Research Article
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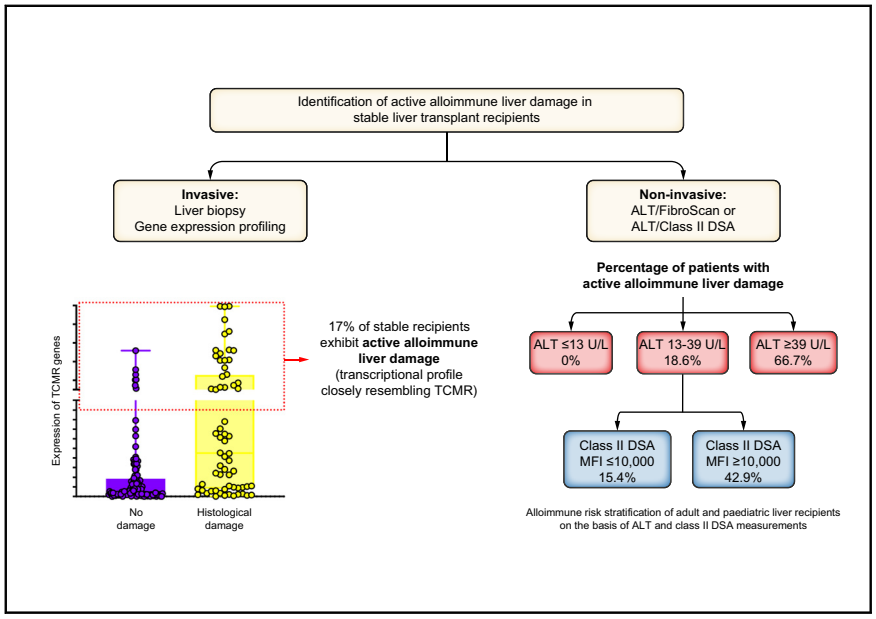
Non-invasive alloimmune risk stratification of long-term liver transplant recipients

Graphical abstract



Highlights

- 22% of stable liver recipients harbour moderate-to-severe subclinical immune allograft damage.
- Subclinical damage is linked to allograft immunogenicity and degree of immunosuppression.
- Recipients with active underlying alloimmunity can be identified using non-invasive markers.

Authors

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Lay summary

A large proportion of liver transplant patients with normal liver tests have inflammatory liver lesions, which in 17% of cases are molecularly indistinguishable from those seen at the time of rejection. ALT, class II donor-specific antibodies and liver stiffness are useful in identifying patients with this form of subclinical rejection. We propose these markers as a useful tool to help clinicians determine if the immunosuppression administered is adequate.

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Non-invasive alloimmune risk stratification of long-term liver transplant recipients

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Background & Aims: Management of long-term immunosuppression following liver transplantation (LT) remains empirical. Surveillance liver biopsies in combination with transcriptional profiling could overcome this challenge by identifying recipients with active alloimmune-mediated liver damage despite normal liver tests, but this approach lacks applicability. Our aim was to investigate the utility of non-invasive tools for the stratification of stable long-term survivors of LT, according to their immunological risk and need for immunosuppression.

Methods: We conducted a cross-sectional multicentre study of 190 adult LT recipients assessed to determine their eligibility to participate in an immunosuppression withdrawal trial. Patients had stable liver allograft function and had been transplanted for non-autoimmune non-replicative viral liver disease >3 years before inclusion. We performed histological, immunogenetic and serological studies and measured the intrahepatic transcript levels of an 11-gene classifier highly specific for T cell-mediated rejection (TCMR).

Results: In this cohort, 35.8% of patients harboured clinically silent fibro-inflammatory liver lesions (13.7% had mild damage and 22.1% had moderate-to-severe damage). The severity of liver allograft damage was positively associated with TCMR-related transcripts, class II donor-specific antibodies (DSAs), ALT, AST, and liver stiffness measurement (LSM), and negatively correlated with serum creatinine and tacrolimus trough levels. Liver biopsies were stratified according to their TCMR transcript levels using a cut-off derived from biopsies with clinically significant TCMR. Two multivariable prediction models, integrating ALT+LSM or ALT+class II DSAs, had a high discriminative capacity for classifying patients with or without alloimmune damage. The latter model performed well in an independent cohort of 156 liver biopsies obtained from paediatric liver recipients with similar inclusion/exclusion criteria.

Conclusion: ALT, class II DSAs and LSM are valuable tools to non-invasively identify stable LT recipients without significant underlying alloimmunity who could benefit from minimisation of immunosuppression.

Lay summary: A large proportion of liver transplant patients with normal liver tests have inflammatory liver lesions, which in 17% of cases are molecularly indistinguishable from those seen at the time of rejection. ALT, class II donor-specific antibodies and liver stiffness are useful in identifying patients with this form of subclinical rejection. We propose these markers as a useful tool to help clinicians determine if the immunosuppression administered is adequate.

Keywords: LT; biomarkers; non-invasive; T cell-mediated rejection; fibrosis; gene-expression profiling; tolerance; FibroScan; immunosuppression minimisation; HLA epitope mismatch; PIRCHE-II score; DSA.
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Introduction

There are currently thousands of clinically stable long-term survivors of adult and paediatric liver transplantation (LT)

worldwide. Pharmacological immunosuppression (IS) remains one of the key modifiable factors influencing their long-term outcomes following LT. Thus, after LT, the incidence of comorbidities such as chronic kidney disease, *de novo* malignancies, metabolic syndrome and cardiovascular diseases, all known to be negatively influenced by prolonged and/or excessive IS, gradually accumulates with time.[1–4] Numerous trials of IS withdrawal have now shown that a large proportion of long-term survivors of LT, with stable allograft function and no significant histological abnormalities, can substantially reduce their overall IS levels, and, in some cases, even completely discontinue IS.[5] Considering that the risk of T cell-mediated rejection (TCMR) following transplantation decreases with time,[6,7] the evidence suggests that a large number of long-term survivors could tolerate lower levels of IS. Conversely, there is ample literature indicating that approximately 30% of well-functioning allografts harbour significant subclinical damage. The molecular profiling of these silent lesions closely overlaps with the transcriptional characteristics of clinically apparent TCMR, which suggests inadequate IS.[8,9] Whereas silent or subclinical liver inflammatory lesions do not appear to compromise short-term outcomes, accumulating evidence indicates that they might threaten long-term allograft longevity, both in paediatric and adult LT recipients.[8,10] Previous work from our team indicates that this is more likely to happen when allografts exhibit high transcript levels of TCMR-related genes.[8]

The dichotomy between too little and too much IS underlines the limitations of the current standard of care, which relies on the empiric use of crude parameters such as liver tests, calcineurin inhibitor trough levels, transplant indication, recipient age and time post-transplantation. The performance of surveillance liver biopsies, particularly when combined with transcriptional profiling tools, has the potential to overcome these limitations, but is championed by only a small number of clinical sites worldwide due to costs, risks, sampling errors and inter-observer variability.

