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Antibody-mediated rejection in pediatric small bowel transplantation: Capillaritis is a major determinant of C4d positivity in intestinal transplant biopsies

Marion Rabant[1,2,3] | **Maud Racapé**[4] | **Laetitia-Marie Petit**[5] | **Jean Luc Taupin**[6] | **Olivier Aubert**[4] | **Julie Bruneau**[1,3] | **Patrick Barbet**[1,3] | **Olivier Goulet**[3,5] | **Christophe Chardot**[3,7] | **Caroline Suberbielle**[6] | **Florence Lacaille**[5] | **Danielle Canioni**[1,3] | **Jean-Paul Duong Van Huyen**[1,3,4]

[1]Pathology Department, Hôpital Necker, Assistance Publique-Hôpitaux de Paris, Paris, France

[2]INSERM U1151, Hôpital Necker, Paris, France

[3]Paris V Descartes University, Sorbonne-Paris-Cité, Paris, France

[4]Paris Translational Research for Organ Transplant INSERM U 970, PARCC, HEGP, Paris, France

[5]Pediatric Gastroenterology Department, Hôpital Necker, Assistance Publique-Hôpitaux de Paris, Paris, France

[6]Immunology and histocompatibility laboratory, Saint Louis Hospital, Paris, France

[7]Pediatric Surgery Department, Hôpital Necker, Assistance Publique-Hôpitaux de Paris, Paris, France

Correspondence
Marion Rabant
Email: marion.rabant@aphp.fr

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The diagnostic criteria for antibody-mediated rejection (ABMR) after small bowel transplantation (SBT) are not clearly defined, although the presence of donor-specific antibodies (DSAs) has been reported to be deleterious for graft survival. We aimed to determine the incidence and prognostic value of DSAs and C4d in pediatric SBT and to identify the histopathologic features associated with C4d positivity. We studied all intestinal biopsies (IBx) obtained in the first year posttransplantation (N = 345) in a prospective cohort of 23 children. DSAs and their capacity to fix C1q were identified by using Luminex technology. Eighteen patients (78%) had DSAs, and 9 had the capacity to fix C1q. Seventy-eight IBx (22.6%) were C4d positive. The independent determinants of C4d positivity were capillaritis grades 2 and 3 (odds ratio [OR] 4.02, $P = .047$ and OR 5.17, $P = .003$, respectively), mucosal erosion/ulceration (OR 2.8, $P = .019$), lamina propria inflammation grades 1 and 2/3 (OR 1.95, $P = .043$ and OR 3.1, $P = .016$, respectively), and chorion edema (OR 2.16, $P = .028$). Complement-fixing DSAs and repeated C4d-positive IBx were associated with poor outcome ($P = .021$ and $P = .001$, respectively). Our results support that capillaritis should be considered as a feature of ABMR in SBT and identify C1q-fixing DSAs and repeated C4d positivity as potential markers of poor outcome.

KEYWORDS
biopsy, classification systems, clinical research/practice, intestinal (allograft) function/dysfunction, intestine/multivisceral transplantation, pathology/histopathology, rejection: antibody-mediated (ABMR)

Abbreviations: ABMR, antibody-mediated rejection; CDC XM, complement-dependent cytotoxicity crossmatch; cMFI, cumulative mean fluorescence intensity; DSA, donor-specific antibody; HE, hematoxylin-eosin; IBx, intestinal biopsies; iDSA, immunodominant DSA; ISBTS, International Small Bowel Transplant Symposium; MFI, mean fluorescence intensity; MI, microvascular inflammation; NA, not available; OR, odds ratio; RBC, red blood cell; SAFB, Luminex single-antigen flow bead; SBT, small bowel transplantation; TCMR, T cell–mediated rejection.

Marion Rabant, Maud Racapé, Danielle Canioni, and Jean-Paul Duong Van Huyen contributed equally.

1 | INTRODUCTION

Small bowel transplantation (SBT) is indicated in patients with total and irreversible intestinal failure, together with complications of parenteral nutrition. Considerable progress has been made during the past several decades, with the development of new immunosuppressive drugs and improvements in surgical techniques. However, several factors, such as acute rejection, infections, and lymphoproliferative disorders, still impair graft and patient survival, with a 60% 5-year patient survival rate and a 5-year graft survival rate of <50%, and most of the graft losses occurring during the first year.[1,2]

In recent years, the use of the Luminex sensitive techniques to detect donor-specific anti-HLA antibodies (DSAs) has allowed antibody-mediated rejection (ABMR) to be increasingly recognized in solid organ transplantation. ABMR is a major cause of allograft loss in heart and kidney transplantation.[3,4] Recently, ABMR has also been identified as an important issue in SBT, and its implication in the poor graft survival after SBT is increasingly suspected. Several groups have shown that the presence of DSAs was associated with a worse outcome[5-7] and with a higher risk of rejection.[8-10] Abu-Elmagd et al[11] reported the role of DSAs in 194 SBT recipients and showed that preformed DSAs were associated with an increased risk of acute rejection, whereas de novo DSAs were associated with a risk of graft loss and chronic rejection. However, the pathology of ABMR in SBT is not currently defined and is not taken into account in the current classification system of rejection in SBT[12] (Table S1). Vascular alterations, including capillary dilatation and congestion, edema, erythrocyte extravasation, and vasculitis, have been reported to be associated with high panel reactive antibodies.[11,13]

The aims of this study were to determine the incidence and prognostic value of DSAs and C4d in pediatric SBT and to identify the histopathologic features associated with C4d positivity. To address these questions, we studied a cohort of pediatric small bowel recipients with analysis of all intestinal biopsies performed in the first year posttransplantation with systematic C4d staining and evaluation of DSAs, capacity to fix C1q, and outcome. We found a high incidence of DSAs and C4d positivity in our pediatric population and identified capillaritis, mucosal erosion/ulceration, lamina propria inflammation, and chorion edema as independent determinants of C4d positivity. Complement fixing capacity of DSAs and repetition of C4d-positive ($C4d^+$) biopsies were associated with poor outcome.

