Prospective randomised marker-based trial to assess the clinical utility and safety of biomarker-guided immunosuppression withdrawal in liver transplantation

Short title: Liver Immunospression Free Trial (LIFT)

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1. STUDY SYNOPSIS

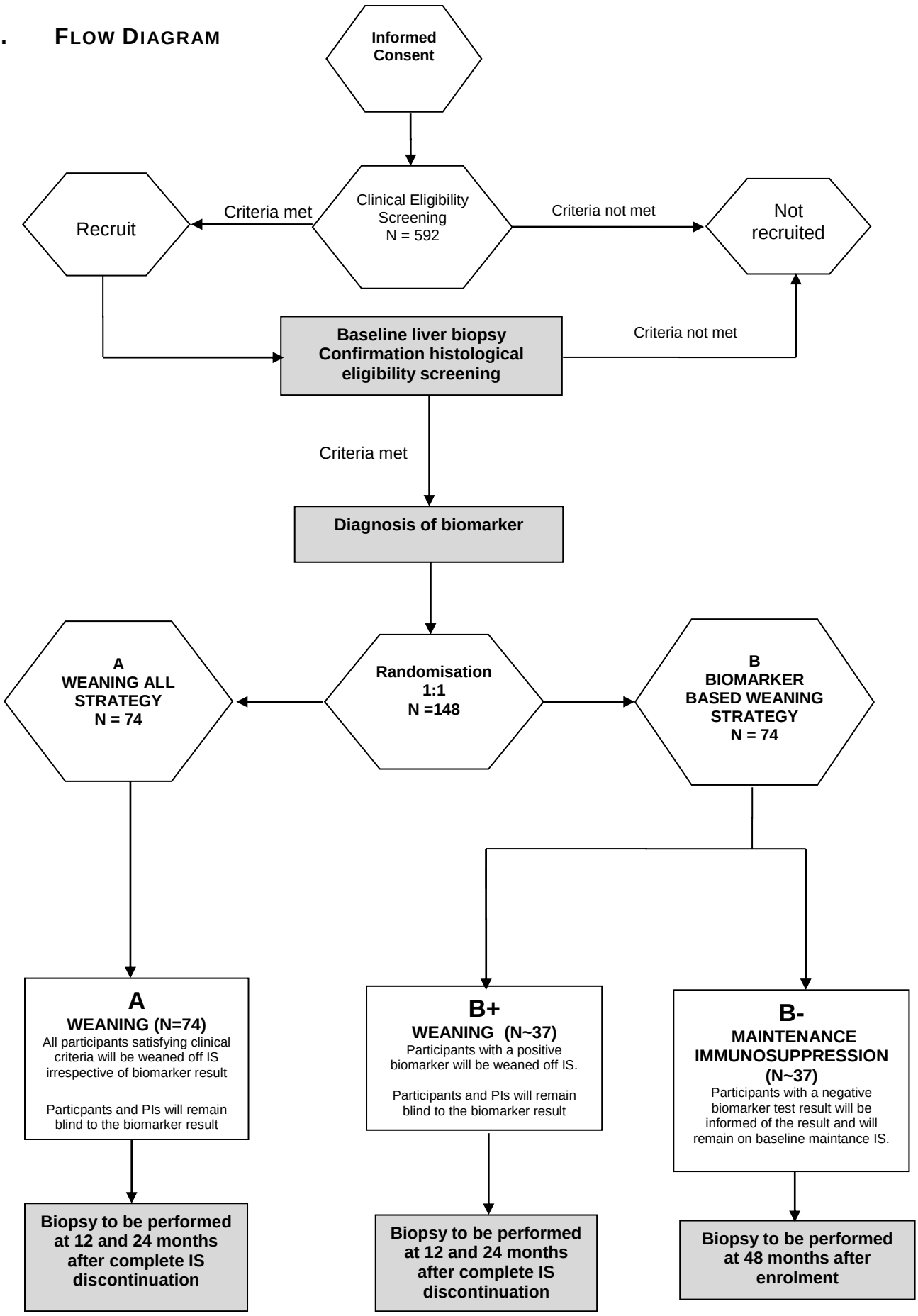
Title of clinical trial	Prospective randomised marker-based trial to assess the clinical utility and safety of biomarker-guided immunosuppression withdrawal in liver transplantation.
Protocol Short Title/Acronym	Liver Immunosuppression Free Trial / LIFT
Study Phase if not mentioned in title	IV
Medical condition or disease under investigation	Liver Transplantation
Chief Investigator	Professor Alberto Sanchez-Fueyo
Sponsor name	King's College London and KCH NHS Foundation Trust
Eudra CT number	2014-004557-14
Purpose of the trial	'LIFT' aims to validate a biomarker test of operational tolerance to stratify liver transplant recipients before withdrawing immunosuppressive medication.
Primary objective	Clinical utility and risk/benefit ratio of employing a transcriptional test of tolerance to stratify liver recipients prior to immunosuppression withdrawal.
Secondary objectives	- Safety of biomarker-guided immunosuppression withdrawal. - Health-economic and quality of life impact of biomarker-guided immunosuppression withdrawal - Improvement in drug-related co-morbidities. - Prevalence of tolerance over time - Role of donor-specific anti-HLA antibodies - Identify mechanisms of liver allograft tolerance
Trial Design	Prospective, multi-centre, phase IV, biomarker-strategy design trial with a randomized control group in which adult liver transplant recipients will undergo immunosuppression withdrawal.
Endpoints	Primary: successful discontinuation of immunosuppression with stable liver biopsy (12 and 24 months after IS withdrawal) and stable liver tests (12, 24 and 36 months after IS withdrawal). Secondary: rejection; graft fibrosis; graft loss; all–cause mortality; participants remaining free of rejection at 3 years post drug withdrawal; renal function; change in co-morbidities associated with drug use; anti-HLA antibodies before and after drug withdrawal; pharmacoeconomic and quality of life changes.
Sample Size	148 participants

Eligibility criteria	Inclusion Criteria: 1) consented adult liver transplant recipients >3 years post-transplant if aged >50 years or ≥6 years post-transplant if aged ≤50 years; 2) deceased or living donor liver transplant; 3) single transplanted organ; 4) direct bilirubin ≤17.1 umol/L and ALT ≤60 IU/L; 5) on calcineurin inhibitor based immunosuppression with or without one of the following: low dose mycophenolic acid, mycophenolate mofetil, azathioprine, sirolimus/everolimus; or on mycophenolic/mycophenolate or sirolimus/everolimus monotherapy; 6) ability to sign informed consent. Exclusion Criteria: 1) serum positivity for HCV-RNA; 2) serum positivity for HIV-1 infection, HBV surface antigen or HBV-DNA; 3) autoimmune liver disease; 4) rejection within the previous year; 5) GFR<30 ml/min; 6) need for chronic anticoagulation that cannot be safely discontinued; 7) baseline liver biopsy showing rejection, advanced fibrosis or moderate-severe inflammation; 8) age <18 years at the time of transplant; 9) pregnant females and females of childbearing age not using effective contraception; 10) curent illicit drug or alcohol use; 11) inability to attend frequent follow-up visits; 12) inability to comply with study directed treatment; 13) medical conditions interfering with safe completion of the trial; 14) participation in another clinical trial.
Active comparator product(s)	NA
Project timetables	Enrollment phase (18 months); patient follow-up (48 months: 6 to 12 months drug weaning, 36 months post-weaning follow-up). Total study duration: 72 months.

2. ABBREVIATION OF TERMS

AE	Adverse event
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
AST	Aspartate aminotransferase
AUC	Area under the curve
CI	Chief Investigator
CNI	calcineurin inhibitor
CRA	Clinical Research Associate
CRF	Case Report Form
CMV	Cytomegalovirus
EBV	Ebstein-Barr Virus
ECG	Electrocardiogram
GCP	Good Clinical Practice
GGT	γ-Glutamyltransferase
CTCAE	Common Terminology Criteria for Adverse Events
Hb	Hemoglobin
HIV	Human immunodeficiency virus
HCV	Hepatitis C virus
HCG	Human Chorionic Gonadotropin
HrQOL	Health-related quality of life
ICF	Informed Consent Form
ICH	International Conference for Harmonisation
IEC	Independent Ethics Committee
IL	Interleukin (eg. IL-1, IL-6, etc)
IS	immunosuppression
i.v.	Intravenous(ly)
KHP-CTO	King's Health Partners Clinical Trials Office
LDH	Lactate Dehydrogenase
LT	Liver Transplant
MDRD eGFR	Estimated glomerular filtration rate
MMF	Mycophenolate mofetil
PBMC	Peripheral blood mononuclear cell
PI	Principal Investigator
PIS	Patient Information Sheet
PTLD	Post-transplant lymphoproliferative disorder
RAI	Rejection Activity Index
SAE	Serious Adverse Event
SDV	Source Data Verification
WBC	White blood cells

3. FLOW DIAGRAM



4. BACKGROUND

4.1 Existing Research:

4.1.1 Liver Tissue Tolerance Biomarker

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 6 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 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-standardized algorithms for patient screening, drug withdrawal, and patient follow-up reduced the generation of truly generalizable information.

The results of the first 2 prospective, multi-centre, and independently monitored clinical trials of IS withdrawal (12, 13) have addressed some of the limitations of previous studies. In the first of these 2 studies, sponsored by the Immune Tolerance Network in the US, 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 Prof. 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. 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 wean from IS, while this occurred in <15% of those transplanted for less than 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 2 studies, preliminary data from an on-going US randomized adult trial were reported in 2011 (14). At that time, 67 adult recipients had been enrolled in the study (53 of them randomized to IS withdrawal and 14 to maintainance IS). In contrast to the two studies described above, IS withdrawal was initiated during the second year post-transplant, and was successful in only 2 out of the 18 participants in whom it was attempted (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 standardized 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 liver transplantation. Consideration of IS withdrawal, however, must carefully weigh the risks of inciting graft rejection. There is a need therefore for precise prospective identification of individuals who have become operationally tolerant to their transplanted liver. This would allow personalized 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.

Recent 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-deltaT cells and NK cells (15, 16). These findings were prospectively validated on samples collected before IS discontinuation in the RISET Consortium trial (17). Microarray and real-time 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 3 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 signficantly associated wtih 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 differred in hepcidin and ferritin serum levels, as well as in hepatocyte iron deposition (higher in liver recipients successfully weaned from IS) (17). The signficant 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 establishement 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 5 genes: *SOCS1, TFRC, PEBP1, MIF, CDHR2*, and predicted the outcome of IS withdrawal with AUC=0.85, SN=89%, SP=86%, PPV=80%, and NPV=92%. The signature was different from those reported from PBMCs or whole blood and was highly reproducible across the 3 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 (Bohne et al. *J Clin Invest* 2012), we conducted a number of additional transcriptional experiments employing the same Applied Biosystems 7900HT real-time PCR platform selected to conduct the current clinical trial. The experiments included a number of commercial and non-commenrcial RNA calibrators, as well as several different housekeeping genes. Reproducibility was optimized by employing a commerical RNA calibrator (liver RNA, Clontech) and both GAPDH and HPRT1 as housekeeping genes. These experiments were used to re-calibrate 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).

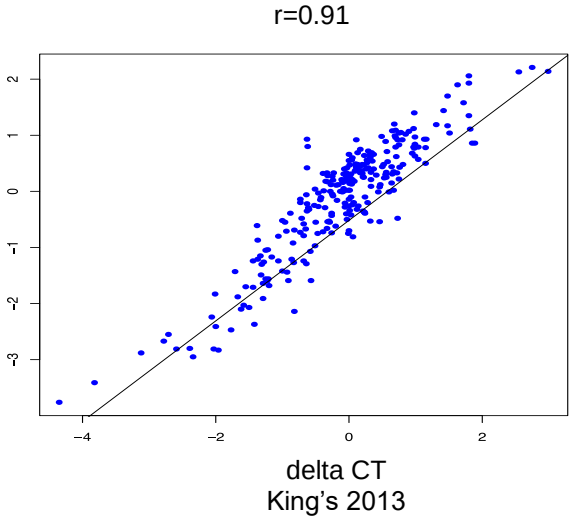


Figure 1: Correlation between the PCR gene expression results for the 5 genes included in the biomarker test of tolerance obtained in the original experiments (Hospital Clinic Barcelona 2011) and in the experiments performed at King's The King's experiments were conducted an Applied Biosystems 7900HT 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.

After re-calibration of the original algorithm to take into account the different experimental conditions, the following equation was defined: 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. The diagnostic performance of the 5-gene biomarker 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 intra-patient variability of real-time PCR 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 retrotranscription 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 righ 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 (Figures 2 and 3). The experimental variability associated with different PCR experiments conducted on the same cDNA samples was negligible (data not shown).

