Claudin-3, Lipopolysaccharide Binding Protein, and Jaundice Clearance in Infants with Biliary Atresia

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Objective To explore the relationship among bacterial translocation, intestinal barrier integrity, and systemic inflammation in early biliary atresia (BA).

Study design Newly diagnosed infants with BA were assessed longitudinally before as well as 6, 12, and 24 weeks after undergoing Kasai portoenterostomy. Plasma immune marker measurement included interleukin (IL)-2, interferon-gamma (IFNγ), IL-4, IL-10, tumor necrosis factor alpha, IL-6, IL-8, IL-1β, IL-17, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Bacterial translocation (lipopolysaccharide binding protein [LBP]; D-lactate), intestinal barrier (claudin-3; intestinal fatty acid binding protein [IFABP], fecal calprotectin) biomarkers, and fecal microbiota genus abundance were analyzed.

Results Fifty-five infants were included, of whom 60% cleared their jaundice. Early post-Kasai, upregulation of plasma adhesion molecules and pro-inflammatory cytokines were associated with poorer jaundice clearance and liver fibrosis and correlated with jaundice severity. Elevated claudin-3 early post-Kasai was associated with poor jaundice clearance [OR 1.02 (1.00, 1.04); $P = .02$] and jaundice severity [$P < .01$]. On multivariable analysis, early ICAM-1 elevation (OR 1.008 [1.002, 1.014]; $P = .01$) and claudin-3 (OR 1.038 [1.009, 1.068]; $P = .02$), represented independent prognostic markers for persistent jaundice. Increased longitudinal trends of LBP (OR 0.79 [0.71, 0.89]; $P < .01$) and IFABP (OR <0.01 [2.4E-43, 0.24]; $P = .04$) were associated with poor jaundice clearance. LBP positively correlated with pro-inflammatory-cytokines (IL-6, $P < .01$: TNFα, $P < .01$) and ICAM-1 ($P < .01$) early post-Kasai. Fecal calprotectin positively correlated with jaundice severity ($P < .01$) by 24 weeks post-Kasai. No correlation between fecal microbiota abundance and bacterial translocation/intestinal integrity markers was demonstrated.

Conclusions Bacterial translocation may be linked to post-Kasai BA-immune pathways. Claudin-3 could represent a novel biomarker of early intestinal permeability in BA; links to the gut microbiota need further exploration. *(J Pediatr 2025;286:114703)*.

Extrahepatic biliary atresia (BA) is a disease characterized by fibro-obliteration of the biliary tree, presenting in infancy with obstructive jaundice that blocks the flow of bile from the liver to the gut.[1] The Kasai portoenterostomy ("Kasai") surgery aims to relieve the bile duct obstruction and re-establish the flow of bile, by creating a bilio-enteric conduit. However, jaundice clearance is only achieved in approximately 60% of infants and progression of liver disease to cirrhosis that requires liver transplantation (LT) often occurs even in infants with successful jaundice clearance.[2] BA remains the most common cause of LT in pediatrics.

Although the exact etiology of BA remains elusive, growing evidence suggests a complex interplay between genetic predisposition and immune dysregulation. Animal and human tissue studies in BA have revealed a prominent role for innate immunity (macrophages, natural killer cells), adaptive immunity (cluster of differentiation 4 [CD4+] T-helper cells [Th1/Th2/Th17]) and cellular adhesion molecule upregulation (ICAM-1 and vascular cell adhesion molecule 1 [VCAM-1]) pathways.[3] The inflammatory process in BA subsequently activates

AST	Aspartate aminotransferase	LT	Liver transplantation
ALT	Alanine transaminase	LPS	Lipopolysaccharide
APRi	AST-to-platelet ratio index	LSM	Liver Stiffness Measurement
BA	Biliary atresia	LBP	Lipopolysaccharide binding protein
GGT	Gamma-glutamyl transferase		
ICAM-1	Intercellular adhesion molecule 1	TNFα	Tumor necrosis factor alpha
IFABP	Intestinal fatty acid binding protein	TB	Total bilirubin
IL	Interleukin	VCAM-1	Vascular cell adhesion molecule 1
IFNγ	Interferon gamma		

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fibrogenic pathways, producing various extracellular matrix proteins, contributing to fibrosis and cirrhosis development[4] and eventually the need for LT. Post-Kasai pharmacotherapy that aims to dampen fibro-inflammatory processes have not convincingly translated into improved native liver survival.

Altered gut microbiota, has been increasingly implicated in chronic liver disease in adult cohorts, with microbiota-modulatory interventional trials showing remarkable promise.[5] In recent years, gut microbiota disturbance has emerged as a significant feature in BA, characterized by an increase in pathobionts—potentially pathogenic organisms—and a decline in beneficial commensal bacteria.[6-12] In our recent work,[13] *higher post-Kasai alpha-diversity, higher relative abundance of Streptococcus and Fusobacterium and lower relative abundance of Dorea, Blautia, and Oscillospira were associated with non-favorable clinical outcomes. Beneficial microbiota, Blautia, and Bifidobacterium, inversely correlated to liver disease severity and fibrosis, respectively.* The gut microbiota and the host immune system are intricately linked, in several ways. One gut microbiota-immune pathway, involves bacterial translocation across a "leaky gut," where potentially harmful bacteria and their products (Pathogen-Associated Molecular Patterns), can regulate the innate immune system by modulating Pattern Recognition Receptors on immune-sensory cells, promoting proinflammatory responses.[14]

The study objective was to assess immune, bacterial translocation and intestinal integrity biomarkers longitudinally in a cohort of newly diagnosed Infants with BA to enhance understanding of potential gut bacteria mechanistic pathways.

Methods

Study Cohort

In this prospective, longitudinal, single-center study, infants with newly diagnosed Infants with BA, confirmed by intraoperative cholangiogram and/or liver biopsy pathology at King's College Hospital were recruited between January 2016 to December 2019. Longitudinal assessment at pre-Kasai, 6 weeks, 12 weeks, and 24 weeks post-Kasai time-points was performed for all infants. All infants underwent standard King's College Hospital BA protocol (**Supplementary Methods**; available at www.jpeds.com). Exclusion criteria included previous bowel resection, primary liver transplant, extrahepatic disease associated with significant morbidity/mortality and international candidates unable to attend longitudinal study visits.

Study Data and Clinical Endpoints

Pre-Kasai, demographic data were recorded for all Infants with BA. Liver wedge biopsies were assessed for the degree of fibrosis with METAVIR staging.[15] METAVIR scoring using the following definitions: F0- absent fibrosis, F1- portal fibrosis without septae/bridging, F2- Portal fibrosis with few septae/bridging, F3- Portal fibrosis with many septae/bridges, F4- cirrhosis. Biochemical parameters were recorded at all time-points; serum total bilirubin (TB μmol/l), aspartate aminotransferase (AST IU/L), alanine transaminase (ALT IU/L), and gamma-glutamyl transferase (GGT IU/L). Mathematical model, AST-to-platelet ratio index (APRi), was calculated at each time-point (**Supplementary Methods**; available at www.jpeds.com). Liver Stiffness Measurement (LSM, kPa) was measured using Transient Elastography (Fibroscan) (**Supplementary Methods**; available at www.jpeds.com). The primary outcome measure was 6-month jaundice clearance as defined by serum TB < 20 μmol/l by 6-months after undergoing the Kasai procedure. For tables and figures, the abbreviations BA-JC and BA-J have been used to define "jaundice clearance" and "persistent jaundice" groups.

