. However, there are many obstacles to implementing such a strategy, as mentioned in the introductory chapter. The main difficulties are the lack of a validated screening method and a lack of consensus on the most effective time to carry out such screening.

Nevertheless, a validated screening tool [the stool colour card (SCC)] does exist for biliary atresia (BA), one of the underlying entities of NC. Universal screening for BA has been implemented in some countries in Asia, where the incidence of BA is higher than in other parts of the world, and has proved cost-effective (43,190). The cost-effectiveness of universal screening in Western countries, where the incidence of BA is lower, has not yet been established (192), and some countries use the SCC to screen for NC in general. There are two problems with this strategy, however. The first is that the method has not yet been validated for this purpose. Second, the information that accompanies the SCC generally refers to the presence of prolonged jaundice. In these cases, the message should be carefully evaluated, as less specialised professionals or caregivers may wrongly assume that the presence of pale stools is mandatory for a diagnosis of cholestasis in a jaundiced newborn or infant.

We requested and obtained permission from both the author (Professor Mei-Hwei Chang) and the Taiwan Ministry of Health and Health Promotion Administration to use the Taiwan SCC as a disclosure and learning tool and for a future BA screening programme in Portugal. We then published a letter to the Editor of *Acta Médica Portuguesa* (233) proposing the use

of the SCC as a learning tool for parents and other caregivers at discharge from the maternity hospitals. We believe that the SCC would also be a valuable tool for General Practitioners (GPs) to use for the first two appointments after discharge from the hospital. Finally, we suggested that the SCC should be included in the digital version of the Portuguese Child and Youth Health Booklet. Moreover, we proposed that the SCC be distributed to both healthcare professionals and caregivers through a web link or an app. Our decision to publish in *Acta Médica Portuguesa* was motivated by our desire to increase the visibility of this type of initiative by reaching both healthcare professionals (i.e., GPs) and decision makers.

1.2. TIMELY AND ACCURATE ETIOLOGICAL DIAGNOSIS – NEW DIAGNOSTIC TOOLS AND PARADIGMS

Another modifiable factor that can affect the outcome and survival of patients with NC is the timing of the etiological diagnosis, which should be performed as soon as possible to facilitate specific and effective treatment.

The identification of the underlying entity is an opportunity not only to determine the most appropriate treatment for the specific patient but also contributes to our knowledge on NC. We highlight the contribution we made long before the thesis period in describing a very rare underlying entity, neonatal sclerosing cholangitis (234), and another two whose association with NC was not well known at the time, namely, argininemia (134) and McCune-Albright syndrome (MAS) (140). Although the latter had been described in previous reports concerning NC (135, 136), we were among the first to prove, through molecular genetic study of liver tissue, that the involvement of the liver was not an occasional association, and we were the first to propose the inclusion of MAS among the list of genetic cholestatic syndromes (140). The patients described in these case reports are included in the cohort published in Article 2.1 (235), except for one of the patients with MAS, a Polish girl.

To add to our old report, published in 1998, of two siblings (a boy and a girl) with sclerosing cholangitis, we recently identified a novel mutation in the girl. This could not be confirmed in her brother, as he declined to be tested (235). Both patients were submitted to OLT as children and transited into adulthood clinically stable and socially integrated. Nevertheless, the boy subsequently developed chronic renal insufficiency (currently at stage 3), while the girl maintained normal renal function and delivered an unaffected offspring.

Concerning argininemia, our report of 2010 was one of few describing NC as the first clinical manifestation of the disease (134). In one patient, the diagnosis of argininemia was made in the course of an NC work-up; liver disease phenotype mimicked Alagille syndrome and progressed to biliary cirrhosis requiring OLT. More recently, we applied a next-generation sequencing (NGS) panel to this patient, but we did not identify any variants other than the one in homozygosity in the ARG1 gene (235). The other patient described in the report was diagnosed through the extended newborn screening (NBS) programme. The development of cholestasis occurred one week after the initiation of medical treatment and had a mild and transitory course.

Finally, the Portuguese patient described in the MAS report (140) had persistently elevated transaminases and gamma glutamyl transferase (GGT) but had no symptoms or signs of progressive liver disease up to the age of 18. Unfortunately, he was lost to follow-up after his transition to adult healthcare (235). In our Article of 2000 (140), we demonstrated the presence of an activating mutation in the gene encoding the alfa-subunit of the G-protein that stimulates adenyl cyclase in the liver tissue of two patients. Similar mutations had previously been identified in other affected tissues and liver specimens of only two patients (138,139). We hypothesised that the defect could lie at the level of hepatocytes and/or the biliary canalicular membrane and that the lower levels of cyclic adenosine monophosphate (AMP) make the secretion of normal biliary components difficult. In 2012, Nault et al. (236) identified a cross-talk between the cyclic AMP and Janus kinase / signal transducer and transcription (JAK/STAT) pathways in liver tumours, but the mechanism by which the same mutation might induce NC remains unexplained.

The development and application of new diagnostic tools, such as the NGS panels, are crucial for improving etiological diagnostic accuracy and timeliness. Large-cohort prospective studies are still needed to prove the cost-effectiveness of the NGS panels. Nicastro et al. (27) conducted a prospective study with 125 patients, 50 of whom underwent a diagnostic protocol including genetic testing and obtained a detection rate of 60%; this rate is much higher than rates previously reported by other authors (26). Nevertheless, a cost-effectiveness analysis must consider aspects other than detection rates, such as reduction of costs, invasiveness with other tests, and speed of etiological diagnosis and therapeutic initiation.

In this respect, we must highlight our more recent illustrative contribution whereby we applied an NGS panel to 13 patients in our series of NC patients [N = 154, including n = 24 (16%) with transient cholestasis and n = 12 (8%) with idiopathic cholestasis], as reported in Article 2.1 (235). We emphasise that this diagnostic tool has only been applied since mid-

2017, with debatable application criteria and a small sample. Therefore, we are still evaluating its applicability to clinical practice. Furthermore, we have not evaluated its cost-effectiveness in our context. Nevertheless, some of our findings resulted in the following contributions:

First, as mentioned above, we achieved molecular confirmation of the diagnosis of neonatal sclerosing cholangitis in a previously described pair of siblings (234, 235), including the description of a novel mutation. Second, we identified some potentially interesting heterozygous variants. 1 - One variant of unknown significance (VOUS) in the *ATP8B* gene in a patient with BA. Given the current acceptance that certain polymorphisms could play a role in the pathogenesis of BA, this report may be relevant. 2 - Some VOUSs in patients with transient cholestasis without known risk factors: a) one patient had a heterozygous variant in the *ABCB11* gene (previously associated with PFIC-2) and b) another had three variants in heterozygosity associated with autosomal recessive diseases (*DCDC,2 DGUOK, and VP5338* genes). 3 - In a patient with transient cholestasis with a risk factor (starvation), we found two heterozygous VOUSs in the *CFTR* gene (cystic fibrosis); both were previously described in association with two different clinical conditions of autosomal recessive inheritance (primary ciliary dyskinesia and bilateral absence of the vas deferens). In this patient, the NC was resolved, and he is currently asymptomatic, with normal liver tests. However, given the short follow-up time, prudence is recommended in interpreting this finding, as the patient has not yet undergone a sweat test.

Over five decades, more entities have been progressively added to the long list of entities underlying NC. With such a long list of possible causes and underlying entities of NC, some systematisation is necessary. On this subject, in early 2000, Balistreri et al. (113), quoting the Chinese proverb 'the principle of wisdom is to call things by their right names', proposed a new categorisation of the entities underlying NC. The main difference was the classification of entitles into familial and sporadic causes instead of extrahepatic and intrahepatic causes.

Although this categorisation poses difficulties for classifying certain entities (e.g., BA, transient cholestasis), in our view, given the current state of knowledge, it is the most appropriate, and we suggest here some adaptations to Balistreri et al.'s original idea (Fig. 21). For example, we propose that based on the current state of the art, BA should be classified as a sporadic and multifactorial entity; however, regarding transient cholestasis, although we think it should be classified as sporadic (and possibly multifactorial), the results we present in Article 3.2 warn us to be cautious in clinical practice (237). Transient cholestasis is an exclusion diagnosis, and it may, although rarely, be the first clinical

manifestation of some metabolic diseases. Moreover, we believe that some distinction should be made between cases of cholestasis with a transient course but not associated with known perinatal risk factors and those cases associated with risk factors.

