NIHR | National Institute for Health and Care Research

Journals Library

Check for updates

Efficacy and Mechanism Evaluation

Volume 12 • Issue 3 • April 2025

ISSN 2050-4373

The clinical utility and safety of biomarker-guided immunosuppression withdrawal in liver transplantation: the LIFT prospective RCT

Julien Vionnet, Rosa Miquel, Juan G Abraldes, Juan-Jose Lozano, Pablo Ruiz, Miquel Navasa, Aileen Marshall, Frederik Nevens, William Gelson, Joanna Leithead, Steven Masson, Elmar Jaeckel, Richard Taubert, Phaedra Tachtatzis, Dennis Eurich, Kenneth Simpson, Eliano Bonaccorsi-Riani, James Ferguson, Alberto Quaglia, Maria Elstad, Marc Delord, Abdel Douiri and Alberto Sánchez-Fueyo

DOI 10.3310/FWXV5380

NIHR | National Institute for Health and Care Research

Efficacy and Mechanism Evaluation

Check for updates

Extended Research Article

The clinical utility and safety of biomarker-guided immunosuppression withdrawal in liver transplantation: the LIFT prospective RCT

Julien Vionnet,[1,2] Rosa Miquel,[1,3] Juan G Abraldes,[4] Juan-Jose Lozano,[5] Pablo Ruiz,[6] Miquel Navasa,[6] Aileen Marshall,[7] Frederik Nevens,[8] William Gelson,[9] Joanna Leithead,[9] Steven Masson,[10] Elmar Jaeckel,[11] Richard Taubert,[11] Phaedra Tachtatzis,[12] Dennis Eurich,[13] Kenneth Simpson,[14] Eliano Bonaccorsi-Riani,[15] James Ferguson,[16] Alberto Quaglia,[7] Maria Elstad,[17] Marc Delord,[17] Abdel Douiri,[17] and Alberto Sánchez-Fueyo[1*]

[1]Institute of Liver Studies, King's College London University and King's College Hospital, London, UK
[2]Transplantation Center and Service of Gastroenterology and Hepatology, University Hospital of Lausanne, Lausanne, Switzerland
[3]Liver Histopathology Laboratory, King's College Hospital, London, UK
[4]Liver Unit, Division of Gastroenterology, Centre of Excellence for Gastrointestinal Inflammation and Immunity Research, University of Alberta, Edmonton, Canada
[5]Bioinformatic Platform, Biomedical Research Center in Hepatic and Digestive Diseases (CIBEREHD), Instituto de Salud Carlos III, Spain
[6]Hospital Clinic, Barcelona, Spain
[7]Royal Free Hospital, London, UK
[8]University Hospital KU Leuven, Belgium
[9]Addenbrooke's Hospital, Cambridge, UK
[10]Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, UK
[11]Medizinische Hochschule Hannover, Hannover, Germany
[12]Leeds Teaching Hospitals NHS Trust, Leeds, UK
[13]Charité, Berlin, Germany
[14]Edinburgh Royal Infirmary, Edinburgh, UK
[15]Liver Transplant Unit, Cliniques Universitaires St-Luc, Brussels, Belgium
[16]Queen Elizabeth Hospital, Birmingham, UK
[17]School of Population Health and Environmental Sciences, King's College London, London, UK

[*]Corresponding author sanchez_fueyo@kcl.ac.uk

Published April 2025
DOI: 10.3310/FWXV5380

This report should be referenced as follows:

Vionnet J, Miquel R, Abraldes JG, Lozano JJ, Ruiz P, Navasa M, *et al.* The clinical utility and safety of biomarker-guided immunosuppression withdrawal in liver transplantation: the LIFT prospective RCT. *Efficacy Mech Eval* 2025;**12**(3). https://doi.org/10.3310/FWXV5380

Efficacy and Mechanism Evaluation

ISSN 2050-4373 (Online)

A list of Journals Library editors can be found on the NIHR Journals Library website

Efficacy and Mechanism Evaluation (EME) was launched in 2014 and is indexed by Europe PMC, DOAJ, Ulrichsweb™ (ProQuest LLC, Ann Arbor, MI, USA) and NCBI Bookshelf.

This journal is a member of and subscribes to the principles of the Committee on Publication Ethics (COPE) (www.publicationethics.org/).

Editorial contact: journals.library@nihr.ac.uk

The full EME archive is freely available to view online at www.journalslibrary.nihr.ac.uk/eme.

Criteria for inclusion in the *Efficacy and Mechanism Evaluation* journal

Manuscripts are published in *Efficacy and Mechanism Evaluation* (EME) if (1) they have resulted from work for the EME programme, and (2) they are of a sufficiently high scientific quality as assessed by the reviewers and editors.

EME programme

The Efficacy and Mechanism Evaluation (EME) programme funds ambitious studies evaluating interventions that have the potential to make a step-change in the promotion of health, treatment of disease and improvement of rehabilitation or long-term care. Within these studies, EME supports research to improve the understanding of the mechanisms of both diseases and treatments.

The programme supports translational research into a wide range of new or repurposed interventions. These may include diagnostic or prognostic tests and decision-making tools, therapeutics or psychological treatments, medical devices, and public health initiatives delivered in the NHS.

The EME programme supports clinical trials and studies with other robust designs, which test the efficacy of interventions, and which may use clinical or well-validated surrogate outcomes. It only supports studies in humans and where there is adequate proof of concept. The programme encourages hypothesis-driven mechanistic studies, integrated within the efficacy study, that explore the mechanisms of action of the intervention or the disease, the cause of differing responses, or improve the understanding of adverse effects. It funds similar mechanistic studies linked to studies funded by any NIHR programme.

The EME programme is funded by the Medical Research Council (MRC) and the National Institute for Health and Care Research (NIHR), with contributions from the Chief Scientist Office (CSO) in Scotland and National Institute for Social Care and Health Research (NISCHR) in Wales and the Health and Social Care Research and Development (HSC R&D), Public Health Agency in Northern Ireland.

This article

The research reported in this issue of the journal was funded by the EME programme as award number 13/94/55. The contractual start date was in April 2015. The draft manuscript began editorial review in February 2023 and was accepted for publication in July 2024. The authors have been wholly responsible for all data collection, analysis and interpretation, and for writing up their work. The EME editors and production house have tried to ensure the accuracy of the authors' manuscript and would like to thank the reviewers for their constructive comments on the draft document. However, they do not accept liability for damages or losses arising from material published in this article.

This article presents independent research. The views and opinions expressed by authors in this publication are those of the authors and do not necessarily reflect those of the NHS, the NIHR, the MRC, the EME programme or the Department of Health and Social Care. If there are verbatim quotations included in this publication the views and opinions expressed by the interviewees are those of the interviewees and do not necessarily reflect those of the authors, those of the NHS, the NIHR, the EME programme or the Department of Health and Social Care.

This article was published based on current knowledge at the time and date of publication. NIHR is committed to being inclusive and will continually monitor best practice and guidance in relation to terminology and language to ensure that we remain relevant to our stakeholders.

Published by the NIHR Journals Library (www.journalslibrary.nihr.ac.uk), produced by Newgen Digitalworks Pvt Ltd, Chennai, India (www.newgen.co).

Abstract

Background: Long-term surviving liver transplant recipients can spontaneously develop operational tolerance, which allows them to completely discontinue their immunosuppression, but we lack validated tools to predict the likelihood of rejection following immunosuppression withdrawal. A previous clinical trial showed that a logistic regression algorithm including the transcript levels of a set of five genes in a liver biopsy could predict the success of immunosuppression withdrawal with high sensitivity and specificity.

Objective: To determine if the use of a liver tissue transcriptional test of tolerance to stratify liver recipients prior to immunosuppression withdrawal accurately identifies operationally tolerant recipients and reduces the incidence of rejection, as compared with a control group in whom immunosuppression withdrawal is performed without stratification.

Design and methods: Prospective, multicentric, phase IV, biomarker-strategy design trial with a randomised control group in which adult liver transplant recipients were randomised 1 : 1 to either: (1) non-biomarker-based immunosuppression weaning (Arm A); or (2) biomarker-based immunosuppression weaning (Arm B).

Setting and participants: Adult liver transplant recipients ≥ 3 years post transplant (≥ 6 years if age ≤ 50 years old) with no history of autoimmunity or recent episodes of rejection, normal allograft function, and no significant histological abnormalities in a baseline screening liver biopsy, recruited from 12 transplant units in United Kingdom, Germany, Belgium and Spain.

Intervention: Enrolled patients underwent a screening liver biopsy to exclude the presence of subclinical allograft damage. Eligible participants randomised to Arm A underwent gradual discontinuation of immunosuppression. Among participants allocated to Arm B, only those found to be biomarker-positive were offered immunosuppression withdrawal, while biomarker-negative participants remained on their baseline immunosuppression. Patients who completely discontinued immunosuppression and maintained stable allograft function underwent protocol liver biopsies at 12 and 24 months after immunosuppression withdrawal.

Main outcome measure: Development of operational tolerance, defined as the successful discontinuation of immunosuppression with maintenance of normal allograft status 12 and 24 months after immunosuppression withdrawal.

Results: One hundred and twenty-two patients were eligible to participate in the trial, 116 were randomised (58 to Arm A and 58 to Arm B), 80 initiated immunosuppression withdrawal and 34 were maintaining on their baseline immunosuppression. Among the 80 patients who initiated withdrawal, 54 (67.5%) developed clinically apparent rejection, 22 (27.5%) successfully discontinued immunosuppression, 21 underwent a liver biopsy and 13 (16.3%) met the histological criteria of operational tolerance at 12 months after immunosuppression discontinuation. The transcriptional tolerance biomarker was not accurate at identifying patients meeting the operational tolerance criteria [odds ratio 1.466, 95% confidence interval (CI) 0.326 to 9.215; $p = 0.744$; Sensitivity (Sn) 54%, Specificity (Sp) 42%, positive predictive value 16%, and negative predictive value 81%, with an accuracy of 44%]. Due to the poor diagnostic performance of the test, the trial was terminated prematurely following an interim analysis of the results. No patients lost their grafts as a result of rejection during the study duration.

Conclusions: In selected liver transplant recipients, immunosuppression withdrawal proved to be feasible, but was successful in a much lower proportion of patients than originally estimated. A previously validated liver tissue transcriptional biomarker test was not considered accurate in predicting the success of immunosuppression withdrawal.

Study registration: Current Controlled Trials ISRCTN47808000 and EudraCT 2014-004557-14.

Funding: This award was funded by the National Institute for Health and Care Research (NIHR) Efficacy and Mechanism Evaluation (EME) programme (NIHR award ref: 13/94/55) and is published in full in *Efficacy and Mechanism Evaluation*; Vol. 12, No. 3. See the NIHR Funding and Awards website for further award information.

Contents

List of tables

List of figures

List of abbreviations

A1AD	alpha-1-antitrypsin deficiency
ALT	alanine aminotransferase
AST	aspartate aminotransferase
AUC	area under the curve
CART	classification and regression tree
cMFI	cumulative mean fluorescence intensity
CNIs	calcineurin inhibitors
DMEC	Data Monitoring and Ethics Committee
DSA	donor-specific anti-HLA antibody
eGFR	estimated glomerular filtration rate
FFPE	formalin-fixed and paraffin-embedded
GGT	γ-glutamyl-transpeptidase
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HRQoL	health-related quality of life
IS	immunosuppression
LFT	liver function tests
LIFT	liver immunosuppression free trial
LSM	liver stiffness measurement
LT	liver transplantation
MFI	mean fluorescence intensity
MIF	macrophage inhibitory factor
MMF	mycophenolate mofetil
NIHR	National Institute for Health and Care Research
NK	natural killer
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
probTCMR	probability of alloimmune damage
ROC	receiver operator characteristics
TCMR	T-cell-mediated rejection
TSC	Trial Steering Committee

Plain language summary

After liver transplantation, the body's immune system may reject the transplanted organ. In order to prevent rejection, the immune system has to be weakened or suppressed by administering anti-rejection medications. The majority of liver transplant patients need to take the anti-rejection drugs for life, which can be problematic due to their many side effects. However, years after transplantation, a small group of patients can stop their anti-rejection drugs without undergoing rejection. This phenomenon is known as transplantation tolerance. In a study completed in 2012, it was possible to identify liver transplant patients who had developed tolerance with high precision by conducting a genetic test in a liver biopsy.

The objective of the current clinical trial was to validate this test of tolerance. This was done by enrolling patients more than 3 years after transplantation and allocating them at random to two groups. All the patients in group A had their anti-rejection medication gradually discontinued, while in group B only those patients who had a positive test result had their anti-rejection medication weaned. The expectation was that more patients would be able to stop their anti-rejection medication in group B than in group A.

One hundred and twenty-two patients were enrolled in the trial, out of whom 80 patients attempted to discontinue the anti-rejection drugs, while 34 patients maintained their normal medications. Among patients who attempted to stop anti-rejection drugs, 67.5% developed rejection, 27.5% completely stopped the anti-rejection drugs, but 16% were considered as truly tolerant after having had a liver biopsy. Overall, drug discontinuation was successful in a much lower proportion of patients than originally predicted. Furthermore, the test of tolerance was not accurate enough to identify tolerant patients before initiating anti-rejection drug discontinuation. As a result of the diagnostic test not performing as expected, the trial had to be terminated prematurely.

Scientific summary

Background

Long-term survival following liver transplantation has not significantly improved over the past 30 years, with long-term sequelae from chronic immunosuppression, including infections and cancer, being the most common causes of death. Long-term surviving liver transplant recipients can spontaneously develop operational tolerance, which allows them to completely discontinue their immunosuppression, but we lack validated tools to predict the likelihood of rejection following immunosuppression withdrawal. A previous clinical trial showed that a logistic regression algorithm including the transcript levels of a set of five genes in a liver biopsy could predict the success of immunosuppression withdrawal with high sensitivity and specificity.

Objective

To determine if the use of a liver tissue transcriptional test of tolerance to stratify liver recipients prior to immunosuppression withdrawal accurately identifies operationally tolerant recipients and reduces the incidence of rejection, as compared with a control group in whom immunosuppression withdrawal is performed without stratification.

Methods

Design

The *liver immunosuppression free trial (LIFT)* was a prospective, multicentre, phase IV, biomarker-strategy design trial with a randomised control group in which adult liver transplant recipients were randomised 1 : 1 to either: (1) non-biomarker-based immunosuppression (IS) weaning (Arm A); or (2) biomarker-based IS weaning (Arm B). Participants: liver transplant recipients ≥ 3 years post transplant (≥ 6 years if age ≤ 50 years old) with no history of autoimmunity or recent episodes of rejection, normal allograft function, and no significant histological abnormalities in a baseline screening liver biopsy, recruited from 12 transplant units in UK, Germany, Belgium and Spain.

Intervention

Enrolled patients underwent a screening liver biopsy to exclude the presence of subclinical allograft damage. Eligible participants randomised to Arm A underwent gradual discontinuation of immunosuppression over a 6 to 9-month period. Among participants allocated to Arm B, only those found to be biomarker-positive were offered immunosuppression withdrawal (as in Arm A), while biomarker-negative participants remained on their baseline maintenance immunosuppression. Patients who completely discontinued immunosuppression and maintained stable allograft function underwent protocol liver biopsies at 12 and 24 months after immunosuppression withdrawal to confirm histological criteria of operational tolerance (as previously described by the Banff Liver Histopathology Group).

Main outcome measure

The primary end point was the development of operational tolerance, defined as the successful discontinuation of immunosuppression with maintenance of normal allograft status, as assessed by liver biopsy and liver tests 12 and 24 months after immunosuppression withdrawal.

Results

Of the 122 patients eligible to participate in the trial, 116 randomised (58 to Arm A and 58 to Arm B), 80 initiated immunosuppression withdrawal and 34 were maintaining on their baseline immunosuppression. Among the 80 patients who initiated immunosuppression withdrawal, 54 (67.5%) developed clinically apparent rejection, 22 (27.5%)

successfully discontinued immunosuppression, 21 underwent a liver biopsy and 13 (16.3%) met the histological criteria of operational tolerance at 12 months after immunosuppression discontinuation. The remaining four patients were withdrawn from the study before developing rejection or reaching the primary end point. Ten out of the 56 (18%) of patients who initiated immunosuppression withdrawal in Arm A achieved operational tolerance at 12 months versus 3 among the 24 patients (13%) who initiated withdrawal in Arm B. The performance evaluation of the transcriptional tolerance biomarker showed that the test was not accurate at identifying patients meeting the operational tolerance criteria (odds ratio 1.466, 95% IC 0.326 to 9.215; $p = 0.744$; Sn 54%, Sp 42%, positive predictive value 16%, and negative predictive value 81%, with an accuracy of 44%). Due to the poor diagnostic performance of the test, the trial was terminated prematurely following an interim analysis of the results.

Following the protocol liver biopsy performed 24 months after withdrawal, 16 out of the 21 who underwent a liver biopsy 12 months after IS discontinuation were considered not to require immunosuppression, 15 (18.8% of the 80 patients who initiated IS withdrawal) of whom met operational tolerance histology criteria. No patients lost their grafts as a result of rejection during the study duration. Subclinical histological abnormalities indicative of active alloimmune damage in patients considered non-eligible to participate in the trial were associated with serum transaminases, donor-specific antibodies and/or transient elastography measurements.

Conclusions

Immunosuppression withdrawal proved to be feasible and safe but was successful in a much lower proportion of subjects than originally estimated. A liver tissue biomarker test previously validated in a population of liver transplant recipients with a much higher prevalence of operational tolerance, was not accurate in predicting the success of immunosuppression withdrawal. As a result, the trial had to be terminated prematurely and did not meet its objectives. The use of non-invasive markers such as serum transaminases, donor-specific antibodies and transient elastography is useful to identify stable liver transplant recipients with active underlying graft alloimmunity despite receiving immunosuppression.

Study registration

Current Controlled Trials ISRCTN47808000 and EudraCT 2014-004557-14.

Funding

This award was funded by the National Institute for Health and Care Research (NIHR) Efficacy and Mechanism Evaluation (EME) programme (NIHR award ref: 13/94/55) and is published in full in *Efficacy and Mechanism Evaluation*; Vol. 12, No. 3. See the NIHR Funding and Awards website for further award information.