In the current study, we analysed liver biopsies and the clinical and laboratory data prospectively collected at the time of screening for participation in the Liver Immunosuppression Free Trial (*LIFT*). Our aims were to identify parameters associated with clinically silent but active immune-mediated liver allograft damage and to design prediction models to non-invasively stratify stable long-term survivors of LT according to their immunological risk and need for IS.

Patients and methods

Study design

We carried out a cross-sectional study in which we included all 190 patients who underwent a liver biopsy between October 2015 and May 2019 as part of the screening procedures to participate in *LIFT* (clinicaltrials.gov NCT024989977), an ongoing multicentre, European randomised controlled trial of IS withdrawal in stable adult long-term LT recipients. The study was approved by the corresponding research ethics committee in each participating country (United Kingdom, Germany, Belgium, Spain) and informed consent was obtained from all participants.

Key inclusion/exclusion criteria

All participants were adult (≥18 years old) stable LT recipients at least 3 years post-transplant and had normal liver function tests at the screening visit, as defined by direct bilirubin <17.1 μmol/L and alanine aminotransferase (ALT) <60 U/L. Patients with active viral infection (HBV, HCV or HIV), pre- or post-transplant autoimmune liver disease, or acute or chronic rejection within the 52 weeks prior to the screening visit were excluded.

Study participant assessments

The following evaluations were performed at inclusion: biochemical, hematological and serological tests; human leukocyte antigen (HLA) typing and alloantibody characterisation; transient elastography-based liver stiffness measurement (LSM); detailed histopathology assessment of screening liver biopsies; and liver tissue gene expression experiments. Detailed methods are provided in the supplementary information.

Statistical analysis

Descriptive statistics included median and range or mean (SD) for continuous variables, and frequencies and percentages for categorical variables. Categorical data were compared by Chi-square or Fischer exact test and continuous variables by *t* test or non-parametric testing (Mann-Whitney), as appropriate. We employed Spearman correlation to generate a similarity matrix of key parameters collected at study entry. We constructed risk prediction models with multivariable binary logistic regression in which the outcome variable was probTCMR >0.09. Selection of this threshold is detailed in the Results section. To assess the performance of the risk prediction models, we assessed their discriminative ability using receiver-operating characteristic (ROC) curve analyses, and their calibration by plotting the comparison between predicted and observed proportions after grouping the patients. Nomograms were based on the corrected logistic regression models. Statistical analyses were performed using SPSS package version 26 and R platform.[11]

Results

Main patient characteristics

The characteristics of the 190 patients enrolled in the study are displayed in Table 1. Patients were mainly male (66.8%) and Caucasian (95.3%). At the time of the screening liver biopsy, the median age of our cohort was 61 (29-78) years and time from LT was 8 (3-26) years. Most patients had received a graft from a deceased donor (97.4%; whole organ 93.7%) and the most frequent indications for LT were alcohol-related cirrhosis (25.8%) and cirrhosis due to chronic HBV or HCV infection (31.6%). At the time of study entry, 96.7% of patients were on calcineurin inhibitor-based IS (69.2% on tacrolimus monotherapy) and the median ALT and direct bilirubin levels were 20 (8-80) U/L and 3 (0-11) μmol/L, respectively. Of note, 2 patients had ALT levels of 63 and 80 U/L at enrolment. This was considered a protocol violation but patients were analysed with the rest of the cohort.

Histological evaluation of protocol liver biopsies

The most frequent histological abnormalities were portal inflammation, fibrosis, and lobular inflammation, present in 144 (75.8%), 119 (62.6%) and 115 (60.5%) biopsies, respectively (Table 2). Portal inflammation was moderate to severe in 34 patients (17.9%), with simultaneous interface hepatitis in 31 of them. Advanced fibrosis was found in 19 patients, including: cirrhosis (Ishak score 6) in 2, incomplete cirrhosis (Ishak score 5) in 6 and advanced fibrosis (Ishak score 3 and 4) in 13. Ordinal logistic regression analysis revealed a significant association between portal inflammation severity and Ishak fibrosis stage.

Table 1. Characteristics of the liver transplant recipients included in the study.

Characteristics	
Age at the time of enrolment (years)	61 (29-78)
Male sex (n, %)	127 (66.8%)
BMI (kg/m^2)	27.4 (18.3-52.8)
Indication for LT (n, %)	
Alcohol-related cirrhosis	49 (25.8%)
Hepatitis B or C-related cirrhosis	60 (31.6%)
Other	81 (42.6%)
Presence of hepatocellular carcinoma	45 (23.7%)
Time from LT to liver biopsy	8 (3-26)
Comorbidities (n, %)	
Diabetes mellitus	49 (25.8%)
Arterial hypertension	96 (50.5%)
Dyslipidaemia	28 (14.7%)
Cardiovascular disease	15 (7.9%)
Malignancy	64 (33.7%)
Immunosuppression (n, %)	
Tacrolimus (±MMF, AZA, sirolimus)	159 (87.4%)
Cyclosporine (±MMF)	17 (9.3%)
Sirolimus (monotherapy)	1 (0.01%)
MMF or AZA (monotherapy)	5 (2.7%)
Tacrolimus trough levels (μg/L)	3.9 (0.5-10.7)
Cyclosporine trough levels (μg/L)	53 (23-223)
AST/ALT/Mean ALT* (U/L)	23 (9-67)/20 (8-80)/20 (8-80)
ALP/GGT (U/L)	77 (33-245)/24 (9-762)
Biochemistry	
Total bilirubin (μmol/L)	8 (2-39)
Direct bilirubin (μmol/L)	3 (0-11)
Creatinine (μmol/L)	88 (44-159)
White blood cell count (x 10^3/mm^3)	6.2 (2.8-31.7)
Platelet count (x 10^3/mm^3)	201 (71-479)
Liver stiffness measurement (Fibroscan)	4.9 (2.4-21.1)
APRI score	0.30 (0.10-0.90)
FIB-4 score	1.57 (0.53-4.43)

ALP, alkaline phosphatase; ALT, alanine aminotransferase; APRI, AST to platelet ratio index; AST, aspartate aminotransferase; AZA, azathioprine; FIB-4, Fibrosis-4 score; GGT, gamma-glutamyltransferase; LT, liver transplantation; MMF, mycophenolate mofetil.
*Mean ALT corresponds to the mean of 1-3 measurements conducted over a median period of 76 days. Continuous data are expressed as median (range).

Thus, the odds of a greater degree of fibrosis in patients with mild, moderate and severe inflammation were 4.3 (95% CI 2.1-8.9, p <0.001), 33.6 (95% CI 12.4-91.0, p <0.001) and 453.3 (95% CI 10.7-19,221.0, p <0.005), when compared to the absence of inflammation. Of note, out of the 144 patients with portal inflammation, 113 had portal or perisinusoidal fibrosis. Forty patients had portal inflammation (mild in 37 and moderate in 3 patients) in the absence of fibrosis, whereas fibrosis without portal inflammation was only observed in 15 patients (mild in all).