2 | PATIENTS AND METHODS

2.1 | Population

We included all children who were transplanted in our institution (Necker-Enfants Malades Hospital, Paris, France) between May 2009 and November 2014. Twenty-six patients were transplanted, but only 23 were included (3 patients who died within the first week were excluded).

Surveillance intestinal biopsies (IBx) were routinely obtained through the ileostomy 3 times a week for 1 month, twice a week for 2 weeks, and, finally, weekly for the next 2 months. Indication biopsies were performed in cases of increased stomal output, fever, abdominal pain, or presence of DSAs. A total of 440 IBx performed during the first year post SBT were reviewed in this study.

In our institution, DSA monitoring has been performed since 2009 by using Luminex single-antigen technology according to a local protocol. Overall, 178 sera were tested for DSAs during the first year posttransplantation, representing a mean of 8.1 ± 3 sera screened per patient.

2.2 | Study design

Pathologic analysis was performed between January 2015 and March 2016 by 2 pathologists (MR and DC) who worked together to review all biopsy specimens included in the study. The morphologic features were assessed and scored with full agreement between the pathologists. Because C4d staining on paraffin section has been systematically performed on all IBx since 2013, all adequate IBx sampled before 2013 and included in the study were retrospectively assessed for C4d staining and blindly reviewed between January 2015 and March 2016. DSA study was reviewed by 2 transplant immunologists (CS and JLT). In accordance with our local protocol, no additional serum has been reassessed for DSAs for the study, except for retrospective evaluation of C1q-binding property.

2.3 | Immunosuppression

Immunosuppression relied on the administration of prednisone and tacrolimus. Interleukin-2 receptor antagonist (basiliximab) induction was used in all patients, except in the 4 patients with repeat transplantation, in whom an intravenous dose (5 mg/kg) of rabbit antithymocyte globulin (Thymoglobulin; Genzyme, Lyon, France) for 5 days was used.

The 12-hour tacrolimus trough levels during the first 3 months were targeted at 12 to 15 ng/mL and then tapered to 8 to 10 ng/mL.

Acute allograft rejection episodes were treated with high-dose corticosteroids. In the presence of DSAs, plasma exchanges were performed 5 times, followed by IVIg (2 g/kg).

2.4 | Detection of DSAs

All assays were performed as recommended by the manufacturers, unless otherwise stated, in the Laboratory of Immunology of Saint-Louis Hospital in Paris. Pretransplant sera were screened for class I or class II HLA antibodies of the IgG isotype by Luminex screening assay (LSM-12; One Lambda). For positive samples, class I and/or class II HLA antibodies were identified with Luminex single-antigen flow bead (SAFB) assays (LS1A04 and LS2A01 from One Lambda, respectively). Posttransplantation sera were drawn every week in

the first 2 months and then once per month. Sensitization was also detected by using the Luminex screening assay, and positive sera were analyzed by using SAFB assays for class I and/or class II HLA. The antibody positivity threshold was set at a normalized mean fluorescence intensity (MFI) of 1000 according to the baseline formula of the Fusion software (One Lambda). For each patient, we recorded the presence or absence and number of DSAs, and for each DSA, its strength was defined as the mean MFI of the beads bearing the donor antigen. De novo DSAs were defined as the appearance of a DSA after transplantation.

2.5 | C1q-fixing capacity

The C1q-fixing capacity of posttransplantation DSAs was measured on posttransplantation serum in the first month posttransplantation by using the classic IgG SAFB assay. The assay was performed using the C1q-Screen® kit (One Lambda), according to the manufacturer's recommendations and read on a Luminex 200® fluoroanalyzer (Luminex Corporation, Austin, TX). The MFI positivity threshold was set at 500 after subtracting the signal obtained for the patient's serum on the negative control bead.