Chapter 1 Introduction

Operational tolerance following clinical liver transplantation

Although life-long immunosuppression (IS) is typically regarded as obligatory for solid-organ recipients to avoid the risk of graft loss from allo-immune attack, evidence that not all liver transplant recipients require perpetual IS has been known for more than two decades.[1] These patients, who maintain normal graft function in the absence of histological signs of progressive graft damage and do not exhibit manifestations of immunocompromise, are conventionally referred to as operationally tolerant. Following the original report from Starzl *et al.* in 1993 describing the cases of six non-compliant liver recipients who discontinued IS and yet maintained normal liver function for 5–13 years,[1] several reports corresponding to retrospective and/or single-centre experiences with IS withdrawal were published.[2–11] On the basis of these studies, a 20% prevalence of operational tolerance in liver transplantation (LT) was proposed,[6] although this estimate did not take into consideration the heterogeneity of the study designs and of the criteria employed to select and enrol patients. The incidence of acute rejection episodes within these studies was very high. These episodes, however, were in most cases mild, and often resolved by return to baseline IS without administration of steroid boluses. Overall, these studies demonstrated the feasibility of discontinuing IS from stable liver recipients, but small sample sizes and/or lack of homogeneous well-standardised algorithms for patient screening, drug withdrawal and patient follow-up reduced the generation of truly generalisable information.

The results of the first two prospective, multicentre and independently monitored clinical trials of IS withdrawal[12,13] addressed some of the limitations of previous studies. In the first of these two studies, sponsored by the Immune Tolerance Network in the USA, IS was prospectively withdrawn in 20 carefully selected paediatric recipients.[13] Drug withdrawal was successful in 12 recipients, who maintained normal graft function after at least 1 year following complete IS discontinuation. Liver biopsies obtained more than 2 years after complete IS withdrawal showed no significant change compared with baseline histology. The most significant clinical factor associated with successful IS withdrawal was an increased time interval between transplantation and initiation of IS weaning (100.6 months in operationally tolerant vs. 73 months in those who failed weaning; $p = 0.03$). No patient developed irreversible graft damage. The second study, led by Professor A. Sanchez-Fueyo, was supported by the European Commission RISET Consortium and enrolled 102 adult liver recipients, at least 3 years after transplantation, from Barcelona, Rome and Brussels.[12] Forty-two participants were successfully weaned, maintained stable graft function for at least 12 months after drug withdrawal, and exhibited no signs of rejection in protocol liver biopsies obtained 12 and 36 months following withdrawal. The successful discontinuation of IS was associated with longer duration after transplantation, more advanced age of the recipients at the time of transplant, and male sex. Furthermore, tolerant patients were more likely to be receiving lower doses of calcineurin inhibitors (CNIs) or no CNIs at all (although this variable was no longer significant following the multivariate analysis). The effect of time after transplantation was surprisingly strong, in that a striking 79% of recipients enrolled in the study more than 11 years after transplant could be successfully weaned from IS, while this occurred in < 15% of those transplanted for < 6 years. In liver recipients who were < 6 years post transplant and older than 50, and in those 6–11 years post transplant, the success rate was 30% and 38%, respectively.[12] In addition to these two studies, data from a US randomised adult trial originally reported in a preliminary form in 2011 and subsequently published in 2019,[14] in which IS withdrawal was initiated during the second year post transplant showed that only 13% managed to discontinue all IS for more than 1 year.[14] This further supports the notion that time after transplantation is a critical parameter associated with tolerance.

Taken together, these results indicate that when liver transplant recipients are carefully selected according to clinical and histological criteria (e.g. > 3 years after transplantation, absence of recent episodes of rejection, no autoimmunity and liver biopsy without significant inflammatory damage), and drug withdrawal is carefully performed following well-standardised protocols, tolerance is observed in approximately 15–40% of recipients, and even more in very long-term surviving patients. This makes IS withdrawal a tangible clinical opportunity in the setting of LT. Consideration of IS withdrawal, however, must carefully weigh the risks of inciting graft rejection. To date, there are no validated biomarkers available to identify patients likely to develop operational tolerance, beyond the clinical parameters outlined above. As a result, clinical trials of IS withdrawal continue to be conducted using a trial-and-error strategy,

whereby patients are carefully monitored while their IS is gradually discontinued, so that whenever liver tests become abnormal IS can be rapidly reinstituted before irreversible graft damage ensues. There is a need, therefore, for precise prospective identification of individuals who have become operationally tolerant to their transplanted liver. This would allow personalised medical patient care by safe drug elimination in select subjects and may also provide clues to the mechanisms accounting for tolerance generation, thereby facilitating the intentional induction of tolerance in those who do not develop it spontaneously. Of note, given that IS withdrawal is not considered standard of care, the real prevalence of operational tolerance in the general population of liver transplant recipients cannot be accurately estimated, but is likely to be significantly lower than what can be extrapolated from the selected group of patients who agree to participate in clinical trials.

Liver tissue transcriptional biomarkers of operational tolerance

Studies from A. Sanchez-Fueyo's group suggest that liver operational tolerance can be predicted employing cellular and/or molecular biomarkers. A gene expression signature indicative of tolerance was first identified in blood from operationally tolerant recipients and appropriate controls. This signature included genes encoding gamma-delta T cells and natural killer (NK) cells.[15,16] These findings were prospectively validated on samples collected before IS discontinuation in the Reprogramming the Immune System for the Establishment of Tolerance (RISET) Consortium trial.[12,17] Microarray and real-time polymerase chain reaction (PCR) experiments conducted on peripheral blood mononuclear cells (PBMC) samples confirmed the over-representation of transcripts preferentially expressed by NK cells in tolerant patients. However, the PBMC molecular signature lacked reproducibility across the three participating clinical centres and could not reliably predict the outcome of IS withdrawal.

In contrast, analyses of liver biopsies by microarray, followed by validation of gene changes by real-time PCR, identified a group of 10 genes (*TFRC*, *PEBP1*, *MIF*, *CDHR2*, *SOCS1*, *IFNG*, *HAMP*, *SLC5A12*, *DAB2*, *HMOX1*) whose differential expression was significantly associated with tolerance, independently from all clinical parameters associated with successful IS withdrawal. An unexpected observation was the over-representation of genes involved in iron metabolism [e.g. transferrin receptor 1 (*TFRC*), hepcidin (*HAMP*), macrophage inhibitory factor (MIF)]. This was consistent with the finding that tolerant and non-tolerant recipients differed in hepcidin and ferritin serum levels, as well as in hepatocyte iron deposition (higher in liver recipients successfully weaned from IS).[17] The significant correlation between intra-hepatic gene expression, serum hepcidin and markers of iron status, provided an indirect validation of the gene expression results, and suggested for the first time that changes in iron metabolism could be involved in the regulation of alloimmune responses and in the establishment of tolerance.

A combination of 5 out of the 10 genes measured at baseline (i.e. before IS was discontinued) was extremely accurate at discriminating those liver recipients who could successfully withdraw IS from those who could not.[17] This predictive signature contained the following five genes: *SOCS1*, *TFRC*, *PEBP1*, *MIF*, *CDHR2*, and predicted the outcome of IS withdrawal with area under the curve (AUC) = 0.85, Sn = 89%, Sp = 86%, positive predictive value (PPV) = 80%, and negative predictive value (NPV) = 92%. The signature was different from those reported from PBMCs or whole blood and was highly reproducible across the three participating clinical sites. Thus, the test was originally identified in the 48 liver transplant recipients enrolled in Barcelona, and validated in an independent cohort of 21 recipients from Brussels and Rome.[17]

In order to confirm the reproducibility of the real-time PCR gene expression results originally performed in Hospital Clinic Barcelona in 2011,[17] we conducted additional transcriptional experiments employing the same Applied Biosystems 7900HT real-time PCR platform selected to conduct the current clinical trial. The experiments included several commercial and non-commercial RNA calibrators, as well as several different housekeeping genes. Reproducibility was optimised by employing a commercial RNA calibrator (liver RNA, Clontech, Mountain View, California, USA) and both GAPDH and HPRT1 as housekeeping genes. These experiments were used to recalibrate the predictive algorithm employing the same exact set-up that will be used in the clinical trial. These experiments included 56 of the original 69 RNA samples used in the 2011 experiments (*Figure 1*).

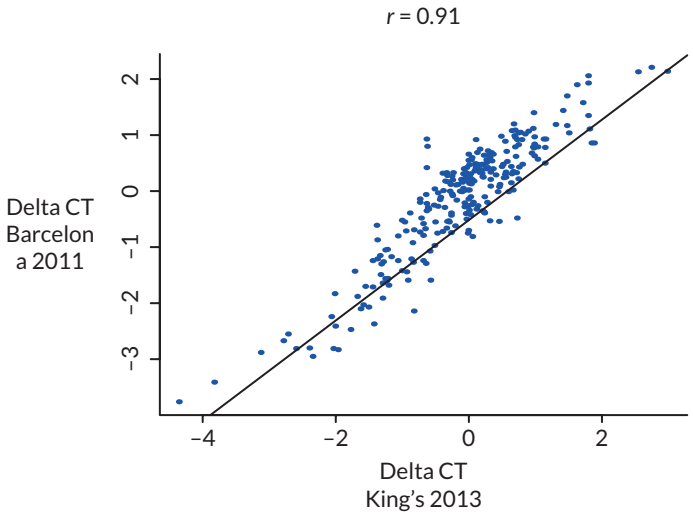

FIGURE 1 Technical replication of liver tissue transcriptional biomarker test. Correlation between the PCR gene expression results for the five genes included in the biomarker test of tolerance obtained in the original experiments (Hospital Clinic Barcelona 2011) and the technical replicates performed at King's. The King's experiments were conducted in an Applied Biosystems 7900HT real-time PCR platform employing optimised low-density PCR arrays, 2 housekeeping genes and 1 commercial RNA calibrator, and using 56 of the original 69 RNA samples employed in the 2011 experiments.

The original algorithm was recalibrated to take into account different experimental conditions, and its diagnostic performance was tested in the set of 56 samples plus an independent set of 9 samples collected from liver recipients in whom IS was discontinued after completion of the original clinical trial. Employing the equation described above, the overall diagnostic performance was SN = 72%, SP = 89%, PPV = 82%, NPV = 83%.

Inter- and intrapatient variability of real-time polymerase chain reaction gene expression measurements

Percutaneous sampling of the liver can be associated with significant errors (e.g. up to 20% sampling error is routinely described in percutaneous liver biopsy histopathology analyses). We conducted additional experiments to quantify the variability associated with biopsies collected from different regions of the liver, as well as with the RNA extraction, RNA retro-transcription and PCR reactions. The expression levels of 24 genes associated with tolerance (including the 5 genes that constitute the biomarker test of tolerance) were measured in samples collected from the right and left lobes of explanted livers under a variety of different experimental conditions. Highly reproducible results were observed between samples collected from the same patients, regardless of the liver lobe, date of RNA extraction and date of RNA retro-transcription (*Figure 2*). The experimental variability associated with different PCR experiments conducted on the same cDNA samples was negligible (data not shown).

Definition of operational tolerance

Operational tolerance is typically defined as stable graft function in a recipient off immunosuppressive drugs and in whom no clinically significant detrimental immune responses and immune deficits are detected.[18] Given that during the performance of intentional IS withdrawal protocols most rejection episodes occur during the period of IS weaning or shortly after having completely discontinued the immunosuppressive drugs, it has been agreed that at least 1 year off IS is required in order to confidently consider that operational tolerance has been established. Standard liver biochemistry tests, however, are an insensitive tool to adequately assess liver allograft damage. For this reason, in addition to the stability of the liver tests, histological proof of a rejection-free allograft is considered an essential component of the clinical definition of operational tolerance.

To provide a consensus guideline on the interpretation of histological findings derived from operationally tolerant liver allografts, and, in particular, on what constitutes a 'rejection-free' allograft, in 2012 the Banff Working Group on Liver Allograft Pathology prepared a report that contained a list of histological findings that could indicate progressive

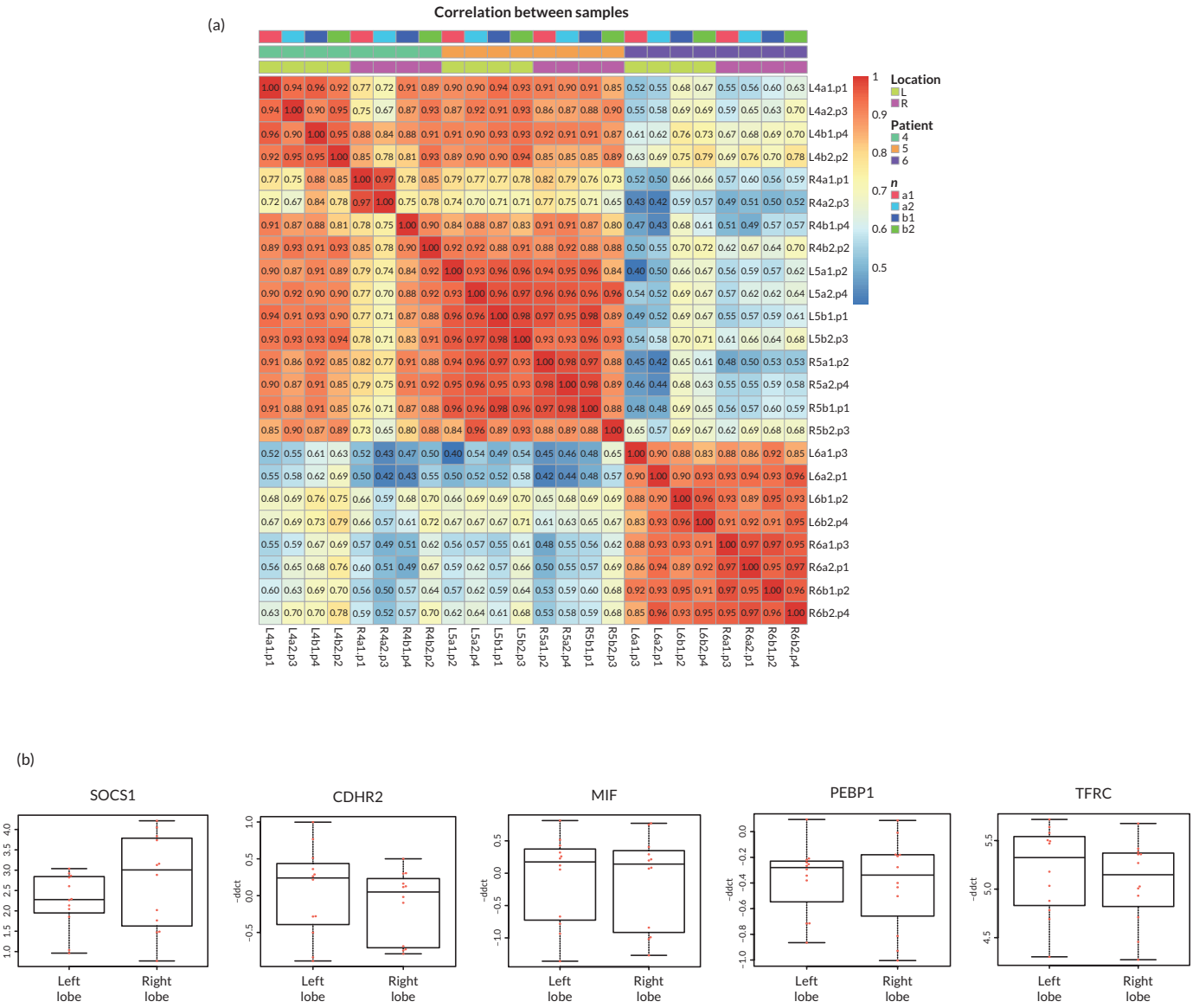

FIGURE 2 Reproducibility of the liver tissue transcriptional biomarker test in liver biopsies collected from different lobes of the liver. (a) Plot showing the correlation coefficients between 24 samples used in a real-time PCR experiment measuring the expression of 24 genes associated with tolerance (including the 5 genes that constitute the biomarker test being assessed). Samples were collected from the right (R) and left (L) lobes of three different livers (patients 4, 5, 6). Two different portions of each liver tissue simple were extracted separately (a, b). For each extraction, two different retro-transcription reactions were performed (a1, a2, b1, b2). (b) Box plots showing the relative expression (ddC_T) of the five genes included in the biomarker test of tolerance in samples collected from the right and left lobes of explanted livers. No significant differences were found for any of the genes tested.

immunological damage and the absence/failure of operational tolerance.[19] This set of criteria, described in *Table 1*, have now been incorporated into the standard definition of operational tolerance in most if not all clinical trials of liver allograft tolerance, and are a component of the primary end point in *liver immunosuppression free trial (LIFT)*.

The use of this histological definition 1 year after complete IS withdrawal, however, does not take into consideration the possibility of tolerant allografts developing transient non-progressive inflammatory changes, a phenomenon very well studied in animal models of spontaneous tolerance and that was also documented in the *RISET* adult multicentre European clinical trial[12,21] (*Figure 3*).

These studies suggest that in patients who develop mild inflammatory changes 1 year after IS withdrawal, remaining off IS is safe provided a careful monitoring with sequential liver biopsies is conducted, and might be preferable to the reinstitution of IS. For this reason, for the current study we followed the Banff Group criteria but included an additional set of criteria in which circumstances IS had to be reinstituted and in which it was considered safe not to do so despite not meeting operational tolerance histological criteria (*Table 2*).

Risks and benefits of immunosuppression discontinuation

Benefits

Chronic IS is associated with a variety of life-threatening side effects following LT, including infection, malignancy, hypertension, diabetes, nephrotoxicity and cardiovascular diseases. CNI-induced nephrotoxicity, in particular, is responsible for a significant rate of chronic renal failure, need for renal replacement therapy and increased mortality.[24–26] Elimination of CNIs may preserve waning renal function and avoid the associated morbidity and mortality risk. Identification of a reproducible and reliable tolerance signature will allow tailoring of IS to individual patient characteristics. It may also identify critical pathways responsible for the tolerant state that can be therapeutically exploited to induce tolerance in those patients who do not achieve it spontaneously. As of today, however, no robust evidences have been generated demonstrating that complete IS withdrawal improves patient survival or favourably modifies the natural history of IS-related comorbidities (probably due to the fact that IS withdrawal is only performed within clinical trials that tend to exclude those patients with severe comorbidities).

