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Hepatic hilar lymph node reactivity at Kasai portoenterostomy for Biliary Atresia: correlations with age, outcome, and histology of proximal biliary remnant.

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Abstract

We hypothesized that if infection is the proximate cause of congenital biliary atresia, an appropriate response to antigen would occur in lymph nodes contiguous with the biliary remnant. We compared the number of follicular germinal centers (GC) in 79 surgically excised hilar lymph nodes (LN) and 27 incidentally discovered cystic duct lymph nodes (CDLN) in 84 subjects at the time of hepatic portoenterostomy (HPE) for biliary atresia (BA) to autopsy controls from the pancreaticobiliary region of non-septic infants > 3 months old at death. All 27 control LN lacked GC a sign in infants of a primary response to antigenic stimulation. GC were found in 53% of 106 LN in 56 of 84 subjects. Presence of GC and number of GC/LN was unrelated to age at onset of

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jaundice but was related to older age at HPE. Absent GC in visible and incidentally removed CDLN predicted survival with the native liver at 2 and 3 years after HPE, p=0.03, but significance was lost at longer intervals. Visible surgically excised LN contiguous with the most proximal biliary remnants had one or more well-formed reactive GC in only 26/51 subjects. No correlation existed between GC in hilar LN and frank inflammatory activity in the proximal hepatic duct, or between the latter and outcome after HPE. GC in hilar LN correlated with generalized neutrophilic pericholangitis in 80 contemporaneous liver biopsies (p=0.04) but not with overall intensity of portal cellular infiltrates.

The absence of consistent evidence of antigenic stimulation in LN contiguous with the biliary remnant argues for a limited role for infection in the etiology of biliary atresia. The intense inflammatory lesions occasionally found in remnants and the acute pericholangitis found in a minority of liver biopsies could be secondary either to bile-induced injury or secondary infection established as obstruction evolves.

Introduction

Proposed etiologies for extra-hepatic biliary atresia (EHBA) include developmental anomaly, viral infection, toxin or ischemia with a possible underlying genetic predisposition (1–5). A foundational premise based on previous histopathological studies of remnant tissue is that a necro-inflammatory process proceeds to fibrous obliteration of extra-hepatic bile ducts beginning at or prior to birth. Although direct evidence for viral etiology of EHBA in human infants is lacking, interest in this theory remains high despite minimal support (6–8). The possibility of a role for infection in human EHBA with or without immune dysregulation or a disordered immune response has been further fueled by histological parallels drawn from reovirus-induced inflammatory cholangitis in newborn BalbC mice (9–11).

Lymphoid tissues in the gastrointestinal tract, spleen and lymph nodes of newborn infants typically lack morphological features associated with an immune response because of lack of previous contact with pathogens. Primary follicles may be present in small numbers, but reactive changes, such as morphological evidence for cell transformation, specialization, expansion of the interfollicular zone and reactive center formation are typically absent.

A humoral response to infection is characterized by antibody formation and morphological features of follicular stimulation with reactive germinal center (GC) formation in lymph nodes and spleen. The GC is a hallmark of the lymph node response to antigen in man. This histological response requires 1–2 weeks after exposure to antigen and has been observed in perinatal autopsies of infants who have persistent viral infections established prior to birth or in the early neonatal period (11–15). GC and mature plasma cells were reported in a significant minority of autopsies of fetuses with congenital rubella syndrome suggesting that the immune system of a normal human fetus, as early as the late second trimester of gestation, is competent to respond with histological features of an acquired humoral immune response. These direct observations in human fetuses are supported by the recent demonstration that in the second trimester, the lymphoid tissues of the rhesus monkey fetus

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have a complete repertoire of properly organized antigen-presenting cells, T cells, and B cells (16).

The lymphoid tissue of infants at the time of diagnosis of EHBA has never been systematically studied. We hypothesize that a local acquired humoral immune response activated during the period of several weeks or more before diagnosis of biliary atresia can be verified by performing a detailed examination of the morphology of the hepatic hilar lymph nodes (LN) removed at the time of the hepatic portoenterostomy (HPE). We compare evidence of hilar lymph node reactivity with three other parameters: inflammatory activity in the excised proximal biliary remnants; the patterns of intrahepatic fibrosis and intrahepatic portal inflammation observed in concurrent liver biopsies; and outcome.

Methods

Patient selection

Study Participants. A diagnosis of biliary atresia was confirmed in 84 subjects either by intra-operative cholangiography or examination of the excised biliary remnant or both. Seventy-nine visible hepatic hilar LN had been surgically excised at the time of HPE including sixty-five LN from 59 ChiLDREN subjects between 2003 and 2013 and 14 hilar LN excised from 25 subjects who underwent HPE for BA at Cincinnati Children's Hospital Medical Center between 1987 and 2003. In addition to these 79 visible surgically excised LN, 27 small LN were incidentally discovered in microscopic sections of proximal biliary remnants at or near the junction of the cystic duct and hepatic duct. Thus, 106 lymph nodes in intimate contact with the extrahepatic biliary tree were available for comparison to 27 autopsy-derived control LN from the same region in infants of comparable age.

The study set of 106 lymph nodes consisted of formalin fixed paraffin embedded sections from 79 surgically excised visible hilar lymph nodes and 27 cystic duct LN identified in microscopic sections of the biliary remnants from the combined groups. Microscopic sections of liver biopsies obtained, at or a few days before surgery, and clinical data related to presentation and outcome were available from 80 subjects.

Biliary remnant data were available from 67 of the 84 subjects: 45 ChiLDREN subjects and 22 archival Cincinnati subjects. The ChiLDREN samples had been oriented by the surgeon and systematically sampled by network pathologists using a ChiLDREN protocol for step-sectioning with alternate slices fixed in formalin and frozen for research. Most remnants were photographed and then step-sectioned perpendicular to the axis of the hepatic duct from the hilum to the proximal common duct (hepatic duct series). A second series of axial sections extended from the cystic duct to the tip of the gall bladder (cystic duct/gall bladder series). In the Cincinnati archival subgroup, the liver hilum and gall bladder ends of the specimens, as identified by the surgeon and pathologist, had guided embedding of remnants; only these transverse slices with information on their relative position were included. For the purpose of this study data from only the first two most proximal levels were correlated with lymph node histology, liver histology and outcome.

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The control group (CG) of 27 peribiliary/peripancreatic lymph nodes were obtained at autopsy from non-septic infants (age range 3–97 days, mean 50 days) at the time of death within 24 hours of corrective surgery for congenital heart defects, or in sudden deaths for which no cause was determined. Controls had no clinical or autopsy evidence of recent infection. All slides were reviewed jointly by KEB and RS using ChiLDREN protocols for evaluation of liver biopsy specimens and step-sectioned biliary remnants.

This study was approved by the Steering Committee of the Childhood Liver Disease Research and Education Network (ChiLDREN), permitting use of data and slides from its ongoing prospective study of infants and children with cholestasis (PROBE; Clinicaltrials.gov NCT00061828). PROBE was conducted with Informed consent on all subjects and under IRB approvals at all clinical sites. The local IRB approved the archival component of the study at the Cincinnati site. PROBE enrollment began June 1 2004 and continues as of 2015. The data cutoff of the current analysis was June 1, 2013.

