EBioMedicine 9 (2016) 346–355

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

Progressive Fibrosis Is Driven by Genetic Predisposition, Allo-immunity, and Inflammation in Pediatric Liver Transplant Recipients

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ARTICLE INFO

Article history:
Received 11 April 2016
Received in revised form 22 May 2016
Accepted 30 May 2016
Available online 1 June 2016

Keywords:
Pediatric liver transplantation
Donor-specific antibodies
HLA antibodies
Humoral immunity
Fibrosis
Portal inflammation
HLA-DRB1

ABSTRACT

Aim: To determine predisposing factors of idiopathic allograft fibrosis among pediatric liver transplant recipients.
Background: Protocol biopsies (PB) from stable liver transplant (LT) recipient children frequently exhibit idiopathic fibrosis. The relation between allograft inflammation, humoral immune response and fibrosis is uncertain. Also the role of HLA-DRB1 genotype has not been evaluated, though it's associated with fibrosis in autoimmune hepatitis.
Patients and Methods: This observational study, included 89 stable LT recipient transplanted between 2004–2012 with mean follow-up of 4.3 years, 281 serial PBs (3.1 biopsy/child) and human leukocyte antigen (HLA) antibody data. PBs were taken 1–2, 2–3, 3–5, 5–7, and 7–10 years post-LT, and evaluated for inflammation and fibrosis using liver allograft fibrosis score (LAFSc). The evolution of fibrosis, inflammation and related predisposing factors were analysed.
Findings: HLA-DRB1*03/04 allele and Class II DSA were significantly associated with portal fibrosis ($p = 0.03$; $p = 0.03$, respectively). Portal inflammation was predisposed by Class II DSA ($p = 0.02$) and non-HLA antibody presence ($p = 0.01$). Non-portal fibrosis wasn't predisposed by inflammation. Lobular inflammation was associated with non-HLA antibodies.
Interpretation: We conclusively demonstrated that allograft inflammation results in fibrosis and is associated with post-LT Class II DSA and non-HLA antibodies. The HLA-DRB1*03/04 allele caused genetic predisposition for fibrosis.
Funding: None.

1. Introduction

Given the long-term survival in pediatric liver transplantation (LT), maintaining stable liver function and preserving allograft histology are paramount. Protocol biopsies (PBs) from LT recipient children revealed frequent allograft inflammation and fibrosis (Evans et al., 2006; Scheenstra et al., 2009; Hubscher, 2011; Venturi et al., 2012, 2014; Gurevich et al., 2015), in stable LT recipients without predisposing factors (Evans et al., 2006; Venturi et al., 2014; Gurevich et al., 2015; Miyagawa-Hayashino et al., 2012). The etiopathogenesis of these "idiopathic" changes is unknown, while graft age, subclinical rejection and medication non-compliance have been considered as possible aetiologies (Hubscher, 2011; Venturi et al., 2014; Miyagawa-Hayashino et al., 2012). Among these, the donor specific class II HLA antibodies have been shown to play an important role in the allograft evolution. This proposition of antibody mediated subclinical inflammation gains

Abbreviations: LT, Liver transplantation; AIH, Auto-immune hepatitis; PB, Protocol biopsy; MFI, Mean fluorescence index; DSA, Donor-specific HLA antibody; ANA, Anti-nuclear antigen antibody; SMA, Smooth muscle actin antibody; LKM, Liver kidney microsomal antibody; ALT, Alanine aminotransferase; GGT, gamma-glutamyl transferase; MMF, Mycophenolate mofetil; DNAIH, De-novo autoimmune hepatitis; LAFSc, Liver allograft fibrosis score; HSC, Hepatic stellate cells.

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http://dx.doi.org/10.1016/j.ebiom.2016.05.040

more emphasis as the C4d deposition in the hepatic tissue has been shown to correlate with the antibody presence (Miyagawa-Hayashino et al., 2012; Kozlowski et al., 2011; Salah et al., 2014). While continuity between uncontrolled inflammation and hepatic fibrosis was documented in hepatitis C and auto-immune hepatitis (AIH), this was not the case for pediatric LT recipients. To establish a temporal association between inflammation and fibrosis, sequential PBs need to be evaluated to verify if inflammation precedes fibrosis histologically. This has not been done in the context of pediatric LT where all the studies have evaluated biopsies cross-sectionally wherein co-existing inflammation and fibrosis could be evaluated but not sequential development (Evans et al., 2006; Scheenstra et al., 2009). Hence the conflictory evidence of inflammation not resulting in fibrosis in pediatric LT versus other inflammatory hepatic diseases. In AIH, the HLA-DRB1*03/04 allele is considered an independent predictor of portal fibrosis (Montano-Loza et al., 2006; de Boer et al., 2014; Liberal et al., 2015). As AIH is the prototype of immune dysregulation-mediated hepatic disease, examining its role in LT recipients is the next logical step: we sought to evaluate "idiopathic" allograft changes by analysing serial PBs in a complication-free LT recipient cohort.

1.1. Hypothesis

We hypothesized that fibrosis at time-point t is influenced by pre-existing inflammation and persistent pre-existing fibrosis at time-point $t - 1$. Other factors that could have an impact on these allograft histological changes are time since LT, presence of HLA and non-HLA antibodies, immunosuppression regime, rejection episodes, HLA-DRB1 status, underlying primary liver disease, donor type (living/deceased), and host and donor demographic characteristics (Fig. 1).

2. Methods

2.1. Patients

This observational study sought to evaluate successive PBs of included children who satisfied the criteria of having undergone LT from 2004 to 2012 (i.e., minimum 3-year post-LT follow-up) and having AST, ALT, GGT levels < 1.5 times upper limit normal at the time of each protocol biopsy. The exclusion criteria were inadequate PBs. i.e. < two successive PBs, inadequate information regarding HLA antibody status (pre-LT and simultaneous to last PB), death of LT recipient, re-transplantation, biliary or vascular complications, chronic rejection, previous hepatocyte treatment or hepatotoxic drug exposure, and combined liver-kidney transplantation. The screening and inclusion, exclusion steps are as per Fig. 2.

2.2. Data Collection Methodology

Data was collected as outlined in Fig. 3.

2.3. PB Details

The protocol stipulated performing PBs 1, 2, 3, 5, 7, and 10 years post-LT. Since LT recipients were all over Europe, not all biopsies followed the same schedule; we considered PBs taken 1–2 years post-LT as PB 1 and those taken at 2–3, 3–5, 5–7, and 7–10 years as PB 2, PB 3, PB 4, and PB 5, respectively. Only PBs were evaluated, and no other biopsies considered.

2.4. Antibody Evaluation

HLA antibody detection was performed using single beads on Luminex platform, with a mean fluorescence index (MFI) cut-off for positivity at 1500 (O'Leary et al., 2014; Reed et al., 2013; Musat et al., 2011). Donor-specific antibody (DSA) detection was conducted for detectable HLA antibodies.