Risks

While there is abundant information in the literature suggesting that in carefully selected liver recipients, IS withdrawal is feasible and safe, the procedure is not without risk, as it can induce immunologically mediated allograft rejection. In

TABLE 1 Banff liver allograft pathology working group criteria of operational tolerance failure in patients off IS[a,19]

Compartment	Findings
Portal inflammation and interface activity	Increased portal inflammation (in comparison with a pre-weaning biopsy sample), especially in association with histopathological evidence of tissue damage manifest as: focally worsening or more prevalent lymphocytic bile duct damage, interface hepatitis, fibrosis, or the appearance of definite venous endotheliitis.
Centrizonal/ perivenular inflammation	New-onset perivenular inflammation (in comparison with a pre-weaning biopsy sample) associated with even mild perivenular necro-inflammatory activity. Note: these changes might be present in the absence of typical portal changes of rejection.
Bile duct changes	New-onset biliary epithelial cell senescence changes or ductopenia when sampling problems and/or an alternative, non-immunological explanation (e.g. biliary strictures) can be reasonably excluded.
Fibrosis[b]	Greater than 1-grade increase in fibrosis in any one compartment: (a) portal/periportal; (b) peri-sinusoidal; or (c) perivenular fibrosis; or new onset bridging fibrosis without an alternative explanation (e.g. biliary strictures) that is reasonably prevalent and not readily explained by a possible sampling error.
Arteries	Any evidence of foam cell or obliterative arteriopathy.

a Patients with underlying autoimmune hepatitis (AIH), hepatitis C virus, primary biliary cholangitis (PBC) or primary sclerosing cholangitis (PSC) are excluded.[20]
b Fibrosis should be graded as follows:[17] portal/periportal: 0–3; peri-sinusoidal: 0–3; perivenular: 0–3.

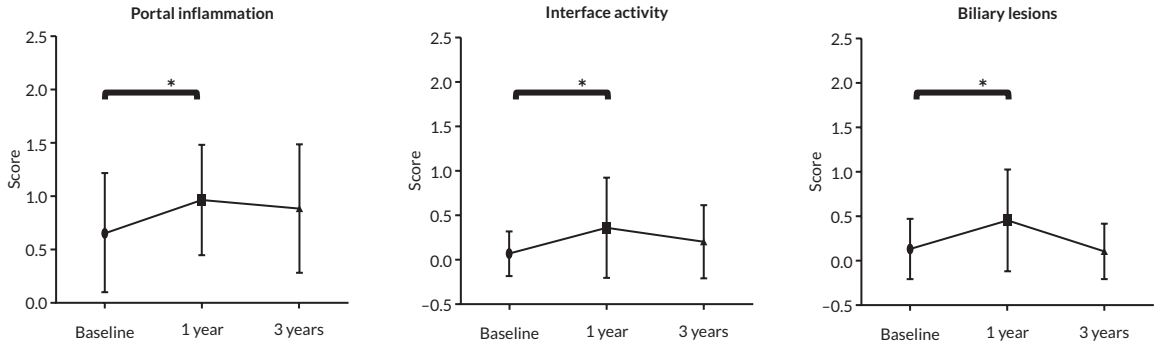

FIGURE 3 Transient histological changes observed after discontinuation of IS. Central blinded review of sequential liver biopsies of liver transplant recipients who successfully discontinued IS (baseline, 1 and 3 years post IS withdrawal) in the RISET adult multicentre European clinical trial, showing that IS withdrawal leads to transient mild increase in inflammatory infiltrates. Data correspond to mean ± standard deviation (SD). No differences between the baseline and the 3-year biopsies were observed.[12,22]

TABLE 2 Follow-up biopsy findings in patients off is that should prompt reinstitution of IS

Compartment	Findings
Portal inflammation and interface activity	Development of moderate portal inflammation or moderate interface activity in most portal tracts
Centrizonal/perivenular inflammation	Central perivenulitis in most central veins
Bile duct changes	New-onset biliary epithelial cell senescence changes or ductopenia when sampling problems and/or an alternative, non-immunological explanation (e.g. biliary strictures) can be reasonably excluded
Fibrosis	At least a 2-grade increase in fibrosis in any one compartment (according to Venturi *et al.*[23])
Arteries	Any evidence of foam cell or obliterative arteriopathy

this regard, the main risks of IS withdrawal are: (1) acute and/or chronic rejection; (2) silent development of allograft fibrosis; (3) potential complications associated with the need to increase IS to treat rejection episodes; and (4) graft loss or patient mortality as a consequence of risks 1–3.

Rejection-associated graft injury or graft loss

A fundamental premise of the current trial is that rejection that develops under the close surveillance of a controlled trial will be easily reversed and will not result in permanent allograft damage. This is based on the results of the recent multicentre clinical trials described above, in which rejection episodes occurring during IS withdrawal were generally mild to moderate in histological severity and easily treated, and no graft losses were observed.[12,14] While theoretically possible, severe rejection leading to graft loss, re-transplantation or patient death is extremely unlikely, and has only been reported in two cases.[3,5] It should be emphasised that these two patients were not enrolled in clinical trials with close surveillance protocols. As such, criteria for patient selection and the process for IS withdrawal were not standardised. Thus, we believe the literature supports the concept that, provided we strictly adhere to the patient monitoring protocols implemented in the recent multicentre clinical trials, we can proceed safely with a trial of IS withdrawal. Given the high rate of expected rejection, the trial is specifically designed to allow early detection of graft dysfunction through frequent monitoring during the period of drug withdrawal and in the period early after IS cessation. Based on our previous experience, we expect that with this approach the majority of rejection episodes will be detected early and reversed readily.

Risk associated with treatment of rejection

The reinstitution of CNIs alone, or in combination with low-dose steroids, to treat the rejection episodes that occur during staged weaning may be associated with transient worsening of certain comorbidities (diabetes, hypertension, hyperlipidaemia, etc.), but is very unlikely to result in irreversible damage. The need to use strong IS regimens to reverse

rejection (e.g. steroid boluses, T-cell depleting antibodies) may increase the risk of infection (e.g. cytomegalovirus reactivation), malignancy and renal dysfunction. Within a carefully monitored clinical trial the development of rejection episodes of such severity is extremely unlikely.

Risk of developing subclinical allograft fibrosis

In most studies, liver recipients off IS have exhibited no obvious progressive liver histological damage. Yoshitomi *et al.*, however, reported slightly increased fibrosis progression in operationally tolerant paediatric liver recipients as compared with recipients under maintenance IS.[27] This case-control study had substantial flaws, as it lacked pre-weaning liver biopsies, and cases and controls significantly differed in the length of their post-transplant follow-up. Reassuringly, neither the *RISET* nor the Immune Tolerance Network trials, that included strict protocols for sequential liver biopsies, observed development of clinically significant fibrosis.

Risk of developing donor-specific antibodies

Several reports in kidney transplantation have described that minimisation or discontinuation of CNIs may promote the generation of donor-specific antibodies, but this has not been universally confirmed in the setting of LT. Thus, in the *RISET* Consortium trial, IS discontinuation did not increase the development of donor-specific antibodies.[12] On the other hand, in the Immune Tolerance Network paediatric trial most participants in whom IS was discontinued developed anti-donor antibodies, but these antibodies were of the immunoglobulin G4 isotype, which are considered non-pathogenic or even protective. Furthermore, none of the paediatric recipients off IS developed significant long-term histological graft damage.[13,22,28] To clarify the role of IS in the development of anti-donor antibodies and long-term histological damage, the current clinical trial stipulates sequential anti-donor antibody monitoring and protocol liver biopsies.

Risks associated with liver biopsy

Liver biopsy remains the gold standard in the diagnosis of rejection and will be employed in all suspected cases of rejection in this trial unless clinically contraindicated or logistically infeasible. The procedure is usually performed percutaneously under ultrasound guidance and local anaesthetic. Occasionally the liver biopsy can be performed through a catheter inserted through the jugular vein (transjugular liver biopsy). It is often associated with mild pain usually lasting only a few hours. The risk of significant bleeding requiring transfusion is 0.5–1% and the risk of bile leak or injury to adjacent organs (pneumothorax, bowel perforation etc.) is even rarer. The risk of one of these complications leading to death is estimated at 0.1–0.01%.[20]

Risk associated with blood draws

Frequent blood draws to allow close monitoring of liver function during and after IS withdrawal is essential for the trial's safe conduct. Peripheral blood draws typically incur mild temporary discomfort. Rare but more serious risks include ecchymosis, thrombophlebitis and infection.

Rationale for conducting immunosuppression withdrawal trials

Long-term survival after solid organ transplantation has increased during the last decades[26] due to improvements in surgical technique, perioperative care and more efficient IS. However, transplant recipients still exhibit higher morbidity and mortality than the general population. One of the main causes is comorbidities negatively influenced by chronic IS drug usage.[29] Minimisation (or complete withdrawal) of IS, particularly CNIs, may overcome these problems and has become a priority goal in transplantation. The clinical opportunity is more tangible in the liver than in other transplantation settings due to the greater capacity of the liver allograft to cope with the cytolytic effects of alloimmune responses. The potential benefits of IS minimisation or withdrawal, however, still need to be balanced with the risks and inconveniences of prompting liver allograft rejection. The recent observation that operational tolerance can be predicted employing a combination of clinical parameters and molecular biomarkers would modify the equipoise in favour of discontinuing IS in previously identified operationally tolerant recipients. Identification of a reproducible and reliable tolerance signature would therefore substantially benefit the liver transplant population. In Europe, approximately 6000 liver transplants are performed every year (700 of them in the UK alone). Under the current standard-of-care indefinite pharmacological IS is prescribed to all of them. The cost of immunosuppressive drugs is £3000–5000/patient/year. In addition to its cost, chronic IS results in substantial side effects (hypertension, diabetes,

renal failure, hyperlipidaemia, cancer, infections) that contribute to patient morbidity and mortality. The implementation of tolerance biomarkers would reduce the cost of medical management in LT, reduce the negative impact of comorbidities associated with the use of chronic IS, and increase the quality of life of liver recipients.

The recent prospective, multicentre drug withdrawal trials conducted in Europe and in the USA as described above have been major breakthroughs in the field.[12,13] First, they have identified the subgroups of liver recipients more likely to benefit from IS withdrawal. Second, they have demonstrated that in hepatitis C negative liver recipients, operational tolerance can be predicted by measuring the expression of a five-gene signature in liver biopsies, while blood transcriptional biomarkers lack sufficient reproducibility. Third, they have identified novel mechanisms likely to be involved in the spontaneous development of immune tolerance (e.g. role of iron metabolism, immunosenescence, influence of anti-HLA antibodies), some of which could have implications beyond LT. These recent studies provide the rationale and clinical opportunity to perform, for the first time, a randomised controlled trial of IS withdrawal to test the clinical utility and safety of a biomarker test of liver transplant tolerance.

Clinically silent allograft damage in stable liver transplant recipients

As outlined above, numerous trials of IS withdrawal have now shown that many stable long-LT recipients in whom surveillance liver biopsies show no significant fibro-inflammatory lesions, can minimise their IS levels or even completely discontinue these drugs.[12,13] These evidences suggest that many LT recipients are being administered excessive IS, particularly those who have been transplanted for a long time given that the risk of rejection following LT is known to decrease with time.[12,30] On the other hand, many other studies that have performed surveillance liver biopsies have shown that there is a significant proportion of LT recipients (around 30%) with normal allograft function who exhibit subclinical histological lesions. From a transcriptional standpoint, these subclinical lesions display molecular features that closely resemble what is detected at the time of T-cell-mediated rejection (TCMR). These data have been interpreted as indicating that these patients are receiving too little IS.[31,32] Whether these subclinical lesions compromise the survival of the allograft is unclear, but there are a number of evidences that suggest that after more than a decade of follow-up they may result in significant allograft damage and even graft loss.[31,33] Data from our research group indicate that those liver biopsies that exhibit the highest transcript levels of TCMR-related genes are more likely to develop clinically significant damage over time.[31] Altogether, these data highlight the difficulties in deciding the optimal amount of immunosuppression for stable LT recipients, when using empirical diagnostic parameters such as serum transaminases or CNI pharmacokinetic measurements. Although these problems could be potentially overcome by using protocol or surveillance liver biopsies and gene expression analyses, very few transplant units worldwide are willing to do so (due to the increased costs, burden and potential risks). In order to optimise the management of LT recipients it is essential therefore to develop non-invasive diagnostic tests and some predictive models to estimate if the amount of IS being administered is adequate, and to determine the likelihood of LT recipients developing clinically significant allograft damage despite normal liver tests.

Chapter 2 Study objectives

The primary objective of the current study was to determine if the use of a liver tissue transcriptional test of tolerance to stratify liver recipients prior to IS withdrawal accurately identifies operationally tolerant recipients and reduces the incidence of rejection, as compared with a control group in whom IS withdrawal is performed without stratification.

Secondary objectives of the study included:

1. to establish the safety of biomarker-guided IS withdrawal
2. to determine the health-economic impact of withdrawing IS in liver transplant recipients and to assess how much this cost is influenced by the use of a diagnostic test of operational tolerance
3. to assess the effect of IS withdrawal on the quality of life of liver transplant recipients
4. to determine the extent to which IS withdrawal improves drug-related comorbidities
5. to investigate if liver transplant recipients under IS become operationally tolerant over time
6. to determine if the presence of donor-specific anti-HLA antibodies (DSA) influences the success of IS withdrawal, and whether IS withdrawal promotes the development of anti-HLA antibodies in liver transplant recipients
7. to explore the association between operational liver transplant tolerance, iron metabolism, immunosenescence and specific gut microbiome profiles
8. to develop non-invasive tools to identify those transplant recipients considered non-eligible to participate in the trial following screening of their liver biopsy, on the basis of the detection of histological signs of alloimmune damage.

Chapter 3 Methods

Clinical trial design

The *LIFT* was a prospective, multicentre, phase IV, biomarker-strategy design trial with a randomised control group in which adult liver transplant recipients underwent IS withdrawal. (*Figure 4*) Enrolled participants were randomised 1 : 1 to either: (1) non-biomarker-based IS weaning (weaning-all; Arm A); or (2) biomarker-based IS weaning (Arm B). In participants allocated to Arm A IS were withdrawn regardless of the result of the biomarker test. Among participants allocated to Arm B, only those found to be biomarker-positive (Arm B+, i.e. potentially tolerant) were offered IS withdrawal, while biomarker-negative participants (Arm B−, i.e. potentially non-tolerant) remained on their baseline maintenance IS. This would allow us to demonstrate that the biomarker is a useful test to personalise IS by offering drug withdrawal only to those participants who are likely to complete the process successfully, avoiding unnecessary rejections among those who have not developed tolerance. Comparing the outcome of IS withdrawal between arms A and B+ would provide direct evidence of the clinical usefulness of the test as a function of its predictive accuracy. We established that for the biomarker to drive safe IS withdrawal its PPV (PPV = Number actually tolerant/Number biomarker-positive) should be no < 0.80, and its sensitivity at least 0.70 To account for centre effects, we used stratified randomisation. Furthermore, to avoid biases, participants undergoing drug withdrawal and their physicians were blinded to the biomarker results. Participants randomised to Arm B− knew their biomarker statuses and were to be maintained in the study until they completed 48 months of follow-up post randomisation, in order to contribute to secondary clinical outcomes.

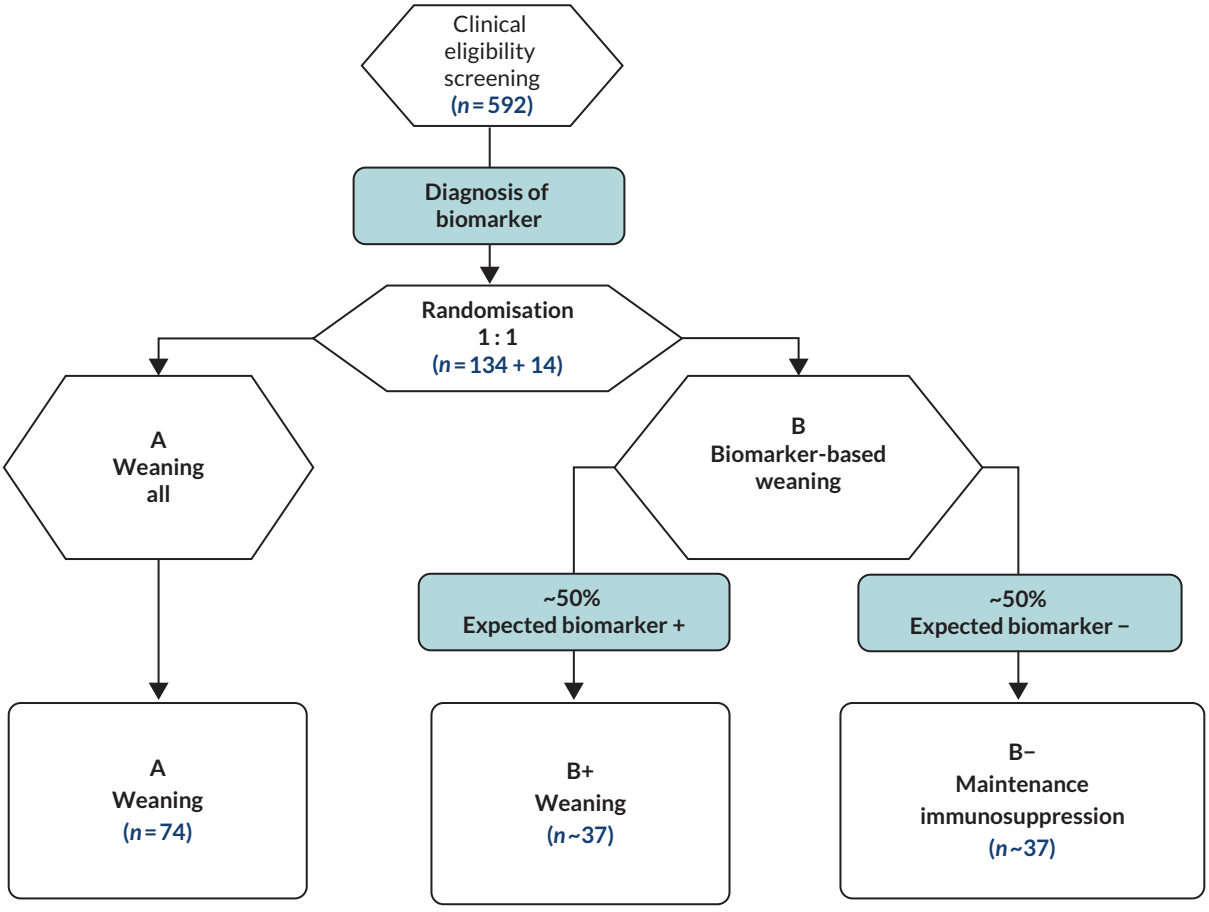

FIGURE 4 Scheme of clinical trial design.

