

## This item is the archived peer-reviewed author-version of:

Experimentally induced biliary atresia by means of rotavirus-infection is directly linked to severe damage of the microvasculature in the extrahepatic bile duct

## **Reference:**

Von Sochaczew ski Christina Oetzmann, Pintelon Isabel, Brouns Inge, Thys Sofie, Deigendesch Nikolaus, Kuebler Joachim F., Timmermans Jean-Pierre, Petersen Claus.- Experimentally induced biliary atresia by means of rotavirus-infection is directly linked to severe damage of the microvasculature in the extrahepatic bile duct The anatomical record: advances in integrative anatomy and evolutionary biology - ISSN 1932-8486 - 302:5(2019), p. 818-824 Full text (Publisher's DOI): https://doi.org/10.1002/AR.23974

To cite this reference: https://hdl.handle.net/10067/1603580151162165141

uantwerpen.be

Institutional repository IRUA



## Experimentally induced biliary atresia by means of rotavirus-infection is directly linked to severe damage of the microvasculature in the extrahepatic bile duct.

Christina **Oetzmann von Sochaczewski<sup>1,4\*</sup>**, Isabel **Pintelon<sup>2\*</sup>**, Inge **Brouns**<sup>2</sup>, Sofie **Thys**<sup>2</sup>, Nikolaus **Deigendesch**<sup>3</sup>, Joachim F. **Kübler**<sup>4</sup>, Jean-Pierre **Timmermans**<sup>2</sup>, Claus **Petersen**<sup>4</sup>

1 Department of Pediatric Surgery, Johannes Gutenberg-Universität Mainz, Mainz, Germany 2 Laboratory of Cell Biology & Histology, Department of Veterinary Sciences, University of Antwerp, Antwerp, Belgium

3 Max-Planck Institute for Infection Biology, Berlin, Germany

4 Department of Pediatric Surgery, Medizinische Hochschule Hannover, Hannover, Germany

\* These authors contributed equally

Running title: Small vessel paucity in biliary atresia

Corresponding author: Isabel **Pintelon**, PhD Laboratory of Cell Biology & Histology, Department of Veterinary Sciences, University of Antwerp, Antwerp, Belgium Campus Drie Eiken - T Building Universiteitsplein 1 BE-2610 Antwerpen Belgium Phone: +323 265 3273 Email: isabel.pintelon@uantwerpen.be

Author contributions:

Study conception and design: Oetzmann von Sochaczewski, Pintelon, Timmermans, Kübler, Petersen.

Acquisition of data: Oetzmann von Sochaczewski, Pintelon, Brouns, Thys, Deigendesch. Analysis and interpretation of the data: Oetzmann von Sochaczewski, Pintelon, Brouns. Drafting of the manuscript: Oetzmann von Sochaczewski, Pintelon, Brouns. Critical revision: Thys, Deigendesch, Timmermans, Kübler, Petersen. All authors approved the final version.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ar.23974

This article is protected by copyright. All rights reserved.

Accepted Articl

**Aim:** Vascular damage has been reported to contribute to atresia formation in several diseases including biliary atresia. This study focused on the extrahepatic biliary plexus in experimental biliary atresia.

**Methods:** Newborn BALB/cAnNCrl-pups were infected with rhesus rotavirus within 24 hours after birth to induce experimental biliary atresia. The extrahepatic biliary plexus was examined by confocal microscopy on whole-mount preparations, scored by three independent researchers and further evaluated at the subcellular level with transmission electron microscopy.

**Results:** Imaging results revealed a progressive destruction of the extrahepatic biliary vascular plexus in the course of experimental biliary atresia induced by rotavirus infection. Endothelial cell damage was already visible as cell swelling and necrosis in the first days after infection and a damaged microcirculation that rapidly deteriorated with progression of obliterative cholangiopathy, was observed in the infected mice as early as 72 hours after birth.

**Conclusions:** In experimental biliary atresia, the destruction of the extrahepatic biliary vascular plexus starts already in the first days post infection and clearly precedes the morphological symptoms of atresia. The deterioration of the vascular bed architecture continues with disease progression. Therefore, we conclude that the (ultra)structural changes in the extrahepatic biliary microvasculature occurring before the visible onset of atresia has a predictive diagnostic value and this impairment in blood supply to the extrahepatic bile duct may be an important contributing factor to the pathogenesis of acquired biliary atresia.