Non-HLA antibodies (ANA, SMA, and LKM) were evaluated by standard immune-fluorescence, and considered positive if detected on the last two PBs with titres > 1:40.

HLA antibodies were tested within 1 month pre-LT and at the last PB; non-HLA antibodies were tested pre-LT and at the last two PBs.

2.5. Immune Suppression

A steroid-free protocol was used, combining tacrolimus and basiliximab (Simulect; Novartis Pharma, 1800, Vilvoorde, Belgium). Two basiliximab doses were administered intravenously at Days 0 and 4 post-LT (10 or 20 mg/dose, for recipient body weight above or below 35 kg, respectively), followed by tacrolimus alone from Day 1

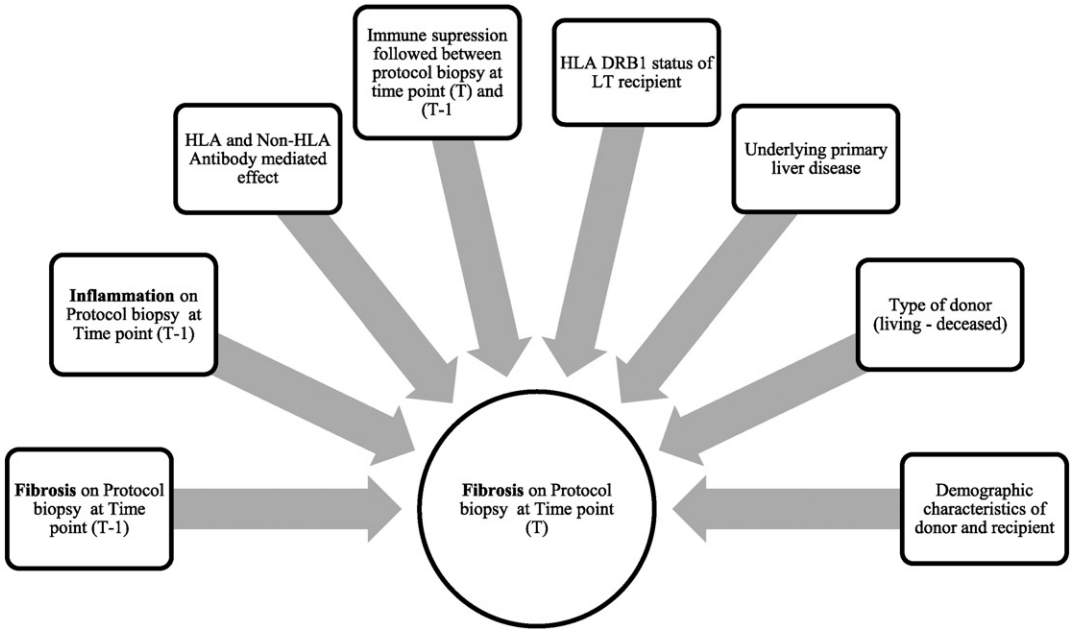

Fig. 1. Hypothesis to test, for development of allograft fibrosis (Our hypothesis for allograft inflammation had same variables other than Fibrosis on Protocol biopsy at time point ($t - 1$)).

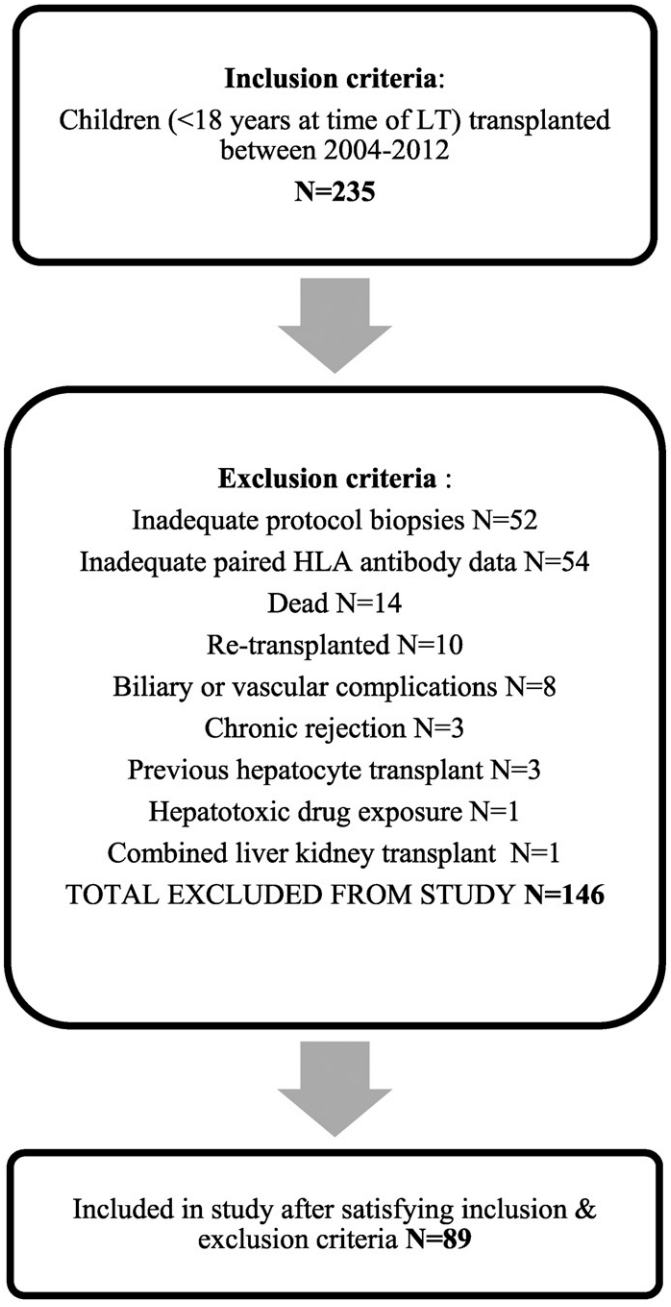

Fig. 2. Screening, inclusion and exclusion steps followed in the study.

post-LT, initially administered at 0.1 mg/kg twice a day, then titrated to target blood trough levels of 8–12 ng/ml during the first month post-LT, 5–8 ng/ml for the next 11 months, and 3–5 ng/ml thereafter. Acute cellular rejection was suspected in patients exhibiting increased liver enzymes (normal range: alanine aminotransferase [ALT]: 5–40 IU/l and c-glutamyl transferase [GGT]: 5–40 IU/l), confirmed by histological signs according to Banff classification. Steroids or mycophenolate mofetil (MMF) were transiently employed during these episodes. Besides rejection, PB observations only induced treatment modifications in cases of de-novo autoimmune hepatitis (DNAIH) or DNAIH-like manifestations. DNAIH was diagnosed from positive autoantibodies, increased serum gamma-globulin concentrations, and histological features of interface and lobular hepatitis or plasma-cell infiltration; steroids, MMF, or azathioprine were then administered. Any immunosuppressive modifications were documented at each visit, with each regime followed between two paired PBs categorized as tacrolimus alone or in combination (MMF, azathioprine, or sirolimus). The combined therapy's impact on allograft histology was compared to that of monotherapy.

