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ABSTRACT

BACKGROUND AND AIMS: Sub-clinical inflammatory changes are commonly described in long-term transplant recipients undergoing protocol liver biopsies. The pathogenesis of these lesions remains unclear. The aim of the study was to identify the key molecular pathways driving progressive sub-clinical inflammatory liver allograft damage.

PATIENTS AND METHODS: All liver recipients followed at Hospital Clínic Barcelona who were >10 years post-transplant were screened to participate in the study. Patients with recurrence of underlying liver disease, biliary or vascular complications, chronic rejection, and abnormal liver function tests were excluded. Sixty-seven patients agreed to participate and underwent blood and serological tests, transient elastography and a liver biopsy. Transcriptome profiling was performed on RNA extracted from 49 out of the 67 biopsies employing a whole genome next generation sequencing platform. Patients were followed for a median of 6.8 years following the index liver biopsy.

RESULTS: Median time since transplantation to liver biopsy was 13 years (10-22). The most frequently observed histological abnormality was portal inflammation with different degrees of fibrosis, present in 45 biopsies (67%). Two modules of 102 and 425 co-expressed genes were significantly correlated with portal inflammation, interface hepatitis and portal fibrosis. These modules were enriched in molecular pathways known to be associated with T cell mediated rejection. Liver allografts showing the highest expression levels for the two modules recapitulated the transcriptional profile of biopsies with clinically

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apparent rejection and developed progressive damage over time, as assessed by non-invasive markers of fibrosis.

CONCLUSIONS: A large proportion of long-term surviving adult liver transplant recipients exhibit subclinical histological abnormalities whose expression profile closely resembles T cell mediated rejection and that may result in progressive allograft damage.

Lay summary: A large proportion of long-term surviving adult liver transplant recipients exhibit subclinical histological abnormalities. Transcriptome profiling of liver tissue showed an expression profile that closely resembles T cell mediated rejection. Liver allografts showing the highest expression levels of rejection-related genes developed progressive damage over time.

INTRODUCTION

Routine serum markers of liver injury such as aspartate and alanine aminotransferases (AST, ALT), gamma-glutamyl-transpeptidase (GGT) or alkaline phosphatase (AP) are known to be insensitive and nonspecific indicators of allograft rejection in liver transplantation (LT) [1,2]. Despite this, the long-term management of LT recipients continues to rely on a combination of serum liver biochemistry tests and calcineurin inhibitor pharmacokinetic markers. The performance of protocol, or surveillance, liver biopsies has been proposed as a more accurate strategy to assess graft function and potentially to personalize the use of immunosuppression [3–5]. This is based on a multiplicity of studies showing that a very large proportion of patients with normal liver biochemistry tests exhibit clinically significant histological lesions, with chronic hepatitis not attributable to recognizable causes such as viral infection or autoimmune hepatitis being the most frequently described abnormality [1,6–8]. The clinical utility of protocol liver biopsies, however, remains contentious [5], and as a result they are not performed in the vast majority of adult liver transplant programs. The controversy stems from an incomplete understanding of the natural history and pathogenesis of the so-called idiopathic inflammatory lesions [2,9]. This is due to the paucity of prospective clinical studies and the lack of in-depth studies comparing the molecular signatures of these lesions with those of well-characterized histological phenotypes.

Employing microarray and real-time PCR analyses, we previously reported that it is possible to identify transcriptional signatures of acute cellular rejection in blood and liver tissue specimens of LT recipients [10,11]. Importantly, these

signatures can be detected even in the midst of the noise caused by other forms of chronic inflammation, such as that induced by hepatitis C virus (HCV). We anticipated that a similar strategy could provide a clue as to the pathogenesis of idiopathic fibro-inflammatory lesions in long-term surviving adult liver transplant recipients. To confirm this premise, we conducted a prospective clinical study in which we approached all surviving LT recipients transplanted at Hospital Clinic Barcelona between 1988 and 1999 without previously diagnosed allograft lesions, and performed liver biopsy, transient elastrography, and next generation sequencing transcriptional studies.

PATIENTS AND METHODS

Patient population

Between December 2007 and December 2009 we screened all patients who had undergone LT at Hospital Clínic Barcelona between 1988 and 1999 and who remained alive and had been followed-up for more than 10 years after transplantation. Exclusion criteria were: 1) diagnosis of *de novo* or recurrent liver disease after transplantation, including HCV-related hepatitis; 2) elevation in AST, ALT or AP ≥2 fold above the upper limit of normality (isolated GGT increases were not considered an exclusion criteria [n=18]); 3) T cell-mediated rejection within 1 year of the inclusion in the study; 4) contraindications for a liver biopsy; 5) severe extra-hepatic co-morbidities (Figure 1). All consented patients underwent a protocol liver biopsy and collection of a blood specimen. Patients included in the study were followed-up until death, re-transplantation, lost to follow-up or December 2017.