Histology and Immunohistochemistry

The cross sectional area and number of primary and reactive follicles were counted in hematoxylin and eosin (H&E) stained slides from 106 LN and 27 controls. The following immunostains were performed: B-cell marker CD79a (RTU; clone; Ventana Medical Systems, Tucson, AZ USA), follicular dendritic cell marker CD21(RTU; clone; Ventana Medical Systems, Tucson, AZ USA) and germinal center B cell marker bcl6 (RTU; Ventana Medical Systems, Tucson, AZ USA). Immunostains were selected to help identify primary follicles (CD79a) and to highlight early signs of antigenic stimulation (CD21 and BCL6) that might antedate obvious follicular center differentiation in H&E stained sections. T cell markers were not used because of limited numbers of slides available from the repository.

For the purpose of this study, the reactive process in each of the two most proximal hepatic duct remnant sections was classified as active or inactive. The designation **active** was further subclassified based upon prevalence of either prominent inflammation/granulation tissue related to a damaged bile duct remnant or a dominant presence of loose cellular reactive fibrous tissue concentrically oriented around ducts and/or periductal glands with little or no inflammatory infiltrate. **Inactive** lesions were defined as little or no inflammation in paucicellular dense fibrous scar tissue, the latter including discrete dense fibrous cords and less well defined fibrous scars. Remnant levels with both active and inactive features were classified as mixed. Alternate categories were as follows: no lesion/no duct, normal duct profile or not assessable because of insufficient tissue, or poor orientation.

Liver biopsy and biliary remnants obtained at HPE were evaluated using the ChiLDREN protocols 10 and 10 B respectively. Hepatic fibrosis was staged based on the Scheuer system (17). The extent of portal inflammatory infiltrates was graded as absent, focal (<50%) or generalized (> 50%) and mild or moderate to severe. Neutrophilic pericholangitis was graded independently using a similar approach.

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Data analysis.

LN area on the glass slide sections was measured using morphometric software included in the Aperio slide scanning system. We assumed that LN had been evenly bisected resulting in approximation of true cross-sectional area. Follicular development in LN was assessed by manually counting the number of primary follicles (PF) and germinal centers (GC) in a representative H&E stained section. Discrete aggregates of intra-nodal lymphoid cells reactive for CD79a, BCL6 and CD21-positive follicular dendritic cell networks were also counted manually. Isolated single immunoreactive cells were not counted. The end point of the analysis consisted of absolute numbers of PF, GC and aggregated cell subtypes per lymph node profile and expressed per unit area of LN. Immunostains were not performed on the control lymph nodes (CLN) because of unpredictable post mortem loss of reactivity and also because Hg-containing autopsy fixative previously used at this institution interferes with immunoreactivity.

Statistical analysis: For binomial proportions and continuous responses, Fisher's exact test and ANOVA were used respectively to determine association of GC in hilar LN with the following: enumerated immunoreactive cellular aggregates; with remnant histology in the proximal two sections of the remnant; hepatic fibrosis stage and portal inflammation assessment in concurrent liver biopsies; age at onset of jaundice and at HPE; and outcome. The outcome analysis compared LN reactivity in subjects who had liver transplantation within two and three years of HPE to those with no liver transplantation to date (minimum follow-up of 5 years) combined with those who had late liver transplantation beyond the age of 5 years. Fisher's exact test was used to assess the relation of GC activity in LN to survival with the native liver at 24 and 36 months post HPE. A Kaplan-Meier survival plot was used to compare overall survival in 74 of 84 subjects for whom LN data and long term native liver survival data were available at the time of HPE. Data on bilirubin levels in the early post HPE period were insufficient to conduct a meaningful analysis of LN reactivity to early successful drainage after HPE.

We applied the zero-inflated Poisson (ZIP) regression model to assess count parameters for which we observed excessive zeros. Our ZIP model also contains a random subject effect to account for intra-patient correlation, since some patients had more than one LN.

Data regarding demographics, age at onset of jaundice, age at HPE and outcome were provided by the CHiLDREN data control center for enrolled patients, and for patients observed prior to 2004, were collected from Cincinnati Children's Hospital medical records.

Results

We compared means using ANOVA for multiple parameters in 106 LN in four groups defined by age at HPE and found that LN size along with specific features of LN reactivity (absolute counts of PF, GC, CD79a, CD21 and BCL6 aggregates) increased with advancing age (Figure 1). Infants with BA and inactive hilar LN (no GC) were significantly younger at HPE (mean age 55.1 d) compared to those with one or more active GC (mean age 71.6 d).

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Data from 106 HPE-LN from 84 subjects, and 27 peribiliary/peripancreatic CLN from 27 autopsies in infants of comparable age appear in Table 1. Twenty-seven of the 106 LN removed at HPE were fortuitously discovered in the biliary remnant adjacent to the cystic duct (CDLN); these are included in the total and also tabulated separately. The 79 surgically visualized hilar LN were significantly larger than either the CDLN or the CLN; the latter two groups were of comparable small size. GC were absent in all CLN. The average number of GC in all 106 HPE-LN was 3.2 per mm^2. CDLN were comparable in size to CLN but often contained at least one GC. Surgically excised visible hilar LN (43/76) were more commonly active (GC positive) than CDLN (9/27), but the difference did not quite achieve statistical significance, p= 0.07.

The 106 HPE-LN were divided into two groups based upon presence or absence of reactive GC in H&E stained sections. Approximately half of all HPE-LN, 52/106 (49%) lacked overt GC formation, identical to the uniformly non-reactive autopsy control LN. Data from 79 visible surgically excised hilar LN, excluding the 27 small CDLN appear in Table 2A; data from all LN appear in Table 2B.

Immunostains listed in Table 2 were selected to help identify primary follicles (CD79a) and to highlight early signs of antigenic stimulation (CD21 and BCL6) that might antedate obvious GC differentiation. In visible hilar LN, all indicators of early follicular activity were significantly greater in LN with detectable GC. Numbers of primary follicles, number of CD79a aggregates, CD21 and BCL6 aggregates paralleled the count of GC as listed in Tables 2A and 2B with p-values from comparisons based upon ANOVA. When data from 27 microscopic CDLN were added (Table 2B), statistical significance increased except for BCL6/mm2 where it was lost. Except for BCL6 aggregates, the positive correlations disappeared when the values were normalized to LN area, a finding that suggests other cellular components of LN reactivity, not measured in this study, had also expanded. The histological features assessed in this study are illustrated in Figure 3. Absent GC in a hilar LN contiguous with an atretic proximal hepatic duct segment exhibiting active fibroplasia without inflammation is shown in Figure 4. Absent GC in a LN contiguous with an atretic cystic duct is shown in Figure 5.

We applied the zero-inflated Poisson (ZIP) regression model to analyze GC and BCL6 counts, because we observed excessive zero counts as demonstrated in a plot of absolute counts of GC and BCL6 aggregates (both absolute counts and normalized counts) in 106 LN vs number observed per LN (Figures 2A, B). For LN that lacked GC (zero count) using the ZIP model for GC, higher age had significantly fewer zero counts compared to lower age. The relationship of absent GC to younger age was significant, p=0.004 for both GC and normalized GC models. The non-zero GC count component was fitted to Poisson regression as a function of age at HPE. The fitted curves are shown in Figures 2C and 2D. For LN that had one or more GC (non-zero), both absolute GC counts and normalized GC counts (expressed per unit area of LN), were significantly increased with age at HPE: p=0.0072 and p=0.0024 respectively. Similar results were obtained for absolute counts of BCL6 aggregates and when normalized to LN area (Figures 2E and F).

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Thus, using the zero-inflated Poisson model that segregates the zero and non-zero counts, the presence of each of the following: at least one GC, GC presence normalized to LN area, absolute aggregated BCL6 cell counts and BCL6 aggregates normalized to LN area, were all significantly related to age at the time of HPE.