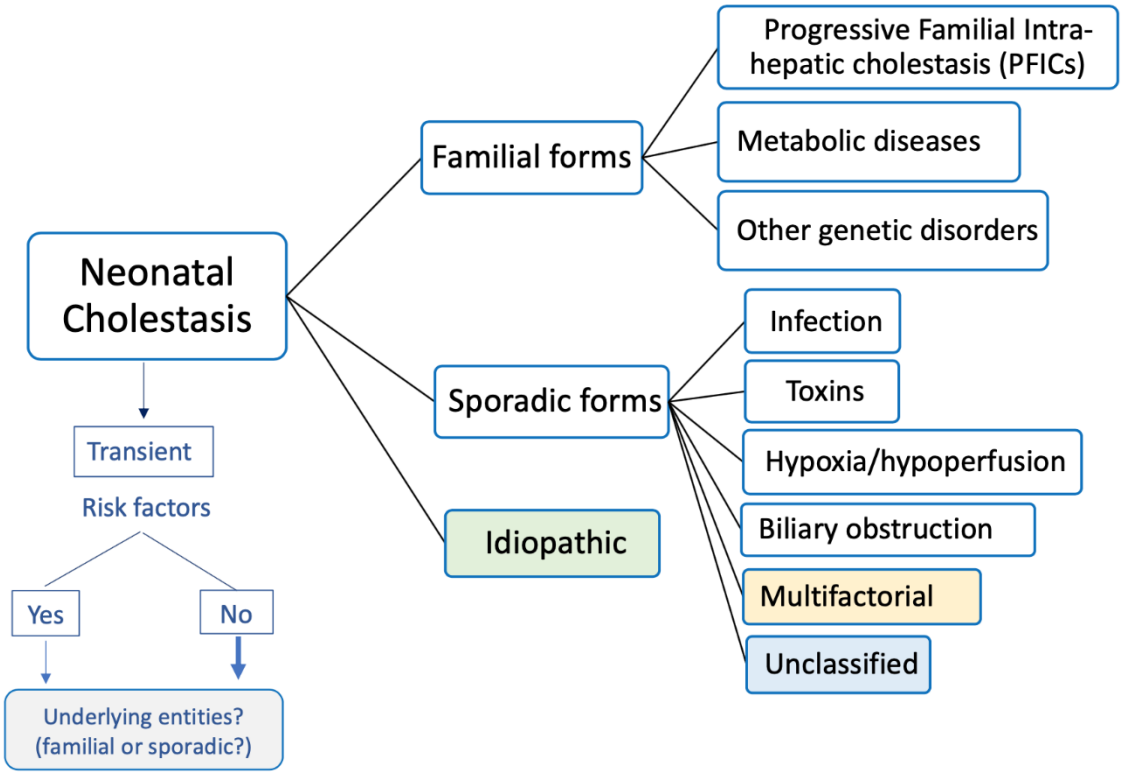

Figure 21 - Underlying entities (schematic classification)

Source: Original

Besides the identification of an extensive list of underlying entities and knowledge of those entities, a timely and accurate etiological diagnosis requires the development of a suitable diagnostic methodology. Currently, we are between two diagnostic paradigms: the old paradigm, whereby we must quickly separate the BA patients from the non-BA ones—a difficult task, based on invasive procedures and followed by a complex and laborious diagnostic process for the non-BA group—and a potential new paradigm, which we are exploring and which integrates new molecular diagnostic tools.

At this crossroads, the building of a diagnostic algorithm may be challenging. This is reflected in the most recent guidelines from scientific societies, the joint ESPGHAN and NASPGHAN guidelines, published in 2017, which have so far failed to provide a decision algorithm (28). Although it is generally accepted that no algorithm will ever be able to

replace clinical skills and expertise applied to each individual patient, it could be a useful tool. We highlight the contribution we made with our proposal of a diagnostic algorithm based on the results of our work, our clinical practice, and a literature review.

To build a diagnostic algorithm, we need to identify discriminators to guide the clinical decision process. Studies in this area are rare and only reported for patients with BA (238). In Article 3.1, we aimed to build a decision algorithm based on discriminators identified by predictive models of diagnosis and prognosis from real-world patients (239). In our study, we were able to build predictive models of diagnosis for some NC underlying entities. In particular, we emphasise an A1ATD diagnostic model, due to the unusually large number of these patients in our sample, and two predictive models of unfavourable prognosis. We also identified three important discriminators out of the five that we used to build the diagnostic algorithm: signs and symptoms of sepsis and/or liver failure, pale stools and GGT >300UI/L. We found that these indicators had predictive value for the diagnosis of metabolic or infectious diseases and BA, respectively, and that GGT > 300UI/L was predictive of both BA and A1ATD diagnosis and unfavourable prognosis. Although these discriminators are in line with clinical evidence and with experts' opinion, our sample was small, so our results must be further validated on larger samples.

Additionally, we retrospectively analysed 16 selected patients with metabolic liver disease who presented with NC (Article 3.2) and identified three main clinical phenotypes of liver injury: NC with liver failure, NC with chronic liver disease, and transient cholestasis (237). In this article, we presented some clinical clues suggestive of metabolic diseases in NC patients. We also proposed a diagnostic algorithm to identify metabolic diseases in NC patients, which includes the use of an NGS genetic panel.

Currently, some authors advocate for a diagnostic paradigm shift in the approach to NC. They propose an initial assessment to identify cases of BA, and in the others, apply the NGS panel immediately (114). Instead, we still find useful some old clinical and biochemical discriminators and advocate for a mix of both paths, a kind of third way to which our algorithms are intended to contribute. Although NGS panels have the potential to significantly increase diagnostic accuracy, this advantage must be carefully weighed against their disadvantages, particularly the difficulty of interpreting them. So far, our clinical practice has shown us that they sometimes bring more questions than answers, which can result in an escalation of anxiety, invasiveness, and costs.

We believe it is important to review the recommendations and guidelines of scientific societies, and to develop a decision algorithm. At the same time, we emphasise the original

and valid contribution our proposals make to this discussion and construction process. We especially highlight our efforts to find some of the discriminators used in the algorithm published in Article 3.1 through the building of diagnostic and prognostic models.

1.3. MANAGEMENT OF UNDERLYING ENTITIES – PATIENTS FIRST

Strategies to improve early recognition of NC and the etiological diagnosis are the first steps towards improving patients' prognosis. After this first end-point has been accomplished, patients' outcomes and survival depend on appropriate management and the availability of supportive and specific treatment. Separate analysis and targeted management of some underlying entities have improved our understanding of these entities and the outcomes of patients with those diseases.

Among these underlying entities, the first to merit a separate analysis was BA. This may be due to historical reasons, the more distinctive clinical picture of BA compared to other entities, and its higher incidence among rare diseases. Regardless of the reasons, a separate analysis makes sense in the search for a deeper understanding of the pathogenesis, diagnosis, and treatment of this entity, as well as for screening and prevention. The results of this strategy over the last few decades, particularly in recent years, have been remarkable (145). At the international level, we highlight the contributions of British (40), French (42) and American (240) multicentre studies, the Japanese registry (JPBAR) (44), and Taiwanese studies (189).

Our contribution, though modest, included the publication in 2010 of a mini-series of patients with BA, to which the *Birth and Growth Medical Journal* awarded the distinction of best original article of the year (241).

BA has an estimated incidence of 1/18,000 births in European countries (42, 242). In Portugal the mean population and the average live birth rate in the period of our study (1992–2007) were 10.2 million people and 10.9‰, respectively. The births rate progressively decreased and in the year 2020 was 8.2‰ in the same population. So, the expected number of new cases per year of BA, in the period of the study was 6.18 and in the year 2020 was 4.65. In the period of our study, Portugal had five paediatric gastroenterology units; thus, the average number of new patients/centre/year was 1.24. In line with what was observed in studies carried out in England and France, this caseload would not allow our centre (or any Portuguese centre) to achieve the best results in the

treatment of BA (40.41). Nevertheless, our data showed that patient outcomes and survival were level with the best of our contemporary peers in other European centres (42).

In Portugal, there is another subgroup of patients with equal or higher incidence among patients with NC than the BA subgroup, namely patients with A1ATD (235). In fact, this subgroup has the highest relative incidence in our series, which was published in 2020 (about 22% versus 16% for BA) (16). This subgroup merited a sub-analysis in 2015, as reported elsewhere (243), aimed at identifying outcome predictors at the time of patient admission and evaluating the effect of treatment with ursodeoxycholic acid (UDCA). Patients with A1ATD who present with NC have mostly been studied by Nordics and British groups, and these studies were mostly outdated. The north of Portugal, which has a genetic heritage dating back to the Celtic invasion of the Iberian Peninsula, has a similar incidence of the disease to that of Northern European countries.