Overall, the liver biopsies of 122 (64.2%) participants had no or minimal allograft damage and met the criteria required by the Banff Working Group on Liver Allograft Pathology to consider IS minimisation.[12] Among the liver biopsies of the 68 (35.8%) participants considered unsuitable for minimisation, those exhibiting portal inflammation grade ≥2, interface hepatitis grade ≥2, or fibrosis ≥2 in 2 of the 3 compartments or ≥3 in 1 compartment (according to Venturi *et al.*[13]) were considered to exhibit moderate-to-severe histological damage. All remaining biopsies were deemed to show mild histological damage. Using these criteria, biopsies were grouped into 3 categories (groups 1, 2 and

Table 2. Histological characteristics of the 190 baseline liver biopsies.

Histological features	
Lobular inflammation	
• Absence	75 (39.5%)
• Mild	108 (56.8%)
• Moderate	6 (3.2%)
• Marked	1 (0.5%)
Portal inflammation	
• Absence	46 (24.2%)
• Mild	110 (57.9%)
• Moderate	33 (17.4%)
• Marked	1 (0.5%)
Interface hepatitis	
• Absence	130 (68.4%)
• Mild	51 (26.8%)
• Moderate	7 (3.7%)
• Marked	2 (1.1%)
Central perivenulitis	
• Absence	159 (83.7%)
• Mild	28 (14.7%)
• Moderate	3 (1.6%)
• Marked	0 (0%)
Portal vein endothelitis	
• Absence	137 (72.1%)
• Mild	52 (27.4%)
• Moderate	1 (0.5%)
• Marked	0 (0%)
Fibrosis (Ishak)	
• Absence	71 (37.4%)
• Mild	87 (45.8%)
• Moderate	11 (5.8%)
• Occasional bridging	10 (5.3%)
• Marked bridging	3 (1.6%)
• Incomplete cirrhosis	6 (3.2%)
• Complete cirrhosis	2 (1.1%)
Portal/Periportal fibrosis#	
• Absence	83 (43.7%)
• Focal	70 (36.8%)
• Marked	24 (12.6%)
• Severe	13 (6.8%)
Perisinusoidal fibrosis#	
• Absence	95 (50.0%)
• Focal	86 (45.3%)
• Marked	9 (4.7%)
• Severe	0 (0.0%)
Perivenular fibrosis#	
• Absence	146 (76.8%)
• Focal	37 (19.5%)
• Marked	6 (3.2%)
• Severe	0 (0.0%)
Steatosis	
• Absence	114 (60.0%)
• Mild	61 (32.1%)
• Moderate	14 (7.4%)
• Severe	1 (0.5%)
Regenerative hyperplasia	
• Absence	104 (54.7%)
• Focal	83 (43.7%)
• Diffuse	3 (1.6%)
Bile duct lesions	
• Absence	138 (72.6%)
• Mild	46 (24.2%)
• Moderate	3 (1.6%)
• Marked	3 (1.6%)
Bile duct loss	
• Absence	178 (93.7%)
• <50% of portal tracts	10 (5.3%)
• >50% of portal tracts	1 (0.5%)

(*continued on next page*)

Table 2. (*continued*)

Histological features	
Ductular reaction	
• Absence	114 (60.0%)
• Presence	76 (40.0%)

Numbers refer to the number of biopsies displaying each feature.
[#]As described by Venturi *et al.*[13]

3 in Table 3): no or minimal allograft damage (64.2%), mild damage (13.7%), and moderate-to-severe damage (22.1%).

To determine which variables influenced allograft damage and to explore their inter-relationships, we constructed a similarity matrix incorporating histological, demographic, clinical and laboratory parameters (Fig. 1). Allograft damage was positively correlated with donor-specific antibodies (DSAs), liver stiffness measurements (LSM) and serum aspartate aminotransferase (AST)/ALT levels, and negatively correlated with estimated glomerular filtration rate (eGFR) and tacrolimus trough levels (p <0.05 for DSA and p <0.01 for all other variables). Of note, in patients without histological damage, the mean eGFR was 10 ml/min lower than in those with moderate-to-severe damage, even though the latter had been transplanted for a longer period of time. A retrospective analysis of sequentially collected creatinine and tacrolimus trough levels revealed that the differences observed between patients with and without histological damage were already significant up to 5 years and 1 year before enrolment, respectively (Fig. S1).

Association between anti-HLA class II DSAs, IS exposure and subclinical liver allograft damage

Out of 185 serum samples obtained at the time of liver biopsy, 91 (49.2%) were positive for either class I and/or class II HLA antibodies, with 37 (20.0%) being positive for class I, 75 (40.5%) for class II and 21 (11.4%) for both. In the 166 participants for whom donor HLA typing information was available, class II DSAs were found in 29 (17.5%) cases, while only 1 patient (0.01%) harboured class I DSAs. Among patients with class II DSAs, 21 (72.4%) had a single DSA, 7 (24.1%) had 2, and 1 patient (3.4%) had 3 or more. Out of the 40 class II DSAs identified, 28 (70.0%) were directed against HLA-DQ antigens, 22 (55.0%) had a mean fluorescence intensity (MFI) >10,000 and 10 (25.0%) an MFI >20,000. Class II DSAs were present in 14 (13.2%) patients without allograft damage and in 15 (25.4%) patients with allograft damage (p = 0.048). Furthermore, the latter exhibited higher cumulative MFI than patients with no allograft damage (p = 0.030) (Table 3). The severity of histological damage was positively associated with both the prevalence of class II DSAs and the cumulative MFI, with the differences being statistically significant when comparing patients with no lesions and those with moderate-to-severe damage. Likewise, we observed positive associations between the strength of the maintenance IS (as defined by either tacrolimus trough levels or the use of double-agent IS regimens), class II DSAs and allograft damage. Conversely, renal function was negatively associated with these parameters (Table 3).

Stratification of liver biopsies on the basis of the transcript levels of TCMR-related genes

To assess the molecular profile of subclinical allograft damage, we conducted transcriptional experiments on RNA extracted from 184 out of the 190 liver biopsies (sufficient quantity of RNA was not

Table 3. Characteristics of patients grouped according to the severity of the baseline histological damage.

