Presence of donor specific HLA class 2 antibodies (DSA class 2) is associated with development of graft fibrosis more than 10 years after liver transplantation—a retrospective single center study

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Abstract
Here the impact of donor specific human leukocyte antigen (HLA) class 2 antibodies (DSA cl 2) on long term outcome after liver transplantation (LT) was investigated. Altogether 156 (44 pediatric and 112 adult) LT recipients were included in the study. Graft fibrosis was assessed by liver elastography and biopsy. DSA cl 2 were determined by Luminex technology. 46% of LT recipients were positive for DSA cl 2 after a median follow-up of 15 years. In the multivariate analysis DSA cl 2 were significantly associated with immunosuppressive monotherapy (OR 5.42; 95% CI: 1.02–28.90; p = .048). Compared to DSA cl 2 negative patients, positive recipients had significantly more graft fibrosis based on the liver stiffness (mean 9.4 ± 9.0 kPa vs. 6.5 ± 6.3 kPa; p < .002) and fibrosis stages determined by liver elastography (p = .016) and the performed liver biopsies (p = .002). Also, a significantly higher incidence of chronic rejections (11% vs. 2%; p = .045) and graft losses (6% vs. 0%; p = .043) were found. In the multivariate regression analysis DSA cl 2 were significantly associated with graft fibrosis (OR 4.57; 95% CI 1.59–13.10; p = .005). So, these data suggest that development of DSA cl 2 occurs more often with immunosuppressive monotherapy and may ultimately result in chronic rejection and graft fibrosis.

KEYWORDS
fibrosis, liver disease: immune/inflammatory, rejection: antibodymediated (ABM)

1 | INTRODUCTION
The role of donor-specific human leukocyte antigen (HLA) class 2 antibodies (DSA cl 2) in orthotopic liver transplantation (LT) has gained increasing attention over the last few years. But due to lacking solid evidence, DSA is not routinely determined during follow-up of LT patients in most transplant centers.

Since the introduction of the bead-based multiplexing technology (Luminex®) for the determination of DSA several studies in adult and...
pediatric LT recipients have assessed the prevalence of pre-existing and de novo DSA cl 2 and evaluated their association with clinical outcome. However, the reported prevalence of DSA cl 2 varies extremely from 8.1% to 78% and depends on several factors such as the cut-off of the mean fluorescence intensity (MFI) used for the definition of positivity, the age, and ethnic heritage of the population and the length of follow-up. With respect to their clinical significance, DSA cl 2 has been suggested to correlate with acute and chronic antibody-mediated rejection (AMR), T-cell mediated rejection (TCMR), and development of biliary complications early after transplantation. In addition, an impact on long-term outcome, that is, development of graft fibrosis, as well as graft and patient survival has been described. In this context, the studies with a long-term follow-up between 10- and 13-years post-transplantation mainly refer to pediatric patients from Asia, while there is only scarce data on adult patients with a long-term follow-up from the Western World.

Therefore, in this single-center study for the first time, a European pediatric and adult LT cohort with a graft survival time of at least 10 years was analyzed to assess the prevalence of DSA and their impact on graft outcome.

2 | MATERIALS AND METHODS

2.1 | Patient population

In this retrospective analysis, patients who underwent their most recent LT between January 1997 and December 2007 at the University Medical Center Hamburg Eppendorf and presented to the outpatient clinic for a yearly check-up during the study period from February 1, 2013 to January 31, 2019. Exclusion criteria were an active or previous hepatitis C virus (HCV) infection or an incomplete set of data. Patients undergoing re-transplantation in the designated time period and patients receiving their graft from a living donor were not excluded. Of 1032 patients undergoing LT between January 1997 and December 2007 at our institution, 156 (15.1%) patients were included in the analysis: Reasons for non-inclusion/exclusion of patients were death (n = 211; 20.5%) or graft loss (n = 50; 4.8%) prior to the study period, no follow-up during the study period at our center (n = 420; 40.7%), HCV infection (n = 65; 6.3%), no prior HLA typing of the donor (n = 53; 5.1%) or incomplete set of data (n = 77; 7.5%).

The study was performed according to the guideline of the local ethic committee (No WF-005/20). For the yearly check-up patients routinely received a laboratory workup, an ultrasound, and liver elastography. A liver biopsy was done in case of a clinical indication in all adult patients and per-protocol every 5 years in pediatric patients until the age of 18 years.

2.2 | HLA typing

Donors and recipients were high-resolution DNA typed (according to EFI standards) for human leukocyte antigen (HLA) HLA-DRB1, DQA1, and DQB1 loci using multiplexed reverse-SSO hybridization technique as described by the manufacturer (LABType™ SSO; One Lambda/Thermo Fisher Scientific).