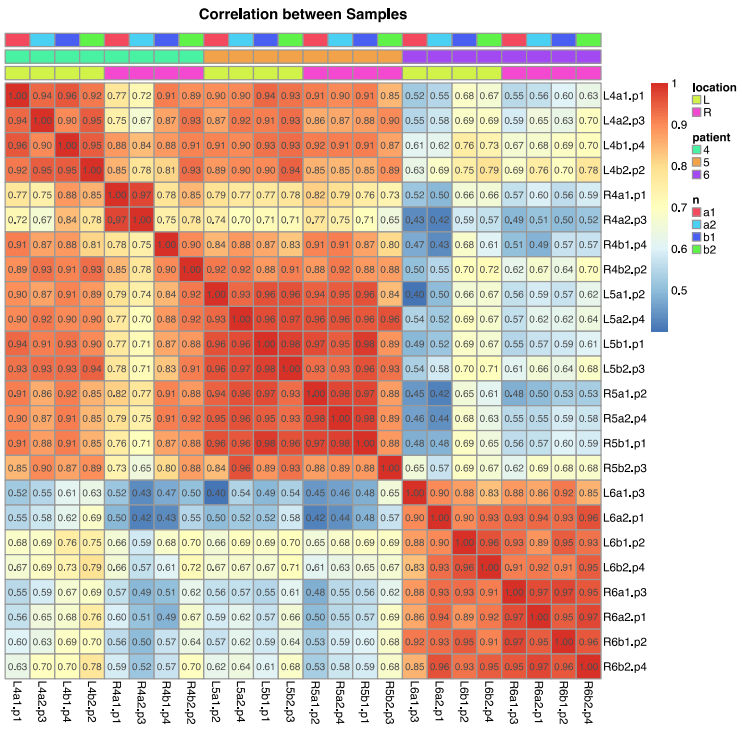
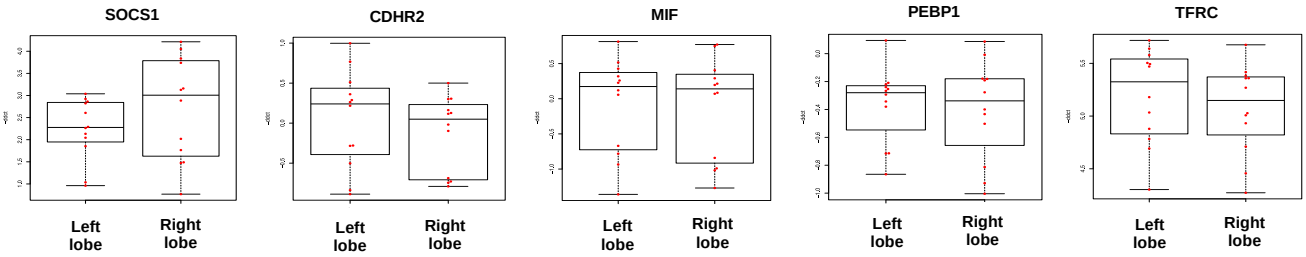


Figure 2: Plot showing the correlation coefficients between 24 samples used in a real-time PCR experiment measuring the expression of 24 genes associated with tolerance (including the 5 genes that constitute the biomarker test being assessed). Samples were collected from the right (R) and left (L) lobes of 3 different livers (patients 4, 5, 6). Two different portions of each liver tissue simple were extracted separatedly (a, b). For each extraction, 2 different retro-transcription reactions were performed (a1, a2, b1, b2).

Figure 3: Box plots showing the relative expression (ddC_T) of the 5 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.



Of note, the biomarkers described above were developed on hepatitis C-negative patients, and have not been adequately studied in patients with active hepatitis C- infection.

4.1.2 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 (33). Given that during the performance of intentional immunosuppression withdrawal protocols the large majority of rejection episodes occur during the period of immunosuppression weaning or shortly after having completely discontinued the immunosuppressive drugs, it has been agreed that at least one year off immunosuppression 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 immunological damage and the absence/failure of operational tolerance (25). This set of criteria, described in Table 4, 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 endpoint in LIFT. The use of this histological definition 1 year after complete immunosuppression 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 multi-centre European clinical trial (12, 34) (Figure 4). 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 re-institution of immunosuppression.

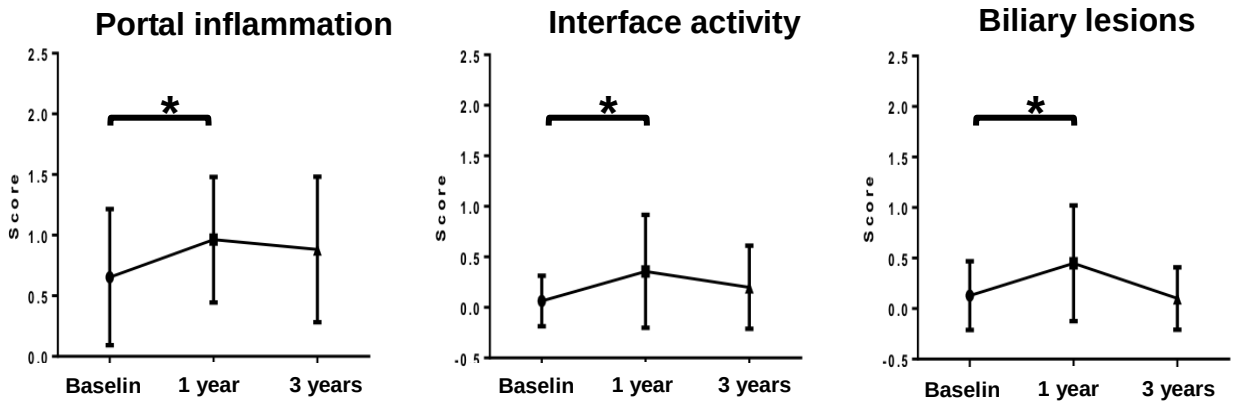


Figure 4: Central blinded review of sequential liver biopsies of liver transplant recipients who successfully discontinued IS (baseline, 1 and 3 years post IS withdrawal) in the RISET adult multi-centre European clinical trial, showing that IS withdrawal leads to transient mild increase in inflammatory infiltrates. Data correspond to mean +/- SD. No differences between the baseline and the 3-year biopsies were observed (12, 34).

4.2 Risks and benefits

Benefits: Chronic IS is associated with a variety of life threatening side effects following liver transplantation, including infection, malignancy, hypertension, diabetes, nephrotoxicity and cardiovascular diseases. Calcineurin inhibitor induced nephrotoxicity, in particular, is responsible for a significant rate of chronic renal failure, need for renal replacement therapy and increased mortality (18-20). Elimination of calcineurin inhibitors 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 who do not achieve it spontaneously.

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 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 multi-centre 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 emphasized that these 2 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 standardized. Thus, we believe the literature supports the concept that, provided we strictly adhere to the patient monitoring protocols implemented in the recent multi-centre 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 readily reversed.

Risk associated with treatment of rejection: The reinstitution of calcineurin inhibitors 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 co-morbidities (diabetes, hypertension, hyperlipidemia, 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. CMV 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 sub-clinical 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 (21). This case-control study had substantial flaws, as it lacked pre-weaning liver biopsies, and cases and controls signficantly differed in the lenght of their post-transplant follow-up. Reassuringly, neither the RISET nor the Immune Tolerance Network trials, that included strict protocols for sequential liver biosies, observed development of clinically-significant fibrosis.

Additional risks:

- *Risk of developing donor-specific antibodies:* Several reports in kidney transplantation have described that minimization or discontinuation of calcineurin inhibitors may promote the generation of donor-specific antibodies, but this has not been universally confirmed in the setting of liver transplantation. 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 a majority of participants in whom IS was discontinued developed anti-donor antibodies, but these antibodies were of the IgG4 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 and S. Feng, personal communication). 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 anesthetic. Occasionally the liver biopsy can be performed through a cathether 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% (22).

- *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.

4.3 Scientific Rationale

Long-term survival after solid organ transplantation has increased during the last decades (23) due to improvements in surgical technique, peri-operative care, and more efficient IS. However, transplant recipients still exhibit higher morbidity and mortality than the general population. One of the main causes are co-morbidities negatively influenced by chronic IS drug usage (24). Minimization (or complete withdrawal) of IS, particular 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 minimization 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). 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 liver transplantation, reduce the negative impact of co-morbidities associated with the use of chronic IS, and increase the quality of life of liver recipients.

The recent prospective, multi-centre drug withdrawal trials conducted in Europe and in the US and 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 5-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 liver transplantation. 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.

5. TRIAL OBJECTIVES AND DESIGN

5.1 Objectives

The overall objective is to assess the clinical utility and risk/benefit ratio of employing a previously validated transcriptional test of tolerance to stratify liver recipients prior to IS withdrawal.

5.2 Primary Objective:

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.

5.3 Secondary Objectives:

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 improve drug-related co-morbidities.
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 influence 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.

5.4 Trial Design

This is a prospective, multi-centre, phase IV, biomarker-strategy design trial with a randomised control group in which adult liver transplant recipients will undergo IS withdrawal. Enrolled participants will be 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 will be 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) will be offered IS withdrawal, while biomarker-negative participants (Arm B-, i.e. potentially non-tolerant) will remain on their

baseline maintenance IS. This will 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+ will provide direct evidence of the clinical usefulness of the test as a function of its predictive accuracy. We have established that for the biomarker to drive safe IS withdrawal its Positive Predictive Value $\left(PPV = {}^{Number\ Actually\ Tolerant}/_{Number\ Biomarker+}\right)$ should be no less than 0.80, and its sensitivity at least 070. To account for centre effects, we will use stratified randomization. Furthermore, to avoid biases, participants undergoing drug withdrawal and their physicians will be blinded to the biomarker results. Participants randomized to Arm B- will know their biomarker status, and will be maintained in the study until they complete 48 months of follow-up post randomisation. They will contribute to secondary clinical outcomes and to the evaluation of the stability of the tolerance signature.

Cost and quality of life (HrQOL) assessments will be conducted alongside the trial to estimate the health-economic implications of the 2 different strategies. Furthermore, sequential biological specimens will be collected to conduct ancillary mechanistic studies. Recruitment will take place in 14 European liver transplant units (King's College Hospital, Royal Free London, Newcastle, Birmingham, Leeds, Edinburgh, Cambridge, Leuven, Hannover, Berlin, Barcelona, Dublin, Cliniques Universitaires Saint-Luc and Palermo-ISMETT).

5.5 Trial Duration

Estimated recruitment period: 18 months.

Individual patient follow-up following enrolment: 48 months (6-12 months drug weaning, 36 months post-weaning follow-up).

Total estimated study duration: 72 months.

6. STUDY POPULATION

6.1 Inclusion Criteria

Participants *must meet all* of the following criteria to be eligible for this study:

1. At the time of screening: more than 3 years post-transplant if participants are ≥50 years old, 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: direct bilirubin ≤17.1 umol/L and ALT ≤60 IU/L at the screening visit.
5. On calcineurin inhibitor (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 six weeks following discontinuation of therapy, see Appendix 6),

6. Ability to sign informed consent.

6.2 Exclusion Criteria

Participants who *meet any* of the following criteria will *not* be eligible for this study:

1. Serum positivity for HCV-RNA
2. Serum positivity for HIV-1 infection, 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. GFR <30 mL/min (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 (Table 1) (25); f) 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 (See Appendix 6).
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 enrollment.

Table 1. Baseline (Pre-weaning) Biopsy Findings Conducive to the Minimization of IS* (25)

Compartment	Findings
Portal inflammation and interface activity	This is preferably absent, but minimal to focal mild portal mononuclear inflammation may be present. Interface necro-inflammatory activity is absent or equivocal/minimal and, if present, involves a minority of portal tracts and not generally associated with fibrosis.
Centrizonal/perivenular inflammation	Negative for perivenular inflammation.
Bile duct changes	Lymphocytic bile duct damage, ductopenia, and biliary epithelial senescence changes are absent unless there is an alternative, non-immunological explanation (e.g. biliary strictures).
Fibrosis**	Fibrosis (if present) should be mild overall, and portal-to-portal bridging should not be more than rare. Perivenular and peri-sinusoidal fibrosis should not be more than mild according to the Banff criteria.
Arteries	Findings for obliterative or foam cell arteriopathy are negative.

* Patients with underlying AIH, HCV, PBC, or PSC are excluded.
** Fibrosis should be graded as follows (26):
Portal/periportal: 0 – 3
Peri-sinusoidal: 0 – 3.
Perivenular: 0 – 3.

6.3 Patient withdrawal criteria

Participants may be prematurely terminated from the study for the following reasons:

1. the participant elects to withdraw consent from all future study activities, including follow-up;
2. the participant is “lost to follow-up”;
3. the participant dies;
4. any participant who fails screening and is deemed ineligible to initiate IS withdrawal;
5. pregnancy.

Participants have the right to voluntarily discontinue study treatment or withdraw from the study at any time for any reason without any consequences

The investigator has the right to discontinue a participant from study treatment or withdraw a participant from the study at any time if it is in the best interest of the participant.

Any medical condition that the investigator or sponsor determines may jeopardize the participant’s safety if she or he continues receiving the study treatment. Screening failures will not be randomized and will not count towards the final sample size.