Nineteen patients had more than 1 LN available for study; 16 had two LN and 3 had three LN. Of these, 12 patients were concordant for active LN status and 7 were discordant. Concordance for presence or absence of GC was independent of whether the LN were surgically excised from the hilum or identified incidentally within the biliary remnant. Six patients had visible hilar LN and incidental CDLN for comparison; four of 6 were concordant for presence/absence of GC. Thus when 2 or more LN were available from a subject, concordance was usual but not universal. In subjects with discordant LN, analyses for correlation with activity in proximal remnant, liver histology and outcome, were based upon the active LN; the inactive LN data was discarded.

Correlative Studies

The presence of one or more GC in HPE-LN, did not correlate with the following: average age at reported onset of jaundice, age at transplant, hepatic fibrosis stage, or when infants less than 14 days old at onset of jaundice were compared to all older infants, Proximal hepatic duct remnants were designated as being active if at least one of the two most proximal levels had active lesions. Of 67 remnants, 51 were in direct contact with an enlarged hilar LN that had been visualized and excised by the surgeon. Only 12/51 remnants were active at both proximal levels of the hepatic duct. Most proximal biliary remnants, 28/51, had active lesions at only one of the two most proximal levels. Inactive lesions at both proximal levels were present in 11/51remnants. Histological classification of the lesion at these most proximal remnant levels was frank inflammation (2), active fibroplasia alone (42) or mixed with inflammation (12), fibrous cords (7), vanishing duct (7), preserved duct lumen with subepithelial fibrosis (3), normal duct (1), and no remnant detected (11). Presence of GC in HPE-LN did not correlate with the presence of active lesions (overt inflammation, active fibroplasia alone, or mixed) in the duct remnant in either of the two proximal levels closest to the hilum, $p=0.73$. Active lesions in the proximal remnant, as defined, also did not predict outcome in 45 patients who had remnant and outcome data.

Two measures of intra-hepatic inflammation, generalized portal cellular infiltrates including myelopoiesis but excluding acute pericholangitis (periduct/ductular polymorphonuclear leukocytes) and acute pericholangitis, evaluated separately, were compared to the presence or absence of reactive follicles in HPE-LN. Moderate or severe generalized portal infiltrates were identified in only 38/318 (12%) centrally reviewed liver biopsies obtained at/near the time of HPE. In this study, we found no correlation between GC in hilar LN and prominent overall intrahepatic portal area cellular infiltrates.

Acute pericholangitis was absent/rare in 26%, focal mild in 23% generalized mild in 32% and generalized moderate/marked in 17% in centrally reviewed liver biopsies obtained at HPE (unpublished data). In the current study, we compared presence or absence of GC in visible excised hilar LN with presence of any degree of generalized intrahepatic acute pericholangitis (Figure 6). In 62 subjects with both visible excised hilar LN and liver biopsy

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data, generalized pericholangitis of any severity, did not correlate with GC activity, 26/41 vs 9/21 with absent or focal mild cholangitis, p= 0.18, (Table 3). Positive correlation of LN reactivity with generalized intrahepatic pericholangitis in liver biopsies from 77 subjects achieved statistical significance (p=0.0026) when CDLN data were included (Table 4).

Outcome analysis: Presence of at least one GC in any LN located in the hepatic hilar region (visible LN plus CDLN) in 74 subjects predicted native liver survival at 24 and 36 months post HPE (p=0.03 at both intervals). However the predictive value gradually declined to statistical insignificance in a Kaplan-Meier plot that included all survival data (Figure 7). Specifically, to test whether the two groups differ in overall survival time with the native liver, we performed both the log-rank test and the Wilcoxon test. P-values were respectively 0.1235 and 0.1074, not significant, although the trend to early favorable survival in subjects without GC is statistically significant in contingency tables using data at 2 and 3 years post HPE. Ten subjects were not included in this analysis because of insufficient follow-up interval (6), lost to follow-up (2), and death before 2 years after HPE.

The meaningful predictor for outcome was the detection of at least one GC. The mean absolute number of GC in all HPE-LN increased with age at HPE but was highly variable within both groups. For example, no difference was observed in the average number of GC in those subjects with early transplant (6.0 GC/LN) compared to those with no transplant (5.3 GC/LN). Furthermore, using a proportional hazard model (Cox regression) the covariates of age at HPE, size of LN and number of GC/LN did not correlate with outcome.

Discussion

The primary purpose of this study was to assess morphological evidence for antigenic stimulation in 106 hepatic hilar lymph nodes contiguous with the atretic extrahepatic biliary tree in 84 infants with BA at the time of HPE. We found that in subjects with BA, reactive germinal centers (GC) were absent in a majority of peribiliary LN but GC increased in frequency with age at the time of HPE. Of 84 subjects, 35 had one or more inactive LN that lacked GC.

Seventy-nine visible hepatic hilar lymph nodes intentionally excised at the time of HPE for biliary atresia were, on average, significantly larger than 27 lymph nodes fortuitously included in step sections of the remnant at the more distal junction of the hepatic and cystic ducts. Autopsy controls from the hepatobiliary region in a group of comparable age in whom infection was absent lacked germinal centers and were significantly smaller than visible hilar LN in BA; cystic duct LN in remnants were comparable in size to control LN. The overall size of hilar LN was not indicative of GC content and was not predictive of outcome. Contributing to hilar LN size was variable expansion of the interfollicular cortical lymphoid tissue plus reactive sinusoidal histiocytosis with admixture of hematopoietic precursors and bile stained macrophages.

Slightly more than one-half of lymph nodes had one or more well-formed GC. Presence of at least one GC in any peribiliary LN was unrelated to age at onset of jaundice but was related to older age at HPE and predicted outcome defined as early transplant at 2 and 3

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years post HPE. We also found a significant relationship between presence of at least one GC in any peribiliary LN and generalized intrahepatic pericholangitis, a concurrence that is reminiscent of the “inflammatory vs fibrotic” intrahepatic phenotype of BA described by Moyer et al (18) and could result from acquired infection. No relationship was found between GC in hilar LN and active lesions in the proximal biliary remnant.

A well-developed GC is the initial morphological response of a regional lymph node to antigen. In experimental studies, GC formation follows a timeline of 7–14 days after exposure to antigen (19). Prevalence of GC-negative peribiliary LN in BA at HPE challenges one of the contemporary theories regarding the etiology of extrahepatic biliary atresia, namely that it results from or is associated with a perinatal viral infection.

CMV, rotavirus, reovirus. HPV and HHV6 have been suggested as primary or secondary agents in biliary atresia (6–8, 20–24). Most recently, CMV-associated biliary atresia has been proposed as a significant etiologic subgroup (24). If BA commonly associates with viral infection, one would expect morphological evidence of an antigenic response in most peribiliary LN in most subjects, as ample time for such a response usually elapses between birth and HPE. We have defined a large subgroup of BA subjects in whom no response to an antigen is detectable in peribiliary LN regardless of age at HPE, and who as a group were younger at HPE than those with reactive hilar LN.

The lymphatic fluid that enters the hepatic hilar LN drains the liver and the region of the hepatic duct where biliary atresia develops. Antigen(s) responsible for local follicular stimulation are unknown in our subjects. Most hilar LN in our BA subjects had not been exposed to antigen because sufficient time had elapsed for a morphological response in LN to be evident at the time of HPE. Interestingly, evidence for LN response to antigen in the youngest infants at the time of onset of jaundice was neither more or less common than in those with later onset of jaundice despite the overall trend to greater lymph node reactivity in older patients at the time of HPE.