Keywords: biliary atresia, mouse, rotavirus, extrahepatic biliary plexus, microcirculation

This article

Biliary atresia or progressive obliterative cholangiopathy is a rare disease of the infant's hepatobiliary system, resulting in subsequent liver cirrhosis and eventually liver failure. This liver disease, which can be either congenital or acquired and is most common in East Asia, leads to an abnormal narrowing, blocking or absence of one or more bile ducts. Although tissue destruction in biliary atresia can be attenuated surgically through a Kasai portoenterostomy, the 20-year survival rate with the native liver in affected children is only 29.6% (± 2% SEM) (Chardot et al. 2013), making biliary atresia the most common indication of pediatric liver transplantation (Rana et al. 2015). Despite intensive research efforts, the causative event for biliary atresia remains unclear. Most results of human and murine studies on acquired biliary atresia point in the direction of a short-lived viral infection of cholangiocytes, which may be followed by an exaggerated auto-inflammatory or auto-immune response that targets bile duct epithelia, resulting in progressive bile duct damage and eradication (Mack 2007; Petersen and Davenport 2013; Mack 2015). An important role in this adaptive immune response seems to be played by lymphocytes (for review *see* (Mack 2015)).

Of all viruses known to be implicated in biliary atresia, reovirus, rotavirus and cytomegalovirus (CMV) have been studied most extensively (for review see (Mack 2007; Mack 2015)). In murine models, experimental biliary atresia is usually induced by intraperitoneal injection of newborn BALB/c pups with rhesus rotavirus (RRV) within the first 24 hours after birth (Petersen et al. 1997; Oetzmann von Sochaczewski et al. 2014). In these models, progressive obstruction of the extrahepatic bile duct accompanied by clinical signs of cholestasis eventually results in complete obliteration of the bile duct's lumen (Riepenhoff-Talty et al. 1993;

Petersen et al. 1997). The presence of RRV particles has been clearly demonstrated in ductal cholangiocytes and in cells of the subepithelial duct layers in an RRV kinetics study using whole mounts of the bile duct, as such providing valuable clues to the pathogenesis of extrahepatic biliary atresia (Oetzmann von Sochaczewski et al. 2014).

Likewise, in biliary atresia following CMV-infection, it has been proposed that direct infection of the cholangiocytes followed by an immune attack against infected biliary epithelial cells is as a possible cause of the disease (Martelius et al. 1997; Op den Dries et al. 2011). However, according to an alternative hypothesis, CMV infection may cause injury to endothelial cells of the peribiliary plexus, followed by microthrombus formation and subsequent inadequate oxygenation of the surrounding tissues (Martelius et al. 1997; Op den Dries et al. 2011). This anoxic damage may result in ischemic injury to the bile duct and development of ischemic cholangiopathy (Hoekstra et al. 2009; Op den Dries et al. 2011).

Ischemia due to blood vessel injury is known to be associated with atresia (Osborne et al. 1991; Gaillard et al. 1996; Evans et al. 1999; Dutta and Harsh 2009), including biliary atresia (Di Sant 'Agnese and Blanc 1976). These observations are further strengthened by recent data on the molecular profile of biliary atresia in livers from affected patients suggesting involvement of hypoxia-ischemia pathways in the pathogenesis of biliary atresia (Fratta et al. 2015).

The hypothesis that the peribiliary vascular plexus is involved in CMV infection, as well as the finding that rotaviridae are able to infect endothelial cells (Morrison et al. 2001; Nuovo et al. 2002), prompted us to investigate the microcirculation in the extrahepatic bile duct in an RVV-infected mouse model for biliary atresia. Blood vessels were visualized in whole-mount preparations of intact extrahepatic bile ducts by using immunohistochemical staining and confocal microscopy. Ultrastructural changes, including the presence of RVV in endothelial cells and the presence of immune cells in the vicinity of blood vessels were examined using transmission electron microscopy.

## **METHODS**

### Animals

Rotavirus-free BALB/cAnNCrl mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and kept in a specific pathogen-free environment in laminar flow cages equipped with a shelter and material to build a nest. Animals were subjected to a 12:12 h light-darkness cycle and sterilized water and food were provided *ad libitum*. Pups were always kept with their mothers and were euthanized by decapitation with scissors. All experiments and procedures were in accordance with both the directive 2010/63/EU, the NIH guidelines for the care and use of laboratory animals as well as with German national regulations. The study protocol was approved by the 'local authority for the protection of animals' (LAVES, Oldenburg, Germany) (permit number 11/360).