2.6. Histological Evaluation

Each PB was evaluated for inflammation and fibrosis by three independent observers, with a consensus made for categorization differences. Fibrosis was assessed using the liver allograft fibrosis score (LAFSc) on Masson's trichrome-stained slides, with scores assigned to portal, sinusoidal, and central areas, as reported (Venturi et al., 2012). This is well accepted and a validated fibrosis scoring system in the context of pediatric allograft fibrosis. Histological inflammation was assessed on hematoxylin-eosin-stained slides. Lobular inflammation and portal-tract infiltration severity was graded as none, mild, moderate, or severe, as detailed in Table 1.

2.7. Data Evaluation and Statistics

Since portal area (LAFSc-P), sinusoidal (LAFSc-S), and central (LAFSc-C) fibrosis are ordinal variables, their respective evolutions were analysed using cumulative logistic mixed-effect models. The fibrosis severity in a given PB at time-point t was modelled using fixed effect variables, including predictors of primary interest (inflammation and fibrosis at previous PB [time-point $t-1$]; Class I and II HLA antibodies; ANA, SMA, and LKM status; additional immunosuppression exposure; HLA-DRB1), predictors of the model's face validity (time since LT and living/deceased donor status), and others additional predictors (donor and recipient ages, recipient gender, underlying primary liver disease, and total rejection number). A random patient effect was introduced into each model for inter-patient differences. Portal tract and lobular inflammation evolution was analysed using cumulative logistic mixed-effect models with the same predictors, except previous PB fibrosis.

Given the number of potential predictors and because our main focus was on clinical and biological variables, a predictor selection procedure was conducted as previously described (Vittinghoff et al., 2012). All primary interest predictors and those required for establishing the model's face validity were systematically included, regardless of their statistical significance. A backward elimination was then applied on additional predictors in order to produce more parsimonious models by removing non-significant ($p > 0.10$) variables.

Mixed-effect models were built using the clmm2 function from ordinal R package, and the maximum likelihood estimations of parameters were computed via adaptive Gauss-Hermite quadrature approximation, as previously recommended (Christensen, 2015). Statistical significance of multi-level variables was computed using a likelihood ratio test.

Predisposing factors for post-LT Class II HLA DSA were assessed using a logistic-regression model, built using the glm R function.

In a spirit of "Reproducible Research", all statistical analyses are reported in R Markdown file which are provided in Supplementary files 3 (LAFSc-P), 4 (LAFSc-C), 5 LAFSc-S), 6 (Portal Inflammation), 7 (Lobular Inflammation), and 8 (predisposing factor to develop Class II HLA-DSA).

3. Funding

No external funding or non-funding support was taken in this study.

4. Ethical clearance

Appropriate ethical clearance was obtained from our institutional board.

5. Results

We included 89 children as described in Fig. 2, with 281 PBs, comprising 189 pairs of successive PBs (average of 3.15 biopsies per

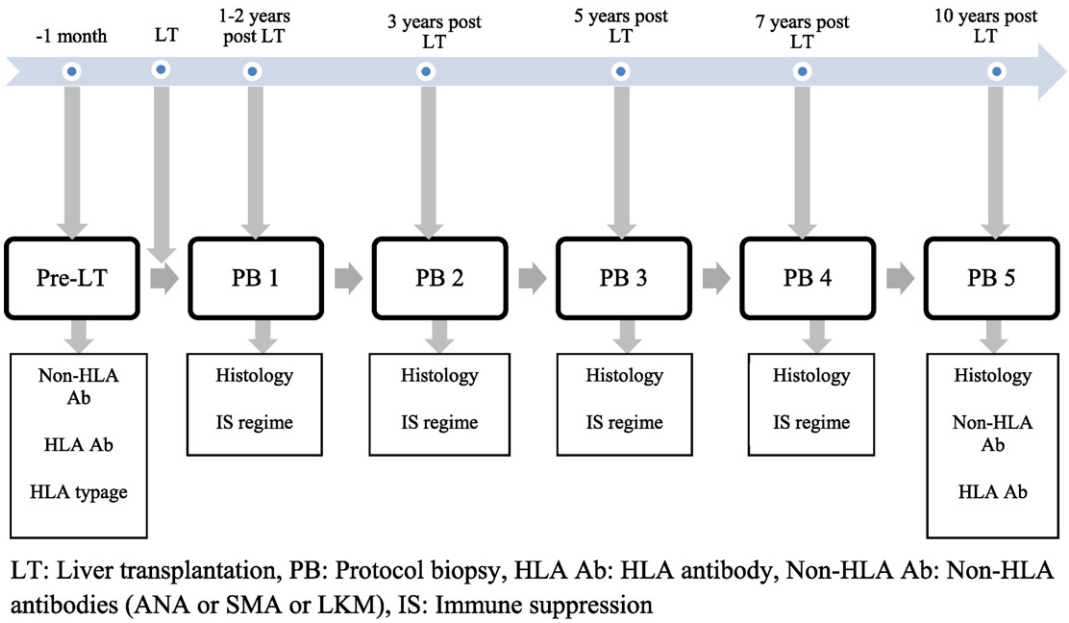

Fig. 3. Data collection time points and schematic, representative of a patient followed up for >10 years. LT: Liver transplantation, PB: Protocol biopsy, HLA Ab: HLA antibody, Non-HLA Ab: Non-HLA antibodies (ANA or SMA or LKM), IS: Immune suppression.

patient). Characterization features are summarized in Table 2 and details provided in Supplementary Tables 1 and 2.

5.1. Allograft Inflammation and Fibrosis Evolution Patterns in Portal, Sinusoidal, and Central Areas Were Patient-specific

Graphical exploration (Fig. 4) of longitudinal allograft inflammation and fibrosis demonstrates our data sets' complex nature. The mean LAFSc total score at baseline (that is PB1) was 3.32 with 54.6% of children having low fibrosis (LAFSc total ≤ 3), 42.0% having moderate fibrosis (LAFSc total between 4 and 5), and only 3.4% having high fibrosis (LAFSc total ≥ 6). Subsequent PB showed that no generalizable patterns can be identified, with progression and regression patterns tending to be patient-specific.

The complex graphical representation rendered it difficult to decipher which histological characteristics present smooth or discontinuous dynamic evolution. Although average fibrosis stabilised over time (black stars in Fig. 4), this may have been biased by the numerous missing data for the last biopsies. Further statistical analysis using cumulative logistic mixed-effect models were thus undertaken to (i) confirm this apparent average fibrosis stability, (ii) detect any correlation between successive PBs from the same patient, and (iii) identify significant predictors of fibrosis and inflammation severity.