Furthermore, we highlight the authors' participation in the GALA study group (Global ALagille syndrome Alliance) during the preparation of this thesis. The results of these studies have been presented in four conference papers (244-247), and a 5th has been accepted for presentation. They are also described in an original article recently submitted to *Gastroenterology* (unpublished data). The GALA study is an international research project, on a global scale, concerned with the retrospective and prospective study of Alagille syndrome (one of the rare genetic diseases that can present as NC). So far, the GALA study has managed to assemble the largest Alagille syndrome (ALGS) cohort ever ascertained (n = 1438 patients, from 67 centres in 29 countries). The Centro Hospitalar Universitário do Porto (CHUP) is the only Portuguese centre in the GALA study group in which the Portuguese author is the local researcher. Currently, 3 Portuguese patients are enrolled in the GALA study. The study found that 86% of patients with ALGS had a history of NC; at patient age 18 years, the estimated cumulative incidence of OLT or death (unfavourable prognosis) was 59.7% (95% CI, 54.6–64.5). Liver-related complications were the leading cause of death (30%). A Kaplan-Meier analysis demonstrated a significantly lower cumulative incidence of survival at 10 and 18 years of age in patients with ALGS presenting with NC ($p < 0.001$) (unpublished data).

The lessons learned over three decades of clinical practice, the results of large international multicentric studies and, more recently, of international research consortia, point out the strategies to improve the diagnosis and prognosis of these patients: training for healthcare professionals, early diagnosis campaigns, universal screening when a cost-effectiveness analysis proves it is warranted, concentration of rare pathologies in the services with the

best skills with reorganisation of services when needed, and the constitution of major international research consortia.

As a corollary of our studies, all patients with BA from our centre are referred to undergo Kasai surgery at the only paediatric liver transplant centre in Portugal. Accordingly, we collaborate closely with that centre in the follow-up of patients and the treatment of complications in the medium and long term. It is our view that the principles that must guide all political decisions on this matter may be summed up in the phrase ‘patients first’.

1.4. ADVANCES IN TREATMENT – AN ILLUSTRATED POSTCARD

The outcome and survival of patients with NC also depends on the existence and application of specific and effective treatment for underlying conditions. Among several specific therapies, enzyme replacement therapy (ERT) is effective for treating some lysosomal diseases. This thesis includes a short report describing two infants with a metabolic disease (EO-LAL-D), whose clinical presentation included progression to cholestasis and liver failure and who were treated innovatively with ERT (248).

The two unrelated patients (one Portuguese and one Polish) presented with hemophagocytic lymphohistiocytosis (HLH) and a diagnosis of a familial form of this entity (FHL); this diagnosis was assumed to be correct while the molecular study was ongoing. They underwent treatment aimed at this entity and progressed to cholestasis and liver failure. Meanwhile, the molecular diagnosis of FHL was negative, and the clinical signs, namely lymphocyte vacuolisation in patient #1 and calcification in the adrenal glands in both patients, suggested another diagnosis. The late diagnosis of EO-LAL-D, also called Wolman's disease, was confirmed, in both patients, by enzymatic assay in leukocytes and by molecular studies; additionally, patient 1 had diagnosis confirmation by an enzymatic assay in fibroblasts.

Treatment with sebelipase alfa was tried in both patients on an off-label regimen after obtaining special permission for use. Unfortunately, the treatment failed to prevent a fatal outcome in both patients, but current evidence shows that it can be life-saving when applied early (211). In fact, two studies have shown that it has resulted in longer survival (79% at 12 months and 68% at 5 years of age) with normal psychomotor milestones and growth, improvement in haematological and liver parameters, and a satisfactory safety profile.

These two cases illustrate how crucial an early and accurate diagnosis is for determining the outcome, as treatment, if started early, can be life-saving.

We also highlight our previous contributions to therapeutic innovation, including our unprecedented proposal to perform liver transplantation in two patients with argininemia. The first patient had NC and progression to liver cirrhosis with no neurological manifestations as far as the date of the liver transplant in 1999 (249). The second patient was transplanted two years later for progressive spastic diplegia and subclinical liver disease. We followed the two patients in the pre-and post-transplant period; in 2013, we described their outcome after a long period of follow-up (12 years and 10 years, respectively), demonstrating that the liver transplant prevented the development of neurological manifestations in the first patient and interrupted the progression of neurological manifestations in the second patient (250). Currently, the condition of both patients remains unchanged.

Our pioneering experience of OLT in argininemia patients (249, 250) has been helpful to other authors in deciding whether to transplant their own patients, as documented by their citations of our publications (251-253).

In both our patients, we demonstrated that argininemia serum levels and serum and urinary levels of guanidine compounds (arginine catabolites) decreased significantly after OLT, which had never been achieved in the period before liver transplant.

Contemporaneously to our first report of OLT in argininemia patients (249), Wyse et al. (254) demonstrated that raised arginine and guanidine compound serum levels increased oxidative stress and reduced antioxidant capacity in the brains of rats. Regarding the possible mechanisms of the disease in the liver, the results of recent studies have been inconclusive (255,256).

More recently, in 2018, Angarita et al. demonstrated that human hepatocyte repopulation in the murine liver can effectively treat arginase deficiency (257), preventing metabolic abnormalities. Moreover, they also demonstrated that such treatment prevents the development of neurological manifestations. In conclusion, advances in knowledge of argininemia have taken place in a translational direction from the patient's bedside to the laboratory bench.

To date, the contributions we have made to the development of innovative therapies are no more than illustrative case studies. The research on innovative therapies for these rare diseases depends on joint efforts across multicentre studies and clinical trials, as occurs with the research on their epidemiology and natural history.

1.5. OUTCOME AND SURVIVAL – A PORTUGUESE CONTRIBUTION

When developing innovative therapies for cholestasis and some of the underlying entities of NC, it is crucial to be able to estimate outcomes and survival prospects so that we can balance the costs of these therapies with the expected benefits for patients. The outcome and survival of patients with NC, as a group, is not well known. Since they depend mainly on the underlying condition, outcome and survival obviously differ for BA, rare genetic and metabolic disorders, infectious diseases, and transient cholestasis. Although data from tertiary centres were available, they were largely outdated for some subgroups of underlying entities. More recently, consortia studies have reported some data (39, 44, 49, 119). In Portugal, however, there have been no studies in this area.

Article 2.1 represents a Portuguese contribution to this important issue (235). In our study, we found that unfavourable outcomes and mortality were considerable (47% and 12%, respectively) and comorbidities were observed in 23%, which is in line with another relatively recent European study (37). Despite the significant improvement in the early recognition of cholestasis and advances in the accuracy of the etiological diagnosis in the last three decades, improvements in outcomes and survival (overall and with native liver) have not been significant. Moreover, there were no significant differences between the period before the year 2000 and the period after 2000 (which were similar in duration) in terms of overall survival (86% versus 88%) or survival with native liver (85% versus 87%). Patients were managed by a medical team, whose core remained stable over the study period, and received supportive and specific treatment in line with the state of the art. We highlight the fact that patients had access to OLT throughout the observation period, initially, in foreign centres and, from January 1994, in a single paediatric centre in our country. OLT was 18.2% (n = 28) and was significantly lower after the year 2000 (18/10, p = 0.020); after the year 2000, the survival rate of patients with OLT reached 100%.

Overall survival rates differed depending on the underlying entities. Patients with metabolic diseases and those with idiopathic cholestasis had significantly lower overall survival rates than the other patients (72% versus 89% and 63% versus 89%, respectively). The survival rate of patients with transient cholestasis was 100% without OLT. The survival rates of patients with BA and A1ATD were not significantly different with or without OLT (93% vs 75% and 100% vs 96%, respectively).

Our results largely support the need for innovative diagnostic tools and therapies to significantly improve outcomes and survival in Portuguese patients. Although our study has some limitations, namely the fact that it is a retrospective, single-centre study, and the sample sizes for some subgroups are small, it also has significant merits. It is a longitudinal

study, conducted over three decades by a medical and research team that maintained its core composition, and the clinical records were of a high standard. To the best of our knowledge, this article is the only one of its kind published in our country. Internationally, it is one of few studies to be published recently that includes a longitudinal analysis of NC outcomes and survival.

Prognostic tools are of enormous value in clinical practice for establishing a therapeutic plan, monitoring patients, and informing parents and caregivers. These tools are scarce in the context of NC. We need prognostic tools to use on admission of patients with NC when an etiological diagnosis has not yet been established. Lu et al. (258) demonstrated the positive predictive value of GGT (serum values of < 75 UI/L and >300 UI/L) for poor prognosis in a sample of 135 patients with intrahepatic cholestasis; this study was based on univariate analysis, without adjustment for the presence of potential confounders or biases.