Plasma and Fecal Sample Collection

Peripheral venous blood (2 ml) was collected in lithium heparin specimen tubes from all infants with Infants with BA at all time-points, and centrifuged at 200 × g for 20 mins at 4C, to provide plasma. Plasma samples were then stored in aliquots of 200 μL at −80C, until further downstream analysis of immune, bacterial translocation and intestinal barrier biomarkers. Fecal samples were collected from all infants with BA at each time-point. Samples were collected directly from the diaper, using a sterile spatula and pot. One fecal aliquot (minimum 200 mg) was stored at room temperature for up to 3 days before being processed for calprotectin. Remaining fecal sample was kept refrigerated during transport at approximately +4C for a maximum of 24 hours, and subsequently aliquoted and stored at −80C for DNA extraction.

Immune Biomarker Measurement. Plasma samples were analyzed using a Human V Plex Custom Proinflammatory panel 1 assay kit [Meso Scale Discovery, Rockville, MD 20850 USA]. for (i) helper T cell-1 (Th)1- associated cytokines; interleukin (IL)-2 and interferon-gamma (IFNγ) (ii) Th2- associated cytokines; IL-4 and IL-10 and (iii) proinflammatory cytokines; tumor necrosis factor alpha (TNFα), IL-6, IL-8 and IL-1β. The Th17-associated cytokine, IL-17 was measured using an ELISA kit [Biotechne]. The soluble cellular adhesion molecules, intercellular adhesion molecule 1 (ICAM-1) and VCAM-1 were analyzed, using ELISA kits [Biotechne, R & D Systems Europe].

Bacterial Translocation and Intestinal Barrier Biomarker Measurement. Plasma samples were analyzed for bacterial translocation and intestinal barrier biomarkers. Lipopolysaccharide binding protein (LBP) was measured using an ELISA kit [Cambridge Bioscience, Cambridge, UK]. D-lactate, was assayed spectrophotometrically [Sigma-Aldrich Company Ltd]. Intestinal barrier biomarkers, claudin-3 [Oxford Biosystems Ltd] and intestinal fatty acid binding protein (IFABP) [Biotechne, R & D Systems Europe], were measured using ELISA kits. Portal and peripheral plasma for "late BA" infants, were measured for LBP only. Fecal calprotectin was measured using ELISA [Diasorin Liaison XL].

DNA Extraction and 16S rRNA Gene Amplicon Sequencing of Fecal Samples. FastDNA Spin Kit for Soil

(MP) was used to extract DNA from feces following manufacturer instructions, with extended 3-minute bead-beating. DNA concentration and quality were quantified using a Qubit 2.0 fluorometer (Invitrogen) (**Supplementary Methods**; available at www.jpeds.com).

Statistical Analysis

Continuous variables were tested for normality (Shapiro–Wilk Normality Test), and as data were non-normally distributed, median values and interquartile ranges were recorded. Categorical variables were reported as frequency and percentages (%). Two independent groups were analyzed using the Mann–Whitney U test, and paired groups were analyzed using the Wilcoxon signed-rank test. Pearson chi-square test was used to analyze categorical variables. Fisher's exact test was used to analyze categorical variables if cell frequencies < 5. Bivariate correlations between continuous variables were calculated using the Spearman's rank correlation. Univariate and multivariable analyses [OR, OR; CI, CI] were performed using binary (for categorical variable) and linear (continuous variables) logistic regression for jaundice clearance and fibrosis associations. Area under the receiver operating characteristic curve (AUROC) analysis was used to calculate the diagnostic accuracy of variables in assessing 6-month jaundice status. AUROC ≥0.75 represented reasonable clinical utility for our cohort. To determine if there was a change in the trend of immune/bacterial translocation/intestinal barrier biomarkers over time, between jaundice outcomes, generalized estimating equations, with an unstructured correlation matrix, were used. This statistical methodology was chosen as it can process factorial, nonparametric, repeated measures data (Wobbrock et al, 2011). A P value <.05 was considered statistically significant for all tests. If multiple testing performed, a Bonferroni correction was applied (accepted P values specified in individual results section). All statistical analyses were performed using SPSS version 29 (IBM), and GraphPad PRISM Version 10.1.0 (Dotmatics).

Ethical Statement

The experimental protocol was approved by the London – Harrow Research Ethics Committee (REC 15/LO/1966) in accordance with the ethical standards established in the 1964 Declaration of Helsinki. Written informed consent was obtained from parent/guardian before specimen collection. Ethical Agreement (REC 18/WA/0009), and access to samples, was granted by the Biobank Access Committee.

Results

Clinical Outcome Groups

Out of the 55 infants with BA recruited to this study, 33 (60%) cleared their jaundice by 6-months post-Kasai and 22 (40%) remained jaundiced at 6-months post-Kasai. At Kasai, demographics were similar between outcome groups (**Table I**). In total 24 (44%) patients underwent listing for liver transplantation by 2 years of age. Of those listed for transplant, 12% (n = 4) had cleared their jaundice and 91% (n = 20) had not (P < .01). Indications for transplant included: jaundice and complications of portal hypertension (n = 12), jaundice (n = 5), complications of portal hypertension (n = 4) and jaundice and recurrent cholangitis (n = 3). Median age at transplant was 10.53 (IQR 7.36, 14.07) months. In total 26 (47%) experienced at least one episode of cholangitis by 6-months post-Kasai, of which 12 (36%) and 14 (64%) constituted the jaundice clearance and jaundiced groups, respectively (P < .06). Antibiotic and steroid administration rates were similar between outcome groups (data not shown).

Table I. Comparison of pre-Kasai baseline characteristics and clinical outcomes, between BA-JC (jaundice clearance) and BA-J (persistent jaundice) groups

Baseline characteristics and clinical outcomes	BA-JC n = 33	BA-J n = 22	P
Age at Kasai (d)	51 (40, 60)	47 (31, 57)	.31
Male n (%)	21 (64)	12 (55)	.58
Birthweight (Kg)	3.11 (2.75, 3.52)	3.06 (2.87, 3.48)	.95
Prematurity n (%)	3 (9)	3 (14)	.67
BASM n (%)	1 (3)	4 (18)	.15
CMV positive	5 (15)	2 (9)	.70
LT- 2 y age n (%)	4 (12)	20 (91)	<.01*
Cholangitis by 6 mo	12 (36)	14 (64)	.06
METAVIR score 3-4	21 (64)	15 (68)	.51

Data are n (%) or median (IQR).
BASM, biliary atresia splenic malformation; *CMV*, cytomegalovirus.
*P < .05.

The number of plasma and fecal samples available at all time-points are stated below, highlighting some missing samples due to practical logistics of obtaining samples in this vulnerable cohort: pre-Kasai, 37 plasma/35 fecal; 6 weeks 41 plasma/42 fecal; 12 weeks 35 plasma/39 fecal; and 24 weeks 42 plasma/42 fecal.

Relationship Among Immune, Bacterial Translocation, and Intestinal Barrier Markers, and Jaundice Clearance

At the pre-Kasai time-point, immune, bacterial translocation and intestinal barrier markers were similar between jaundice outcome groups (**Table II**). Early post-Kasai immune, bacterial translocation and intestinal barrier marker comparison between clinical outcome groups are illustrated in **Table II** and **Figure 1, A-I**. As early as 6 weeks post-Kasai, ICAM-1 (P < .01), IL-4 (P = .02), and claudin-3 (P < .01) were elevated in the jaundiced group. By 12 weeks post-Kasai, ICAM-1 (P = .01), claudin-3 (P = .04), IL-8 (P < .01), and IL-1β (P = .01) were elevated in the jaundiced group. By 24 weeks post-Kasai (**Table II**), ICAM-1, VCAM-1, IL-2, IL-6, IL-8, TNFα, and IL-1β were elevated in the jaundiced group [Supplementary Table includes data for 2 year native liver survival and liver transplant outcome groups]. A univariate analysis was performed at 6 weeks post-Kasai; TB [OR 1.03 (1.02, 1.05);