2.3 | DSA/HLA antibody detection

In our institution, HLA antibody determination has been available since 2013. It was implemented in the yearly check-up investigations of patients and in addition, was performed in case of elevated liver function tests (LFTs).

For the analysis, the earliest and the latest results available were included. Luminex single antigen beads (LABScreen® Single Antigen Assays; One Lambda/Thermo Fisher Scientific Inc.) were used to detect anti-DR and -DQ antibodies (Ab) as described previously. Antibodies detected with MFI of ≥1500 were considered positive.

2.4 | Assessment of liver fibrosis by liver elastography

For non-invasive assessment of liver parenchymal integrity and liver stiffness, all patients received ultrasound-based liver elastography (TE) (FibroScan®; EchoSens) under fasting conditions. The target area of the right liver lobe was determined by ultrasonography to be 6 cm in depth without major vascular structures. Only procedures with at least 10 valid measurements, a success rate of at least 60% and an inter-quartile range (IQR)/median ratio of less than 30% were included. The average IQR/median ratio was 1.3% (range 0.2%-10.4%).

In children, a hepatic 2D shear wave elastography (2D SWE) (GE Logiq 9 ultrasound system; GE Medical Systems) was used, as previously reported. In patients with a split liver graft, the left lobe was examined via the epigastric abdominal wall. 12 successful elastograms were considered as a successful measurement. The average IQR/median ratio was 2.2% (range 0.9%-8.8%).

Four fibrosis stages were defined according to the mean kPa value determined: stage 1 (F0/1): 1–6 kPa; stage 2 (F2): 6.1–10 kPa; stage 3 (F3): 10.1–15.9 kPa; stage 4 (F4): ≥16 kPa.

2.5 | Assessment of liver biopsies

All liver biopsies stating in the report signs of chronic rejection were reevaluated by a pathologist blinded to the results of the DSA. Fibrosis was assessed according to the Desmet fibrosis score, diagnosis of T-cell mediated rejection and antibody-mediated rejection was based on the Banff criteria. For immunohistochemical Anti-C4d antibody (Rabbit polyclonal antibody; abcam) were used.

2.6 | Statistical analyses

Continuous variables are described as means and standard deviations or median with minimum and maximum. Categorical data are given as absolute and relative frequencies.
### TABLE 1  Patient characteristics

| Variable                                      | Entire study population (n = 156) | Adult patients (n = 112; 72%) | Pediatric patients (n = 44; 28%) | Descriptive p-value |
|-----------------------------------------------|----------------------------------|-------------------------------|---------------------------------|---------------------|
| Time of follow-up, years<sup>b</sup>         | 15 (10–21)                      | 14 (11–21)                    | 16 (10–21)                      | .075<sup>‡</sup>    |
| Recipient age at TX, years<sup>b</sup>       | 40 (0–72)                       | 49 (18–72)                    | 2 (0–17)                        | <.001<sup>‡</sup>   |
| Donor age, years<sup>b</sup>                 | 34 (1–70)                       | 39 (10–70)                    | 23 (1–59)                       | <.001<sup>‡</sup>   |
| Recipient BMI, kg/m<sup>2</sup> <sup>a</sup> | 24.9 ± 5.0                      | 25.9 ± 5.0                    | 22.3 ± 3.7                      | .001<sup>‡</sup>    |
| Donor BMI, kg/m<sup>2</sup> <sup>a</sup>     | 23.6 ± 3.3                      | 24.0 ± 3.2                    | 22.5 ± 3.5                      | .012<sup>‡</sup>    |
| Recipient sex (female), n (%)                | 77 (49)                         | 59 (53)                       | 18 (41)                         | <.001**             |
| Split liver graft, n (%)                     | 74 (47)                         | 38 (34)                       | 36 (82)                         | <.001**             |
| Warm ischemia time, minutes<sup>a</sup>     | 40 ± 14                         | 40 ± 15                       | 39 ± 13                         | .001**             |
| Cold ischemia time, minutes<sup>a</sup>     | 590 ± 168                       | 608 ± 164                     | 544 ± 170                       | .031<sup>‡</sup>    |

