ORIGINAL ARTICLE

Donor-specific antibodies after pediatric liver transplantation: a cross-sectional study of 50 patients

Jesper M. Kivelä[1], Silja Kosola[2], Juha Peräsaari[3], Heikki Mäkisalo[4], Hannu Jalanko[1], Christer Holmberg[1], Mikko P. Pakarinen[2] & Jouni Lauronen[3]

1 Pediatric Nephrology and Transplantation, Children's Hospital, Helsinki University Central Hospital, University of Helsinki, Helsinki, Finland
2 Pediatric Surgery, Children's Hospital, Helsinki University Central Hospital, University of Helsinki, Helsinki, Finland
3 Finnish Red Cross Blood Service, Helsinki, Finland
4 Transplantation and Liver Surgery Clinic, Department of Surgery, Helsinki University Central Hospital, University of Helsinki, Helsinki, Finland

Correspondence
Jesper M Kivelä, Biomedicum 2C, Room 605, PL 705 00290 HUS, Helsinki, Finland.
Tel.: +358 9 471 757 26;
fax: + 358 9 471 757 37;
e-mail: jesper.m.kivela@helsinki.fi

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SUMMARY

The role of donor-specific HLA antibodies (DSAs) after pediatric liver transplantation (LT) is inadequately established. We conducted a cross-sectional study on the prevalence of DSAs and their association with liver histology and biochemical variables after pediatric LT. Serum samples were drawn for HLA antibody analyses from 50 patients (76% of 66 eligible patients) operated on at age <18 years between 1987 and 2007 with a median of 10.0 (interquartile range 4.0–16.4) years after deceased donor LT. Mixed and single-antigen beads with Luminex were used for HLA antibody screening and detection. A mean fluorescence intensity (MFI) value of 1000 was used for positive cutoff. Twenty-six patients (52%; 95% confidence interval (CI) 39% to 65%) had DSAs. In 22 (85%) patients, DSAs were against class II HLA antigens with a mean (standard deviation) MFI of 13 481 (4727). The unadjusted prevalence ratio for portal inflammation in DSA-positive compared to DSA-negative patients (n = 47; 9/24 vs. 1/23) was 8.6 (95% CI 1.6 to 50.9). Laboratory values at the time of study were comparable between DSA-positive and DSA-negative patients. In conclusion, approximately half of patients studied had DSAs after pediatric LT. Portal inflammation was associated with DSA positivity although the wide confidence interval around the ratio estimate warrants cautious interpretation.

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Key words
HLA antibodies, liver histology, liver transplantation, pediatric, portal inflammation, prevalence

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Introduction

Donor-specific HLA antibodies (DSAs) increase the risk for antibody-mediated rejection and graft failure in both adult and pediatric kidney transplant patients [1,2]. Their role, however, in a liver transplantation (LT) setting is in a continuous flux. A recent meeting report concluded that DSAs have been associated with a range of injury in LT patients [3].

The proportion of patients with DSAs after LT have varied between 8 and 67% depending on the study setting, and the mean fluorescence intensity (MFI) cutoff employed [4–12]. A majority of these DSA-positive patients showed antibodies against class II HLA antigens [4–10].

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A pediatric study after living-related donor LT showed high MFI (over 17 000) for class II DSAs and DSA positivity were associated with liver fibrosis, inflammation and C4d staining in protocol liver biopsies [6]. In another study of 50 pediatric LT patients, DSA-positive patients were younger at the time of LT than DSA-negative patients [9]. In addition, DSAs against HLA-DQ antigens were associated with late graft dysfunction, including *de novo* autoimmune hepatitis [9]. In a third pediatric study, all chronic rejection patients with DSAs had *de novo* DSAs and there was no difference in fibrosis grade between DSA-positive and DSA-negative patients with chronic rejection [10].

Our purpose was to investigate the prevalence of DSAs and their association with liver histology and biochemical variables based on serum samples taken in a cross-sectional manner without clinical indication after pediatric LT. Additional factors associated with DSAs were also analyzed.

The guidelines for reporting observational studies recommended by STROBE Statement [13] were followed.

Methods

Patients and study design

Our study is part of a research project launched in 2008 in which we have cross-sectionally studied several outcomes and their associations with clinical characteristics in pediatric LT patients. Serum samples for HLA antibody analyses were also a part of the original research plan.

All pediatric patients (under 18 years of age) who received deceased donor liver grafts in 1987–2007 at the Children's Hospital or Transplantation and Liver Surgery Clinic (both part of Helsinki University Central Hospital) were evaluated for study eligibility (Fig. 1). A serum sample was taken from 50 patients (76%) and analyzed for HLA antibodies. Sixteen patients (24%) did not participate in the study due to refusal or logistic reasons. These 16 nonparticipants were older than study participants [median (interquartile range) age at LT 11.2 (2.1–16.1); 95% confidence interval (CI) for difference in medians 1.9 to 15.4; $P = 0.012$], and were more often transplanted with whole livers than reduced liver grafts (10/16 vs. 15/50; Fisher's exact test $P = 0.036$).

The serum sample for HLA antibody analysis was taken once from each study patient, in a cross-sectional manner, as a part of the study protocol during 2009–2011. HLA antibody findings (focus on DSAs) were analyzed in relation to the study patients' cross-sectional biochemical variables and liver histology (taken as a part of our routine clinical follow-up protocol). In addition, we analyzed different baseline factors (e.g., gender and graft type) and their association with DSA positivity.