7. STUDY MEDICATION

At the time of enrolment participants will be treated with tacrolimus, cyclosporine, azathioprine, everolimus/sirolimus and/or mycophenolate mofetil/mycophenolic acid. All specified immunosuppressive pharmacological drugs are commercially available and are licensed and have marketing authorisations as part of the standard of care in liver transplantation. For the purpose of this trial these drugs are defined as IMPs (Investigational Medicinal Products). Each IMP is specified

by active substance only. The objective of the trial is not to gain further information about the background treatment, but to assess the safety and promise of the diagnostic biomarker test of tolerance as a tool to stratify liver transplant recipients prior to an attempt to immunosuppression discontinuation. For reference safety information for IMPs, Investigators should refer to the relevant current summary of product characteristics (SmPC). Patients on mycophenolate mofetil or mycophenolic acid will need to receive effective contraception that will be maintained until 6 weeks following the discontinuation of these drugs (please see Appendix 6 for details on acceptable forms of contraception).

8. PLANNED INTERVENTIONS

8.1 Screening and Biomarker Test

All participants will undergo a Screening Visit during which informed consent will be obtained, the medical records will be reviewed to assess potential eligibility, a screening liver biopsy will be conducted, and bloods will be drawn for mechanistic studies. The screening liver biopsy will be 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 will undergo the analysis of the liver tolerance transcriptional biomarker test. This will be conducted on a 3 mm fraction of the screening liver biopsy cylinder that will be preserved in RNAlater reagent and frozen prior to shipment. The study will collect data on the percentage of stable participants identified as clinically suitable but who do not qualify for IS withdrawal and the reason for disqualification. This will clarify the trial's relevance to the broader liver transplant population. Biological specimens obtained from patients considered non-eligible on the basis of the screening liver biopsy will be kept for future research provided specific consent has been obtained.

8.2 Randomisation

Participants will be randomized 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 will be withdrawn regardless of the result of the biomarker test. Participants allocated to Arm B will be offered IS withdrawal only if they are classified as tolerant (Arm B+), while they will remain on maintenance IS if classified as non-tolerant (Arm B-).

8.2.1 Randomisation Procedure

A patient identification number (PIN) will be generated by registering the patient on the MACRO eCRF system (InferMed Macro), after consent has been signed. This unique PIN will be recorded on all source data worksheets and used to identify the patient throughout the study. Authorised site staff will be allocated a username and password for the randomization system. Once a patient is consented, all baseline data collected and eligibility confirmed, the staff member will log into the randomization system (www.ctu.co.uk) and click 'randomisation – advanced' and select LIFT and

enter the participants details (including MACRO PIN). The 'help' section of the system has video demonstrations to aid new staff in using the system. Once randomized, the system automatically generates confirmation emails to key staff, with or without treatment allocation information, depending on their role in the study. Participants that withdraw will not be replaced; levels of attrition have formed part of the sample size calculation to accommodate this.

8.2.2 Randomisation method

Randomisation will be via a 24 hour bespoke web based randomisation system hosted at the KCTU on a secure server. 148 adult liver transplant recipients will be randomised 1:1 at the level of the individual using the method of minimisation, stratified by site (11) and by the result of the Biomarker Test. The use of a random component in the minimisation algorithm will ensure that treatment allocation does not become deterministic and thus will protect pre-randomisation allocation concealment.

8.3 Immunosuppression Withdrawal Protocol (Arms A and B+)

- ***Weaning from calcineurin inhibitor (CNI) or mycophenolate/mycophenolic acid monotherapy:***
  - Participants will initiate IS weaning after at least 3 weeks of stable liver function (as documented by 2 separated laboratory exams).
  - Weaning will occur in eight 3-week intervals with each subsequent reduction based on liver function test stability over the prior 3-week interval.
  - No single reduction should exceed 50% of the daily dose except the final reduction. Withdrawal will proceed as follows:
    - Reduce total daily dose to 75% current total dose x 3 weeks
    - Reduce total daily dose to 75% current total dose x 3 weeks
    - Reduce total daily dose to 50-75% current total dose x 3 weeks
    - Give above dose 5x weekly x 3 weeks with consolidation to once daily dose
    - Give current dose 4x weekly x 3 weeks
    - Give current dose 3x weekly x 3 weeks
    - Give current dose 2x weekly x 3 weeks
    - Give current dose 1x weekly x 3 weeks and discontinue


Effective contraception must be used before beginning mycophenolate/mycophenolic acid therapy, during therapy, and for six weeks following discontinuation of therapy (please see Appendix 6 for details on acceptable forms of contraception).

- Weaning from 2 IS drugs:

- Participants on 2 IS drugs will first undergo withdrawal of the CNI as described above. Once the participant has discontinued the CNI, at least 3 weeks of stable liver function documented by 2 sequential blood tests will be required before initiating withdrawal of the second drug.

- Weaning of the mycophenolate/mycophenolic or azathioprine will occur in three 3-week intervals as follows:

  -Reduce daily dose to approximately 66% of initial total dose x 3 weeks (e.g. in patients initially receiving MMF 1500mg daily dose, reduce to 1000mg daily dose).

  -Reduce daily dose to approximately 33% of initial total dose x 3 weeks (e.g. in patients initially receiving MMF 1500mg daily dose, reduce to 500mg daily dose).

  - Discontinue

- Pausing of IS weaning:

- In the case of adverse events that could compromise safety, IS withdrawal can be temporarily paused for up to 4 weeks. During this time interval the participant will remain at the current dose. Participants are allowed up to 3 non-consecutive pauses. IS withdrawal will also be temporarily paused during the investigation of allograft dysfunction.

- Discontinuation of IS Weaning/Resumption of IS:

- Participants undergoing IS weaning who experience rejection will be re-started on IS and will not be allowed a second attempt.
- Participants who have successfully completed IS weaning and who subsequently experience rejection will be re-started on IS.
- Participants who cannot complete the IS weaning protocol and do not experience rejection will remain in the study but will be considered as 'failures'.

8.4 Maintenance Immunosuppression (Arm B-)

Participants randomized to Arm B and who are biomarker-negative will have no reduction 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 will be managed according to each centre's standard-of-care.

8.5 Management of Allograft Dysfunction

Allograft dysfunction is defined as any unexplainable elevation in ALT and GGT relative to baseline and above the upper limit of normality.

- When ALT and GGT are >100 IU/L a liver biopsy must be performed. Before indicating the liver biopsy, liver tests can be repeated for verification and re-assessment within 7 days. During this period of time IS withdrawal will be termporarily paused and participants will remain at their current IS doses.
- When ALT and/or GGT are <100 IU/L IS withdrawal will be temporarily paused and liver tests will be repeated within 7 days. In case of persistent allograft dysfunction with ALT and/or GGT <100 IU/L a liver biopsy can be performed or alternatively IS withdrawal can remain

paused for up to 4 weeks. Within 4 weeks IS must resume or a liver biopsy needs to be performed.

If a biopsy for cause has been performed and is non-diagnostic, then additional, follow-up biopsies will be performed at the discretion of the site investigator.

8.6 Diagnosis and treatment of Rejection Episodes

Rejection episodes will be diagnosed on the basis of liver biopsy findings according to Banff criteria. Clinical decision will be made on the basis of local biopsy readings. Investigators can treat rejection episodes according to each centre's standard-of-care. The following are however the recommended guidelines:

- **Allograft dysfunction with *no acute rejection or indeterminate for acute rejection*,** and without other explanatory diagnosis should be treated, if the investigator chooses, with reinstitution of the baseline IS (regimen employed before initiation of IS withdrawal). If liver tests do not improve within 4 weeks, repeat biopsy should be considered prior to further escalation of treatment.
- ***Mild acute rejection*** should be treated initially with reinstitution of baseline IS. If liver tests do not improve within 2 weeks, dose increase or addition of 20 mg oral prednisolone (or equivalent) should be considered. Corticosteroids will be rapidly tapered down over a 4 week period. A second biopsy can be performed at any time at the investigator's discretion.
- ***Moderate acute rejection without jaundice and with mild biochemical abnormalities*** should be treated with reinstitution of baseline IS and 20 mg oral prednisolone (or equivalent) with rapid taper down of steroid doses over a 4 week period. If liver tests do not improve within 2 weeks, conversion or addition of another agent should be considered prior to corticosteroids. A second biopsy can be performed at any time at the investigator's discretion.
- ***Moderate acute rejection with marked biochemical abnormalities and/or jaundice*, *severe acute rejection***, or *chronic rejection*, should be treated according to site standard of care. Antibody treatment should be reserved for steroid-resistant acute rejection proven by repeat liver biopsy.

8.7 Reinstitution of IS following successful IS withdrawal

Patients who do not develop allograft dysfunction but in whom a protocol biopsy performed at 12 or 24 months after complete IS discontinuation reveales moderate inflammatory changes or other changes suggestive of progressive histological damage (as described in Table 5) will be treated with reinstitution of the baseline IS. Patients who do not exhibit any of the changes described in Table 5 but fail to meet the primary endpoint on the basis of the histological criteria defined in Table 4 will be maintained off IS until the end of the study.

9. ENDPOINTS

9.1 Primary endpoint

The primary endpoint is defined as the successful discontinuation of IS with maintainance of normal allograft status as assessed by liver biopsy and liver tests 12 and 24 months after IS withdrawal (operational tolerance) and stable liver tests (12, 24 and 36 months after IS withdrawal). For the purposes of validating the clinical usefulness of the tolerance biomarker, successful IS withdrawal is considered the Gold Standard. Since this outcome is strictly restricted to the IS withdrawal process and by definition cannot be observed in Arm B-, the analysis of the primary outcome will be restricted to Arms A and B+.

9.2 Secondary endpoints

9.2.1 The secondary clinical endpoints of the trial are defined as:

- Rejection (incidence, severity, timing, steroid resistant rejection, chronic rejection).
- Reasons for failure of IS withdrawal.
- Requirement for IS re-institution despite successful IS withdrawal on the basis of the histological criteria described in Table 5.
- 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 enrollment.
- Change in co-morbidities associated with IS use (hypertension, cardiovascular risk profile, diabetes mellitus, hyperlipidemia, malignancy).
- Stability of the biomarker signature between baseline and last study visit.
- HrQOL changes associated with IS withdrawal.
- Pharmacoeconomic impact of IS withdrawal.
- Fibroscan:

  1- To assess if drug withdrawal results in sequential changes in liver stiffness over time in patients considered tolerant.

  2- To evaluate if development of an episode of rejection in patients who do not successfully discontinue immunosuppression increases liver stiffness measurements.

  3- To compare changes in liver stiffness over time in patients who remain on maintenance immunosuppression and those who undergo drug weaning.

9.2.2 The secondary mechanistic endpoints of the trial are:

- Intra-hepatic and systemic iron parameters.

- Time post-transplant, age, sex and type of IS.
- Markers of immune-exhaustion in blood and liver tissue.
- Gut microbiome profile.
- Blood and intra-hepatic lymphocyte subsets (including regulatory T cells).
- Development of anti-HLA antibodies (before and after initiation of IS withdrawal).

10. ASSESSMENTS AND FOLLOW-UP

10.1 Evaluation of eligibility

All participants meeting the inclusion criteria will be identified from the local transplant clinics. At the start of the trial, the entire population of transplant clinic attendees who are >3 years post-transplantation are potentially eligible for recruitment. On subsequent screening rounds, participants who reach 3 years post-transplantation after the start of the trial will become eligible.

10.2 Informed consent

Eligible participants will be approached at a routine clinic appointment by a trial investigator or research nurse, and given printed and verbal information about the trial. They will have the opportunity to return for a second consultation within a few days to give informed consent for recruitment into the study.

10.3 Timing of visits

10.3.1 Baseline visit

Consenting participants will have the following baseline assesments completed during their visit:

- Informed consent
- Physical exam (including height and weight measurement)
- Vital signs;
- Gender
- Ethnicity
- Date of birth
- Pre- and post-transplant medical history.
- Urine or blood βhCG (females)
- HLA type and that of donor liver (if available)
- Viral serology
- Autoantibody panel (ANA, AMA, SMA, LKM, quantitative IgG)
- IS drug 12-hour trough levels (as appropriate).
- MDRD eGFR

- Biochemistry panel (including creatinine, urea, electrolytes (sodium, potassium), and glucose).
- Full blood count (FBC) (including differential WBC count).
- Liver function tests (LFT) (including ALT, AST, GGT, alkaline phosphatase, total bilirubin (total and conjugated fraction at baseline visit)).
- Review of inclusion & exclusion criteria
- Concomitant medication

10.3.2 Screening Visit (3 weeks + /- 1 week)

The following assessments will be performed and recorded.

- Review of inclusion and exclusion criteria.
- Physical examination and vital signs
- Full blood count (FBC)
- Biochemistry panel
- Liver function tests (LFT)
- Baseline liver biopsy (see section 10.8)
- Blood draw for mechanistic studies
- Gut microbiome (stool)
- Diagnosis of the biomarker test
- Advesrse event (AE) since time of consent.
- Concomitant medications

10.3.3 Randomisation Visit (4-6 weeks post liver biopsy)

Subjects identified as eligible for the clinical trial based on all screening procedures including liver biopsy results will attend a Randomisastion Visit that needs to occur within 6 weeks of performing the biopsy, and within 2 weeks of receiving the email from LIFT with the randomisation results In participants randomised to IS withdrawal the Randomisation Visit will mark the beginning of the IS withdrawal protocol.