Cost and health-related quality of life (HRQoL) assessments were conducted alongside the trial to estimate the health-economic implications of the two different strategies. Furthermore, sequential biological specimens were collected to conduct ancillary mechanistic studies.

We carried out a cross-sectional study in which we included all 190 patients who gave informed consent to participate in the trial and had a screening liver biopsy done were included in a cross-sectional study to investigate the pathogenesis of subclinical allograft damage and to identify non-invasive tools of active alloimmunity.

Fourteen European liver transplant units participated in the trial: King's College Hospital, Royal Free London, Newcastle, Birmingham, Leeds, Edinburgh, Cambridge, Leuven, Hannover, Berlin, Barcelona, and Cliniques Universitaires Saint-Luc. The study was approved by the corresponding research ethics committee in each participating country (United Kingdom, Germany, Belgium, Spain). The original trial timelines included enrolment phase: 18 months; patient follow-up: 48 months (6–12 months drug weaning, 36 months post-weaning follow-up); total study duration: 72 months.

Study population

Inclusion criteria

1. At the time of screening: more than 3 years post transplant if participants are ≥ 50 years old, odds ratio (OR) ≥ 6 years post transplant if participant age is ≤ 50 years old.
2. Recipient of either deceased or living donor liver transplant.
3. Recipient of single organ transplant only.
4. Liver function tests (LFT): direct bilirubin ≤ 17.1 µmol/l and alanine aminotransferase (ALT) ≤ 60 IU/l at the screening visit.
5. On CNI IS with or without one of the following: low-dose mycophenolic acid (≤ 1080 mg daily), mycophenolate mofetil (MMF ≤ 1500 mg daily), azathioprine (≤ 150 mg daily), sirolimus/everolimus; or on monotherapy with sirolimus/everolimus or mycophenolate/mycophenolic acid monotherapy (effective contraception must be used before beginning mycophenolate therapy, during therapy, and for 6 weeks following discontinuation of therapy).
6. Ability to sign informed consent.

Exclusion criteria

1. Serum positivity for hepatitis C virus (HCV)-RNA.
2. Serum positivity for human immunodeficiency virus (HIV)-1 infection, hepatitis B virus (HBV) surface antigen or HBV-DNA.
3. Immune-mediated liver disease in which IS discontinuation is inadvisable (autoimmune hepatitis, primary sclerosing cholangitis, primary biliary cirrhosis).
4. Acute or chronic rejection within the 52 weeks prior to screening.
5. Glomerular filtration rate < 30 ml/minute (to mitigate the risk of worsening renal failure should rejection occur and high level of CNI be required).
6. The need for chronic anti-coagulation that cannot be safely discontinued to safely perform for a liver biopsy.
7. Baseline (screening) liver biopsy showing any of the following: (a) acute rejection according to Banff criteria; (b) early or late chronic rejection according to Banff criteria; (c) inflammatory activity and/or fibrosis in excess of permissive criteria;[20] (d) any other findings that might make participation in the trial unsafe. Eligibility will be determined by the central pathologist.
8. Patient age < 18 years old at the time of transplant.
9. Pregnant females and females of childbearing age not using effective contraception.
10. Current illicit drug or alcohol abuse.
11. Inability to participate in frequent monitoring of liver function (every 3 weeks) and clinical visits during IS withdrawal.
12. Inability to comply with study-directed treatment.
13. Any medical condition that in the opinion of the principal investigator would interfere with safe completion of the trial.
14. Participation in another clinical trial during the month prior to enrolment.

Patient screening and biomarker test

All participants underwent a screening visit during which prior informed consent was obtained, the medical records were reviewed to assess potential eligibility, a screening liver biopsy was conducted and blood samples were drawn for mechanistic studies. The screening liver biopsy was shipped to King's and employed to confirm histologic eligibility by the central pathologist. Subjects identified as eligible for the clinical trial based on all screening procedures underwent the analysis of the liver tolerance transcriptional biomarker test. This was conducted on a 3-mm fraction of the screening liver biopsy cylinder that was preserved in RNAlater reagent and frozen prior to shipment. The study collected clinical and histological data as well as blood samples from the stable participants identified as clinically suitable but who did not qualify for IS withdrawal.

Randomisation

Participants were randomised 1 : 1 to either (1) non-biomarker-based IS weaning (Arm A) or (2) biomarker-based IS weaning (Arm B). In participants allocated to Arm A, IS was withdrawn regardless of the result of the biomarker test. Participants allocated to Arm B were offered IS withdrawal only if they were classified as tolerant (Arm B+), while they remained on maintenance IS if classified as non-tolerant (Arm B−).

Immunosuppression withdrawal protocol (Arms A and B+)

Weaning from calcineurin inhibitor or mycophenolate/mycophenolic acid monotherapy

Participants initiated IS weaning after at least 3 weeks of stable liver function (as documented by two separated laboratory exams). Weaning occurred in eight 3-week intervals with each subsequent reduction based on LFT stability over the prior 3-week interval. No single reduction should exceed 50% of the daily dose except the final reduction.

Withdrawal proceeded as follows:

- Total daily dose was reduced to 75% current total dose × 3 weeks.
- Total daily dose was reduced to 75% current total dose × 3 weeks.
- Total daily dose was reduced to 50–75% current total dose × 3 weeks.
- Above dose was given 5 × weekly × 3 weeks with consolidation to once daily dose.
- The same dose was given 4 × weekly × 3 weeks.
- The same dose was given 3 × weekly × 3 weeks.
- The same dose was given 2 × weekly × 3 weeks.
- The same dose was given 1 × weekly × 3 weeks and then discontinued.

Weaning from two immunosuppression drugs

Participants on two IS drugs first underwent withdrawal of CNI as described above. Once the participant had discontinued the CNI, at least 3 weeks of stable liver function documented by two sequential blood tests was required before initiating withdrawal of the second drug. Weaning of the mycophenolate/mycophenolic or azathioprine occurred in three 3-week intervals as follows:

- Daily dose was reduced to approximately 66% of initial total dose × 3 weeks (e.g. in patients initially receiving MMF 1500 mg daily dose, reduced to 1000 mg daily dose).
- Daily dose was reduced to approximately 33% of initial total dose × 3 weeks (e.g. in patients initially receiving MMF 1500 mg daily dose, reduced to 500 mg daily dose).
- Medication was then discontinued.

Pausing of immunosuppression weaning

In the case of adverse events that could compromise safety, IS withdrawal was temporarily paused for up to 4 weeks. During this time interval, the participant remained at the current dose. Participants were allowed up to three non-consecutive pauses. IS withdrawal was also temporarily paused during the investigation of allograft dysfunction.

Discontinuation of immunosuppression weaning/resumption of immunosuppression

Participants undergoing IS weaning who experienced rejection were re-started on IS and were not allowed a second attempt. Participants who successfully completed IS weaning and who subsequently experienced rejection were re-started on IS. Participants who could not complete the IS weaning protocol and did not experience rejection remained in the study but were considered as 'failures'.

Maintenance immunosuppression (Arm B−)

Participants randomised to Arm B and who were biomarker-negative were not allowed reductions in their IS for the first 12 months of study participation, except for the management of toxicity attributed to IS (as determined by the local investigator). For the remaining of the study, IS was managed according to each centre's standard of care.

Management of allograft dysfunction

Allograft dysfunction was defined as any unexplainable elevation in ALT and γ-glutamyl-transpeptidase (GGT) relative to baseline and above the upper limit of normality. When ALT and GGT were > 100 IU/l the study protocol stipulated pausing IS withdrawal and performing a liver biopsy. When ALT and/or GGT are < 100 IU/l, IS withdrawal was to be temporarily paused and liver tests were repeated within 7 days. In case of persistent allograft dysfunction with ALT and/or GGT < 100 IU/l, a liver biopsy should be performed or alternatively IS withdrawal remain paused for up to 4 weeks. Within 4 weeks IS should resume or a liver biopsy performed.

Diagnosis and treatment of rejection episodes

Rejection episodes were diagnosed based on liver biopsy findings according to Banff criteria. Clinical decisions, including treatment of acute rejection, were made based on local biopsy readings and each centre's standard of care.

Reinstitution of immunosuppression following successful immunosuppression withdrawal

Patients who did not develop allograft dysfunction but in whom a protocol biopsy performed at 12 or 24 months after complete IS discontinuation revealed moderate inflammatory changes or other changes suggestive of progressive histological damage (as described in *Table 2*) were treated with reinstitution of the baseline IS. Patients who did not exhibit any of the changes described in *Table 2* but failed to meet the primary end point based on the histological criteria defined in *Table 1* were maintained off IS until the end of the study.

Primary end point

The primary end point was defined as the successful discontinuation of IS with maintenance of normal allograft status as assessed by liver biopsy and liver tests 12 and 24 months after IS withdrawal (operational tolerance; see histological criteria in *Table 1*). For the purposes of validating the clinical usefulness of the tolerance biomarker, successful IS withdrawal was considered the gold standard. Since this outcome was strictly restricted to the IS withdrawal process and, by definition, could not be observed in Arm B−, the analysis of the primary outcome was restricted to Arms A and B+. Of note, the histological criteria of operational tolerance employed in *LIFT* (*Table 1*) differ from those employed in the RISET Consortium trial from which the tolerance biomarker was derived.

Secondary end points

- Rejection (incidence, severity, timing, steroid-resistant rejection, chronic rejection).
- Reasons for failure of IS withdrawal.
- Requirement for IS reinstitution despite successful IS withdrawal based on the histological criteria described in *Table 2*.
- Progression of graft fibrosis in tolerant participants and those on maintenance IS.
- Graft loss.
- All-cause mortality.
- Proportion of tolerant participants remaining free of rejection at 3 years post IS withdrawal.
- Renal function at 1, 2 and 3 years after enrolment.
- Change in comorbidities associated with IS use (hypertension, cardiovascular risk profile, diabetes mellitus, hyperlipidaemia, malignancy).
- Stability of the biomarker signature between baseline and last study visit.
- HRQoL changes associated with IS withdrawal.
- Pharmacoeconomic impact of IS withdrawal.
- Sequential transient elastography (FibroScan®, Echosens, Paris, France) measurements.
- Liver tissue transcriptional analyses.
- Blood immune cell flow cytometric phenotyping.
- Alloreactivity assays.
- Intra-hepatic and systemic iron parameters.
- Time post transplant, age, sex and type of IS.
- Gut microbiome profile.
- Development of anti-HLA antibodies (before and after initiation of IS withdrawal).

Liver histopathology

All liver biopsies were performed percutaneously under local anaesthesia except for one biopsy obtained using the transjugular route. Out of the 20 mm of liver core, a 5-mm portion was immediately preserved in RNAlater reagent (Ambion), kept at 4 °C for 24 hours, and then cryopreserved at −80 °C after removal of the RNAlater reagent. The remaining 15-mm cylinder was formalin-fixed and paraffin-embedded (FFPE) for histological assessment. FFPE haematoxylin-eosin and Masson's trichrome stained 4-mm tissue sections of all liver biopsies were prospectively assessed and scored for 18 histopathologic criteria by a central liver histopathologist (RM) without knowledge of any clinical or serological data (*Table 3*).

Liver tissue transcriptional analyses

Cryopreserved liver tissue samples were homogenised in 1 ml TRIzol reagent (Invitrogen) using a bead-beating technology (TissueLyser, Qiagen) in 1.5-ml reaction tubes (Eppendorf). Total RNA was extracted following the manufacturers, guidelines. The concentration, purity and quality of the RNA were measured and assessed using Nanodrop (ThermoScientific) as well as 4200 TapeStation System (Agilent). The liver tissue biomarker test was assessed employing commercially available primer/probe sets, low-density PCR arrays and an Applied Biosystems 7900HT real-time PCR platform. We employed a commercial RNA calibrator (liver RNA, Clontech), two housekeeping genes (GAPDH and HPRT1) and the following equation: 2.132 + 0.442 (CDHR2 ddC_T) −1.148 (MIF ddC_T) + 1.247 (SOCS1 ddC_T) −1.373 (TFRC ddC_T) + 3.065 (PEBP1 ddC_T) with an optimal diagnostic score cut-off of 0.458.

In addition, we employed a customised 20-gene panel and the NanoString® platform (NanoString Technologies, Inc., Seattle, WA, USA) to investigate the expression levels of genes previously shown to be diagnostic of T-cell-mediated rejection following LT.[34] This panel included 11 genes (*CXCL9*, *TOP2A*, *MMP9*, *GBP2*, *GPNMB*, *HMMR*, *CCL19*, *HLA-DMA*, *CD74*, *PLA2G7*, *MMP7*). NanoString data were processed using the geometric mean from previously selected housekeeping genes (*CNOT10*, *MRPS5*, *MTMR14*) using NanoStringNorm. We then used the Predictive Analysis of Microarrays statistical software to derive a gene expression classifier from the transcript levels of the 11 gene set.

TABLE 3 Semiquantitative scoring for central liver histopathology assessments

1. Number of fragments/length (mm);
2. Number of complete portal tracts;
3. Number of central veins;
4. Lobular inflammation: 0 = no inflammation, 1 = rare inflammatory foci, 2 = multiple inflammatory foci, 3 = confluent/bridging areas of necrosis;
5. Central perivenulitis: 0 = no inflammation, 1 = mild (patchy, focal perivenulitis), 2 = moderate (perivenulitis in most central veins), 3 = severe (confluent or bridging hepatocellular necrosis);
6. Portal inflammation: 0 = no inflammation, 1 = mild, 2 = moderate, 3 = severe;
7. Interface hepatitis: 0 = no interface activity, 1 = mild, 2 = moderate, 3 = severe;
8. Bile duct lesions: 0 = no, 1 = minimal (intraepithelial inflammatory cells or cholangiocytes injury), 2 = moderate (epithelial lesions in most portal tracts, without destruction), 3 = severe (destructive epithelial lesions in most portal tracts);
9. Bile duct loss: 0 = no loss; 1 = loss of bile ducts in < 50% of the portal tracts, 2 = loss in > 50% of the portal tracts;
10. Portal vein branches: 0 = present in all portal tracts, 1 = absent in a minority of portal tracts, 2 = absent in most portal tracts;
11. Portal vein endotheliitis: 0 = absent, 1 = mild (present in a minority of portal veins), 2 = moderate (present in most portal veins), 2 = severe;
12. Fibrosis (Ishak): 0–6;
13. Portal and periportal fibrosis (Venturi *et al.*[23]): 0–3;
14. Perisinusoidal fibrosis (Venturi *et al.*[23]): 0–3;
15. Perivenular fibrosis (Venturi *et al.*[23]): 0–3;
16. Ductular reaction: yes or no;
17. Cholestasis: hepatocanalicular yes or no, cholangiolar yes or no;
18. Regenerative hyperplasia: 0 = absent, 1 = occasional foci of hepatocellular regenerative 2 = diffuse (classical) nodular regenerative hyperplasia.

Human leucocyte antigen typing and alloantibody characterisation

Human leucocyte antigen (HLA) typing data from donor and recipients were retrieved from national (e.g. National Health System Blood and Transplant for the centres in the UK) and local databases or obtained from cryopreserved DNA using low-resolution molecular HLA typing via PCR-sequence specific oligonucleotide probe hybridisation in combination with Luminex (Luminex Corporation, Austin, TX, USA) technology. Screening and specificity analysis for DSA was determined using LABScreen® Mixed Bead followed by LABScreen® Single Antigen assay (OneLambda Inc., Canoga Park, CA, USA). All anti-HLA antibody measurements were performed on a single batch at Guy's Clinical Transplantation Laboratory and mean fluorescence intensity (MFI) ≥ 1000 was considered positive as per laboratory guidelines. In patients with more than one DSA, the cumulative MFI was calculated by addition of the single MFI. In patients without detectable DSA, cumulative MFI was considered as 0.

Statistical analyses and sample size estimation

The study was originally designed and powered to test the hypothesis that the IS weaning according to the 'biomarker-based strategy' (Arm B+) is superior to the 'weaning all strategy' (Arm A), with respect to the proportion of participants who, having started the IS withdrawal, protocol, complete it successfully without undergoing allograft rejection. We established that for the biomarker to drive safe IS withdrawal its positive predictive value should be no < 0.8. Additionally, for the biomarker test to be considered successfully validated for clinical use, its sensitivity as estimated within Arm A should be at least 0.7. The sample size was based on an estimated positive biomarker rate of 50% in the

overall cohort, with 90% power and 5% type I error rate. With this constellation, 100 participants were calculated to be sufficient to show superiority of Arm B + compared to Arm A. The final sample size, including the participants in Arm B− and 10% drop-out, was of 148 participants.

To validate the clinical utility of the biomarker, the following conditions were stipulated: (1) superiority of Arm B+ compared to Arm A; (2) the confidence interval (CI) of the proportion of successes in Arm B+ should include 0.80 and exclude 0.50; and (3) the CI of the test's sensitivity as estimated in Arm A should include 0.70 and exclude 0.50.

Two interim analyses were planned: when 33% and 50% of participants reached the primary outcome. At 33% the plan was to calculate the 95% CI of the PPV of the test, that included the expected 80% PPV, and excluded a PPV of 50% with 90% power. Were the CI to exclude 80%, and shown not to be significantly different from 50%, the trial was due to stop as a result of poor accuracy of the biomarker test. At 50% the plan was to validate the sensitivity of the test using data from the 37 participants randomised to Arm A only (weaning all), which would allow to estimate with 80% power the 95% CI of the sensitivity that included 70% and excluded 50%. To test for superiority of Arm B+, compared to Arm A, we compared the proportion of successfully weaned participants in each arm using a chi-squared test. The probability of rejection over time was estimated using Kaplan–Meier survival curve. Descriptive statistics included median and range or mean and SD for continuous variables, and frequencies and percentages for categorical variables. Categorical data were compared by chi-squared or Fischer exact test and continuous variables by t-test or non-parametric testing (Mann–Whitney), as appropriate. Statistical analyses were performed using SPSS (IBM, New York, USA) package version 26 and R platform (The R Foundation for Statistical Computing, Vienna, Austria).

To determine the parameters associated with the presence of subclinical rejection in the baseline protocol biopsies, we constructed risk prediction models with multivariable binary logistic regression in which the outcome variable was probability of alloimmune damage (probTCMR) > 0.09. 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.