Long-term results after pediatric LT of our core study population ($n = 66$) have been published previously [14–17]. Based on our previous study [17] considering late hepatic artery thrombosis diagnosed with MRI, 32 patients from the MRI study were also involved in the HLA study at hand. We utilized collected data to test for a difference in number of late hepatic artery thromboses between DSA-positive and DSA-negative patients.

The characteristics of 50 study patients are shown in Table 1. Of 50 patients, eight (16%) received combined liver–kidney transplantation (CLKT). One patient (2%) received ABO incompatible LT. In addition, three of 50 patients underwent re-LT 4, 19 and 20 years before this HLA antibody analysis. Indications for re-LT were primary nonfunction, hepatic artery thrombosis and chronic rejection. The patients' medical records and the national LT registry were utilized to retrieve all clinical data. Additionally, the Finnish Red Cross Blood Service's clinical laboratory database was utilized.

This study was approved by the Ethics Committee for Pediatrics, Adolescent Medicine, and Psychiatry of the Hospital District of Helsinki and Uusimaa (application number 345/13/03/03/2008). Informed consent was obtained from patients and for minors also from parents or guardians.

Immunosuppression

The immunosuppression protocol was based on triple medication with cyclosporine, azathioprine and methylprednisolone. If clinically indicated, cyclosporine was replaced with tacrolimus, and azathioprine with mycophenolic acid. Baseline immunosuppression was similar for CLKT and LT patients.

Basiliximab was initiated as an induction therapy in 1999. Basiliximab was given during LT and on the postoperative day depending on the patient's body weight (either 10 mg < 35 kg or 20 mg > 35 kg). Cyclosporine and tacrolimus trough target levels were 80 to 100 μg/l and 4 to 6 μg/l after one year, respectively. Methylprednisolone was switched to every other day usage at 6 months with aim to discontinuation when the patient reached adulthood. At our center, the mean methylprednisolone dose was 0.19 mg/kg/day

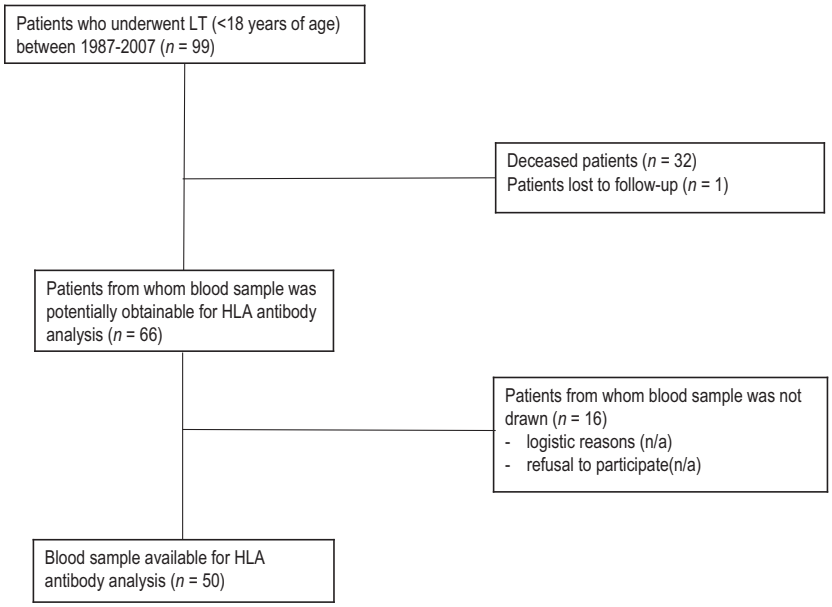

Figure 1 Flow diagram of patient selection process.

at 6 months and 0.07 mg/kg/day at median follow-up of 7 years [18]. Acute rejection episodes were mainly treated with a 5-day increased dose of methylprednisolone.

At the time of serum sample collection for HLA antibody analysis, 35 patients (70%) were treated with cyclosporine and 13 patients (26%) with tacrolimus. Two patients were neither on cyclosporine nor on tacrolimus (one on monotherapy with sirolimus and the other on double immunosuppression with azathioprine and methylprednisolone). Thirty-three patients (66%) still used methylprednisolone when the blood sample for HLA antibody analysis was drawn. Of these 33 patients, 8 patients were aged $\geq$ 18 years at the time of HLA antibody sample.

HLA antibodies

One Lambda Labscreen® mixed and single-antigen beads with Luminex® were used for HLA antibody screening and identification with the use of HLA Fusion 2.0 software (One Lambda Inc., Canoga Park, CA). A normalized MFI cutoff point of 1000 was used for positivity in single-antigen analyses. MFI values were combined if a patient had DSAs against more than one HLA antigen (n = 3). Antibodies against HLA-A, -B, -C, -DR and -DQ were analyzed.

All HLA antibody analyses were conducted at the Finnish Red Cross Blood Service by one of the authors (JL) who was unaware of patient information at the time of the analyses.

Pre-LT HLA antibody analysis was not a part of the routine protocol prior to LT in all patients, and these analyses were available for 20 patients (40%). Therefore, we were unable to analyze in most patients whether the detected antibodies were preformed or *de novo*. T-cell cross-match was analyzed at the time of LT with the complement-dependent cytotoxicity (CDC) technique, and cross-match data were available from 48 patients.

Blood group antibodies

Blood group antibody screening was performed prior to any operation or blood transfusion with commercial or Finnish Red Cross Blood Service (FRCBS) in-house screening cells.