5.2. Portal Fibrosis Correlated with Preceding Portal Inflammation, Presence of Class II DSA, and Recipient HLA-DRB1 Status

Portal fibrosis at any time-point t significantly correlated with portal inflammation severity at $t-1$. OR strength and significance were higher for severe inflammation (OR = 148) or moderate (OR = 10.3) than mild (OR = 3.64) (Table 3), implying that preceding portal inflammation and also its severity significantly impact portal fibrosis in a given biopsy. Class II DSA antibody status (OR = 5.84, p = 0.02) and HLA-DRB1*03/04 genotype (OR = 2.28, p = 0.03) significantly correlated with higher fibrosis.

5.3. Sinusoidal Fibrosis Did Not Correlated with Preceding Inflammation

Sinusoidal, unlike portal, fibrosis at a given time-point t did not correlate with inflammation at previous time-point $t-1$ (Table 4). The association with recipient HLA DRB1 status was also weaker (OR = 1.93, p = 0.08) in the sinusoidal area than in the portal area. Regarding sinusoidal fibrosis (Table 4), underlying primary liver disease and total number of rejections were removed from the model as both variables were not associated (p-value > 0.10) with the outcome. On the other hand, recipient age (p-value = 0.08) and gender (p-value = 0.07) were not eliminated, as a liberal criterion is usually recommended during the backward elimination process in order to rule out confounding factors more effectively.

5.4. Central Fibrosis Correlated with Pre-existing Fibrosis and Deceased-donor Transplant

Central fibrosis at any time-point t significantly (p< 0.01) correlated with central fibrosis severity at $t-1$ (Table 5). ORs correlated with LAFSc-C severity, confirming that fibrosis persists over time, with higher fibrosis in a previous PB indicating increased fibrosis persistence risk in the current PB. Among other predictors, the strongest association was found with deceased donors (OR = 2.89, p = 0.01).

Table 1
Histological inflammation scoring system.

	Mild	Moderate	Severe
Portal tract inflammation	Discreet infiltration of lympho-plasmocytes or lymphoid aggregates	Lympho-plasmocytic infiltrates but no interface hepatitis	Presence of interface hepatitis
Lobular inflammation	Discreet infiltration of lympho-plasmocytes or lymphoid aggregates	Lympho-plasmocytic infiltrates but no lobular necrosis	Presence of lobular necrosis

Table 2
Demographic and clinical characteristics of patients at baseline.

Characteristic.	Clinical series (n = 89).
Underlying primary liver disease (no./%).	.
Biliary atresia	52/58.4%
Alagille	8/9.0%
Familial cholestasis	5/5.6%
Ductal plate abnormality (CHF)	3/3.4%
Metabolic liver disease	9/10.1%
Malignant disease	8/9.0%
Criggler Najjar	1/1.1%
Indeterminate cirrhosis	3/3.4%
Number of acute rejection episodes (no./%)	
None	22/24.7%
1	30/33.7%
2	24/27.0%
3	11/12.4%
4	2/2.2%
Age at LT (yrs.) (mean/SD)	
Recipient	3.2/4.1
Donor	30.9/10.02
Recipient gender distribution	
Males (no./%)	37/41.6%
Donor type	
Living (no./%)	74/83.1%
Duration of follow up since LT (yrs)	
Mean/SD	4.3/2.5
Recipient HLA DRB1*03/04 status	
Positive/Negative	39/50
Pre-LT HLA antibody status (no./%)	
Class I HLA antibodies	
Neg	75/84.3%
Non DSA	9/10.1%
DSA	5/5.6%
Class II HLA antibodies	
Neg	83/93.3%
Non DSA	4/4.5%
DSA	2/2.2%
Post-LT HLA antibody status (no./%)	
Class I HLA antibodies.	
Neg	63/70.8%
Non DSA	18/20.2%
DSA.	8/9.0%
Class II HLA antibodies	
Neg	62/69.7%
Non DSA	12/13.5%
DSA	15/16.8%
Number of patients with successive PBs (no./%)	.
2 successive PBs	29/32.6%
3 successive PBs	29/32.6%
4 successive PBs	22/24.7%
5 successive PBs	9/10.1%

LT: liver transplant; PB: protocol biopsy; PB 1: protocol biopsy taken between 1–2 years post LT; PB 2: protocol biopsy taken between 2–3 years post LT; PB 3: protocol biopsy taken between 3–5 years post LT; PB 4: protocol biopsy taken between 5–7 years post LT; PB 5: protocol biopsy taken between 7–10 years post LT; SD: standard deviation; HLA: human leukocyte antigen; DSA: donor-specific (HLA) antibodies.

5.5. Portal Inflammation Correlated with Pre-existing Inflammation, Class II DSA, and Non-HLA Antibodies

Portal inflammation at any time-point t significantly correlated ($p < 0.01$) with portal inflammation severity at $t - 1$ (Table 6). OR strength and significance correlated with severity, suggesting a strong persistence of severe inflammation. Other significant predictors of portal inflammation included Class II DSA (OR = 4.77, p = 0.02) and non-HLA antibodies (OR = 2.31, p = 0.01).

5.6. Lobular Inflammation Correlated with Pre-existing Lobular Inflammation and Non-HLA Antibodies

A strong persistence of lobular inflammation was detected via highly significant ORs positively correlated with previous PB inflammation severity. Lobular inflammation severity was significantly impacted by non-HLA antibodies (OR = 2.28, p-value = 0.05).

5.7. Post-LT Class II DSA Were Determined By Donor and Recipient Age and Impacted Allograft Evolution

Post-LT Class II DSAs were observed in 15/89 (16.8%) children (Table 2). Of these, 13 developed them "de-novo", i.e., no pre-LT expression (Fig. 5). The strongest predictor of post-LT Class II DSAs was donor age (Table 8). For each 1-year increase in donor age, the odds of developing post-LT Class II DSAs decreased by over 10% (OR = 0.89, $p < 0.01$). On other hand, Post-LT DSAs was positively and significantly associated with recipient age (OR = 1.21, p = 0.03). Both results indicate that older donor age decreases the risk of developing class II DSA while older recipient age increases this risk.

While time since transplantation was not significant (p = 0.37), longer time (>5 years) correlated with higher ORs, post-LT Class II DSA tending to increase over time. Antibody presence significantly correlated with portal inflammation (OR = 4.77, p = 0.02) and fibrosis (OR = 5.84, p = 0.02) evolution (Tables 3 and 6).

*5.8. HLA-DRB1*03/04 Allele in Recipients Was Associated with Allograft Portal and Sinusoidal Fibrosis*

HLA-DRB1*03/04 allele in recipients was positively correlated (OR > 1) with allograft fibrosis in both portal and sinusoidal areas. The association was stronger in the portal (OR = 2.28, p = 0.03) than in the sinusoidal (OR = 1.93, p = 0.08) part, where the association was found not to be significant.