Variable	No damage (group 1) n = 122	Mild damage (group 2) n = 26	Moderate-to-severe damage (group 3) n = 42	Overall p values[*,§]	Group 1 *vs.* 2 p values[#,§]	Group 1 *vs.* 3: p values[#,§]	Group 2 *vs.* 3: p values[#,§]
Age at enrolment (years)	61 (31-78)	59 (32-77)	60 (29-75)	0.101	0.118	0.079	0.950
Time after LT (years)	7 (3-26)	11 (3-25)	10 (3-23)	0.144	0.198	0.083	0.960
BMI (kg/m^2)	27.4 (18.3-52.8)	27.6 (21.6-39.5)	26.5 (19.3-38.3)	0.287	0.742	0.117	0.373
Male (n, %)	88 (72.1%)	14 (53.8%)	25 (59.5%)	0.104	0.067	0.128	0.645
AST (U/L)	21 (9-67)	26 (13-45)	26 (15-56)	**0.001**	**0.044**	**0.001**	0.735
ALT (U/L)	18 (8-63)	26 (13-54)	22 (11-80)	**0.001**	**0.001**	**0.028**	0.126
ALP (U/L)	75 (33-245)	86 (56-149)	74 (44-122)	**0.047**	**0.014**	0.497	0.101
GGT (U/L)	23 (9-762)	22 (11-155)	28 (9-126)	0.421	0.913	0.191	0.430
Platelets (x10^9/L)	201 (99-479)	216 (71-345)	193 (116-422)	0.761	0.966	0.508	0.488
Creatinine (μmol/L)	93 (44-159)	85 (59-124)	80 (52-151)	**0.002**	0.076	**0.001**	0.357
eGFR, MDRD formula (ml/min/1.73 m^2)	68 (36-115)	67 (38-90)	78 (42-92)	**0.008**	0.280	**0.002**	0.195
APRI	0.30 (0.10-0.70)	0.30 (0.20-0.70)	0.40 (0.20-0.90)	**0.034**	**0.042**	**0.043**	0.824
FIB-4	1.58 (0.53-4.43)	1.48 (0.90-3.27)	1.57 (0.59-3.91)	0.981	0.845	0.980	0.872
Tacrolimus trough level (μg/L)	4.1 (0.5-10.7)	3.0 (1.0-5.8)	3.3 (1.1-7.7)	**0.023**	**0.022**	0.058	0.732
CNI monotherapy (n, %)	69 (58.5%)	17 (68.0%)	32 (82.1%)	**0.026**	0.377	**0.008**	0.195
Liver stiffness measurement (kPa)	4.5 (2.4-8.2)	4.8 (3.3-8.6)	7.3 (3.1-21.1)	**<0.0005**	0.104	**<0.0005**	**0.009**
Presence of class II DSAs (n, %)	14 (13.1%)	4 (17.4%)	11 (30.6%)	0.061	0.739	**0.018**	0.361
Cumulative MFI of class II DSAs	1,779 (5,665)	3,876 (8,701)	6,388 (12,317)	**0.045**	0.487	**0.012**	0.325

Significant p values (p <0.05) are indicated in **bold**. Continuous data are expressed as median (range), except for MFI, which is expressed as mean (SD). Categorical data are expressed as frequencies and percentages.
ALT, alanine aminotransferase; ALP, alkaline phosphatase; APRI, AST to platelet ratio index; AST, aspartate aminotransferase; CNI, calcineurin inhibitor; DSAs, donor-specific antibodies; eGFR, estimated glomerular filtration rate; FIB-4, Fibrosis-4 score; GGT, gamma-glutamyltransferase; LT, liver transplantation; MFI, mean fluorescence intensity.
[*]Overall independent-sample Kruskal-Wallis test.
[#]Non-parametric independent-sample Mann-Whitney test.
[§]Chi-square or Fisher exact test for categorical variables, as appropriate.

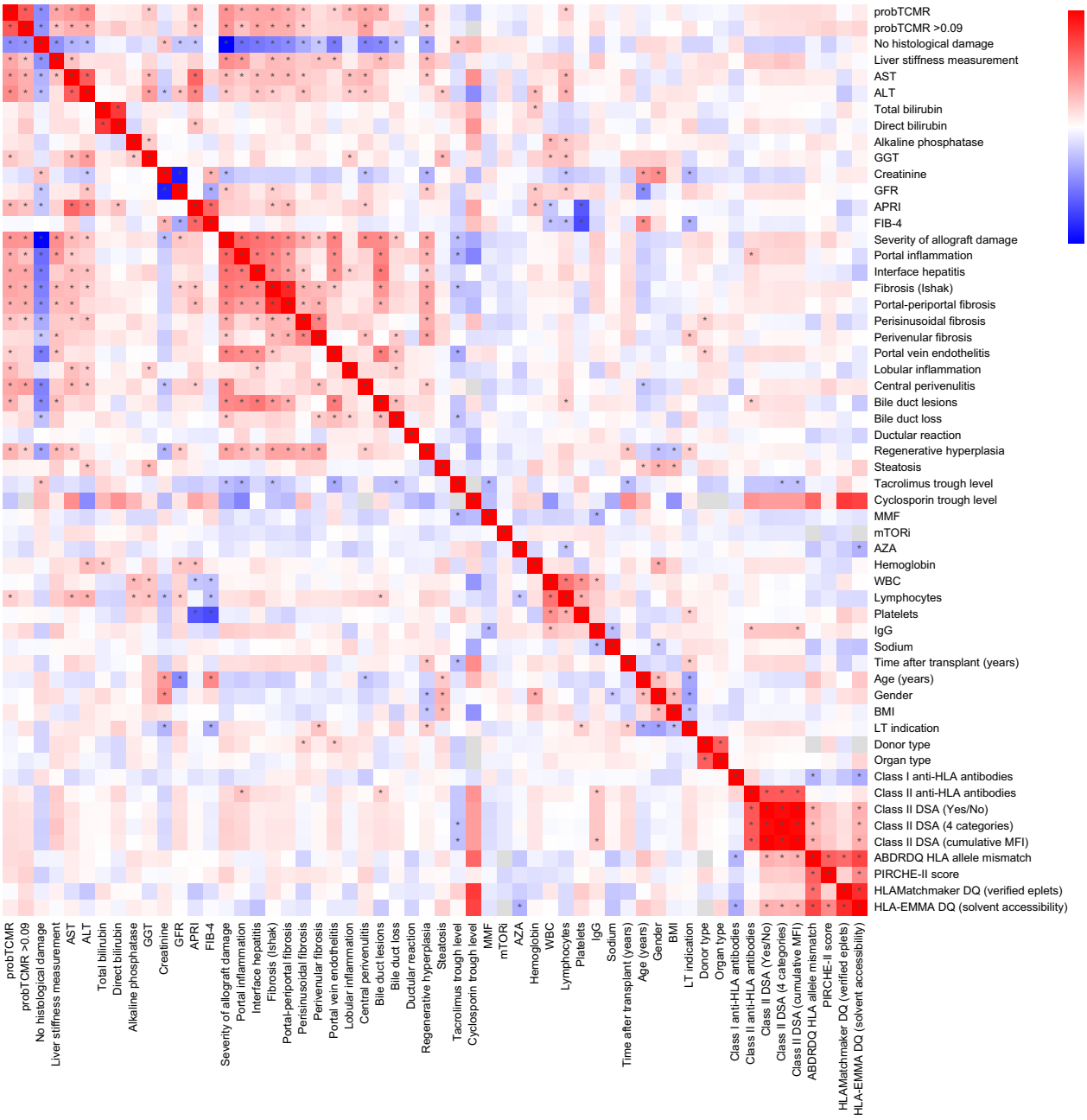

Fig. 1. Similarity correlation matrix displaying the relationships between demographic, clinical, biochemical, histological and transcriptional parameters collected at study entry. The intensity of the color in each box is proportional to the Spearman correlation coefficient of the corresponding variable pair. *p <0.01. ALP, alkaline phosphatase; ALT, alanine aminotransferase; APRI, AST to platelet ratio index; AST, aspartate aminotransferase; AZA, azathioprine; DSAs, donor-specific antibodies; FIB-4, Fibrosis-4 score; GGT, gamma-glutamyltransferase; LT, liver transplantation; MMF, mycophenolate mofetil; mTORi, mammalian target of rapamycin inhibitor; probTCMR, probability of TCMR; TCMR, T cell-mediated rejection; WBC, white blood cell. (This figure appears in color on the web.)