We interpret our findings to indicate that an unknown foreign antigen may contribute to evolution of extrahepatic atresia in almost half of all subjects but is not necessarily etiologic for the atresia. Zani et al have shown that about 10% of infants with BA have an early immunological response to CMV and that this subgroup has a poor outcome after HPE. Antigen(s), presumably related to either transient bacteremia or ascending microbial infection, may enter the liver in those subjects with prominent intrahepatic pericholangitis, possibly as the obstruction progresses. Germinal centers, when present in peribiliary LN may result from secondary infection, such as cholangitis, or chemical alteration of tissue antigens due to bile leakage from a damaged duct prior to complete obstruction. A third possibility that may explain our findings is that biliary atresia may be divided into at least two etiologic groups of roughly equal size, only one of which involves local antigenic stimulation.

The histopathology of the biliary remnant in BA has been described before, almost exclusively in tissue from the most proximal portion of the hepatic duct near the hilum (25–30). Our findings of both active and inactive lesions in the proximal hepatic duct with no

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apparent consistent intra-remnant pattern are similar to those previously described. However, our definition of “active” included both the uncommon overt inflammatory lesions and, much more commonly what we interpret as active cellular fibroplasia, a non or minimally inflammatory feature that is circumferential to epithelialized duct remnants and peribiliary glands. Active fibroplasia, though readily distinguishable from dense end-stage cicatrix (the fibrous cord), is a likely precursor to dense fibrosis.

We conclude that absent evidence for a response to antigen in many hepatic hilar LN suggests that biliary atresia is commonly not associated with infection, but is consistent with multiple pathways to a common phenotype. Our series shows that In a subgroup of substantial size, either primary or secondary infection initiated at least several weeks prior to recognition of biliary atresia at HPE, stimulates GC formation and may also play a significant role in progressive liver injury through a complicating intrahepatic pericholangitis. Absence of GC in a majority of LN contiguous with the atretic biliary tree and in a large minority of subjects with BA bolsters the concept that BA is often an evolving congenital anomaly independent of infection. Histological findings in most proximal biliary remnant sections suggest a non-inflammatory progressive regression of the extra-hepatic bile ducts that typically is accompanied by circumferential active fibroplasia with little or no inflammation.

Subjects with a visible LN response to antigen were more likely to require liver transplantation within 2 and 3 years after HPE than those with non-reactive LN; however survival of the native liver beyond that time point was not predicted. Search for antigens that may relate to progression of BA and to outcome after HPE should be pursued. Possibly antimicrobial therapy may be more efficacious than steroid therapy (31) in improving the outcome after HPE. Because histological response to antigen was related to older age at HPE, exposure and response to antigen is likely to be a post-natal event. Therefore our findings emphasize the importance of ongoing efforts to develop a better screening method for biliary atresia in early infancy (32).

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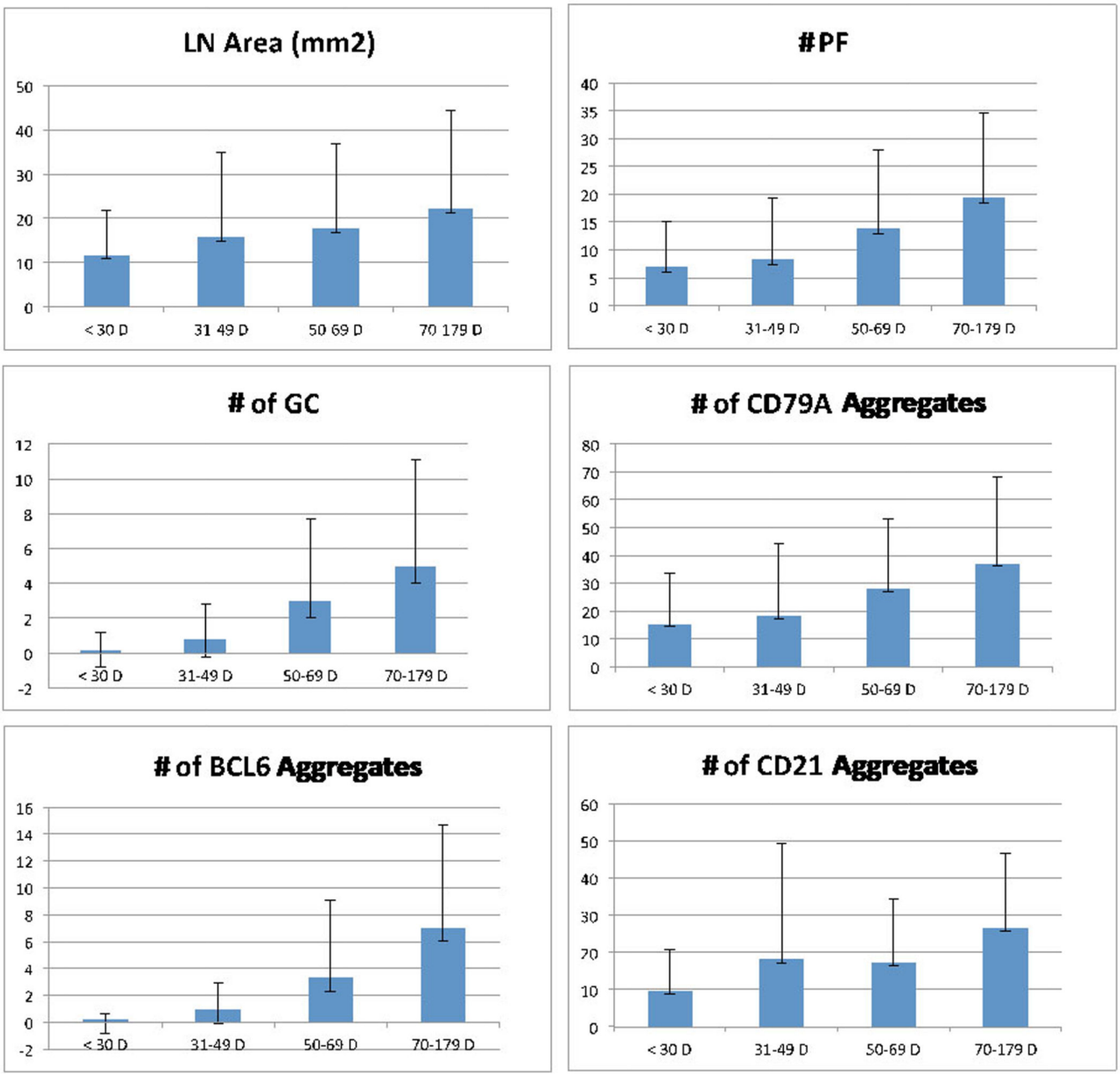

Figure 1.

Bar graphs demonstrate absolute values of 6 parameters of 106 hilar lymph nodes in subjects grouped by age at the time of Kasai procedure for biliary atresia.