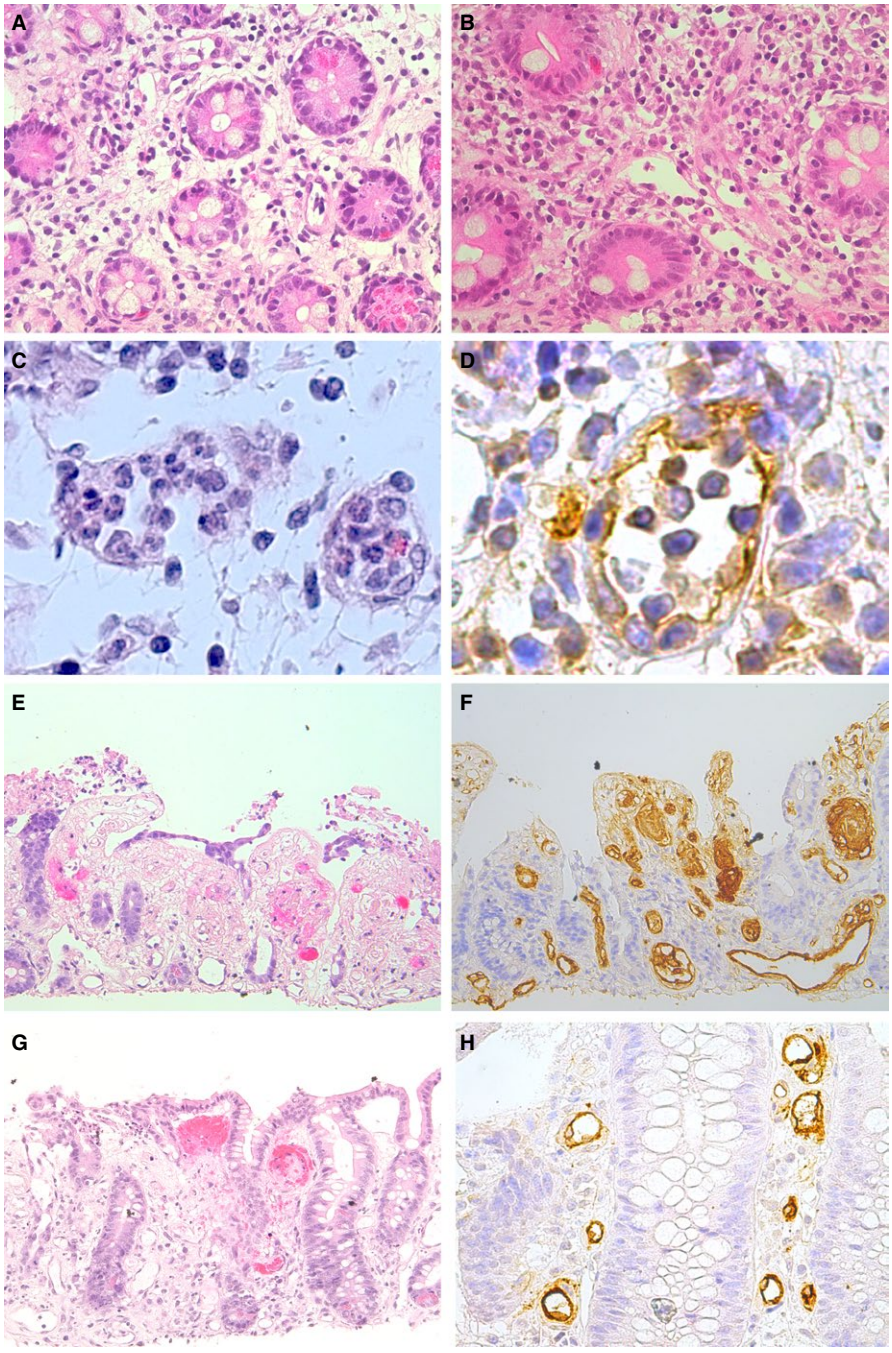

FIGURE 1 Histologic features associated with antibody-mediated rejection of intestinal allograft. A and B. Edema (A, HE X100) and lamina propria inflammation (B, HE X200), with the presence of capillaritis. C. Capillaritis with intravascular polymorphonuclear and mononuclear cells (HE X400). D. Diffuse positive C4d staining in an inflamed capillary (immunoperoxidase ×600, same case as C). E. Mucosal erosion with thrombosis (HE X50). F. Diffuse positive C4d staining (immunoperoxidase X50, same case as E). G. Thrombosis (HE X50). H. Diffuse positive C4d staining on chorion capillaries (C4d3) (immunoperoxidase ×100, same case as G). HE, hematoxylin and eosin stain [Color figure can be viewed at wileyonlinelibrary.com]

2.6 | Pathology

IBx were fixed in formalin and stained with hematoxylin-eosin for routine microscopy.

Only adequate IBx were included in the statistical analysis. Adequacy was defined by the presence of ≥10 crypts. The following histologic features were noted: villous architecture, mucosal erosion and ulceration, inflammation of the lamina propria, edema, apoptosis, focal glandular necrosis or defect, and mitosis. Particular attention was paid to vascular changes: thrombosis, capillary dilatation, and microvascular inflammation (MI) characterized by circulating cells in capillaries (capillaritis) (Figure 1). Capillaritis was characterized by the presence of leukocytes (mononuclear cells and polymorphonuclear cells) in the capillaries of the lamina propria. Capillaritis was graded as 2 separate scores that were then merged into a unique score for analysis purposes. The E score ("Extent') evaluated the percentage of the area with MI in capillaries, with a score of 0 indicating <10% of MI and a score of 1 indicating >10% of MI on the area. The C score ("Cellularity") evaluated the maximum number of cells in the most-affected capillary, with a score of 0 indicating 0 to 2 cells in the capillary, a score of 1 indicating 3 or 4 cells in the capillary and a score of 2 indicating ≥5 cells in a capillary. The composite score was defined as follows: grade 0 for E0C0 and E1C0, grade 1 for E0C1 plus E0C2, grade 2 for E1C1, and grade 3 for E1C2. Inflammation of the lamina propria was graded from 0 to 3, according to the intensity of inflammation (no inflammation, focal, moderate, and severe inflammatory infiltrates). IBx graded with scores of 2 and 3 were pooled for analysis purposes due to a small number of score 3 IBx (n = 5) (Figure 1). A summary of the definitions of the histologic lesions is given in Table S2.

Intestinal T cell–mediated rejection (TCMR) was diagnosed and graded from undetermined to severe based on the scheme proposed at the VIIIth International Small Bowel Transplant Symposium[12] (Table S1).

2.7 | C4d staining

Immunohistochemical C4d staining was performed on all adequate protocol or indication biopsies, on paraffin-embedded tissue sections using monoclonal rabbit anti-human C4d antibody (DB-107, dilution 1:200; DB Biotech, Kosice, Slovakia). C4d staining was scored by the semiquantitative evaluation of mucosal capillary staining, according to the Banff classification for renal allografts, from 0 to 3 (negative, minimal, focal, and diffuse) depending on the percentage of capillaries having a circumferential linear staining pattern: no staining = score 0, <10% = score 1 (minimal), 10% to 50% = score 2 (focal), and >50% = score 3 (diffuse) (Figure 1). A C4d staining score of 0 or 1 was considered negative, and only C4d staining scores of 2 and 3 were considered positive for further analysis.

Positive controls were renal allograft biopsies with documented ABMR or ABO incompatible renal allografts. Negative controls were normal IBx. A control group of patients with inflammatory bowel disease (n = 10) was also tested for C4d staining (data not shown), with a negative C4d for all the biopsies.

2.8 | Follow-up

The median follow-up period post SBT was 3.8 years (IQR 0.75-5.63 years), ranging from 43 days to 6.82 years. IBx were studied during the first year post SBT. Graft and patient survival rates were reported.