Commercial or FRCBS in-house panel cells were used for antibody identification in cases with a positive screening test. Only data from FRCBS and the transplant center were retrospectively reviewed. Thus, some patients may have had blood group antibodies detected at other hospitals.

Liver histology

Some of the liver biopsy methods and findings from our study population have been reported previously [16]. Liver biopsies were taken in a cross-sectional manner during routine follow-up visits, and biopsy specimens were fixed with formalin, embedded in paraffin and stained using routine histochemical stainings. In

Table 1. Patient characteristics of 50 study patients and their division into two groups based on DSA findings.

	All patients (n = 50)	DSA negative (n = 24)	DSA positive (n = 26)	P-value
Gender, n (%)				
Male	26 (52)	10 (42)	16 (62)	0.257
Female	24 (48)	14 (58)	10 (38)	
Median age at the time of LT, years	2.6 (1.3–12.0)	6.0 (1.4–12.7)*	2.0 (1.1–9.9)*	0.164
Median age at the time of DSA, years	17.3 (10.1–21.0)	16.6 (7.8–20.4)†	18.1 (10.1–21.3)†	0.641
Median follow-up time at DSA, years	10.0 (4.0–16.4)‡	5.6 (3.1–15.8)§	11.3 (4.0–17.6)§	0.140
Diagnosis, n (%)				
Biliary atresia	17 (34)	6 (25)	11 (42)	0.263
Metabolic disease¶	10 (20)	4 (17)	6 (23)	
Malignancy	6 (12)	2 (8)	4 (15)	
Hepatitis	6 (12)	4 (17)	2 (8)	
Other**	11 (22)	8 (33)	3 (12)	
Transplantation type, n (%)				
LT††	42 (84)	17 (71)	25 (96)	0.021
CLKT	8 (16)	7 (29)	1 (4)	
LT era, n (%)‡‡				
Before year 2001	25 (50)	9 (38)	16 (62)	0.156
After year 2001	25 (50)	15 (63)	10 (38)	
Graft type, n (%)				
Reduced liver	35 (70)	16 (67)	19 (73)	0.760
Whole liver	15 (30)	8 (33)	7 (27)	
Arterial anastomosis to, n (%)§§				
Aorta	21 (42)	8 (33)	13 (50)	0.259
Hepatic artery	27 (54)	15 (63)	12 (46)	
Not available	2 (4)	1 (4)	1 (4)	
Biliary reconstruction, n (%)				
Roux-en-Y	37 (74)	16 (67)	21 (81)	0.339
Duct-to-duct	13 (26)	8 (33)	5 (19)	
CNI immunosuppression at DSA, n (%)¶¶				
Cyclosporine	35 (70)	14 (58)	21 (81)	0.106
Tacrolimus	13 (26)	9 (38)	4 (15)	
No CNI	2 (4)	1 (4)	1 (4)	
Acute cellular rejection, n (%)				
Rejection	31 (62)	16 (67)	15 (58)	0.570
No rejection	19 (38)	8 (33)	11 (42)	
Late hepatic artery thrombosis, n (%)***				
Thrombosis	15 (47)	7 (47)	8 (47)	1.0
No thrombosis	17 (53)	8 (53)	9 (53)	

ARPKD, autosomal recessive polycystic kidney disease; CLKT, combined liver kidney transplantation; DSA, donor-specific antibody; HUS, hemolytic uremic syndrome; LT, liver transplantation.

For continuous variables, values in parentheses are standard deviation (SD) for mean and interquartile range (IQR; 25th and 75th percentile) for median. Non-normally distributed variables are as medians expect age at the time of DSA (see footnote b). P-values are derived from comparisons between DSA groups. Cutoff for positive DSA MFI >1000.

*Difference (95% CI) in medians 4.1 (−1.6 to 9.7) years.

†Difference (95% CI) in medians – 1.5 (−7.6 to 4.7) years. Age at the time of DSA was normally distributed variable as per Shapiro–Wilk test. Mean (SD) age at the time of DSA 15.8 (7.9) for DSA-negative and 16.8 (7.7) for DSA-positive groups (P = 0.652; Student's t-test). Mean (SD) age for whole group 16.4 (7.7) years.

‡Median follow-up time for LT patients (n = 42) 11.2 years and CLKT patients (n = 8) 4.6 years.

§Difference (95% CI) in medians – 5.8 (−13.5 to 1.9) years.

¶Tyrosinemia 4, Wilson disease 1, hyperoxaluria 2, familial hypercholesterolemia 1, ornithine transcarbamylase deficiency 1, mitochondrial encephalopathy lactic acidosis with stroke-like episodes 1.

**ARPKD 4, Budd–Chiari syndrome 2, atypical HUS 2, familial congenital liver cirrhosis 1, sclerosing cholangitis 1, drug-induced liver failure 1.

††Re-LT (n = 3; 1 DSA negative and 2 DSA positive).

‡‡Categorization based on median LT year 2001.

§§Two anastomoses not retrievable from medical records; missing anastomoses not included in analysis.

¶¶CNI immunosuppression at the time of DSA; not baseline immunosuppression, patients without CNI (n = 2) not included in analysis.

***n = 32 patients based on our previous study (ref. 17).

addition, specimens were immunostained for complement component 4d (C4d) deposits with the use of polyclonal rabbit anti-human antibody (dilution 1 to 50) (Cat.no BI-RC4D, Biomedica, Vienna, Austria), and cytokeratin 7 (CK7) with the use of SP52 monoclonal antibody and ultraView Universal DAB Detection Kit (Ventana, Tucson, Arizona, USA).