available for 6 specimens), and employed an 11-gene signature previously found to accurately identify clinically apparent TCMR in adult and paediatric LT recipients.[8,14,15] The analysis included a subset of indication liver biopsies from 18 LT recipients enrolled in *LIFT* who developed acute TCMR following IS withdrawal. First, we trained the 11-gene classifier in the subset of 18 TCMR biopsies and 120 biopsies with no histological damage collected at study entry, and we selected a threshold of 0.09 to classify samples as having a low or high probability of TCMR (probTCMR). This conservative threshold, which corresponded to the point on the ROC curve with the highest Youden index, had a high sensitivity to detect samples with a molecular profile in keeping with TCMR and showed a good balance between sensitivity and specificity (Fig. 2A). Next, we assigned a probTCMR to each of the 184 liver biopsies on the basis of their corresponding transcript levels. The probTCMR values significantly correlated with the severity of the underlying histological damage as well as with AST, ALT, and AST-to-platelet ratio index (APRI) score (Fig. 1). Among the 184 liver biopsies analysed, 31 (16.8%) had a gene expression profile that conferred a probTCMR >0.09. Among liver biopsies with no, mild, and moderate-to-severe

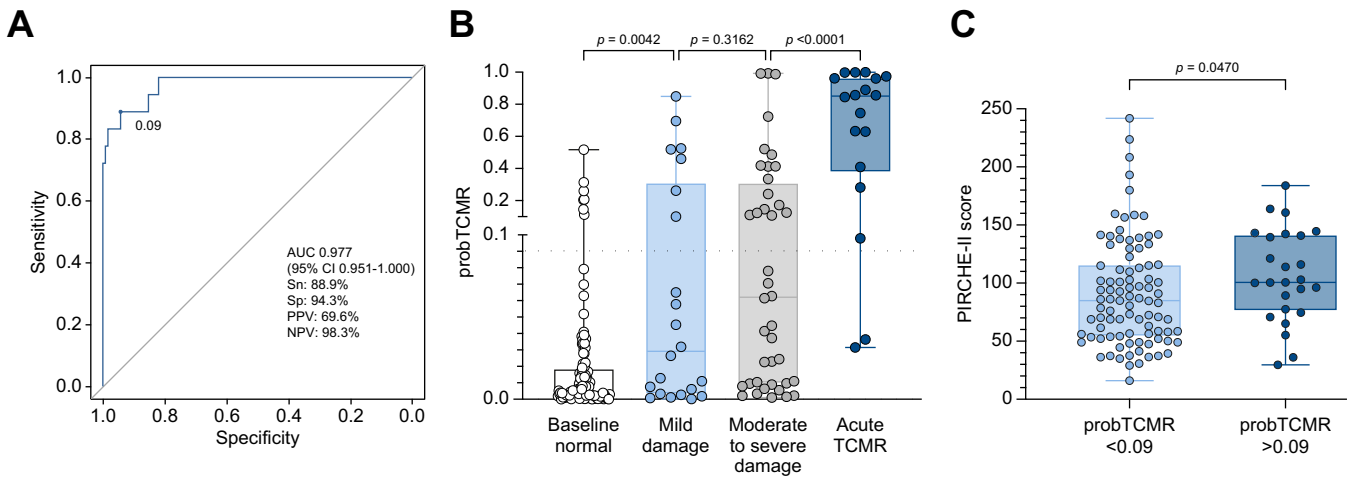

Fig. 2. Transcript levels of TCMR related genes in liver biopsies with or without silent allograft damage. (A) Receiver-operating characteristic curve displaying the overall diagnostic performance of the 11-gene transcriptional signature in discriminating liver biopsies with no damage *vs.* those with TCMR. A cut-off corresponding to a probTCMR of 0.09 provided the greatest discriminative capacity. (B) probTCMR on the basis of the transcript levels of 11 genes assessed in liver biopsies from stable patients with no liver allograft damage (white; n = 121), mild damage (light blue; n = 26), and moderate-to-severe damage (grey; n = 42), and in patients with allograft dysfunction due to TCMR (dark blue; n = 18). (C) Predicted epitope HLA mismatches between donor and recipient as assessed by *PIRCHE-II* scores were higher in patients with high probTCMR (>0.09) than in those with low probTCMR (<0.09). HLA, human leukocyte antigen; NPV, negative predictive value; PPV, positive predictive value; probTCMR, probability of TCMR; TCMR, T cell-mediated rejection.

histological damage, 5.8% (7/120), 29.2% (7/24) and 42.5% (17/40) had probTCMR >0.09, respectively (Fig. 2B).

PIRCHE-II score is associated with the transcriptional signature of TCMR and class II DSAs

HLA typing data were available for 121 donor-recipient pairs. ABDR, ABDRDQ and ABCDRDQ loci were typed in 121, 92 and 87 pairs, respectively. Both global ABDRDQ allele mismatch and the degree of ABDRDQ molecular mismatch, as assessed by either eplet,[16] amino acid mismatch,[17] or *PIRCHE-II*,[18] were associated with class II DSAs, regardless of whether these were expressed as a categorical, ordinal or continuous variable. A separate analysis of eplet and amino acid mismatches in individual class I and class II loci revealed that the association between molecular HLA mismatches and DSAs was restricted to the DQ loci. Neither eplet nor amino acid mismatches were associated with subclinical liver allograft damage or with probTCMR scores (Table S2). In contrast, there was a trend towards higher *PIRCHE-II* scores in patients with some degree of subclinical liver allograft damage (p = 0.064), and a significant association between *PIRCHE-II* and probTCMR scores (p = 0.047) (Fig. 2C).