2.9 | Statistical analysis

We used the mean and SD values or median and IQR values to describe continuous variables. We compared the means and proportions between groups by using Mann–Whitney or Fisher exact tests. Multiple logistic regression analysis was performed to identify which variables were associated with the presence of C4d staining on the IBx. Delay from SBT to biopsy was log transformed to fit the normality assumption. The variables selected in the multivariable logistic regression model were based on $P < .2$ in the univariable analysis. For all other tests, statistical significance was set at $P \leq .05$. Survival analyses were performed by using the Surv function of the survival package in R, and conditional distributions of histologic variables were plotted by using the cdplot function of the vcd package in R. Graft or patient survival (mixed outcome) according to the percentage of $C4d^+$ IBx group or to the DSA/C1q status was plotted by using Kaplan–Meier curves and compared by using the log-rank test. All tests were 2-sided. Statistical analyses were performed with the use of STATA 14.0 software (Stata Corporation, College Station, TX) and R software version 3.1.2 (R Development Core Team, 2008; R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org).

3 | RESULTS

3.1 | Population

Twenty-three patients (14 boys) received an SBT from an ABO blood type–identical deceased donor, and this was combined with a liver in 9 cases or with multivisceral transplantation in 2 cases. Four patients received a second SBT.

The characteristic demographic data and clinical features are summarized in Table 1. The mean recipient age was 6.6 ± 3.7 years and ranged from 1.1 to 16.2 years. The main cause of intestinal failure was congenital enteropathy (microvillous inclusion disease or tufting enteropathy) in 52.2% of the patients, followed by short bowel syndrome in 7 patients (30.4%) and motility disorder in 4 patients (17.4%).

All patients received red blood cell (RBC) infusions on the day of transplantation (mean 3.4 ± 2.6, range 1-11 infusions) and all but 6 patients received RBC infusions during the first month posttransplant (mean 5.5 ± 4.7). At least 7 patients received RBC and platelet

TABLE 1 Demographic data and clinical features

Variables	Total population N = 23
Recipient	
Male, n (%)	14 (60.9)
Age, year (mean ± SD)	6.6 ± 3.7
First transplantation	19 (82.6)
Cause of intestinal failure, n (%)	
Short bowel	7 (30.4)
Congenital enteropathy	12 (52.2)
Dysmotility	4 (17.4)
Type of transplantation, n (%)	
Small bowel alone	12 (52.2)
Liver plus small bowel	9 (39.1)
Multivisceral	2 (8.7)
Immunologic characteristics, n (%)	
Presence of DSAs (MFI >1000)	18 (78.3)
Preexisting DSAs, n (%)	6 (26.1)
De novo DSAs, n (%)	12 (52.2)
No DSAs, n (%)	5 (21.7)
HLA A/B mismatch, n (%)	3.4 (0.6)
HLA DQ/DR mismatch, n (%)	2.3 (1.1)
HLA A/B/DQ/DR mismatch, n (%)	5.7 (1.5)
Immunosuppression, n (%)	
Standard tacrolimus plus steroids	23 (100)
Basiliximab induction	19 (87.5)
Thymoglobulin induction	4 (12.5)
Biopsies	
Number/patient, mean ± SD	15 ± 5.2
Follow-up, years, median (IQR)	3.8 (0.75-5.63)

infusions before transplantation; information was not available for the other patients.

3.2 | DSAs

The characteristics of DSAs are summarized in Table 2. DSA status was considered positive (DSA^+) when the MFI was >1000. Among the 23 patients, 18 patients had an immunodominant DSA (iDSA) with a MFI >1000 (78%) (DSA^+ patients). Five patients were DSA negative (DSA^-) during the first year post SBT.

The median iDSA MFI was high, at 4923 (IQR 3629-6865). The mean cumulative MFI of DSA (all reactivities) was 11 369 (IQR 6739-19 223). DSA^+ patients had a median of 4 DSA reactivities (range 1-8).

Among the 18 DSA-positive patients, 6 (33.3%) had preexisting DSAs and 12 (66.7%) developed de novo DSAs. Two patients were considered to have de novo DSAs based on a negative assay performed >6 months before transplantation, in the absence of a more recent pretransplantation serum. De novo DSAs were identified very early, after a median of 13 days (IQR 12-16) posttransplantation.

Thirteen patients had both class I and II DSAs, 5 had class II DSAs only, and none had class I DSAs only. The iDSA was class II in 11 patients (61%).

C1q-fixing capacity was determined in a serum drawn after a median time of 16 days (IQR 10-22) after SBT. Among the 18 DSA-positive patients, 9 (50%) had a C1q-fixing DSA. The median MFI of the strongest C1q-fixing DSA (C1q iDSA) was 12 748 (IQR 2186-15 665). C1q-fixing DSAs were mainly class II (n = 5). Details are provided in Table S3.

3.3 | Histologic parameters associated with positive C4d

A total of 440 IBx performed during the first year post SBT were reviewed, and 345 adequate IBx were included in the analysis (mean 15 ± 5 per patient). Ninety percent of the IBx were performed during the first 3 months. The detailed pathology findings are reported in Table 3. A total of 267 IBx were considered negative for C4d staining, and 78 were considered positive (C4d ≥2, $C4d^+$) in 16 patients. Seven recipients had no $C4d^+$ biopsies, and 1 recipient had <15% of positive biopsies. Seven patients had >30% of $C4d^+$ biopsies, whereas 8 patients had between 15% and 30% of positive biopsies.

According to the International Small Bowel Transplant Symposium (ISBTS) classification, 192 IBx displayed no acute rejection, whereas 76 IBx were graded as undetermined for rejection (22.1%), 33 were graded as mild rejection (9.6%), 21 were graded as moderate rejection (6.1%), and 22 were graded as severe rejection (6.4%).