#### **Experimental biliary atresia**

Virus production and induction of experimental biliary atresia were described elsewhere (Oetzmann von Sochaczewski et al. 2014). In brief, rhesus rotavirus (strain MMU 18006) was grown in MA-104 cells and titrated for an adequate concentration to induce experimental biliary

atresia. Pups received intraperitoneal injections of  $50\mu$ L with  $2.5\times10^6$  focus-forming units of RRV or  $50\mu$ L of saline. Eight out of 119 pups (6.7%) that died within 72 hours post injection were considered as injection-related mortality cases and hence excluded from the analysis.

Animals were assigned to three groups based on the respective age group that correlated to the key points in the development of the disease: postnatal day 3 marks the start of the early phase of experimental biliary atresia, which progresses from inflammatory obstruction of the extrahepatic bile duct at postnatal day 7 to complete atresia at postnatal day 14 (Bessho and Bezerra 2011). Accordingly, group I contained animals from postnatal day 1 to 5 (n=33), group II consisted of 6- to 10-day-old animals (n=41) and group III included the remaining animals from postnatal day 11 to 14 (n=22).

A total of 119 mice were used: 96 mice (including 35 non-infected controls) were evaluated with confocal microscopy and 23 mice were analyzed with transmission electron microscopy.

## Examination of the vascular plexus using whole-mounts of extrahepatic bile ducts *Immunohistochemistry*

Preparation of extrahepatic bile ducts for microscopy was carried out as described before (Oetzmann von Sochaczewski et al. 2014). Bile duct whole mounts were immersion-fixed with 4% paraformaldehyde (in 0.1 M phosphate buffer; pH 7.4) for 30 minutes after isolation. Immunohistochemical incubations were carried out at room temperature on free-floating bile ducts. Primary and secondary antisera were diluted in phosphate-buffered saline (PBS; 0.01 M; pH 7.4) containing 10% normal horse serum, 0.1% bovine serum albumin, 0.05% thimerosal, 0.01% NaN<sub>3</sub> and 1%Triton-X-100. Prior to incubation with the primary antisera, whole mounts

were incubated for 1 h with the antibody diluent. Whole mounts were incubated overnight with a monoclonal primary antibody raised in rat against the endothelial marker CD31 (1:50; Abcam ab56299, Cambridge, UK). To visualize immunoreactivity for CD31, whole mounts were further incubated for 4 h with Cy3-conjugated donkey anti-rat immunoglobulins (DARa-Cy3; 1:200; Jackson ImmunoResearch, 712-165-150, West Grove, PA, USA).

#### Microscopy

High-resolution images were obtained using a microlens-enhanced dual spinning disk confocal microscope (UltraVIEW VoX; PerkinElmer, Seer Green, UK) equipped with a 561-nm diode lasers for excitation of Cy3. Images were processed and analyzed using Volocity software.

## **Statistics**

To assess possible impairment of the extrahepatic biliary vascular plexus, whole-mount, preparations of the extrahepatic biliary duct of all animals were randomly and independently scored by three researchers (I.P., I.B. and S.T.) as 'healthy' or 'impaired', based on 4 parameters (capillary density, vessel size, number of anastomoses within the plexus and the distance between them). Agreement was examined with Krippendorff's  $\alpha$  (Hayes and Krippendorff 2007), yielding a moderate agreement (based on the rating proposed by (Landis and Koch 1977) of  $\alpha$ =0.571 (95% confidence interval: 0.446 to 0.6941) for the whole dataset. Krippendorff's  $\alpha$  was calculated using (version 3.4.2) with the rel-package (version 1.3.1). Confidence intervals were calculated using the bootstrap approach with 1,000 iterations. Mice were assigned to a certain group, if there was disagreement between the observers, results were debated until a consensus was found and the extrahepatic biliary plexus was rated as either 'healthy' or 'impaired'.

Barnard's test (Barnard 1947) was used for comparison of the frequencies in the assessment of the extrahepatic biliary plexus and calculated with the Barnard-package (version 1.8). Two-sided P values <0.05 were considered significant.