- Review of inclusion / exclusion criteria
- Liver function tests (LFT)
- Blood draw for mechanistic studies
- Metabolic panel (HbA1c, fasting glucose, fasting total colesterol, LDL, HDL)
- HLA antibodies
- Treatment-related diseases history – infection (CMV, fungal, other), biliary conditions, type II diabetes, kidney failure (possibly due to CNI), PTLD, cancer.
- Randomisation
- Adverse events
- Concomitant medications
- HrQOL assessment

Please note: Start decreasing the IS doses as per section 8.3 of this protocol.

10.3.3 Withdrawal visit from study

- Adverse events
- Concomitant medications
- Confirm the participant's agreement for the data and samples collected up to this point to be used for analysis.

10.4 Monitoring of Liver Function Tests

Participants undergoing IS withdrawal will have liver function tests performed every 3 weeks during the weaning period and the 6 months after complete IS withdrawal, monthly during the following 6 months, every 2 months during the following 6 months, and every 3 months thereafter. Participants who undergo rejection will have liver function tests every 1-2 weeks during the first month after the diagnosis, and at least every 3 months thereafter. Participants receiving maintenance IS (Arm B-) will have liver function tests every 3 months.

10.5 Transplant Centre Visits

Transplant Centre Visits will be performed every 6 months in all participants. The following will be collected and recorded:

- Interval history
- Treatment-related diseases history (every 6 months)
- Physical examination and vital signs
- Full blood count Biochemistry panel
- Liver function tests
- Metabolic panel
- HrQOL (SF-36v2 and NIDDK questionnaires every year and at last study visit, EQ-5D questionnaire every 6 months and prior to a for-cause biopsy required during or following IS weaning)
- HLA antibodies (at last study visit)
- Diagnosis of biomarkers test (last study visit)
- Blood draw for mechanistic studies (to be collected every 3 months in patients attending routine liver function tests at the Transplant Centre, if feasible)
- Gut microbiome (stool) (at 18 month from randomisation and at last study visit)
- Adverse events
- Concomitant medications

10.6 Telephone Visits

During study participation, and between Transplant Centre visits, telephone visits will be conducted to assess compliance with the study protocol. These will be conducted following the performance of each liver function test assessment, and will include:

- Interval history
- Adverse events
- Concomitant medications.

10.7 Visit Windows

- Laboratory evaluations should be completed within ± 5 days of the scheduled dates during the weaning period and the 6 months after complete IS withdrawal; within ± 7 days during months 6-12 after complete IS withdrawal; and within ± 2 weeks thereafter.
- All Transplant Centre Visits throughout the study should be completed within ± 2 weeks of the scheduled time points.

10.8 Liver Biopsies

Protocol liver biopsies will be performed:

1. At screening (to determine eligibility for study participation and to measure the transcriptional biomarker of tolerance);
2. 12 and 24 months after complete IS discontinuation for all recipients in Arms A and B+ who successfully withdraw IS;
3. 48 months after enrolment for participants on maintenance IS (Arm B-).

Patients who completely discontinue IS but require IS reinstitution on the basis of the 12-month post-withdrawal liver biopsy findings will still undergo a follow-up biopsy 24 months after IS withdrawal. In addition, “for-cause” biopsies will be performed to evaluate allograft dysfunction.

10.9 Liver biopsy procedure

Consent specific for the procedure will be obtained before each protocol or for cause liver biopsy. Liver biopsies will be performed employing 18-gauge or larger needle using a percutaneous technique. Depending on each participating centre’s standard practice, transjugular liver biopsies will also be acceptable. A minimum of 20 mm of core tissue will be obtained. Patients will be informed that the liver biopsy procedure may require 2 passes to obtain the required amoung of tissue.

For all biopsies performed during the subject’s study participation, the tissue should be preserved for the following 2 purposes: 1) 15 mm (1.5 cm) will be formalin fixed and paraffin imbedded; 2) the remaining (at least 5 mm) will be placed in RNAlater and sent to King’s College Hospital Liver Histopathology Laboratory.

Each site's local Pathology laboratory will keep 1 H&E slide after local reading, and the rest of the slides bearing stained and unstained tissue sections as well as the paraffin block will be sent to King's College Hospital Histopathology Laboratory. Appendix 4 contains a detailed protocol on how to process the formalin-fixed and paraffin embedded sample.

10.10 Assessment of liver biopsies

All liver biopsies performed within the trial will be evaluated both locally and by a central pathologist (blinded to biomarker status). Eligibility criteria, definition of primary endpoint (Table 4), and indication for reinstitution of IS in patients without allograft dysfunction (Table 5) will be determined on the basis of the central pathologist read. In addition, the central read will be used for all study data analyses. Analysis of for-cause biopsies for the purposes of clinical management will be conducted by the local pathologists. The central pathology read of four-cause biopsies will be made available to the sites for consideration at their discretion.

All biopsies read by the central pathology core will be scored employing a pre-defined histopathology review form (Appendix 5). Biopsies will be assessed for adequacy, length, and the total number of portal tracts and central veins. Necro-inflammatory activity and fibrosis will be graded according to the Ishak scale and according to Venturi et al. (26). AR- and CR-related activity will be graded and staged according to Banff criteria.

10.11 Transient elastography assessment (FibroScan®)

All participants will undergo transient elastography assessments using FibroScan at screening and every 12 months (visits M12, M24, M36, M48). Elastography results in isolation should not be employed to modify the management of study participants (e.g. modifying immunosuppressive doses or indicating a for-cause biopsy).

11. VISITS AND PROCEDURES SCHEDULE

11.1 Table 2: Transplantation Centre Visits Schedule

Assessment / Visit	Baseline - 7 Wks	Screening - 4 Wks	M 0	M 6	M 12	M 18	M 24	M 30	M 36	M 42	M 48
Informed consent	X										
Demographics: gender, DOB, ethnicity	X										
Height and weight	X										
Medical history (pre & post transplantation)	X										
Interval history [1]		X	X	X	X	X	X	X	X	X	X
Treatment-related diseases history			X	X	X	X	X	X	X	X	X
Physical examination	X	X	X	X	X	X	X	X	X	X	X
Vital signs	X	X	X	X	X	X	X	X	X	X	X
Liver biopsy [2]		X[2]									X[2]
Diagnosis of biomarker test		X									X
Biochemistry panel	X	X	X	X	X	X	X	X	X	X	X
Hematology (FBC)	X	X	X	X	X	X	X	X	X	X	X
Liver function tests [3]	X	X	X	X	X	X	X	X	X	X	X
Blood/ urine HCG test *(females only)*	X										
HLA type of recipient and of donor *(if available)*	X										
Autoantibody panel	X										
IS drug 12-hour trough levels *(as appropiate)*	X	X	X	X	X	X	X	X	X	X	X
Viral serology	X										
MDRD eGRF	X										
Inc. / Exc. Criteria	X	X	X								
Metabolic panel			X	X	X	X	X	X	X	X	X
Blood draw for mechanistic studies [4]		X	X	X	X	X	X	X	X	X	X
Gut microbiome		X				X					X
Health Economics Questionnaire				X	X	X	X	X	X	X	X
EQ-5D Questionnaire			X	X	X	X	X	X	X	X	X
SF-36 and NIDDK Questionnaires			X		X		X		X		X
Randomisation			X								
Adverse Events [1]	X	X	X	X	X	X	X	X	X	X	X
Concomitant Medication [1]	X	X	X	X	X	X	X	X	X	X	X
Fibroscan		X			X		X		X		X

[1] Information will also be collected during telephone visits (following each liver function test assessment).
[2] Protocol liver biopsies additional to screening one will be performed at 12 and 24 months post sucessful IS weaning or at 48 months post randomization for patients on maitenance IS treatment.
[3] Additional arm-specific schedule of liver function test monitoring is outlined in section 10.4.
[4] Performed every three months for patients undergoing liver function test monitoring at the Transplant Centre.
Wks weeks prior to randomization
M months from randomization
M0 randomisation visit

11.2 Table 3: Mechanistic laboratory assessments

Assessments	Volume (mL)	Screening	Period																	Reiection
			M 0	M 3	M 6	M 9	M 12	M 15	M 18	M 21	M 24	M 27	M 30	M 33	M 36	M 39	M 42	M 45	M 48	
Frozen PBMC (flow cytometry, epigenetic studies)	**50**	X	X		X		X		X		X				X				X	X
Whole blood Tempus tubes (gene expression profiling)	**6**	X	X		X		X		X		X		X		X		X		X	X
Serum (HLA alloantibodies)	**5**	X	X				X												X	X
Plasma (miRNA/microvesicles)	**5**	X	X	X[1]	X	X[1]	X	X[1]	X	X[1]	X	X[1]	X	X[1]	X	X[1]	X	X	X	X
Serum (iron metabolism)	**5**	X	X	X[1]	X	X[1]	X	X[1]	X	X[1]	X	X[1]	X	X[1]	X	X[1]	X	X	X	X
Plasma (cytokine assays)	**5**	X	X		X		X		X		X		X		X		X		X	X
Serum (metabolomic profile)	**5**	X	X		X		X		X		X		X		X		X		X	X
Stool sample (gut microbiome)		X							X										X	
Urine sampes (metabolomic profile)			X						X										X	
Liver biopsy (gene expression)[2]		X[2]							X[2]										X[2]	X

[1] Optional: To be collected only in patients undergoing liver function test monitoring at the Transplant Centre.
[2] Protocol liver biopsies will be performed at the screening visit and also at 12 and 24 months post successful IS weaning or at 48 months post randomization for patients on maintenance IS treatment.
M months from randomisation
M0 randomisation visit
R To be collected in patients undergoing a for-cause biopsy for suspicion of rejection.

12. EFFICACY ASSESSMENT

12.1 Definition of efficacy outcome measures

- ***Allograft dysfunction*:** any unexplainable elevation in ALT and GGT relative to baseline and above the upper limit of normality.

- ***Allograft rejection***: will be defined on liver biopsy according to Banff criteria. In rare cases in which it may be not feasible or safe to await the performance of a liver biopsy, episodes of severe allograft dysfunction impairment occurring during weaning that cannot be attributed to any other etiology and that rapidly respond to the empirical re-initiation of immunosuppressive drug therapy will be considered as indicative of presumed allograft rejection.

- ***Histopathology of acute allograft rejection***: Acute rejection will be defined by a liver biopsy exhibiting one of the following:

a) Presence of at least 2 of the following 3 features features according to Banff criteria: 1) predominantly mononuclear portal tract inflammation containing lymphocytes, neutrophils and eosinophils; 2) inflammatory bile duct damage; 3) venous subendothelial inflammation of portal or central veins.

b) Portal and/or lobular inflammation without bile duct damage and subendothelial inflammation provided that: 1) the inflammatory lesions were not present in the baseline (pre-weaning) biopsy; 2) they cannot be attributed to any other etiology (drug toxicity, autoimmunity, viral hepatitis); and 3) they occur in the presence of allograft dysfunction that rapidly normalizes by the re-initiation of immunosuppressive drug therapy. This takes into consideration the fact that in long-term surviving liver transplant recipients acute rejection tends to exhibit less subendothelial inflammation and greater interface and lobular activity resembling chronic hepatitis).

- ***Failure of IS withdrawal*:** failure to complete IS withdrawal for any reason.

- ***Operational tolerance*:** successful withdrawal of IS and maintainance of normal allograft status, as assessed by the absence of allograft dysfunction 12 months after complete IS discontinuation, and by the lack of clinically-significant changes in the liver biopsy obtained 12 months after withdrawal as described in Table 4.

Table 4. Banff Liver Allograft Pathology Working Group Criteria of Operational Tolerance Failure in Patients Off Immunosuppression

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.

Table 4. Banff Liver Allograft Pathology Working Group Criteria of Operational Tolerance Failure in Patients Off Immunosuppression

Compartment	Findings
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**	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

*Patients with underlying AIH, HCV, PBC, or PSC are excluded (25).
** Fibrosis should be graded as follows (26):Portal/periportal: 0 – 3
Peri-sinusoidal: 0 – 3.
Perivenular: 0 – 3.

Table 5. Follow-Up Biopsy Findings in Patients Off IS that Should Prompt Re-Institution 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 (26)).
Arteries	Any evidence of foam cell or obliterative arteriopathy

12.2 Pharmaco-economic and quality of life assessments

Health related Quality of Life (HrQOL) Assessment: we will employ the Short-Form 36 Health Survey (SF-36) and/or the EQ-5D as generic surveys. SF-36 represents the most prominent generic measure in assessing patient's HrQOL following liver transplantation (27). In contrast, EQ-5D is the preferred generic measure for assessing QOL in adults, according to the Reference Case by NICE (28). By applying preference values from population studies to EQ-5D health states we will be able to generate utility values that will be used in quality-adjusted life years (QALY) and cost-per-QALY-analyses (28, 29). In addition, we will also assess QOL using disease-specific questionnaires (29, 30). The NIDDK Liver Transplant Database QOL questionnaire contains 63 items on 6 subscales that cover general health, role function, social function, psychological status, personal function, and measures of liver disease (27). Participants will be asked to complete the stated questionnaires on the following time points: inclusion, months 3, 6, 9, 12, 18, 24, 36, 48 (final study visit), and in case of rejection episodes. We expect differences in QOL to occur between arm A and B and between arm B+ and B-. Due to the higher risk of rejection, during the initial 6-9 months of the study QOL of B+ participants could be worse than those in arm B-. In the long run, they might be better off due to the fact that they are not dependent on IS and may have a reduction in IS side effects.