In a comparison of the histology of $C4d^+$ and $C4d^-$ IBx (Table 3), $C4d^+$ IBx were performed significantly earlier (28.2 ± 28.2 vs 51.1 ± 66.6 days, $P = .011$) and were associated with significantly more histologic features of rejection (apoptosis, $P < .0001$; lamina propria inflammation, $P < .0001$; crypt necrosis, $P = .001$; and mucosal erosion/ulceration $P < .0001$). Capillaritis and thrombosis were also found more frequently in $C4d^+$ IBx ($P < .0001$ and $P = .005$, respectively). According to ISBTS classification, rejection episodes in $C4d^+$ IBx were significantly more severe than in $C4d^-$ IBx ($P < .0001$). Thirty-nine percent of biopsies with TCMR (including mild, moderate, and severe TCMR, n = 76) showed C4d positivity (n = 30).

3.4 | Determinants of C4d positivity

Seven histologic parameters were independently associated with positive C4d staining after univariable analysis (mucosal erosion/ulceration, capillaritis, lamina propria inflammation, gland necrosis, thrombosis, apoptosis, and chorion edema; $P < .2$, Table 4). Moreover, associated liver transplantation and the delay from SBT to biopsy were independently associated with a positive C4d in univariable analysis. Finally, after multivariable logistic regression analysis, 4 histologic parameters were independently and significantly associated with the presence of positive C4d staining: capillaritis grades 3 and 2 (odds ratio [OR] 5.17 and 4.02, $P = .003$ and .047, respectively), lamina

TABLE 2 Characteristics of iDSA >1000 MFI posttransplantation

	CDC XM	*HLA A/B* mismatches	*HLA DQ/DR* mismatches	De novo/ preexisting DSAs	Delay transplantation-detection (days)	iDSA class	iDSA	iDSA MFI	Number of reactivities (class)	cMFI (mean)	C1q-binding DSAs (Y/N)
1	T and B negative	4	4	De novo	13	II	DQ7	6241	3 (II)	12091	Y
2	NA	3	2	Negative							
3	T and B negative	3	4	De novo	13	II	DQ6	4963	2 (II)	6541	N
4	NA	3	2	Preexisting		I	B8	4851	6 (I and II)	16601	Y
5	NA	3	2	De novo	12	II	DQ6	2658	6 (I and II)	8824	Y
6	NA	3	2	Negative							
7	T and B negative	4	2	De novo	13	II	DQA05/01	2032	1 (II)	2032	N
8	NA	NA	NA	De novo	20	II	DQ4	1300	3 (I and II)	3286	N
9	T and B negative	3	0	De novo	13	II	DQ7	6805	1 (II)	6805	N
10	T and B negative	4	3	De novo	30	I	B37	2375	6 (I and II)	7586	N
11	T and B negative	3	2	Preexisting		II	DQA04/05/06	5851	4 (I and II)	10646	N
12	T and B negative	4	2	Pre-existing		II	DR53	10543	4 (I and II)	20445	Y
13	T and B negative	2	2	Negative							
14	T and B negative	3	4	De novo	12	I	A25	3953	8 (I and II)	16895	N
15	T and B negative	3	2	De novo	15	I	A2	4882	4 (I and II)	18586	Y
16	NA	4	4	De novo	13	II	DQ2	11266	5 (I and II)	32844	Y
17	NA	4	3	Negative							
18	T and B negative	4	3	Preexisting		II	DQ7	7045	8 (I and II)	20266	Y
19	T and B negative	3	2	Preexisting		I	B49	4678	3 (I and II)	10275	Y
20	T and B negative	3	0	De novo	18	I	A26	6161	5 (I and II)	18875	N
21	NA	4	3	De novo	7	I	B7	9382	7 (I and II)	46612	Y
22	NA	4	2	Preexisting		II	DQ7	3971	3 (II)	6171	N
23	NA	3	1	Negative							

NA, not available; CDC XM, complement-dependent cytotoxicity crossmatch; iDSA, immunodominant donor-specific antibodies; cMFI, cumulative mean fluorescence intensity.

TABLE 3 Histologic and immunologic characteristics according to the C4d status (<2 vs ≥2)

		n	All biopsies (n = 345)	n	C4d negative (<2) (n = 267)	n	C4d positive (≥ 2) (n = 78)	*P*
Time since transplantation (days), mean (SD), y		345	45.9 (60.8)	267	51.1 (66.6)	78	28.2 (28.2)	**.0136**
Mucosal erosion/ulceration, No. of positive biopsies (%)		345	40 (11.6)	267	20 (7.5)	78	20 (25.6)	**<.0001**
Lamina propria inflammation, No. of positive biopsies (%)	Grade 1	338	93 (27.5)	264	67 (25.4)	74	26 (35.1)	**<.0001**
	Grade 2/3		30 (8.9)		16 (6.1)		14 (18.9)	
Chorion edema, No. of positive biopsies (%)		341	63 (18.5)	265	45 (17)	76	18 (23.7)	.184
Apoptosis, No. of positive biopsies (%)		337	64 (19)	264	42 (15.9)	73	22 (30.1)	**.011**
Gland necrosis, No. of positive biopsies (%)		337	55 (16.3)	263	33 (12.5)	74	22 (29.7)	**.001**
Fibrosis, No. of positive biopsies (%)		345	19 (5.5)	267	14 (5.2)	78	5 (6.4)	.778
Thrombosis, No. of positive biopsies (%)		345	25 (7.2)	267	13 (4.9)	78	12 (15.4)	**.005**
Capillary dilatation and congestion hemorrhage, No. of positive biopsies (%)		345	175 (50.7)	267	132 (49.4)	78	43 (55.1)	.440
Capillaritis, No. of positive biopsies (%)	Grade 1	326	166 (50.9)	252	134 (53.2)	74	32 (43.2)	**<.0001**
	Grade 2		22 (6.7)		15 (5.9)		7 (9.5)	
	Grade 3		75 (23)		45 (17.9)		30 (40.5)	
Mitosis, No. of positive biopsies (%)		337	61 (18.1)	264	49 (18.6)	73	12 (16.4)	.734
Acute cellular rejection[a], No. of positive biopsies (%)	No rejection	344	192 (55.8)	266	159 (59.8)	78	33 (42.3)	**<.0001**
	Undetermined		76 (22.1)		61 (22.9)		15 (19.2)	
	Mild rejection		33 (9.6)		26 (9.8)		7 (9)	
	Moderate rejection		21 (6.1)		11 (4.1)		10 (12.8)	
	Severe rejection		22 (6.4)		9 (3.4)		13 (16.7)	

Statistically significant results are in bold.