All patients signed an informed consent. The study was approved by the ethical committee at Hospital Clínic Barcelona.

Laboratory tests and fibrosis evaluation

The following tests were performed at the time of inclusion: liver tests (serum AST, ALT, GGT, AP, total bilirubin, INR); platelet count; serologies for hepatitis B (surface antigen HBsAg, anti-core antibodies), hepatitis C, and hepatitis E (IgG anti-hepatitis E antibodies and RNA) viruses; and serum autoantibodies (anti-nuclear antibodies ANA, anti-smooth muscle antigen SMA, anti-liver-kidney microsomal antibodies anti-LKM1/2). Liver tests and platelet counts were also analyzed at the last follow-up visit. Indirect fibrosis scores (APRI and FIB-4) were calculated using the standard formulas at the time of inclusion and at the last follow-up [12,13]. In addition, 30 patients underwent a liver stiffness measurement (LSM) with Fibroscan® transient elastography (Echosens, France) at the time of liver biopsy.

Liver histopathology

Hematoxylin-eosin and Masson's trichrome stained sections were blindly assessed by two liver histopathologists (RM and LNS). A semi-quantitative score was used to evaluate and grade different parameters as follows: 1) Architectural abnormalities: 0= absent; 1= minimal abnormalities consistent with irregular regenerative foci; and 2= moderate-marked with parenchymal atrophy and centrilobular collapse (with or without inflammation) or nodular regenerative hyperplasia; 2) Lobular inflammation: 0= no inflammation; 1= mild (sinusoidal inflammatory cells and/or mild focal necrosis); 2= moderate (multiple necro-

inflammatory foci); and 3= marked (confluent or bridging necrosis); 3) Central perivenulitis (with or without endotheliitis): 0= no inflammation; 1= mild (patchy, focal perivenulitis); 2= moderate (perivenulitis in most central veins); and 3= marked (confluent or bridging hepatocellular necrosis); 4) Portal inflammation: 0= no inflammation; 1= mild (small groups of inflammatory cells); 2=moderate (expansive inflammatory infiltrate in >50% of portal tracts); and 3=marked (severe inflammatory infiltrate in most portal tracts), 5) Interface hepatitis: 0= no interface activity; 1= mild; 2= moderate; and 3=severe; 6) Bile duct lesion: 0= no; 1= minimal (intraepithelial inflammatory cells or abnormal colangiocytes); 2= moderate (epithelial lesions in most portal tracts, no destruction); and 3= marked (destructive epithelial lesions in most portal tracts); 7) Bile duct loss**:** 0= no loss; 1= loss of bile ducts in <50% of the portal tracts; and 2= loss in ≥ 50% of the portal tracts; 8) Portal vein branches: 0= present in all portal tracts; 1= absent in a minority of portal tracts; and 2=absent in most portal tracts; 9) Portal vein endotheliitis: 0=absent; 1= mild (present in a minority of portal veins); 2=moderate (present in most portal veins); and 3= marked; 10) Portal fibrosis: 0= absent; 1=minimal fibrosis (minority of portal tracts); 2= moderate (periportal expansion in most portal tracts); 3= bridging fibrosis; 4= cirrhosis; 11) Peri-sinusoidal fibrosis: 0=absent; 1= focal; 2=marked; 12) Steatosis: 0= mild (< 30% of hepatocytes); 1= moderate (30-60%); and 2= severe (> 60% of hepatocytes).

Statistical analysis

Categorical data were compared by Chi-squared or Fischer's test as appropriate. Continuous variables were compared by non-parametric testing (Mann-Whitney). A cumulative odds ordinal logistic regression with

proportional odds was run to determine the effect of severity of portal inflammation on the risk of having portal fibrosis. We employed non-parametric analysis of covariance (ANCOVA) adjusting for a smooth nonlinear effect of baseline using a spline function to rigorously explore the effects of module gene expression assignment on the following continuous variables: bilirubin, AST, ALT, INR, platelet count, APRI and FIB4. Data are reported as adjusted mean difference and 95% confidence interval (95%CI). Statistical analyses were performed using SPSS statistical package version 23 and R.

