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**OR 7337**

# Long-term outcomes and clinical impact of anti-HLA donor-specific antibodies (DSA) after liver transplantation: prospective study in a pilot cohort.

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## ABSTRACT

The presence of donor-specific antibodies (DSA) has been considered to affect survival of allograft and patient after liver transplantation (LT). However, their significance is not well understood.

We performed a prospective study of 32 adult patients who underwent LT in 2011 to analyse the existence of DSA, associated risk factors, and medium-term impact. Immunological determinations were performed immediately before LT and at 3, 6, 12 months and 5 years after LT.

Eight patients (24.2%) presented preformed DSA. However, titres were negative in all patients 5 years after LT and there were no events associated. Eight out of 24 patients (33.3%) developed *de novo* DSA. After 5 years, only 2 remained positive; both were class II with high MFI values at diagnosis (over 15 000). No association was found between development of DSA and risk of rejection, graft loss, or death. However, an increase in liver stiffness values was observed in patients with persistent DSA; in one, we recorded focal sinusoidal deposition of C4d and moderate liver fibrosis.

In conclusion, the incidence of DSA is high after LT. In addition, the persistence of *de novo* DSA could be associated with silent liver fibrosis with potential impact on graft outcomes.

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**Abbreviations**

LT: Liver transplantation; DSA: donor-specific antibodies; AMR: antibody-mediated rejection; HCV: hepatitis C virus; MMF: Mycophenolate mofetil

**Keywords**

Liver transplantation, donor-specific antibodies, antibody-mediated rejection

## INTRODUCTION

The prevalence and incidence of anti-HLA donor-specific antibodies (DSA) and their impact on clinical outcomes after liver transplantation (LT) are poorly characterized. In the last decade, the interest in the potential negative consequences of DSA after LT is growing. Their presence has been related to various patterns of liver injury (acute and chronic AMR) and also to a negative impact in allograft survival and patient mortality (1–3) in cross-sectional studies. However, there are no prospective, long-term studies evaluating the presence and clinical relevance of DSA after LT. Therefore, the aim of our study was to prospectively analyse the epidemiology, long-term outcome, and clinical impact of DSA after LT.

## PATIENTS AND METHODS

We performed an observational, prospective, single-centre study including all LT performed in recipients aged ≥18 years in 2011 in the Liver Transplant Unit of Gregorio Marañón Hospital. The only exclusion criterion was to have previously undergone non-liver solid organ transplantation, patients with combined organ transplantation were excluded. This is a pilot study and as such, a sample size calculation was not performed.

All patients were managed and followed up according the pre-defined protocol of the Liver Transplant Unit. Clinical evaluation consisted of targeted, symptom-specific examinations and laboratory tests, which were weekly during the first month, every 15 days until the third month, and every month during the first year. Clinical evaluations were then performed every 3 to 6 months. The initial immunosuppressive regimen

was based on tacrolimus, with targeted tacrolimus trough levels of 8 ng/ml and corticosteroids, descending progressively from 200 mg until its withdrawal at 3-4 months after transplantation. Basiliximab was used as induction therapy in patients with pretransplant renal failure. Mofetil mycophenolate was introduced with an initial dose of 1000 mg every 24 hours up to a maximum dose of 2000 mg every 24 hours, to minimize the dose of calcineurin inhibitors in patients with renal failure, diabetes mellitus, arterial hypertension, or neurotoxicity. Patients were followed up until August 2016.

To detect graft fibrosis, liver stiffness measurements (Fibroscan 502 Touch, Echosens Paris) were routinely performed in all patients at 5 years after transplantation. Blood samples for DSA determinations were obtained immediately before LT and every 3 months during the first year. After 5 years of LT, another blood sample was obtained. The presence, class (anti-HLA class I, anti-HLA class II, and anti-major histocompatibility complex class I-related chain A [anti-MICA]) and mean fluorescence intensity (MFI) of DSA were analysed in a fluoro analyser LABScan 100 (Luminex, Austin, Tx). Donors and recipients' HLA were also typed (PCR-SSO reverse, One Lambda, Canoga Park, CA). Liver biopsy was performed in patients who showed *de novo* DSA positivity with MFI over 5000 associated to an increase in liver stiffness measurement over 7.5 kPa at 5 years after LT.