Biopsy specimens were re-evaluated and scored for the purpose of our cross-sectional research project (see Patients and study design) using a semiquantitative scoring system by two experienced liver pathologists blinded to clinical data to reach consensus. The scoring framework was as follows: portal inflammation 0–3, fibrosis 0–4, cytokeratin 7 (CK7) for periportal hepatocytes 0–3 and bile duct proliferation 0–2. C4d deposits were coded as present (> 10% of microvascular endothelium) or absent.

Data for portal inflammation and fibrosis analyses were available for 47 patients (94%), and CK7 for periportal hepatocytes and bile duct proliferation analyses for 46 patients (92%). C4d immunostaining was available for 44 patients (88%).

Nine patients' liver biopsies were taken at a different time from blood samples for HLA antibody analysis with a median (interquartile range) 294 days (71–353) between these two time points.

Statistics

Statistical analyses were performed with Stata 12.1 (StataCorp LP, College Station, TX). JMP Pro 10.0.2 (SAS Institute, Gary, NC) was used for exact Cochran–Armitage trend test. On the basis of DSA presence (MFI >1000) in serum samples, groups with DSAs (n = 26) and without DSAs (n = 24) were formed. Shapiro–Wilk test was used to check normality with alpha-level at 0.05. Continuous variables are presented as medians (non-normally distributed) and interquartile ranges (IQR) or as means (normally distributed) and standard deviations (SD) unless otherwise indicated. Laboratory values of under (n = 5) or over (n = 1) the detection level were set to lowest (e.g., bile acids < 2 were set to 2) or highest reported level, respectively. Missing values were treated as missing in analyses.

The Bonett–Price method [19] with Stata module *bpmedian* [20] was used for comparisons between medians with 95% confidence interval (CI) for difference, and unpaired Student's t-test was used for comparisons between means with 95% CI for difference. Ninety-five percent confidence interval for single binomial proportion (e.g., proportion of DSA-positive patients) was calculated with the method by Wilson. Fisher's exact test was used for comparisons between categorical variables. Additionally, odds ratios (ORs) and prevalence ratios (PRs) for various dichotomous variables and DSA grouping were calculated. Ninety-five percent confidence interval for OR and PR were calculated with Baptista–Pike mid-P-value method and Koopman asymptomatic score method, respectively, for small sample size as recommended by Fagerland *et al.* [21]. User-written Stata modules for both of these aforementioned methods were used [22,23]. Ordered histological factors (portal inflammation, fibrosis, CK7 for periportal hepatocytes and bile duct proliferation) and DSA grouping were analyzed with an exact version of Cochran–Armitage trend test due to small sample size. Spearman rank correlation with 95% CI was reported with the use of Stata user-written module [24]. All P-values are two-tailed.

Results

HLA antibodies

Of 50 studied patients, 33 (66%, 95% CI 52% to 78%) had HLA antibodies and 26 (52%, 95% CI 39% to 65%) had either class I or class II DSAs (Table 2). Most DSA-positive patients had antibodies against class II HLA antigens (n = 22; 85%), and most often against one HLA locus (n = 19; n = 16 HLA-DQ and n = 3 HLA-DR). In the remaining 3 of 22 patients, antibodies were against two class II HLA antigens: HLA-DQ and HLA-DQ (n = 2), and HLA-DQ and HLA-DR (n = 1). None of the patients had both class I and II DSAs. Mean (SD) MFIs for class I and II DSAs were 4236 (3110) and 13 481 (4727), respectively. Median MFI (IQR) for class II DSAs was 14 647 (9227–17 092).

Of 20 patients for whom pre-LT HLA antibody analyses were available, 11 (55%) had DSAs at the time of this study. DSAs were considered *de novo* in ten of these 11 (91%) DSA-positive patients based on their negative antibody finding in pre-LT test. Therefore, prevalence of *de novo* DSAs was 50% (10/20) in patients with a pre-LT sample available, but this estimate is based on a subsample (n = 20; 40%) of all patients.

The HLA-A, HLA-B and HLA-DR mismatch classification (combined up to 6 mismatches; 4/2) is shown in Fig. 2. Most of the patients (66%) had four or more HLA mismatches, and none of the patients had a zero mismatch. Two HLA-DR mismatches were present in 9 DSA-positive and 13 DSA-negative patients.

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In DSA-positive patients, MFI for class II DSAs showed no association with the combined mismatch classification [Spearman rho – 0.09 (95% CI – 0.49 to 0.34); $P = 0.686$, $n = 22$]. In 45 patients, LT was performed after a negative T-cell cross-match and in three patients after a positive cross-match ($n = 48$; 2 not available). All of these three cross-match positive patients were DSA negative at the time of study.

Baseline and other factors, and DSAs

Baseline factors of DSA-positive and DSA-negative patients are presented in Table 1. DSA-positive patients tended to be younger at the time of LT than DSA-negative patients, and their follow-up period from LT to blood sample drawn tended to be longer. There was no association between age at LT and follow-up period [Spearman rho – 0.20 (95% CI – 0.46 to 0.08); $P = 0.159$, $n = 50$].

Transplantation type (LT vs. CLKT) was associated with DSA positivity (25/42 vs. 1/8; Fisher's exact test $P = 0.021$). As shown in Table 3, the prevalence of DSA-positive patients was almost fivefold higher within the LT compared to the CLKT group although the 95% CI was wide. None of the other factors shown in Table 3 were markedly associated with DSAs.

