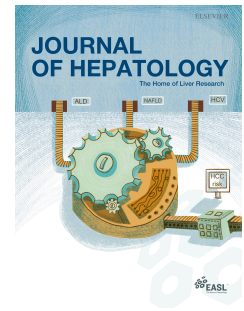


Journal Pre-proof



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

Julien Vionnet, Rosa Miquel, Juan G. Abraldes, Jurate Wall, Elisavet Kodela, Juan-Jose Lozano, Pablo Ruiz, Miguel Navasa, Aileen Marshall, Frederik Nevens, Will Gelson, Joanna Leithead, Steven Masson, Elmar Jaeckel, Richard Taubert, Phaedra Tachtatzis, Dennis Eurich, Kenneth J. Simpson, Eliano Bonaccorsi-Riani, Sandy Feng, John Bucuvalas, James Ferguson, Alberto Quaglia, Julia Sidorova, Maria Elstad, Abdel Douiri, Alberto Sánchez-Fueyo

PII: S0168-8278(21)02001-8

DOI: <https://doi.org/10.1016/j.jhep.2021.08.007>

Reference: JHEPAT 8398

To appear in: *Journal of Hepatology*

Received Date: 9 March 2021

Revised Date: 26 July 2021

Accepted Date: 3 August 2021

Please cite this article as: Vionnet J, Miquel R, Abraldes JG, Wall J, Kodela E, Lozano JJ, Ruiz P, Navasa M, Marshall A, Nevens F, Gelson W, Leithead J, Masson S, Jaeckel E, Taubert R, Tachtatzis P, Eurich D, Simpson KJ, Bonaccorsi-Riani E, Feng S, Bucuvalas J, Ferguson J, Quaglia A, Sidorova J, Elstad M, Douiri A, Sánchez-Fueyo A, Non-invasive alloimmune risk stratification of long-term liver transplant recipients, *Journal of Hepatology* (2021), doi: <https://doi.org/10.1016/j.jhep.2021.08.007>.

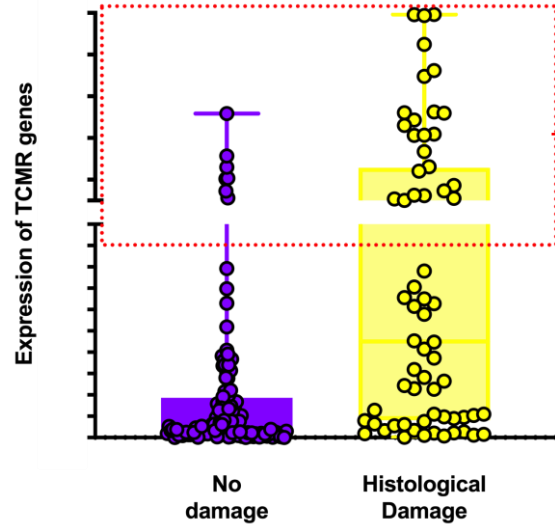
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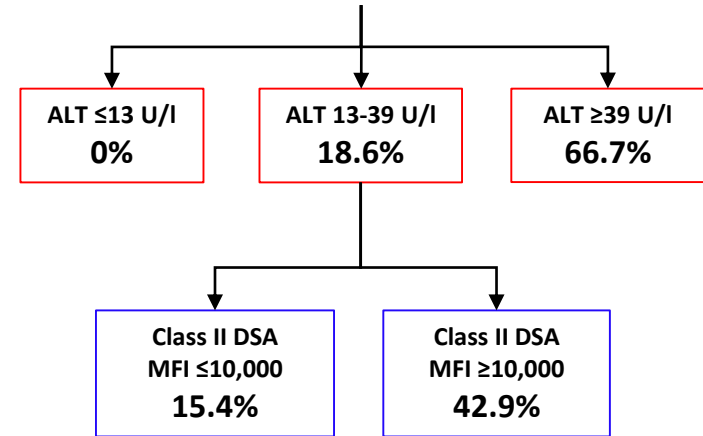
IDENTIFICATION OF ACTIVE ALLOIMMUNE LIVER DAMAGE IN STABLE LIVER TRANSPLANT RECIPIENTS

Invasive:
Liver biopsy
Gene expression profiling

Non-invasive:
ALT/FibroScan or
ALT/Class II DSA



Percentage of patients with active alloimmune liver damage



Alloimmune risk stratification of adult and paediatric liver recipients on the basis of ALT and class II DSA measurements

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

Running title: Alloimmune risk stratification in liver transplantation

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Word count: 6123.