No significant difference (p = 0.45) was found between the prevalence of HLA-DRB1*03/04 allele between patients with biliary atresia (25/52, 48.1%) versus patients with others indications for LT (14/37, 37.8%).

5.9. Age, Gender, Underlying Primary Liver Disease, and Number of Rejections Were Not Associated with Fibrosis and Inflammation

Regarding portal and central fibrosis and portal and lobular inflammation (Tables 3, 5, 6, 7), non-significant (p-value > 0.10) associations were found for donor and recipient ages, recipient gender, underlying primary liver disease, and total number of rejections. Considering that these non-significant variables are neither of primary interest nor required to guarantee the face validity of the model, they were removed during backward elimination in order to produce more parsimonious models. Regarding sinusoidal fibrosis (Table 4), underlying primary liver disease and total number of rejections were removed from the model as both variables were not associated (p-value > 0.10) with the outcome. On the other hand, recipient age (p-value = 0.08) and gender (p-value = 0.07) were not eliminated, as a liberal criterion is usually recommended during the backward elimination process in order to rule out confounding factors more effectively.

6. Discussion

Allograft histological inflammation was shown not to be innocuous, eventually resulting in fibrosis, and associated with post-LT Class II DSA and persistent non-HLA antibodies. HLA-DRB1*03/04 allele in LT recipients was an independent predisposing factor for developing fibrosis, without influencing inflammation.

This is a unique study, involving an uncomplicated patient cohort, which focused on serial PBs to delineate idiopathic fibrosis and inflammation in allografts and explored their predictors, using paired PBs, studying the impact of pre-existing fibrosis and inflammation on current fibrosis and inflammation, assessing correlations with various HLA and non-HLA antibodies, and applying statistical models enabling the representation of each patient's evolution pattern. We also focused

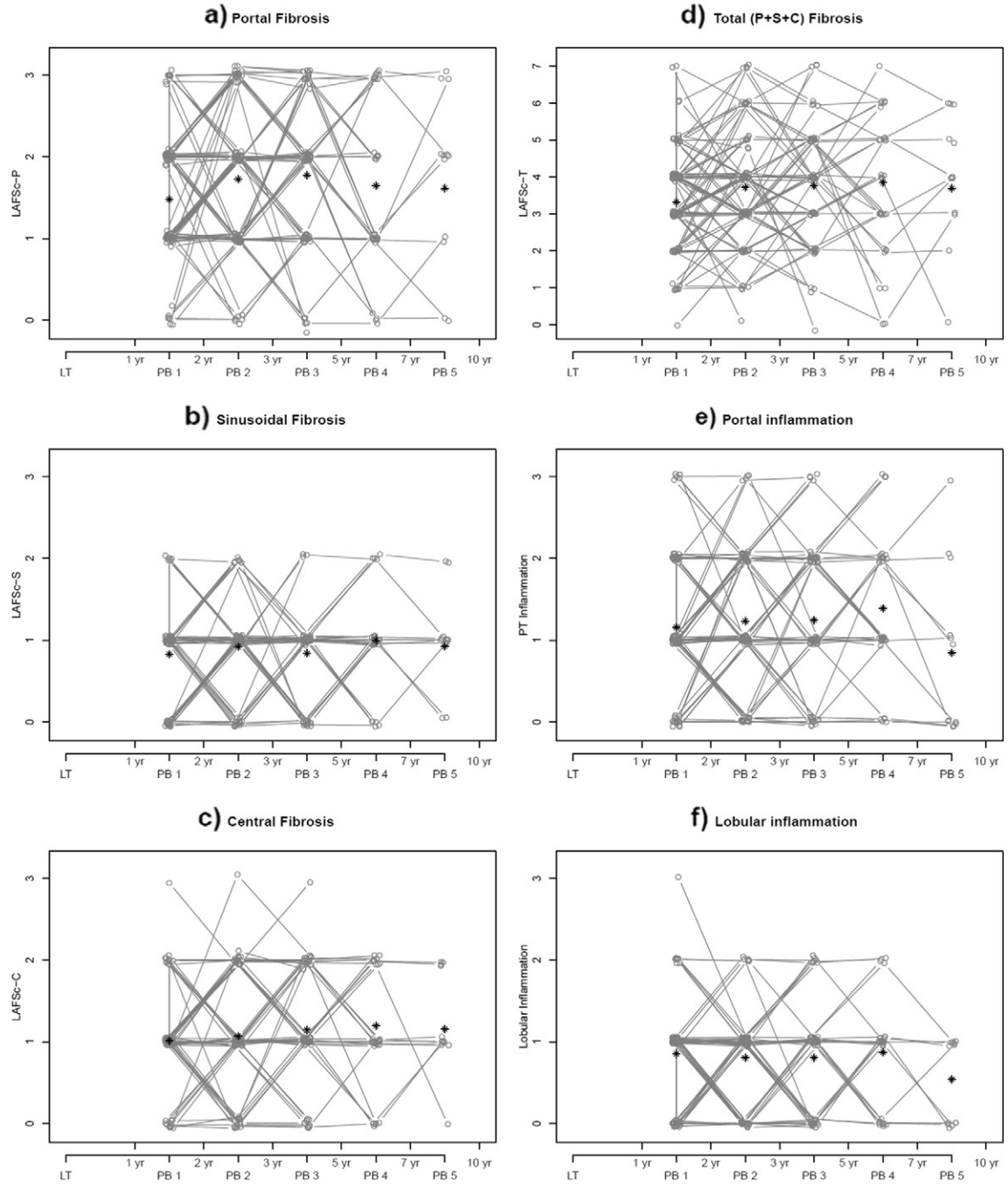

Fig. 4. Individual (gray circles) and averaged (black stars) evolutions of fibrosis across serial protocol biopsies are shown. Longitudinal evolution of each patient is plotted independently using the gray circles at different time-points and connected via solid lines. Longitudinal evolution of fibrosis (a,b,c,d) and inflammation (e,f) across the 5 protocol biopsies. Each graphic reports the longitudinal evolution of one specific histological feature (a: Portal Fibrosis, b: Sinusoidal Fibrosis, c: Central Fibrosis, d Total (i.e., Portal + Sinusoidal + Central) Fibrosis, e: Portal inflammation, f: lobular inflammation). Within each graphic, individual evolution of each patient is reported with connected gray circles while averaged evolution is reported with black stars. Based on these graphics, no generalizable patterns were identified for each histological feature.

on different hepatic parenchyma zones using LAFSc (Venturi et al., 2012). This zone-specific analysis enabled us to decipher cause-specific inflammation and fibrosis patterns, resulting in a scheme for PB interpretation (Fig. 6).