The frequency of acute cellular rejection (biopsy proven, fine-needle aspiration or clinically suspected and treated) episodes between DSA-positive and DSA-negative patients was similar (Fisher's exact test $P = 0.570$). This was also true for methylprednisolone usage at the time of study (17/26 vs. 16/24, Fisher's exact test $P = 1.0$). Frequency of late hepatic artery thrombosis was comparable between DSA-positive and DSA-negative patients (Fisher's exact test $P = 1.0$).

Table 2. The HLA antibody status of 50 pediatric liver transplantation patients at cross-sectional study period.

- HLA antibodies, $n = 50$ (% of total)
- Negative, $n = 17$ (34%)
- Positive, $n = 33$ (66%)
  - Non-DSA, $n = 7$ (21% of HLA antibody positive)
  - DSA, $n = 26$ (79% of HLA antibody positive)
    - Class I, $n = 4$ (15% of DSA positive)
      - MFI 1000–3000, $n = 2$
      - MFI 5000–10 000, $n = 2$
    - Class II, $n = 22$ (85% of DSA positive)
      - MFI 5000–10 000, $n = 6$*
      - MFI > 10 000, $n = 16$†

DSA, donor-specific antibody.

Some MFI categories were omitted because no observations fell within particular MFI range.

*$n = 1$ with combined MFI of 5532 (1528 + 4004; HLA-DR + HLA-DQ).

†$n = 2$ with combined MFI of 16 720 (3596 + 13 124; HLA-DQ + HLA-DQ) and 13 818 (2553 + 11 265; HLA-DQ + HLA-DQ).

Liver histology, biochemical markers and DSAs

Liver histology findings between DSA-positive and DSA-negative patients are shown in Fig. 3. Patients with DSAs had more pronounced portal inflammation in their liver biopsies compared to patients without DSAs ($P = 0.009$; exact Cochran–Armitage trend test). No linear trend was observed for other liver histology factors between DSA positivity and severity of histological grading ($P = 0.195$ for fibrosis, $P = 0.582$ for CK7 for periportal hepatocytes and $P = 0.638$ for bile duct proliferation; exact Cochran–Armitage trend test).

Only one DSA-positive patient was within the most severe (i.e., 3) portal inflammation category. Association with DSA positivity was also evident when portal inflammation was analyzed dichotomously (9/24 vs. 1/23; Fisher's exact test $P = 0.010$). The unadjusted prevalence ratio for portal inflammation in patients with DSAs compared to patients without was 8.6 (95% CI 1.6 to 50.9; Koopman method). Unadjusted prevalence ratios (Koopman method for all) for other liver histology were as follows: (DSA positive compared to DSA negative): fibrosis 1.9 (12/24 vs. 6/23; 95% CI 0.9 to 4.3), CK7 for periportal hepatocytes 2.0 (6/23 vs. 3/23; 95% CI 0.6 to 6.7) and bile duct proliferation 0.8 (8/23 vs. 10/23; 95% CI 0.4 to 1.6). Four patients (9% of 44 patients) had C4d deposits in their biopsy specimens (DSA positive 1/23 vs. DSA negative 3/21, Fisher's exact test $P = 0.335$). Of these four C4d-positive patients, two had sinusoidal and two had nonspecific C4d staining in their respective biopsy specimens.

In sensitivity analysis, after excluding 9 patients whose blood sample and liver biopsy were not taken at the same time, portal inflammation remained more frequent in DSA-positive compared to DSA-negative patients although association attenuated (7/21 vs. 1/17; Fisher's exact test $P = 0.053$, unadjusted PR 5.7 95% CI 1.1 to 33.9; Koopman method).

The possibility of chronic rejection was not completely excluded in one DSA-negative patient due to a bile duct loss over 50% in biopsy specimen. However, a loss of portal arterioles was not evident.

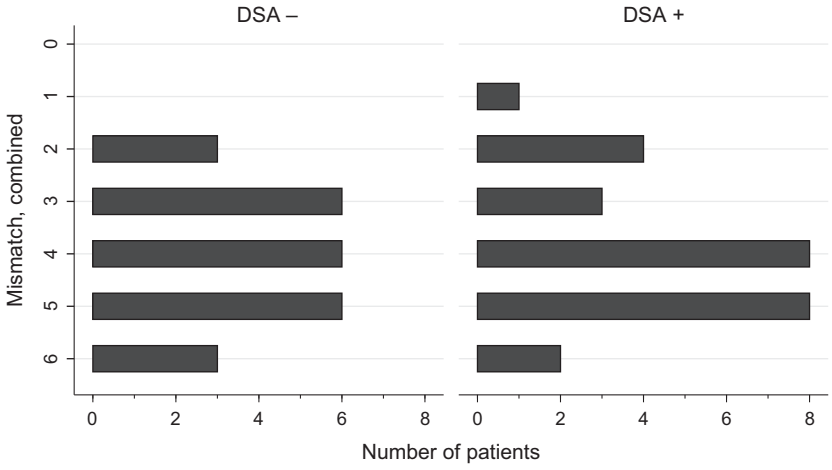

Figure 2 Combined HLA-A, -B and -DR mismatches in DSA-negative (n = 24; DSA −) and DSA-positive (n = 26; DSA +) patients. Combined mismatch category is shown in y-axis and number of patients in each category in x-axis.

Laboratory values are presented in Table 4. They were comparable between groups.

Blood group antibodies

Based on information from two databases, a total of seven patients (14% of 49 patients; one not available) had blood group antibodies. Among DSA-positive patients (n = 25), four were blood group antibody positive compared to three in DSA-negative group (Fisher's exact test P = 1.0).