RNA extraction

RNA was extracted from formalin-fixed, paraffin-embedded liver biopsy specimens. First, excessive paraffin surrounding the tissue was manually removed from the blocks and 8 sections of 5um each were obtained and placed in Eppendorf tubes. Tissue RNA was then isolated following deparaffinization, incubation with lysis buffer and DNase treatment according to the RNeasy® FFPE kit's instructions (Qiagen, UK). RNA was eluted using a RNeasy MinElute® spin column following ethanol addition. The concentration and purity of the RNA was measured using Nanodrop (ThermoScientific) and Qubit RNA BR Assay kit (Bio-Rad, Hercules, CA), and the RNA stored at -80°C.

RNA Sequencing (RNASeq) experiments

Out of the 67 protocol liver biopsies performed as part of the study, 49 were employed to conduct RNASeq experiments, while in the remaining cases this could not be completed due to insufficient liver tissue material, poor quality RNA or sub-optimal sequenced reads. RNASeq was done in collaboration with

Genetracer Biotech using the Ion Torrent Proton II (Thermo Fisher), which employs custom designed primers targeting multiple exons for all known protein coding genes as primary transcript detection strategy. Briefly, 50ng RNA was used to generate sequencing libraries using the Ion AmpliSeq™ Transcriptome Human Gene Expression Kit (Thermo Fisher), and barcoded using Ion Express barcodes. cDNA library quality was assessed using the Agilent® 2200 TapeStation System and the High Sensitivity D1000 ScreenTape System (Agilent Technologies, Santa Clara, CA). Templates were run on the Ion PI™ v3 chips using the Ion Chef system and Hi-Q™ Chef kits. Sequencing depth per sample was 12 million genome-aligned reads. Sequenced reads were mapped against the reference sequences including 20,800 different genes present in the AmpliSeq Human Gene Expression panel using Novalign software V3.02.08 (http://www.novocraft.com). Novoalign finds global optimum alignments using full Needleman–Wunsch algorithm showing better sensitivity than other aligners [14]. After mapping, unique mapped reads were summarized as counts representing the gene expression levels for over 20,800 different genes present in the AmpliSeq Human Gene Expression panel. Low expressed genes were excluded from downstream analysis if the sum of counts were lower than 100. Transformation of count data to log2-counts per million (logCPM) was accomplished estimating the mean-variance relationship and using this parameter to compute appropriate observational-level weights. The resulting data was then used for linear modeling and differential expression by means of the Limma R-package [15]. Fold-changes, moderated *P* values, and false discovery rates (FDR) obtained by adjusting *P* values for multiple testing using Benjamini-Hochberg, were calculated. Additional RNASeq experiments were

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performed on RNA samples extracted from 18 archived diagnostic liver biopsies conducted at Hospital Clinic Barcelona during 2009. These for-cause biopsies were performed in patients with allograft dysfunction and corresponded to 8 samples of early T cell mediated rejection (within 3 months post-transplant); 6 samples of late (>1 year post-transplant) T cell mediated rejection; and 4 samples of post-transplant recurrent HCV infection. Transcriptional data derived from the 18 for-cause biopsies were employed exclusively for functional pathway analysis and were not included in the remaining analyses.

The raw data from Ion-Torrent ampliseq transcriptome were deposited in the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) Short Read Archive data-base (Bioproject accession no. PRJNA437250; https://www.ncbi.nlm.nih.gov/sra).

Analysis of gene expression data

To explore the correlation patterns among genes across the RNASeq data derived from the 49 protocol biopsies we employed the *Weighted Gene Correlation Network Analysis* (WGCNA) software package available from R [16]. To perform WGCNA, correlations between all pairs of genes in the expression dataset were quantified and transformed into measures of connection strength by emphasizing strong correlations and minimizing the noise in the pairwise measurements. A hierarchical clustering algorithm was then used to identify modules of highly interconnected genes. Next, to reduce multi-dimensionality, consensus eigengenes were defined for each module and used to relate the modules to external data (e.g. clinical traits).

The statistical significance of *a priori* defined sets of genes representing

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biological pathways on the whole transcriptional dataset, which included the 49 protocol biopsies plus the 18 for-cause biopsies, was computed employing *Quantitative Set Analysis for Gene Expression* (QuSAGE) [17], using the *Kyoto Encyclopedia of Genes and Genomes* (KEGG) pathway database and the transplantation-related *Pathogenesis-Based Transcript* (PBT) gene sets from the Alberta Transplant Applied Genomics Centre (ATAGC; http://atagc.med.ualberta.ca/Research/GeneLists). For QuSAGE analysis the threshold of statistical significance was set at FDR<0.10. To identify pathways enriched in modules of co-expressed genes we employed the *enricher* function from *ClusterProfiler* R-package for hypergeometric tests.