Our Article 3.1 (239) adds knowledge in this area. Based on our sample of 154 patients (the cohort characterised in Article 2.1), including cases of intra and extrahepatic cholestasis, we analysed clinical and biochemical variables at admission. Using statistical tools for univariate, bivariate, and multivariate analysis, we built two valid logistic regression models, in which GGT had positive predictive value for unfavourable prognosis. Interestingly, the GGT cut-off values were similar to those obtained by Lu et al. (259). In a model with GGT ≤ 100 UI/L, the OR was 4.566 (p = 0.016), and in a model with GGT > 300 UI/L, the OR was 3.772 (p = 0.002). The bilirubin serum levels also had predictive value for an unfavourable prognosis in both models (total bilirubin positive and unconjugated bilirubin negative predictive value). As previously mentioned, our results need to be externally validated in larger samples.

Once the etiological diagnosis has been established, some predictive factors for prognosis can be established for some subgroups of patients. For example, once a diagnosis of BA is established, we know that the prognosis depends on how early Kasai surgery is performed and the skills of the medical team. We also know that if Kasai surgery is unsuccessful, the disease will progress to end-stage liver disease, requiring OLT before 2 years of age (242). However, the prognostic indicators for medium- and long-term outcomes in patients for whom Kasai surgery has been successful are not well established. Furthermore, in patients with other underlying entities (e.g., ALGS), the predictive indicators of prognosis are not well known. In this respect, we emphasize the upcoming results of the GALA study group.

In this section, we have discussed early recognition of NC, the etiological diagnosis (underlying entities), and management and treatment as determinants of the outcome and survival of patients with NC. We have suggested some strategies to improve these determinants and the patients' diagnosis and prognosis, and presented our attempts to apply these strategies in Portugal.

Next, we address the other determining factor for the patient's diagnosis and prognosis, namely, the pathophysiology of NC, and underline our contributions on this matter. We highlight that in some underlying entities (e.g., BA, A1ATD), liver disease may progress to fibrosis and cirrhosis even after the resolution of cholestasis. This reinforces the need to understand the pathophysiology of cholestasis and that of its underlying entities to improve patients' prognosis and to move towards the development of preventive strategies.

2. FROM THE BEDSIDE TO THE BENCH. A LINK TO THE PATHOPHYSIOLOGY OF NEONATAL CHOLESTASIS.

The mechanisms of cholestasis remain largely unknown, but the involvement of oxidative stress has been suggested by some authors, mainly as a consequence (21). However, Champion et al. (23) also suggested that oxidative stress could be the cause of transient cholestasis in neonates, instead of its consequence. Neonates are especially prone to oxidative stress due to their reduced antioxidant defences. Additionally, they are often exposed to situations associated with increased oxidative stress, either physiological (e.g. labour and change to hyperoxia environment after birth) or pathological (foetal growth restriction, asphyxia, sepsis, etc), some of which, in turn, have also been associated with transient cholestasis.

Transient cholestasis has been described in the literature since the end of the 20th century (22). In our series, published in Article 2.1, we reported an increase in patients with transient cholestasis, especially from the year 2000 onwards (235). Transient cholestasis has been associated with clinical risk factors, such as prematurity, low birth weight, sepsis, and surgery (especially abdominal) (23). However, the best known and most studied risk factor in this subgroup of patients is parenteral nutrition, which has been explored in dedicated studies (166,173). Risk factors for transient cholestasis combine greater immaturity (prematurity) with greater exposure to oxidative stress (chronic or acute) and, thus, their exploitation may potentially provide a clue to the pathophysiology of neonatal cholestasis – this was the starting point for our translational investigation from the 'bedside to the bench'.

Our main research question was if there is a link between the mechanisms of bile formation and secretion and the mechanisms that regulate the redox balance in a life stage in which both systems are immature.

Suchy et al (17) have demonstrated that serum total bile acids are raised in neonates and small infants, and its profile is immature. Bile acids are molecules with signaling properties (similar to hormones), with a wide range of actions, from nutrient sensors to metabolic regulators. Their role in hepatocellular functions is critical, and their enterohepatic circulation is tightly regulated. To date, the full implications of an immature bile acid profile in neonates and young infants are unknown. As far as we know, our research is a pioneer exploring a potential link between bile acids profile and redox status in healthy infants. We performed an exploratory study in two-month old healthy infants and chose erythrocytes as a minimally invasive model of cumulative oxidative stress damage, and considering that it has been shown that bilirubin (259) and bile acids, namely GCDCA and TCDCA (260), may cause eryptosis (erythrocyte's suicidal death) in healthy individuals.

Our article 4.1 displays the first results of our research and intends to contribute to the knowledge of liver physiology and redox balance in early postnatal life. Our findings are in line with previous animal (261, 262) and human studies (17), with high plasma levels of total bile acids and an immature profile. However, our main finding was a strong negative correlation between the CDCA percentage in plasma and the membrane-bound haemoglobin (MBH) (a biomarker of erythrocyte's oxidative damage) reinforced by the correlations with glutathione. This finding is in opposition to observations in some cholestatic liver diseases, in which an increase in plasma CDCA concentration was associated with increased oxidative stress in the plasma and liver (89, 263).

As far as we know, our finding is original and was not previously reported. We speculate the immature bile acid profile leads to adaptive/compensatory mechanisms which may involve a better redox status in full-term healthy infants to activate compensatory circuits and improve bile acid homeostasis (a full-circle). Future studies should confirm our finding that may achieve clinical applicability in preventive and therapeutic strategies in transient cholestasis and in other non-familial forms of neonatal cholestasis.

Our secondary research question was whether the type of diet might have any influence in a potential link between bile acid profile and redox balance in healthy infants.

The liver is an organ that matures progressively after birth and during the first year of life. This maturation process is influenced by several determining factors, both internal and external. One of the external (environmental) factors may be the diet. Studies performed in

breastfed animals showed higher levels of total biliary bile acids and increased concentrations of conjugated CDCA than in the formula-fed ones (261, 262). Recently, a study using an animal model showed that early weaning affected several hepatic metabolic enzymatic pathways, offering new insights into how diet can disrupt liver metabolic maturation (264). Additionally, there is some evidence that breastfeeding may improve the redox balance in preterm infants (265) and in infants affected by some medical conditions (266).

On this issue, the results presented in Article 4.1 are most likely hindered by small sample size, and though not achieving statistical significance are in line with previously described studies in animal models, namely a trend towards a delayed maturation of the bile acid profile in infants exclusively breastfed compared to formula-fed ones; and, also as in these studies, they pointed to the exception to this immaturity, characterized by serum levels of GCDCA tending to be higher in those who are exclusively breastfed. Interestingly, GCDCA was associated with cytotoxicity, mitochondrial dysfunction, and oxidative stress in liver tissue from human patients with obstructive-type cholestatic liver disease (267). However, our results also showed a trend towards a better redox balance in those exclusively breastfed. Furthermore, a finding that we consider very important, the diet factor reinforced the predictive capacity of linear regression models that demonstrated that oxidative stress parameters (MBH, GSSG, and GSH) can predict the percentage of CDCA conjugates concentration. Future studies should confirm the results observed in our study, breastfeeding could be seen as a preventive measure in the setting of neonatal cholestasis.

Finally, we intend to question here the concept of 'physiological cholestasis' from Suchy et al (17). In our opinion, this nomenclature is inaccurate and may confuse the clinicians. Healthy neonates and infants have higher total bile acid values than older children and adults, and have a different bile acid profile, but as long as their conjugated bilirubin is below the threshold considered pathological (< 1 mg/dl), they do not meet the criteria for clinical designation of 'cholestasis'.

All we need, in clinical practice, is to define the reference values of bile acids for these age groups. Currently, the available data are sparse (72, 268), and there is no consensus on reference values due to variations in methodologies and physiological conditions under which studies were performed. Our Article 4.1 contributes with the results from a sample of two-month-old Portuguese healthy infants, which may be used as reference values for our population and as a control group for further studies. We underline that the diet factor must be taken in to account whenever establishing those reference values.

In summary, the neonatal liver is prone to develop cholestasis due to its immaturity, both morphological (biliary tree) and functional (enzymatic systems). Additionally, neonates have immature antioxidant defences. Also, neonates and young infants have an innate pro-inflammatory bias of their immune system, and there is increasing evidence of the participation of this condition in the pathogenesis of cholestasis (a more detailed discussion on this subject is beyond the scope of this thesis). When to this background is added an internal (e.g., genetic disease, especially involving one of the enzymatic systems responsible for bile formation or excretion) or external factors (e.g., infectious, drug, or toxic agent), pathological cholestasis seems inevitable. Likewise, in the presence of certain clinical conditions (e.g., hypoxia, hemodynamic disturbance, fasting, surgery) or treatments (e.g., parenteral feeding), the same clinical expression is expected, although with a transient course (Fig. 22). Preliminarily, we suggest that the transient cholestasis be considered on the list of clinical manifestations of oxygen radical disease in newborns (112).

Our article 4.1 is an original contribution to the knowledge of this complex scenario, although our results may need to be validated in larger samples. We hope that the progress of this research may lead to therapeutic and preventive strategies for NC.