Research governance

Sponsorship

This was an investigator-initiated, non-commercial clinical trial funded by a research grant awarded by the National Institute of Health Research (NIHR) UK. The trial was jointly sponsored by King's College London and King's College Hospital NHS Foundation Trust. King's Health Partners Clinical Trials Office assisted with regulatory submissions and pharmacovigilance and provided sponsor Quality assurance oversight of trial processes such as consent and TMF maintenance.

Trial Steering and Data Monitoring and Ethics Committees

The Trial Steering Committee (TSC) included a senior transplantation physician from the UK unconnected to the study. Members included the chief investigator, two other principal investigators from the trial, two representatives of the liver transplant patient organisation LISTEN, as per INVOLVE recommendations, and two other senior independent transplant physician/surgeons. In addition, a Data Monitoring and Ethics Committee (DMEC) was established comprising a senior UK-based transplant physician/surgeon as chair, a liver transplant physician from overseas and a biostatistician. All the members were independent of the trial. The DMEC met every 3 months throughout the duration of the trial. In addition, the DMEC was due to meet in the occurrence of any of the following [or at any time required by the chief investigator, the sponsor or the Efficacy and Mechanism Evaluation (EME) board]: (1) death or graft loss in any study subject; and (2) composite incidence of severe acute rejection, steroid-resistant acute rejection or chronic rejection > 5%. The TSC met on a 6 monthly basis throughout the period of the trial, and at other times deemed necessary by the CI, the sponsor or by the EME board. The trial was reviewed by the Institute of Liver Studies R&D Governance Board at King's College Hospital, which provides research governance oversight for all liver trials instituted at King's College Hospital, as well as by representatives from the liver transplant patient association LISTEN.

Insurance and indemnity

Indemnity was provided by the Clinical Negligence Scheme for Trust and insurance is provided by King´s College London.

Patient and public involvement

The current project benefited from the active involvement of liver transplant patients, both at national and international levels. Between 2005 and 2010, the chief investigator participated in several meetings with European transplant patient associations, organised by the European Union FP6 *RISET* Consortium, in which tolerance and drug minimisation were highlighted as priority research topics. In 2013 information regarding the current proposal was discussed in detail with members of the liver transplant patient support group *LISTEN* at King's College Hospital, who attended an ad hoc meeting focused in the proposal. To elucidate patients' willingness to participate in this type of research, a questionnaire was distributed among all LISTEN members and patients attending LT clinics. Results revealed that 79% of patients were highly concerned about the side effects of IS, 100% agreed that research in tolerance is highly important, and 91% stated that they would be willing to participate in a drug withdrawal trial. *LISTEN* representatives reviewed the clinical trial protocol and participated in the TSC.

Equality and diversity strategy statement

The co-sponsors of the study, King's College London University and King's College Hospital NHS Trust, have strong equality and diversity strategies in place. Given that ethnic minorities remain under-represented among liver transplant patients, the study monitored ethnicity among the trial participants. All trial documents were translated, or native translators were employed, to cater for trial participants who did not have English as their native language. The co-sponsors and the trial investigators promoted an inclusive, nurturing, accessible and flexible research environment for all staff involved in delivering the project.

Chapter 4 Results

Patient demographic and clinical characteristics

Between October 2015 and May 2019 190 patients were consented to participate in the trial and underwent a screening liver biopsy (see *Table 4* and *Figure 5*).

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

TABLE 4 Characteristics of the 190 liver transplant recipients who were included in the study and underwent a screening liver biopsy

Characteristics	Value
Age at the time of enrolment (years)	61 (29–78)
Male gender (*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%)
IS (n, %)	
Tacrolimus (± MMF, AZA, sirolimus)	159 (87.4%)
Ciclosporin (± 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)

TABLE 4 Characteristics of the 190 liver transplant recipients who were included in the study and underwent a screening liver biopsy (*continued*)

Characteristics	Value
Ciclosporin trough levels (µg/l)	53 (23–223)
AST/ALT/mean ALT[a] (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 (× 10^3/mm^3)	6.2 (2.8–31.7)
Platelet count (× 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)

AP, alkaline phosphatase; APRI, AST to platelet ratio index; AST, aspartate aminotransferase; AZA, azathioprine; BMI, body mass index; FIB-4, Fibrosis-4 score; MMF, mycophenolate mofetil.
a 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).

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 CNI-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.

Histological evaluation of protocol liver biopsies obtained at screening

Among the 190 patients who underwent a screening liver biopsy, 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 5*). 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 to 8.9; $p < 0.001$), 33.6 (95% CI 12.4 to 91.0; $p < 0.001$) and 453.3 (95% CI 10.7 to 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).

Altogether, the liver biopsies of 122 participants (64.2%) exhibited no or minimal allograft damage and were deemed to be within the criteria stipulated by the Banff Working Group on Liver Allograft Pathology to consider IS withdrawal.[19] 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.*[23]) 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 6*): no or minimal allograft damage (64.2%), mild damage (13.7%) and moderate to severe damage (22.1%).

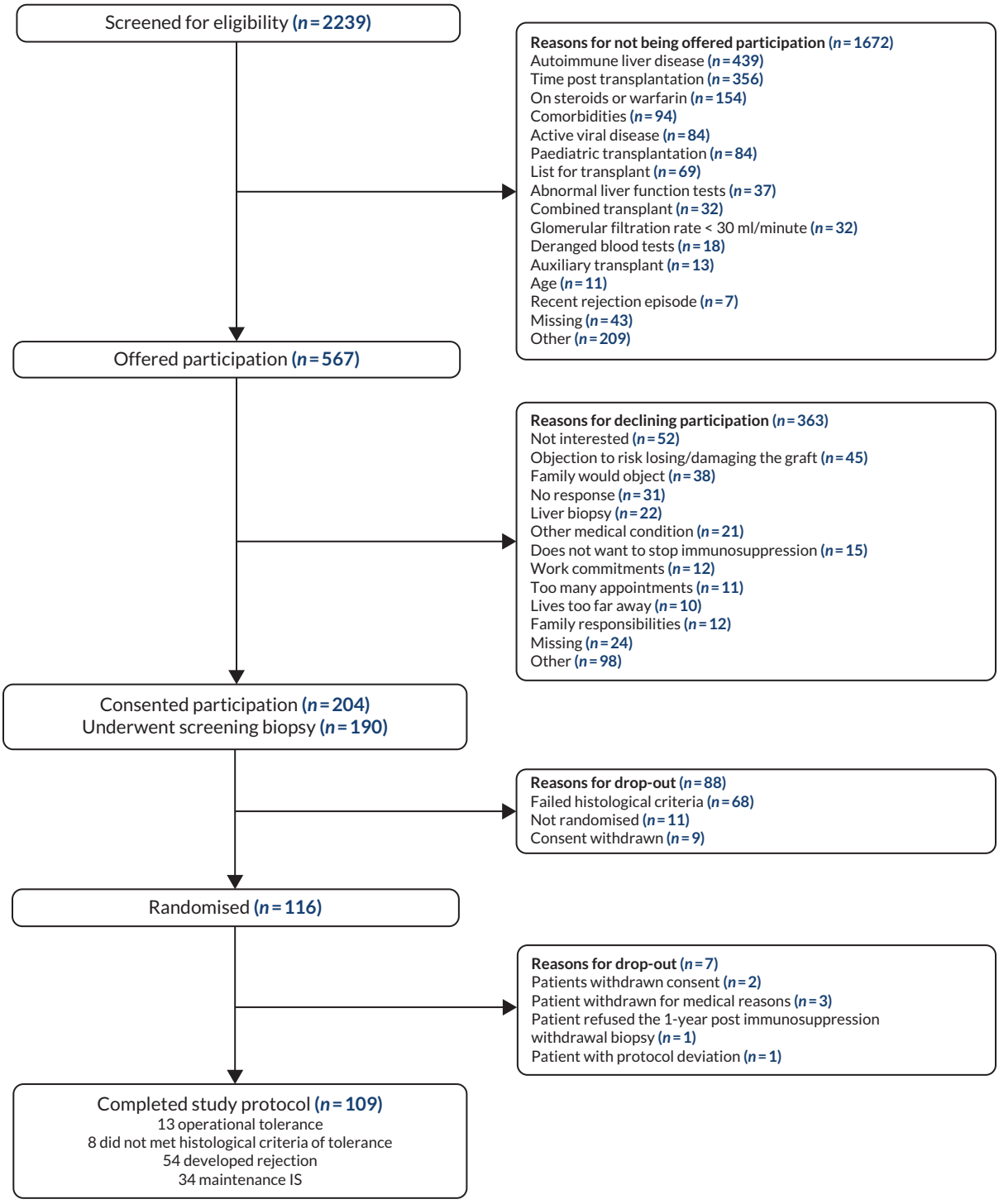

FIGURE 5 Consolidated Standards of Reporting Trials flow diagram *LIFT*.

Eligibility for randomisation

Of the 122 study participants who were considered eligible to participate in the trial after undergoing the screening liver biopsy, 116 were randomised to either '*IS withdrawal*' (*n* = 82) or '*maintenance IS*' (*n* = 34) (*Figure 5*). The demographic and clinical characteristics of the two study groups are shown in *Table 6*. Two patients randomised to IS withdrawal withdrew from the study before initiating weaning.

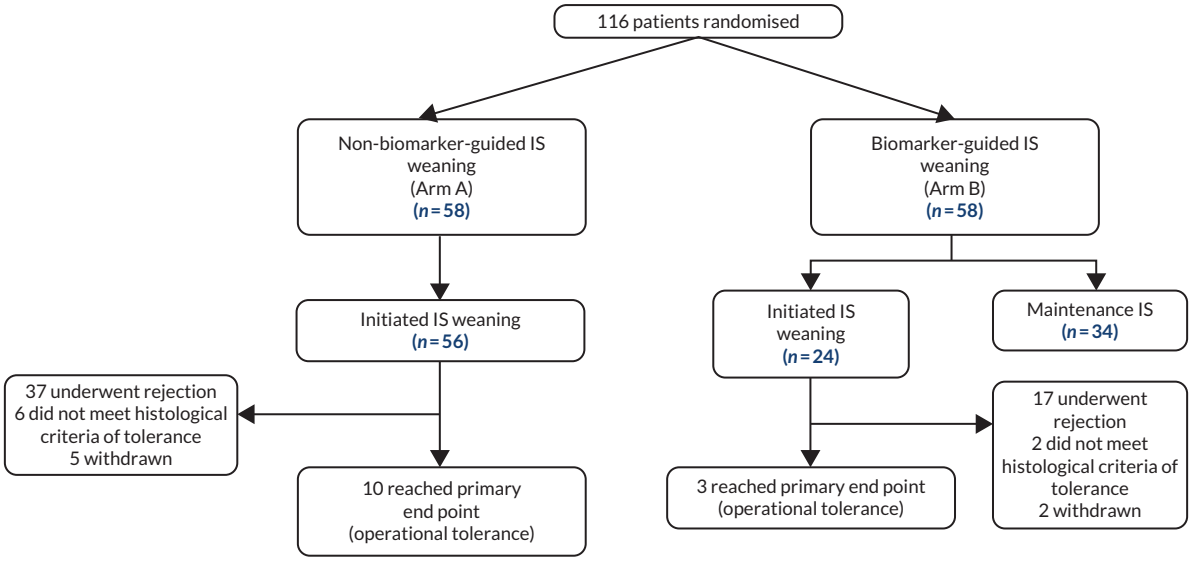

FIGURE 5 Continued

TABLE 5 Histological characteristics of the 190 baseline liver biopsies (at screening)

Histological features		Histological features (continued)	
Number complete portal tracts	8 (1–36)[a]		
Number central veins	5 (0–30)[a]		
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 endotheliitis		***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%)

continued

TABLE 5 Histological characteristics of the 190 baseline liver biopsies (at screening) (*continued*)

Histological features		Histological features (continued)	
Fibrosis (Ishak)		***Perisinusoidal fibrosis***[b]	
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***[b]	
Incomplete cirrhosis	6 (3.2%)	Absence	146 (76.8%)
Complete cirrhosis	2 (1.1%)	Focal	37 (19.5%)
Portal/Periportal fibrosis[a]		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%)

a Median (range); otherwise, numbers refer to the number of biopsies displaying each feature.
b As described by Venturi *et al.*[23]

TABLE 6 Demographics of patients allocated to IS maintenance and IS weaning

IS weaning status		Maintenance	Weaning	Total	*p*-value
Age	Median (IQR)	62.0 (10.4)	61.1 (13.3)	61.1 (10.9)	0.522
Time since transplantation	Median (IQR)	7.0 (5.8)	8.2 (6.6)	7.9 (5.4)	0.086
Ethnicity	Caucasian	33 (97.1)	80 (97.6)	113 (97.4)	0.651
	Black	0 (0.0)	1 (1.2)	1 (0.9)	
	Other	1 (2.9)	1 (1.2)	2 (1.7)	
Primary liver disease *N* (%)	Alcohol	10 (29.4)	27 (32.9)	37 (31.9)	0.997
	HCC	2 (5.9)	2 (2.4)	4 (3.4)	
	Hepatitis C	7 (20.6)	14 (17.1)	21 (18.1)	
	Hepatitis B	2 (5.9)	7 (8.5)	9 (7.8)	
	A1AD	1 (2.9)	2 (2.4)	3 (2.6)	
	Haemochromatosis	2 (5.9)	2 (2.4)	4 (3.4)	
	Wilson's disease	0 (0.0)	1 (1.2)	1 (0.9)	
	DILI	1 (2.9)	3 (3.7)	4 (3.4)	
	PCLD	1 (2.9)	2 (2.4)	3 (2.6)	
	Vascular	0 (0.0)	1 (1.2)	1 (0.9)	
	NAFLD	1 (2.9)	4 (4.9)	5 (4.3)	
	Cryptogenic	1 (2.9)	4 (4.9)	5 (4.3)	

TABLE 6 Demographics of patients allocated to IS maintenance and IS weaning (*continued*)

IS weaning status		Maintenance	Weaning	Total	*p*-value
	Amyloidosis	0 (0.0)	0 (0.0)	0 (0.0)	
	Other	6 (17.6)	13 (15.9)	19 (16.3)	
Secondary liver disease *N* (%)	HCC	3 (8.8)	12 (14.6)	15 (12.9)	0.774
	Haemochromatosis	0 (0.0)	2 (2.4)	2 (1.7)	
	Hepatitis B	2 (5.9)	2 (2.4)	4 (3.4)	
	Hepatitis C	1 (2.9)	1 (1.2)	2 (1.7)	
	Cryptogenic	1 (2.9)	1 (1.2)	2 (1.7)	
	Alcohol	0 (0.0)	2 (2.4)	2 (1.7)	
	Other	0 (0.0)	2 (2.4)	2 (1.7)	
	A1AD	0 (0.0)	1 (1.2)	1 (0.9)	
IS treatment at enrolment *N* (%)					
Azathioprine	0	29 (85.3)	74 (90.2)	103 (88.8)	0.520
	1	5 (14.7)	8 (9.8)	13 (11.2)	
Tacrolimus	0	7 (20.6)	7 (8.5)	14 (12.1)	0.113
	1	27 (79.4)	75 (91.5)	102 (87.9)	
Ciclosporin	0	29 (85.3)	76 (92.7)	105 (90.5)	0.295
	1	5 (14.7)	6 (7.3)	11 (9.5)	
Sirolimus	0	33 (97.1)	81 (98.8)	114 (98.3)	0.502
	1	1 (2.9)	1 (1.2)	2 (1.7)	
Everolimus	0	33 (97.1)	80 (97.6)	113 (97.4)	1.000
	1	1 (2.9)	2 (2.4)	3 (2.6)	
Total *N* (%)		34 (29.3)	82 (70.7)	116	

A1AD, alpha-1-antitrypsin deficiency; DILI, drug-induced liver injury; HCC, hepatocellular carcinoma; PCLD, polycystic liver disease.

Outcomes of immunosuppression withdrawal

Fifty-four (67.5%) out of the 80 trial participants who started IS withdrawal developed clinically apparent rejection after initiating the discontinuation of IS (see *Tables 7*, *8* and *Figure 6*).

Twenty-two patients (27.5%) successfully discontinued IS without developing allograft dysfunction and maintained stable liver tests off IS for 12 months. Twenty-one of them underwent the 12-month post-IS withdrawal protocol liver biopsy (one patient with normal liver tests refused the protocol biopsy and withdrew from the study). This revealed that 13 (16.3%) met the histological criteria of operational tolerance, while the remaining eight exhibited histological lesions not present in the baseline biopsy (out of whom only two required IS reinstitution). Following the performance of the second follow-up liver biopsy 24 months post IS withdrawal, 17 (21.3%) patients were considered not to require IS reinstitution, 15 (18.8%) of whom met operational tolerance histology criteria and two exhibited mild histological changes (*Table 9*).