The study was performed according to the International Guidelines for Ethical Review of Epidemiological Studies (Council for the International Organizations of Medical Sciences [CIOMS], Geneva, 2008) and the Declaration of Helsinki (Seul, October 2008). The study was reviewed and approved by the local Clinical Research Ethics Committee (Code of approval 112/10) All patients gave their written informed consent prior to enrolment.

**STATISTICAL ANALYSIS**

Quantitative variables are expressed as mean (SD) or median (range), as appropriate, and were compared using the *t* test or its non-parametric equivalent. Qualitative variables are expressed as percentages (%) and were compared using the $\chi^2$ or Fisher exact test, as appropriate. The correlation between quantitative variables was

analysed using Pearson's correlation index. Univariate and multivariate regression analysis were performed to explore factors associated with the development of preformed and *de novo* DSA. To compare graft and patient survival in patients with and without DSA, Kaplan-Meier curves were constructed and compared using the log-rank test. An alpha value < 0.05 (2-sided) was considered statistically significant. All statistical analyses were performed using STATA 12.1.

**RESULTS**

We included 36 out of 40 LT performed during the study period that met the inclusion criteria and had no exclusion criteria. All patients included in the study were transplanted with whole liver grafts from brain-dead donors. DSA determinations were not possible in 4 patients for technical reasons. Four patients did not complete the study owing to early death associated with surgical complications (n=2) or loss to follow-up (n=2). Therefore, the study cohort comprised 32 patients.

Demographic and clinical baseline data are shown in table 1. Decompensated cirrhosis (53.1%) was the main reason for LT, followed by hepatocellular carcinoma (40.6%). All patients received tacrolimus as the main immunosuppressive drug. Mycophenolate mofetil (MMF) was also used in 18 patients (56.3%). Basiliximab was used as induction therapy in 14 patients (43.8%).

**Pre-formed DSA**

Eight patients (24.2%) had pre-formed DSA: 4 were class I DSA, 3 class II, and 1 anti MICA. The mean fluorescence intensity was 10250 (range 2800-23000). Preformed DSA remained positive in 4 out of 8 patients (50%) 12 months after LT. However, persistence of preformed DSA after 5 years was not recorded in any cases (Figure 1).

Patients with pre-formed DSA positivity were younger (46.2 (4.6) vs. 53.5 (1.8) years, p=0.08) and predominantly female (50 vs. 16%, p=0.06) compared to patients without pre-formed DSA, though not statistically significant. Most of the patients who presented pre-formed DSA had previously received a transfusion (62.5% vs. 36%, p=0.236). There were no differences in the presence of DSA related to previous pregnancy, HCV infection, or donor age.

Univariate analysis only showed a trend for the association between female sex (OR 5.0; 95% CI 0.87-28.86; p=0.072) and the presence of pre-formed DSA (Table 2).

No differences were found for graft dysfunction, clinical events, or 5-year liver stiffness (8.9 [3.4] kPa vs 8.3 [1.1] kPa) among patients with or without pre-formed DSA. Furthermore, there were no differences in clinical outcomes among patients who lost pre-formed DSA during the first year after LT or later.