[a]Acute cellular rejection according to the VIIIth International Small Bowel Transplantation Symposium held in Miami, FL, on September 13, 2003. Capillaritis was graded as 2 separate scores that were merged as a unique grade for analysis purposes. The E score ("Extent') evaluated the percentage of the area with microvascular inflammation in capillaries, with a score 0 representing <10% of MI and a score 1 representing >10% of MI on the area. The C score ("Cellularity") evaluated the maximal number of cells in the most affected capillary, with a score of 0 indicating 0-2 cells in the capillary, a score of 1 indicating 3 or 4 cells in the capillary, and a score of 2 indicating >5 cells in a capillary. The composite score was defined as follows: E0C0 (grade 0), E0C1 + E0C2 (grade 1), E1C1 (grade 2), and E1C2 (grade3).

propria inflammation grades 2/3 and 1 (OR 3.10 and 1.95, *P* = .016 and .043, respectively), mucosal erosion/ulceration (OR 2.80, *P* = .019), and chorion edema (OR 2.16, *P* = .028). Liver transplantation was of borderline significance, with an OR of 0.56 (*P* = .051) (Table 4).

3.5 | Kinetics and association of C4d positivity with the 4 histologic parameters (capillaritis, lamina propria inflammation, mucosal erosion/ulceration, and chorion edema)

We studied the relationship among the 4 histologic features significantly associated with C4d positivity from the multivariable analysis. The conditional density plot in Figure 2 shows how the conditional distribution of the 5 histologic lesions changes during the first 3 months when the majority of IBx were performed. The probability of lesions increased around the same time after SBT for all of the lesions and coincided with the median delay of appearance of DSAs (median 13 days, IQR 12-16) (Figure 2). However, among the 71 $C4d^+$ IBx (for which all other 4 lesions were evaluated), none were positive for the 5 lesions at the same time (1 + 2 + 3 + 4 + 5, Table S4). The most prevalent association of lesions with C4d staining was lamina propria inflammation plus capillaritis (1 + 3 + 5, 19.7% of $C4d^+$ IBx). Additionally, 14.1% of $C4d^+$ IBx displayed capillaritis (1 + 5). Isolated C4d was present in 14.1% of IBx (1, Table S4).

TABLE 4 Factors associated with C4d-positive (C4d ≥2) biopsy in univariate and multivariate analyses

			OR	95% CI	*P*
Univariate analysis					
Histology	Log (delay biopsy-transplantation)	No	1	—	
		Yes	0.476	(0.250-0.902)	**.023**
	Ulceration	No	1	—	
		Yes	4.259	(2.152-8.428)	**<.0001**
	Lamina propria inflammation	No	1	—	
		Grade 1	2.066	(1.154-3.699)	**.015**
		Grade 2	4.658	(2.082-10.423)	**.000**
	Chorion edema	No	1	—	
		Yes	1.517	(0.817-2.816)	**.186**
	Apoptosis	No	1	—	
		Yes	2.280	(1.253-4.150)	**.007**
	Gland necrosis	No	1	—	
		Yes	2.949	(1.590-5.469)	**.001**
	Fibrosis	No	1	—	
		Yes	1.238	(0.431-3.550)	.692
	Thrombosis	No	1	—	
		Yes	3.552	(1.549-8.147)	**.003**
	Capillary dilatation and congestion hemorrhage	No	1	—	
		Yes	1.256	(0.757-2.085)	.377
	Capillaritis (composite)	No	1	—	
		Grade 1	2.770	(1.028-7.464)	**.044**
		Grade 2	5.413	(1.504-19.477)	**.010**
		Grade 3	7.733	(2.778-21.525)	**.000**
	Mitosis	No	1	—	
		Yes	0.863	(0.432-1.725)	.677
Clinical	No. of transplantations	No	1	—	
		Yes	1.48	(0.799-2.742)	.213
	Liver transplantation	No	1	—	
		Yes	0.599	(0.360-0.997)	**.049**
Multivariate analysis					
Histology	Lamina propria inflammation	No	1	—	
		Grade 1	1.954	(1.021-3.741)	**.043**
		Grade 2	3.095	(1.239-7.736)	**.016**
	Ulceration	No	1	—	
		Yes	2.796	(1.185-6.597)	**.019**
	Chorion edema	No	1	—	
		Yes	2.160	(1.089-4.283)	**.028**
	Capillaritis	No	1	—	
		Grade 1	2.004	(0.717-5.602)	.185
		Grade 2	4.016	(1.019-15.829)	**.047**
		Grade 3	5.169	(1.781-15.002)	**.003**
Clinical	Liver transplantation	No	1	—	
		Yes	0.556	(0.308-1.003)	**.051**

In the univariate analysis, variables in bold were included in the multivariate analysis.