#### Etiology of liver disease

| Etiology                         | Entire study population (n = 156) | Adult patients (n = 112; 72%) | Pediatric patients (n = 44; 28%) |
|----------------------------------|----------------------------------|-------------------------------|---------------------------------|
| Autoimmune disease (AIH, PSC, PBC), n (%) | 35 (22)                         | 29 (26)                       | 6 (14)                          | <.001**             |
| Viral hepatitis, n (%)           | 17 (11)                          | 16 (14)                       | 1 (2)                           |                    |
| Alcoholic liver disease, n (%)   | 23 (15)                          | 23 (21)                       | 0 (0)                           |                    |
| Retransplantation, n (%)         | 22 (14)                          | 13 (11)                       | 9 (21)                          |                    |
| Other, n (%)                     | 59 (38)                          | 31 (28)                       | 28 (64)                         |                    |

#### Immunosuppressive maintenance therapy

| Therapy          | Entire study population (n = 156) | Adult patients (n = 112; 72%) | Pediatric patients (n = 44; 28%) |
|------------------|----------------------------------|-------------------------------|---------------------------------|
| Tacrolimus, n (%)| 78 (50)                          | 59 (53)                       | 19 (43)                         | .374**             |
| Cyclosporine A, n (%) | 68 (44)                         | 47 (42)                       | 21 (48)                         | .591**             |
| Mycophenolate, n (%) | 74 (47)                         | 62 (55)                       | 12 (27)                         | .002**             |
| mTOR inhibitors, n (%) | 20 (13)                         | 15 (14)                       | 5 (11)                          | 1.000**            |
| Azathioprine, n (%)   | 7 (5)                           | 7 (6)                         | 0 (0)                           | .192**             |
| Steroids, n (%)      | 23 (15)                         | 18 (16)                       | 5 (11)                          | .617**             |
| mTOR inhibitor, >6 months, n (%) | 38 (24)                         | 30 (27)                       | 8 (18)                          | .305**             |
| Immunosuppressive monotherapy, n (%) | 52 (33)                         | 24 (21)                       | 28 (64)                         | <.001**             |

Note: p-Value refers to differences between adult and pediatric patients.

Abbreviations: AIH, autoimmune hepatitis; BMI, body mass index; PBC, primary biliary cirrhosis; PSC, primary sclerosing cirrhosis.

<sup>a</sup>Mean ± SD.

<sup>b</sup>Median (min-max.).

**Fisher’s exact test.

†Unpaired student’s t-test.

‡Mann–Whitney U test.

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**FIGURE 1** The prevalence of donor-specific antibodies (DSA) cl 2 according to the number of years after LT.
The assumption of normally distributed data was assessed graphically (not shown). Right skewed variables were logarithmically transformed with basis 10. For two-group comparisons of continuous variables, Student’s t-test or Wilcoxon–Mann–Whitney U test was used. Fisher’s exact test or Chi-square test was performed for comparison of dichotomous or categorical variables. Multivariable logistic regression analysis was performed to assess factors associated with the present of DSA cl 2 including all variables with a p-value <0.1 in the univariable analysis. Multivariable logistic regression was used to assess the influence of DSA cl 2 positivity vs. negativity on the occurrence of fibrosis (based on elastography stiffness; >7.5 kPa) with the following covariate adjustment: that is, adjusting for recipient’s age at LT and time since LT. The statistical analysis was performed with SPSS version 25.0 (SPSS Inc.).

3 | RESULTS

3.1 | Study population

Altogether 156 patients – 44 (28%) pediatric and 112 (72%) adult recipients – were included in the study (Table 1). The median follow-up of all patients was 15 years (min.–max. 10–21) and not significantly different between pediatric and adult recipients. Pediatric patients