Table II. Comparison of biochemistry and fibrosis/immune/BT/intestinal barrier markers between BA-JC (jaundice clearance) and BA-J (persistent jaundice) groups, pre-Kasai and (6 wks, 12 wks, 24 wks) post-Kasai time-points. Data are n(%) or median (IQR)

	Pre-Kasai			6 wks post-Kasai			12 wks post-Kasai			24 wks post-Kasai		
Parameter	BA-JC n = 33	BA-J n = 22	*P*	BA-JC n = 33	BA-J n = 22	*P*	BA-JC n = 33	BA-J n = 22	*P*	BA-JC n = 33	BA-J n = 22	*P*
Age at visit (wks)	NA	NA	NA	13.30 (11.92, 15.63)	13.10 (10.82, 18.63)	.99	20.30 (17.57, 22.14)	19.29 (17.93, 21.97)	.83	33.20 (31.26, 36.23)	32.00 (28.23, 35.21)	.13
Biochemistry												
TB (μmol/L)	132.00 (104.00, 156.00)	123.50 (99.25, 141.75)	.47	18.50 (8.25, 42.75)	123.00 (49.50, 156.00)	<.01*	13.00 (6.00, 26.00)	124.00 (43.25, 207.25)	<.01*	6.5 (4.00, 10.00)	203.00 (36.00, 273.00)	<.01*
GGT (IU/L)	430.00 (200.00, 723.50)	362.50 (284.50, 794.25)	.92	1125.00 (785.5, 1520.5)	1009.50 (893.75, 634.5)	.80	913.00 (510.0, 1329.0)	980.50 (393.00,1436.00)	.82	387.50 (200.50, 685.75)	451.00 (238.00, 1047.00)	.43
AST (IU/L)	140.00 (85.50, 204.50)	132.50 (94.00, 268.00)	.55	102.00 (76.50, 133.00)	187.50 (125.00, 86.50)	<.01*	103.00 (65.75, 140.50)	177.50 (133.75, 243.00)	<.01*	85.50 (52.25, 101.50)	177.00 (123.00, 243.00)	<.01*
ALT (IU/L)	119.00 (47.00, 163.50)	104.00 (57.25, 151.75)	.84	101.00 (63.50, 139.50)	121.00 (96.00, 192.50)	.05	91.50 (50.00, 116.50)	123.50 (88.75, 164.25)	.04*	71.00 (49.00, 87.00)	113.00 (74.00, 177.00)	<.01*
Fibrosis markers												
LSM (Kpa)	10.50 (6.70, 14.30)	10.10 (6.40, 16.80)	.71	15.40 (9.30, 28.40)	22.40 (9.70, 39.10)	.15	25.55 (13.58, 61.23)	56.30 (14.53, 75.00)	.18	29.45 (14.83, 55.08)	75.00 (75.00-75.00)	<.01*
APRi	0.67 (0.50, 1.07)	0.72 (0.45, 1.92)	.48	0.67 (0.47, 1.42)	1.45 (0.91, 2.25)	<.01*	0.97 (0.41, 1.75)	1.76 (1.42, 3.32)	<.01*	1.12 (0.58, 1.55)	2.12 (1.43, 4.10)	<.01*
Immune markers												
IL-2 (ng/L)	0.31 (0.11, 0.51)	0.37 (0.16, 0.60)	.65	0.23 (0.00, 0.51)	0.49 (0.00, 1.07)	.25	0.51 (0.28, 0.95)	0.59 (0.27, 1.61)	.58	0.383 (0.20, 0.67)	13.33 (0.65, 1.79)	<.01*
IL-4 (ng/L)	0.08 (0.00, 0.12)	0.07 (0.06, 0.13)	.97	0.11 (0.06, 0.18)	0.20 (0.09, 0.30)	.02*	0.14 (0.01, 0.24)	0.21 (0.15, 0.34)	.11	0.13 (0.07, 0.22)	0.13 (0.09, 0.61)	.37
IL-6 (ng/L)	1.19 (0.97, 3.32)	1.51 (0.95, 2.30)	.87	1.88 (1.40, 3.54)	2.60 (1.31, 5.32)	.55	2.19 (1.44, 4.82)	3.98 (2.15, 6.45)	.13	2.19 (1.38, 3.64)	3.78 (2.54, 10.11)	.02*
IL-8 (ng/L)	106.16 (77.33, 349.10)	109.21 (63.24, 194.55)	.39	183.05 (58.98, 368.44)	234.20 (137.24 650.34)	.16	156.98 (110.75, 284.11)	564.99 (270.95, 1674.00)	<.01*	98.95 (48.49, 255.49)	502.85 (294.38, 2000.87)	<.01*
IL-10 (ng/L)	2.33 (1.62, 6.18)	2.21 (1.46, 3.29)	.24	2.33 (1.54, 5.09)	2.50 (1.93,4.31)	.48	3.70 (2.24, 6.75)	3.41 (1.93, 4.45)	.54	2.79 (2.04, 3.49)	3.55 (1.98, 4.72)	.28
IFNy (ng/L)	6.06 (2.87, 8.76)	6.74 (3.84, 12.75)	.57	8.77 (5.33, 20.54)	9.91 (7.86, 16.58)	.32	8.87 (6.04, 15.33)	8.30 (5.56, 9.23)	.29	10.34 (6.32, 14.59)	13.15 (10.65, 44.84)	.06
TNFα (ng/L)	7.31 (5.37, 9.55)	7.84 (6.10, 8.33)	.92	6.52 (5.19, 8.69)	7.16 (6.32, 12.66)	.08	9.11 (6.05, 11.75)	8.80 (5.96, 10.81)	.93	7.25 (5.76, 8.94)	8.94 (8.02, 11.83)	.04*
IL-1β (ng/L)	0.56 (0.26, 1.25)	0.54 (0.30, 0.77)	.60	0.52 (0.27, 1.05)	0.90 (0.43, 2.18)	.06	0.60 (0.31, 0.97)	1.46 (0.67, 3.22)	.01*	0.47 (0.29, 0.66)	1.61 (0.87, 7.84)	<.01*
IL-17 (ng/L)	4.88 (1.65, 6.80)	5.87 (2.80, 9.11)	.24	5.73 (3.66, 7.99)	6.86 (2.11, 11.54)	.84	5.87 (4.10, 12.51)	5.77 (3.49, 12.24)	.804	7.36 (4.58, 9.39)	9.25 (5.35, 10.40)	.24
VCAM-1 (μg/L)	1392.54 (1061.00, 1700.52)	1469.80 (957.38, 1852.38)	.91	1659.47 (1206.21 970.42)	1846.46 (1389.62,2818.32)	.39	1689.64 (1439.06,2035.96)	2547.95 (1617.23,2746.98)	.05	1552.96 (1153.48, 1883.04)	1965.64 (1803.41, 2359.86)	.02*
ICAM-1 (μg/L)	1019.17 (723.08, 1306.25)	848.80 (729.80, 1297.00)	.77	1178.56 (915.10, 1491.22)	1812.02 (1440.17, 2264.80	<.01*	1125.60 (807.12, 1599.56)	1842.20 (1376.60,2191.00)	.01*	989.52 (597.37, 1343.80)	2019.59 (1352.97, 2297.45)	<.01*
BT/Intestinal barrier markers												
LBP (mg/L)	8.47 (5.35, 11.56)	8.02 (0.00, 9.66)	.40	9.33 (7.49, 13.29)	8.94 (6.95, 17.89)	.89	10.20 (7.89, 12.84)	9.27 (6.30, 10.26)	.27	8.35 (0.00, 12.06)	11.07 (9.14, 13.10)	.11
D-Lactate (ng/μl)	17.70 (13.13, 24.96)	16.76 (9.30, 18.80)	.42	10.65 (8.97, 23.64)	14.87 (10.40, 20.25)	.48	9.37 (6.80, 15.82)	15.21 (11.66, 20.11)	.05	14.97 (967.46, 2088.31)	17.58 (9.41, 22.95)	.47
IFABP (ng/L)	3165.53 (2656.89, 5611.30)	3213.73 (2056.40, 5347.81)	.36	2139.03 (1501.72,3621.62)	1508.18 (1108.26,2415.66)	.13	2080.66 (1245.89,3099.55)	2300.61 (1139.15,3584.49)	.74	1500.35 (967.46, 2088.31)	2883.72 (981.58, 3206.44)	.23
Claudin-3 (ng/L)	66.08 (45.99, 97.62)	67.04 (3.65, 89.18)	.41	57.51 (26.45, 80.36)	112.27 (58.72, 152.19)	<.01*	66.41 (29.52, 100.62)	105.91 (59.76, 156.34)	.04*	55.75 (31.09, 106.69)	101.91 (38.26, 192.78)	.15
Fecal Calprotectin (μg/g)	289.00 (88.00, 585.00)	145.00 (95.00, 294.00)	.35	264.00 (165.00, 476.00)	367.00 (123.00, 840.00)	.60	288.00 (109.25, 497.00)	315.00 (130.00, 405.00)	.76	48.00 (15.00, 273.50)	135.00 (96.25, 173.75)	.09