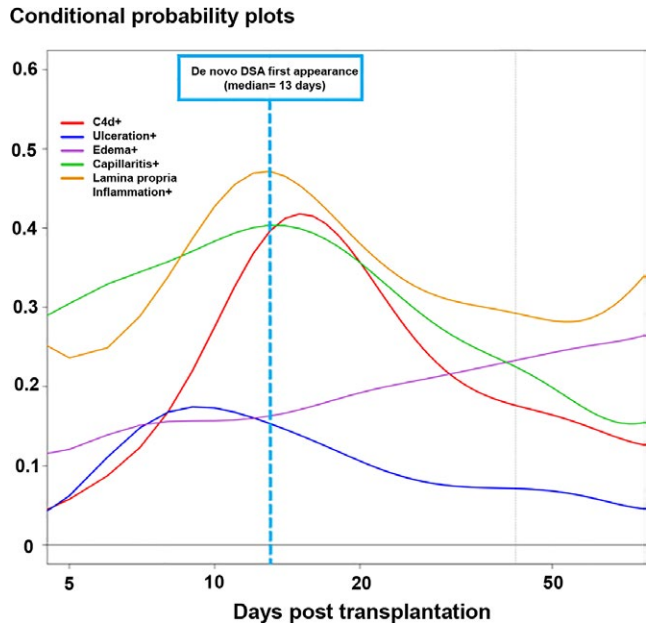

FIGURE 2 Conditional probability plot of histologic lesions. The conditional probability plot shows the conditional distribution of C4d staining and the 4 histologic features associated with C4d staining in the multivariable analysis (C4d, mucosal erosion/ulceration, capillaritis, chorion edema, and lamina propria inflammation) during the first 3 months. The probability of detecting lesions on the IBx increased around the same time after SBT for all the lesions and coincided with the median delay of appearance of DSA

3.6 | Relationship between C4d status, DSAs, and C1q-fixing capacity of DSA and outcome

The mean incidence of positive C4d biopsies in the 18 DSA^+ patients was 27.1%, whereas it was 14.7% in the 5 DSA^- patients. Two of those DSA^- patients had no $C4d^+$ biopsies. The 3 other patients had 35.3%, 21.4%, and 16.7%, respectively, of $C4d^+$ biopsies with no other histologic features of rejection.

During follow-up, 5 patients lost their graft and 4 patients died. We compared the 2-year graft/patient survival rates among patients with <15% of $C4d^+$ IBx (n = 8), 15% to 30% of $C4d^+$ IBx (n = 8), or >30% of $C4d^+$ IBx (n = 7).

Patients with <15% of $C4d^+$ IBx had a better survival (87.5% at 2 years) compared with patients with 15% to 30% of $C4d^+$ IBx (75% at 2 years) and patients with >30% of $C4d^+$ IBx (14.3% at 2 years, log-rank test, $P = .001$) (Figure 3A). Patients with >30% of $C4d^+$ IBx (n = 7) displayed a significantly higher percentage of IBx with mucosal erosion/ulceration, capillaritis, and lamina propria inflammation (Figure S1).

Patients who had no DSAs and no C1q-fixing DSAs (DSA^-/$C1q^-$, n = 5) had a better graft survival (80% at 2 years) than did those who had DSAs with no C1q-fixing DSA (DSA^+/$C1q^-$, n = 9, 77.8% 2-year graft survival) and those with DSAs and concomitant C1q-fixing DSAs (DSA^+/$C1q^+$, n = 9, 33.3% 2-year graft survival) (Figure 3B).

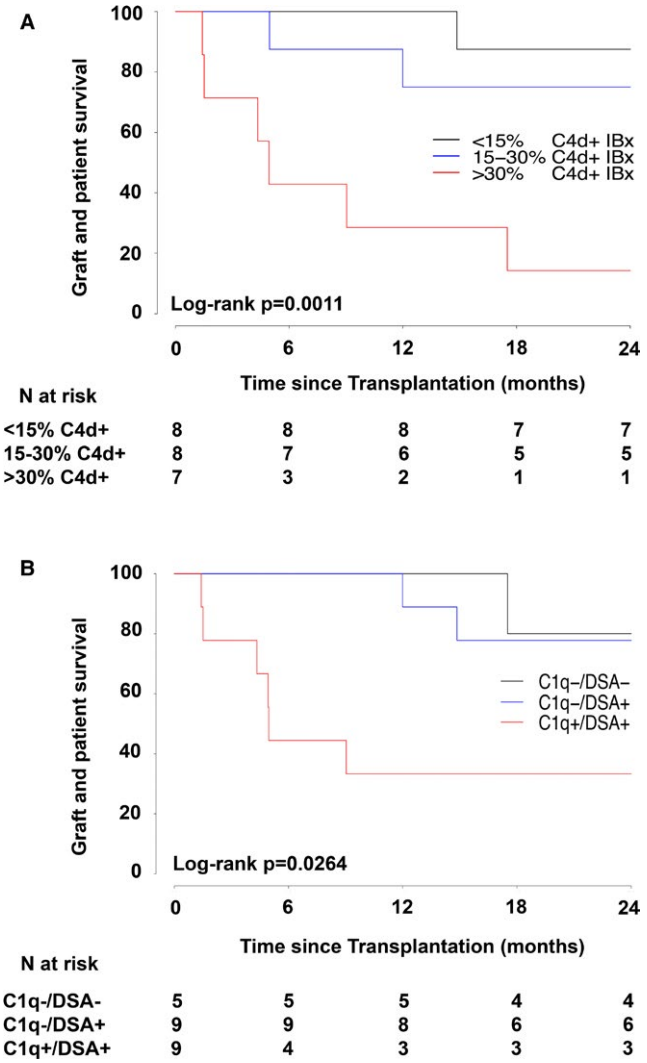

FIGURE 3 Graft and patient survival according to the percentage of C4d positive biopsy per patient and to C1q status. A. Patients were divided in 3 groups according to the percentage of $C4d^+$ IBx during the first year post SBT (<15%, 15-30%, >30%). Estimates were obtained using the Kaplan-Meier method and compared using the log-rank test. B. Graft and patient survival analysis according to the presence of DSA and to the capacity of DSA to fix C1q. Estimates were obtained using the Kaplan-Meier method and compared using the log-rank test

4 | DISCUSSION

Relying on a large number of IBx from a series of pediatric recipients, this study recognizes the pattern of histologic allograft injury associated with C4d positivity in SBT. Capillaritis, mucosal erosion/ulceration, lamina propria inflammation, and chorion edema were significantly and independently associated with complement deposition and thus may represent histologic features associated with intestinal ABMR. Additionally, C4d positivity and C1q-fixing DSAs were associated with worse graft and patient survival rates at 2 years post SBT.