# Examination of the blood vessels of the extrahepatic duct using transmission electron microscopy

Bile ducts were immersion-fixed after isolation in 2.5% glutaraldehyde solution for 30 min, rinsed in 0.1 M sodium cacodylate buffer (pH 7.4) and postfixed in 1% OsO4 solution for 2 h. After dehydration in an ethanol gradient (70% ethanol for 20 min, 96% ethanol during 20 min, 100% ethanol for  $2 \times 20$  min), whole mounts were embedded in EMbed 812 (Electron Microscopy Sciences, Hatfield, PA, USA). Ultrathin sections were stained with 2% uranyl acetate and lead citrate, and examined in a Tecnai G2 Spirit Bio Twin Microscope (FEI, Eindhoven, the Netherlands) at 120 kV.

## RESULTS

# Characteristics of the extrahepatic biliary microvascular network during progression of experimental biliary atresia

Confocal microscopy of CD31-immunostained whole-mounts revealed that in noninfected animals the peribiliary vascular plexus consisted of a dense network of similarly sized capillaries that evenly surrounded the extrahepatic bile duct (figure 1a) and contained multiple anastomoses (figure 1a). This pattern was already discernible in the first postnatal days (postnatal days 1-5; Group I) and became more pronounced as the density of the capillary network increased between postnatal days 6 and 10 (group II; figure 1b). This increase in density was no longer observed after postnatal day 10 (group III) (figure 1c).

In contrast, the capillary network of RRV-infected animals already showed a number of alterations in the first days post infection (Group I), i.e., the gaps between the collaterals were wider compared to non-infected controls, anastomoses were scarce and the capillaries differed in size (figure 1d). The presence or absence of structural changes in the extrahepatic biliary microvascular bed, blindly scored by three independent researchers, allowed us to differentiate between non-infected control and infected animals: all 10 non-infected control animals (100%) were identified based on a normal appearance of the microvasculature (= healthy vascular plexus) whereas 12 out of 23 infected samples in Group I (52.2%) could be recognized (P=0.0049) ( $\alpha$ =0.709, 95% confidence interval: 0.446 to 0.879) (figure 2a).

The pathological alterations observed in the extrahepatic biliary plexus coincided with further progression of the disease in postnatal days 6 to 10 (Group II): extrahepatic bile duct areas deprived of capillary supply expanded, the number of anastomoses decreased further and the size variation of the capillaries increased (figure 1e). In line with this development, the number of infected mice scored with an impaired microcirculation increased to 19 of 24 (79.2%), while only two (11.8%; n=17) non-infected control was scored as having an impaired microcirculation (P<0.0001) ( $\alpha$ =0.452, 95% confidence interval: 0.252 to 0.638) (figure 2b).

The capillary network vanished in the last days before the pups perished (Group III). The number and density of the capillaries and their anastomoses gradually decreased; only straight vessels of a larger caliber persisted at the site of atresia and anastomoses between them were only very sporadically observed (figure 1f). As such, 13 of 14 (92.9%) mice were correctly assigned to the infected group and all five of six (83.3%) non-infected controls were correctly scored as well (P=0.0058) ( $\alpha$ =0.53, 95% confidence interval: 0.226 to 0.803) (figure 2c).

## The endothelium of the extrahepatic biliary vascular plexus is progressively damaged in the course of acquired biliary atresia.

In non-RRV-infected animals, the peribiliary vascular plexus showed a normal endothelium on all postnatal days examined: the endothelium was composed of typical squamous cells, with a thin rim of cytoplasm surrounding the flattened nucleus. Non-condensed cytoplasm was present in the rest of the endothelium lining the vessel wall (figure 3a,b). The inner surface of the endothelial cells had a smooth appearance (figure 3a,b).

In the early days post RRV (Group I), the capillaries surrounding the extrahepatic bile ducts showed endothelial cells that were edematous (figure 3d) and contained swollen nuclei (figure 3d). Some endothelial cells became necrotic (figure 3e,f), while others still seemed to be intact (figure 3c).

Endothelial damage worsened with disease progression (Group II), as the endothelial edema became more pronounced and started to narrow the vessel lumen (figure 3g). In addition, necrosis of endothelial cells became more extensive (figure 3h).

The vascular destruction progressed further in the end stage (Group III) of experimental biliary atresia when endothelial necrosis outweighed the rare intact capillaries (figure 3i,j).

Interestingly, careful examination of the capillary endothelium revealed, in contrast to the vessel lumen, no virus particles in any of the infected groups (figures 3a-j).

## Progressive endothelial damage is accompanied by changes in the inflammatory infiltrate

While immune cells surrounding the microvascular bed of the extrahepatic biliary duct were scarce in non-infected animals (figure 4a,b), the inflammatory infiltrate consisted predominantly of neutrophils in the first five days post infection (Group I; figure 4c,d). The cellular composition of the inflammatory infiltrate changed between postnatal day 6 and 10 (Group II): a reduction in the number of neutrophils coincided with an increase in the number of lymphocytes (figure 4e, f).