Discussion

Approximately half of the pediatric LT patients in our cross-sectional study had DSAs, especially against class II HLA antigens with high MFI values. Portal inflammation was more common in DSA-positive than DSA-negative patients. We observed no clear differences in laboratory tests between DSA-positive and DSA-negative patients. In additional analyses, the prevalence of DSAs was higher among LT compared to CLKT patients.

Our finding that around 50% of patients had DSAs is in line with other pediatric LT studies [6,9]. Miyagawa-Hayashino *et al.* reported that 32 patients (48%) had DSAs after living donor LT [6]. Similarly, Wozniak *et al.* reported that 28 patients (56%) had DSAs after LT [9]. In both of these studies, follow-up time was comparable to ours, and the same MFI cutoff value (1000) for positivity was also used. Recently, Grabhorn *et al.* reported that 20 pediatric patients (47%) had DSAs after deceased or living donor LT, and almost all had *de novo* DSAs [10]. Prevalence of DSAs was 33% in 24 patients considered to have excellent graft function (i.e., group 1) with a median follow-up of 10 years after LT [10].

Among those patients with pre-LT HLA antibody sample available, half had *de novo* DSAs. The true prevalence of *de novo* DSAs in our population remains unknown, as we could not determine whether DSAs were preformed or *de novo* in the DSA-positive patients without a pre-LT HLA antibody sample.

The prevalence of portal inflammation in protocol liver biopsies was almost ninefold higher in DSA-positive compared to DSA-negative patients. There was, however, uncertainty regarding the prevalence ratio estimate as made evident by the wide confidence interval. Miyagawa-Hayashino *et al.* [6] showed that DSA-positive patients had more indeterminate inflammation than DSA-negative patients in their respective protocol liver biopsies although approximately 63% of DSA-positive and 54% of DSA-negative patients had some form of inflammation [6]. In our study, all patients except one had mild inflammation and therefore more detailed analysis of inflammation between DSA-positive and DSA-negative patients was not feasible.

In a multicenter immunosuppression withdrawal trial, nine of 18 pediatric patients had DSAs and at least three of these patients with DSAs had portal inflammation [25]. Our portal inflammation estimate of 38% within DSA-positive patients is more or less in line with Feng *et al.*'s study [25], although their focus was on immunosuppression withdrawal (i.e., operational tolerance) in a subsample of a larger pediatric patient population with parental living donor liver transplants.

Considering fibrosis, our study is in line (at least to some extent) with Miaygawa-Hayashino *et al.*'s study [6]. Of 32 DSA-positive patients, all had some form of fibrosis in their respective liver biopsies, compared to 24 of 35 DSA-negative patients, and fibrosis tended to

Table 3. Unadjusted odds ratios (ORs) and prevalence ratios (PRs) for DSAs and their association with different factors.

	OR (95% CI)*	PR (95% CI)†
Gender		
Male	2.2 (0.7–6.4)	1.5 (0.9–2.7)
Female (reference)		
Transplantation type		
LT	10.3 (1.5–119.8)	4.8 (1.2–26.9)
CLKT (reference)		
LT era		
LT before year 2001	2.7 (0.9–7.8)	1.6 (0.9–2.9)
LT after year 2001 (reference)		
Graft type		
Reduced graft	1.4 (0.4–4.6)	1.2 (0.7–2.3)
Whole liver (reference)		
Arterial anastomosis		
Aorta	2.0 (0.6–6.2)	1.4 (0.8–2.4)
Hepatic artery (reference)		
Biliary reconstruction		
Roux-en-Y	2.1 (0.6–7.2)	1.5 (0.8–3.3)
Duct-to-duct (reference)		
CNI immunosuppression‡		
Cyclosporine	3.4 (0.9–11.2)	2.0 (0.95§–4.9)
Tacrolimus (reference)		
Acute cellular rejection		
Rejection	0.7 (0.2–2.2)	0.8 (0.5–1.5)
No rejection (reference)		
Late hepatic artery thrombosis¶		
Thrombosis	1.02 (0.3–3.6)	1.01 (0.5–2.0)
No thrombosis (reference)		

For simplicity, reference categories were chosen so that increased odds ratios (OR > 1) for all variables are presented unless otherwise indicated. Unadjusted ORs and PRs were calculated using one variable at the time (i.e., univariate analysis) for DSA positivity (MFI >1000). Confidence intervals (CIs) for ORs and PRs were calculated with the mid-*P*-value method by Baptista–Pike and with the method by Koopman, respectively. Number of patients in each category can be derived from Table 1.

*Odds ratio can be defined as odds of being DSA positive within particular group compared to reference group. For example, in gender category: OR = (16 DSA + men/10 DSA − men)/(10 DSA + women/14 DSA − women) = 2.24.

†Prevalence ratio can be defined as risk ratio. For example, in gender category: PR = (16 DSA + males/total 26 males)/(10 DSA + females/total 24 females) = 1.48.

‡Calcineurin inhibitor (CNI) at the time of blood sample drawn for HLA antibody analysis.

§Shown as 2 decimal places as rounding would yield 1.0.

¶*n* = 32 patients based on our previous study (ref. 17).

be graded as severe for DSA-positive patients [6]. The prevalence ratio for fibrosis in their DSA-positive compared to DSA-negative patients would be 1.5 (95% CI 1.2 to 1.9) if calculated. However, in our study, considering fibrosis, the confidence interval of PR included 1 (95% CI 0.9 to 4.3) although the confidence intervals of two prevalence ratios, when informally assessing, markedly overlap. Half of our DSA-positive patients had fibrosis compared to 26% of DSA-negative patients. However, fibrosis was typically deemed as mild when present. This is contrary to the findings by Miaygawa-Hayashino *et al.* [6].