***Assessment of Costs*:** costs will be assessed both from a healthcare and a societal perspective. For the healthcare perspective, only direct costs that accrue during the study period will be assessed (i.e. costs for IS medication, clinical visits, admissions, biomarker stratification). For the societal perspective, additional costs such as patient's absence from work, among others, will be accounted for. Resource utilization will be measured during the trial using a specific questionnaire, that will capture drug utilization, hospitalization, and diagnostic, therapeutic, and laboratory services consumed by patients. Resource utilization will be valued using prices relevant to the NHS. To calculate expected costs for the biomarker-guided weaning strategy, a micro-costing of the stratification process will be conducted.

- Health Economic Modelling: a health-economic decision-analytic model will be developed with the trial's data to investigate the long-term effects of the two IS withdrawal strategies. We will apply 2 different time horizons: (a) 5 years, and (b) a lifetime horizon, starting with the date of initial IS withdrawal. In line with the NICE reference case all costs and benefits will be discounted by 3.5% for the base case analysis, and the effects of varying discount rates will be investigated using appropriate sensitivity analyses. The 2 different perspectives described above will be analyzed.

- Cost-Utility Analyses: we will evaluate QALYs, costs and the incidence of downstream events including rejection episodes. We will calculate incremental cost-utility ratios (ICERs)

based on differences in patient outcomes and costs. The cost-utility analyses will be conducted using: (a) the trial data and the patient follow-up time frame; and (ii) a 10 year and lifetime horizon. To investigate uncertainty related to the model results we will employ deterministic sensitivity analyses to uncertainty connected to the data inputs, and probabilistic Monte-Carlo-Simulation to assess uncertainty connected to variability. Participants in study arm B+ (IS withdrawal, biomarker-positive) are expected to use lower amounts of drugs than participants in arm B- (biomarker-negative), and to undergo fewer rejection episodes than participants in arm A. The health economic model will allow us to determine if these reduced costs outweigh the costs of biomarker stratification.

12.3 Ancillary mechanistic analyses

The trial incorporates the collection, preparation and storage of biological specimens for mechanistic analyses. The overarching objective of these analyses is to idenfity the key mechanism/s involved in development of liver allograft tolerance, with a particular emphasis in pathways that could be manipulated to intentionally induce transplantation tolerance. The specific aims will be:

a) to confirm the influence of iron homeostasis in the development of liver transplant tolerance, and how iron homeostasis interacts with clinical and immunological parameters associated with tolerance;

b) to investigate if immune exhaustion/senescence plays a role in the establishment of transplantation tolerance;

c) to determine the impact of donor-specific anti-HLA antibodies on the success of IS withdrawal;

d) to study how the gut microbiome influences the immunogenicity of the transplanted liver and the development of tolerance;

e) to investigate how these factors might influence changes in the biomarker-status of participants over time.

12.4 Sampling Volumes and biological specimen storage

The biological specimen collection schedule is described in detail in Table 3. Samples will be used to conduct the following studies:

1. Quantification of cytokines, micro-RNA, iron and metabolomics parameters in serum.

2. Measurement of anti-HLA class I and class II antibodies.

3. Flow cytometry analyses and epigenetic/genomic studies on cryopreserved peripheral blood mononuclear cells (PBMCs). PBMCs will be isolated in a Ficoll gradient and cryopreserved in freezing medium.

4. Gut microbiome sequencing: approximately 1g of fresh stool will be collected at randomisation visit, 18 and 48 months after randomisation. Stool samples will be cryopreserved in RNAlater reagent.

6. Liver tissue gene expression studies: a portion of all protocol and liver tissue samples will be cryoprerved in RNAlater reagent. Gene expression studies will be performed employing microarray and/or digital PCR technology.
7. Liver tissue immunostaining: formalin-fixed and paraffin embedded liver tissue excess clinical material collected as a result of the for-cause or protocol biopsies will be employed to investigate the accumulation of specific lymphocyte subsets using multiplex immunostaining techniques.

12.5 Future/Unplanned studies:

As research tests are developed over time, specimens stored during the trial may be used in future assays to address the mechanistic goals of the study. Additionally, samples may be used for assays/ experiments outside the scope of the current study proposal, such as study of differences in the TCR sequence repertoire, proteomics, epigenetics, or other explorations that may emerge during the trial period. Reevaluations or new assays will only be performed on samples of participants who have consented for future research. This will apply as well to consented patients considered non-eligible on the basis of the screening biopsy.

13. PROCEDURES FOR RECORDING AND REPORTING ADVERSE EVENTS

Following review by the MHRA, the proposed trial has been considered a test of efficacy of the IS, and therefore a Clinical Trial of an Investigational Medicinal Product (CTIMP) according to the EU Clinical Trial Directive 2001/20/EC. For the purposes of this trial these drugs are defined as IMPs (Investigational Medicinal Products) but they will not require special labelling/accountability/storage etc.

For assessment of safety, we will follow the definitions given by the Medicines for Human Use Regulations 2004 and Amended Regulations 2006:

- **Adverse Event (AE):** Any untoward medical occurrence in a subject to whom a medicinal product has been administered including those that are not necessarily caused by or related to that product.
- **Adverse Reaction (AR):** Any untoward and unintended response in a subject to an investigational medicinal product, which is related to any dose administered to that subject.
- **Unexpected Adverse Reaction (UAR):** An adverse reaction the nature and severity of which is not consistent with the information about the medicinal product in question set out in the SmPCs for the IS medications employed by the participants enrolled in the study.
- **Serious adverse Event (SAE), Serious Adverse Reaction (SAR)** or Unexpected Serious Adverse Reaction (USAR): Any adverse event, adverse reaction or unexpected

adverse reaction, respectively, that results in death; is life-threatening; required hospitalisation or prolongation of existing hospitalisation; results in persistent or significant disability or incapacity; consists of a congenital anomaly or birth defect. Although not a serious adverse event, any unplanned pregnancy will also be reported via the SAE reporting system.

13.1 Reporting Responsibilities in the United Kingdom, Germany, Spain, Italy and Belgium

The delivery of the sponsor's responsibility for Pharmacovigilance (as defined in Regulation 5 of the Medicines for Human Use Regulations 2004) has been delegated to King's Health Partners Clinical Trials Office (KHP-CTO). The Chief Investigator (CI) will immediately (and no later than 24hrs) report to the KHP-CTO all SAEs, SARs and SUSARs in accordance with the current Pharmacovigilance Policy. Important Medical Events that may not be immediately life-threatening or result in death or hospitalisation but may jeopardise the patient or may require intervention to prevent one of the other outcomes listed in the SAE definition should also be considered serious and reported using the SAE form. The KHP-CTO will report SUSARs to the regulatory authorities (MHRA). The CI will report to the relevant ethics committee. Reporting timelines are as follows:

- SUSARs that are fatal or life threatening must be **reported within 7 days** after the sponsor is first aware of the reaction. Any additional relevant information must be reported within a further 8 days.

- SUSARs that are not fatal or life threatening must be **reported within 15 days** of the sponsor first becoming aware of the reaction.

The CI and KHP-CTO (on behalf of the sponsor), will submit annually to the MHRA and REC, a Development Safety Update Report (DSUR). The CI will submit annually to the main REC an Annual Progress Report. For the purposes of this study, graft rejection does not constitute an SAE, and will be recorded in a dedicated section in the case report form (CRF). Pregnancy is not considered to be an AE or SAE. However, the trial centre should report any pregnancy in a trial patient to the Sponsor within 24 hours of becoming aware of the pregnancy, using the dedicated paper form supplied by the Sponsor. A pregnancy should be carefully monitored and the Investigator should track the progress of the mother and the foetus as if the pregnancy were an SAE, providing detailed information to the Sponsor as it becomes available. During gestation, any occurrences that result in a SAE should be reported on the paper SAE Form as per the SAE reporting procedure. The Investigator must follow all patient pregnancies to term and report the outcome to the Sponsor. When the outcome of a pregnancy falls under the criteria for an SAE (e.g. spontaneous or induced abortion, stillbirth, death of newborn, congenital anomaly, birth defect) the Investigator should

respond by submitting an SAE report. The Sponsor will report pregnancy outcomes to the regulatory authorities.

13.2 Reporting Responsibilities in Ireland

The delivery of the sponsor's responsibility for Pharmacovigilance (as defined in Regulation 5 of the Medicines for Human Use Regulations 2004) has been delegated to King's Health Partners Clinical Trials Office (KHP-CTO). The Chief Investigator (CI) will immediately (and no later than 24hrs) report to the KHP-CTO all SAEs and SARs in accordance with the current Pharmacovigilance Policy. Important Medical Events that may not be immediately life-threatening or result in death or hospitalisation but may jeopardise the patient or may require intervention to prevent one of the other outcomes listed in the SAE definition should also be considered serious and reported using the SAE form. SUSARs occurring in Ireland will be reported by the HPRA to the Eudravigilance (EVCTM) on the Sponsor's behalf. The HPRA will require SUSAR reports in an expedited fashion to ensure the HPRA and reporting timelines are as follows:

- SUSARs that are fatal or life threatening must be **reported within 3 days** after the sponsor is first aware of the reaction. Any additional relevant information must be reported within a further 8 days.

- SUSARs that are not fatal or life threatening must be **reported within 9 days** of the sponsor first becoming aware of the reaction.

The CI and KHP-CTO (on behalf of the sponsor), will submit annually to the HPRA, a Development Safety Update Report (DSUR). For the purposes of this study, graft rejection does not constitute an SAE, and will be recorded in a dedicated section in the case report form (CRF). Pregnancy is not considered to be an AE or SAE. However, the trial centre should report any pregnancy in a trial patient to the Sponsor within 24 hours of becoming aware of the pregnancy, using the dedicated paper form supplied by the Sponsor. A pregnancy should be carefully monitored and the Investigator should track the progress of the mother and the foetus as if the pregnancy were an SAE, providing detailed information to the Sponsor as it becomes available. During gestation, any occurrences that result in a SAE should be reported on the paper SAE Form as per the SAE reporting procedure. The Investigator must follow all patient pregnancies to term and report the outcome to the Sponsor. When the outcome of a pregnancy falls under the criteria for an SAE (e.g. spontaneous or induced abortion, stillbirth, death of newborn, congenital anomaly, birth defect) the Investigator should respond by submitting an SAE report. The Sponsor will report pregnancy outcomes to the regulatory authorities.

14. STOPPING CRITERIA

The Sponsor and CI reserve the right to stop the trial at any time, for any justifiable reason. These include, among others:

1) request from the Data Monitoring & Ethics Committee (DMEC) or a regulatory authority;
2) failure to meet patient recruitment targets;
3) serious and/or persistent non-compliance with the trial protocol;
4) non-compliance with ethical standards, regulatory requirements or GCP;
5) when the Sponsor/chief investigator is aware of important new information that adversely affects the conduct of the study;
6) Findings uncovered during monitoring visits, trial audits or inspections that compromise the suitability of the site to act as a trial centre.

In the event of premature discontinuation, the Sponsor will promptly notify the responsible regulatory authorities, and provide a detailed written explanation of the reasons for early termination. The affected trial participants will also be informed promptly and appropriate follow-up will be arranged. The CI will also inform the REC.

During the course of the study any of the following will pause the study and trigger a review by the DMEC: 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% (this composite end point will be monitored on a regular basis by the Study Statistician, who will estimate its incidence and exact one-tailed 90% CI and check whether the lower bound of the CI is above the pre-set threshold of 5%.).

15. PROPOSED SAMPLE SIZE

Primary Hypothesis

To demonstrate the superiority of biomarker-led IS withdrawal over maintenance IS for hard clinical outcomes such as long term survival or incidence of IS-related side effects, the trial would require an unrealistically large sample size and a very lengthy follow-up. Notwithstanding, given the well known potential long-term benefits of IS withdrawal and the health-economic implications, the current study has been designed and powered to test the hypothesis that the IS weaning under the novel "Biomarker-based" strategy (Arm B+) is superior to Weaning All (Arm A), with respect to the proportion of participants who, having started the IS withdrawal protocol, complete it successfully without undergoing allograft rejection. Comparing arms A and B+ will show the utility of the biomarker as a clinical decision tool, as a function of its predictive accuracy. We have established that for the biomarker to drive safe IS withdrawal its Positive Predictive Value $\left(PPV = {}^{Number\ Actually\ Tolerant}/_{Number\ Biomarker+}\right)$ should be no less than 0.80, which would result into a statistically significant difference in the proportion of successfully weaned participants between arms A and B+. If the biomaker's PPV was 0.50, then participants in arm B+ would be no

better than taken at random, and there would be no difference with respect to arm A. Additionally, for the biomarker test to be considered successfully validated for clinical use, its sensitivity as estimated within arm A should be at least 0.70.