In Group III, the inflammatory infiltrate consisted predominantly of lymphocytes (figure 4g,h).

This changing inflammatory process was paralleled by an increasing obstruction of the extrahepatic bile duct that resulted in a complete obliteration at day 14 post infection (figure 4c-g).

## DISCUSSION

Thorough investigation of our RRV-induced model of experimental biliary atresia (Oetzmann von Sochaczewski et al. 2014) revealed a progressive destruction of the peribiliary vascular plexus, prior to and along with progressive obliterative cholangiopathy leading to severe

Accepted Articl

hepatobiliary injury. Diminishing of the blood vessel density was accompanied by progressive endothelial destruction, as could be detected using TEM microscopy.

Although rotaviridae are able to infect endothelial cells (Morrison et al. 2001; Nuovo et al. 2002), no signs of rotavirus were detected in the endothelial cells of the extrahepatic biliary vascular plexus of infected mice in any of the post-infection time periods investigated, whereas extrahepatic cholangiocytes of infected mice do harbor viral particles in the same time period, as was shown in an earlier study of our lab (Oetzmann von Sochaczewski et al. 2014). Given this absence of viral particles in the endothelium, the progressive destruction of endothelial cells of the extrahepatic vascular plexus cannot be attributed to direct RRV infection.

Apart from the progressive deterioration of the blood supply and ongoing biliary atresia, a bystander inflammatory response located in the bile duct lamina propria and submucosa was observed in the present as well as in a previous study by our group (Oetzmann von Sochaczewski et al. 2014). Our TEM results are in accordance with those of Shivakumar and colleagues (Shivakumar et al. 2009), who reported that neutrophils were the predominant immune cell type in the bile duct wall during the first days after RRV infection. These authors further showed that the neutrophils were later gradually replaced by natural killer cells and, to a lesser extent, also by macrophages (Shivakumar et al. 2009). Our results indicate that the inflammatory infiltrate in Group I (postnatal days 1-5) merely consisted of neutrophils that were preferentially located underneath the extrahepatic bile duct epithelium. In Group II (postnatal days 6-10), the majority of the neutrophils were replaced by mononuclear cells, especially lymphocytes, while in Group III, lymphocytes, and to a lesser extent macrophages, made up the entire inflammatory infiltrate

(postnatal days 11-14). Since the peribiliary vascular plexus is situated in the wall of the biliary duct, several cytokines released from the inflammatory cells (Bessho et al. 2014; Klemann et al. 2016; Shivakumar et al. 2017) may also contribute to endothelial injury.

Accepted Articl

Although the exact cause of endothelial damage during RRV infection and biliary atresia remains unclear, we could show that endothelial damage results in obvious blood vessel destruction, even prior to the visible onset of atresia. The increasing impairment of the microcirculation in the present study parallels the damage of the cholangiocytes by natural killer cells (Shivakumar et al. 2009). Whether these NK cells, as well as other cell types contributing to biliary atresia such as regulatory T cells, macrophages, dendritic cells and CD8+ T cells are involved in the destruction of the microvascular endothelial cells remains to be determined. It has been reported that a different blood supply of the extrahepatic bile ducts, may render them more susceptible to ischemia (Deltenre and Valla 2008). Progressive destruction of the extrahepatic biliary vascular plexus, similar to the one observed in the present study, has also been reported in autoimmune-mediated biliary diseases, where it was found to lead to vasopenia, and progressive destruction of the bile ducts (Washington et al. 1997; Matsunaga and Terada 1999). Moreover, ischemic injury has been put forward as one of the contributing factors in the pathogenesis of biliary atresia (Di Sant 'Agnese and Blanc 1976; Lee et al. 2010; Fratta et al. 2015).

In conclusion, the present study has shown that the microcirculation of the extrahepatic peribiliary region is damaged in a model of RRV-induced biliary atresia prior to the onset of clear hepatobiliary injury. Next to obvious other factors that contribute to the disease, we propose that an impaired microcirculation due to the damaged extrahepatic biliary microvascular

network might be an aggravating factor in the progression of acquired biliary atresia.

## ACKNOWLEDGEMENTS

We thank Janina Rudolph for excellent technical assistance.