TABLE 7 Demographics of patients allocated to IS maintenance and IS weaning

IS weaning status		Maintenance	Weaning	Total	*p*-value
Age	Median (IQR)	62.0 (10.4)	61.1 (13.3)	61.1 (10.9)	0.522
Time since transplantation	Median (IQR)	7.0 (5.8)	8.2 (6.6)	7.9 (5.4)	0.086
Ethnicity	Caucasian	33 (97.1)	80 (97.6)	113 (97.4)	0.651
	Black	0 (0.0)	1 (1.2)	1 (0.9)	
	Other	1 (2.9)	1 (1.2)	2 (1.7)	
Primary liver disease *N* (%)	Alcohol	10 (29.4)	27 (32.9)	37 (31.9)	0.997
	HCC	2 (5.9)	2 (2.4)	4 (3.4)	
	Hepatitis C	7 (20.6)	14 (17.1)	21 (18.1)	
	Hepatitis B	2 (5.9)	7 (8.5)	9 (7.8)	
	A1AD	1 (2.9)	2 (2.4)	3 (2.6)	
	Haemochromatosis	2 (5.9)	2 (2.4)	4 (3.4)	
	Wilson's disease	0 (0.0)	1 (1.2)	1 (0.9)	
	DILI	1 (2.9)	3 (3.7)	4 (3.4)	
	PCLD	1 (2.9)	2 (2.4)	3 (2.6)	
	Vascular	0 (0.0)	1 (1.2)	1 (0.9)	
	NAFLD	1 (2.9)	4 (4.9)	5 (4.3)	
	Cryptogenic	1 (2.9)	4 (4.9)	5 (4.3)	
	Amyloidosis	0 (0.0)	0 (0.0)	0 (0.0)	
	Other	6 (17.6)	13 (15.9)	19 (16.3)	
Secondary liver disease *N* (%)	HCC	3 (8.8)	12 (14.6)	15 (12.9)	0.774
	Haemochromatosis	0 (0.0)	2 (2.4)	2 (1.7)	
	Hepatitis B	2 (5.9)	2 (2.4)	4 (3.4)	
	Hepatitis C	1 (2.9)	1 (1.2)	2 (1.7)	
	Cryptogenic	1 (2.9)	1 (1.2)	2 (1.7)	
	Alcohol	0 (0.0)	2 (2.4)	2 (1.7)	
	Other	0 (0.0)	2 (2.4)	2 (1.7)	
	A1AD	0 (0.0)	1 (1.2)	1 (0.9)	
IS treatment at enrolment *N* (%)					
Azathioprine	0	29 (85.3)	74 (90.2)	103 (88.8)	0.520
	1	5 (14.7)	8 (9.8)	13 (11.2)	
Tacrolimus	0	7 (20.6)	7 (8.5)	14 (12.1)	0.113
	1	27 (79.4)	75 (91.5)	102 (87.9)	
Ciclosporin	0	29 (85.3)	76 (92.7)	105 (90.5)	0.295
	1	5 (14.7)	6 (7.3)	11 (9.5)	
Sirolimus	0	33 (97.1)	81 (98.8)	114 (98.3)	0.502
	1	1 (2.9)	1 (1.2)	2 (1.7)	

TABLE 7 Demographics of patients allocated to IS maintenance and IS weaning (*continued*)

IS weaning status		Maintenance	Weaning	Total	*p*-value
Everolimus	0	33 (97.1)	80 (97.6)	113 (97.4)	1.000
	1	1 (2.9)	2 (2.4)	3 (2.6)	
Total *N* (%)		34 (29.3)	82 (70.7)	116	

A1AD, alpha-1-antitrypsin deficiency; DILI, drug-induced liver injury; HCC, hepatocellular carcinoma; NAFLD, Non-alcoholic fatty liver disease; PCLD, polycystic liver disease.

TABLE 8 Demographics of patients randomised to Arm A and Arm B

		Biomarker-based IS weaning (group B) (*N* = 58)	Non-biomarker-based IS weaning (group A) (*N* = 58)	Total	*p*-value
Age	Median (IQR)	61.5 (57–66.8)	60.0 (53–67)	61.0 (55.8–67)	0.914
Gender	Male	45 (77.6)	39 (67.2)	84 (72.4)	0.299
	Female	13 (22.4)	19 (32.8)	32 (27.6)	
Ethnicity	Caucasian	57 (98.3)	56 (96.6)	113 (97.4)	1.000
	Black	0 (0.0)	1 (1.7)	1 (0.9)	
	Other	1 (1.7)	1 (1.7)	2 (1.7)	
Time since transplant	Median (IQR)	7.8 (4.9–10.7)	8.1 (6.2–12.5)	7.9 (5.7–11.1)	0.331
BMI	Median (IQR)	27.4 (23.7–30.8)	27.6 (24.2–29.9)	27.5 (24.1–30.7)	0.757
Primary liver disease	Alcohol	20 (34.5)	17 (29.3)	37 (31.9)	0.890
	HCC	2 (3.4)	3 (5.1)	5 (4.4)	
	Hepatitis C	10 (17.2)	11 (19.0)	21 (18.1)	
	Hepatitis B	2 (3.4)	7 (12.1)	9 (7.8)	
	A1AD	1 (1.7)	2 (3.4)	3 (2.6)	
	Haemochromatosis	3 (5.2)	1 (1.7)	4 (3.4)	
	Wilson's disease	0 (0.0)	1 (1.7)	1 (0.9)	
	DILI	2 (3.4)	2 (3.4)	4 (3.4)	
	PCLD	2 (3.4)	1 (1.7)	3 (2.6)	
	Vascular	1 (1.7)	0 (0.0)	1 (0.9)	
	NAFLD	2 (3.4)	3 (5.2)	5 (4.3)	
	Cryptogenic	2 (3.4)	3 (5.2)	5 (4.3)	
	Other	11 (18.9)	8 (13.8)	19 (16.4)	
Secondary liver disease	Alcohol	0 (0.0)	2 (3.4)	2 (1.7)	0.103
	HCC	5 (7.6)	10 (17.2)	14 (13)	
	Hepatitis C	2 (3.4)	0 (0.0)	2 (1.7)	
	Hepatitis B	2 (3.4)	2 (3.4)	4 (3.4)	

continued

TABLE 8 Demographics of patients randomised to Arm A and Arm B (*continued*)

		Biomarker-based IS weaning (group B) (*N* = 58)	Non-biomarker-based IS weaning (group A) (*N* = 58)	Total	*p*-value
	A1AD	0 (0.0)	1 (1.7)	1 (0.9)	
	Haemochromatosis	2 (3.4)	0 (0.0)	2 (1.7)	
	DILI	0 (0.0)	0 (0.0)	0 (0.0)	
	NAFLD	0 (0.0)	0 (0.0)	0 (0.0)	
	Cryptogenic	1 (1.7)	1 (1.7)	2 (1.7)	
	Other	0 (0.0)	2 (3.4)	2 (1.7)	
Type of donor	Cadaveric	57 (98.3)	58 (100.0)	115 (99.1)	1.000
	Living	1 (1.7)	0 (0.0)	1 (0.9)	
Type of transplant	Whole organ	57 (98.3)	52 (89.7)	109 (94.0)	0.114
	Partial organ	1 (1.7)	6 (10.3)	7 (6.0)	
Tac monotherapy	FALSE	32 (55.2)	42 (72.4)	74 (63.8)	0.082
	TRUE	26 (44.8)	16 (27.6)	42 (36.2)	
CsA monotherapy	FALSE	56 (96.6)	58 (100.0)	114 (98.3)	0.496
	TRUE	2 (3.4)		2 (1.7)	
MMF monotherapy	FALSE	57 (98.3)	56 (96.6)	113 (97.4)	1.000
	TRUE	1 (1.7)	2 (3.4)	3 (2.6)	
Ever monotherapy	FALSE	57 (98.3)	58 (100.0)	115 (99.1)	1.000
	TRUE	1 (1.7)		1 (0.9)	
CNI + MMF or Aza	FALSE	36 (62.1)	32 (55.2)	68 (58.6)	0.572
	TRUE	22 (37.9)	26 (44.8)	48 (41.4)	
AST (IU/l)	Median (IQR)	21.0 (19.0–24.0)	23.0 (20–26.5)	22.0 (19–25)	0.154
ALT (IU/l)	Median (IQR)	19.0 (15.0–25.0)	20.0 (15–26)	19.0 (15–25)	0.697
ALP (IU/l)	Median (IQR)	77.5 (62.5–91.8)	75.5 (64–88.8)	77.0 (64–89.5)	0.943
GGT (IU/l)	Median (IQR)	20.5 (16.0–31.0)	24.0 (16–38)	22.0 (16–34)	0.260
Bilirubin (µmol/l)	Median (IQR)	7.0 (0.8–11.0)	6.0 (0.9–12.0)	7.0 (0.8–11.0)	0.878
Randomisation status	IS maintenance	34 (58.6)	0 (0.0)	34 (29.3)	< 0.001
	IS weaning	24 (41.4)	58 (100.0)	82 (70.7)	

A1AD, alpha-1-antitrypsin deficiency; Aza, azathioprine; CsA, ciclosporin A; DILI, drug-induced liver injury; Ever, everolimus; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease; PCLD, polycystic liver disease; Tac, tacrolimus.

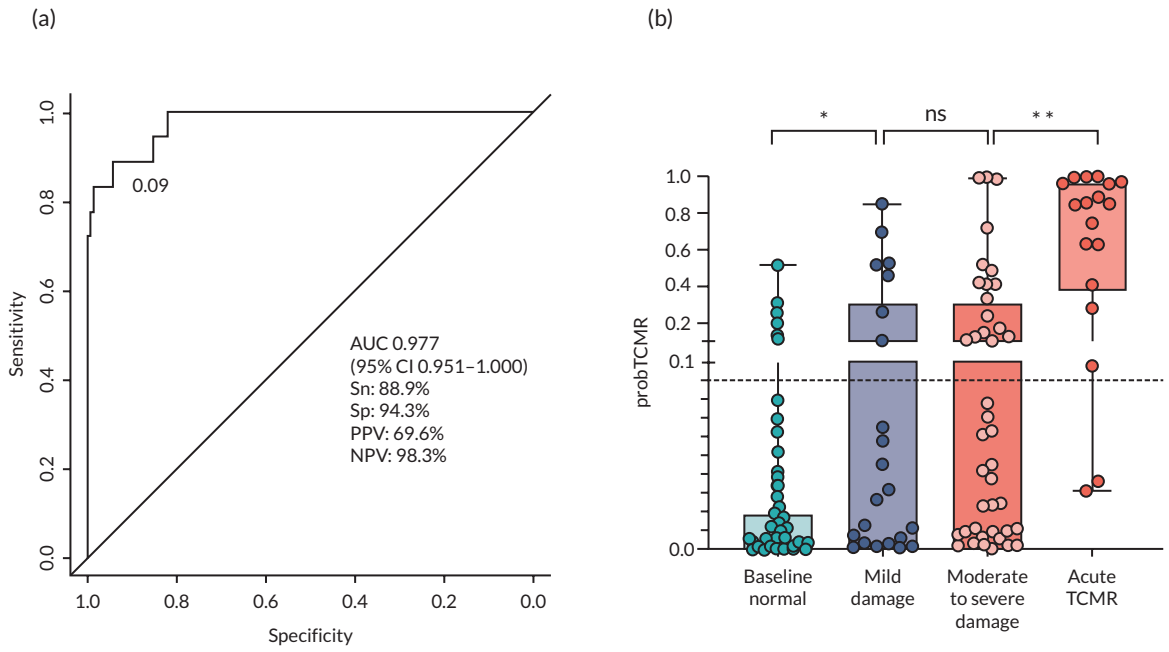

FIGURE 6 Transcript levels of T-cell-mediated rejection-related genes in liver biopsies with or without silent allograft damage. (a) ROC curve displaying the overall diagnostic performance of the 11-gene transcriptional signature in discriminating liver biopsies with no damage versus those with TCMR. 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 (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). **p* < 0.01; ***p* < 0.0001.

TABLE 9 Demographics of patients who successfully discontinued IS

Status		Rejection	Meeting HTC 1-year post IS weaning	Not meeting HTC 1-year post IS weaning	Total
Age	Median (IQR)	60.8 (11.7)	67.1 (11.0)	59.6 (17.0)	61.1 (13.1)
Time since transplantation	Median (IQR)	7.1 (4.3)	13.3 (4.2)	10.4 (5.2)	8.4 (7.5)
Ethnicity	Caucasian	53 (98.1)	12 (92.3)	8 (100.0)	73 (97.3)
	Black	1 (1.9)	0 (0.0)	0 (0.0)	1 (1.3)
	Other	0 (0.0)	0 (0.0)	1 (7.7)	1 (1.3)
Primary liver disease	Alcohol	18 (33.3)	3 (23.1)	3 (37.5)	24 (32.0)
	HCC	0 (0.0)	1 (7.7)	1 (12.5)	2 (2.7)
	Hepatitis C	10 (18.5)	2 (15.4)	0 (0.0)	12 (16.0)
	Hepatitis B	1 (1.9)	5 (38.5)	1 (12.5)	7 (9.3)
	A1AD	1 (1.9)	0 (0.0)	0 (0.0)	1 (1.3)
	Haemochromatosis	2 (3.7)	0 (0.0)	0 (0.0)	2 (2.7)
	Wilson's disease	1 (1.9)	0 (0.0)	0 (0.0)	1 (1.3)
	DILI	2 (3.7)	1 (7.7)	0 (0.0)	3 (4.0)
	PCLD	2 (3.7)	0 (0.0)	0 (0.0)	2 (2.7)
	Vascular	0 (0.0)	0 (0.0)	1 (12.5)	1 (1.3)

continued

TABLE 9 Demographics of patients who successfully discontinued IS (*continued*)

Status		Rejection	Meeting HTC 1-year post IS weaning	Not meeting HTC 1-year post IS weaning	Total
	NAFLD	3 (5.6)	1 (7.7)	0 (0.0)	4 (5.3)
	Cryptogenic	3 (5.6)	0 (0.0)	0 (0.0)	3 (4.0)
	Other	11 (20.4)	0 (0.0)	2 (25.0)	13(17.4)
Secondary liver disease diagnosis	HCC	9 (16.7)	2 (15.4)	0 (0.0)	11 (14.7)
	None	39 (72.2)	9 (69.2)	6 (75.0)	54 (72.0)
	Haemochromatosis	2 (3.7)	0 (0.0)	0 (0.0)	2 (2.7)
	Hepatitis B	1 (1.9)	1 (7.7)	0 (0.0)	2 (2.7)
	Hepatitis C	1 (1.9)	0 (0.0)	0 (0.0)	1 (1.3)
	Cryptogenic	0 (0.0)	0 (0.0)	1 (12.5)	1 (1.3)
	Alcohol	1 (1.9)	0 (0.0)	0 (0.0)	1 (1.3)
	Other	1 (1.9)	1 (7.7)	0 (0.0)	2 (2.7)
	A1AD	0 (0.0)	0 (0.0)	1 (12.5)	1 (1.3)
IS treatment at enrolment N (%)					
Azathioprine	0	49 (90.7)	13 (100.0)	6 (75.0)	68 (90.7)
	1	5 (9.3)		2 (25.0)	7 (9.3)
Tacrolimus	0	6 (11.1)	1 (7.7)		7 (9.3)
	1	48 (88.9)	12 (92.3)	8 (100.0)	68 (90.7)
Ciclosporin	0	48 (88.9)	13 (100.0)	8 (100.0)	69 (92.0)
	1	6 (11.1)			6 (8.0)
Sirolimus	0	53 (98.1)	13 (100.0)	8 (100.0)	74 (98.7)
	1	1 (1.9)			1 (1.3)
Everolimus	0	52 (96.3)	13 (100.0)	8 (100.0)	73 (97.3)
	1	2 (3.7)			2 (2.7)
Total *N* (%)		54 (68.4)	13 (17.3)	8 (10.7)	75

A1AD, alpha-1-antitrypsin deficiency; DILI, drug-induced liver injury; HCC, hepatocellular carcinoma; HTC, histological tolerance criteria; PCLD, polycystic liver disease.

Diagnostic performance of the transcriptional tolerance biomarker test

A pre-planned interim analysis to estimate the 95% CI of the PPV of the biomarker test on the first 48 patients reaching the primary outcome was completed in December 2018. Among the 48 patients, 12 (25%) successfully discontinued IS and 8 (17%) met the primary end point. The stipulated PPV target (95% CI of the PPV including the expected 80% and excluding a PPV of 50%) was not met. Thus, the performance of the test was: Sn 75%, Sp 44%, PPV 31%, NPV 84% in predicting the success of IS withdrawal; and Sn 63%, Sp 41%, PPV 18%, NPV 84% in the predicting the primary end point. The decision threshold used with the gene expression test was considered to be incorrect, since it over-predicted successful IS discontinuation. This was in keeping with the much lower than expected prevalence of successful drug withdrawal: since there were few true positives, the false positives are large as compared to the number of true positives and the resulting PPV is low. After reviewing the results of the pre-planned interim analysis, the NIHR EME

Board made the decision to stop the study in light of the impossibility of answering the original question posed by the trial with the biomarker as configured, while continuing the follow-up of those patients randomised and still in the study (the overall follow-up was reduced by 1 year).

Following completion of follow-up, the analysis of the 75 patients who either developed rejection or met the primary end point revealed that 10 out of the 56 (18%) of patients who initiated immunosuppression withdrawal in Arm A achieved operational tolerance at 12 months versus 3 among the 24 patients (13%) who initiated withdrawal in Arm B+. The performance evaluation of the transcriptional tolerance biomarker showed that the test was not accurate at identifying patients meeting the operational tolerance criteria (OR 1.466, 95% IC 0.326 to 9.215; $p = 0.744$; Sn 54%, Sp 42%, PPV 16%, and NPV 81%, with an accuracy of 44%).

Variables associated with immunosuppression withdrawal outcomes

Time since transplantation at the time of enrolment in the trial was the variable most strongly associated with the outcome of IS weaning, with patients who had been transplanted for longer periods of time being more likely to successfully discontinue IS (*Table 9*). Among patients who did not develop clinically apparent rejection, time since transplantation was also associated with the likelihood of meeting histological criteria of operational tolerance at the 1-year post-IS withdrawal liver biopsy (*Table 9*).

Mechanistic and health economic analysis

These could not be completed as originally planned due to the premature termination of the trial as a result of the suboptimal performance of the biomarker test.

Variables associated with subclinical allograft damage

To determine which variables influenced subclinical allograft damage and to explore their inter-relationships, we constructed a similarity matrix incorporating histological, demographic, clinical and laboratory parameters of all 190 patients who underwent a liver biopsy as part of the screening to participate in *LIFT*.

Allograft damage was positively correlated with donor-specific antibodies, 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/minute lower than in those with moderate to severe damage, even though the latter had been transplanted for a longer period of time.

Out of 185 serum samples obtained at the time of the screening liver biopsies, 91 (49.2%) were positive for either class I and/or class II HLA antibodies, with 37 (20.0%) being positive for anti-class I, 75 (40.5%) for anti-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 > 10,000 and 10 (25.0%) an MFI > 20,000. 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 6*). 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 10*).

TABLE 10 Characteristics of all screened 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[a,b]	Group 1 vs. 2: *p*-values[b,c]	Group 1 vs. 3: *p*-values[b,c]	Group 2 vs. 3: *p*-values[b,c]
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 (×10^9/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/minute/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
LSM (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 (12,317)	**0.045**	0.487	**0.012**	0.325

AP, alkaline phosphatase; APRI, AST to platelet ratio index; FIB-4, Fibrosis-4 score; MDRD, modification of diet in renal disease.
a Overall independent-sample Kruskal–Wallis test.
b Chi-squared or Fisher exact test for categorical variables, as appropriate.
c Non-parametric independent-sample Mann–Whitney test.

Note
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.