Expectations:

- **Biomarker Allocation:** Approximately 50% of participants will be positive for the biomarker (17).
- **Arm A:** 50% Successfully weaned participants **vs.**
- **Arm B+**: Under H_0: 50% successfully weaned participants (if PPV = 0.50); under H_1 : 80% or more successfully weaned participants (if PPV ≥ 0.80).

100 participants would be sufficient to show superiority of Arm B+ compared to Arm A, with 90% power and 5% type I error rate, assuming an allocation ratio of 2:1 between arms A and B+ (given 50% biomarker positive). The resulting sample size including participants allocated to arm B- would be 134 subjects. To account for dropouts, the final sample size will be increased in 10%, resulting in a total of 148 participants.

16. STATISTICAL ANALYSIS

16.1 Primary Analysis

To test for superiority of arm B+, compared to arm A, we will compare the proportion of successfully weaned participants in each arm using generalized linear mixed models (GLMM) (logistic regression) with random effects for study centre, as a factor used in the minimisation procedure. Potential confounding will be examined, as part of sensitivity analysis, for the following covariates: (study centre, age at randomization, time from transplantation at randomization, a binary indicator of whether the participant was receiving a second drug at baseline). The primary outcome will be analysed per Intention-to-Treat, i.e. participants allocated to arms A or B+ who do not start the weaning protocol or initiate weaning but do not complete it, will be treated as 'failures'. To ultimately validate the clinical utility of the biomarker, 2 further conditions will need to be met: 1) the confidence interval of the proportion of successes in arm B+ should include 0.80 and exclude 0.50; and 2) the confidence interval of the test's sensitivity as estimated in arm A should include 0.70 and exclude 0.50.

16.2 Secondary Analysis

For the exploratory analysis of secondary outcomes, we will use GLMM to adjust stated outcome(s) for study site as a random effect. Multivariate models will include, as fixed effects, the main effects of treatment strategy arm and biomarker status, as well as the interaction term. Secondary analyses are considered exploratory and will not be adjusted for multiple comparisons. A survival analysis approach will additionally be adopted to examine times to acute rejection in patients assigned to IS weaning. For the analysis of serial measures we will use mixed effects regression with random

effects for study subject, as well as for study centre. The stability of the biomarker signature at the final study visist will be tested with GLMM: logistic regression for a binary classification signature and linear regression for a continuous diagnostic score before classification cut-off. The model will include, as fixed effects, the baseline classification or, correspondingly, the baseline estimated probability of tolerance, the main effect of treatment strategy arm and their interaction, as well as study centre, as a random effect. The effect of co-variates (same as for the analysis of the primary outcome and additionally baseline drugs and duration of weaning) will be examined as part of sensitivity analysis. The effect of potential mechanistic mediators on primary and secondary outcomes will be evaluated using generalized latent mixed models (31). This will allow us to consider different models of causation (32). Missing baseline data required for covariate adjustment should not represent an issue for the primary analysis. Patters of missingness of post-randomisation assessments will be examined in relation to the co-variates used for adjustment. If there are two or more outcome time points, missing post-randomisation assessments will be dealt with by fitting GLMMs to all the available data using maximum likelihood methods. If post-treatment variables are found to be predictive of drop-out, multiple imputation will be considered. (R statistics software will be used for statistical analyses (http://www.R-project.org).

16.3 Interim analyses and stop/go decisions

We will carry out interim analyses when 33% and 50% of participants reach the primary outcome. At 33% we will calculate the 95% confidence interval 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 show not to be significantly different from 50%, the trial will stop due to poor accuracy of the biomarker test. At 50% we will validate the sensitivity of the test using data from the 37 participants randomized to arm A only (weaning all), which will allow us to estimate with 80% power the 95% confidence interval of the sensitivity that included 70% and excluded 50%.

17. ETHICAL ARRANGEMENTS

All parties involved in this study should conduct the trial in accordance with the ethical principles that have their origin in the Declaration of Helsinki on Ethical Principles for Medical Research Involving Human Subjects, adopted by the General Assembly of the World Medical Association (1996), and in agreement with ICH-GCP and applicable local regulatory requirements.

17.1 Ethics Committee Approval

Prior to initiation of the trial, the protocol, patient information sheets (PIS), consent forms (CFs) and any other requested documentation will be submitted to the concerned Research Ethics Committee (REC). Patient recruitment will not commence before a written favourable opinion has been issued by the REC. A copy of the REC approval letter will be filed in the TMF (Trial Master File). Substantial

amendments to the trial protocol and changes to documentation will be approved in writing by the REC. The CI shall inform the concerned REC promptly of any new information that may adversely affect the safety of participants or the trial conduct. The CI will conform with any requirement for providing periodic progress reports to the concerned REC. Upon completion of the trial, the CI will provide the REC with a brief report of the outcome of the trial, if required. All correspondence between the CI and the REC will be filed in the TMF and copies sent to the Sponsor.

17.2 Subject Information and Consent

The trial team will prepare a master copy of the PIS and CF approved by the REC. The trial team will ensure that any changes to these documents are approved by the REC before being used in the study.

17.3 Consent Procedure

All trial Investigators seeking consent must have received specific training in the taking of consent and be up-to-date on their annual refresher training. For each trial participant, an authorised trial Investigator must obtain written informed consent prior to conducting a trial-related procedure involving the subject. If a prospective recipient has signed informed consent more than three months prior to initiation of the study, it is recommended to obtain a second signed CF to ensure that the patient still agrees to study participation. The Investigator must always provide the subject with a copy of the completed consent form (CF) and participant information sheet (PIS) and store the original in the TMF. Signed, original consent forms must be retained in the TMF at all times and made available (for review) to study monitors, auditors and inspectors, upon request. It is recommended that copies of the signed patient consent form(s) should also be kept in the patient notes. A comprehensive verbal explanation by the Investigator will accompany the written information sheet given to potential trial participants. The Investigator should explain the aims, methods, anticipated benefits and potential risks of the study, including any discomfort it may entail. They will be told of the serological tests that must be undertaken and their right to receive the results. Subjects should be given sufficient time to read the information sheet thoroughly and the opportunity to clarify any points that they do not understand. At the end of the discussion, the subject should be granted as long as they feel necessary to digest the information provided and to consider their involvement in the trial. They should be free to discuss their participation with others outside the clinical trial team (e.g. family, friends, general practitioner) and must not feel pressure to provide an immediate decision. Subjects should also be allowed a second opportunity to ask the Investigator and/or research nurse questions regarding their participation, after the initial interview. All queries or concerns about the trial should be answered to the satisfaction of the subject. When obtaining informed consent, the Investigator should respect the free will of the individual and must not exert undue influence on the subject or enforce compulsory enrolment into the study. The

language and expressions used by the Investigator to explain the nature and purpose of the study should be as plain and understandable as possible. The verbal information should not contain specialist terminology used with the deliberate intention of confusing or misleading the subject. If a subject is unable to read and/or write, but capable of understanding the oral information provided and consenting to trial participation, an impartial witness should be present during the entire discussion and should sign the consent form on behalf of the subject, if consent is indicated. Subjects unable to freely give their informed consent are excluded from this trial, as specified by the eligibility criteria (see Eligibility Criteria). If a potential trial participant cannot understand the native language version of the PIS/ICF, these documents will be translated on request. Subjects who decline to give written informed consent must not be enrolled in the trial or involved in trial-related activities.

17.4 Consent Withdrawal

A trial participant has the liberty to withdraw their consent at any time and for any reason, without penalty or loss of benefits to which the individual would otherwise be entitled. Participants who withdraw consent will discontinue their participation in the trial. Future trial follow-up will be cancelled and immunosuppressive treatment according to protocol specifications will no longer be imposed. These participants will end their involvement with the immunomonitoring components of the trial and will not be asked to provide biological samples beyond the date of withdrawal. The Sponsor will retain and use all data collected up to the point of patient withdrawal. Liver recipients will be informed during the consent procedure that, if they withdraw, they will not be able to ask for previously collected trial data to be destroyed. Prior to giving consent, recipients will be informed that they are able to request the destruction of stored biological samples (e.g. blood/urine for IM assays) upon withdrawal, and that this will only be possible for samples that have not been tested at the time of withdrawal. Participants will not be able to request the deletion of data generated from tested samples.

17.5 Personal Data Privacy Protection

To protect the identities of trial patients, each transplant recipient will be assigned a unique patient trial identifier upon enrolment in the order in which they are enrolled in the study. All electronic and paper records containing patient data generated by the study will be encoded with the appropriate patient trial identifier. Patient names will not be used to report or record trial data. Only Investigators and authorised staff at the trial centre will be in possession of documents that link patient names to patient trial identifiers (i.e. IS/ICF and Patient Identification Log). It is the responsibility of the PI to ensure that these documents are treated in a confidential manner and stored securely. Regarding any paper records containing subject data (e.g. laboratory results, medical reports), the trial team will delete the subject's name and mark the document with the appropriate encoded identifiers.

Safety reports transmitted by the Sponsor to the responsible authorities and ECs will use encoded subject identifiers. All data collected by the study will be regarded as strictly confidential. Access to the trial eCRF platform will be password protected and electronic login credentials will be issued only to named authorised individuals. The Sponsor requires the PI to permit the Sponsor, designated trial monitors, and when necessary, members of the EC or representatives of the regulatory authorities to inspect and/or copy medical records relevant to the study and trial documents bearing patient names. During such activities, the confidentiality of personal data will be respected at all times. By signing the IS/ICF, the recipient will specifically consent to direct access to his/her medical records and source documentation for the purpose of source data verification (SDV) and regulatory inspection.

18. RESEARCH GOVERNANCE

18.1 Sponsorship

This is an investigator-initiated, non-commercial clinical trial funded by a research grant awarded by the National Institute of Health Research (NIHR) UK. The trial will be jointly sponsored by King's College London and King's College Hospital NHS Foundation Trust. KHP-CTO will assist with regulatory submissions and pharmacovigilance and will provide sponsor QA oversight of trial processes such as consent and TMF maintenance.

18.2 Trial Steering and Data Monitorning and Ethics Committees

A Trial Steering Committee (TSC) will be convened. The membership will be decided by the CI in partnership the sponsor. The chair will be a senior transplantation physician or surgeon from the UK who is unconnected to the study. Members will include the CI, two other PIs from the trial, 2 representatives of the liver transplant patient organisation LISTEN, as per INVOLVE recommendations, and 2 other senior independent transplant physician/surgeons (at least one of them from overseas). In addition, a Data Monitoring and Ethics Committee (DMEC) will be established comprising a senior UK-based transplant physician/surgeon as chair, a liver transplant physician/surgeon from overseas and a biostatistician. All the members will be independent of the trial. The DMEC will meet every 3 months throughout the duration of the trial. In addition, the DMEC will meet in the occurrance of any of the following (or at any time required by the CI, the sponsor or the 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% (this composite end point will be every 3 months by the Study Statistician, who will estimate its incidence and exact one-tailed 90% CI and compare it to pre-defined thresholds). The TSC will meet on a six monthly basis throughout the period of the trial, and at other times deemed necessary by the CI, the sponsor or by the EME board. A Trial Management group will be established, to include the CI, statisticians and the project manager. This will meet monthly to manage the set up and ongoing conduct of the trial. In addition, there will be regular (6 monthly) meetings of the PIs, alternating

face-to face with videoconferences. The trial has been 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.

18.3 Insurance and Indemnity

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

18.4 Publication Policy

All information, data and results obtained from study are confidential. Agreement from the Sponsor will be required prior to the public disclosure of any study-related data. It is expected that results from the study will be published in peer-reviewed scientific/medical journals and presented at scientific/clinical symposia and congresses. All publications and presentations relating to the study must be authorised by the CI.

19. QUALITY ASSURANCE

19.1 Trial Monitoring

Monitoring of this trial will be to ensure compliance with Good Clinical Practice and scientific integrity will be managed and oversight retained, by the KHP-CTO Quality Team.

19.2 Data Handling

The Chief Investigator will act as custodian for the trial data. The following guidelines will be strictly adhered to:

• Patient data will be anonymised

• All anonymised data will be stored on a password protected computer.

• All trial data will be stored in line with the Medicines for Human Use (Clinical Trials) Amended Regulations 2006 and the Data Protection Act.

• All trial data will be archived in line with the Medicines for Human Use (Clinical Trials) Amended Regulations 2006 as defined in the Kings Health Partners Clinical Trials Office Archiving SOP. An electronic Case Report Form (eCRF) will be created using the InferMed Macro system. Source data will be entered by authorised staff onto the eCRF with a full audit trail. This system is regulatory compliant (GCP, 21CRF11, EC Clinical Trial Directive). The eCRF will be created in collaboration with the trial statisticians and the investigators and maintained by the King's Clinical Trials Unit. It will be hosted on a dedicated secure server within KCL (Trial Database website address: www.ctu.co.uk and click the link to MACRO EDC V4).