| TABLE 2 | Comparison of characteristics of DSA cl 2 positive and negative patients (total population) |
|-----------|---------------------------------------------------------------|
| Variable | DSA cl 2+ (n = 72; 46%) | DSA cl 2− (n = 84; 54%) | Descriptive p-Value |
| Time of follow-up, years | 16 (10–21) | 14 (10–21) | .002† |
| Recipient age at TX, years | 25 (0–67) | 46 (0–72) | <.001‡ |
| Recipient BMI, kg/m² | 24.3 ± 5.1 | 25.3 ± 4.8 | .292§ |
| Indication for transplantation, n (%) | | | |
| Autoimmune disease (AIH, PSC, PBC), n (%) | 15 (21) | 20 (24) | .227** |
| Viral hepatitis, n (%) | 6 (8) | 11 (13) | |
| Alcoholic liver disease, n (%) | 7 (10) | 11 (13) | |
| Retransplantation, n (%) | 11 (15) | 11 (13) | |
| Other, n (%) | 33 (46) | 26 (31) | |
| Donor age, years | 28 (1-70) | 36 (6–64) | .018‡ |
| Donor BMI, kg/m² | 23.3 ± 3.9 | 23.9 ± 2.7 | .304† |
| Recipient sex (female), n (%) | 35 (49) | 42 (50) | .874** |
| Warm ischemia time, minutes | 40 ± 14.6 | 39 ± 14.1 | .671† |
| Cold ischemia time, minutes | 568 ± 175 | 608 ± 159 | .141† |
| Full organ transplantation, n (%) | 32 (44) | 50 (59) | .077** |
| Living donation, n (%) | 3 (4) | 1 (1) | .336** |
| Split liver graft, n (%) | 40 (56) | 34 (41) | .077** |
| Right | 20 (50) | 26 (76) | .030** |
| Left | 20 (50) | 8 (24) | .030** |
| Combined kidney transplantation, n (%) | 6 (8) | 7 (9) | 1.000** |
| Maintenance immunosuppressive therapy | | | |
| Tacrolimus, n (%) | 34 (48) | 44 (52) | .630** |
| Cyclosporine A, n (%) | 33 (46) | 35 (42) | .630** |
| Mycophenolate, n (%) | 30 (42) | 44 (52) | .630** |
| mTOR inhibitor, >6 months, n (%) | 13 (18) | 25 (29) | .201** |
| Azathioprine, n (%) | 2 (3) | 5 (6) | .452** |
| Steroids, n (%) | 13 (18) | 10 (12) | .366** |
| Immunosuppressive monotherapy, n (%) | 31 (43) | 21 (25) | .026** |

Abbreviations: AIH, autoimmune hepatitis; BMI, body mass index; DSA, donor-specific antibodies; PBC, primary biliary cirrhosis; PSC, primary sclerosing cirrhosis.

*Mean ± SD.

†Median (min-max.).

**Fisher’s exact test.

†Unpaired student’s t-test.

‡Mann–Whitney U test.
received grafts from significantly younger donors and more often split grafts with a shorter cold ischemic time. Also, pediatric patients significantly more often received a maintenance immunosuppressive monotherapy with either cyclosporine or tacrolimus ($p < .001$) and less often mycophenolate ($p = .002$; Table 1).

### 3.2 Prevalence of HLA antibodies (HLA-Ab) and DSA

After a median follow-up of 15 years, the prevalence of DSA cl 2 was 46%. DSA cl 2 were more often directed against DQ loci ($n = 62; 40\%$) than against DR loci ($n = 29; 19\%$) and mean MFI was significantly higher against DQ loci (MFI 13 603 vs. 7295; $p = .002$). Furthermore, 26% of the DSA cl 2 positive patients had DSA against DQ-, and against DR loci. Altogether, the prevalence of DSA was significantly higher in pediatric as compared to adult patients (73% vs. 36%; $p < .001$).

Figure 1 illustrates the prevalence of DSA cl 2 as a function of the number of years a patient has been transplanted. In the study population, there was a significant increase ($p = .042$) in the prevalence of DSA cl 2 from 17% to 67% between the group of patients with 10 years as compared to those with 21 years of follow-up post-LT. In addition, we performed a longitudinal analysis for individual patients. During follow-up, 152 of the 156 (97.5%) patients received