***De Novo* DSA**

No patients with preformed DSA developed *de novo* DSA during follow-up. Eight out of 24 patients without pre-formed DSA (33.3%) developed *de novo* DSA (1 class I DSA, 6 class II, and 1 anti MICA); this was within the first year after LT in 7 cases. *De novo* DSA remained positive at 5 years in only 2 cases. Both cases had class II DSA and showed higher MFI values (over 15000) at diagnosis than patients who lost *de novo* DSA. (Figure 1). There were no differences in age, sex, aetiology of liver disease, or history of past transfusions in patients who developed *de novo* DSA. Interestingly, patients with *de novo* DSA less frequently received MMF as part of their immunosuppressive therapy (37.5% vs. 62.5%, p=0.217) although these differences did not reach statistical significance. No differences were found between patients with persistent *de novo* DSA and non-persistent *de novo* DSA for tacrolimus levels at one and three months after liver transplantation [(9.8 vs 14.1, p=0.126) and (8.5 vs 10.1, p=0.632), respectively]. No differences were found between the groups for administration of basiliximab. Univariate regression analysis did not reveal any variables associated with the development of *de novo* DSA (Table 3). No association was detected between the appearance of *de novo* DSA and graft damage or liver-related clinical outcomes. However, the two patients who had persistent *de novo* DSA showed an increase in liver stiffness values (10.1 and 10.2 kPa respectively) at the fifth year after LT. That was not observed in patients with transient DSA (10.15 (0.05) kPa vs 6.7, (1.2) kPa, p=0.114) although these differences did not reach statistical significance. Interestingly, there were no alterations in the laboratory profile of the 2 patients.

Liver biopsy was performed in the two patients who presented *de novo* DSA with MFI over 5000 and an increase in liver stiffness measurement over 7.5 kPa at 5 years after

LT. In one patient with a very high MFI value (21000) at 5 years, we detected focal sinusoidal deposition of C4d and moderate liver fibrosis with isolated porto-portal incomplete bridges associated with mild to moderate lymphocytic inflammatory infiltrate without lobular inflammation (Figure 2). Histological findings of chronic rejection or ductular damage were absent. There were no alterations in the liver function tests at this time point (ALT 13 U/L, AST 11 U/L, Bilirubin 0.6 mg/dL, GGT 40 U/L, FA 85 U/L, Creatinine 1.30 mg/dL, Albumin 4.9 g/dL, INR 1.03, APTT 28.5 seconds, Fibrinogen 379 mg/dL, Na 139 mmol/L). Immunosuppression consisted of cyclosporine 50mg every 12 hours with cyclosporine levels of 124 ng/mL. Immunosuppression was changed from tacrolimus to cyclosporine because of poorly controlled diabetes mellitus. The patient was compliant with the treatment. Given the findings described above, MMF 500mg every 12 hours was added to treatment.

The second patient with abnormal stiffness had a moderate MFI value (6000); we only observed non-specific portal inflammation with mild to moderate inflammatory infiltrate in portal spaces comprising mainly lymphocytes associated with mild portal tract expansion and without fibrosis. No C4d deposition was observed. Histological findings of chronic rejection or ductular damage were absent. There were no alterations in the liver function tests at this time point (ALT 18 U/L, AST 20 U/L, Bilirubin 0.4 mg/dL, GGT 26 U/L, FA 85 U/L, Creatinine 1.39 mg/dL, Albumin 4.2 g/dL, INR 0.98, APTT 35.7 seconds, Fibrinogen 602 mg/dL, Na 140 mmol/L). Immunosuppression regimen consisted on tacrolimus 1mg every 24 horas with tacrolimus levels of 3.3 ng/mL. The patient was compliant with the treatment. No changes were made on immunosuppression management.

There was a third patient who presented *de novo* DSA at 5 years after LT, showing a liver stiffness measurement of 5.7 kPa and completely normal liver function tests.

None of the three patients were treated with interferon or direct antiviral (AAD) treatment against hepatitis C.

Patient and graft survival were similar in patients with and without DSA (figure 3).

Two patients died during the study period: one patient who presented not persistent *de novo* DSA died due to an intracerebral hemorrhage; the second patient who did not had pre-formed or *de novo* DSA died due to recurrence of hepatocellular carcinoma.

There were no cases of graft loss.