Stratification of liver biopsies based on the transcript levels of T-cell-mediated rejection-related genes

To assess the molecular profile of subclinical allograft damage, we conducted transcriptional experiments on RNA extracted from 184 liver biopsies obtained at screening employing an 11-gene signature previously found to accurately identify clinically apparent TCMR in adult and paediatric LT recipients.[28,31,34] The analysis included a subset of indication liver biopsies from 18 LT recipients who developed acute TCMR following IS withdrawal as part of the trial. First, we trained the 11-gene classifier in the subset of 18 TCMR biopsies and the 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 probTCMR. This conservative threshold had a high sensitivity to detect samples with a molecular profile in keeping with TCMR and showed a good balance between sensitivity and specificity (*Figure 6a*). Next, we assigned a probTCMR to each of the 184 liver biopsies based on 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 score. Among the 184 liver biopsies analysed, 31 (17%) 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), 31.8% (7/22) and 42.5% (17/40) had probTCMR > 0.09, respectively (*Figure 6b*).

Non-invasive detection of clinically significant alloimmune liver damage

Considering the previously reported association between liver tissue TCMR-related gene expression and progressive liver allograft damage,[31] we hypothesised that a probTCMR = 0.09 (which corresponds to the transcript levels observed at the time of clinically apparent TCMR; *Figure 6b*), would constitute an optimal threshold of clinically significant active alloimmune damage. We employed binary logistic regression analysis to select the optimal set of non-invasive parameters capable of identifying patients meeting this criterion. Baseline ALT and LSM were both independently associated with alloimmune damage (*Table 10*). The model's discrimination was evaluated through a ROC curve, which yielded an AUC = 0.82 (*Figure 7a*). 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 three 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 study cohort) were associated with an extremely low likelihood of alloimmune damage. Conversely, patients with ALT > 34 had a 45% chance of having alloimmune damage. LSM did not improve the predictive capacity of the model in these two 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) (*Figure 7b*).

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; categorised as cMFI < 10,000, 10,000 ≤ cMFI < 20,000 and cMFI ≥ 20,000), and the corresponding ROC curve had an AUC = 0.76 (95% CI 0.66 to 0.85) (*Table 11*). The results of a CART analysis including ALT and class II DSA are depicted in *Figure 7c* (*Table 12*). Similar results were obtained when mean ALT was employed (data not shown).

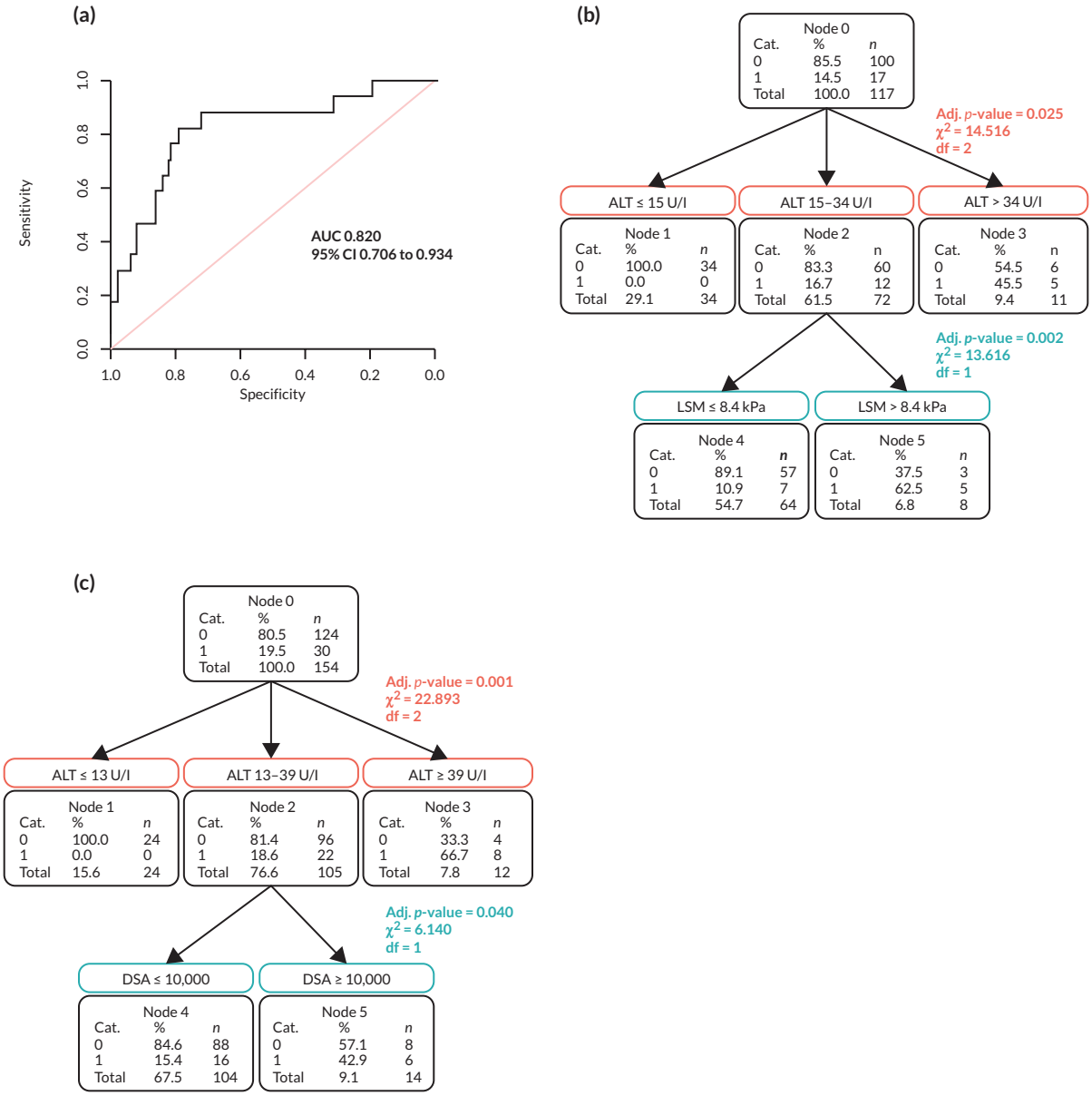

FIGURE 7 Performance of predictive models, integrating baseline ALT and LSM and baseline ALT and class II DSA, respectively, in identifying subclinical alloimmune liver allograft damage. (a) ROC curve corresponding to the performance of the logistic regression model comprising ALT and LSM in discriminating between patients with a probTCMR above and below 0.09 on the basis of liver tissue gene expression. (b) Prognostic analysis generated by 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.

TABLE 11 Logistic regression model including ALT and LSM to predict clinically significant alloimmune liver damage

Significant of the variables in the model (Wald test)	χ^2	df	*p*-value
LSM	7.73	1	0.0054
ALT	8.35	1	0.0038
Performance metrics (corrected with bootstrap)			
R^2	0.267		
c-statistic	0.809		
Model logit (coefficients corrected with bootstrap)			
Logit = −5.1058647 + 0.29947708*LSM + 0.066825492*ALT			

df, degrees of freedom.

Note
One hundred and seventeen patients (17 with significant alloimmune liver damage) had reliable LSM and were included in the regression model. Model was corrected with bootstrap (300 replications) for optimism. Individual probabilities of alloimmune damage can be calculated as 1/[1+exp(-logit)].

TABLE 12 Logistic regression model including ALT and class II DSA to predict clinically significant alloimmune liver damage in the liver biopsies conducted at screening

Significance of the variables in the model (Wald test)	χ^2	*p*-value
ALT	3.878	0.0001
DSA negative or < 10,000 (reference)	NA	NA
DSA 10,000–20,000	0.97	0.3297
DSA > 20,000	2.10	0.0356
Performance metrics (corrected with bootstrap)		
R^2	0.1682	
c-statistic	0.732	
Model logit (coefficients corrected with bootstrap)		
Logit = −2.9901276 + 0.064810848*ALT + 0.70892136*1(if DSA 10,000–20,000) + 1.3758922*1 (if DSA > 20,000)		

Note
One hundred and fifty-four patients (34 with significant alloimmune liver damage) had values of class II DSA. DSA values were categorised in: negative or cMFI < 10,000, cMFI 10,000–20,000, cMFI > 20,000. Model was corrected with bootstrap (300 replications) for optimism. Individual probabilities of alloimmune damage can be calculated as 1/[1 +exp (−logit)].

Chapter 5 Discussion

Surgical advances together with the advent of potent and relatively selective pharmacological immunosuppressants have resulted in striking improvements in short-term outcomes following clinical LT, with 1-year patient survival above 90%. In contrast, improvements in long-term survival and patient quality of life have been more modest. Multiple lines of evidence indicate that in order to impact long-term outcomes it will be essential to develop tools to minimise, and if at all possible, completely withdraw, immunosuppressive drugs. The clinical opportunity to develop such tools in the setting of clinical LT is real, as demonstrated by the fact that a subset of liver allograft recipients is found to be operationally tolerance upon IS withdrawal. Although carefully monitored IS weaning is feasible, it is costly, cumbersome and potentially risky, in particular in patients who have not been transplanted for many years, in whom the prevalence of operational tolerance appears to be very low. The identification of accurate biomarkers of operational tolerance remains therefore an important research priority in LT.

In 2012, our research group published data from a prospective, multicentre trial of IS withdrawal in which, out of the 75 recipients completing the trial, 42 (56%) underwent rejection, while 33 (44%) were successfully weaned off IS and were considered to have reached spontaneous operational tolerance (*RISET* Consortium trial).[12,17] Both flow cytometric and gene-expression analyses of PBMCs confirmed the over-representation of NK cells and NK-related gene sets in the tolerant recipients. However, the PBMC molecular signature lacked reproducibility across the three participating clinical sites and could not reliably predict the outcome of IS withdrawal. The most accurate and reproducible predictor of withdrawal outcome proved to be the liver tissue-derived transcriptional profile obtained at baseline. This intra-graft expression profile, generated using cDNA microarrays and subsequently validated employing real-time PCR, showed no overlap with genes identified from PBMC. Somehow intriguingly, among the 10 genes showing transcriptional differences of greatest magnitude in relation to tolerance *(TRFC*, *PEBP1*, *MIF*, *CDHR2*, *SOCS1*, *IFNG*, *HAMP*, *SLC5A12*, *DAB2*, *HMOX1)*, there was an overrepresentation of those involved in iron metabolism. These included transferrin receptor 1 *(TRFC)*, hepcidin *(HAMP)* and *MIF*. A combination of 5 of the 10 biopsy-derived genes *(SOCS1*, *TFRC*, *PEBP1*, *MIF*, *CHDR2)*, when measured prior to the initiation of IS withdrawal, was extremely accurate at discriminating those recipients who could successfully withdraw IS from those who could not. Importantly, in the population of patients analysed, the transcriptional signature had a better predictive capacity than, and not influenced by, clinical/demographic parameters also associated with the outcome of IS withdrawal. In the univariate analysis, the parameters significantly associated with the success of IS withdrawal were: 'time from transplantation to enrolment', 'male gender', 'older recipient age at transplantation' and 'absence of calcineurin inhibitor immunosuppressants at enrolment'. In the multivariate analysis, only 'time from transplantation' (OR 1.353, 95% CI 1.166 to 1.570; $p < 0.0001$), 'age at transplantation' (OR 1.073, 95% CI 1.018 to 1.130; $p = 0.009$) and 'male gender' (OR 4.375, 95% CI 1.315–14.412; $p = 0.016$) were independently associated with the probability of being tolerant (*c*-statistic 0.816, 95% CI 0.733 to 0.9). Patients enrolled more than 10.6 years after transplantation had a 79% rate of successful withdrawal, while in those transplanted for more than 5.7 years withdrawal was successful in 38% of cases. In these two subgroups of recipients with high and intermediate likelihood of tolerance, incorporation of age or gender did not improve the model's predictive capacity. In contrast, among recipients enrolled 3–5.7 years after transplantation, age at transplantation helped to identify subgroups of recipients with very low (0%) or intermediate (30%) likelihood of success. On the other hand, of those patients who were taking no CNIs at the time of enrolment, 60% managed to completely withdraw IS.

The *LIFT* trial was designed to validate the capacity of the five-gene liver tissue transcriptional signature described above to identify operationally tolerant liver recipients before initiating IS withdrawal. Patient selection criteria mirrored the criteria employed in the *RISET* Consortium trial,[12] with the exception that patients had to be > 3 years post transplant or > 6 years post transplant if age < 50 years old. These slightly more stringent criteria were introduced in order to enrol a population of transplant recipients with an estimated likelihood of spontaneous operational tolerance > 40%. A second difference with the *RISET* Consortium trial was the histological definition of operational tolerance, which in the *LIFT* trial followed the Banff Group recommendations, published after the *RISET* trial had been completed,[19] and which stipulated stricter criteria both for considering a patient eligible to undergo IS weaning and for confirming the establishment of operational tolerance. Hence, while in *RISET* all patients who maintained stable allograft function

after discontinuing IS were also labelled as operationally tolerant on histological grounds, in *LIFT* 38% (8/21) of them exhibited histological changes in the 1-year post-IS withdrawal liver biopsy that exceeded the stipulated criteria for operational tolerance.

The biomarker test proved to be inaccurate in identifying operationally tolerant recipients before initiating IS withdrawal. As a result, the trial had to be prematurely terminated and some of the secondary objectives could not be fulfilled.

There are a number of potential explanations to account for these poor diagnostic performance results of the biomarker test. First, the prevalence of operational tolerance in *LIFT* was substantially lower than expected based on the results of the *RISET* Consortium trial from which the diagnostic test had been derived (16.3% vs. 44%, respectively). The drastically different prevalence of operational tolerance in the *RISET* and *LIFT* trials likely explains the poor diagnostic performance of the transcriptional biomarker. This difference was not attributable to the use of different histological cut-offs, given that there was still a marked difference in the proportion of recipients successfully discontinuing IS regardless of the histological outcome (27.5% vs. 44%, respectively). The populations of liver transplant recipients enrolled in the two trials were very similar in what regards variables known to be associated with successful IS discontinuation, such as recipient age and time from transplantation at the time of enrolment. The only clear difference was the proportion of recipients who were receiving at enrolment an IS regimen without CNI (30% in *RISET* vs. 2.6% in *LIFT*). This could represent a selection bias (i.e. patients in whom CNI discontinuation resulted in rejection might not have been selected to participate in the trial). Alternatively, it could also indicate that CNIs prevent the development of operational tolerance, which is supported by abundant pre-clinical and clinical literature,[36] or, alternatively, that pharmacological immunosuppression influences the transcript levels of the genes included in the diagnostic test. The latter is supported by the results observed when using the exact same biomarker test in two independent IS withdrawal clinical trials. In the first one, patients were enrolled when they had already discontinued CNI and were kept on a mammalian target of rapamycin inhibitor, and in this group of patients the transcriptional biomarker proved to be very accurate in predicting the outcome of IS withdrawal.[37] In contrast, the biomarker test failed to predict tolerance in the *iWITH* trial, which was conducted in paediatric liver transplant recipients who were all taking CNIs at study entry.[28]

The fact that patients who discontinued IS without developing clinically apparent rejection, but did not meet histological criteria of operational tolerance at the 1-year post-IS withdrawal protocol biopsy, were kept off IS (provided they did not exhibit severe histological lesions), allowed us to confirm that in some cases operational tolerance is associated with transient histological changes. This is in keeping with what was found during the long-term follow-up of the *RISET* patients who successfully discontinued IS, in whom a transient histological deterioration was observed.[12,21] This observation indicates that the Banff Group criteria of operational tolerance should probably be modified, to avoid the prompting the reinstitution of IS in patients who could safely be kept off with adequate sequential monitoring.

From the standpoint of the analysis of the screening liver biopsies, the results of *LIFT* confirm the findings from the *iWITH* trial indicating that even in highly selected recipients with normal or minimally increased liver tests, there is a high prevalence of subclinical damage.[32] Furthermore, *LIFT* 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.[31,32,38–43] Based on our results, it could be concluded that LT recipients who exhibit subclinical histological lesions require more IS. On the other hand, it is noteworthy that these patients with subclinical liver damage are also deriving a clinical benefit from their lower IS, given that they display better renal function. Hence, strengthening maintenance IS in an indiscriminate manner might not be an optimal approach from them. This is where the availability of non-invasive markers to identify those recipients likely to have developed the most active forms of subclinical alloimmunity would be highly valuable. To develop such a tool, we hypothesised that those biopsies exhibiting TCMR-related transcript levels of comparable magnitude to what is observed at the time of clinically apparent TCMR, would represent those more likely to eventually result in significant damage. By stipulating this transcriptional cut-off, we removed the difficulties of identifying a histological threshold of clinical significance which has not been developed in the literature yet. Furthermore, in so doing we provided a solution to internally normalise the gene expression data derived from *LIFT* and *iWITH*. The cut-off we selected (0.09) had high sensitivity to classify samples as having TCMR. Thus, we considered that all surveillance liver biopsies obtained as part of the study that had TCMR-related transcript

levels above this cut-off, displayed clinically significant alloimmune damage. Of note, given that it is still unclear the extent to which subclinical histological damage negatively impacts graft survival, our non-invasive predictive models were intentionally designed to prioritise high negative predictive value over positive predictive value. Thus, our proposal would be to use these models not to strengthen IS (which could result in side effects), but to potentially minimise IS in those LT recipients who do not exhibit significant active alloimmunity above the pre-defined cut-off. On the other hand, for those LT recipients at risk of having developed active alloimmunity, the non-invasive models could be used to inform the decision of performing a surveillance liver biopsy to adequately assess the degree of liver damage and the need for stronger IS.