In the aforementioned immunosuppression withdrawal trial, three of four DSA-positive patients had portal fibrosis compared to five of eight DSA-negative patients before immunosuppression withdrawal. None of these 12 tolerant patients displayed central fibrosis [25]. Grabhorn *et al.* [10] reported that 14 of 15 patients with excellent graft function and protocol liver biopsies available had no fibrosis or it was grade 1 [10].

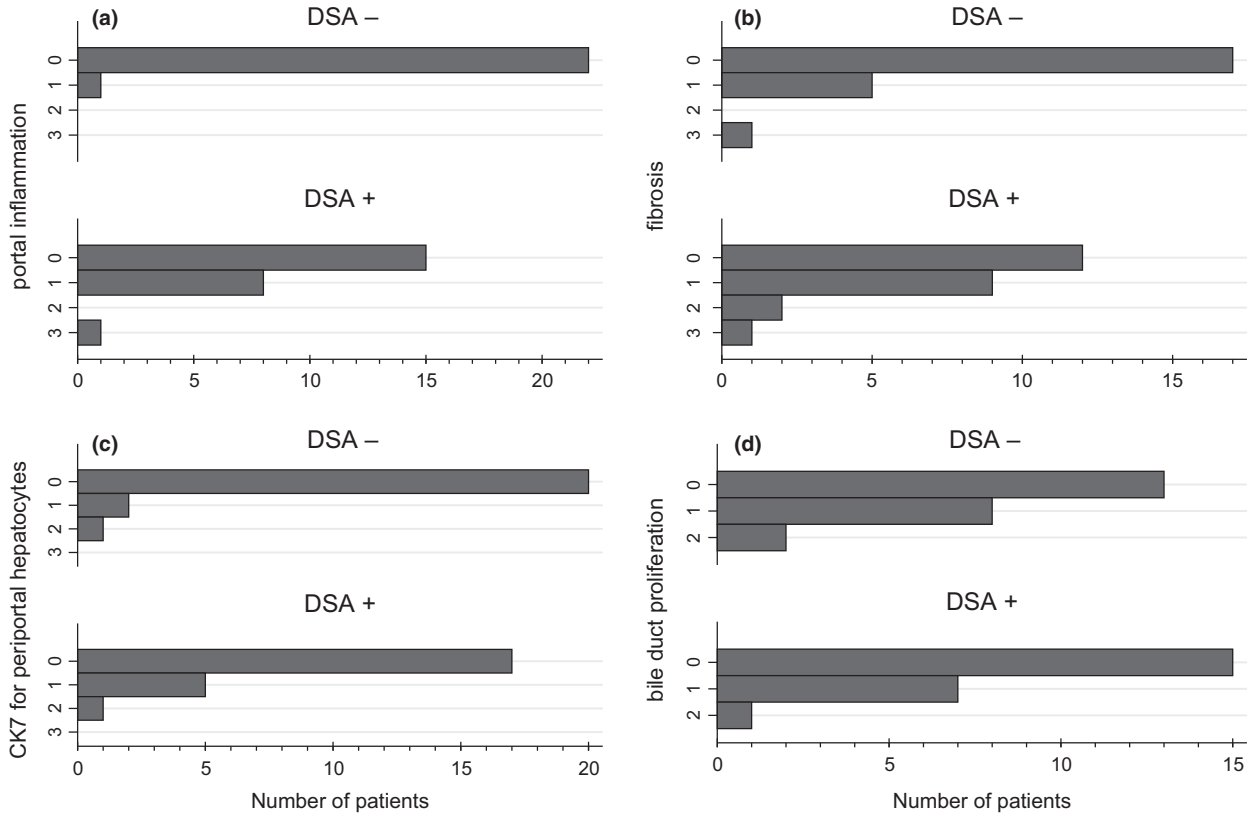

Figure 3 Liver histology findings in DSA-negative (DSA −) and DSA-positive (DSA +) patients considering (a) portal inflammation, $n = 47$; (b) fibrosis, $n = 47$; (c) periportal hepatocytes, $n = 46$; and (d) bile duct proliferation, $n = 46$. Semiquantitative grading classification is shown in *y*-axis and number of patients in each category in *x*-axis. *P*-values of exact Cochran–Armitage trend test were as follows: $P = 0.009$ for portal inflammation, $P = 0.195$ for fibrosis, $P = 0.582$ for periportal hepatocytes and $P = 0.638$ for bile duct proliferation.

However, the number of patients with grade 1 fibrosis was not specified [10].

We observed C4d endothelial staining in three DSA-negative patients and one DSA-positive patient. Others have found that C4d staining was not significantly higher in DSA-positive (34%) versus DSA-negative (20%) patients when the last biopsies were analyzed [6]. However, when more biopsies per patient were analyzed, C4d staining was higher in DSA-positive compared to DSA-negative patients [6]. In the absence of DSAs, C4d deposits can be caused by numerous other factors, for example, autoimmune hepatitis and biliary complications [26].