LSM and ALT levels are useful non-invasive tools to detect active alloimmune liver damage

Considering the previously reported association between liver tissue TCMR-related gene expression and progressive liver allograft damage,[8] we hypothesised that a probTCMR = 0.09 (which corresponds to the transcript levels observed at the time of clinically apparent TCMR; Fig. 2A), would constitute an optimal threshold for active alloimmune damage, likely to be clinically significant. We employed binary logistic regression analysis to select the optimal set of non-invasive parameters capable of identifying patients meeting this criteria. Baseline ALT and LSM were both independently associated with alloimmune damage (Fig. S2 and Table S3). The model's discrimination was evaluated through a ROC curve, which yielded an AUC = 0.82 (Fig. 3A). The results of the logistic regression analysis were similar regardless of whether ALT was entered as a single measurement or as the mean of up to 3 measurements conducted over a median period of 76 days (data not shown). As a complementary strategy, and to provide a simpler model, we conducted a classification and regression tree (CART) analysis, identifying baseline ALT as the stronger predictor of alloimmune damage. ALT values ≤15 U/L (present in 22% of the *LIFT* cohort) were associated with an extremely low likelihood of alloimmune damage. Conversely, patients with ALT >34 U/L had a 45% chance of having alloimmune damage. LSM did not improve the predictive capacity of the model in these 2 groups of patients, but it was very useful in identifying subgroups of patients with high and low likelihood of alloimmune liver damage among those with intermediate ALT levels (15-34 U/L) (Fig. 3B).

Non-invasive detection of active alloimmune liver damage using ALT and DSAs in an independent cohort of paediatric transplant recipients

Given that LSMs are not routinely performed in many transplant centres, we sought alternative predictive models by excluding LSM from the logistic regression analysis. The resulting model included ALT and the cumulative MFI of class II DSAs (cMFI; categorized as cMFI <10,000, 10,000≤cMFI<20,000 and cMFI ≥20,000), and the corresponding ROC curve had an AUC of 0.76 (95% CI 0.66-0.85) (Fig. S3 and Table S4). The results of a CART analysis including ALT and class II DSAs are depicted in Fig. 3C. Similar results were obtained when mean ALT was employed (data not shown).

To assess the external validity of this model, we re-analysed the results from the *iWITH* trial (NCT01638559)[9,15], a prospective multicentre IS withdrawal study in stable long-term paediatric LT recipients that collected clinical, laboratory, histological and transcriptional data from 157 recipients, but not LSM data. To avoid experimental batch effects, the 11-gene TCMR transcriptional signature was re-trained on *iWITH* liver biopsies with and without TCMR (*i.e.* 15 biopsies with clinically apparent TCMR

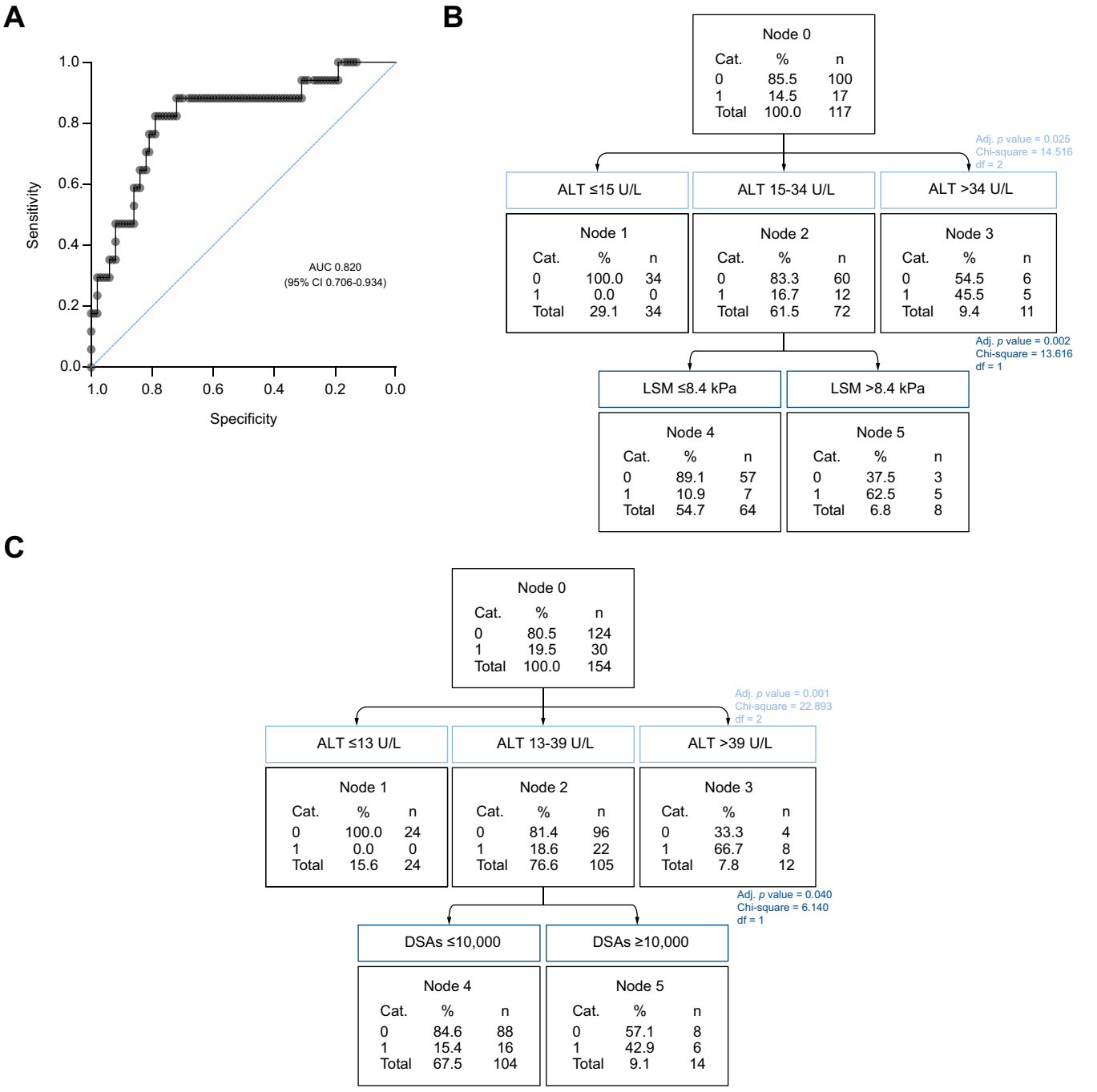

Fig. 3. Performance of predictive models for the identification of subclinical alloimmune liver allograft damage. (A) Receiver-operating characteristic curve corresponding to the performance of the logistic regression model comprising ALT and LSM in discriminating between patients with a probTCMR above and below 0.09 on the basis of liver tissue gene expression. (B) Prognostic analysis generated by classification and regression tree analysis for the ALT and LSM predictive model. Category (Cat.) 0 corresponds to probTCMR <0.09; Cat. 1 corresponds to probTCMR >0.09. (C) Classification and regression tree analysis for the ALT and class II DSA predictive model. Cat. 0 corresponds to probTCMR <0.09; Category 1 corresponds to probTCMR >0.09. ALT, alanine aminotransferase; DSAs, donor-specific antibodies; LSM, liver stiffness measurement; probTCMR, probability of TCMR; TCMR, T cell-mediated rejection.