In previous studies evaluating PBs, fibrosis prevalence was >70% at 5 years post-LT, with none exhibiting normal histology after 10 years (Evans et al., 2006; Scheenstra et al., 2009; Venturi et al., 2014). In a clinical series with similar mean fibrosis evolution as in our cohort (LAFSc total = l2.91 vs 3.32 at baseline, and 3.31 vs 3.76 at 3 years post LT), Venturi et al. demonstrated that fibrosis in pediatric LT recipients is not a "one-way street" and regression frequently occurs (Venturi et al., 2014; Chevallier et al., 1994). Computing mean fibrosis would thus not provide information about a patient-specific evolution, as in our study (Fig. 4). We thus implemented cumulative logistic mixed-effect models for statistical analysis, enabling representation of each patient's course.

Portal tracts are immunologically-active zones with high hepatic stellate cell (HSC) levels (Maia et al., 2010). HSCs can be either fibrogenic when activated by persistent inflammation or fibrinolytic in quiescent state (Kisseleva et al., 2012; Troeger et al., 2012; Mallat and Lotersztajn, 2013). Portal inflammation, which would thus be pro-fibrogenic, was shown to persist across serial PBs (Table 6), correlating

Table 3
Multivariate analysis using cumulative logistic mixed effect model to determine correlation between current portal fibrosis and various variables listed in the table.

Variable	Odds-ratio (95% CI)[a]	p-value[a]
Previous portal fibrosis (LAFSc-P) ($t-1$)		0.39
0	1.0	–
1	2.20 (0.51–9.48)	0.29
2	3.45 (0.76–15.6)	0.11
3	4.17 (0.73–23.8)	0.11
Previous portal inflammation ($t-1$)		<0.01
none	1.0	–
mild	3.64 (1.44–9.18)	<0.01
moderate	10.3 (3.41–30.9)	<0.01
severe	148 (11.8–1870)	<0.01
Class I HLA		0.64
Neg	1.0	–
Not DSA	0.67 (0.25–1.76)	0.41
DSA	1.39 (0.23–8.39)	0.72
Class II HLA		<0.01
Neg	1.0	–
Not DSA	3.56 (0.92–13.8)	0.07
DSA	5.84 (1.36–25.0)	0.02
Other antib (ANA or SMA or LKM)		
Neg	1.0	–
Pos	0.60 (0.30–1.18)	0.14
Additional immunosuppression exposure		
No	1.0	–
Yes	0.86 (0.34–2.21)	0.76
Recipient HLA DRB1*03/04		
Neg	1.0	–
Pos	2.28 (1.06–4.89)	0.03
Time since transplantation		0.40
2–3 yr	1.0	–
3–5 yr	0.82 (0.41–1.64)	0.58
5–7 yr	0.54 (0.21–1.37)	0.19
> 7 yr	0.36 (0.08–1.58)	0.17
Donor status		
Living	1.0	–
Deceased	1.90 (0.76–4.80)	0.17

CI: confidence interval; LAFSc: liver allograft fibrosis scoring; HLA: human leukocyte antigen; DSA: donor-specific (HLA) antibodies.
[a] Odds-ratio, 95% confidence intervals (CI), and p-value were obtained from a cumulative logistic mixed effect model. This model included all predictors of primary interest and all predictors of the model's face validity. This model resulted from a backward elimination process which eliminated non-significant (p-value > 0.10) additional predictors including donor and recipient ages, recipient gender, underlying primary liver disease, and total number of rejections.

Table 4
Multivariate analysis using cumulative logistic mixed effect model to determine correlation between current sinusoidal fibrosis and various variables listed in the table.

Variable	Odds-ratio (95% CI)[a]	p-value[a]
Previous sinusoidal fibrosis (LAFSc-S) ($t-1$)		0.26
0	1.0	–
1	1.36 (0.56–3.27)	0.50
2	3.55 (0.77–16.5)	0.10
Previous lobular inflammation ($t-1$)		0.59
None	1.0	–
Mild	1.72 (0.78–3.82)	0.18
moderate	1.18 (0.27–5.04)	0.83
severe	1.69 (0.02–166.2)	0.82
Class I HLA		0.58
Neg	1.0	–
Not DSA	0.79 (0.26–2.37)	0.67
DSA	0.41 (0.07–2.40)	0.32
Class II HLA		0.78
Neg	1.0	–
Not DSA	1.45 (0.39–5.37)	0.58
DSA	0.79 (0.19–3.23)	0.74
Other antib (ANA or SMA or LKM)		
Neg	1.0	–
Pos	0.60 (0.28–1.30)	0.20
Additional immunosuppression exposure		
No	1.0	–
Yes	1.47 (0.60–3.59)	0.40
Recipient HLA DRB1*03/04		
Neg	1.0	–
Pos	1.93 (0.93–3.98)	0.08
Time since transplantation		0.26
2–3 yr	1.0	–
3–5 yr	0.58 (0.26–1.27)	0.17
5–7 yr	1.45 (0.53–3.93)	0.47
> 7 yr	0.47 (0.11–2.07)	0.32
Donor status		
Living	1.0	–
Deceased	1.94 (0.76–4.94)	0.17
Recipient age (yr)	0.92 (0.83–1.01)	0.08
Recipient gender		
Male	1.0	–
Female	2.02 (0.96–4.29)	0.07

CI: confidence interval; LAFSc: liver allograft fibrosis scoring; HLA: human leukocyte antigen; DSA: donor-specific (HLA) antibodies.
[a] Odds-ratio, 95% confidence intervals (CI), and p-value were obtained from a cumulative logistic mixed effect model. This model included all predictors of primary interest and all predictors of the model's face validity. This model resulted from a backward elimination process which eliminated non-significant (p-value > 0.10) additional predictors including recipient ages, underlying primary liver disease, and total number of rejections.

with its severity. Portal inflammation emerged as the strongest predictor of portal fibrosis in the next PB (Table 3). Despite the well-documented link between inflammation and fibrosis in hepatitis C, such link remained inconclusive in pediatric LT. A Birmingham study on PBs 1, 5, and 10 years post-LT demonstrated greater fibrosis in biopsies with co-existing chronic hepatitis, inflammatory activity being not predictive of consequent fibrosis (Evans et al., 2006). The authors evaluated fibrosis and inflammation cross-sectionally at three time-points over 10 years.

While other studies had similar findings (Herzog et al., 2008), our results contradict these, as preceding inflammation and its intensity correlated with subsequent fibrosis. To explain this discrepancy, we correlated fibrosis at time-point t to inflammation at $t-2$ (Supplementary Table 9) to mimic the PB intervals of the Birmingham study, revealing no correlation. Hence, the discrepancies were likely produced by our shorter PB intervals rather than between-cohort differences. These findings suggest our PB schedule to be appropriate for further prospective studies on allografts.