Missing sample record; from the total recruited cohort, plasma samples are available in [pre-Kasai] n = 37 (BA-JC, 22; BA-J, 15) [6 wks Post-Kasai] n = 41 (BA-JC, 26; BA-J, 15) [12 weeks Post-Kasai] n = 35 (BA-JC, 21; BA-J, 14) [24 wks post-Kasai] n = 42 (BA-JC, 22; BA-J, 20). From the total recruited cohort, fecal samples are available in [pre-Kasai] n = 35 (BA-JC, 20; BA-J, 15) [6 wks Post-Kasai] n = 42 (BA-JC, 23; BA-J, 19) [12 weeks Post-Kasai] n = 39 (BA-JC, 20, BA-J, 19) [24 wks post-Kasai] n = 42 (BA-JC, 24; BA-J, 18). *$P < .05$. NA-not applicable as age at Kasai stated in **Table I**.

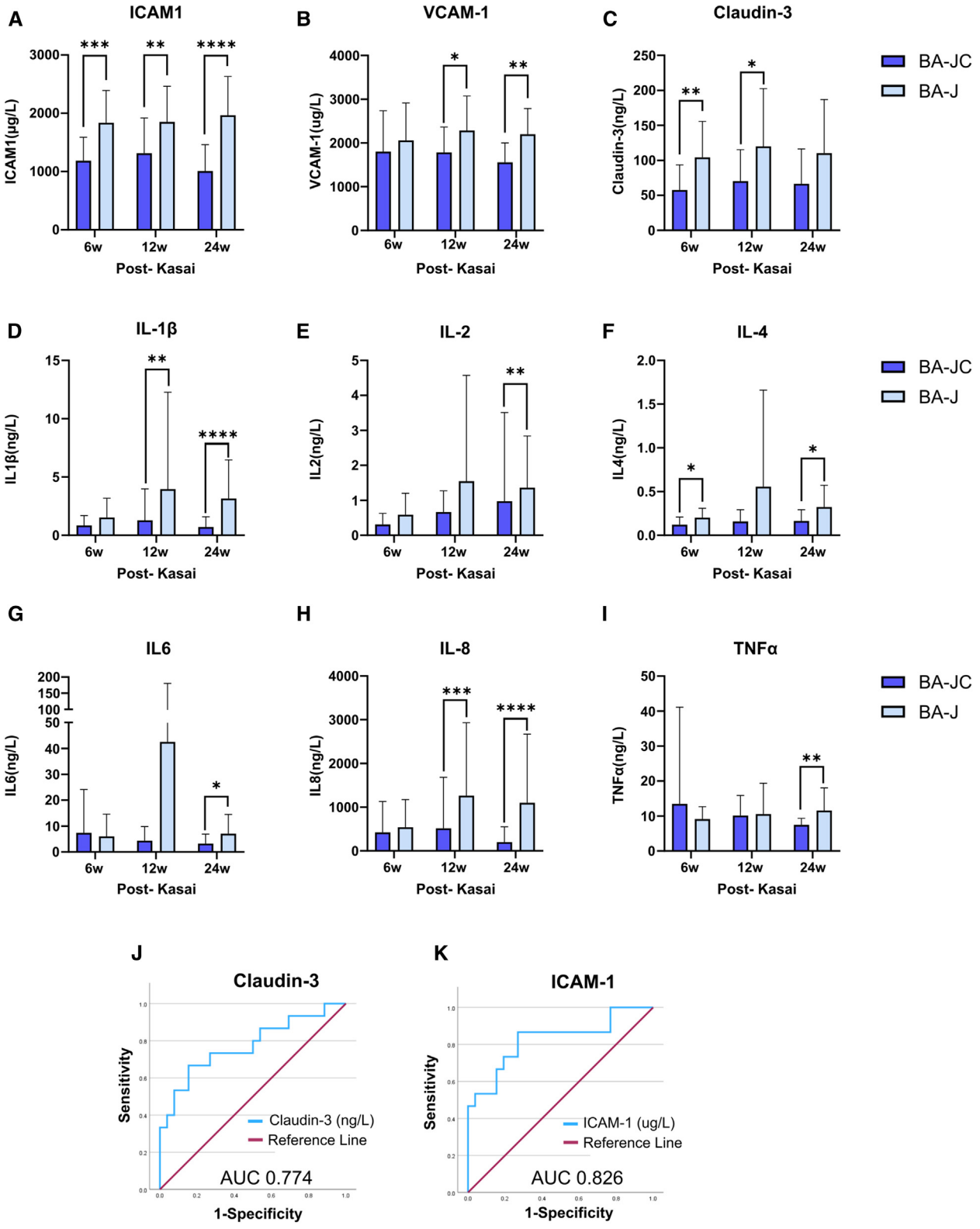

Figure 1. Comparison of longitudinal (**A**) ICAM-1 (**B**) VCAM-1 (**C**) Claudin-3 (**D**) IL-1β (**E**) IL-2 (**F**) IL-4 (**G**) IL-6 (**H**) IL-8 (**I**) TNFα between BA-JC (jaundice clearance) and BA-J (persistent jaundice) groups post-Kasai. Receiver-operating characteristic (ROC) curves for (**J**) Claudin-3 and (**K**) ICAM-1 at (6 wks time-point) as predictors of jaundice at 6 mo post-Kasai.