elicited during IS weaning and 83 screening biopsies with normal histology). The resulting gene model was employed to assign a probTCMR value to each of the 148 *iWITH* biopsies collected at study entry with available NanoString gene expression data, which were then stratified into high or low probTCMR groups using the same conservative threshold employed in *LIFT* to define alloimmune damage (corresponding to a probTCMR of 0.09) (Fig. 4A). The logistic regression model, including DSAs and ALT, showed good discrimination (AUC = 0.73) and excellent calibration (Fig. 4B), with similar performance on CART analysis (Fig. S4), when applied to the *iWITH* dataset. On a final note, a model containing ALT alone provided useful discriminative value

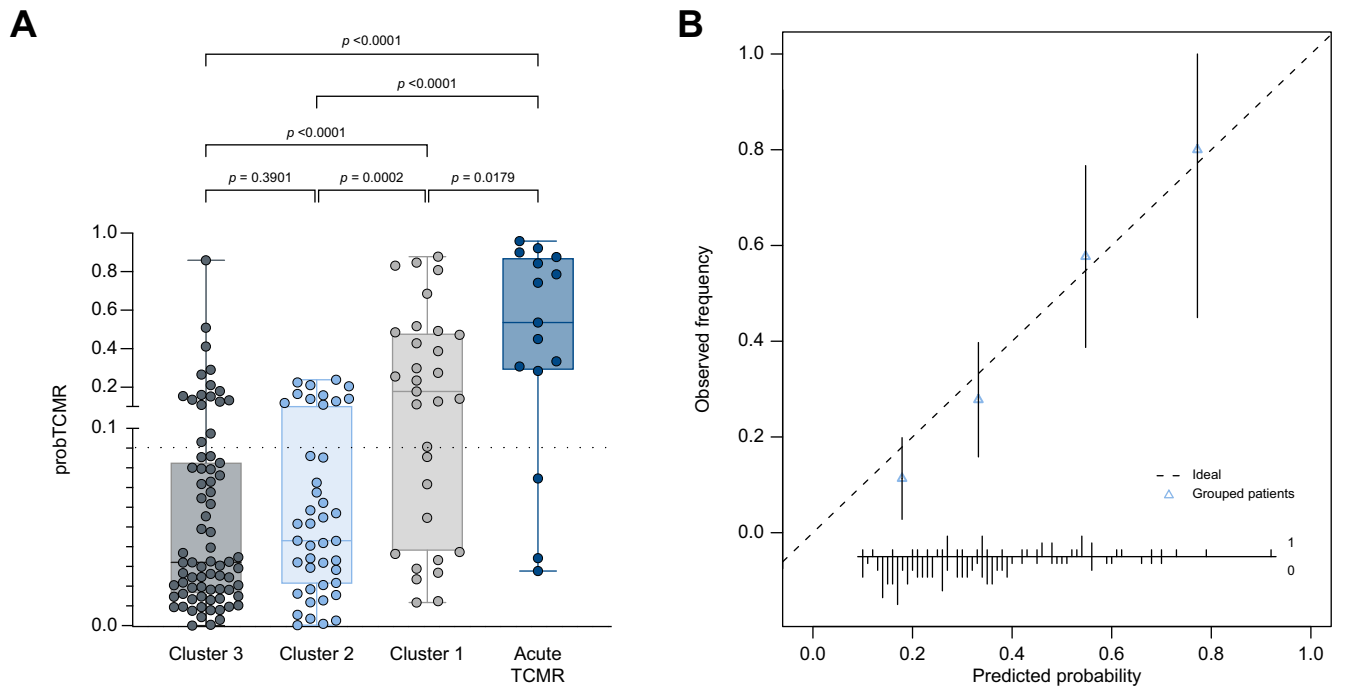

Fig. 4. Performance of baseline ALT and class II DSAs for the identification of subclinical alloimmune liver allograft damage in paediatric recipients. (A) probTCMR on the basis of the transcript levels of 11 genes assessed in liver biopsies collected at study entry from *iWITH* patients with minimal or no liver allograft damage (Cluster 3; n = 74), fibrosis without interface activity (Cluster 2; n = 43), and interface activity with/without fibrosis (Cluster 1; n = 31). An additional group of samples corresponds to patients who developed allograft dysfunction due to TCMR (n = 15). The dashed line corresponds to a probTCMR diagnostic cut-off of 0.09. (B) Calibration plot of the ALT and class II DSAs predictive model illustrating the outcome of validating the ALT and class II DSAs predictive model on *iWITH* patients. The dotted line represents the smooth non-parametric fit of predicted *vs.* observed probabilities. The histogram on the x axis corresponds to the distribution of predicted probabilities. ALT, alanine aminotransferase; DSA, donor-specific antibody; probTCMR, probability of TCMR; TCMR, T cell-mediated rejection.

for high or very low ALT levels in both adult and paediatric recipients, but was inferior to the models incorporating LSM or class II DSAs for ALT levels in the range of 16-37 U/L (Fig. S5).

Discussion

Precision medicine strategies have transformed diagnostic systems and disease classifications across a variety of disciplines but have had no impact on the long-term management of LT recipients, which remains imprecise and highly empirical. The realisation that well-functioning grafts often harbour silent immune-mediated damage, with a transcriptional profile closely resembling that of TCMR, suggests that decisions into the adequacy of maintenance IS should include detailed histological assessments. However, this clashes with the reluctance of many centres to implement surveillance liver biopsy programmes and the limitations of conventional histopathology scoring systems. In the current study we combined non-invasive biochemical and serological markers, liver elastography and pathogenesis-based liver tissue gene expression markers to define the most accurate screening tool to identify alloimmune-driven subclinical liver allograft injury. We analysed cross-sectional data and biological specimens from a cohort of stable adult LT recipients enrolled in *LIFT*, a multicentre clinical trial of protocolised IS withdrawal. The generalizability of the results was assessed by comparison with a dataset derived from *iWITH*, a similar IS withdrawal trial conducted in paediatric LT recipients.[9,15]

The analysis of the *LIFT* liver biopsies confirm the findings from *iWITH* indicating that even in highly selected recipients with normal or minimally increased liver tests there is a high prevalence of subclinical damage.[9] Furthermore, our results corroborate the observations made by our group and others on the association between subclinical liver allograft damage, circulating class II DSAs and increased intrahepatic TCMR-related transcripts.[8,9,19–24] Of note, while our study cannot establish causality, it clearly delineates the relationships existing between IS exposure, renal function and silent allograft damage. This is an important finding that illustrates the risk benefit balance of long-term maintenance IS in LT and which previous studies had failed to uncover.[8,9,19,20,22–24]