Chapter 6 Conclusions

- In selected liver transplant recipients, IS withdrawal proved to be feasible. Although 68% of patients developed rejection, this was only graded as severe in 9% of cases, and no cases of graft loss were observed.
- The proportion of patients who successfully discontinue IS and meet the criteria of operational tolerance was much lower than originally estimated (based on the results of previous multicentre *RISET* Consortium trial). Thus, only 16% of patients who initiated immunosuppression withdrawal met the histological criteria of operational tolerance 12 months after complete IS discontinuation.
- A liver tissue transcriptional biomarker test, previously validated in the population of liver transplant recipients enrolled in the *RISET* trial (where the prevalence of tolerance was much higher than in *LIFT*), had a poor diagnostic performance and was not considered accurate in predicting the success of IS withdrawal in *LIFT*.
- The analysis of the demographic and clinical characteristics of the patients enrolled in *LIFT* revealed they differed from the population of patients enrolled in the clinical trial from which the biomarker was originally derived (*RISET*) in the type of immunosuppression medication administered at the time of study entry. This limitation could explain the poor performance of the biomarker test.
- Time since transplantation at the time of enrolment in the trial was the variable most strongly associated with the outcome of IS weaning, with patients who had been transplanted for longer periods of time being more likely to successfully discontinue IS.
- Current histological criteria of operational tolerance are likely to be too strict and should be refined to account for patients who develop transient histological abnormalities upon IS discontinuation.
- The very low prevalence of operational tolerance even in a selected subgroup of liver transplant patients with normal underlying liver histology and considered of low immunological risk, indicates that immunosuppression withdrawal should only be performed within carefully monitored clinical trials, ideally involving experimental treatments designed to promote allograft tolerance.
- The use of non-invasive markers such as serum transaminases, donor-specific antibodies and transient elastography is useful to identify stable liver transplant recipients with active underlying graft alloimmunity despite receiving IS. These non-invasive markers could be useful in a large number of liver transplant patients for whom no tools are currently available to assess the adequacy of their maintenance immunosuppressive regimens.

Additional information

Contributions of authors

Julien Vionnet (Clinical Research Fellow in Liver Transplantation) was responsible for data collection, study supervision, data analysis, manuscript writing, critical review for intellectual content and approval of the manuscript.

Rosa Miquel, (Consultant Liver Histopathologist) contributed to the concept and design of the study, conducted the histological analysis, collected data, and provided critical review for intellectual content and approval of the manuscript.

Juan G Abraldes (https://orcid.org/0000-0003-3421-937X) (Professor of Hepatology) provided advice on statistical analysis and manuscript writing, provided critical review for intellectual content and approval of the manuscript.

Juan-Jose Lozano (Senior Bioinformatician) conducted bioinformatic analysis and provided critical review of the study for intellectual content and approved the manuscript.

Pablo Ruiz (https://orcid.org/0000-0002-5810-8802) (Consultant Liver Transplant Hepatologist) conducted data collection, critically reviewed the study for intellectual content and approved the manuscript.

Miquel Navasa (Consultant Liver Transplant Hepatologist) conducted data collection, critically reviewed the study for intellectual content and approved the manuscript.

Aileen Marshall (Consultant Liver Transplant Hepatologist) conducted data collection, critically reviewed the study for intellectual content and approved the manuscript.

Frederik Nevens (Consultant Liver Transplant Hepatologist) conducted data collection, critically reviewed the study for intellectual content and approved the manuscript.

William Gelson (https://orcid.org/0000-0002-8100-8703) (Consultant Liver Transplant Hepatologist) conducted data collection, critically reviewed the study for intellectual content and approved the manuscript.

Joanna Leithead (Consultant Liver Transplant Hepatologist) conducted data collection, critically reviewed the study for intellectual content and approved the manuscript.

Steven Masson (https://orcid.org/0000-0003-1041-9844) (Consultant Liver Transplant Hepatologist) conducted data collection, critically reviewed the study for intellectual content and approved the manuscript.

Elmar Jaeckel (https://orcid.org/0000-0003-4108-7192) (Consultant Liver Transplant Hepatologist) conducted data collection, critically reviewed the study for intellectual content and approved the manuscript.

Richard Taubert (https://orcid.org/0000-0001-9270-2496) (Consultant Liver Transplant Hepatologist) conducted data collection, critically reviewed the study for intellectual content and approved the manuscript.

Phaedra Tachtatzis (Consultant Liver Transplant Hepatologist) conducted data collection, critically reviewed the study for intellectual content and approved the manuscript.

Dennis Eurich (Consultant Liver Transplant Hepatologist) conducted data collection, critically reviewed the study for intellectual content and approved the manuscript.

Kenneth Simpson (Consultant Liver Transplant Hepatologist) conducted data collection, critically reviewed the study for intellectual content and approved the manuscript.

Eliano Bonaccorsi-Riani (Consultant Liver Transplant Hepatologist) conducted data collection, critically reviewed the study for intellectual content and approved the manuscript.

James Ferguson (https://orcid.org/0000-0002-2529-0409) (Consultant Liver Transplant Hepatologist) conducted data collection, critically reviewed the study for intellectual content and approved the manuscript.

Alberto Quaglia (https://orcid.org/0000-0002-3333-3579) (Consultant Liver Histopathologist) contributed to the concept and design of the study, conducted the histological analysis, collected data, and provided critical review for intellectual content and approval of the manuscript.

Maria Elstad (Junior Statistician) supervised all statistical analysis.

Marc Delord (Junior Statistician) supervised all statistical analysis.

Abdel Douiri (https://orcid.org/0000-0002-4354-4433) (Reader Statistician) supervised all statistical analysis.

Alberto Sánchez-Fueyo (https://orcid.org/0000-0002-8316-3504) (Professor of Hepatology) responsible for concept and design of the study, obtaining funding, proving overall study supervision, data collection, data analysis, manuscript writing, critical review for intellectual content and approval of the manuscript.

Disclosure of interests

Full disclosure of interests: Completed ICMJE forms for all authors, including all related interests, are available in the toolkit on the NIHR Journals Library report publication page at https://doi.org/10.3310/FWXV5380.

Primary conflicts of interest: The authors report no conflicts of interest relevant to the contents of the present report.

Patient data statement

This work uses data provided by patients and collected by the NHS as part of their care and support. Using patient data is vital to improve health and care for everyone. There is huge potential to make better use of information from people's patient records, to understand more about disease, develop new treatments, monitor safety and plan NHS services. Patient data should be kept safe and secure, to protect everyone's privacy, and it's important that there are safeguards to make sure that they are stored and used responsibly. Everyone should be able to find out about how patient data are used. #datasaveslives You can find out more about the background to this citation here: https://understandingpatientdata.org.uk/data-citation

Confidentiality and anonymity

All data contained in this report have been managed and shared in a way that safeguards the confidentiality and anonymity of participants and are consistent with the terms of consent signed by participants.

Data-sharing statement

All data requests should be submitted to the corresponding author for consideration. Access to anonymised data may be granted following review.

Ethics statement

All research was conducted in accordance with the World Medical Association Declaration of Helsinki. The project was approved by the Bristol Research Ethics Committee (Health Research Authority, NRES Committee London-South East), 9/2/2015, Reference 14/LO/2172.

Information governance statement

King's College London University is committed to handling all personal information in line with the UK Data Protection Act (2018) and the General Data Protection Regulation (EU GDPR 2016/679). Under the Data Protection legislation, King's College London University is the Data Controller, and you can find out more about how we handle personal data, including how to exercise your individual rights and the contact details for our Data Protection Officer here: www.kcl.ac.uk/research/support/rgei/research-ethics/kings-college-london-statement-on-use-of-personal-data-in-research.

Publication

Vionnet J, Torres-Yaguana J, Miquel R, Abraldes JG, Wall J, Kodela E, *et al*. Randomized trial investigating the utility of a liver tissue transcriptional biomarker in identifying adult liver transplant recipients not requiring maintenance immunosuppression [published online ahead of print 17 Dec 2024]. *Am J Transplant* 2024. https://doi.org/10.1016/j.ajt.2024.12.002

References

1. Starzl TE. Cell migration and chimerism – a unifying concept in transplantation – with particular reference to HLA matching and tolerance induction. *Transplant Proc* 1993;**25**:8–12.
2. Mazariegos GV, Reyes J, Marino IR, Demetris AJ, Flynn B, Irish W, *et al*. Weaning of immunosuppression in liver transplant recipients. *Transplantation* 1997;**63**:243–9.
3. Devlin J, Doherty D, Thomson L, Wong T, Donaldson P, Portmann B, Williams R. Defining the outcome of immunosuppression withdrawal after liver transplantation. *Hepatology* 1998;**27**:926–33.
4. Orlando G, Manzia T, Baiocchi L, Sanchez-Fueyo A, Angelico M, Tisone G. The Tor Vergata weaning off immunosuppression protocol in stable HCV liver transplant patients: the updated follow up at 78 months. *Transpl Immunol* 2008;**20**:43–7.
5. Tryphonopoulos P, Tzakis AG, Weppler D, Garcia-Morales R, Kato T, Madariaga JR, *et al*. The role of donor bone marrow infusions in withdrawal of immunosuppression in adult liver allotransplantation. *Am J Transplant* 2005;**5**:608–13.
6. Lerut J, Sanchez-Fueyo A. An appraisal of tolerance in liver transplantation. *Am J Transplant* 2006;**6**:1774–80.
7. Eason JD, Cohen AJ, Nair S, Alcantera T, Loss GE. Tolerance: is it worth the risk? *Transplantation* 2005;**79**:1157–9.
8. Pons JA, Revilla-Nuin B, Baroja-Mazo A, Ramírez P, Martínez-Alarcón L, Sánchez-Bueno F, *et al*. FoxP3 in peripheral blood is associated with operational tolerance in liver transplant patients during immunosuppression withdrawal. *Transplantation* 2008;**86**:1370–8.
9. Pons JA, Yélamos J, Ramírez P, Oliver-Bonet M, Sánchez A, Rodríguez-Gago M, *et al*. Endothelial cell chimerism does not influence allograft tolerance in liver transplant patients after withdrawal of immunosuppression. *Transplantation* 2003;**75**:1045–7.
10. Takatsuki M, Uemoto S, Inomata Y, Egawa H, Kiuchi T, Fujita S, *et al*. Weaning of immunosuppression in living donor liver transplant recipients. *Transplantation* 2001;**72**:449–54.
11. Assy N, Adams PC, Myers P, Simon V, Ghent CN. A randomised controlled trial of total immunosuppression withdrawal in stable liver transplant recipients. *Gut* 2007;**56**:304–6.
12. Benítez C, Londoño MC, Miquel R, Manzia TM, Abraldes JG, Lozano JJ, *et al*. Prospective multicenter clinical trial of immunosuppressive drug withdrawal in stable adult liver transplant recipients. *Hepatology* 2013;**58**:1824–35.
13. Feng S, Ekong UD, Lobritto SJ, Demetris AJ, Roberts JP, Rosenthal P, *et al*. Complete immunosuppression withdrawal and subsequent allograft function among pediatric recipients of parental living donor liver transplants. *JAMA* 2012;**307**:283–93.
14. Shaked A, DesMarais MR, Kopetskie H, Feng S, Punch JD, Levitsky J, *et al*. Outcomes of immunosuppression minimization and withdrawal early after liver transplantation. *Am J Transplant* 2019;**19**:1397–409.
15. Martinez-Llordella M, Puig-Pey I, Orlando G, Ramoni M, Tisone G, Rimola A, *et al*. Multiparameter immune profiling of operational tolerance in liver transplantation. *Am J Transplant* 2007;**7**:309–19.
16. Martinez-Llordella M, Lozano JJ, Puig-Pey I, Orlando G, Tisone G, Lerut J, *et al*. Using transcriptional profiling to develop a diagnostic test of operational tolerance in liver transplant recipients. *J Clin Invest* 2008;**118**:2845–57.
17. Bohne F, Martínez-Llordella M, Lozano JJ, Miquel R, Benítez C, Londoño MC, *et al*. Intra-graft expression of genes involved in iron homeostasis predicts the development of operational tolerance in human liver transplantation. *J Clin Invest* 2012;**122**:368–82.

18. Demetris AJ, Lunz GJ 3rd, Randhawa P, Wu T, Nalesnik M, Thomson AW. Monitoring of human liver and kidney allograft tolerance: a tissue/histopathology perspective. *Transpl Int* 2009;**22**:120–41.
19. Banff Working Group on Liver Allograft Pathology. Importance of liver biopsy findings in immunosuppression management: biopsy monitoring and working criteria for patients with operational tolerance. *Liver Transpl* 2012;**18**:1154–70.
20. Rockey DC, Caldwell SH, Goodman ZD, Nelson RC, Smith AD; American Association for the Study of Liver Diseases. Liver biopsy. *Hepatology* 2009;**49**:1017–44.
21. Taubert R, Danger R, Londoño MC, Christakoudi S, Martinez-Picola M, Rimola A, *et al*. Hepatic infiltrates in operational tolerant patients after liver transplantation show enrichment of regulatory T cells before proinflammatory genes are downregulated. *Am J Transplant* 2016;**16**:1285–93.
22. Feng S, Demetris AJ, Spain KM, Kanaparthi S, Burrell BE, Ekong UD, *et al*. Five-year histological and serological follow-up of operationally tolerant pediatric liver transplant recipients enrolled in WISP-R. *Hepatology* 2017;**65**:647–60.
23. Venturi C, Sempoux C, Bueno J, Ferreres Pinas JC, Bourdeaux C, Abarca-Quinones J, *et al*. Novel histologic scoring system for long-term allograft fibrosis after liver transplantation in children. *Am J Transplant* 2012;**12**:2986–96.
24. Ojo AO, Held PJ, Port FK, Wolfe RA, Leichtman AB, Young EW, *et al*. Chronic renal failure after transplantation of a nonrenal organ. *N Engl J Med* 2003;**349**:931–40.
25. Karie-Guigues S, Janus N, Saliba F, Dumortier J, Duvoux C, Calmus Y, *et al*. Long-term renal function in liver transplant recipients and impact of immunosuppressive regimens (calcineurin inhibitors alone or in combination with mycophenolate mofetil): the TRY study. *Liver Transpl* 2009;**15**:1083–91.
26. Sharma P, Welch K, Eikstadt R, Marrero JA, Fontana RJ, Lok AS. Renal outcomes after liver transplantation in the model for end-stage liver disease era. *Liver Transpl* 2009;**15**:1142–8.
27. Yoshitomi M, Koshiba T, Haga H, Li Y, Zhao X, Cheng D, *et al*. Requirement of protocol biopsy before and after complete cessation of immunosuppression after liver transplantation. *Transplantation* 2009;**87**:606–14.
28. Feng S, Bucuvalas JC, Mazariegos GV, Magee JC, Sanchez-Fueyo A, Spain KM, *et al*. Efficacy and safety of immunosuppression withdrawal in pediatric liver transplant recipients: moving toward personalized management. *Hepatology* 2021;**73**:1985–2004.
29. European Liver Transplant Registry. *Data Analysis Booklet*. 2012. URL: www.eltr.org (accessed 15 May 2022).
30. Halloran PF, Chang J, Famulski K, Hidalgo LG, Salazar ID, Merino Lopez M, *et al*. Disappearance of T cell-mediated rejection despite continued antibody-mediated rejection in late kidney transplant recipients. *J Am Soc Nephrol* 2015;**26**:1711–20.
31. Londoño MC, Souza LN, Lozano JJ, Miquel R, Abraldes JG, Llovet LP, *et al*. Molecular profiling of subclinical inflammatory lesions in long-term surviving adult liver transplant recipients. *J Hepatol* 2018;**69**:626–34.
32. Feng S, Bucuvalas JC, Demetris AJ, Burrell BE, Spain KM, Kanaparthi S, *et al*. Evidence of chronic allograft injury in liver biopsies from long-term pediatric recipients of liver transplants. *Gastroenterology* 2018;**155**:1838–51.e7.
33. Neves Souza L, de Martino RB, Sanchez-Fueyo A, Rela M, Dhawan A, O'Grady J, *et al*. Histopathology of 460 liver allografts removed at retransplantation: a shift in disease patterns over 27 years. *Clin Transplant* 2018;**32**:1–7.
34. Bonaccorsi-Riani E, Pennycuick A, Londoño MC, Lozano JJ, Benítez C, Sawitzki B, *et al*. Molecular characterization of acute cellular rejection occurring during intentional immunosuppression withdrawal in liver transplantation. *Am J Transplant* 2016;**16**:484–96.
35. Vionnet J, Miquel R, Abraldes JG, Wall J, Kodela E, Lozano JJ, *et al*. Non-invasive alloimmune risk stratification of long-term liver transplant recipients. *J Hepatol* 2021;**75**:1409–19.

36. Whitehouse G, Gray E, Mastoridis S, Merritt E, Kodela E, Yang JHM, *et al*. IL-2 therapy restores T-cell dysfunction induced by calcineurin inhibitors. *Proc Natl Acad Sci U S A* 2017;**114**:7083–8.

37. Levitsky J, Burrell BE, Kanaparthi S, Turka LA, Kurian S, Sanchez-Fueyo A, *et al*. Immunosuppression withdrawal in liver transplant recipients on sirolimus. *Hepatology* 2020;**72**:569–83.

38. Miyagawa-Hayashino A, Yoshizawa A, Uchida Y, Egawa H, Yurugi K, Masuda S, *et al*. Progressive graft fibrosis and donor-specific human leukocyte antigen antibodies in pediatric late liver allografts. *Liver Transpl* 2012;**18**:1333–42.

39. Ohe H, Uchida Y, Yoshizawa A, Hirao H, Taniguchi M, Maurya E, *et al*. Association of anti-human leukocyte antigen and anti-angiotensin II type 1 receptor antibodies with liver allograft fibrosis after immunosuppression withdrawal. *Transplantation* 2014;**98**:1105–11.

40. Vionnet J, Sempoux C, Pascual M, Sánchez-Fueyo A, Colmenero J. Donor-specific antibodies in liver transplantation. *Gastroenterol Hepatol* 2019;**43**:34–45. https://doi.org/10.1016/j.gastrohep.2019.09.010

41. Kelly D, Verkade HJ, Rajanayagam J, McKiernan P, Mazariegos G, Hübscher S. Late graft hepatitis and fibrosis in pediatric liver allograft recipients: current concepts and future developments. *Liver Transpl* 2016;**22**:1593–602. https://doi.org/10.1002/lt.24616

42. Höfer A, Jonigk D, Hartleben B, Verboom M, Hallensleben M, Manns MP, *et al*. Non-invasive screening for subclinical liver graft injury in adults via donor-specific anti-HLA antibodies. *Sci Rep* 2020;**10**:1–12.

43. Höfer A, Jonigk D, Hartleben B, Verboom M, Hallensleben M, Hübscher SG, *et al*. DSA are associated with more graft injury, more fibrosis, and upregulation of rejection-associated transcripts in subclinical rejection. *Transplantation* 2020;**104**:551–61.

EME
HSDR
HTA
PGfAR
PHR

Part of the NIHR Journals Library
www.journalslibrary.nihr.ac.uk

Published by the NIHR Journals Library