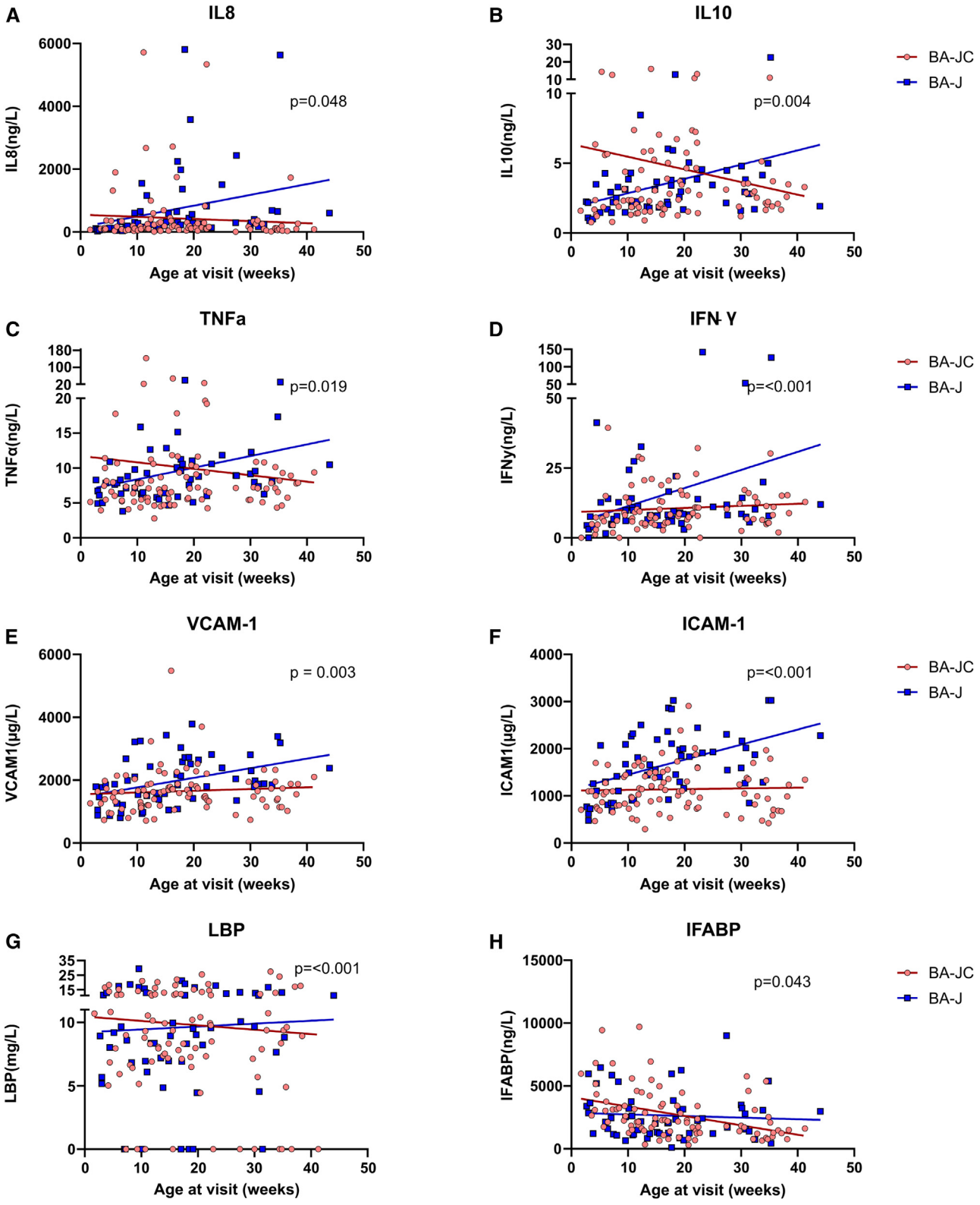

Figure 2. Longitudinal scatter plots to illustrate significantly different immune/BT/intestinal barrier marker over 6 mo post-Kasai period, based on 6 mo jaundice status [BA-JC, jaundice clearance; BA-J, persistent jaundice]; (**A**) IL-8 (**B**) IL-10 (**C**) TNFα (**D**) IFNy (**E**) VCAM-1 (**F**) ICAM-1 (**G**) LBP (**H**) IFABP. Generalized estimated equation (GEE) analysis performed. Dots represent a single patient sample from each of the outcome groups. Lines represent Pearson regression lines showing correlation between each marker over time. The lines which overlap show divergent trends from their starting points.

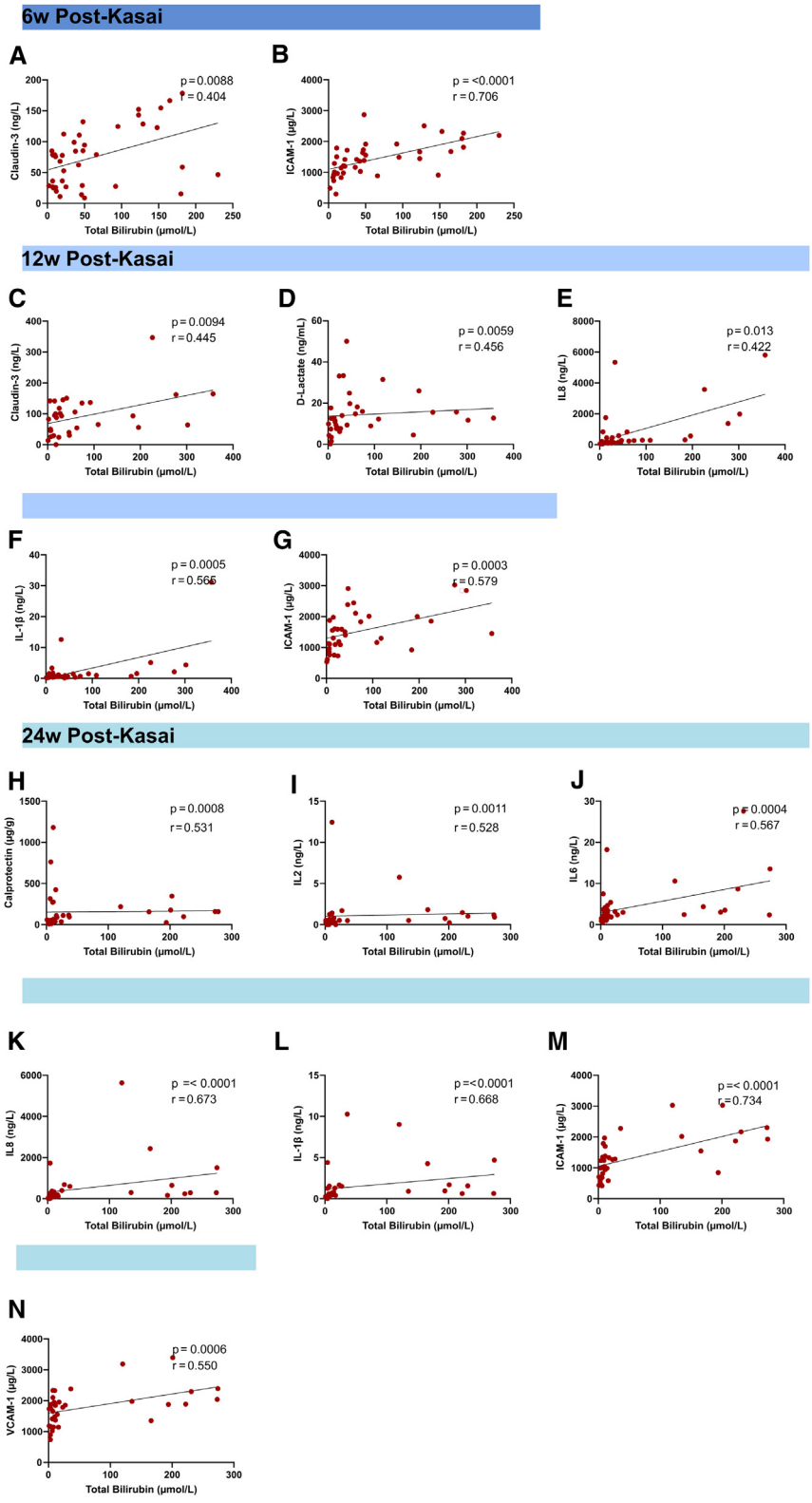

Figure 3. Scatter plots to illustrate correlation between immune/BT/intestinal barrier marker and jaundice severity 6w- (**A, B**), 12w- (**C-G**), 24w- (**H-N**) post-Kasai time-points. A Bonferroni correction for multiple testing was applied; **P* < .005 for immune markers; **P* < .01 for BT/intestinal barrier markers. Only significant correlations illustrated; see **Supplementary Table 6** (available at www.jpeds.com) for all correlations.