**DISCUSSION**

DSA have been implicated in asymptomatic progressive liver graft damage characterized by mild to moderate fibrosis and C4d deposition(4). Importantly, data suggesting a pathological role of DSA after LT are based on cross-sectional studies, thus limiting knowledge regarding epidemiology and influence on natural history after transplantation. Furthermore, the applicability of DSA determinations in clinical practice is unclear. Therefore, our objective was to prospectively evaluate the impact of DSA after LT in a cohort of recipients followed up in the long term.

We found that positivity of pre-formed DSA at the time of LT is not an infrequent event, but that it is transient and not associated with liver allograft damage. In fact, we observed that, similar to previous studies(5,6), pre-formed DSA became negative after 5 years of follow-up in all cases. Furthermore, we did not find any association between preformed DSA and graft damage. Importantly, DSA clearance and the lack of clinical consequences were observed even though MFI levels were greater than 5000 in all cases. In contrast, some reports have suggested that pre-formed DSA with MFI levels > 5000 may be persistent and could be associated with C4d deposits in liver biopsy(7). We confirmed that the prevalence of factors predisposing to the presence of pre-formed DSA was related to female gender, as previously described(8). However, we did not find any association between pre-formed DSA and previous transfusions or pregnancy, both of which have been implicated in the positivity of pre-formed DSA(9).

In our study, a significant proportion of patients developed *de novo* DSA (33.3%) during the first year; this incidence was greater than that reported in other studies. For instance, Kaneku et al. described among a cohort of 749 LT recipients, an incidence of *de novo* DSA (with an MFI ≥ 5,000) during the first year after LT of 8.1% (10). Del Bello et al detected de novo DSA (with an MFI ≥ 1,000) in 14% of 152 patients in a cross sectional study with a median time of follow-up of 34 months(11). Furthermore, in the French cross-sectional study recently reported by Vandevoorde et al., 19.9% presented de novo DSA after LT: 20/164 (12.2%) at one year, 29/217 (13.4%) at five years, and 26/133 (19.5%) at ten years(12). This difference in the incidence of *de novo* DSA is

likely to be due to the prospective design and the systematic determination in all LT recipients in our series, and probably reflects the real incidence of the phenomenon. Interestingly, the appearance of *de novo* DSA after the first year is unlikely. A relevant finding is that there were no clinically predictive factors such as age, sex, aetiology, or previous transfusions for the development of *de novo* DSA. However, the type of immunosuppressive regimen could condition *de novo* appearance of DSA. Although previous studies have reported the use of cyclosporine and low doses of calcineurin inhibitors as risk factors for occurrence of DSA(10–12), there were no differences in average tacrolimus levels or the use of basiliximab induction therapy in our study. However, patients who received MMF developed de novo DSA less frequently (37.5% vs. 62.5), though not statistically significant. However, data on the effect of MMF on *de novo* DSA formation are controversial. Studies in kidney transplantation have shown inconsistent results regarding the influence of MMF on the development of DSA [23,24]. Retrospective studies in LT recipients, have also shown varying results(10,15). Although some studies suggest a modest benefit of administering MMF, this effect may simply result from increased intensity of immunosuppressive regimens that include MMF therapy, rather than a specific mechanism to reduce the risk of *de novo* DSA.

The most important finding of our study is probably that the impact of DSA on liver damage seems to be restricted to those cases with persistent *de novo* DSA, as suggested by the increased stiffness and histological changes that were only observed in the 2 patients in whom DSA remained positive (with MFI over 5000) after the first year. Remarkably, the MFI value doubled in patients with persistent DSA. Our results suggest that further DSA determinations are unnecessary in patients in whom *de novo* DSA did not appear within the first year. In contrast, the evaluation of persistence and titers of DSA after the first year is mandatory in patients with early positivity. Furthermore, and owing to the fact that it is non-invasive, regular measurement of liver stiffness may help in the evaluation of these patients.