Tables: 3; **Figures:** 4.

Conflict of interest: The authors have no conflict of interest to declare.

Financial support statement: The work was supported by an award from the National Institute for Health Research (NIHR) Efficacy and Mechanism Evaluation (EME) Programme (reference 13/94/55; to ASF), the Medical Research Council Centre for Transplantation, and the NIHR Biomedical Research Centre at Guy's and St Thomas' National Health Service (NHS) Foundation Trust and King's College London. JV was supported by the Swiss National Science Foundation (Early Postdoc.Mobility P2LAP3_181318 and Postdoc.Mobility P400PM_194501 grants), by the Fondation genevoise de bienfaisance Valeria Rossi di Montelera (Eugenio Litta grant), by the Société Académique Vaudoise and the Lausanne University Hospital.

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.

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ABSTRACT

Background and 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 has low applicability. Our aim was to investigate the utility of non-invasive tools in stratifying stable long-term LT recipients according to their immunological risk and need for immunosuppression.

Methods: We conducted a cross-sectional multicentre study of 190 adult liver 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: 36% patients harboured clinically-silent fibro-inflammatory liver lesions, which were scored as moderate-to-severe in 22%. The severity of liver allograft damage was positively associated with TCMR-related transcripts, class II donor-specific antibodies (DSA), ALT/AST and liver stiffness measurements (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 DSA, respectively, had a high discriminative capacity in classifying patients with or without alloimmune damage. The latter model had a good performance in an independent cohort of 156 liver biopsies obtained from paediatric liver recipients with similar inclusion/exclusion criteria.

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

Word count: 275.

Lay summary:

A large proportion of liver transplant patients with normal liver tests have liver inflammatory 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.

INTRODUCTION

There are currently thousands of clinically-stable long-term surviving adult and paediatric liver transplant recipients worldwide. Pharmacological immunosuppression (IS) remains one of the key modifiable factors influencing their long-term outcomes following liver transplantation (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 surviving LT recipients with stable allograft function and no significant histological abnormalities can substantially reduce their overall IS levels, and, in some cases, even completely discontinue IS⁵. Considering that the risk of T cell-mediated rejection (TCMR) following transplantation decreases with time^{6,7}, these evidences suggest that a large number of long-term surviving LT recipients 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 evidences indicate 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 genes⁸.

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 the 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 surviving LT recipients 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 subjects.

Subjects: Key inclusion/exclusion criteria

All participants were adult (≥ 18 years old) stable liver transplant recipients at least three years post-transplant and had normal liver function tests (LFT) at the screening visit, as defined by direct bilirubin $< 17.1 \mu\text{mol/l}$ and ALT $< 60 \text{ U/l}$. Patients with active viral infection [hepatitis B (HBV), hepatitis C (HCV) or human immunodeficiency virus (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; HLA typing and alloantibody characterisation; transient elastography liver stiffness measurements (LSM); detailed histopathology assessment of screening liver biopsies; and liver tissue gene expression experiments. Detailed methods are provided as **Supplementary Data**.

Statistical analysis

Descriptive statistics included median and range or mean and standard deviation (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 $\text{probTCMR} > 0.09$. 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 operator characteristics (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 platform¹¹.

RESULTS

Main patients 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, patient median age 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 the 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) $\mu\text{mol/l}$, respectively. Of note, 2 subjects 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. 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 10.7-19221.0,

$p < 0.005$), 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 immunosuppression 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 three categories (groups 1, 2 and 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 (DSA), liver stiffness measurements (LSM) and serum 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 (**Supplementary Fig.1**).