In our study, 60% of LT patients had DSAs compared to 12.5% of CLKT patients. In 123 pediatric kidney transplantation patients, the prevalence of DSAs (MFI >1000) at our center was 29% [27]. This estimate falls between the LT and CLKT estimates in our study. In one study of 65 adult simultaneous liver–kidney transplantation patients with post-transplantation sample available, nine had *de novo* DSAs [28]. Most of these post-transplantation serum samples were taken from six months to two years [28]. In contrast, the follow-up time of our pediatric CLKT patients ranged from 3 to 9 years (median 4.6 years). However, our finding that prevalence is higher among LT than CLKT patients should be confirmed by other pediatric studies in the future.

Our study has limitations. First, we could only reflect our results in the context of a cross-sectional snapshot, that is, we cannot draw conclusions about the evolution of DSAs before and after the studied time point or the clinical relevance of DSAs in general. Second, a semiquantitative histology grading system is always prone to subjectivity, although we tried to diminish this using more than one pathologist. The timing of liver histology and blood sampling for HLA antibody analysis was clearly not "matched" for some patients, that is, cross-sectional period varied between these two factors for the same patient. Third, from a statistical point of view, wide confidence intervals around the ratio estimates warrant cautious interpretation. No regression analyses were conducted as low number of events per variable and small sample size might bias estimates derived from

Table 4. Biochemical variables in DSA-negative and DSA-positive patients.

	DSA negative (n = 24)	DSA positive (n = 26)	Difference (95% CI)	P-value
Median ALT (U/l)	21 (14–32)	24 (17–39)	−3 (−14 to 8)	0.583
Median AST (U/l)*	29 (25–36)	33 (26–41)	−4 (−11 to 3)	0.276
Median GGT (U/l)†	21 (10–40)	22 (15–34)	−1 (−15 to 13)	0.890
Median ALP (U/l)	141 (87–230)	129 (88–215)	12 (−68 to 91)	0.777
Median total bilirubin (μmol/l)	9 (6–13)	10 (8–12)	−1 (−4 to 2)	0.540
Median bile acids (μmol/l)‡	4.9 (3.4–10.1)	6.6 (3.9–11.8)	−1.7 (−5.1 to 1.8)	0.342
Mean albumin (g/l)§	37.6 (3.4)	37.9 (3.0)	−0.3 (−2.2 to 1.6)	0.738
Mean pre-albumin (mg/l)§	211 (52)	196 (58)	15 (−17 to 48)	0.347
Mean TT (%)¶	110 (29)	102 (24)	9 (−6 to 24)	0.252
Mean APTT (sec)‡	26.2 (2.1)	27.0 (2.1)	−0.8 (−2.1 to 0.4)	0.176
Mean galactose half-life (min)**	10.6 (1.7)	10.9 (1.8)	−0.3 (−1.6 to 1.0)	0.617

ALP, alkaline phosphatase; ALT, alanine transaminase; APTT, activated partial thromboplastin time; AST, aspartate transaminase; GGT, gamma-glutamyl transferase; TT, thromboplastin time.

Values in parentheses are standard deviation (SD) for mean and interquartile range (IQR; 25th and 75th percentile) for median. Normally distributed variables are as means and non-normally distributed as medians.

Laboratory tests are plasma or serum based. Conversion of the laboratory values to conventional unit: bilirubin (mg/dl) divided by 17.104, bile acids (μg/ml) divided by 2.448, albumin (g/dl) divided by 10, pre-albumin (mg/dl) divided by 10.

*N = 49 (n = 24, DSA negative and n = 25, DSA positive).

†Four values <10 were set to 10.

‡N = 48 (n = 24 in both DSA negative and DSA positive); one value <2 for bile acids was set to 2.

§N = 47 (n = 24, DSA negative and n = 23, DSA positive).

¶One value > 170 was set to 170, normal reference range defined 70–130%, difference 9 due to rounding.

**N = 32 (n = 18, DSA negative and n = 14, DSA positive), normal value defined as half-life <15 min.

the logistic regression models [29,30]. Although regression modeling with small sample sizes can be performed, for example, with penalized likelihood logistic regression, this method was not employed due to lack of a clear theoretical background for multimodeling. In addition, we did not adjust P-values or confidence intervals for multiple comparisons.

Our study has strengths. First, we studied 76% of all potential pediatric LT patients at our center, which is the only transplant center in our country. Although patients who were not studied were older at LT than study patients, especially DSA-positive patients, no any strict selection criteria were used for patient inclusion. However, selection bias is possible if all patients are not studied or patient selection is not based on random sampling. Second, treatment protocol at our center is unified, and all patients are treated by the same transplant physicians and operated on by the same transplant surgeons, which might at least reduce systematic variability in patient care.

A myriad of open questions remain considering DSAs in the pediatric LT setting, for example, pre-LT DSA testing [31]. Ultimately, DSA testing should be studied under randomized controlled situations to establish whether patient outcomes are improved.

In conclusion, our study shows that around half of the patients studied here after pediatric LT with a median follow-up of 10 years had DSAs, especially class II DSAs with high MFI values. Particularly, portal inflammation was more common in DSA-positive than in DSA-negative patients, but uncertainty around the ratio estimate warrants cautious interpretation of this finding. No drastic differences were seen between DSA-positive and DSA-negative patients in terms of biochemical laboratory tests within our cross-sectional study period.

Authorship

JMK: participated in data collection, analyzed data and wrote the manuscript. SK: participated in data collection (major contribution) and manuscript revision. JP: supervised HLA antibody analyses. HM: contributed to the study in the adult LT unit. HJ: contributed to the study in the pediatric LT unit. CH, MPP and JL: designed the study and wrote the manuscript. JL:

conducted HLA antibody analyses. All authors accepted the final version of manuscript.

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Conflicts of interest

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