20. DATA MANAGEMENT

20.1 Data Collection

Trial data will be collected in a study-specific electronic database. The database will use a web-based platform for electronic data capture, enabling the investigators to enter data remotely into electronic Case Report Forms (eCRFs) approved by the Sponsor. The main person responsible for data entry will be trained in the use of the system by the CI and Sponsor. Data reported in the eCRFs should be consistent with the corresponding source data, or the discrepancies should be explained. All applicable fields in an eCRF page should be completed and if data are not available, this should be clearly indicated on the form. The eCRF platform automatically creates a protected audit trail for all data entries and changes. Amendments to eCRF data will be recorded in the audit trail with a time and date stamp, along with a user-specified reason for the implemented change. The PI is responsible for submitting a complete set of eCRFs for each enrolled patient. Any supportive paper documentation (including details of any SAE) transmitted from the investigators to the Sponsor should be clearly marked with the trial name, patient trial identifier and patient date of birth. Any personal information, including the name of the patient, should be removed or rendered illegible to preserve individual confidentiality. Prior to patient recruitment, the CI should provide the Sponsor with a complete list of the normal ranges for each clinical laboratory test specified by the protocol, accompanied by an accreditation certificate. Updated copies of the laboratory test ranges will be requested by the Sponsor during the trial, according to regulatory requirements.

20.2 Specification of Source Data

Source data are defined as all the information in original records (and certified copies of original records) of clinical findings, observations, or other activities that are necessary for the complete reconstitution and evaluation of the trial. Source data must be available at the trial centre, to authenticate the existence of the study participants and substantiate the integrity of the data in the trial database. An eCRF is a data entry screen and does not constitute source data, unless otherwise specified. The data entered into an eCRF will be verifiable with original source records.
Source documentation for the study includes, but is not limited to:

- Completed patient resource use logs
- Completed biopsy assessment forms (trial-specific form to be given to the local pathology department)
- Informed consent forms
- Medical records/clinical reports/laboratory reports/hospital correspondence

The CI is responsible for producing a clean data set for the final statistical analysis. Inconsistencies in the trial data will be investigated using data queries that prompt the trial centre to clarify or confirm discrepant items. The eCRF system will incorporate automated and manual query generation tools. The CI will systematically check incoming trial data for consistency, omissions and compliance with

the protocol. The CI will also oversee the adherence of the trial to the official schedule of follow-up visits. The database will be locked at the end of the study when all protocol-specified data have been collected and cleaned.

20.3 Database Access Privileges

Database access will be strictly restricted through passwords to the authorised research team. The trial manager will request usernames and passwords from the KCTU administrator. It is a legal requirement that passwords to the eCRF are not shared, and that only those authorised to access the system are allowed to do so. If new staff members join the study, a personalized username and password should be requested via the Trial Manager.

20.4 Archiving

At the end of the trial, the KCL Clinical Trials Unit will provide the CI with a copy of the dataset in CSV format on a CD-ROM, which will be archived in the TMF. The TMF will be archived as per current KHP CTO SOPs. To enable peer review and/or audits from health authorities, all essential source and study documentation will be securely archived after study completion, in accordance with current regulatory requirements. Essential documents should be archived in a way that ensures that they are readily available, upon request, to the concerned authorities.

21. SIGNATURES

Chief Investigator: Prof. Alberto Sanchez-Fueyo

Signature 

Date: 12 June 2018

Statisician: Dr Abdul Douiri

Signature 

Date: 12 June 2018

22. PRINCIPAL INVESTIGATOR SIGNATURE PAGE

I agree to comply with study protocol version 9 dated 12 June 2018 the principles of GCP all regulatory requirements including the Medicines for Human Use (Clinical Trial) Regulations and Declaration of Helsinki (1996 Version), the Research Governance Framework for Health & Social Care and appropriate reporting requirements.

Principal Investigator:

Signature:

Date

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24. APPENDICES

24.1 Appendix 1. HrQoL

Quality of Life Form – NIDDK
GENERAL INFORMATION

What is your name?

1. What is today's date?
2. What is your date of birth?
3. What is your sex? (Male; Female)
4. What is your current marital status?
(check one of Never married; Married/cohabitating; Separated; Divorced; Widowed)
5. With whom do you live? (Alone; Spouse or partner; Spouse/partner and children; Children only; Parents only; Other family members or friends; Other)
6. Besides yourself, how many other people live in your household?
7. How many years of education have you completed?
8. What is the highest education degree you have obtained?
(Never graduated from high school; High School diploma; Trade School degree beyond high school; College/University degree; Advanced degree [M.A., M.S., Ph.D., M.D., J.D., etc.])
9. Do you currently smoke cigarettes?
IF YES what is the average number of cigarettes that you smoke each day? (Less than 1/2 pack; 1/2 to 1 pack; 1 to 2 packs; More than 2 packs)
10. Do you currently drink alcohol?
IF YES how many drinks of alcohol do you have in a typical week (one drink 5 1 bottle beer or 1 glass of wine or one mixed drink)?

B. WORK

Many patients with liver problems are not able to work or to take care of their household. These questions are meant to record your work experience.

11. Since finishing school (high school, college or trade school) how many years have you worked either full-time or part-time?
12. What is your current occupation?

Page **1** of **5**

Quality of Life Form – NIDDK
GENERAL INFORMATION

13. What is your current work activity or employment status?

(Employed full-time; Employed part-time; Employed, but temporarily laid off; Unemployed, looking for work; Unemployed, not looking for work; Unemployed, unable to work because of health; Homemaker; Student full-time; Student part-time; Retired; Other)

14. Does your health keep you from working for pay or from being a homemaker or from going to school?

15. Are you limited in the *kind* of work for pay, housework, or schoolwork you can do because of your health?

16. Are you limited in the *amount* of work for pay, housework or schoolwork you can do because of your health?

17. How satisfied are you with your present work situation or your present ability to function as a homemaker or a student?

(Completely satisfied; Very satisfied; Satisfied; Neutral; Dissatisfied; Very dissatisfied; Completely dissatisfied; Doesn't apply)

C. HEALTH

These questions deal with your general health and how it affects your everyday life and ability to work.

18. How would you rate your overall health at the present time? (Excellent; Good; Fair; Poor)

19. Compared to one year ago, how would you rate your health? (Better; About the same; Worse)

20. During the last month, how much bodily pain have you had? (None; Mild; Moderate; Severe)

21. During the past month, how many days have you been sick in bed for at least part of the day?

22. During the past year, how many days would you estimate that you have been in the hospital as an inpatient?

23. During the past year, how many days would you estimate that you have been out of work because of your health?

Quality of Life Form – NIDDK
GENERAL INFORMATION

24. Does your health currently limit the kind of vigorous activities that you can do, such as running, heavy lifting, sports?

25. Do you now have any trouble walking several blocks or climbing a few flights of stairs because of your health?

26. Do you now have any trouble walking a single block or climbing one flight of stairs because of your health?

27. Do you currently have trouble bending, lifting, or stooping because of your health?

28. Overall, how satisfied are you with your health at the present time? (Completely satisfied; Very satisfied; Satisfied; Neutral; Dissatisfied; Very dissatisfied; Completely dissatisfied)

29. Which of the eight following statements best describes your state of health, how you feel and your level of activity?

(Normal, no complaints, no evidence of disease; Able to carry out normal activity, minor symptoms; Able to carry out normal activity with effort, some symptoms; Able to care for myself but unable to carry on normal activity or do active work; Requiring occasional assistance but able to care for most of my own needs; Requiring considerable assistance and frequent medical care; Disabled, requiring special care and assistance; Worse off than any of these statements suggest)

30. Is your present state of health causing problems with your:
    30.1 Job or work (that is: paid employment);
    30.2 Looking after the home (examples: cleaning, cooking, doing odd jobs);
    30.3 Social life (examples: going out, seeing friends, going to a show);
    30.4 Home life (that is: relationships with other people in your home);
    30.5 Sex life;
    30.6 Interests and hobbies (examples: sports, arts and crafts, do-it-yourself);
    30.7 Vacations (examples: summer or winter vacations, weekends away)

D. SYMPTOMS

Below is a list of problems and complaints that people sometimes have. Please read each item carefully and indicate how much you were distressed by each symptom during past *month.* (Not at all, A little bit, Moderately, Quite a bit, Extremely)

31. Fatigue or lack of energy;
32. Muscle weakness;
33. Poor appetite;
34. Excess appetite or overeating;
35. Nausea or vomiting;

Quality of Life Form – NIDDK
GENERAL INFORMATION

36. Abdominal pains or discomfort;
37. Abdominal swelling or bloating;
38. Bowel problems (diarrhea/constipation);
39. Muscle aches or pains;
40. Joint aches or pains;
41. Back pains;
42. Head- aches;
43. Difficulty concentrating;
44. Sleeplessness or insomnia;
45. Nervousness, anxiety;
46. Mood swings;
47. Feeling depressed, sad or blue;
48. Trembling or shakiness;
49. Decreased interest in sex;
50. Impotence (men only);
51. Poor or blurred vision;
52. Change in facial appearance;
53. Bruising or fragile skin;
54. 54 Warts;
55. Itching of skin;
56. Fluid retention or swelling of ankles;
57. Jaundice (yellow tinge to eyes);
58. Darkening of the urine.

E. QUALITY OF LIFE

The following questions are aimed at evaluating your quality of life in general including your satisfaction with your life.

59. Taking all things together, how would you say things are these days? Would you say you're: (Very happy; Pretty happy; Not too happy)

60. All things considered, how satisfied are you with your life as a whole these days? (Completely satisfied Very satisfied Satisfied Neutral; Dissatisfied Very dissatisfied Completely dissatisfied)

61. All things considered how satisfied are you with your family life— the time you spend and the things you do with members of your family? (Completely satisfied Very satisfied Satisfied Neutral; Dissatisfied Very dissatisfied Completely dissatisfied Doesn't apply, I have no family)

62. How satisfied are you with your marriage? (Completely satisfied Very satisfied Satisfied Neutral; Dissatisfied Very dissatisfied Completely dissatisfied Doesn't apply, not married)

Quality of Life Form – NIDDK
GENERAL INFORMATION

63. Here are some words and phrases which we would like you to use to best describe how you feel about your present life. For example, if you think your present life is *very* "boring," record as 1. If you think it is *very* "interesting," record as a 7. If you think it is somewhere in between record a number between 2 and 6.

1 2 3 4 5 6 7

Boring	Interesting
Enjoyable	Miserable
Easy	Hard
Useless	Worthwhile
Friendly	Lonely
Full	Empty
Discouraging	Hopeful
Tied Down	Free
Disappointing	Rewarding
Brings out the best in me	Doesn't give me much of a chance

THIS FORM WAS FILLED OUT (check one)

- ☐ by the patient without assistance
- ☐ by the patient with assistance from the LTD study coordinator
- ☐ by the patient shortly after the transplant, without assistance
- ☐ by the patient shortly after the transplant, with assistance from the LTD study coordinator
- ☐ by the next of kin of the patient (Name_____, Relationship_____)

(This is a compressed version of Quality of Life form used for data collection by the NIDDK-LTD. Copies of the form are available by request to the author.)

24.2 Appendix 2. SF36 Version 2

Your Health and Well-Being

This survey asks for your views about your health. This information will help keep track of how you feel and how well you are able to do your usual activities. *Thank you for completing this survey!*

For each of the following questions, please mark an ☒ in the one box that best describes your answer.

1. In general, would you say your health is:

Excellent	Very good	Good	Fair	Poor
☐1	☐2	☐3	☐4	☐5

2. Compared to one year ago, how would you rate your health in general now?

Much better now than one year ago	Somewhat better now than one year ago	About the same as one year ago	Somewhat worse now than one year ago	Much worse now than one year ago
☐1	☐2	☐3	☐4	☐5

3. The following questions are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much?

	Yes, limited a lot	Yes, limited a little	No, not limited at all
a. Vigorous activities, such as running, lifting heavy objects, participating in strenuous sports	☐1	☐2	☐3
b. Moderate activities, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf	☐1	☐2	☐3
c. Lifting or carrying groceries	☐1	☐2	☐3
d. Climbing several flights of stairs	☐1	☐2	☐3
e. Climbing one flight of stairs	☐1	☐2	☐3
f. Bending, kneeling, or stooping	☐1	☐2	☐3
g. Walking more than a mile	☐1	☐2	☐3
h. Walking several hundred yards	☐1	☐2	☐3
i. Walking one hundred yards	☐1	☐2	☐3
j. Bathing or dressing yourself	☐1	☐2	☐3

SF-36v2™ Health Survey © 1996, 2000 by QualityMetric Incorporated and Medical Outcomes Trust. All Rights Reserved.
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(SF-36v2 Standard, US Version 2.0)

4. During the past 4 weeks, how much of the time have you had any of the following problems with your work or other regular daily activities as a result of your physical health?

	All of the time	Most of the time	Some of the time	A little of the time	None of the time
a. Cut down on the amount of time you spent on work or other activities	☐1	☐2	☐3	☐4	☐5
b. Accomplished less than you would like	☐1	☐2	☐3	☐4	☐5
c. Were limited in the kind of work or other activities	☐1	☐2	☐3	☐4	☐5
d. Had difficulty performing the work or other activities (for example, it took extra effort)	☐1	☐2	☐3	☐4	☐5

5. During the past 4 weeks, how much of the time have you had any of the following problems with your work or other regular daily activities as a result of any emotional problems (such as feeling depressed or anxious)?