Association between anti-HLA class II donor-specific antibodies (DSA), IS exposure and subclinical liver allograft damage

Out of 185 serum samples obtained at the time of the 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 subjects from

whom donor HLA typing information was available, class II DSA were found in 29 (17.5%) cases, while only 1 patient (0.01%) harboured class I DSA. Among patients with class II DSA, 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 DSA identified, 28 (70.0%) were directed against HLA-DQ antigens, 22 (55.0%) had an MFI >10000 and 10 (25.0%) an MFI >20000. Class II DSA 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 DSA 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 two-agent IS regimens), class II DSA 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 available for 6 specimens), and employed an 11-gene signature previously found to accurately identify clinically-apparent T cell-mediated rejection (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 APRI

score (**Fig. 1**). Among the 184 liver biopsies analysed, 31 (16.8%) had a gene expression profile that conferred them a probTCMR >0.09 . Among liver biopsies with no, mild, and moderate-to-severe 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 T cell-mediated rejection and class II DSA

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 eplet¹⁶, amino acid mismatch¹⁷, or PIRCHE-II¹⁸, were associated with class II DSA, 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 DSA was restricted to the DQ loci. Neither eplet nor amino acid mismatches were associated with subclinical liver allograft damage or with probTCMR scores (**Supplementary Table 2**). 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**).

Liver stiffness measurements (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 damage⁸, 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 of 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 (**Supplementary Fig. 2 and Supplementary Table 3**). 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 DSA 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 class II DSA cumulative MFI (cMFI; categorized as cMFI <10000 , $10000 \leq \text{cMFI} < 20000$ and cMFI ≥ 20000), and the corresponding ROC curve had an AUC=0.76 (95%CI=0.66-0.85) (**Supplementary Fig. 3 and Supplementary Table 4**). The results of a CART analysis including ALT and class II DSA is 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 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**). Applying the logistic regression model including DSA and ALT derived from *LIFT* to the *iWITH* dataset showed good discrimination

(AUC=0.73) and excellent calibration (**Fig. 4B**), with similar performance of the CART decision tree (**Supplementary Fig. 4**). On a final note, a model containing ALT alone provided useful discriminative value for high or very low ALT levels in both adult and paediatric recipients, but was inferior to the models incorporating LSM or class II DSA for ALT levels in the range of 16-37 U/l (**Supplementary Fig. 5**).

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 damage⁹. Furthermore, our results corroborate the observations made by our group and others on the association between subclinical liver allograft damage, circulating class II DSA 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 study²⁷. The fact that in our study all three HLA molecular mismatch algorithms predicted the development of class II DSA 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 DSA in the pathogenesis of silent allograft damage remains unclear¹⁵, the consistently strong positive association between class II DSA strength, reflected by MFI level, and TCMR-related transcripts, noted both in *iWITH* and in our study, suggests that DSA, 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 what our results indicate²⁸. A nuanced delineation of the interactions between IS, DSA 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 DSA model in the *LIFT* and *iWITH* studies is remarkable considering the immunological differences existing between the two cohorts of patients. First, the prevalence of class II DSA (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 discussed⁹. We believe these are significantly alleviated by the striking parallelisms 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% clinically-stable long-term surviving adult and paediatric LT recipients, 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, DSA, 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; AP, alkaline phosphatase; APRI, AST-to-platelet ratio index; AUC, area under the curve; DSA, donor-specific anti-HLA antibody; eGFR, estimated glomerular filtration rate; FIB-4, fibrosis-4 score; GGT, γ -glutamyl-transpeptidase; HBV, hepatitis B virus, HCV, hepatitis C virus; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; IS, immunosuppression; LFT, liver function tests; LIFT, Liver Free Immunosuppression Trial; LSM, liver stiffness measurement; LT, liver transplantation; MFI, mean fluorescence intensity; probTCMR, probability of alloimmune damage; ROC, receiver operator characteristics; SD, standard deviation; TCMR, T cell-mediated rejection.

ACKNOWLEDGEMENTS

We are grateful to all the patients who participated in the study. We thank Dr Maria Meneghini and Dr Oriol Bestard (Hospital Bellvitge, Barcelona, Spain) for guidance regarding HLA molecular mismatch analyses, Dr Olivia Shaw (Clinical Transplantation Laboratory, Guy's Hospital, London, United Kingdom) for her help in the interpretation of HLA typing and anti-HLA antibody data, and Matthias Niemann (PIRCHE) and Cynthia Kramer (HLA-EMMA) for helpful discussions. AD acknowledges funding support from the NIHR Applied Research Collaboration (ARC) South London at King's College Hospital NHS Foundation Trust and the Royal College of Physicians.

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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TABLES

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

Characteristics	Value
Age at the time of enrolment (years)	61 (29-78)
Male gender (n, %)	127 (66.8%)
BMI (kg/m²)	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)
AP / 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)

*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).