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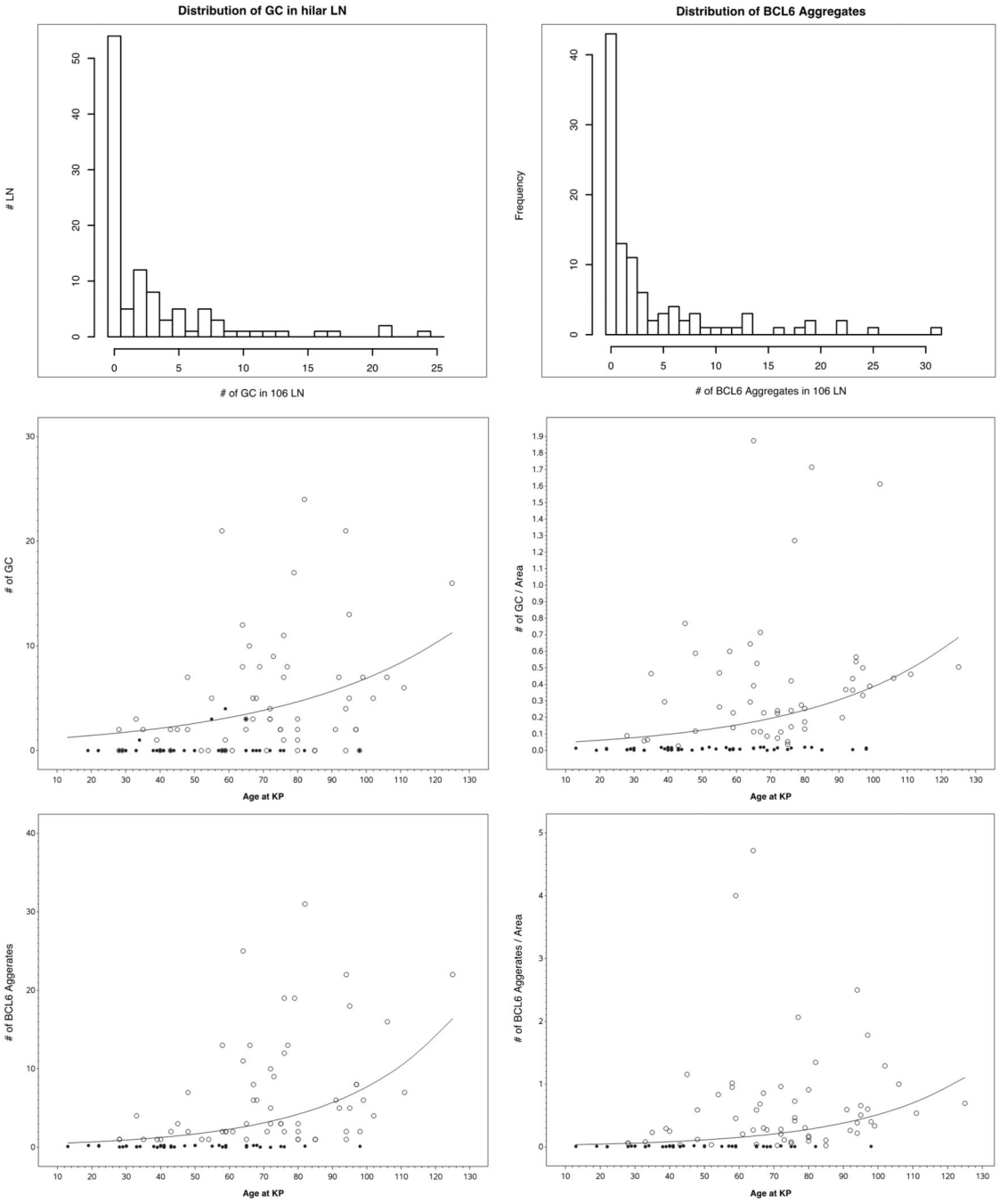

Figure 2.

Top panels show distribution of GC and BCL6 aggregates in 106 LN. Both were absent in a majority of LN. Substantial variation in the enumerated features was observed in the large minority of LN. In bottom panels are zero-inflated Poisson regression curves that show a significant relationship of absolute and normalized counts of GC and BCL6 aggregates to age at HPE

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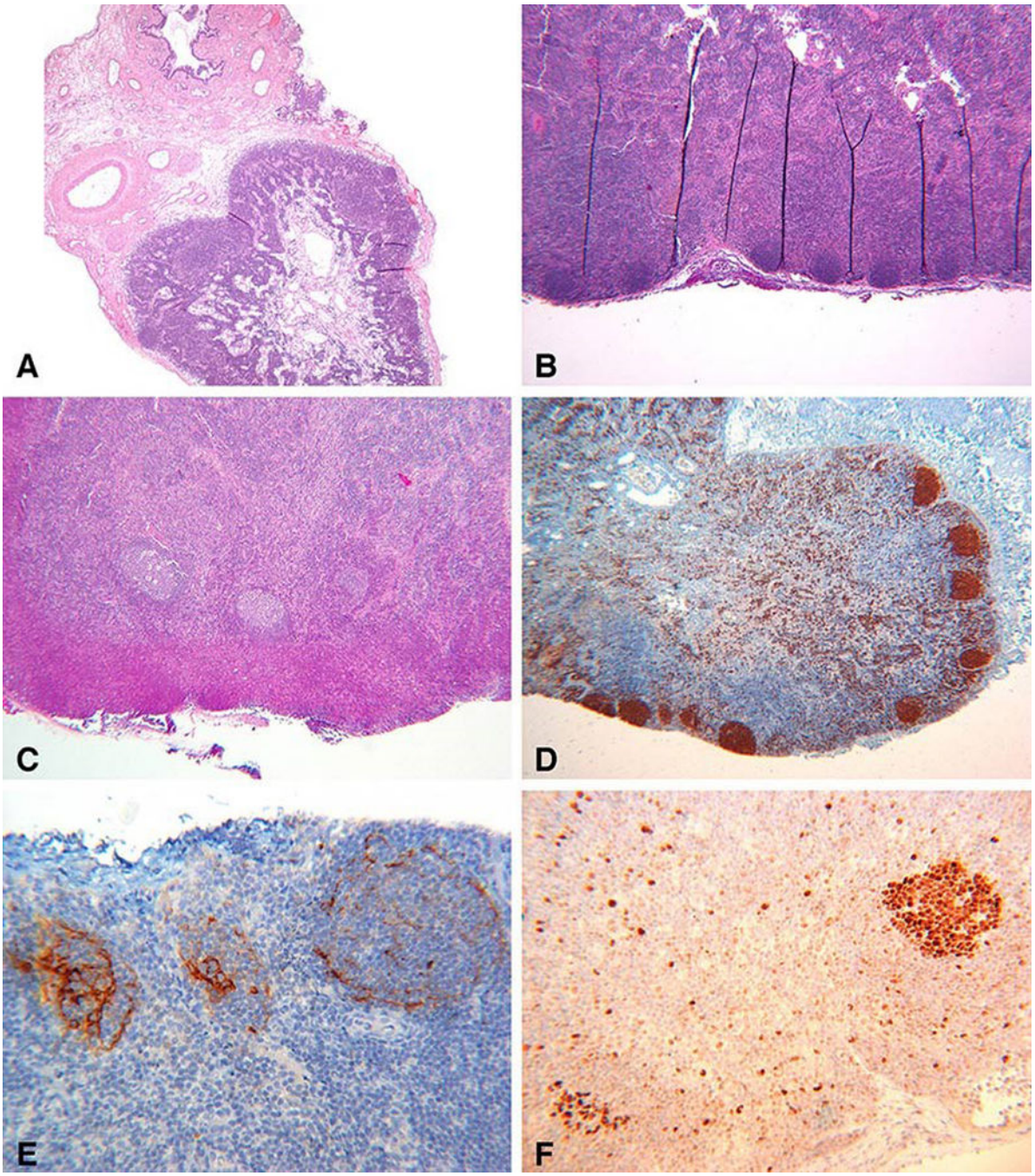

Figure 3.