| TABLE 3 Comparison of characteristics of DSA cl 2 positive and negative patients (adult patients) |
|--------------------------------------------------|
| Variable                          | DSA cl 2+ ($n = 40; 36\%$) | DSA cl 2− ($n = 72; 64\%$) | Descriptive p-Value |
|-----------------------------------|----------------------------|---------------------------|---------------------|
| Time of follow-up, years$^b$      | 16 (10–20)                 | 14 (10–21)                | .017‡               |
| Recipient age at TX, years$^a$    | 44 ± 13                    | 48 ± 11                   | .052†               |
| Recipient BMI, kg/m$^2$           | 26.3 ± 5.3                 | 25.6 ± 4.9                | .451†               |
| Indication for transplantation, n (%) |                            |                           |                     |
| Autoimmune disease (AIH, PSC, PBC), n (%) | 10 (25)                    | 19 (26)                   | .975*               |
| Viral hepatitis, n (%)            | 6 (15)                     | 10 (14)                   |                     |
| Alcoholic liver disease, n (%)    | 7 (18)                     | 16 (22)                   |                     |
| Retransplantation, n (%)          | 5 (12)                     | 8 (11)                    |                     |
| Other, n (%)                      | 12 (30)                    | 19 (27)                   |                     |
| Donor age, years$^a$              | 38 ± 16                    | 38 ± 13                   | .964†               |
| Donor BMI, kg/m$^2$               | 24.3 ± 4.1                 | 23.8 ± 2.6                | .365§               |
| Recipient sex (female), n (%)     | 38 (53)                    | 21 (53)                   | .977**              |
| Warm ischemia time, minutes$^a$   | 40 ± 15                    | 40 ± 14                   | .872†               |
| Cold ischemia time, minutes$^a$   | 604 ± 174                  | 611 ± 160                 | .840†               |
| Full organ transplantation, n (%) | 26 (65)                    | 48 (67)                   | 1.000*              |
| Living donation, n (%)            | 0 (0)                      | 1 (1)                     | 1.000*              |
| Split liver graft, n (%)          | 14 (35)                    | 24 (33)                   | 1.000*              |
| Right                             | 13 (93)                    | 24 (100)                  | .368**              |
| Left                              | 1 (7)                      | 0 (0)                     | .368**              |
| Combined kidney transplantation, n (%) | 6 (15)                    | 7 (10)                    | .539**              |
| Maintenance immunosuppressive therapy |                          |                           |                     |
| Tacrolimus, n (%)                 | 22 (55)                    | 37 (52)                   | .844***              |
| Cyclosporine A, n (%)             | 16 (40)                    | 31 (43)                   | .843***              |
| Mycophenolate, n (%)              | 20 (50)                    | 42 (58)                   | .432**              |
| mTOR inhibitor, >6 months, n (%)  | 9 (23)                     | 21 (29)                   | .509**              |
| Azathioprine, n (%)               | 2 (5)                      | 5 (7)                     | 1.000**              |
| Steroids, n (%)                   | 10 (25)                    | 8 (11)                    | .065*                |
| Immunosuppressive monotherapy, n (%) | 10 (25)                    | 14 (19)                   | .631**              |

Abbreviations: AIH, autoimmune hepatitis; BMI, body mass index; DSA, donor-specific antibodies; PBC, primary biliary cirrhosis; PSC, primary sclerosing cirrhosis.

$^a$Mean ± SD.

$^b$Median (min-max.).

$^c$Chi-squared test.; **Fisher’s exact test.

$^d$Unpaired student’s t-test.

$^e$Mann–Whitney U test.
more than one DSA cl 2 screening with an average time interval of 3.2 years. Twenty (20%) of the 99 initially DSA cl 2 negative patients developed DSA cl 2 within the study period, while only 5 (9%) of the 53 initially DSA cl 2 positive patients lost their DSA cl 2.

### 3.3 Comparison of characteristics of DSA cl 2 positive and negative patients

Characteristics of DSA cl 2 positive and negative patients at the time of LT are given in Tables 2-4 for the total, the adult and pediatric study population, respectively. Looking at the total population, time of follow-up post-LT was significantly longer in DSA cl 2 positive as compared to DSA cl 2 negative patients (16 vs. 14 years; \( p = .002 \)).

Also, DSA cl 2 positive patients significantly more often had received an immunosuppressive monotherapy as compared to DSA cl 2 negative patients (43% vs. 25%, \( p = .026 \)).

Table 5 shows the results of the multivariable logistic regression analysis which was performed to identify risk factors for development of DSA cl 2 in the total study population. The adjusted odds ratio (with 95% confidence interval) for recipient's age, time of follow-up since LT, and immunosuppressive monotherapy were 0.95 (0.91–0.98), 1.41 (1.12-1.79), and 5.42 (1.02-28.90), respectively.
3.4 Comparison of clinical outcome parameters of DSA cl 2 positive and negative patients

Clinical outcome parameters are given in Tables 6-8 for the total, the adult, and the pediatric study population. Looking at the total population, there was a significantly higher rate of biopsy-proven chronic rejections (11% vs. 2%; \( p = .045 \)) in DSA cl 2 positive as compared to negative patients, but not of early or late acute rejections. The latest liver biopsies of all patients with chronic rejection were retrospectively stained for the expression of C4d. The biopsies from all eight DSA cl 2 positive patients were found to show histological characteristics of chronic antibody-mediated rejection (cAMR) and to express C4d immunoreactivity with a combined score of at least four out of six points. On the other hand, the liver biopsies of the two DSA cl 2 negative patients with chronic rejection had a score of only two out of six points based on the cAMR Banff criteria (Table 9). In addition, during the 6-year study period all four graft losses occurred in DSA cl 2 positive patients (6% vs. 0%; \( p = .043 \); Tables 6-8). All four patients had very high titers of DSA cl 2 and after re-transplantation explant histologies revealed the typical characteristics of cAMR with C4d immunoreactivity on endothelial cells and sinusoids (Table 9).