Abbreviations: ALT, alanine aminotransferase; AP, alkaline phosphatase; APRI, AST to platelet ratio index; AST, aspartate aminotransferase; AZA, azathioprine; BMI, body mass index; FIB-4, Fibrosis-4 score; GGT, γ -glutamyl-transferase; LT, liver transplantation; MMF, mycophenolate mofetil.

Table 2. Histological characteristics of the 190 baseline liver biopsies

Histological features		Histological features (continued)	
Lobular inflammation		Portal inflammation	
• Absence	75 (39.5%)	• Absence	46 (24.2%)
• Mild	108 (56.8%)	• Mild	110 (57.9%)
• Moderate	6 (3.2%)	• Moderate	33 (17.4%)
• Marked	1 (0.5%)	• Marked	1 (0.5%)
Central perivenulitis		Interface hepatitis	
• Absence	159 (83.7%)	• Absence	130 (68.4%)
• Mild	28 (14.7%)	• Mild	51 (26.8%)
• Moderate	3 (1.6%)	• Moderate	7 (3.7%)
• Marked	0 (0%)	• Marked	2 (1.1%)
Portal vein endothelitis		Bile duct lesions	
• Absence	137 (72.1%)	• Absence	138 (72.6%)
• Mild	52 (27.4%)	• Mild	46 (24.2%)
• Moderate	1 (0.5%)	• Moderate	3 (1.6%)
• Marked	0 (0%)	• Marked	3 (1.6%)
Regenerative hyperplasia		Bile duct loss	
• Absence	104 (54.7%)	• Absence	178 (93.7%)
• Focal	83 (43.7%)	• <50% of portal tracts	10 (5.3%)
• Diffuse	3 (1.6%)	• >50% of portal tracts	1 (0.5%)
Fibrosis (Ishak)		Perisinusoidal fibrosis[#]	
• Absence	71 (37.4%)	• Absence	95 (50.0%)
• Mild	87 (45.8%)	• Focal	86 (45.3%)
• Moderate	11 (5.8%)	• Marked	9 (4.7%)
• Occasional bridging	10 (5.3%)	• Severe	0 (0.0%)
• Marked bridging	3 (1.6%)	Perivenular fibrosis[#]	
• Incomplete cirrhosis	6 (3.2%)	• Absence	146 (76.8%)
• Complete cirrhosis	2 (1.1%)	• Focal	37 (19.5%)
Portal/Periportal fibrosis[#]		• Marked	6 (3.2%)
• Absence	83 (43.7%)	• Severe	0 (0.0%)
• Focal	70 (36.8%)	Ductular reaction	
• Marked	24 (12.6%)	• Absence	114 (60.0%)
• Severe	13 (6.8%)	• Presence	76 (40.0%)
Steatosis			
• Absence	114 (60.0%)		
• Mild	61 (32.1%)		
• Moderate	14 (7.4%)		
• Severe	1 (0.5%)		

Numbers refer to the number of biopsies displaying each feature.

[#]As described by Venturi *et al.*¹³

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
Body mass index (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 gender (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
AP (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/l)	93 (44-159)	85 (59-124)	80 (52-151)	0.002	0.076	0.001	0.357
Estimated glomerular filtration rate, 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 DSA (n, %)	14 (13.1%)	4 (17.4%)	11 (30.6%)	0.061	0.739	0.018	0.361
Class II DSA cumulative MFI	1779 (5665)	3876 (8701)	6388 (12317)	0.045	0.487	0.012	0.325

*Overall independent-sample Kruskal-Wallis test.

#Non-parametric independent-sample Mann-Whitney test.

§Chi-square or Fisher exact test for categorical variables, as appropriate.

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.

Abbreviations: ALT, alanine aminotransferase; AP, alkaline phosphatase; APRI, AST to platelet ratio index; AST, aspartate aminotransferase; CNI, calcineurin inhibitor; DSA, donor-specific antibodies; FIB-4, Fibrosis-4 score; GGT, γ-glutamyl-transferase; LT, liver transplantation; MFI, mean fluorescence intensity; SD, standard deviation

FIGURE LEGENDS

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. * $p < 0.01$

Fig. 2. Transcript levels of T cell-mediated rejection related genes in liver biopsies with or without silent allograft damage.