$P < .01$], AST [OR 1.03 (1.01, 1.05) $P < .01$], ICAM-1 [OR 1.003 (1.001, 1.005); $P = .01$], and claudin-3 [OR 1.02 (1.00, 1.04); $P = .02$], were associated with persistent jaundice at 6-months post-Kasai. A multivariable analysis incorporating the above parameters revealed ICAM-1 [OR 1.008 (1.002, 1.014); $P = .01$], and claudin-3 [OR 1.038 (1.009, 1.068); $P = .02$] as independent prognostic markers of persistent jaundice with AUROC's of 0.83 and 0.77, respectively (Figure 1, J-L).

Longitudinal Relationship Among Immune, Bacterial Translocation, and Intestinal Barrier Markers and Jaundice Clearance

Statistical analysis using GEE (for longitudinal data) was performed to determine a difference in the trend of immune, bacterial translocation and intestinal barrier biomarkers from pre-Kasai to 24 weeks post-Kasai, between jaundice outcome groups. The following markers revealed an increased trend (Figure 2, A-H) in the jaundiced group; VCAM-1 ($P < .01$), ICAM-1 ($P < .01$), IL-10 ($P < .01$), IFNγ ($P < .01$), TNFα ($P = .02$), LBP ($P < .01$), and intestinal fatty acid binding protein [IFABP] ($P = .04$).

Relationship Among Immune, Bacterial Translocation, and Intestinal Barrier Markers and Jaundice Severity

A Spearman's rank correlation test was performed for immune markers and jaundice severity (TB), at each time-point. At 6 weeks post-Kasai, ICAM-1 positively correlated with TB (r = 0.7, $P < .001$). At 12 weeks post-Kasai, ICAM-1 (r = 0.6, $P < .001$), IL-8 (r = 0.6, $P < .001$), IL-1β (r = 0.6, $P < .001$), positively correlated with TB. By 24 weeks post-Kasai, ICAM-1 (r = 0.7, $P < .001$), IL-8 (r = 0.7, $P < .001$), IL-1β (r = 0.7, $P < .001$), IL-6 (r = 0.6, $P = .001$), IL-2 (r = 0.5, $P < .001$), VCAM-1 (r = 0.6, $P = .001$) positively correlated with TB. A separate Spearman's rank correlation test was performed for bacterial translocation and intestinal barrier biomarkers and jaundice severity (TB), at each time-point. At 6 weeks (r = 0.4, $P = .009$) and 12 weeks post-Kasai (r = 0.4, $P = .009$), claudin-3 positively correlated with TB. D-lactate positively correlated (r = 0.5, $P = .008$) with TB at 12-week time-point only. By 24 weeks post-Kasai, fecal calprotectin positively correlated with TB (r = 0.5, $P = .001$). All significant correlations are illustrated in Figure 3.

Relationship Among Inflammation, Bacterial Translocation, and Intestinal Barrier Biomarkers and Liver Fibrosis

At the pre-Kasai time-point, ICAM-1 was associated with fibrosis severity, as represented by APRi [OR 0.56 (115.08, 354.56) $P < .001$] and LSM [OR 0.70 (26.65, 58.76) $P < .001$) but not histological fibrosis. By 6 weeks post-Kasai, ICAM-1 [OR 0.57 (204.08, 574.74) $P < .001$] and VCAM-1 [OR 0.57 (333.74, 945.57) $P < .001$] was associated with APRi and ICAM-1 associated with LSM [OR 0.45 (6.23, 26.88) $P = .003$). By 12 weeks post-Kasai, IL-1β [OR 0.68 (0.09, 0.22) $P < .001$], TNFα [OR 0.55 (0.04, 0.16) $P = .001$], IL-6 [OR 0.72 (0.007, 0.02) $P = .001$], IL-8 [OR 0.51 (0.00, 0.001) $P = .003$], IL-4 [OR 0.72 (0.86, 1.81) $P < .001$], IL-2 [OR 0.74 (0.32, 0.66) $P < .001$] were associated with APRi. By 24 weeks post-Kasai, ICAM-1 [OR 0.6 (0.01, 0.04) $P = .003$] was associated with LSM. D-lactate was the only bacterial translocation/intestinal barrier biomarker which was associated with fibrosis [LSM 12-week time-point: OR 0.51 (0.58, 2.84) $P = .004$]. All significant correlations are illustrated in Figure 4.

Relationship Among Bacterial Translocation and Systemic Inflammation

A Spearman's rank correlation (Figure 5, A) was performed for bacterial translocation and systemic inflammatory components. At 6 weeks post-Kasai, LBP positively correlated with IL-6 (r = 0.5; $P = .001$) and TNFα (r = 0.5; $P = .002$). At 12 weeks post-Kasai, LBP (r = 0.5, $P < .001$) and D-lactate (r = 0.6, $P < .001$) positively correlated with ICAM-1. At 24 weeks post-Kasai, LBP positively correlated with IL-6 (r = 0.5, $P = .002$) and IL-17 (r = 0.5, p0.004). All significant correlations are illustrated in Figure 5.

Relationship Among Gut Microbiota, Bacterial Translocation, and Intestinal Barrier Biomarkers

We previously described[13] the top 13 most abundant bacterial genera (defined as median relative abundance >0.01%) on 16S rRNA amplicon sequencing of BA fecal samples, across all time-points, were as follows; *Enterococcus*, *Clostridium*, *Fusobacterium*, *Pseudomonas, Bifidobacterium, Streptococcus, Blautia, Actinomyces, Hemophilus, Bacteriodes. Dorea, Oscillopira, Lactobacillus.* A Spearman's rank-order correlation (Figure 6, A) was run to determine the relationship between BA microbiota and bacterial translocation/intestinal barrier biomarkers. Lactobacillus positively correlated with calprotectin at pre-Kasai; but no other correlations were demonstrated at post-Kasai time-points.

Discussion

This prospective, longitudinal cohort study characterizes immune dysregulation, along with potential pathways of bacterial translocation and intestinal barrier dysfunction in BA.

This study contributes to the understanding of inflammation in BA. The BA liver is infiltrated by cluster of differentiation 4+ T cells, natural killer cells, and macrophages, with upregulated adhesion molecules on biliary and vascular endothelium.[3,16] We found a significant early systemic pro-inflammatory state post-Kasai, linked to poor jaundice clearance. This state is marked by increased soluble adhesion molecules (ICAM-1, VCAM-1) and pro-inflammatory cytokines (IL-8, IL-6, IL-1β, and TNFα) from various immune cells, along with Th1/Th2-associated cytokines (IL-2, IFNγ, IL-4, IL-10). Pro-inflammatory cytokines correlated with jaundice severity from 12 weeks post-Kasai. Immune pathways early