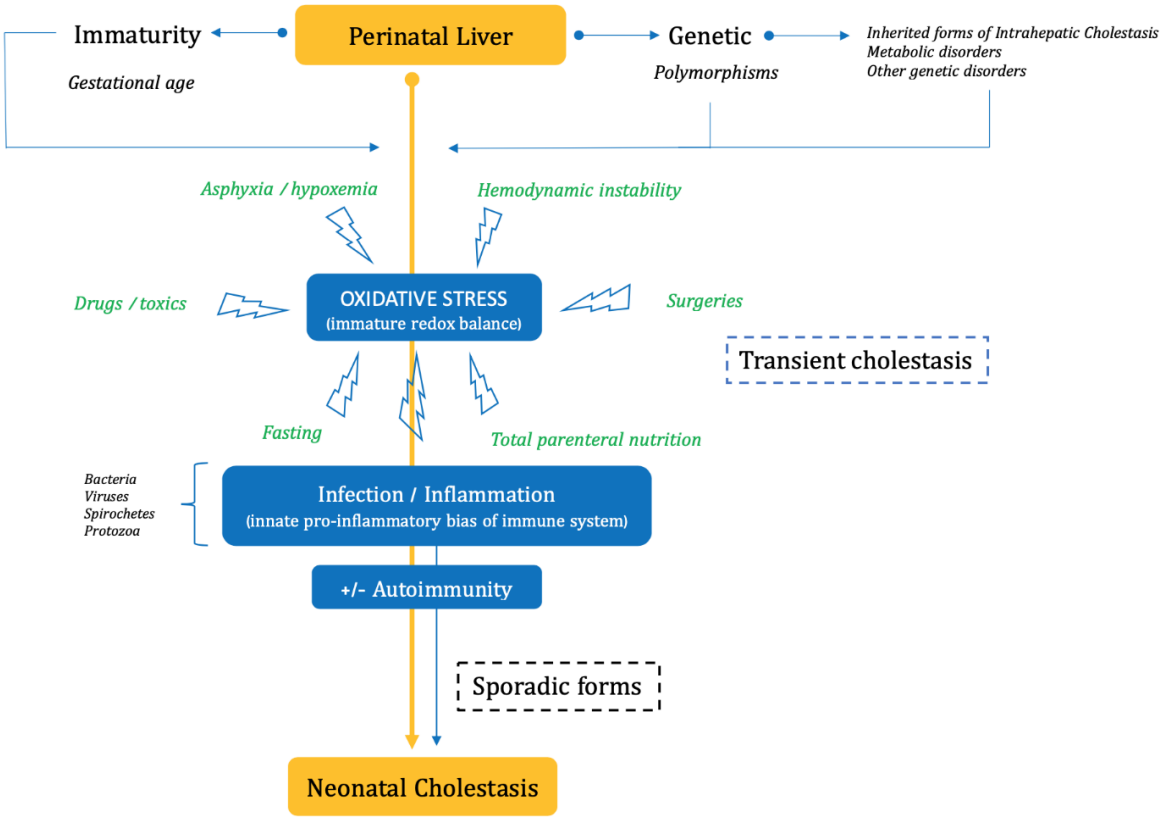

Figure 22 – A draft on neonatal cholestasis pathophysiology

Source: Original

3. LIMITATIONS OF THE STUDIES

Although our studies have made some valuable contributions, they also have some limitations, which must be acknowledged.

- Study 1.1 is regional in scope which does not allow knowing any nationwide asymmetries. Our instruments were a questionnaire and a card with stool photographs. Both were locally validated, which means they lack some robustness.
- Studies 2.1 and 2.2 were retrospective, although the core team who diagnosed and treated patients remained stable and kept high-quality clinical records.
- Studies 3.1 and 3.2 were based on retrospective data, albeit of a high quality; the sample in Study 3.2 was small.
- Study 4.1 was performed on a smaller sample than initially scheduled due to difficulties in recruiting healthy infants for a study involving blood collection.

4. CONCLUSIONS

Major findings and author contributions of this thesis

CLINICAL STUDIES

- We found that healthcare professionals in institutions at the three levels of the NHS in northern Portugal maintain clinical practices that prevent early recognition of NC. Our study on this issue is the first of its nature to be conducted in Portugal.
- We proposed the use of the SCC of Taiwan (adapted version) as a learning tool in maternity departments and for early ambulatory screening in primary healthcare. We also proposed its incorporation into the Portuguese Child and Youth Health Booklet (digital version). Permission to use the SCC for this purpose was obtained from Professor Mei-Hwei Chang and the Health Promotion, Ministry of Health and Welfare, Taiwan.
- We contribute with Portuguese epidemiological data, obtained through a retrospective, longitudinal study conducted over ~35 years in a tertiary centre. The author was directly involved in the diagnosis and management of most patients through the years. This is the first study to have been published in Portugal with this characterisation.
- Over time, the age of cholestasis recognition decreased significantly ($p < 0.001$) and changes in epidemiology occurred, with a significant decrease in A1ATD ($p < 0.001$)

and an increase in transient cholestasis (p = 0.004). Surprisingly, the number of cases of idiopathic cholestasis did not vary significantly across the long study period, nor was there any significant change in the outcome (p = 0.116). There were no significant improvements in either overall survival or survival with native liver. However, after the year 2000, the survival rate in patients with OLT was 100%.

- An NGS panel was applied to 13 of 154 patients, with contributory results in 7. However, so far, the panel led to a conclusive diagnosis of underlying entities in only two patients.
- We constructed diagnostic and prognostic models of NC based on our analysis of a cohort diagnosed and treated over three decades. We developed a diagnostic algorithm based on these models, personal experience, and current knowledge. The latest ESPGHAN and NASPGHAN guidelines are from 2017 and do not include an algorithm; the most recent algorithm from these societies is from 2004.
- Based on our clinical practice and literature review, we developed another decision algorithm, integrating the NGS panel and aimed specifically at the diagnosis of metabolic diseases among patients with NC.
- We treated a Portuguese patient suffering from a very rare cholestatic disease with innovative medical treatment. This was the first patient in Portugal to be treated with this drug. This led to a joint publication, which included another patient treated at another European centre.
- We offered a Portuguese contribution to an international consortium established to study a rare cholestatic entity, ALGS. The work of the consortium has led to the characterisation of a large international cohort, with important data on outcome, survival, and prognostic factors.

TRANSLATIONAL STUDY – FROM THE CLINICS TO PHYSIOLOGY

As far as we know, we conducted the first clinical study exploring an association between bile acid profile and redox status in healthy infants and investigated a possible impact of type of diet:

- Our data suggest that the bile acid profile in early postnatal life could have an influence on redox state regulation (and/or vice versa) and the turnover of erythrocytes.
- We offered a preliminary suggestion that, in humans, exclusive breastfeeding may be associated with higher concentrations of bile acids and delayed maturation of the bile

acid profile and, through this or other mechanisms, could contribute to a better redox state in healthy full-term infants.

- We contribute with data, stratified by gender, of a sparsely described population within the target age of NC onset, which may be used as a control group in future studies.
- Our data may turn to be a contribution to the development of therapeutic and preventive strategies. For example, preventive and therapeutic measures addressing oxidative stress, as well as the type of diet, might be part of preventive strategies, especially for transient cholestasis and sporadic forms of NC.

CHAPTER VI

FUTURE PERSPECTIVES

The main clinical challenge to be addressed in NC syndrome is lowering the mortality and morbidity of patients and increasing their survival while reducing their dependency on OLT.

Nowadays, to answer this challenge, basic and translational research seek new directions which will allow us to understand better the causes and the mechanisms of the disease(s) underlying NC.

1. REMAINING QUESTIONS AND GLOBAL RESEARCH DIRECTIONS

Currently, research on NC is taking place on several fronts, and among the main driving forces of present and future research are the following: a) the constitution of international research consortia for disease subgroups allowing the construction of large cohorts of well-studied patients and with high-quality specimen samples, b) cooperation between basic and clinical sciences and partnerships with industry; c) the use of new experimental models with an emphasis on organoids technology.

Either considering NC syndrome as a whole or considering the several underlying entities in particular, including transient cholestasis or the idiopathic cholestasis 'bags', many issues remain, involving both clinical and translational features, as follows:

#1 Beginning

-A foetal disease? In addition to GALD, are there more underlying entities with a foetal onset that can be detected in the prenatal period or immediately after birth (e.g. BA)? Can transient cholestasis also start to be determined before birth based on the evaluation of certain some perinatal risk factors?