Portal inflammation was significantly associated with Class II DSA and non-HLA antibodies, as expected, since DSAs have been implicated in allograft inflammation, fibrosis, and biliary and vascular complications (O'Leary et al., 2014). Although MHC Class II (major histocompatibility complex) is expressed by specialised antigen-presenting cells and not hepatocytes, hepatocytes facing persistent insult were recently shown to exhibit these antigens (Yamagiwa et al., 2014), especially in the periportal regions. This could explain the association between portal inflammation and Class II DSAs. A study collecting serial HLA antibody and inflammation data at each PB is required to investigate whether Class II DSA precedes or follows inflammation.

Most of our DSAs were "de-novo", and their development was predicted by donor and recipient ages. Other studies reported younger age at LT and medication non-compliance to be predisposing factors, which was not confirmed in our cohort (Del et al., 2014). Only three children developed both Class II DSA and non-HLA antibodies, hinting at different etiopathogeneses. In line with previous results (Venturi et al., 2014), we found non-HLA antibodies to be associated with portal and lobular inflammation. Non-HLA antibodies were detected in 40–75% of post-LT cases (Chen et al., 2013), associated with acute rejections, chronic rejections, and DNAIH. Since transient positivity can occur with rejection episodes, we considered non-HLA antibodies as positive when detected at the last two PBs.

HLA-DRB1*03/04 allele in LT recipients was an independent risk factor for portal fibrosis, with no significant association with inflammation. This corresponds to the AIH scenario Montano-Loza et al., 2006; Liberal et al., 2015. These alleles are believed to result in faulty antigen processing, with antigenic mimicry resulting in hepatic injury. Given that, within 4 weeks, the recipient's Kuffer cells were shown to completely

Table 5
Multivariate analysis using cumulative logistic mixed effect model to determine correlation between central fibrosis and various variables listed in the table.a*

Variable	Odds-ratio (95% CI)[a]	p-value[a]
Previous central fibrosis (LAFSc-C) ($t-1$)		<0.01
0	1.0	–
1	4.51 (1.61–12.6)	<0.01
2	9.41 (2.71–32.7)	<0.01
3	92.5 (3.50–2449)	<0.01
Previous lobular inflammation ($t-1$)		0.17
none	1.0	–
mild	1.55 (0.72–3.31)	0.26
moderate	0.44 (0.12–1.58)	0.21
severe	1.36 (0.03–72.1)	0.88
Class I HLA		0.43
Neg	1.0	–
Not DSA	1.39 (0.52–3.72)	0.51
DSA	0.42 (0.08–2.14)	0.30
Class II HLA		0.34
Neg	1.0	–
Not DSA	1.75 (0.51–6.01)	0.37
DSA	2.42 (0.61–9.60)	0.21
Other antib (ANA or SMA or LKM)		
Neg	1.0	–
Pos	0.55 (0.27–1.11)	0.09
Additional immunosuppression exposure		
No	1.0	–
Yes	2.21 (0.96–5.09)	0.06
Recipient HLA DRB1*03/04		
Neg	1.0	–
Pos	1.42 (0.73–2.76)	0.30
Time since transplantation		0.66
2–3 yr	1.0	–
3–5 yr	1.14 (0.54–2.39)	0.73
5–7 yr	1.35 (0.54–3.36)	0.51
> 7 yr	0.52 (0.13–2.18)	0.37
Donor status		
Living	1.0	–
Deceased	2.89 (1.25–6.67)	0.01

CI: confidence interval; LAFSc: liver allograft fibrosis scoring; HLA: human leukocyte antigen; DSA: donor-specific (HLA) antibodies.

[a] Odds-ratio, 95% confidence intervals (CI), and p-value were obtained from a cumulative logistic mixed effect model. This model included all predictors of primary interest and all predictors of the model's face validity. This model resulted from a backward elimination process which eliminated non-significant (p-value > 0.10) additional predictors including donor and recipient ages, recipient gender, underlying primary liver disease, and total number of rejections.

Table 6
Multivariate analysis using cumulative logistic mixed effect model to determine correlation between portal inflammation and various variables listed in the table.a*

Variable	Odds-ratio (95% CI)[a]	p-value[a]
Previous portal inflammation ($t-1$)		<0.01
none	1.0	–
mild	4.91 (2.00–12.1)	<0.01
moderate	29.6 (10.2–85.4)	<0.01
severe	78.9 (13.8–451)	<0.01
Class I HLA		0.54
Neg	1.0	–
Not DSA	0.73 (0.27–1.96)	0.53
DSA	0.48 (0.11–2.15)	0.34
Class II HLA		0.02
Neg	1.0	–
Not DSA	2.98 (0.90–9.29)	0.07
DSA	4.77 (1.22–18.6)	0.02
Other antib (ANA or SMA or LKM)		
Neg	1.0	–
Pos	2.31 (1.18–4.49)	0.01
Additional immunosuppression exposure		
No	1.0	–
Yes	0.71 (0.29–1.73)	0.45
Recipient HLA DRB1*03/04		
Neg	1.0	–
Pos	1.42 (0.76–2.67)	0.28
Time since transplantation		0.01
2–3 yr	1.0	–
3–5 yr	0.76 (0.39–1.51)	0.43
5–7 yr	1.10 (0.47–2.56)	0.82
> 7 yr	0.09 (0.02–0.45)	<0.01
Donor status		
Living	1.0	–
Deceased	1.91 (0.91–4.02)	0.09

CI: confidence interval; LAFSc: liver allograft fibrosis scoring; HLA: human leukocyte antigen; DSA: donor-specific (HLA) antibodies.

[a] Odds-ratio, 95% confidence intervals (CI), and *P*-value were obtained from a cumulative logistic mixed effect model. This model included all predictors of primary interest and all predictors of the model's face validity. This model resulted from a backward elimination process which eliminated non-significant (*P*-value >0.10) additional predictors including donor and recipient ages, recipient gender, underlying primary liver disease, and total number of rejections.

repopulate the allograft (Manns & Mix, 2013 Nov), any host antigen-presenting cell defects would be evident in the new graft, explaining our findings.

Among other factors that can potentially cause histological idiopathic changes in the allograft, immunosuppression medication compliance and adequacy is important. The methods to verify compliance should include evaluation of sudden changes in blood levels of the drug, unexplained late rejection episodes and patient questionnaires. However as this study included only patients who were stable and under close follow up, this aspect could have been minimized. Another facet that needs to be considered before generalization of these results is that this study included a much selected cohort of stable pediatric LT recipients; hence universal application of these findings would be a big extrapolation.

Topographic fibrosis distribution was a unique aspect of this study. This enabled us to identify a stronger persistence versus a discontinuous evolution in central and sinusoidal area, respectively. We found deceased donor to be the only predisposing factor for central fibrosis. While vascular and biliary complications, chronic rejection have been reported in earlier studies (Venturi et al., 2014), these were exclusion criteria in the current study as they are known fibrogenic complications. Inflammation in non-portal, unlike portal, areas was neither predictive of fibrosis nor associated with Class II DSA, but rather associated with non-HLA antibodies. We speculate that inflammation-driven fibrosis, when mediated by DSA, is limited to portal areas.