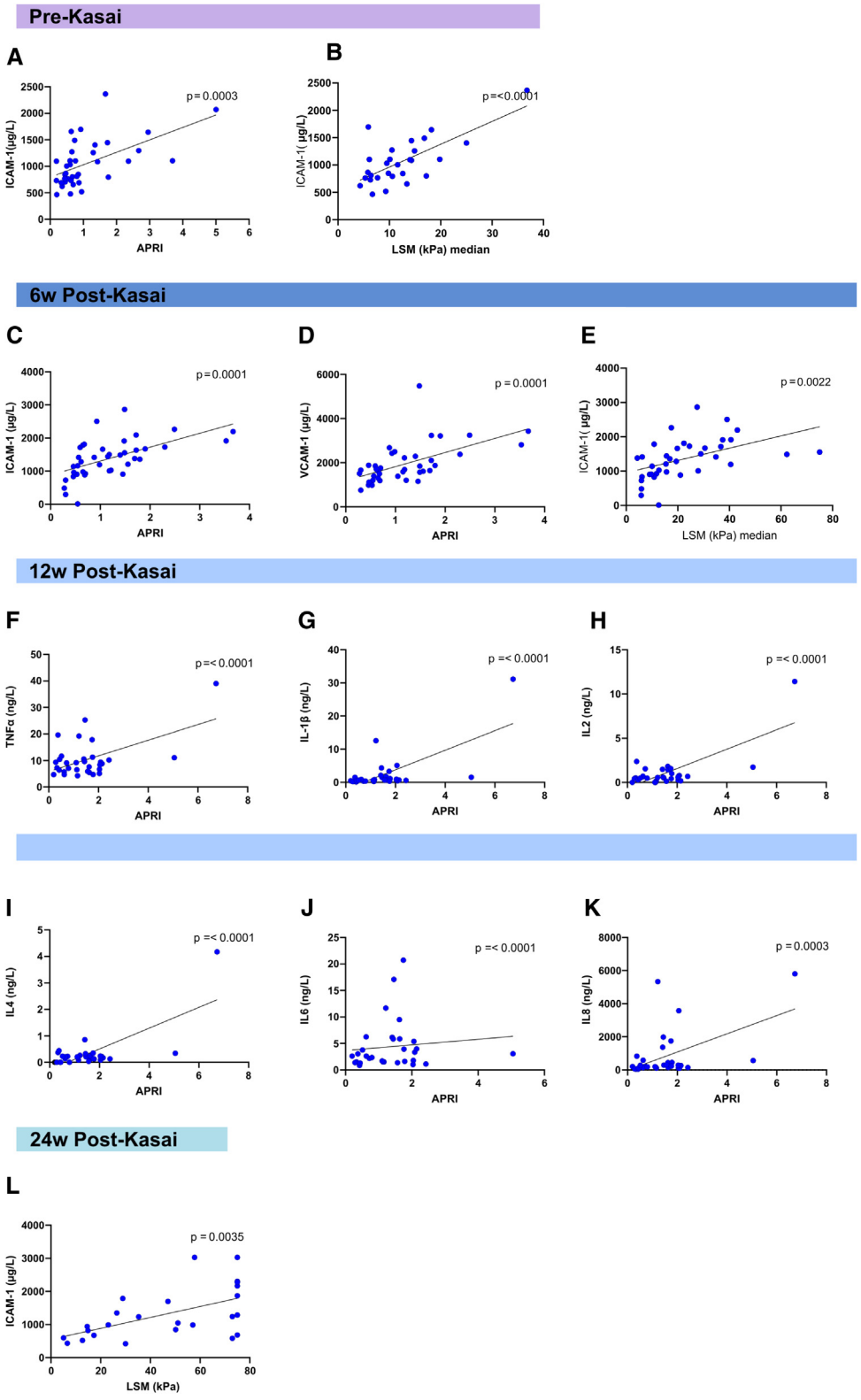

Figure 4. Scatter plots to illustrate linear regression between immune/BT/intestinal barrier marker and fibrosis biomarkers at pre- (**A**, **B**) and 6w- (**C-E**), 12w- (**F-K**) and 24w- (**L**) post-Kasai time-points. A Bonferroni correction for multiple testing was applied; **P* < .005 for immune markers; **P* < .01 for BT/intestinal barrier markers.

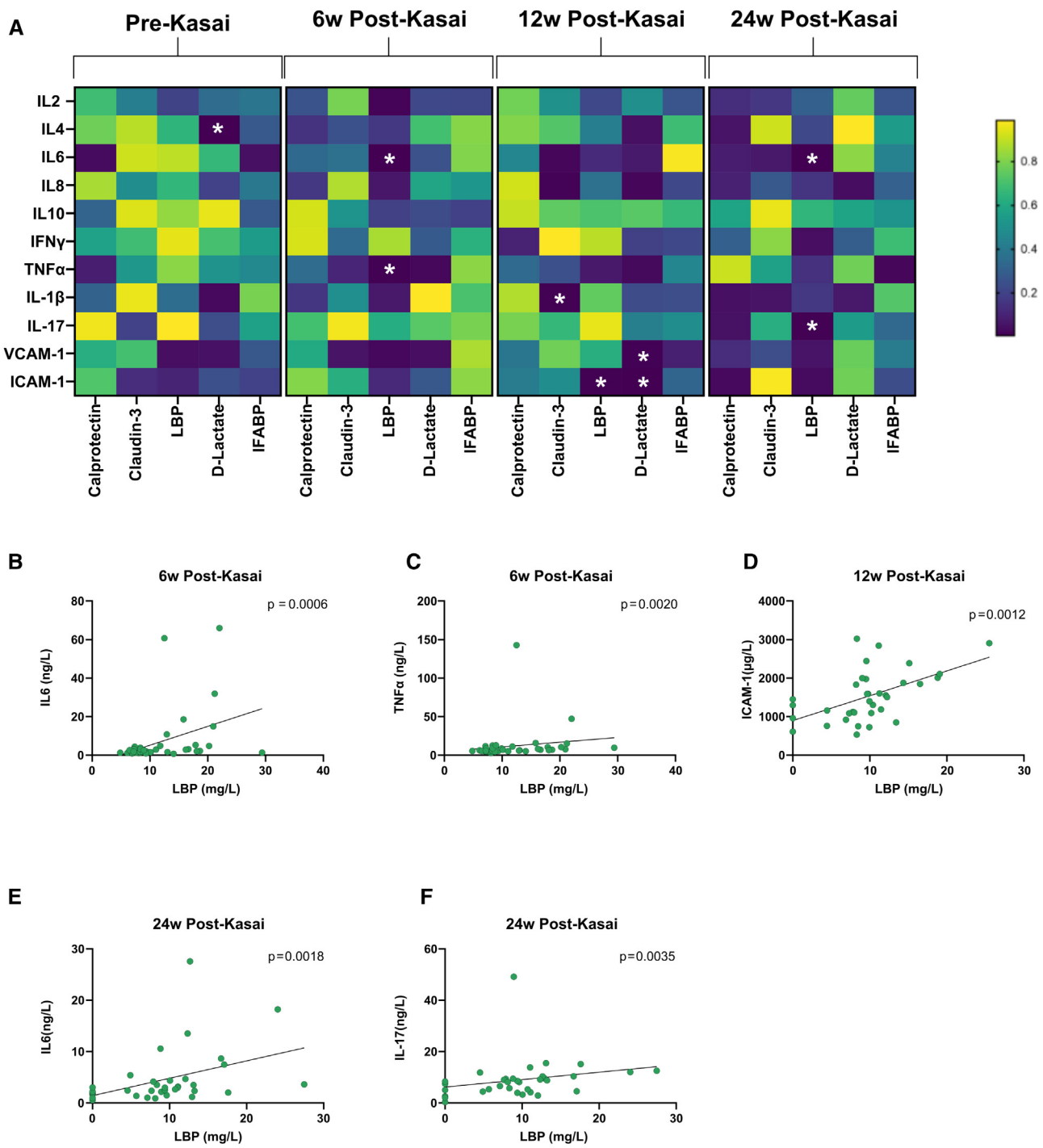

Figure 5. (**A**) Heatmap to illustrate correlations between immune and BT/intestinal barrier markers at pre- and post-KPE timepoints. (**B-F**) Scatterplots to illustrate significant correlations between LBP and immune markers. A Bonferroni correction for multiple testing was applied; *$P < .005$.

post-Kasai may help predict clinical outcomes. Like other studies, ICAM-1 was the earliest post-Kasai predictor of poor jaundice clearance.[16-18] Although ICAM-1 promotes immune cell migration, its role in triggering ductal destruction vs reflecting liver inflammation remains unclear.