## LITERATURE CITED

- Bessho K, Bezerra JA. 2011. Biliary atresia: will blocking inflammation tame the disease? Annu. Rev. Med. 62:171–185.
- Bessho K, Mourya R, Shivakumar P, Walters S, Magee JC, Rao M, Jegga AG, Bezerra JA. 2014. Gene expression signature for biliary atresia and a role for interleukin-8 in pathogenesis of experimental disease. Hepatology 60:211–223.
- Chardot C, Buet C, Serinet M-O, Golmard J-L, Lachaux A, Roquelaure B, Gottrand F, Broué P, Dabadie A, Gauthier F, et al. 2013. Improving outcomes of biliary atresia: French national series 1986-2009. J. Hepatol. 58:1209–1217.
- Deltenre P, Valla D-C. 2008. Ischemic cholangiopathy. Semin. Liver Dis. 28:235–246.
- Di Sant 'Agnese PA, Blanc WA. 1976. Infarction of the common bile duct in an infant after surfical exploration for jaundice: a possible cause of extrahepatic biliary atresia. J. Pediatr. Surg. 11:543–545.
- Dutta HK, Harsh S. 2009. Embryogenesis of esophageal atresia: Is localized vascular accident a factor? J. Indian Assoc. Pediatr. Surg. 14:73–75.
- Evans JA, Greenberg CR, Erdile L. 1999. Tracheal agenesis revisited: analysis of associated anomalies. Am. J. Med. Genet. 82:415–422.
- Fratta LXS, Hoss GRW, Longo L, Uribe-Cruz C, da Silveira TR, Vieira SMG, Kieling CO, Dos Santos JL. 2015. Hypoxic-ischemic gene expression profile in the isolated variant of biliary atresia. J. Hepato-Biliary-Pancreat. Sci. 22:846–854.
- Frelon DG. 2010. ReCal: Intercoder reliability calculation as a web service. Int. J. Internet Sci. 5:20–33.
- Gaillard D, Bouvier R, Scheiner C, Nessmann C, Delezoide AL, Dechelotte P, Leheup B, Cordier MP, Carles D, Lallemand A. 1996. Meconium ileus and intestinal atresia in fetuses and neonates. Pediatr. Pathol. Lab. Med. J. Soc. Pediatr. Pathol. Affil. Int. Paediatr. Pathol. Assoc. 16:25–40.
- Hayes A, Krippendorff K. 2007. Answering the call for a standard reliability measure for coding data. Commun. Methods Meas. 1:77–89.