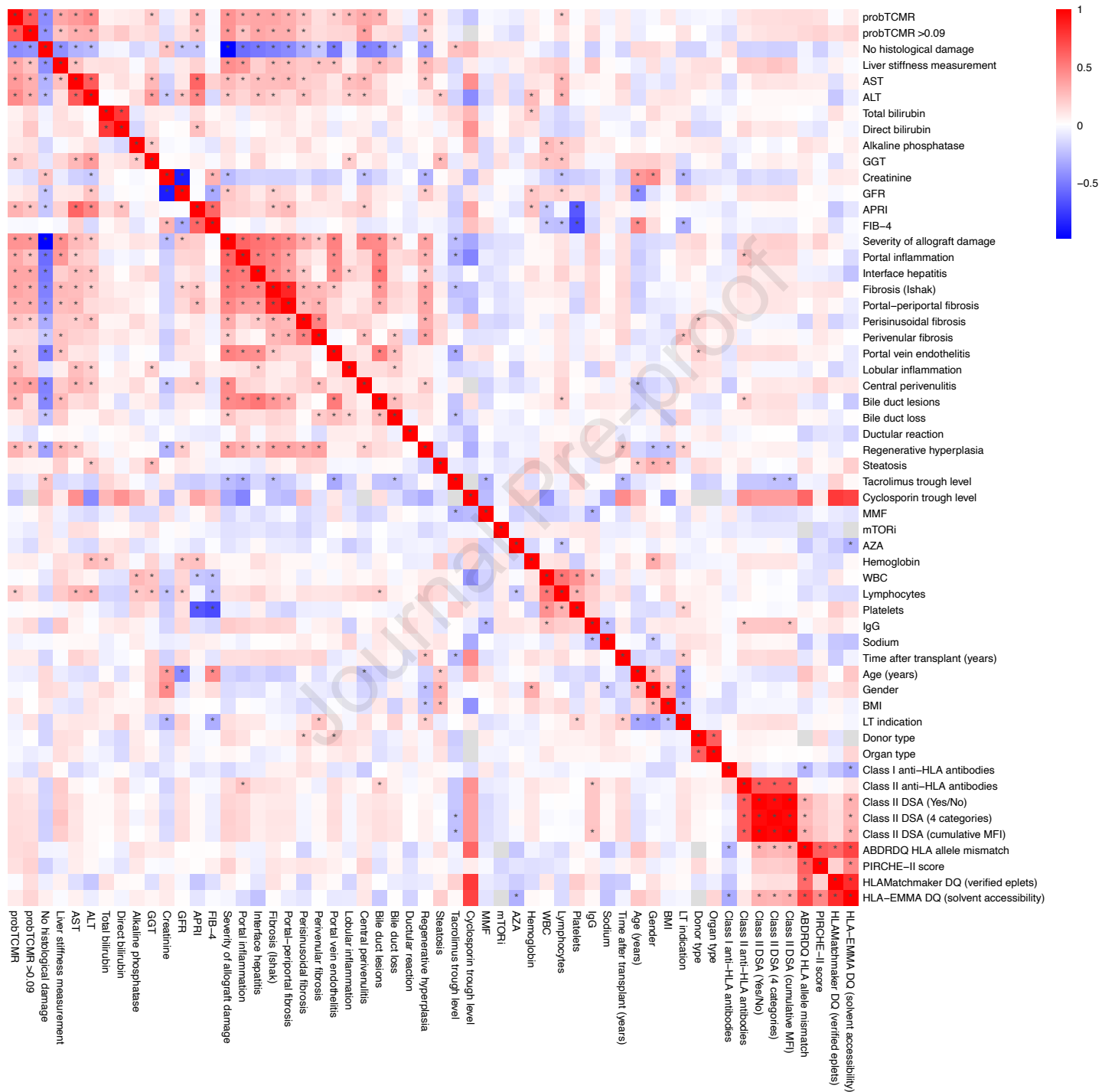
(A) Receiver operating characteristics (ROC) curve displaying the overall diagnostic performance of the 11-gene transcriptional signature in discriminating liver biopsies with no damage versus those with T cell-mediated rejection (TCMR). A cut-off corresponding to a probability of TCMR (probTCMR) of 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 (**purple**; $n=121$), mild damage (**yellow**; $n=26$), and moderate-to-severe damage (**orange**; $n=42$), and in patients with allograft dysfunction due to TCMR (**red**; $n=18$). (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).