Although it has been reported that graft and patient survival are lower in patients with persistent *de novo* DSA (10,16), we did not confirm this finding. In fact, graft and patient survival were similar in patients with and without DSA in our series. However,

it should be emphasized that chronic AMR typically appears as asymptomatic progression of fibrosis in patients with normal or near normal liver chemistry. Furthermore, it has been suggested that AMR may be more common than previously reported[29–32]. We partially confirmed this aspect; the 2 patients who had persistent *de novo* DSA had abnormal liver stiffness values without alterations in liver chemistry at 5 years. Interestingly, both patients had class II DSA with elevated MFI titres; this finding is consistent with previous reports(21). According to our results, in selected patients who develop persistent *de novo* DSA with high MFI after 1 year of LT may be monitored with periodic DSA testing and regular evaluation of liver stiffness aimed to unmask subclinical alloimmune mediated graft dysfunction, aligning with previous observations. This approach may help to optimize immunosuppression and ultimately preventing irreversible damage and graft loss. When liver stiffness is elevated (>7.5 kPa), a confirmatory liver biopsy should be performed to detect C4d deposition and to confirm liver fibrosis or inflammation. However, additional studies with larger number of patients are needed to confirm the utility of liver stiffness for monitoring patients with de novo DSA.

Our study is subject to limitations. First, the small number of patients limits the strength of our conclusions and decreases their generalizability. Moreover, the absence of protocol liver biopsies impedes the identification of silent liver damage and the evaluation of any potential association with the existence of DSA. Finally, since antibody subclasses were not determined, the impact of the different antibody subclasses on tissue injury patterns and on the mechanisms of liver damage cannot be ascertained.

In conclusion, our results suggest that the incidence of DSA is frequent after LT. Even though DSA are usually cleared after LT without any impact on long term survival, the persistence of *de novo* DSA could be associated with silent liver fibrosis with potential impact on graft outcomes. However, further prospective studies with a larger number of patients are needed to fully characterize the influence of DSA on the natural history of LT.

**CONFLICTS OF INTEREST**

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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**Table 1. Patients' characteristics n = 32.**

| | |
|---|---|
| Female gender (%) | 8/32 (25) |
| Age (mean (SD); years) | 51.69 (10.36) |
| Transfusion before LT (%) | 13/32 (40.63) |
| **Etiology (%)** | |
| Alcohol abuse | 8/32 (25) |
| HCV | 15/32 (46.88) |
| HBV | 4/32 (12.50) |
| Cholestatic liver disease | 4/32 (12.50) |
| Autoimmune | 1/32 (3.13) |
| **Transplant indication (%)** | |
| Decompensated cirrhosis | 17/32 (53.13) |
| Hepatocellular carcinoma | 13/32 (40.63) |
| Fulminant hepatic failure | 1/32 (3.13) |
| Late retransplant | 1/32 (3.13) |
| **Initial immunosuppression (%)** | |
| Tacrolimus | 32/32 (100) |
| Mycophenolate mofetil | 18/32 (56.25) |
| Esteroids | 30/32 (93.75) |
| Basiliximab | 14/32 (43.75) |
| **Tacrolimus levels (mean (SD); ng/ml)** | |
| First month | 12.04 (4.30) |
| Third month (31/33) | 9.59 (3.15) |
| Sixth month (30/33) | 8.36 (2.64) |

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| | OR | 95%CI | p |
|---|---|---|---|
| **Female gender** | 5.00 | (0.87-28.86) | 0.072 |
| **Age at transplantation** | 0.94 | (0.86-1.01) | 0.112 |
| **Previous blood transfusions** | 3.33 | (0.63-17.60) | 0.156 |
| **HCV positivity** | 0.28 | (0.05-1.69) | 0.166 |
| **Hepatocellular carcinoma** | 0.84 | (0.16-4.35) | 0.835 |
| **Donor's gender** | 1.18 | (0.24-5.86) | 0.838 |
| **Donor's age** | 0.96 | (0.91-1.02) | 0.166 |
| **Gender missmatch** | 1.66 | (0.33-8.37) | 0.535 |