#2 Susceptibility

-Genetic susceptibility: Beyond underlying genetic diseases, are there genetic variants that may contribute to increasing the susceptibility to sporadic forms of NC? Genetic studies in large cohorts of rare diseases using NGS (whole-exome sequencing) technology are ongoing. This technology can be applied to identify alleles associated with specific phenotypes or with more aggressive disease progression and to study mutations or epigenetic modifications in the involved tissue.

-Developing bile duct anatomy increases vulnerability: To what extent might the anatomical features of the developing biliary tract contribute to increase the susceptibility to biliary injury

and progression to obliterative fibrosis (e.g. BA)? Organoid technology allows the building of models of artificial bile ducts and their reconstruction after injury and may be used in research projects.

#3 Exposure

-Environmental toxins: Are there important environmental toxins that may work as triggers to sporadic cases of NC? If so, which? Organoid technology allows the building of models of artificial bile ducts and their reconstruction after injury. These models can also allow the testing of environmental toxins and the experimentation of new therapies.

#4 Mechanisms of disease(s)

-Role of oxidative stress in the scenario of NC: What is the role of the redox state in the onset and progression of NC? Is the development of oxidative stress a cause or a consequence of the disease?

-Inflammatory and immune response: What is the role of the inflammatory and immune responses? Do they play a remodelling/adaptive mechanism? And/or are they involved in the progression of biliary fibrosis and liver dysfunction?

-Is there a role for the intestinal microbiome? In patients with chronic cholestasis, is there a change in the intestinal microbiome, and can this interfere with the progression of inflammation and liver fibrosis?

- Players/mechanism(s) and molecular targets: How can we identify the players, their cross-talk and the most important molecular targets to block disease mechanisms? High-density immunotyping and cell-sequencing panels of mononuclear cells in the peripheral blood, liver and biliary tree may be used for this purpose.

-Transient cholestasis as a model for NC pathogenesis enlightenment: Future lines of research may include the study of oxidative stress as an underlying mechanism and/or an important feature in disease progression. Future research may try to support the inclusion of transient cholestasis in the Oxygen Radical Related Disease of the Newborn.

#5 Biomarkers of disease(s) and clinical end-points

Are there other clinically relevant end-points (liver stiffness? serum biomarkers?) besides liver failure, OLT or death? Some studies are assessing liver stiffness in certain subgroups of patients by using new imaging technologies, to search for monitoring tools of disease progression and new clinical end-points.

#6 Targeted therapies

-Novel medical therapies: There are currently some clinical trials with bile acid analogues (FXR agonists and others) and with agents that decrease the bile acid burden in the liver (ASBT inhibitors, NTCP inhibitors, and others). There are also ongoing studies with several anti-fibrotic, anti-inflammatory, immunosuppressive, anti-oxidants, including agents that increase reduced glutathione (145). May any of these novel therapies will allow the slowing of progression or even the reversibility of, for example, BA?

-Gene therapy: For patients with certain rare metabolic diseases requiring special diets hard to comply with, the search for novel therapies which may turn their outcomes to being less 'diet-dependent' is also required. Gene therapy may become an alternative for these patients in the near future.

- Cell-free therapy: Instead of cell therapy (infusion of mesenchymal stem cells, or others), a technique with some problems that have limited its application in clinical practice in the last 20 years, cell-free therapy (infusion of extracellular vesicles (EV) or exosomes) seems promising to induce liver fibrosis reversal, and many clinical trials are expected to be initiated in the near future (269). May cell-free therapy be useful to patients with NC with progression to liver cirrhosis?

#7 Screening

- Studies are underway to validate the ‘old’ method to evaluate conjugated bilirubin in the first days of life as a universal screening tool for NC (especially for prenatal-onset diseases). Studies are also underway to validate the SCC as a cost-effective screening method for BA in other continents beyond Asia.

#8 Prevention

In our view, the prevention of NC may soon become the ultimate goal.

-The prenatal diagnosis and abortion of affected embryos is already possible for some genetic determined conditions underlying NC with very dark prognosis. Advances in diagnostic tools with the possibility of being applied to prenatal diagnosis may expand the offer of this type of intervention.

-Nevertheless, based on current knowledge, we may foresee some more interesting preventive strategies, namely directed towards NC modifiable risk factors (e.g. type of diet, exposure to environmental toxins, …), and, since NC is a syndrome in which many insults share a favourite form of clinical expression, the full comprehension of disease mechanisms might allow us to intervene and reverse it preventing some underlying entities, such as for example, BA.

In the future, the clinical challenge may turn to be 'how short are we able to cut the current extensive list of underlying entities of NC if we act preventively?'

2. ONGOING STUDIES AND FUTURE PROJECTS

Within the scope of this thesis, there are still some ongoing studies concerning both clinical and translational research.

As to clinical research, regarding objective #1, a cross-sectional, prospective, nationwide study is in progress to assess the knowledge and the clinical practices of healthcare professionals at the three levels of the NHS (public and private services), to assess the knowledge and clinical practices regarding neonatal jaundice and pale stools.

A national expert panel (physicians and nurses) was formed to validate the questionnaire used in this process. The first round of experts for the validation of a short questionnaire (maximum 5 minutes) is completed. We recently obtained permission from Prof Mei-Hwei Chang and the Taiwan Ministry of Health and Health Promotion to use the images of the Taiwan SCC. After completing the validation of the questionnaire, the next task to be carried out will be sending a request to the Medical Council of Physicians and the Nurses Board for permission to access the professionals' email addresses to perform a nationwide survey.

The results from this study may eventually strengthen the conclusions and the proposals we have made in our Articles 1.1 and 1.2.

Concerning translational research, objective #4, we are preparing a manuscript based on the data from a cross-sectional prospective study performed in blood cord samples from healthy neonates to compare the results of the same outcome measures with those obtained in two-month-old healthy infants described in article 4.1. The main aim is to better characterise, and compare, the role of GGT as a biomarker in both conditions - in the mother-child binomial at the moment of delivery and in the healthy two-month old infant.

Another manuscript is being prepared to report on a single specific-control study concerning two infants with NC (one with A1ATD, ZZ phenotype and another with E. coli urinary tract infection) comparing their data with those from the control group, presented in Article 4.1.

The results from these studies may contribute to strengthening the rationale for finding new therapeutic and preventive strategies for NC.

Many maternal conditions are associated with oxidative stress and inflammation, and their effects can pass to the foetus through the placenta. Childbirth, by itself, is a moment of great stress, for both mother and the child, even when it is an uneventful vaginal delivery.

The pathogenesis of GALD is an excellent illustration of a disease that originates in the mother-child binomial. What has been discovered about the pathogenesis of BA, at least in part, points in the same direction (270).

MicroRNAs are bioactive molecules that can cross the placenta (in both directions) and are present in breast milk with functions that have been the subject of much research. One of these functions could be the regulation of bile acid metabolism, such as through GPBAR1 gene expression. There is recent evidence suggesting that environmental factors may influence the expression of this gene (271).

We hypothesise that maternal, as well as congenital and childbirth-associated factors may have impact on the neonatal liver with the clinical expression of cholestasis in early postnatal life. Additionally, breast milk feeding may prolong the cross-talk between mother and child and, thus, may play an important role in this scenario. Our next project is to test this hypothesis, namely by exploring the bile acid profile, oxidative stress and inflammation biomarkers, and studying miRNAs subtypes in the blood of pregnant women, in the

umbilical cord, breast milk, and the blood of newborns/infants, in mother-infant pairs, both healthy, with maternal disease and/or with a child with NC.

Currently, we think that the 'squaring of the circle' in NC (Fig. 23) towards the ultimate goal of prevention, will not be achieved without studying the mother-child binomial, in both the physiological and disease mother-child scenarios, instead of studying the neonate/infant with cholestasis alone.

Only this new concept of approach will allow to us identify all modifiable risk factors and understand the disease mechanisms, and win this challenge.