These findings pave the way for further intervention studies wherein additional drugs could be used to diminish the inflammation and consequent fibrosis. The anti-inflammation strategies could be customised depending on the probable mechanisms, which could be Azathioprine

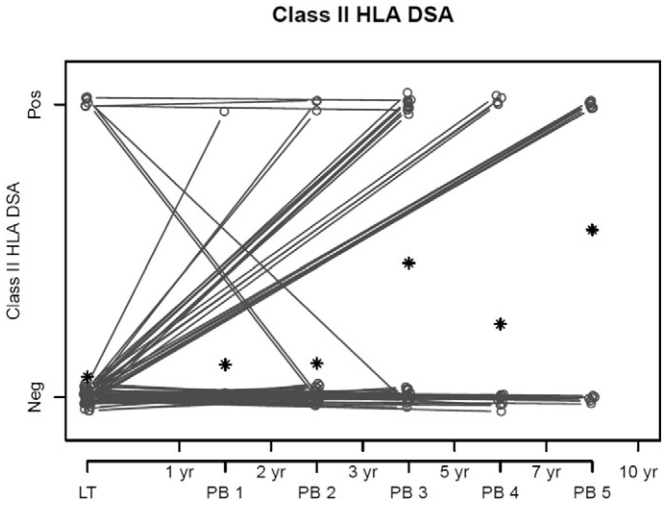

Fig. 5. Demonstrates the fate of pre-LT class II DSA and the development of "de-novo" antibodies post LT in all cases except two. Evolution of Class II HLA DSA status recorded 1 month pre-LT and at the last PB. Individual evolution of each patient is reported with connected gray circles while averaged evolution is reported with black stars.

Table 7
Multivariate analysis using cumulative logistic mixed effect model to determine correlation between lobular inflammation and various variables listed in the table.

Variable	Odds-ratio (95% CI)[a]	p-value[a]
Previous lobular inflammation ($t-1$)		<0.01
none	1.0	–
mild	3.19 (1.49–6.83)	<0.01
moderate-severe	27.4 (6.46–116.1)	<0.01
Class I HLA		0.11
Neg	1.0	–
Not DSA	0.37 (0.13–1.02)	0.05
DSA	1.84 (0.34–9.96)	0.48
Class II HLA		0.31
Neg	1.0	–
Not DSA	2.18 (0.61–7.78)	0.23
DSA	2.14 (0.49–9.30)	0.31
Other antib (ANA or SMA or LKM)		
Neg	1.0	–
Pos	2.28 (1.01–5.16)	0.05
Additional immunosuppression exposure		
No	1.0	–
Yes	1.27 (0.54–2.97)	0.58
Recipient HLA DRB1*03/04		
Neg	1.0	–
Pos	1.21 (0.62–2.39)	0.57
Time since transplantation		0.30
2–3 yr	1.0	–
3–5 yr	0.84 (0.40–1.79)	0.66
5–7 yr	1.33 (0.52–3.36)	0.55
> 7 yr	0.30 (0.07–1.32)	0.11
Donor status		
Living	1.0	–
Deceased	0.96 (0.43–2.15)	0.92

CI: confidence interval; LAFSc: liver allograft fibrosis scoring; HLA: human leukocyte antigen; DSA: donor-specific (HLA) antibodies.

[a] Odds-ratio, 95% confidence intervals (CI), and p-value were obtained from a cumulative logistic mixed effect model. This model included all predictors of primary interest and all predictors of the model's face validity. This model resulted from a backward elimination process which eliminated non-significant (p-value > 0.10) additional predictors including donor and recipient ages, recipient gender, underlying primary liver disease, and total number of rejections.

or MMF in presence of antibody mediated inflammation or T-reg infusions when there is suspicion of decreased regulation. Also recipients with HLA DRB1*03/04 could be regarded as high risk of fibrosis and be monitored more closely.

7. Conclusion

Genetic predisposition, allo-antibodies and allograft inflammation contribute to long term graft fibrosis. The portal fibrosis is strongly associated with presence and severity of the preceding inflammation, while this is not seen in the non-portal areas. These factors should thus be monitored post-LT and could be used in customization of immune suppression.

Table 8
Predisposing factors to development of class II DSA.a

Variable	Odds-ratio (95% CI)[a]	p-value[a]
Gamma globulin level	0.99 (0.84–1.16)	0.87
Total number of rejections	0.62 (0.28–1.34)	0.22
Time since LT (years)		0.37
1–3	1.0	–
3–5	2.99 (0.44–20.4)	0.26
5–7	4.62 (0.62–34.3)	0.13
> 7	4.38 (0.51–37.9)	0.18
Recipient age (years)	1.21 (1.02–1.44)	0.03
Donor age (years)	0.89 (0.83–0.96)	<0.01

CI: confidence interval; LT: liver transplant; ACR: acute cellular rejection; HLA: human leukocyte antigen; DSA: donor-specific (HLA) antibodies.

[a] Odds-ratio, 95% confidence intervals (CI), and p-value were obtained from a logistic regression model. This model included all predictors of primary interest and all predictors of the model's face validity and resulted from a (backward) elimination of one non-significant additional predictor (Recipient gender, p > 0.10).

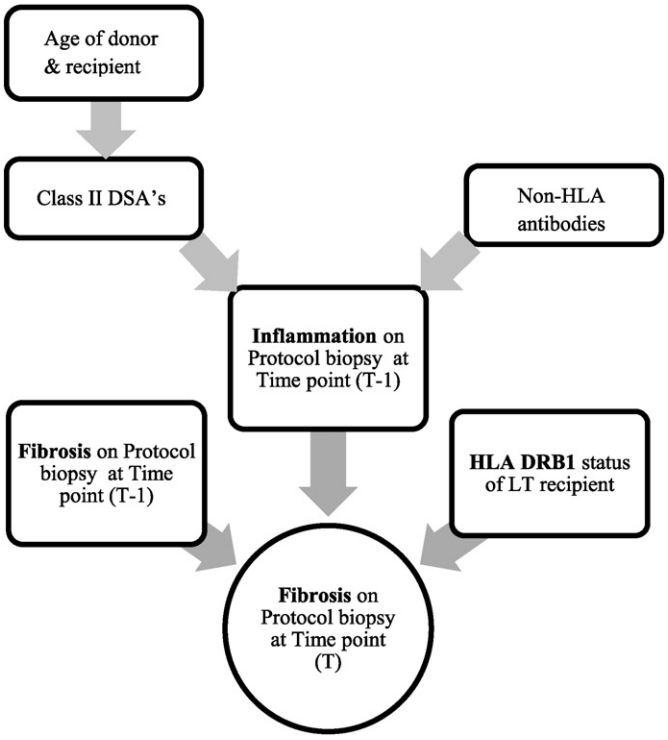

Fig. 6. Mechanism of "idiopathic" allograft histological changes.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2016.05.040.

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Financial support

None.

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