**Table 2. Univariate logistic regression for the presence of preformed DSA.**

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**Table 3. Univariate logistic regression for the presence of de Novo DSA.**

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| | OR | 95%CI | p |
|---|---|---|---|
| **Female gender** | 0.62 | (0.05-7.12) | 0.700 |
| **Age at transplantation** | 0.97 | (0.88-1.07) | 0.590 |
| **Previous blood transfusions** | 3.00 | (0.50-17.95) | 0.229 |
| **HCV positivity** | 1.67 | (0.29-9.45) | 0.564 |
| **Hepatocellular carcinoma** | 0.77 | (0.14-4.39) | 0.770 |
| **Donor's gender** | 1.29 | (0.23-7.05) | 0.772 |
| **Donor's age** | 1.02 | (0.94-1.09) | 0.677 |
| **Gender missmatch** | 1.00 | (0.17-5.77) | 1.000 |
| **MMF** | 0.36 | (0.06-2.08) | 0.253 |
| **Tacrolimus 1 month** | 1.06 | (0.88-1.28) | 0.538 |
| **Tacrolimus 3 months** | 1.09 | (0.79-1.50) | 0.606 |
| **Tacrolimus 6 months** | 0.93 | (0.65-1.35) | 0.710 |
| **Basiliximab** | 1.67 | (0.30-9.27) | 0.560 |

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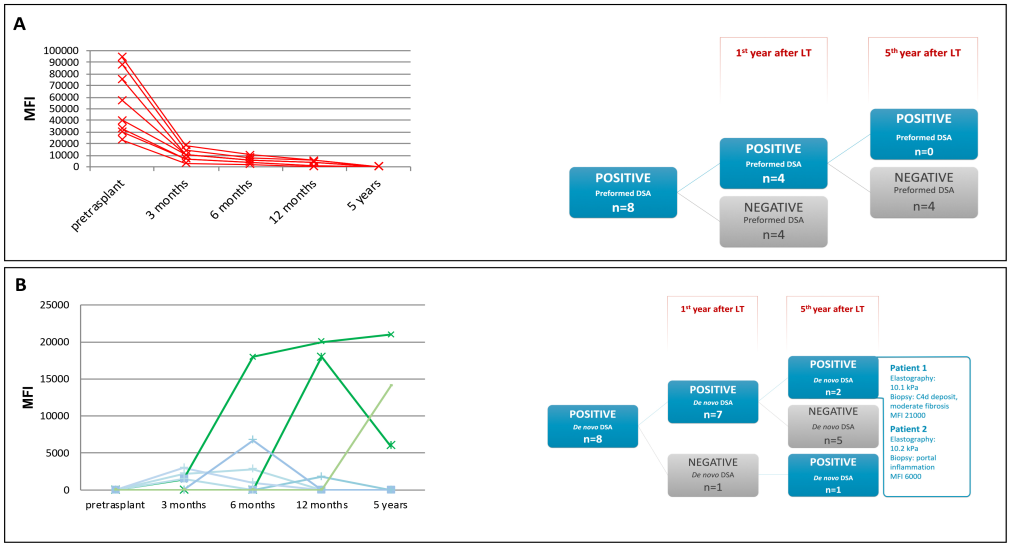


Figure 1. A) Evolution of Preformed DSA during follow-up. B) Evolution of *De novo* DSA during follow-up.