To interrogate whether biopsies exhibiting idiopathic inflammatory lesions were likely to correspond to unrecognized forms of rejection, we re-analyzed a liver tissue microarray dataset derived from two immunosuppression withdrawal trials previously reported by our group, which contained expression data from 20 pairs of liver biopsies obtained before drug weaning was initiated and at the time of rejection [11]. A gene classifier of T cell mediated rejection was identified using *Predictive Analysis of Microarrays* (PAM). Of note, 7 of the 20 biopsy pairs were obtained from patients who had chronic hepatitis due to hepatitis C virus at the time of initiating drug withdrawal [11]. All samples were analyzed together to be able to develop classifiers that would identify rejection on top of underlying inflammatory lesions. The diagnostic accuracy of the resulting rejection-associated signature was evaluated by 4-fold cross-validation, in which the original sample is randomly partitioned into 4 equal size subsamples. Of the 4 subsamples, a single subsample is retained as the validation data for testing the model, and the remaining 4-1 sub-samples are

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used as training data, and the procedure is repeated 40 times.

RESULTS

Patients' characteristics

Between January 1988 and December 1999, 654 LTs were performed in 589 patients at Hospital Clinic Barcelona. At the time of initiating the study (December 2007), 312 patients were dead and 38 were lost to follow-up. One hundred eighteen patients were excluded due to a previous diagnosis of recurrent liver disease or chronic rejection. Among the 121 evaluable patients, 67 were finally included in the study and underwent a surveillance liver biopsy between December 2007 and December 2009. The remaining 54 patients were excluded for the presence of severe co-morbidities, contraindications for a liver biopsy, or refused consent (Figure 1). The characteristics of the patients enrolled in the study are shown in Table 1. Briefly, the majority of patients were male (n=52, 77.6%), with a median age (at the time of liver biopsy) of 60.4 years (29.5-75.5). The median time from LT to liver biopsy was 13 years (10-22). The most common indication for LT was alcoholic cirrhosis (n=23, 34%). The most frequent maintenance immunosuppressive regimen at the time of biopsy was based on cyclosporine (53.8%; n=36). Independently of the immunosuppressive regimen, all patients had low immunosuppressive drug levels (below the limit of quantification in 12 [18%] patients) at the time of liver biopsy. Following the performance of the surveillance liver biopsy, enrolled patients underwent routine follow-up clinical visits every 6 months for a median time of 6.8 [range 2-10] years. During the follow-up, 11 patients died (5 died of

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cardiovascular complications, 3 due to neoplasms, 2 died of end-stage liver disease, and 1 patient died of respiratory tract infection) and 4 patients were lost to follow-up.

Histological evaluation

The results of the central histological analysis are shown in Table 2. Histological abnormalities were detected in 51 (76%) liver biopsies. Six patients (9%) had cirrhosis, 5 (8%) had chronic rejection and 8 (12%) patients had moderate to severe hepatic steatosis. The most frequently observed histological abnormality was portal inflammation, present in 45 biopsies (67%). Sixteen (35%) out of the 45 patients exhibited moderate to severe portal inflammation with interface hepatitis in 11 patients. Patients with portal inflammation were negative for hepatitis B, hepatitis C, and hepatitis E serological markers. While 11 of the 45 patients with moderate to severe portal inflammation displayed positive antinuclear antibodies and/or anti-smooth muscle antibodies in titles ranging between 1:40 to 1:640, none of them met criteria of the International Autoimmune Hepatitis Group for the diagnosis of autoimmune hepatitis. Furthermore, auto-antibodies were also positive in 27 patients with no evidence of significant portal inflammation. Of note, 32 (71%) out of the 45 patients with portal inflammation had portal or perisinusoidal fibrosis (4 of these patients had cirrhosis). On the contrary, among the 22 patients without portal inflammation, only 7 (32%) had portal or perisinusoidal fibrosis (p=0.02). By ordinal logistic regression analysis, we observed that the severity of inflammation was significantly associated with the severity of portal fibrosis. The odds of portal fibrosis in patients with moderate or severe inflammation were 6.5 (95%CI 1.48-

28.17; p=0.013) and 15.2 (95%CI 1.34-172.18; p=0.028), respectively, when compared to the absence of inflammation. Centrilobular fibrosis was observed in 5 patients, all of whom exhibited moderate to marked portal inflammation although concomitant portal fibrosis (F1) was only apparent in 1.