Our main result is the identification of capillaritis as a major determinant of C4d positivity. Indeed, microcirculation inflammation is

also an important criterion of ABMR after kidney, heart, pancreas, and liver transplantation,[14,15] and our results suggest that this is also true in SBT. Here, capillaritis grades 2 and 3 were significantly and independently associated with a positive C4d. Interestingly, capillaritis grades 2 and 3 correspond to the current threshold for the diagnosis of peritubular capillaritis in kidney transplantation. This threshold of >10% of capillaries with >2 cells in the most affected capillary seems to be valid for the definition of microcirculation inflammation in SBT. Edema, which reflects increased permeability in an injured microvascular bed, was also an independent determinant of C4d positivity. Microthrombosis was more frequent in $C4d^+$ IBx and significantly associated with C4d positivity, although it was not independently associated in the multivariable analysis. However, thrombosis and erosions/ulcerations were strongly correlated, suggesting ischemic injury as one of the potential mechanisms of mucosal erosions/ulcerations in SBT. Interestingly, Ruiz et al also reported microvascular lesions such as vascular dilatation, erythrocyte congestion, extravasated erythrocytes, and edema in patients with high panel reactive antibodies.[13]

Systematic evaluation of all IBx allowed us to describe associations and kinetics of the lesions. As illustrated by conditional probability analysis, pathologic lesions appeared simultaneously, together with C4d positivity and DSAs, within the first 2 weeks posttransplantation. Such precocity of the onset of ABMR is mainly seen in kidney and heart transplantation in cases of preformed DSAs in the absence of preventive ABMR therapeutics. However, we found various combinations of C4d positivity, edema, capillaritis, mucosal erosion/ulceration, and lamina propria inflammation, with almost half of $C4d^+$ IBx displaying only 1 lesion. Edema, capillaritis, mucosal erosion/ulceration, and lamina propria inflammation in the absence of C4d staining were found in 27 (7.8%), 28 (8.1%), 5 (1.5%), and 42 (12.2%) IBx, respectively, which suggests that as in other solid organ transplantation, ABMR is a fluctuant process that often presents with incomplete features. This observation underscores the need for repeated protocol biopsies to detect ABMR.

Features of TCMR were often found in C4d+ biopsies. Few studies have indeed reported the association of DSAs with more frequent and more severe TCMR.[6] Here, 39% of biopsies with TCMR showed also C4d positivity, suggesting that a substantial proportion of IBx with rejection displayed features of mixed rejection. These results raise the questions of the prevalence of mixed rejection, associating features of TCMR and ABMR, which seems frequent in SBT, and of the specificity of histologic features of TCMR. The mechanisms underlying TCMR and ABMR are distinct but are not mutually exclusive. Reciprocal cognate interactions between T and B cells play indeed a key role in the generation of alloimmune responses, most likely after SBT, as the intestine contains a tremendous amount of lymphoid tissue.[16,17] This may account for the high prevalence of DSAs in SBT recipients and the coexistence of ABMR and TCMR. Molecular studies with the determination of the transcriptomic signatures of ABMR and TCMR and exhaustive DSA testing may help better clarify the pathogenesis of intestinal rejection.

De novo DSAs and persistent preexisting DSAs have been associated with a significantly worse survival compared with patients with no DSAs or patients with preformed DSA dropping to undetectable levels after SBT.[11] In our pediatric cohort, DSAs were found in a high percentage of patients (87.0%) either preformed (35%) or early de novo (65%), with a class II iDSA in 65% of DSA+ patients, which is higher than previously described.[18,19] The high incidence of DSAs in our cohort may be in part explained by the induction therapy consisting only of basiliximab in most patients compared with rabbit antithymocyte globulin or alemtuzumab in the other studies[20] and by the high rate of RBC transfusion. Interestingly, in our study, the C1q-fixing capacity of the DSAs was associated with a poorer 2-year graft survival compared with DSAs with no C1q-fixing capacity, as previously reported in kidney transplantation.[21] Finally, we found that the repetition of C4d positivity, as assessed by the percentage of $C4d^+$ IBx, was associated with a poor prognosis. As expected, the incidence of edema, capillaritis, erosion/ulceration, and lamina propria inflammation increased with the percentage of $C4d^+$ IBx, but the size of our series did not allow us to build reliable models to investigate an independent role of histopathology on graft survival.

In summary, we identified for the first time capillaritis, lamina propria inflammation, mucosal erosion/ulceration, and chorion edema as histologic features associated with in situ C4d complement deposition in SBT. These histologic features as potential criteria of ABMR in SBT need to be confirmed with further studies. In particular, capillaritis scoring, including the extent (<10% or >10%) and the number of cells in the most affected capillary, needs to be prospectively evaluated. Our study highlights several issues that will need to be considered for future working formulation for the definition of ABMR in intestinal transplantation: (1) the high incidence of DSA, at least in pediatric population and the importance of C1q testing, (2) the importance of lesions of the microcirculation and vascular disorders, (3) the fluctuation of C4d status, and (4) the potential high incidence of mixed rejection.

Beyond the traditional acute TCMR, the recognition of the humoral arm as a major component of acute intestinal allograft outcome may lead to a better identification of potential targets for investigation and intervention, which may improve the SBT outcomes. Molecular studies with the determination of the transcriptomic signatures of ABMR and TCMR and exhaustive DSA testing may help better clarify the pathogenesis of intestinal rejection.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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