- Hoekstra H, Buis CI, Verdonk RC, van der Hilst CS, van der Jagt EJ, Haagsma EB, Porte RJ.
  2009. Is Roux-en-Y choledochojejunostomy an independent risk factor for nonanastomotic biliary strictures after liver transplantation? Liver Transplant. Off. Publ. Am. Assoc. Study Liver Dis. Int. Liver Transplant. Soc. 15:924–930.
- Klemann C, Schröder A, Dreier A, Möhn N, Dippel S, Winterberg T, Wilde A, Yu Y, Thorenz A, Gueler F, et al. 2016. Interleukin 17, Produced by γδ T Cells, Contributes to Hepatic Inflammation in a Mouse Model of Biliary Atresia and Is Increased in Livers of Patients. Gastroenterology 150:229–241.e5.
- Landis JR, Koch GG. 1977. An application of hierarchical kappa-type statistics in the assessment of majority agreement among multiple observers. Biometrics 33:363–374.
- Lee H-C, Chang T-Y, Yeung C-Y, Chan W-T, Jiang C-B, Chen W-F, Chan H-W, Liu H-F, Lin M, Lee Y-J. 2010. Genetic variation in the vascular endothelial growth factor gene is associated with biliary atresia. J. Clin. Gastroenterol. 44:135–139.
- Mack C. 2007. The Pathogenesis of Biliary Atresia: Evidence for a Virus-Induced Autoimmune Disease. Semin. Liver Dis. 27:233–242.
- Mack CL. 2015. What Causes Biliary Atresia? Unique Aspects of the Neonatal Immune System Provide Clues to Disease Pathogenesis. Cell. Mol. Gastroenterol. Hepatol. 1:267–274.
- Martelius T, Krogerus L, Höckerstedt K, Mäkisalo H, Bruggeman C, Lautenschlager I. 1997. CMV causes bile duct destruction and arterial lesions in rat liver allografts. Transplant. Proc. 29:796–797.
- Matsunaga Y, Terada T. 1999. Peribiliary capillary plexus around interlobular bile ducts in various chronic liver diseases: An immunohistochemical and morphometric study. Pathol. Int. 49:869–873.
- Morrison C, Gilson T, Nuovo GJ. 2001. Histologic distribution of fatal rotaviral infection: an immunohistochemical and reverse transcriptase in situ polymerase chain reaction analysis. Hum. Pathol. 32:216–221.
- Nuovo GJ, Owor G, Andrew T, Magro C. 2002. Histologic Distribution of Fatal Rotaviral Pneumonitis: An Immunohistochemical and RT In Situ PCR Analysis. Diagn. Mol. Pathol. 11.
- Oetzmann von Sochaczewski C, Pintelon I, Brouns I, Dreier A, Klemann C, Timmermans J-P, Petersen C, Kuebler JF. 2014. Rotavirus particles in the extrahepatic bile duct in experimental biliary atresia. J. Pediatr. Surg. 49:520–524.
- Op den Dries S, Sutton ME, Lisman T, Porte RJ. 2011. Protection of bile ducts in liver transplantation: looking beyond ischemia. Transplantation 92:373–379.
- Osborne MP, Haddon SJ, Worton KJ, Spencer AJ, Starkey WG, Thornber D, Stephen J. 1991. Rotavirus-induced changes in the microcirculation of intestinal villi of neonatal mice in relation to the induction and persistence of diarrhea. J. Pediatr. Gastroenterol. Nutr. 12:111–120.
- Petersen C, Biermanns D, Kuske M, Schäkel K, Meyer-Junghänel L, Mildenberger H. 1997. New aspects in a murine model for extrahepatic biliary atresia. J. Pediatr. Surg. 32:1190– 1195.
- Petersen C, Davenport M. 2013. Aetiology of biliary atresia: what is actually known? Orphanet J. Rare Dis. 8:128.
- Rana A, Pallister ZS, Guiteau JJ, Cotton RT, Halazun K, Nalty CC, Khaderi SA, O'Mahony CA, Goss JA. 2015. Survival Outcomes Following Pediatric Liver Transplantation (Pedi-

SOFT) Score: A Novel Predictive Index. Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg. 15:1855–1863.

- Riepenhoff-Talty M, Schaekel K, Clark HF, Mueller W, Uhnoo I, Rossi T, Fisher J, Ogra PL. 1993. Group A rotaviruses produce extrahepatic biliary obstruction in orally inoculated newborn mice. Pediatr. Res. 33:394–399.
- Shivakumar P, Mizuochi T, Mourya R, Gutta S, Yang L, Luo Z, Bezerra JA. 2017. Preferential TNFα signaling via TNFR2 regulates epithelial injury and duct obstruction in experimental biliary atresia. JCI Insight 2. [accessed 2017 May 14]. http://dx.doi.org/10.1172/jci.insight.88747
- Shivakumar P, Sabla GE, Whitington P, Chougnet CA, Bezerra JA. 2009. Neonatal NK cells target the mouse duct epithelium via Nkg2d and drive tissue-specific injury in experimental biliary atresia. J. Clin. Invest. 119:2281–2290.
- Washington K, Clavien PA, Killenberg P. 1997. Peribiliary vascular plexus in primary sclerosing cholangitis and primary biliary cirrhosis. Hum. Pathol. 28:791–795.

### **FIGURE LEGENDS**

#### Figure 1. Progressive destruction of the extrahepatic biliary plexus in the course of

#### experimental biliary atresia

The extrahepatic biliary plexus was examined by confocal microscopy of whole-mounts of the biliary duct stained with the endothelial marker CD31. Group I consisted of 1- to 5-day-old pups (A, D), group II was composed of animals aged 6 to 10 days (B, E) and group III included 11- to 14-day-old pups with end-stage disease (C, F).

A, B and C (postnatal days 3, 7 and 10 respectively): In non-infected animals, a dense network of capillaries is seen to surround the extrahepatic bile duct. Capillaries are evenly sized and connected by anastomoses in all three groups.

D, E and F (postnatal days 3, 7 and 14 respectively): (D) A reduction in capillary numbers and size differences are already visible in RRV-infected animals of group I of experimental biliary atresia. In addition, the gaps between the anastomoses of the microvascular bed are wider in these infected animals compared to non-infected controls. (E) The size variation between the

capillaries is increased and the number of anastomoses between the capillaries is further reduced.(F) The capillary network is nearly vanished and only few larger vessels are remaining.Anastomoses between capillaries are scarce.