Furthermore, grafts of DSA cl 2 positive patients were characterized by higher liver fibrosis stages as determined by elastography (Figure 2A,B). This finding was noted for the entire population (Figure 2A; \( p = .016 \)) and the subgroup of patients transplanted at adult age (Figure 2B; \( p = .025 \)). Also, the mean liver stiffness values

| Model (Variable)                | Odds ratio | Lower bound | Upper bound | \( p \)-Value |
|--------------------------------|------------|-------------|-------------|--------------|
| Recipient’s age                | 0.946      | 0.911       | 0.983       | .005         |
| Time of follow-up              | 1.413      | 1.115       | 1.791       | .004         |
| Immunosuppressive monotherapy  | 5.420      | 1.016       | 28.902      | .048         |
| Treatment with mTOR inhibitor  | 1.384      | 0.367       | 5.225       | .632         |
| Split graft                    | 1.005      | 0.189       | 5.347       | .996         |

Note: \( n = 156 \) patients; Outcome 0: DSA cl 2 negative; Outcome 1: DSA cl 2 positive.

| Variable                                 | DSA cl 2 + (\( n = 72; 46\% \)) | DSA cl 2 – (\( n = 84; 54\% \)) | \( p \)-Value |
|------------------------------------------|--------------------------------|--------------------------------|---------------|
| Graft loss, \( n \) (%)                  | 4 (6)                          | 0 (0)                          | .043**        |
| Retransplantation during follow-up, \( n \) (%) | 4 (6)                          | 0 (0)                          | .043**        |
| Blood parameters at last follow-up       |                                |                                |               |
| ALAT: 1.5× above the standard value, \( n \) (%) | 4 (6)                          | 2 (2)                          | .416**        |
| ASAT: 1.5× above the standard value, \( n \) (%) | 4 (6)                          | 1 (1)                          | .182**        |
| GGT: 1.5× above the standard value, \( n \) (%) | 19 (26)                        | 12 (14)                        | .071**        |
| Platelet, Mrd/L\(^a\)                   | 231 ± 136                      | 232 ± 95                       | .945†         |
| Albumin, g/L\(^a\)                      | 36.1 ± 5.2                     | 37.3 ± 3.9                     | .102†         |
| Bilirubin, mg/dl\(^b\)                  | 0.6 (0.2–25.2)                 | 0.5 (0.2–7.6)                  | .064‡         |
| Biliary complications, \( n \) (%)       | 20 (28)                        | 21 (25)                        | .718**        |
| ITBL, \( n \) (%)                       | 3 (4)                          | 3 (4)                          | 1.000**       |
| AS, \( n \) (%)                         | 6 (8)                          | 12 (14)                        | .318**        |
| NAS, \( n \) (%)                        | 2 (3)                          | 3 (4)                          | 1.000**       |
| Rejection episodes                      |                                |                                |               |
| Acute rejection within 90 days post TX, \( n \) (%) | 5 (7)                          | 10 (12)                        | .415**        |
| Late acute rejection >90 days post OL, \( n \) (%) | 13 (18)                        | 12 (14)                        | .662**        |
| Chronic rejection, \( n \) (%)          | 8 (11)                         | 2 (2)                          | .045**        |
| Deceased, \( n \) (%)                   | 3 (4)                          | 2 (2)                          | .663**        |

Abbreviations: ALAT, alanine transaminase; AS, anastomotic strictures; ASAT, aspartate transaminase; CAP, controlled attenuation parameter; DSA, donor-specific antibodies; GGT, gamma-glutamyl transferase; ITBL, Ischemic Type Biliary Lesions; NAS, nonanastomotic strictures.

\(^a\)Mean ± SD.

\(^b\)Median (min-max.).

**Fisher’s exact test.

†Unpaired student’s \( t \)-test.

‡Mann–Whitney U test.
were significantly higher in DSA cl 2 positive as compared to negative patients in the entire population (9.5 ± 9.0 vs. 6.5 ± 6.3 kPa; \( p = .002 \)) and the subgroup of adults (10.3 ± 11.7 vs. 6.1 ± 5.6 kPa; \( p = .014 \)). For the subgroup of pediatric patients, the liver stiffness was not significantly different between DSA positive and negative cases (Figure 2C; \( p = .692 \)).

When assessing liver biopsy specimen there was also a shift toward significantly higher fibrosis stages in the DSA cl 2 positive as compared to the negative patients in the entire population (9.5 ± 9.0 vs. 6.5 ± 6.3 kPa; \( p = .002 \)) and the subgroup of adults (10.3 ± 11.7 vs. 6.1 ± 5.6 kPa; \( p = .014 \)). For the subgroup of pediatric patients, the liver stiffness was not significantly different between DSA positive and negative cases (Figure 2C; \( p = .692 \)).