There were no significant differences among patients with or without portal inflammation regarding the type and trough levels of immunosuppressive drugs, liver tests, or liver stiffness measurements neither at the time of the protocol liver biopsy nor at the last follow-up visit. When specifically analyzing the group of patients with portal inflammation, there were not significant differences in the baseline characteristics between patients with or without portal/perisinusoidal fibrosis. The only variable significantly associated with the presence of fibrosis was liver stiffness measurement, which was significantly higher in patients with fibrosis than in patients without fibrosis (11.8 vs. 4.8 kPa; p=0.049).

Identification of gene expression modules associated with histological abnormalities

To explore the mechanisms potentially driving the histological abnormalities observed in the protocol biopsies, we employed *Weighted Gene Correlation Network Analysis* (WGCNA) to identify modules of genes whose expression is coordinated in a similar manner across the whole group of samples. We summarized the results in a correlation heatmap, in which the Y-axis corresponds to groups of genes (modules) with similar expression, and the X-axis includes the clinical, serological and histological variables of interest (Figure 2). We chose to focus on 2 specific modules, named *Cyan* and *Pink*, containing 102 and 425 genes respectively, because they showed the most

significant positive correlation with portal inflammation, the most prevalent histological abnormality, as well as with interface activity and portal fibrosis, which are considered markers of progressive histological damage (Figure 2 and Supplementary Table 1). The *Pink* module was most significantly enriched in KEGG canonical pathways involved in extracellular matrix remodeling (Supplementary Table 2). In contrast, among the genes contained in the *Cyan* module we identified an over-representation of KEGG canonical pathways known to be regulated by IFN**γ,** as well as ATAGC PBT gene sets previously reported to be associated with T cell mediated rejection (Supplementary Table 3). These results suggested a potential link between active portal inflammation, fibrosis and T cell mediated rejection.

The gene expression module associated with portal inflammation overlaps with the transcriptional signature observed at the time of T cell mediated rejection

To further explore whether the sub-clinical portal inflammatory lesions might correspond to unrecognized forms of rejection, we defined a transcriptional signature of T cell mediated rejection (TCMR) by re-analyzing the previously reported microarray gene expression data derived from 20 liver transplant recipients who developed rejection while undergoing protocolized immunosuppression withdrawal [11]. Using *Predictive Analysis of Microarrays* (PAM) on the gene expression profiles derived from the 20 pairs of liver biopsies (i.e. collected before initiating drug weaning and at the time of rejection), we identified a parsimonious group of 13 genes that classified samples as either rejecting or non rejecting with high accuracy (Figure 3A and B

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and Supplementary Table 4). We interrogated the RNASeq expression dataset from the protocol biopsy set and noted a significant correlation between the eigengenes of the group of 13 genes and those of the *Cyan* and *Pink* gene expression modules (Figure 3C).

To better understand the biological implications of the changes in the expression of these two modules, we decided to concentrate on those samples exhibiting extreme transcriptional phenotypes. To do so, we assigned biopsies into 2 groups, *Cyan&Pink_High* and *Cyan&Pink_Low*, according to whether their expression levels were above or below the median level of expression for both modules. We then compared the enrichment in rejection-associated gene sets between the transcriptome of the *Cyan&Pink_High* group and the groups of post-transplant biopsies with chronic hepatitis C, early TCMR and late TCMR, employing the *Cyan&Pink_Low* group as the common comparator for all groups. To conduct this analysis we used the QuSAGE method, which allows direct 2-way comparisons between several groups of samples. The *Cyan&Pink_High* group transcriptome was significantly enriched in a number of KEGG canonical pathways associated with allograft rejection and immunopathology (Table 3). Furthermore, among the ATAGC PBT gene sets, the following gene sets known to be associated with T cell mediated rejection were also up-regulated in the *Cyan&Pink_High* group: *QCAT* (cytotoxic T lymphocyte associated transcripts), *QCMAT* (macrophage transcripts associated with rejection), *GRIT1* (IFNg dependent rejection-associated transcripts), *AMAT1* (alternative macrophage associated transcripts correlating with T cell mediated rejection), *ENDAT* (endothelial cell associated transcripts), *BAT* (B cell associated transcripts), IRITD3 and IRITD5 (injury and rejection