# Figure 2. Scoring of microcirculation impairment in the extrahepatic biliary vascular plexus

The extrahepatic biliary vascular plexus was analyzed using whole-mount confocal microscopy. The appearance of the network was blindly scored by three independent researchers as 'disturbed impaired' or 'healthy', and the results for infected and non-infected pups were compared. The visible check of the status of the microcirculatory network allowed differentiating between non-infected and infected pups in all three groups. The inter-rater agreement was measured according to Krippendorff's  $\alpha$ -value (0= no agreement; 1= full agreement) and showed a moderate agreement of  $\alpha$ =0.571 (95% confidence interval: 0.446 to 0.694) for the whole dataset. The Y-axis depicts the number of evaluated mice per subgroup.

(A) Group I 52.5% of the infected mice (n=23) were scored correctly as 'impaired', while all non-infected animals (n=10) were scored as 'healthy' ( $\alpha$ =0.709, 95% confidence interval: 0.446 to 0.879); (B) Group II: 79.2% of the infected mice (n=24) were scored correctly as 'impaired', while 11.8% (n=2 out of 17) of the non-infected animals was scored as 'impaired' ( $\alpha$ =0.452, 95% confidence interval: 0.252 to 0.638); (C) Group III: 92.9% of the infected mice (n=14) were scored correctly as 'impaired', while five of six (83.3%) were correctly scored as 'healthy' ( $\alpha$ =0.53, 95% confidence interval: 0.226 to 0.803). Statistical significance was calculated using Barnard's unconditional test.

## Figure 3. Progressive damage of endothelial cells during development of experimental biliary atresia

Endothelial damage and presence of viral particles were evaluated with transmission electron microscopy. No viral particles were found in any of the endothelial cells examined.

A, B (postnatal day 7): In non-infected control animals, capillaries show a normal architecture, i.e. an undamaged endothelium, open lumen and normal nuclear appearance.

Group I: In RRV-infected animals (C, postnatal day 3; D, postnatal day 5; E, postnatal day 3; F, postnatal day 5), the endothelium has either a normal appearance (C), is swollen (D) or is undergoing necrosis (E, F). Irregularly shaped endothelial nuclei are sometimes found (E). Neutrophil influx is shown by neutrophil presence in the capillary lumen (D) or by obvious diapedesis (C).

Group II: RRV-infected animals at postnatal day 8. (G) Destroyed capillary due to endothelial necrosis. (H) Endothelial cells with extremely swollen nuclei, narrowing the capillary lumen and inducing stasis.

Group III: RRV-infected animals at postnatal day 14. (I) Capillary remnant with necrotic endothelial cells. (J) Rare irregularly shaped capillaries surrounded by necrotic cells.

## Figure 4. Development and composition of the inflammatory infiltrate surrounding the extrahepatic peribiliary vascular plexus

The inflammatory infiltrate of the extrahepatic biliary duct was assessed by transmission electron microscopy. Submucosal regions contained several blood vessels (BV). If possible the extrahepatic bile duct lumen (*asterisk*) and epithelium (E) are included in the pictures as reference points.

(A, B): In non-infected animals, immune cells surrounding the capillaries are scarce and diverse. Capillaries do not display any abnormalities. The lumen of the extrahepatic bile duct is patent.

(C, D) Group I: In RRV-infected animals, the connective tissue layer surrounding the extrahepatic bile duct and hence also the capillaries, contain a high number of neutrophils. Most neutrophils appear as clusters situated immediately underneath the ductal epithelium. Some macrophages are also found. Cholangiocytes in infected animals appear more swollen than in non-infected ones.

(E, F) Group II: Severe inflammation is present with decreased neutrophil numbers and increased macrophage numbers. Lymphocytes are abundantly present, especially in the submucosal region of the extrahepatic bile duct wall. Note that the lumen of the extrahepatic bile duct is further narrowed and made up of a reduced number of cholangiocytes.

(G, H) Group III: Immune cells populate all layers of the atretic extrahepatic bile duct. The inflammatory infiltrate is solely composed of mononuclear cells represented by lymphocytes and macrophages. The lumen of the extrahepatic bile duct is obliterated.





This article is protected by copyright. All rights reserved.

control v rticl 5 µm 2 µm group I infected um G Accepte group II group III 2 µm

This article is protected by copyright. All rights reserved.

**vrticl** Accepte