3A. Autopsy control hepatic hilar LN is contiguous with a normal hepatic duct and hilar vessels. A few scattered non-reactive primary follicles are located in the cortex (arrows). H&E stain. X20

3B. Hilar lymph node in BA contains many non-reactive primary follicles (arrows). H&E stain, X40.

3C.Hilar lymph node in BA contains three follicles with well-formed reactive centers (arrow). H&E stain, X40

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3D. Hilar lymph node in BA contains multiple primary follicles that are immunoreactive for immature B cells. CD79a IHC, X40.

3E. Hilar lymph node in BA contains three primary follicles with early signs of activation in the form of aggregated dendritic cells but lacking features of well- developed reactive centers. CD21 IHC, X100.

3F. Hilar lymph node in BA contains cell aggregates reactive for BCL6, located in immature follicular centers. BCL6 IHC, X100.

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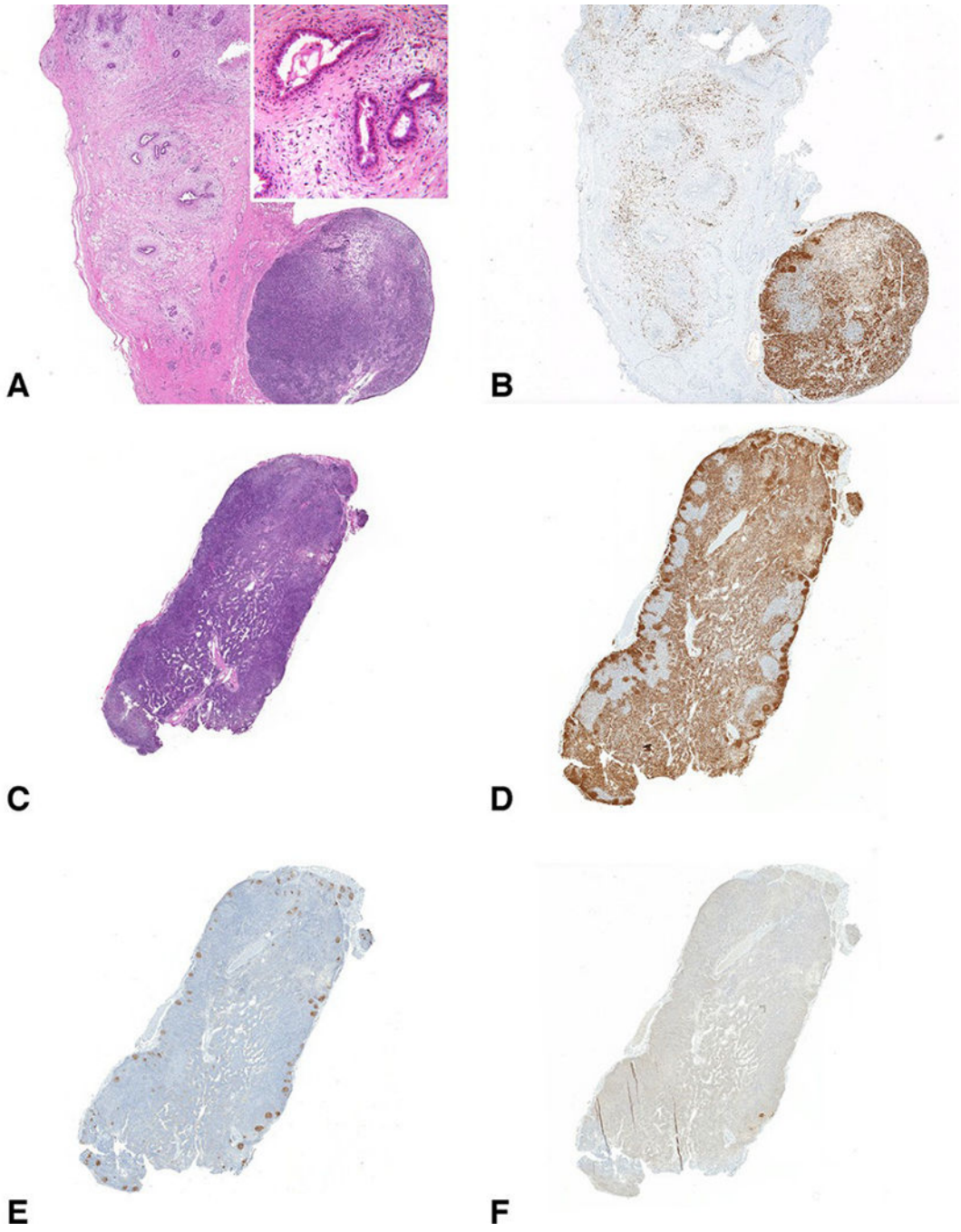

Figure 4.

4A. Proximal hilar level in BA with non-inflammatory uniform concentric active fibroplasia related to remnants of ducts and peribiliary glands (insert). The fortuitously included contiguous small hilar LN contains rare scattered primary follicles and no well-formed germinal centers. H&E stain, X20. Insert, X100.

4B. Serial section to 4A. The immunostain highlights rare primary follicles (arrow) and patchy presence of reactive cells in portions of the interfollicular cortex of this small hilar LN. Scattered reactive interstitial mononuclear B cells, inconspicuous in Figure 4A, are

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located within the zone of active fibroplasia surrounding bile duct remnants. CD79, IHC, X20

4C. Surgically-excised (visible) hilar lymph node, same subject as 4A-B. No primary follicles or germinal centers are visible. H&E stain, X7

4D. Surgically-excised (visible) hilar lymph node, same subject as 4A-B. Numerous primary follicles are visible in the superficial cortex (arrows). The interfolliclular zone is the seat of variable scattered mononuclear cells of B-lineage. CD79a IHC. X7

4E. Surgically-excised (visible) hilar lymph node, same subject as 4A-B. All primary follicles in the superficial cortex show early signs of activation. CD21 IHC, X7

4F. Surgically-excised (visible) hilar lymph node, same subject as 4A-B. Rare primary follicles in the superficial cortex contain aggregates of immunoreactive cells signifying the early stage of GC formation. BCL6 IHC, X7.