Persistent immune activation in BA drives hepatocyte apoptosis and the release of proinflammatory and fibrogenic cytokines, promoting hepatic stellate cell activation and fibrosis.[4] In our study, APRi outperformed liver stiffness as an early predictor of outcomes, likely due to technical limitations in infants. ICAM-1 correlated with fibrosis markers early on, and by 12 weeks post-Kasai, multiple immune pathways (eg, IL-8, IL-6, and TNFα) were linked to fibrosis. Despite attempts to modulate post-Kasai immune responses (eg, steroids,

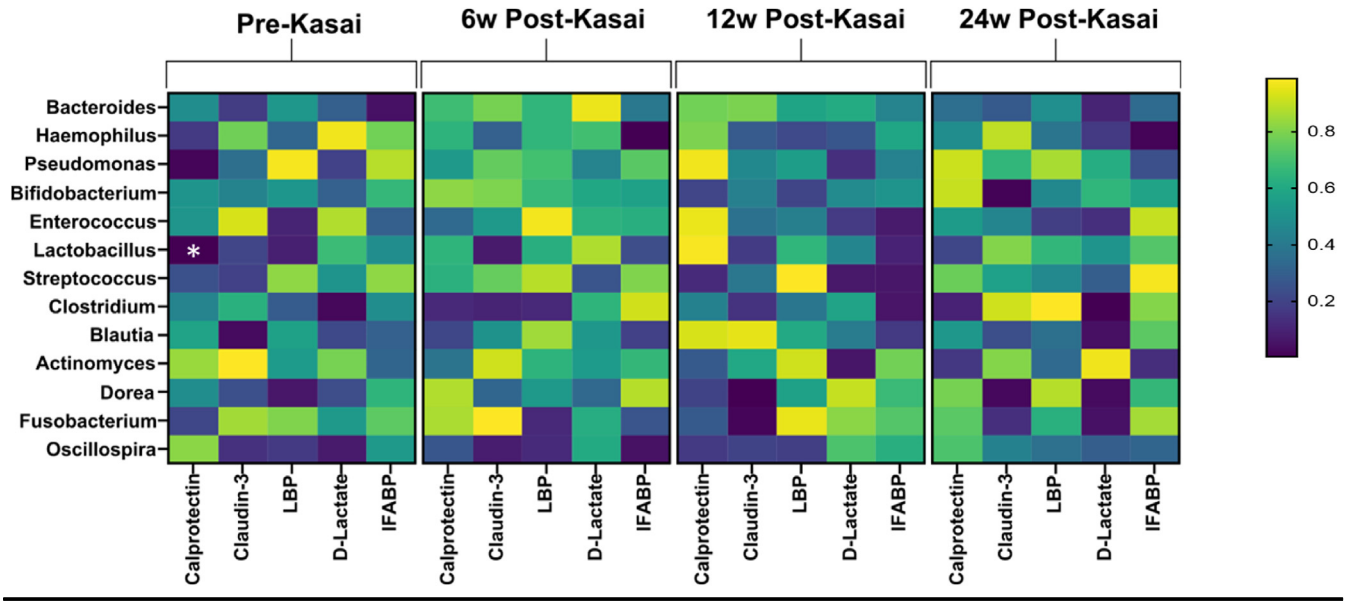

Figure 6. Heatmap to illustrate the correlations between most abundant BA microbiota genera and BT/intestinal barrier biomarkers at pre- and post-Kasai timepoints. A Bonferroni correction for multiple testing was applied; *$P < .003$.

immunoglobulin), these interventions have not clearly improved native liver survival and carry potential risks.[19-21]

Gut microbiota–immune interactions, particularly bacterial translocation, play a key role in disease. lipopolysaccharide (LPS), a gram-negative bacterial Pathogen-Associated Molecular Pattern, and its binding partner LBP, are commonly used biomarkers. Due to its longer half-life, LBP better reflects sustained bacterial exposure. The LPS-LBP-cluster of differentiation 14 complex activates toll-like receptor 4, triggering inflammation via monocytes, macrophages, and endothelium.[22] Although this pathway is implicated in cirrhosis and cholestasis,[23] its role in BA remains unclear.[24,25] In our study, elevated LBP was linked to poor jaundice clearance and correlated with ICAM-1 and early monocyte-driven cytokines (IL-6, TNFα), suggesting LPS-LBP may be relevant in BA pathogenesis. However, as LPS reflects only gram-negative bacteria, it may not fully capture the broader BA microbiota landscape.

D-lactate, a fermentative product of D-lactate-producing gut microbiota, is also considered a bacterial translocation marker, and has been implicated in alcoholic liver disease[26] and necrotizing enterocolitis.[27] D-lactate did not reveal any consistent findings in our study, similar to a previous cross-sectional study.[28]

Compromise of the intestinal barrier can increase permeability, or "leaky gut," facilitating bacterial translocation. Barrier integrity relies on structural, biochemical, and immune components. A key finding in this study was the association between elevated plasma claudin-3 and poor jaundice clearance early post-Kasai, with strong prognostic value. Claudin-3, a tight junction protein in intestinal epithelial and hepatic cells,[29] has been linked to increased permeability in cirrhosis and necrotizing enterocolitis. However, its dual origin limits its specificity as a gut permeability marker in BA, highlighting the need for in vitro validation. Although claudin-3's link to jaundice clearance weakens by 6 months, this may reflect early postsurgical changes influenced by factors like antibiotics or steroids. Its lack of correlation with immune or fibrosis markers suggests more complex, possibly nonlinear, mechanisms.

Fecal calprotectin reflects neutrophil migration into the gut and has been linked to cirrhosis complications.[30] Even though its use in infants is limited by factors like diaper absorption, we observed a correlation between calprotectin and bilirubin by 6 months post-Kasai, suggesting a potential role for intestinal inflammation in advanced cholestasis. IFABP, a protein released upon small intestinal mucosal damage, was previously shown to be elevated in cholestatic infants.[28] In our study, rising IFABP levels were associated with poor jaundice clearance ($P = .04$). In vitro models of intestinal injury and inflammation could help validate these findings.

We found no direct correlation between dominant bacterial genera and markers of bacterial translocation or intestinal barrier dysfunction in BA. Rather than weakening the gut-liver-immune axis hypothesis, this likely reflects the complexity of microbiota involvement or the need for more detailed species-level analysis.

This study has several limitations. First, healthy and non-BA cholestatic infant controls were not included due to the study's focus on immune, bacterial translocation, and intestinal barrier pathways in relation to jaundice clearance and disease severity. In addition, prior studies have already established immune differences among these groups, and controlling for microbiota-confounding factors (eg, diet, antibiotics, choleretics) would introduce complexity and limit

interpretability. Second, only genus-level 16S rRNA microbiota data were available; species- or strain-level insights from whole-genome sequencing would allow deeper analysis. Third, plasma and fecal samples served as proxies for bacterial translocation and intestinal inflammation, and they have inherent limitations, as discussed. Future studies incorporating intestinal and liver tissue analysis (eg, immunohistochemistry or gene expression of markers like claudin-3) would strengthen mechanistic insights. Finally, some data were missing due to the inherent challenges of longitudinal sampling in a vulnerable infant population, with subgroup sizes clearly reported.

In summary, this study highlights the potential roles of bacterial translocation and intestinal barrier disruption in the early post-operative course of BA. Future research can expand on these preliminary findings through tissue analysis and whole-genome sequencing to explore underlying mechanistic pathways, potentially paving the way for much-needed therapeutic interventions. ■

CRediT authorship contribution statement

Vandana Jain: Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Jessica Nulty:** Writing – review & editing, Methodology, Formal analysis. **Emma C. Alexander:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Charlotte Burford:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Mark Davenport:** Writing – review & editing, Methodology. **Shilpa Chokshi:** Writing – review & editing, Formal analysis. **Antonio Riva:** Writing – review & editing, Formal analysis. **Matthew J. Dalby:** Writing – review & editing, Methodology, Formal analysis. **Anita Verma:** Writing – review & editing. **Lindsay J. Hall:** Writing – review & editing, Formal analysis. **Muhammed Yuksel:** Writing – review & editing, Formal analysis. **Anil Dhawan:** Writing – review & editing, Supervision, Conceptualization.

Declaration of Competing Interest

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