The molecular pathogenesis of subclinical liver allograft damage is known to be driven by an interferon gamma-orchestrated cellular immune response[8,9] closely resembling what is observed in clinically apparent 'classical' TCMR.[14,25,26] Our results indicate that both reduced IS exposure and increased number of HLA epitope mismatches between donor and recipient are implicated in this response. The relevance of HLA molecular mismatch, and in particular *PIRCHE-II* scores, to the pathogenesis of inflammatory liver allograft damage elicited by low tacrolimus exposure is supported by the results of a recent IS minimisation study.[27] The fact that in our study all 3 HLA molecular mismatch algorithms predicted the development of class II DSAs but only *PIRCHE-II* scores were associated with TCMR-related transcripts, suggests that indirectly-alloreactive CD4+ T cells (*i.e.* those recognising donor HLA epitopes processed and presented by recipient antigen-presenting cells on class II HLA molecules) might have a pathogenic role in silent

alloimmune liver damage. This mechanism is fundamentally different from either direct or semi-direct allorecognition, in which recipient T cells recognise intact donor HLA molecules on donor or recipient antigen-presenting cells, respectively, and which drive 'classical' TCMR early after transplantation. The involvement of different pathways of allorecognition could explain the much more indolent nature of silent liver allograft damage as compared to 'classical' TCMR, despite sharing a similar transcriptional fingerprint. Indirect CD4+ T-cell alloreactivity is also key in the development of antibody responses against allogeneic HLA molecules. Although the exact role of DSAs in the pathogenesis of silent allograft damage remains unclear,[15] the consistently strong positive association between class II DSAs strength, reflected by MFI level, and TCMR-related transcripts, noted both in *iWITH* and in our study, suggests that DSAs, rather than just being a marker of CD4+ T-cell sensitisation, likely contribute to enhance and/or perpetuate the alloimmune inflammatory response. Of note, we estimated donor/recipient HLA epitope mismatching by applying imputation and inference tools to low resolution HLA typing data. Consequently, the impact of HLA epitope mismatching could be far greater than our results indicate.[28] A nuanced delineation of the interactions between IS, DSAs and HLA epitope mismatching will require access to high resolution HLA genotyping data, which is not readily available in cadaveric donor LT.

Our data could be interpreted as proof that in LT recipients with silent damage more immunosuppression is needed. On the other hand, the fact that these same patients exhibit better renal function clearly illustrates the potential drawbacks of strengthening IS indiscriminately. In this regard, a means to non-invasively identify recipients with or without underlying active alloimmunity, as reflected by our pathogenesis-based transcriptional marker, would be highly useful. We chose to train the transcriptional signature on biopsies from patients with clinically significant alloimmunity (*i.e.* allograft dysfunction due to TCMR in the setting of protocolised IS discontinuation). This eliminated the inherent arbitrariness of selecting a histological threshold and provided a mechanism to internally normalize the gene expression data derived from *LIFT* and *iWITH*. We selected a conservative cut-off (0.09) with high sensitivity to operationally classify samples as having TCMR, and considered all samples collected at study entry with a molecular profile over this cut-off as exhibiting clinically silent but active alloimmune damage. Since the non-invasive models were fitted to predict the molecular classifier, their use would result in a very high negative predictive value for alloimmune damage and a low positive predictive value. Considering that the impact of silent liver damage on allograft longevity is uncertain and that there are risks associated with strengthening IS, we envision the non-invasive models being used as a triage tool to reliably exclude the presence of significant active alloimmunity. This would not only reassure patients and clinicians but could also enable the implementation of IS minimisation strategies, both by facilitating patient selection and by informing on the impact of modifications to IS dosing through repeated assessments of the models over time. However, whether the iterative use of the models is capable of adequately predicting and monitoring the outcome of IS minimisation will need to be investigated in a prospective randomised controlled trial. On the opposite side of the spectrum, the predictive models could also be useful in identifying patients at risk of progressive alloimmune liver damage in whom further investigations including a liver biopsy might be warranted. The simplified CART algorithms provided, which are supported by robust logistic regression analyses, should facilitate these clinical applications.

The similar performance of the ALT and class II DSAs model in the *LIFT* and *iWITH* studies is remarkable considering the immunological differences existing between the 2 cohorts of patients. First, the prevalence of class II DSAs (50% *vs.* 18%) and the proportion of biopsies with high probTCMR scores (31.8% *vs.* 16.8%) were substantially higher in paediatric than in the adult recipients, respectively. Second, their IS regimens differed (all *iWITH* patients were on calcineurin inhibitor monotherapy while 30% of *LIFT* participants were on combined IS regimens). Finally, the presence of moderate-to-severe fibrosis in the absence of concomitant inflammation, observed in 17% of *iWITH* participants, was absent in the adult *LIFT* cohort.

The limitations inherent to the study of highly selected LT patients within a rigorously controlled clinical trial have been previously discussed.[9] We believe these are significantly alleviated by the striking parallels observed between the *LIFT* and *iWITH* cohorts, which altogether encompass adult and paediatric recipients from 23 clinical sites across 6 countries in 2 continents. Nonetheless, the strict inclusion/exclusion criteria employed to select study participants for both *LIFT* and *iWITH* will need to be taken into consideration when applying our findings to the general population of LT patients. Likewise, the under-representation of biopsies exhibiting moderate-to-severe steatosis and/or steatohepatitis could compromise the performance of the risk stratification models in LT patients with alcoholic or non-alcoholic fatty liver disease.

In conclusion, approximately 17% and 32% of clinically stable long-term adult and paediatric survivors of LT, respectively, harbour significant alloimmune liver graft damage. In adults, this is associated with humoral sensitisation, lower IS levels and higher donor immunogenicity but also with better renal function. Readily available non-invasive assessments such as ALT, DSAs, and LSM can be used to determine the risk of underlying alloimmunity. Our results will help clinicians evaluate the adequacy of IS both in adult and paediatric LT recipients.

Abbreviations

AST, aspartate aminotransferase; ALT, alanine aminotransferase; APRI, AST-to-platelet ratio index; CART, classification and regression tree; DSAs, donor-specific antibodies; eGFR, estimated glomerular filtration rate; HLA, human leukocyte antigen; IS, immunosuppression; LIFT, Liver Free Immunosuppression Trial; LSM, liver stiffness measurement; LT, liver transplantation; MFI, mean fluorescence intensity; probTCMR, probability of alloimmune damage; ROC, receiver-operating characteristics; TCMR, T cell-mediated rejection.

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Conflict of interest

The authors have no conflicts of interest to declare.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

JV: data collection, study supervision, data analysis, manuscript writing, critical review for intellectual content and approval of the manuscript; JGA: statistical analysis, manuscript writing, critical review for intellectual content and approval of the manuscript; RM: concept and design of the study, histological analysis, data collection, critical review for intellectual content and approval of the manuscript; JW: data collection, clinical trial management, approval of the manuscript; EK: laboratory data analysis, approval of the manuscript; JJL: statistical analysis, critical review for intellectual content and approval of the manuscript; PR, MN, AM, FN, WG, JL, SM, EJ, RT, PT, DE, KJS, EBR, JF, AQ: data collection, critical review for intellectual content and approval of the manuscript; SF, JB: manuscript writing and critical review for intellectual content and approval of the manuscript; ME, AD: statistical analysis; ASF: concept and design of the study, obtained funding, study supervision, data collection, data analysis, manuscript writing, critical review for intellectual content and approval of the manuscript.

Data availability statement

To ensure the privacy of the participating patients, further research data remains confidential. The anonymized dataset is available on request.

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Supplementary data

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Author names in bold designate shared co-first authorship

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