A multivariable logistic regression analysis for assessment of risk factors for development of liver fibrosis was performed. All variables that showed a significant difference between patients with and without fibrosis were included. So, ‘immunosuppressive monotherapy’ (\( p = .494 \)) was not included in the analysis. An adjusted odds ratio of 4.57 (95% CI: 1.59–13.10) was estimated for the independent variable ‘presence of DSA cl 2’ (Table 10). For recipient’s age and time since transplant, the adjusted odds ratios were 0.96 (95% CI: 0.84–1.11) and 0.96 (95% CI: 0.97–1.02), respectively.

### DISCUSSION

This retrospective analysis in a population of Caucasian adult and pediatric long-term liver transplant recipients supports the hypothesis that very low immunosuppressive treatment is associated with the emergence of DSA cl 2. Furthermore, during long-term follow-up the presence of DSA cl 2 significantly increased the risk of developing liver fibrosis.

In the long-term LT recipients studied here the prevalence of DSA cl 2 with the MFI >1500 was high with 46% after a mean follow-up of 15 years. The majority of DSA cl 2 were directed against DQ loci. Both findings are in principle in agreement with previous investigations.\(^3,17\) However, comparison with published data is limited due to the varying MFI cut-off levels used, different lengths of patient follow-up, different patients’ ethnic heritage, and living-related vs. post-mortem donation. Previously, the prevalence of DSA cl 2 was reported to vary in long-term pediatric patients between 45% and
51% after a follow-up of 9.7 to 13.1 years. In adult patients, the prevalence was reported to be much lower with 18% to 22%, but after a much shorter follow-up period of only 4 to 6.4 years.3–6,8,37 Here, we present the first single-center study with direct comparison of pediatric and adult patients. The prevalence of DSA cl 2 of 73% in pediatric patients after a median period of 16 years post-LT was significantly higher than the prevalence of 36% in adult recipients after a median follow-up of 14 years (p < .001).

The reason for this difference is unknown. It may be due to a smaller graft size with lower resistance mechanisms against humoral rejection or under-immunosuppression.9,38

Furthermore, there was a significant increase in the prevalence of DSA cl 2 from 17% in patients transplanted 10 years compared to 67% for patients transplanted more than 20 years (p = .048). Also, looking at the development of DSA cl 2 longitudinally over a mean period of 3.2 years, the MFI of DSA was found to increase over time in the majority of patients, while on the other hand DSA was rarely lost and MFIs only rarely decreased over time.

Notably, in this population more DSA cl 2 developed in the second decade post-LT as compared to the first one. It is conceivable that this is related to the steady decrease in the immunosuppressive medication over time which is part of the protocol of our center. Indeed, further findings support the hypothesis that the occurrence of DSA may be due to under-immunosuppression or patients’ non-adherence. In comparison to DSA cl 2 negative patients, DSA cl 2 positive long-term LT recipients were significantly more often found to be treated with a maintenance immunosuppressive monotherapy (p = .026) consisting of either cyclosporine or tacrolimus than with a dual or triple combination therapy (Tables 2 and 5).

Another important finding of our study is the significant association between the presence of DSA cl 2 and the development of graft fibrosis in long-term LT recipients. In the pediatric subpopulation the significant association was shown when assessing fibrosis by liver histologies (p = .037), but not when assessing liver elastographies (p = .172). This is most likely due to the fact that only a few biopsies were available in the adult population.
Our data are well in agreement with previous smaller studies from Asia that suggest a correlation between DSA cl 2 and development of fibrosis in pediatric patients.\textsuperscript{5,6,8,10,21,22} But so far there have been hardly any data on adult and Caucasian LT recipients. Only San Segundo et al\textsuperscript{8} revealed in a small retrospective analysis of 28 adult patients from Spain an association between DSA cl 2 and liver stiffness on elastography after the mean follow-up of 6.4 years. None of the previous studies had a follow-up of 15 years.