**Figure 1. A) Evolution of Preformed DSA during follow-up. B) Evolution of De novo DSA during follow-up.**

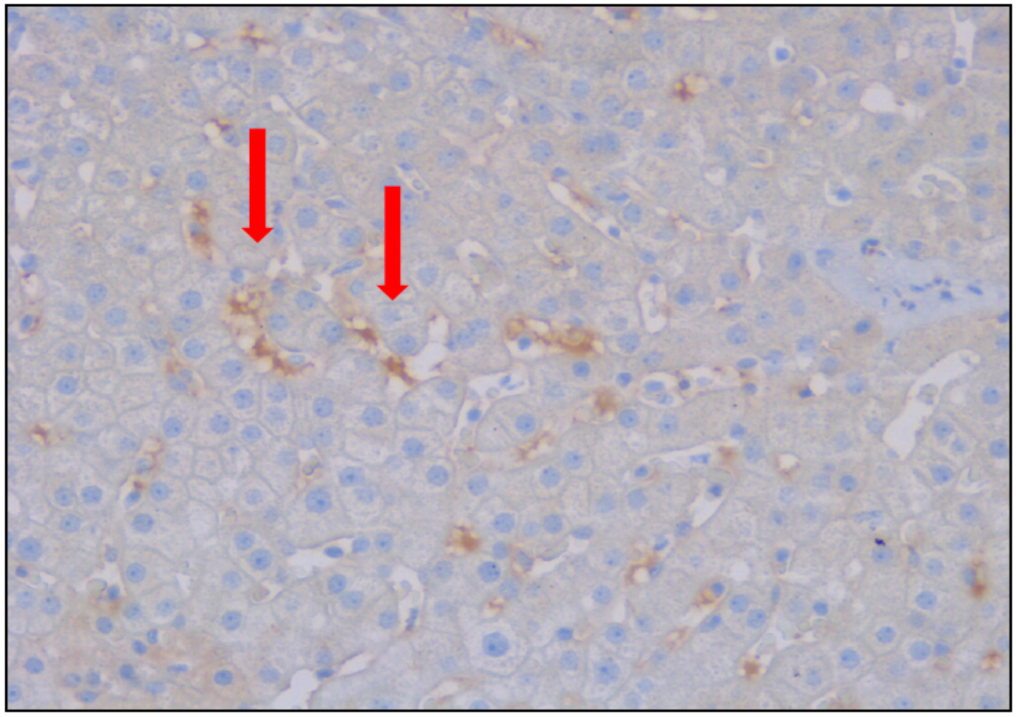

Figure 2. Patient 1 liver biopsy: C4d focal sinusoidal staining (red arrows).

**Figure 2. Patient 1 liver biopsy: C4d focal sinusoidal staining (red arrows).**

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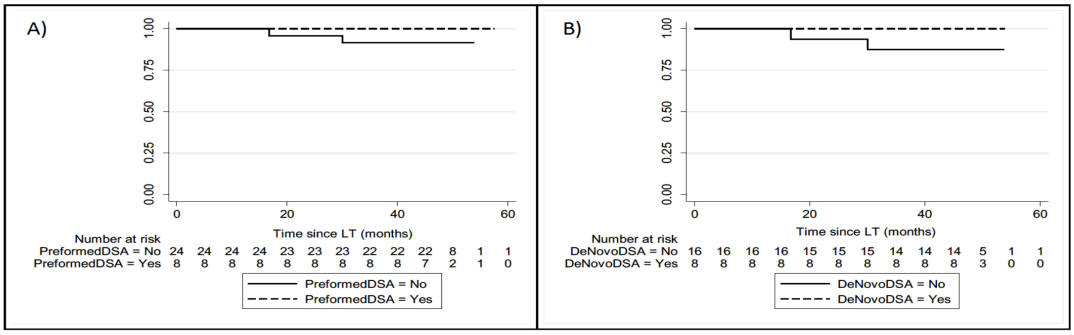


**Figure 3. A) Kaplan Meier survival analysis in patients with and without pre formed DSA. Log rank test p=0.41. B) Kaplan Meier survival**

**Figure 3. A) Kaplan Meier survival analysis in patients with and without pre-formed DSA. Log rank test p=0.41. B) Kaplan Meier survival analysis in patients with and without de novo DSA (8 patients with pre-formed DSA excluded). Log rank test p=0.31.**