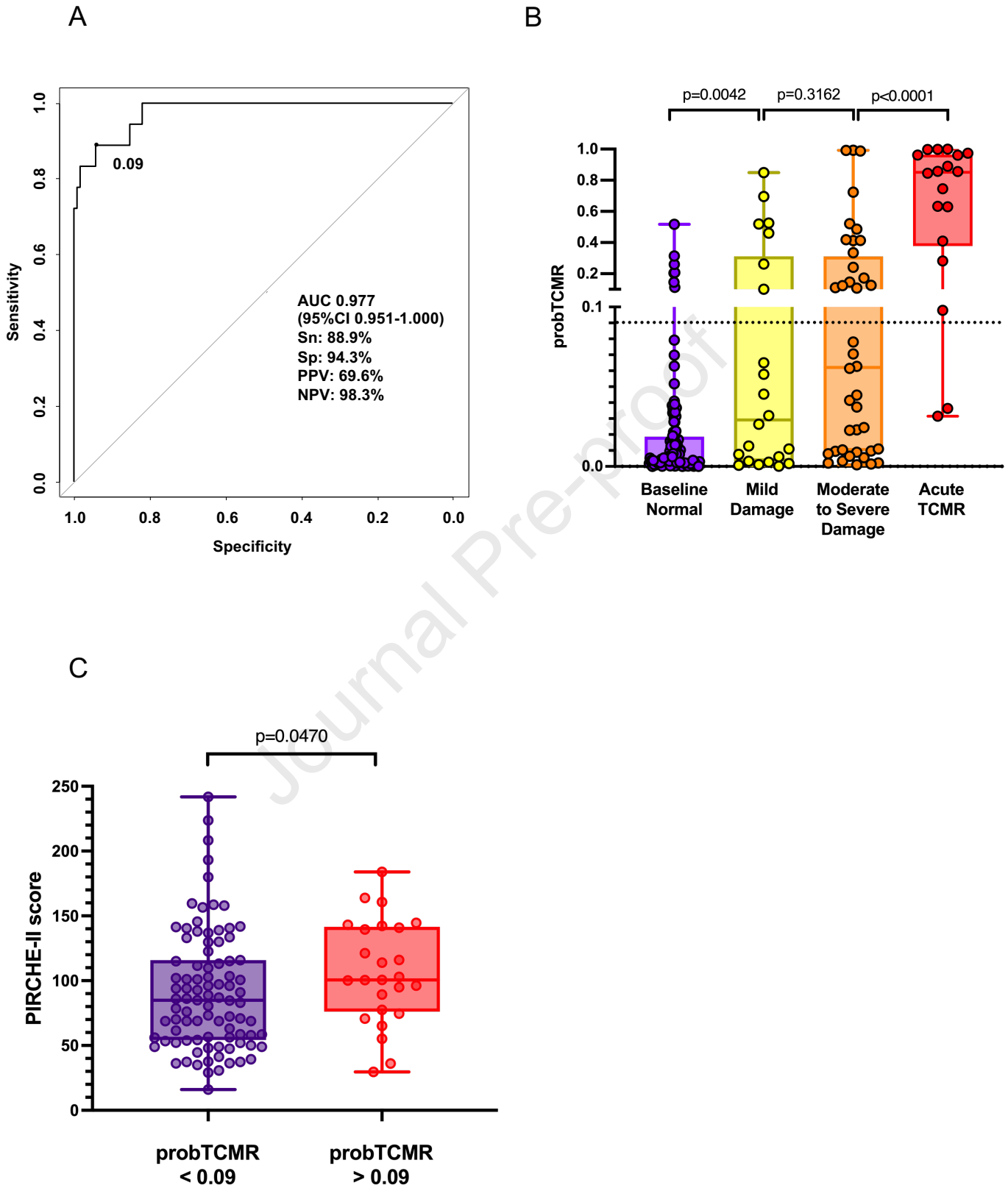
Fig. 3. Performance of predictive models, integrating baseline ALT and liver stiffness measurement (LSM) and baseline ALT and class II donor-specific antibodies (DSA), respectively, in identifying subclinical alloimmune liver allograft damage.