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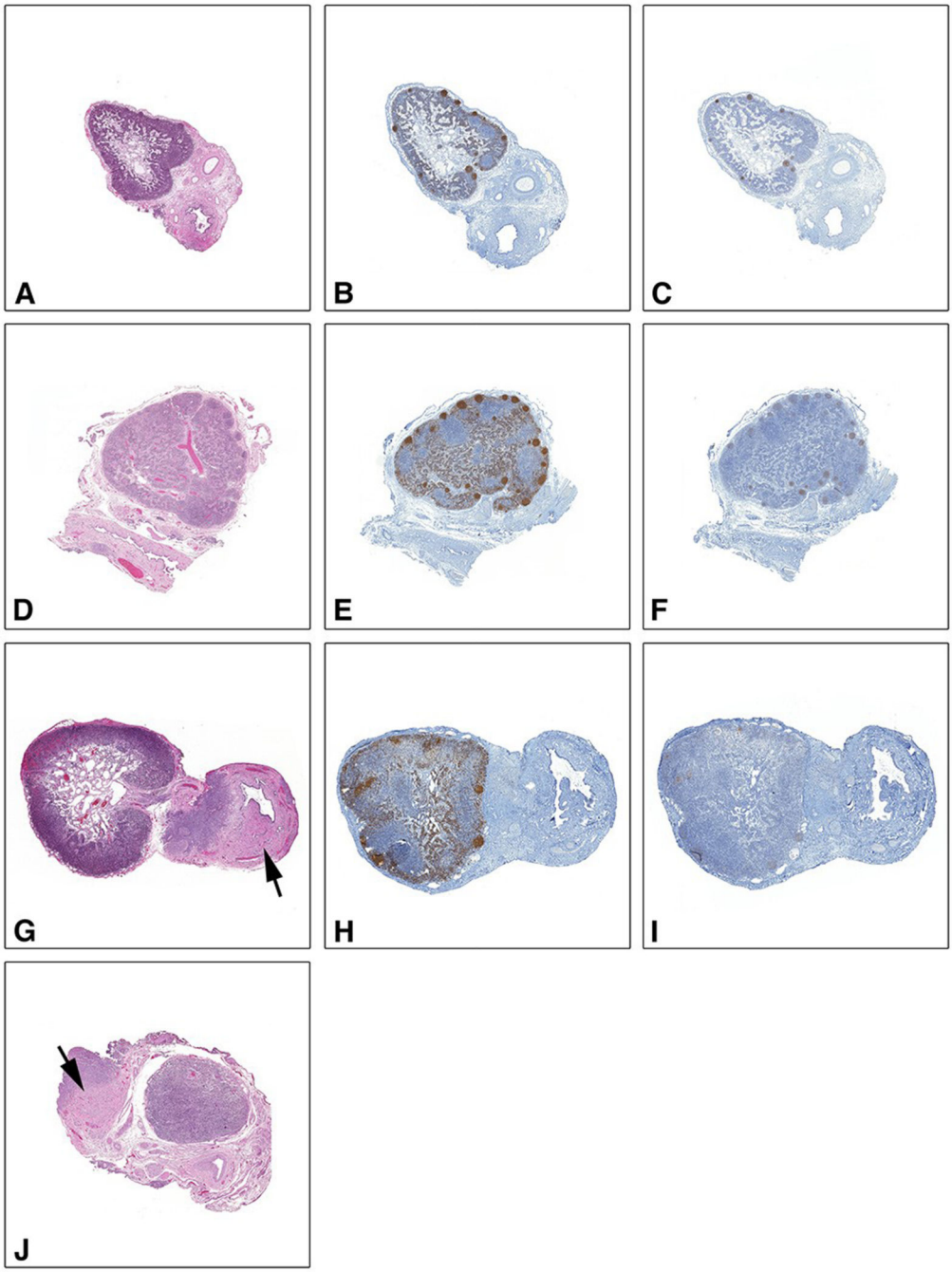

Figure 5.

Panels A-J. Examples of four cystic duct lymph nodes with contiguous cystic duct discovered in four excised biliary atresia remnants

A-C. Lymph node adjacent to patent cystic duct with minimal subepithelial fibrosis and intact epithelium lacks visible reactive germinal centers. H&E stain X7. B. Serial section of A. Condensed zones of immunoreactive cells in cortex are non-reactive primary follicles. CD79a IHC. X7. C. Serial section of A. A minority of the primary follicles contain small

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aggregates of immunoreactive cells consistent with early response to antigen. CD21 IHC, X7.

D-F. Lymph node adjacent to patent longitudinally oriented cystic duct with minimal subepithelial fibrosis and intact epithelium lacks visible reactive germinal centers. H&E stain X7. E. Serial section of D. Condensed zones of immunoreactive cells in cortex are non-reactive primary follicles. CD79a IHC. X7. F. Serial section of D. A minority of the primary follicles contain small aggregates of immunoreactive cells consistent with early response to antigen. CD21 IHC, X7.

G-I. Lymph node adjacent to patent longitudinally oriented cystic duct with substantial eccentric subepithelial fibrosis and intact epithelium lacks visible reactive germinal centers. H&E stain X7. H. Serial section of G. Condensed zones of immunoreactive cells in cortex are non-reactive primary follicles. CD79a IHC. X7. I. Serial section of G. Rare primary follicles contain small aggregates of immunoreactive cells (arrow) consistent with early response to antigen. CD21 IHC, X7.

J. Small lymph node contiguous with discrete dense fibrous cord representing atresia of the cystic duct, has no visible reactive germinal centers. H&E stain, X7.

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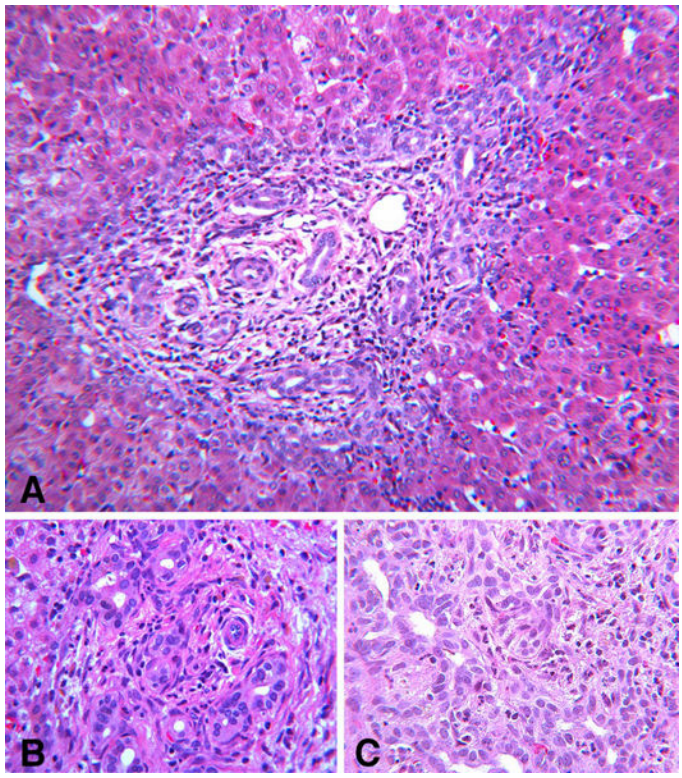

Figure 6.

A. Portal inflammatory infiltrates in biopsies obtained concurrent with HPE were graded for prevalence, overall cellularity and for any component of acute pericholangitis. This is an example of moderate portal cellularity with a detectable component of acute pericholangitis. H&E stain, X100.