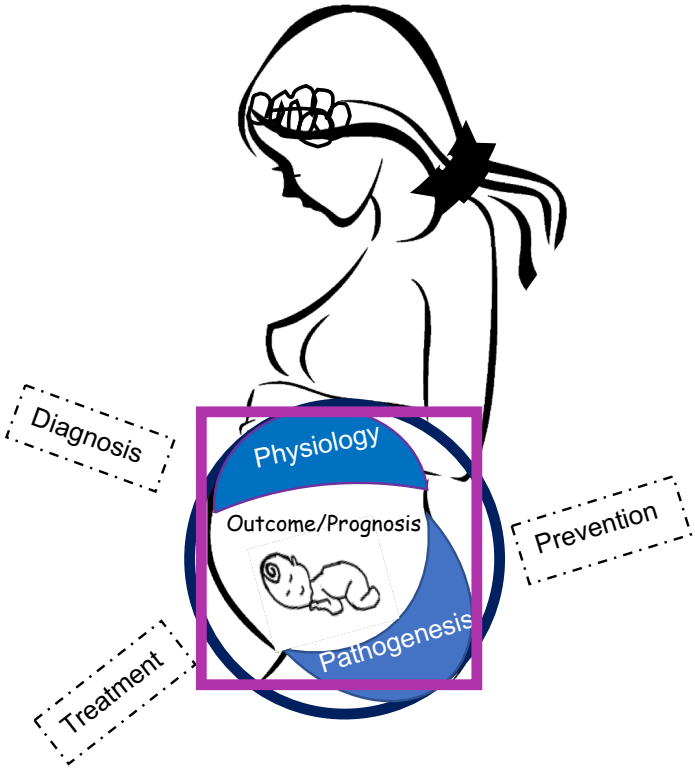

Figure 23 – Squaring the circle of NC

Source: Original

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APENDICES

QUESTIONÁRIO PARA MÉDICOS

1. LOCAL DE TRABALHO

Cuidados primários -------- ☐

Hospital nível B ---------- ☐

Hospital nível A ---------- ☐

2. ESPECIALIDADE:

Médico de Família ------ ☐

Pediatra ---------- ☐

Neonatologista ---------- ☐

3.TEMPO TOTAL DE EXERCÍCIO PROFISSIONAL:

< 5 anos ---------- ☐

5-10 anos ---------- ☐

11-20 anos ---------- ☐

>20 anos ---------- ☐

4. Na sua pratica profissional costuma **observar** a cor das fezes dos RN ictéricos?

Sim ---------- ☐

Não ---------- ☐

5. Na sua prática profissional costuma **observar** a cor da urina dos RN ictéricos?

Sim ---------- ☐

Não ---------- ☐

6. Num RN ictérico, e **com outros sinais / sintomas de doença**, quando pede um doseamento de bilirrubinas, incluindo a bilirrubina conjugada?

- Em qualquer idade, pois provavelmente trata-se de uma situação patológica ---------- ☐
- A partir dos 14 dias de idade -------- ☐
- A partir dos 28 dias de idade -------- ☐
- A partir dos 2 meses de idade ------- ☐

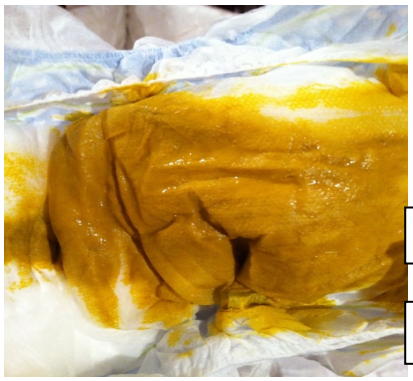
7. Num RN ictérico, amamentado ao seio materno, e **sem outros sinais / sintomas de doença**, quando pede um doseamento de bilirrubinas incluindo a bilirrubina conjugada?

- Não peço, pois em princípio trata-se de uma icterícia do aleitamento materno ---------- ☐
- A partir dos 14 dias de idade --------- ☐
- A partir dos 28 dias de idade --------- ☐
- A partir dos 2 meses de idade -------- ☐

8. Em relação à **cor das fezes** de um RN por favor classifique-as como **Normais** (N) ou como **Suspeitas (S)** de o não serem: *(assinale com uma cruz em cima de N ou S)*

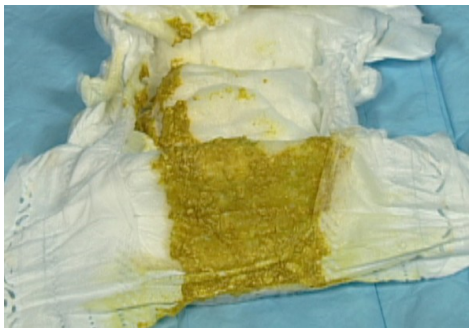
N

S



N

S



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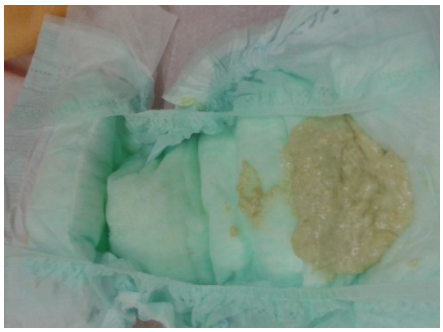

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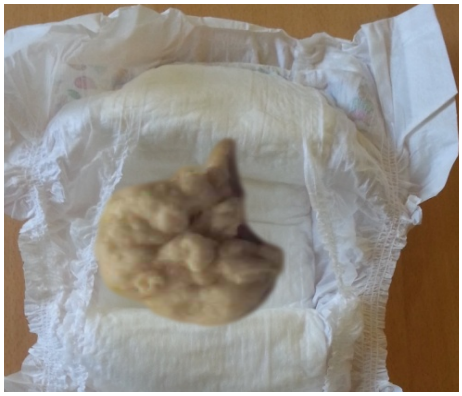

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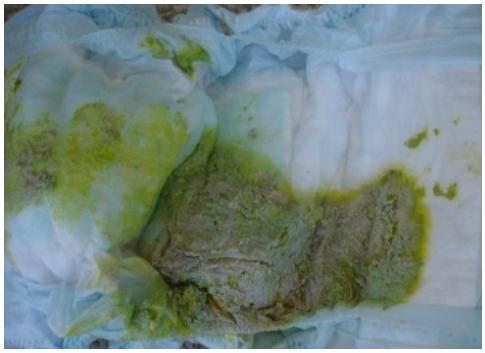

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QUESTIONNAIRE FOR PHYSICIANS

1. WORKPLACE

Primary care ---------- ☐

B-Level hospital ---------- ☐

A-Level hospital -------- ☐

2. SPECIALIZATION

General practice ---------- ☐

Paediatrics ---------- ☐

Neonatology ---------- ☐

3. YEARS OF PROFESSIONAL EXPERIENCE

< 5 years ---------- ☐

5–10 years ---------- ☐

11–20 years ---------- ☐

> 20 years ---------- ☐

4. In your professional practice, do you usually OBSERVE THE STOOL COLOUR of jaundiced newborns / infants?

YES ---------- ☐

NO ---------- ☐

5. In your professional practice, do you usually OBSERVE THE URINE COLOUR of jaundiced newborns/infants?

YES --------- ☐

NO ---------- ☐

6. In a jaundiced newborn, with other signs/symptoms of disease, when do you request a conjugated bilirubin assay?

- At any age, as it is probably a pathological situation ---------- ☐
- Beyond 14 days of age ---------- ☐
- Beyond 28 days of age ---------- ☐
- Beyond 2 months of age ---------- ☐

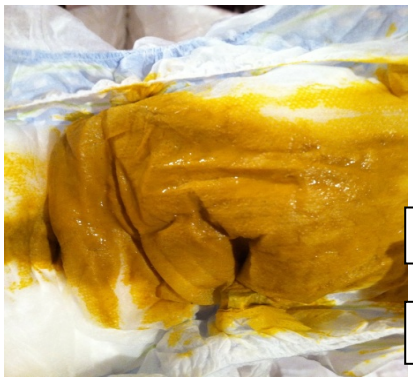
7. In a jaundiced newborn, breastfed, without other signs/symptoms of disease, when do you request a conjugated bilirubin assay?

- I do not request one, as jaundice is probably related to breast-feeding ---------- ☐
- Beyond 14 days of age ---------- ☐
- Beyond 28 days of age ---------- ☐
- Beyond 2 months of age ---------- ☐

8. Regarding the **stool colour** of a newborn, please classify it as **Normal** (N) or **Suspects** (S) *(mark with a cross over N or S)*:

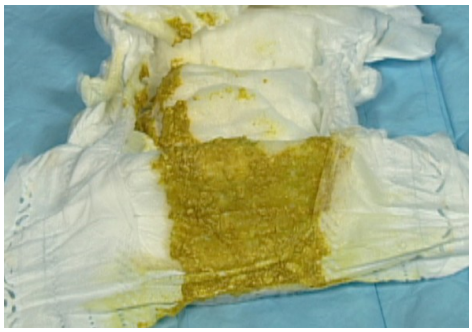
N

S



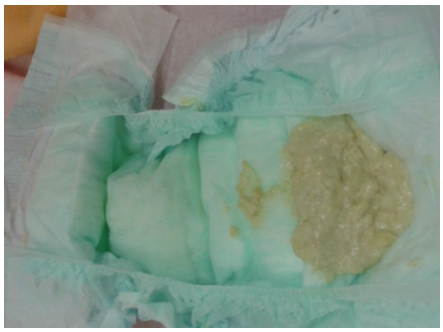
N

S



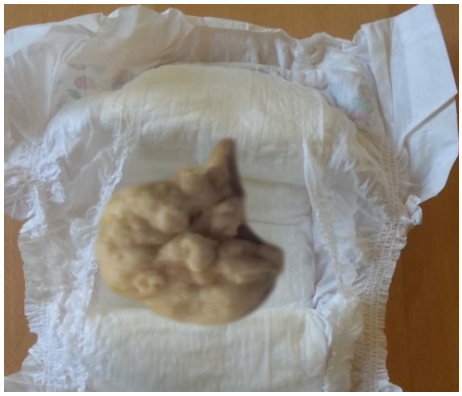
N

S



N

S

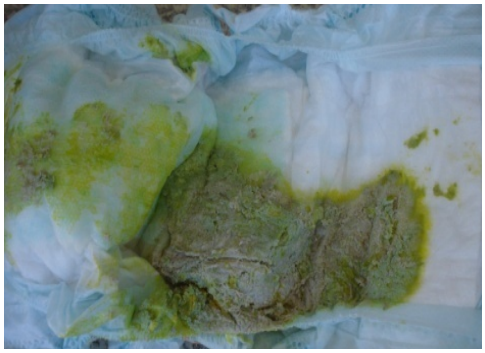


N

S

N

S



N

S

N

S

QUESTIONÁRIO PARA ENFERMEIROS

1. LOCAL DE TRABALHO

Cuidados primários --------- ☐

Hospital nível B ---------- ☐

Hospital nível A ---------- ☐

2. Enfermeiro ESPECIALISTA em PEDIATRIA:

Sim --------- ☐

Não --------- ☐

3. TEMPO TOTAL DE EXERCÍCIO PROFISSIONAL:

< 5 anos ---------- ☐

5-10 anos -------- ☐

11-20 anos -------- ☐

>20 anos --------- ☐

4. Na sua prática profissional costuma **observar** a cor das fezes dos recém-nascidos (RN) ictéricos? ☐

Sim -------

Não ------- ☐

5. Na sua prática profissional costuma **observar** a cor da urina dos RN ictéricos?

Sim ------- ☐

Não ------- ☐

6. Perante um RN ictérico, <u>com menos de 7 dias</u>, **quando** pede observação por um médico?

- De imediato ---------- ☐
- Depende da intensidade da icterícia ---------- ☐
- Depende da presença de outros sinais/sintomas de doença ---------- ☐
- Depende da intensidade da icterícia + presença outros sinais/sintomas de doença ------ ☐

7. a) Num RN ictérico, <u>com mais de 7 dias</u>, amamentado ao seio materno, e aparentemente <u>sem outros sinais / sintomas de doença,</u> **quando** pede observação por um médico?

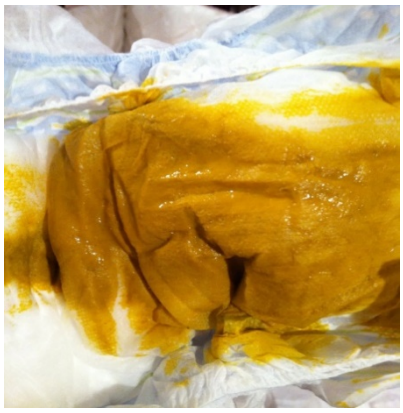
- De imediato ---------- ☐
- Depende da intensidade da icterícia ---------- ☐
- Quando a icterícia persiste para além dos 14 dias de idade ---------- ☐
- Quando a icterícia persiste para além dos 28 dias de idade ---------- ☐
- Quando a icterícia persiste para além dos 2 meses de idade --------- ☐

7. b) Se na pergunta anterior respondeu *"depende da intensidade da icterícia"*, se a mesma persiste, apesar da intensidade não lhe parecer preocupante, a partir de que idade pede observação por um médico? *(se na pergunta anterior escolheu outra opção ignore esta pergunta)*

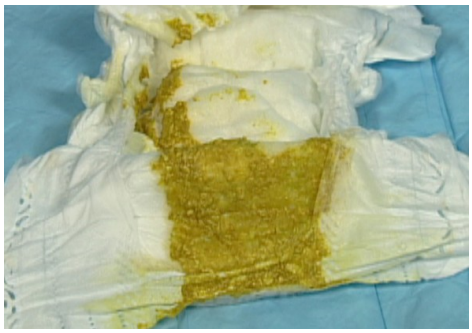
- A partir dos 14 dias de idade ----------☐
- A partir dos 28 dias de idade ----------☐
- A partir dos 2 meses de idade ----------☐

8. Em relação **à cor das fezes** de um RN por favor classifique-as como **Normais** (N) ou como **Suspeitas (S)** de o não serem: *(assinale com uma cruz em cima de N ou S)*

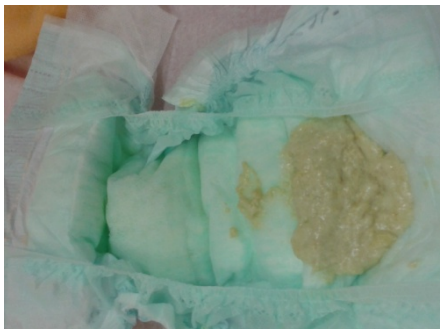
N
S



N
S



N
S



N
S

N

S

N

S

N

S

N

S

QUESTIONNAIRE FOR NURSES

1. WORKPLACE

Primary care ---------- ☐

B-level hospital -------- ☐

A-level hospital -------- ☐

2. Nurse SPECIALIST in PAEDIATRICS

YES ------- ☐

NO --------- ☐

3. YEARS OF PROFESSIONAL EXPERIENCE

< 5 years ---------- ☐

5–10 years -------- ☐

11–20 years ------- ☐

> 20 years -------- ☐

4. In your professional practice, do you usually OBSERVE THE STOOL COLOUR of jaundiced newborns / infants?

YES --------- ☐

NO ---------- ☐

5. In your professional practice, do you usually OBSERVE THE URINE COLOUR of jaundiced newborns / infants?

YES ------ ☐

NO -------- ☐

6. When do you send a jaundiced newborn, LESS THAN 7 DAYS OLD, for medical observation?

- Immediately ----------
- Depends on the jaundice intensity ---------- ☐
- Depends on the presence of other signs/symptoms of disease -- ☐
- Depends on the jaundice intensity + presence of other signs/symptoms of disease ------ ☐

7. a) When do you send a jaundiced newborn, MORE THAN 7 DAYS OLD, breastfed, and apparently without other signs/symptoms of disease, for medical observation?

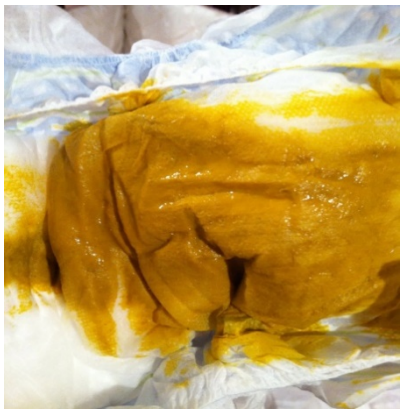
- Immediately ---------- ☐
- Depends on the jaundice intensity ---------- ☐
- When jaundice persists beyond 14 days of age ---------- ☐
- When jaundice persists beyond 28 days of age ---------- ☐
- When jaundice persists beyond 2 months of age ---------- ☐

7. b) If, in the previous question, you answered 'depends on the jaundice intensity', **if it persists**, although the intensity does not seem to worry you, BEYOND what age do you send the newborn for medical observation? *(If, in the previous question, you chose another option, ignore this question.)*

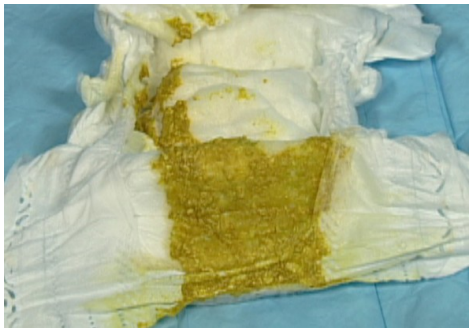
- Beyond 14 days of age ---------- ☐
- Beyond 28 days of age ---------- ☐
- Beyond 2 months of age ---------- ☐

8. Regarding newborns' **stool colour**, please classify it as **Normal** (N) or **Suspects** (S) *(mark with a cross over N or S)*:

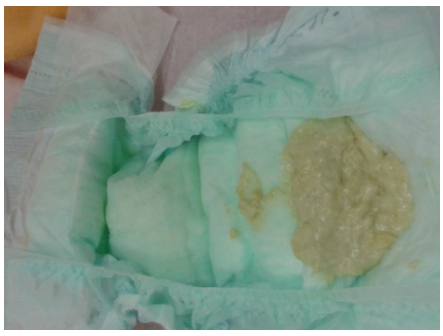
N S



N S



N S



N S

N

S

N

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N

S

N

S