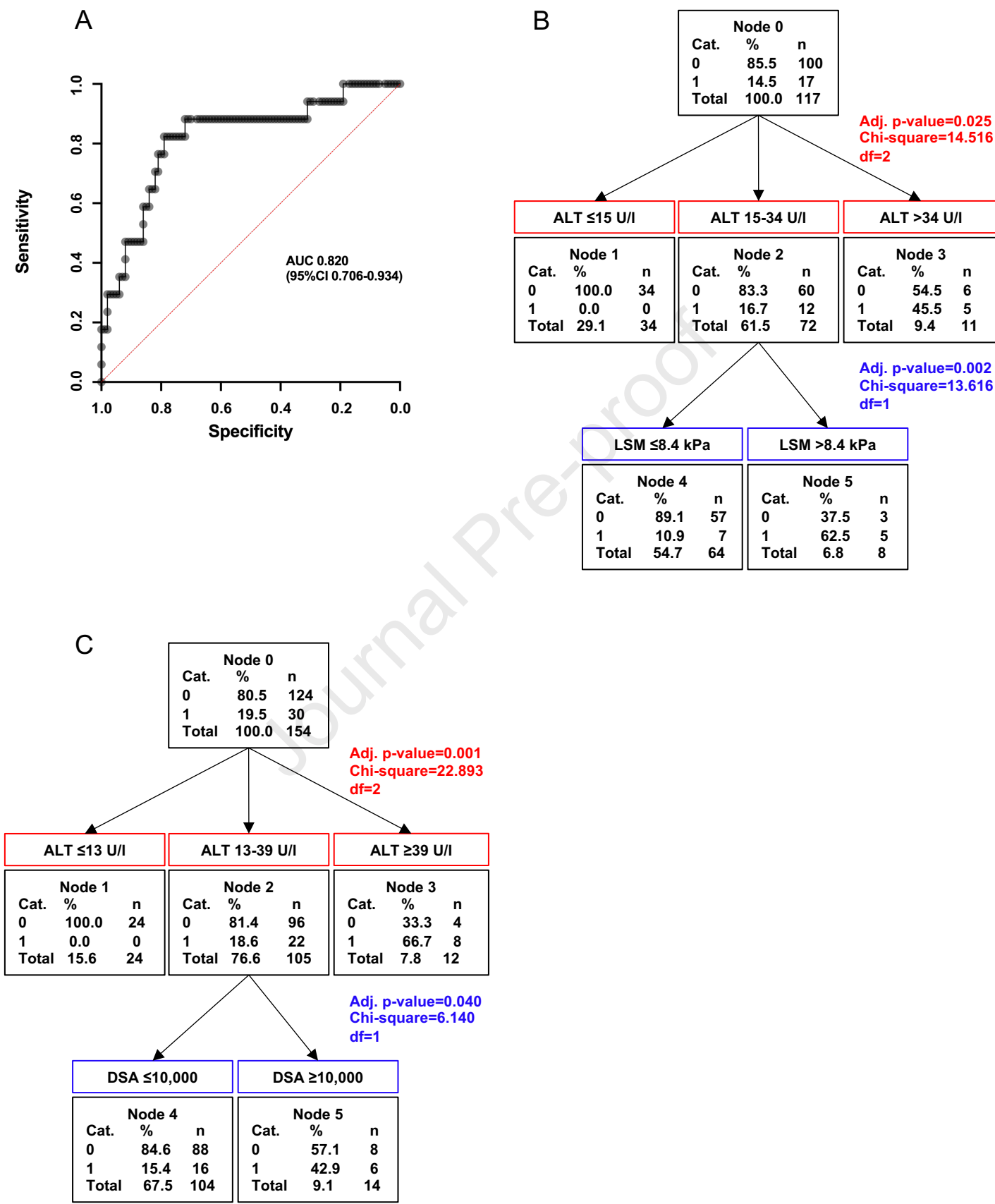
(A) Receiver operating characteristics (ROC) curve corresponding to the performance of the logistic regression model comprising ALT and LSM in discriminating between patients with a probability of T cell-mediated rejection (probTCMR) above and below 0.09 on the basis of liver tissue gene expression. (B) Prognostic analysis generated by classification and regression tree (CART) analysis for the ALT and LSM predictive model. Category (Cat.) 0 corresponds to probTCMR <0.09 ; Category 1 corresponds to probTCMR >0.09 . (C) CART analysis for the ALT and class II DSA predictive model. Category (Cat.) 0 corresponds to probTCMR <0.09 ; Category 1 corresponds to probTCMR >0.09 .