Furthermore, the data demonstrated here also support the hypothesis that fibrosis development may be due to cAMR. Significantly more DSA cl 2 positive than negative patients were found to have a chronic rejection on liver biopsy (11% vs. 2%; \(p = .045\); Tables 6-8). In addition, all four patients requiring re-transplantation during the study period were found to have DSA cl 2 with a very high MFI of \(>10,000\) and showed typical features of cAMR in their explants.\textsuperscript{34,39} Previously, O Leary et al\textsuperscript{40} showed a relation between DSA and cAMR. However, in her study only DSA with the MFI of \(>10,000\) were regarded as clinically relevant. In addition, a recent investigation from Hannover supports our hypothesis of a relation between the presence of DSA and subclinical rejection as well as graft fibrosis.\textsuperscript{22} They analyzed gene expression of 93 transcripts for graft injury, tolerance, and immune regulation in 77 liver biopsies, 36 of them with subclinical rejection. In this analysis DSA positivity was associated with histological evidence of more severe graft inflammation and fibrosis and also with an upregulation of rejection-associated transcripts.

However, there are limitations in our study. First, we did not differentiate between pre-formed and de novo DSA cl 2. But our longitudinal analysis reveals that the majority of DSA cl 2 newly emerged during follow-up. Furthermore, we looked only at a selected patient population with graft survival of at least 10 years. Many of the patients transplanted in the time period studied were lost of follow-up, but only 4.8% of patients are known to have lost their graft.

Also, although very suggestive, our data does not imply a causality between under-immunosuppression, presence of DSA cl 2, and fibrosis in the graft. A true causality is very difficult to prove even in future prospective studies due to many parameters influencing fibrosis progression and DSA development in a real-life setting. Nevertheless, in our multivariate analysis, the presence of DSA cl 2 was associated with immunosuppressive monotherapy (odds ratio: 5.420; \(p = .048\)). Furthermore, in the multivariate analysis for graft fibrosis, DSA class 2 was a risk factor (odds ratio: 4.57; \(p = .005\)) in this study population. So, further studies in larger populations with prospective DSA cl 2 determination and regular fibrosis assessment over a period of many years might give further insight.

Therefore, so far the clinical consequences of DSA positivity in LT are unclear. We recommend yearly monitoring of DSA cl 2 as well as fibrosis development by elastography. In case of progressing fibrosis and high titers of DSA cl 2, a liver biopsy should be considered to exclude early cAMR and to re-evaluate patient’s adherence and immunosuppressive therapy.
FIGURE 2  Comparison of the fibrosis stage between donor-specific antibodies (DSA cl 2 positive and DSA cl 2 negative patients based on transient elastography results. A: The entire study population; B: The adult population; C: The pediatric population.

(A) Total population

- DSA cl 2 positive (n = 58)
- DSA cl 2 negative (n = 66)

(B) Adult patients

- DSA cl 2 positive (n = 29)
- DSA cl 2 negative (n = 60)

(C) Pediatric patients

- DSA cl 2 positive (n = 29)
- DSA cl 2 negative (n = 6)
FIGURE 3 Comparison of the fibrosis stages between donor-specific antibodies (DSA) cl 2 positive and DSA cl 2 negative patients based on liver biopsy evaluation. A: The entire study population; B: The adult population; C: The pediatric population

(A) Total population

n = 49
p = 0.002 (χ²-test)

(B) Adult patients

n = 15
p = 0.172 (χ²-test)

(C) Pediatric patients

n = 34
p = 0.037 (χ²-test)
### Table 10: Multivariable binary logistic regression for fibrosis progression based on elastography stiffness (>7.5 kPa)

| Model (Variable) | Odds Ratio  | 95% Confidence interval | **p**-Value |
|------------------|-------------|-------------------------|-------------|
| Recipient’s age at LT | 0.964 | 0.835 - 1.113 | .598 |
| Time since LT | 0.964 | 0.973 - 1.016 | .617 |
| DSA cl 2 positive | 4.570 | 1.594 - 13.103 | .005 |

Note: n = 124 patients; Outcome 0: No Fibrosis (<7.5 kPa); Outcome 1: Fibrosis (>7.5 kPa).

### Author Contributions
B Sultani collected the data, performed research/study, designed figures, and wrote the manuscript. M Marget performed the laboratory analysis and analyzed the laboratory results. A Briem-Richter recruited and followed up the paediatric transplanted patients, and added ideas for improving the manuscript. J Hermann recruited and followed up the paediatric transplanted patients and analyzed the sonographic/elastographic findings. S Meisner recruited and followed up the adult transplanted patients and analyzed the sonographic/elastographic findings. E Grabhorn recruited and followed up the paediatric transplanted patients, and added ideas for improving the manuscript. A Ozga performed the statistical analysis. S Weidemann performed immunohistology, analyzed and classified the biopsy samples. U Herden and L Fischer recruited, operated, and followed up the paediatric and adult patients, and added ideas for improving the manuscript. M Sterneck designed the research/study, recruited, and followed up the adult transplanted patients, performed research/study, analyzed the results, and wrote the manuscript.

### Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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