B. Mild acute pericholangitis accompanies florid proliferation of ducts and ductules. H&E stain, X250.

C. Severe pericholangitis accompanies florid proliferation of ducts and ductules. H&E stain, X250.

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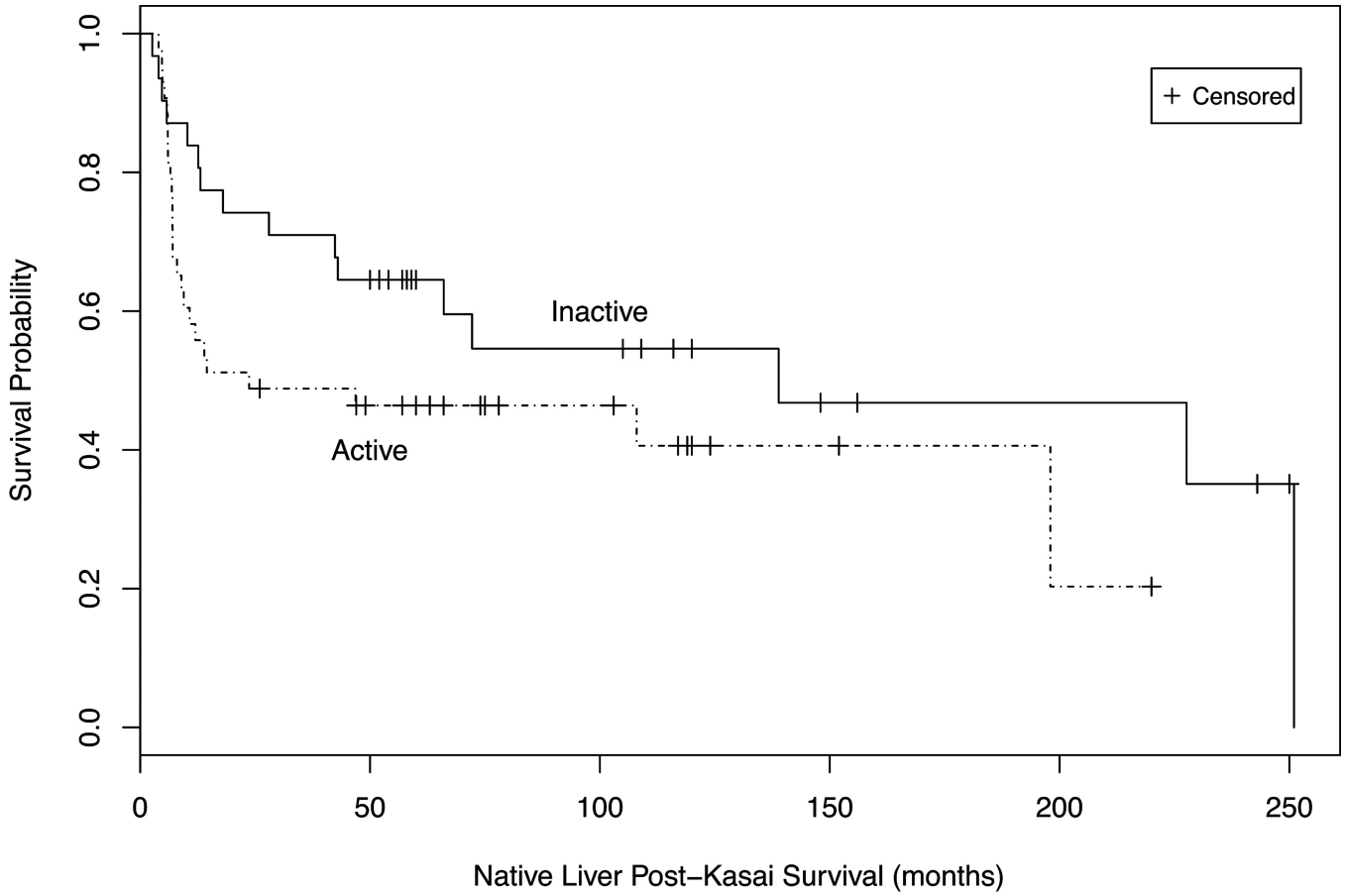

Figure 7.

Kaplan-Meier plot of survival of the native liver in months after HPE in 74 subjects with adequate followup information. Non-censored data indicate liver transplantation. The two plot lines designate subjects with no reactive centers vs one or more reactive centers in any hilar LN. Ten subjects with hilar LN data are excluded because of death (2), lost to followup (2) or followup < 2 years (4).

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Table 1.

106 Hepatic hilar lymph nodes in BA vs autopsy controls

	Visible Hilar LN	p value	Control LN	p value	Cystic duct LN
N	79		27		27
Mean Age	65d	0.02	51d	ns	57d
Area/mm2	22.9	0.00001	4.4	ns	5.2
# PF	17.1	0.00001	3.9	ns	4.8
PF/mm2	0.9	0.01	1.6	ns	1.5
#GC	3.5	0.001	0	0.004	2.3
#GC/mm2	0.2	0.002	0	0.005	0.3

Analysis of variance (ANOVA) was used to compare means and standard deviations of control LN to surgically excised hilar LN and to cystic duct LN. PF, primary follicles; GC, germinal centers.

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Table 2A.

LN reactivity vs BA demographics in 68 subjects with 76* visible hilar LN and biliary atresia

	Active hilar LN n =43 (40 pts)	Inactive hilar LN n=33 (28 pts)	P value**
Onset Jaundice	29 d	24 d	ns
Age HPE	72 d	55 d	0.002
Age transplant	16.8 mo	60.3 mo	0.04
LN area/mm2	28.6	16.6	0.01
# GC	6.4 +/−5.9	0	0.00000
#PF	22.2	11.3	0.001
PF/mm2	1.0	0.9	ns
# CD79a	38.9	23.3	0.01
CD79a/mm2	2.0	2.0	ns
#CD21	33.5	16.3	0.002
CD21/mm2	1.8	1.6	ns
#BCL6	7.7	0.3	0.00000
BCL6/mm2	0.4	0.02	0.00000

* In three subjects with two hilar LN discordant for presence or absence of GC, the inactive LN was excluded for this tabulation

** Analysis of variance (ANOVA) was used to compare means and standard deviations in assessment of visible hilar LN. PF, primary follicles; GC, germinal centers.

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Table 2B.

LN reactivity vs BA demographics in 99/106* hilar LN from 84 subjects with biliary atresia

	Active LN n =52 (46 pts)	Inactive LN n= 47 (38 pts)*	P value**
Onset Jaundice	29 d	24 d	ns
Age HPE	71 d	51 d	0.0001
Age transplant	24 mo	60.3 mo	0.07
LN area/mm2	24.6	13.1	0.004
# GC	5.9	0	0.00000
GC/mm2	0.4	0	0.00000
#PF	19.9	8.7	0.00005
PF/mm2	1.1	1.0	ns
# CD79a	39.7	17.9	0.0001
CD79a/mm2	2.2	2.3	ns
#CD21	29.2	12.7	0.0002
CD21/mm2	1.8	1.8	ns
#BCL6	7.5	0.4	0.00000
BCL6/mm2	0.6	0.2	ns

*In seven subjects with two hilar LN discordant for presence or absence of GC, the inactive hilar LN was excluded from this analysis.

**Analysis of variance (ANOVA) was used to compare means and standard deviations in assessment of surgically excised hilar LN. PF, primary follicles; GC, germinal centers. Compared to values in Table 2A for visible hilar LN only, significance increased for all values except BCL6/mm2 when data from CDLN were included.

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Table 3.

Relationship of GC reactivity in visible hilar LN to intrahepatic acute pericholangitis in 62 subjects*.

	Active hilar LN (35pts)	Inactive hilar LN (27pts)	P value**
Generalized pericholangitis (any degree:> 50%portal areas)	n=31	n=17	0.18
No or focal mild Pericholangitis	n=9	n=15	

*Liver biopsy data was available in 64/84 subjects with visible hilar LN; two with severe focal acute pericholangitis are excluded from this table

**2-tailed Fisher exact test was used to calculate significance.

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Table 4.

Relationship of GC reactivity in 99/106[*] hilar LN to intrahepatic acute pericholangitis in 77[**] subjects

	Active LN 40 pts	Inactive LN 37 pts[*]	P value[**]
Generalized pericholangitis (any degree: > 50%portal areas)	n=31	n=16	0.0026
No or focal mild Pericholangitis	n=10	n=27	

[*] Seven inactive lymph nodes excluded due to discordant positive results for GC in a second hilar LN.

[**] 80/84 subjects had liver biopsy data; three with focal severe pericholangitis excluded.