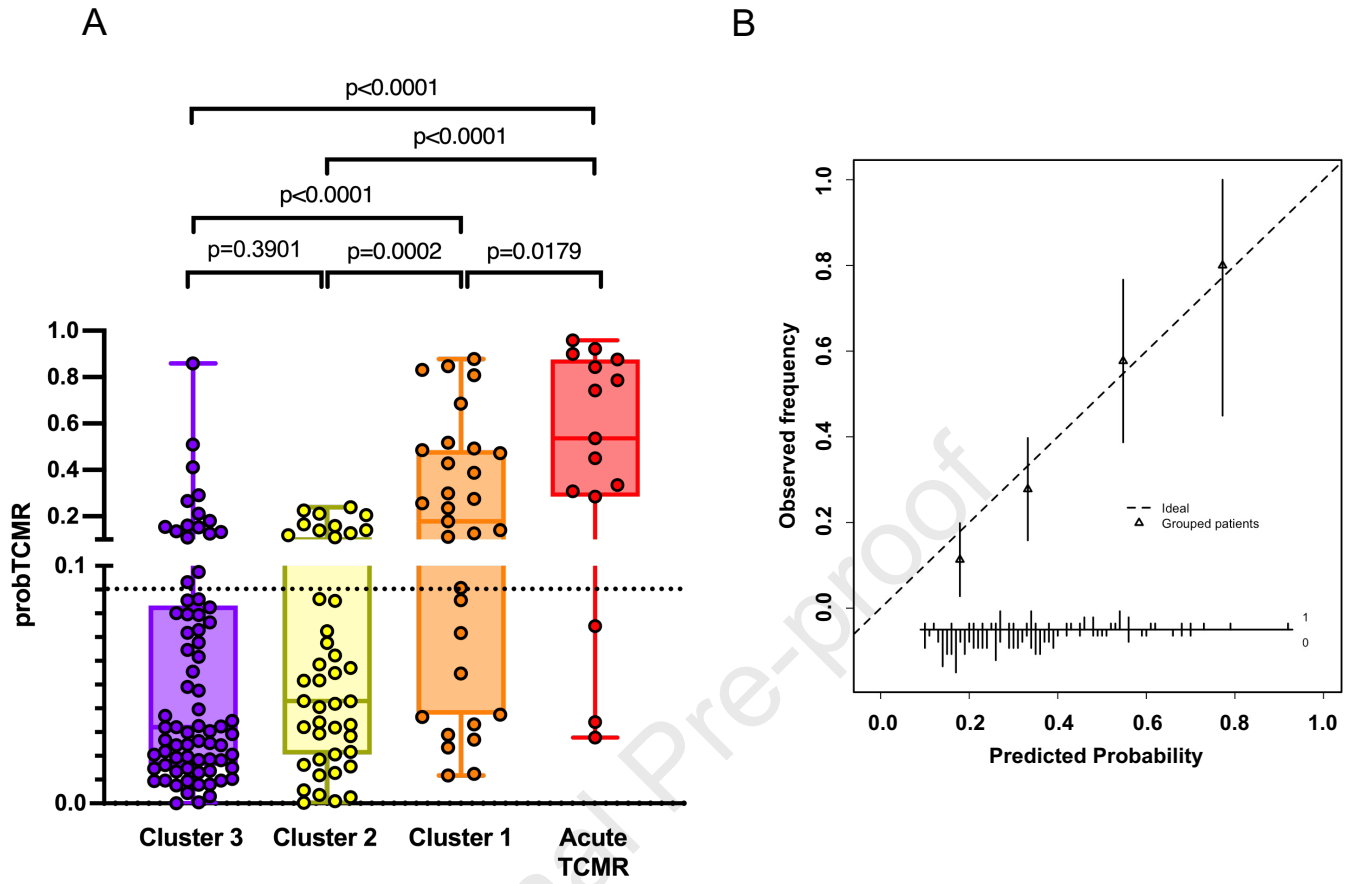
Fig. 4. Performance of baseline ALT and class II donor-specific antibodies (DSA) in identifying subclinical alloimmune liver allograft damage in paediatric recipients.

(A) Probability of T cell-mediated rejection (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 T cell-mediated rejection (n=15). The dashed line corresponds to a probTCMR diagnostic cut-off of 0.09. (B) Calibration plot of the ALT and class II DSA predictive model illustrating the outcome of validating the ALT and class II DSA predictive model on *iWITH* patients. The dotted line represents the smooth non-parametric fit of predicted versus observed probabilities. The histogram on the x axis corresponds to the distribution of predicted probabilities.









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