	All of the time	Most of the time	Some of the time	A little of the time	None of the time
a. Cut down on the amount of time you spent on work or other activities	☐1	☐2	☐3	☐4	☐5
b. Accomplished less than you would like	☐1	☐2	☐3	☐4	☐5
c. Did work or other activities less carefully than usual	☐1	☐2	☐3	☐4	☐5

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6. During the past 4 weeks, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbors, or groups?

Not at all	Slightly	Moderately	Quite a bit	Extremely
☐1	☐2	☐3	☐4	☐5

7. How much bodily pain have you had during the past 4 weeks?

None	Very mild	Mild	Moderate	Severe	Very Severe
☐1	☐2	☐3	☐4	☐5	☐6

8. During the past 4 weeks, how much did pain interfere with your normal work (including both work outside the home and housework)?

Not at all	A little bit	Moderately	Quite a bit	Extremely
☐1	☐2	☐3	☐4	☐5

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9. These questions are about how you feel and how things have been with you during the past 4 weeks. For each question, please give the one answer that comes closest to the way you have been feeling. How much of the time during the past 4 weeks...

	All of the time	Most of the time	Some of the time	A little of the time	None of the time
a. Did you feel full of life?	☐1	☐2	☐3	☐4	☐5
b. Have you been very nervous?	☐1	☐2	☐3	☐4	☐5
c. Have you felt so down in the dumps that nothing could cheer you up?	☐1	☐2	☐3	☐4	☐5
d. Have you felt calm and peaceful?	☐1	☐2	☐3	☐4	☐5
e. Did you have a lot of energy?	☐1	☐2	☐3	☐4	☐5
f. Have you felt downhearted and depressed?	☐1	☐2	☐3	☐4	☐5
g. Did you feel worn out?	☐1	☐2	☐3	☐4	☐5
h. Have you been happy?	☐1	☐2	☐3	☐4	☐5
i. Did you feel tired?	☐1	☐2	☐3	☐4	☐5

10. During the past 4 weeks, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting friends, relatives, etc.)?

All of the time	Most of the time	Some of the time	A little of the time	None of the time
☐1	☐2	☐3	☐4	☐5

11. How TRUE or FALSE is each of the following statements for you?

	Definitely true	Mostly true	Don't know	Mostly false	Definitely false
a. I seem to get sick a little easier than other people	☐1	☐2	☐3	☐4	☐5
b. I am as healthy as anybody I know	☐1	☐2	☐3	☐4	☐5
c. I expect my health to get worse	☐1	☐2	☐3	☐4	☐5
d. My health is excellent	☐1	☐2	☐3	☐4	☐5

THANK YOU FOR COMPLETING THESE QUESTIONS!

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24.3 Appendix 3: EQ-5D 5L Health Questionnaire

Under each heading, please tick the ONE box that best describes your health TODAY

1.	**Mobility**	**1**	**I have no problems in walking about**
		2	**I have slight problems in walking about**
		3	**I have moderate problems in walking about**
		4	**I have severe problems in walking about**
		5	**I am unable to walk about**
		999	*Unknown*

2.	**Self-Care**	**1**	**I have no problems washing or dressing myself**
		2	**I have slight problems washing or dressing myself**
		3	**I have moderate problems washing or dressing myself**
		4	**I have severe problems washing or dressing myself**
		5	**I am unable to wash or dress myself**
		999	*Unknown*

3.	**Usual Activities** (*e.g. work, study, housework, family or leisure activities)*	**1**	**I have no problems doing my usual activities**
		2	**I have slight problems doing my usual activities**
		3	**I have moderate problems doing my usual activities**
		4	**I have severe problems doing my usual activities**
		5	**I am unable to do my usual activities**
		999	*Unknown*

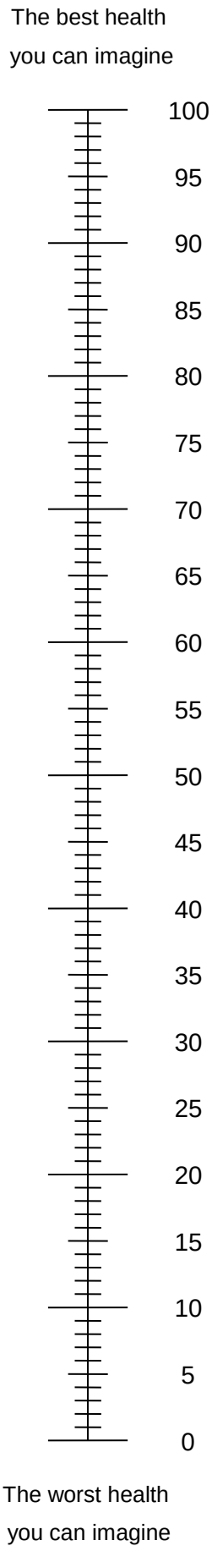
4.	**Pain/ Discomfort**	**1**	**I have no pain or discomfort**
		2	**I have slight pain or discomfort**
		3	**I have moderate pain or discomfort**
		4	**I have severe pain or discomfort**
		5	**I have extreme pain or discomfort**
		999	*Unknown*

5.	**Anxiety/ Depression**	**1**	**I am not anxious or depressed**
		2	**I am slightly anxious or depressed**
		3	**I am moderately anxious or depressed**
		4	**I am severely anxious or depressed**
		5	**I am extremely anxious or depressed**
		999	*Unknown*

We would like to know how good or bad your health is TODAY.

- This scale is numbered from 0 to 100.
- 100 means the best health you can imagine. 0 means the worst health you can imagine.
- Mark an X on the scale to indicate how your health is TODAY.
- Now, please write the number you marked on the scale in the box below.

6.	**Your Health Today:** **Min = 0** **Max = 100**	☐☐☐ *999* *Unknown*



24.4 Appendix 4: Processing of formalin-fixed and paraffin embedded liver biopsy samples

The following protocol has been designed with the purpose of facilitating the diagnostic assessment at the local centre and the following central pathology review at King's. It should give a degree of flexibility in the choice of staining according to local practice. The panel of stains listed below should be sufficient to cover most of the diagnostic requirements for an initial local review. This protocol also gives the opportunity to carry out additional staining if necessary, minimising tissue waste. The sections stained locally will be returned to the sender in due course.

Fixation and embedding:

Formalin fixation and paraffin embedding should be carried out according to the standard local procedure. There is no particular preference for the type of formalin to be used (10 % formal-saline or 10% neutral buffered formalin).

Sectioning of liver biopsy samples for diagnosis and trial:

Trim away excess paraffin wax from around tissue to give a *mesa* shape to the block – this helps with ribboning for cutting serial sections

Cut 20 serial sections at 4 microns, and pick up 2 sections per slide

Stain as follows:

Slide 1: H&E
Slide 2: Perls
Slide 3: Unstained
Slide 4: Reticulin stain – untoned
Slide 5: Masson trichrome
Slide 6: Orcein
Slide 7: Unstained
Slide 8: Diastase Periodic acid – Schiff
Slide 9: Unstained
Slide 10: H&E

Unstained sections may be used for additional staining or IHC if required. Send all stained slides, (except for 1 H&E slide), together with unstained slides and paraffin block to:

Dr Alberto Quaglia/Dr Rosa Miguel
Institute of Liver Studies
King's College Hospital
London, SE5 9RS

24.5 Appendix 5: Central Pathology Review Form: Central Pathology Review Form

1. Number of fragments / length (mm)	
2. Number of complete portal tracts	
3. Number of central veins	
4. Lobular inflammation	
	0 No 1 Sinusoidal cells and/or mild focal necrosis 2 Moderate, multiple necro-inflammatory foci 3 Marked, confluent or bridging necrosis
5. Central perivenulitis (with or without endothelitis)	
	0 No 1 Patchy, focal perivenular inflammation 2 Perivenulitis is most of the central veins 3 Marked (confluent or bridging hepatocellular necrosis)
6. Portal inflammation	
	0 No 1 Mild (some or all portal tracts; small groups of inflammatory cells) 2 Moderate (some or all portal tracts; expansive) 3 Moderate/marked (all portal tracts) 4 Marked (all portal tracts)
7. Interface hepatitis	
	0 No 1 Mild (focal, few portal tracts) 2 Mild/moderate (focal, most portal tracts) 3 Moderate (continuous around <50% portal tracts) 4 Severe (continuous around >50% portal tracts)
8. Bile duct lesions	
	0 No 1 Minimal (intraepithelial inflammatory cells or abnormal cholangiocytes) 2 Moderate (epithelial lesions in most portal tracts, no destruction) 3 Marked (destructive lesions of the bile ducts)
9. Bile duct loss	
	0 No 1 < 50% 2 ≥ 50%
10. Portal vein branches	
	0 Present in all portal tracts 1 Absent in a minority of portal tracts 2 Absent in most of the portal tracts
11. Portal vein endothelitis	
	0 No 1 Mild, in the minority of portal veins 2 Mild, in most of the portal veins 3 Marked
12. Fibrosis (Ishak)	
	0 No fibrosis 1 Fibrous expansion of some portal areas, with or without thin fibrous septa 2 Fibrous expansion of most portal areas, with or without thin fibrous septa 3 Fibrous expansion of most portal areas with occasional portal to portal (P-P) fibrous septa 4 Fibrous expansion of portal areas with marked bridging fibrosis (portal to portal (P-P) as well as portal to central (P-C)) 5 Marked bridging fibrosis (P-P and/or P-C) with occasional nodules (incomplete cirrhosis) 6 Cirrhosis, probable or definite
12. Fibrosis (Venturi et al.) (26)	
	0 – 3: Portal/periportal. 0 – 3: Peri-sinusoidal. 0 – 3: Perivenular.
13.	Ductular Reaction
14. Cholestasis	

	Hepatocanalicular: Yes/No Cholangiolar: Yes/No
15. Regenerative hyperplasia	
	0 Absent 1 Focal nodular regenerative hyperplasia (occasional foci of hyperplastic regeneration of the liver plates) 2 Diffuse nodular regenerative hyperplasia (classical NRH).

24.6 Appendix 6: Acceptable methods of contraception

Female subjects must agree to one of the following during the duration of the study:

• Complete abstinence from intercourse of reproductive potential. Abstinence is an acceptable method of contraception only when it is in line with the subject's preferred and usual lifestyle.

• Consistent and correct use of 1 of the following methods of birth control: a) intrauterine device (IUD) with a failure rate of <1% per year; b) tubal sterilization; c) vasectomy in the male partner; d) hormonal methods (oral contraceptives, injectable progesterone, implants of levonorgestrel, transdermal contraceptive patch, contraceptive vaginal ring). In case of essure micro-insert system, this will need to be used in association with another method of contraception;

Male subjects with female partners of childbearing potential must use condoms during the trial.

25. PROTOCOL VERSIONS AND AMENDMENTS

25.1.1 Non substantial amendments

Version / Date/ Reason for Amendment	Date Submitted	Approved
1.0 dated 24 September 2015 PIS number changed to 3.1 to incorporate the changes to the study duration and the blood volume collected	24 September 2015	28 September 2015
2.0 dated 17 March 2016 Maternity leave cover by Dr Rebecca Jones	17 March 2016	17 March 2016
3.0 dated 24 November 2016 to change the collection of stool sample from M0 to screening.	24 November 2016	8 December 2016

25.1.2 Substantial amendments

Version / Date/ Reason for Amendment	Date Submitted	Approved
Sub. Amendment 1 : 2.0 dated 5 January 2015 – to include the IMP azathioprine and administrational changes	13 March 2015	26 March 2015
Sub. Amendment 2: 4.0 dated 17 July 2015- to include additional sample collection at screening visit, to update statistical analysis to incorporate adjustment for confouders in the primary, as well as the secondary outcome and correction of typos	21 July 2015	27 July 2015
Sub. Amendment 3: 5.0 dated 05/05/2016- to allow transjugular biopsies, to include the collection of urine and addition of Fibroscan, clarification of the inclusion criteria, addition of new staff and correction of typos	12 May 2016	22 June 2016
Sub. Amendment 4: 6.0 dated 24 January 2017- PI change for Dr James O'Beirne to Dr Aileen Marshall. Addition of a new Belgian site with the PI Dr Eliano BONACCORSI RIANI and Ireland site with the PI Dr Diarmuid Houlihan. Statistical analysis amended. Fibroscan secondary outcomes added	26 January 2017	02 March 2017
Sub. Amendment 5: 7.0 dated 29 June 2017- PI change from Dr Graeme Alexander to Dr Joanna Leithead.	11 July 2017	17 July 2017
Sub Amendment 6 (IRE only): 8.0 dated 13 October 2017- updated with the responses to HPRA	30 August 2017	3 November 2017
Sub. Amendment 7: 9.0 dated 12 June 2018 – PI change for Berlin from Dr Andreas Pascher to Dr Dennis Eurich. Addition of new Italian site with the PI Dr Riccardo Volpes. Addition of Sirolimus and		

Everolimus in the inclusion criteria. Change of the GFR in the exclusion criteria. Clarification of primary endpoint and operational tolerance. Change of the trial statistician to Dr Abdel Douiri.		