associated transcripts). A direct comparison between the transcriptional profile of the TCMR, late TCMR, and *Cyan&Pink_High* groups revealed that they were very similar at a functional pathway level, with analogous enrichment scores in what regards immunopathology pathways and rejection-associated gene sets. In contrast, the transcriptomes of these 3 groups differed from the transcriptional profile observed in the chronic hepatitis C group, in which, with the exception of the *GRIT1* and *BAT* gene sets, no significant enrichment in rejection-associated transcript sets was noted (Table 3). The potential for progressive liver damage was investigated by quantifying the enrichment in a set of 122 genes preferentially expressed by liver stellate cells and known to be correlated with the extent of liver fibrosis and with the survival of patients with liver cirrhosis [18]. Over-representation of this gene set was only noted on the *Cyan&Pink_High* expression module (Table 3).

Clinical parameters associated with liver tissue gene expression changes

We next investigated whether the transcriptional differences between the *Cyan&Pink_High* and *Cyan&Pink_Low* groups were translated into changes in clinically-relevant biochemical and hematological parameters, either at baseline (at the time of the index protocol biopsy) or at the last follow-up visit. At baseline, *Cyan&Pink_High* patients had significantly higher INR and APRI fibrosis score values than the *Cyan&Pink_Low* patients (p=0.032 and 0.014, respectively; Supplementary Table 5). Furthermore, they showed more advanced portal inflammation, interface hepatitis and portal fibrosis (Supplementary Table 5). At the last follow-up visit, as compared to patients in the *Cyan&Pink_Low* group, patients from the *Cyan&Pink_High* group continued

to have a significantly higher APRI score and in addition they exhibited higher FIB-4 fibrosis score, AST and bilirubin, and lower platelet count (Supplementary Table 5). To better understand the significance of the changes observed during patient follow-up, and to clarify whether *Cyan&Pink* group assignment exerted an effect that was independent from the changes seen at baseline, we re-analyzed the data employing a non-parametric ANCOVA adjusted by the length of the follow-up. This confirmed the significant association between *Cyan&Pink_High* group assignment and the development of higher bilirubin level (p=0.0475), lower platelet count (p=0.0025), higher ALT (p=0.0124), and higher APRI score (p=0.0277), at the end of the follow-up (Table 4).

DISCUSSION

It is now well established that subclinical chronic allograft injury is very prevalent in long-term surviving LT recipients. Previous studies have reported histological abnormalities in 5% to 90% of late protocol liver biopsies. In children, the prevalence appears to be higher higher ranging between 67% and 95% [6,7,21]. The reasons for this variability are not entirely clear but they are probably related to differences in the indications for transplantation, follow-up duration, immunosuppression protocols, and thresholds for liver test levels [1,19,20]. In most studies, portal inflammation with or without interface activity and/or fibrosis, often referred to as idiopathic post-transplant hepatitis, was the most frequent finding [6,7,20,19,21–24].

The objective of our study was to investigate the mechanisms associated with sub-clinical graft deterioration by conducting an unbiased analysis of clinical,

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histological, biochemical and high-throughput liver tissue transcriptional data. In particular, we questioned whether biopsies displaying idiopathic post-transplant hepatitis corresponded to unrecognized forms of rejection. To reduce the risk of bias, provide adequate clinical context and ensure the generalizability of the observations, we designed a prospective study in which we interrogated all patients transplanted at Hospital Clinic Barcelona over a 10-year period and we conducted protocol liver biopsies in those meeting pre-defined inclusion/exclusion criteria.

We first employed an exploratory systems biology strategy to identify, in an unsupervised manner, the key mechanistic underpinnings of the histology lesions. This was conducted using a modular analysis that explored the correlations between groups of co-expressed genes and semi-quantitative histological scores across the whole set of liver tissue samples. Out of the 23 modules of genes identified, we selected 2 for further analyses, on the basis of their significant correlation with portal inflammation and fibrosis. By representing each of the 2 expression modules by an 'eigengene' we could show a significant correlation between the modules and a 13 gene set specific for TCMR in LT that we derived from a re-analysis of two previously published microarray studies conducted by our group [10,11]. Furthermore, the two modules were enriched in gene sets previously identified as being associated with allograft rejection across a variety of experimental and clinical settings, which again closely resembled what is observed at the time of clinically apparent early and late TCMR.

A non-rejection related form of liver allograft inflammation (recurrent HCV-induced chronic hepatitis) also exhibited transcriptional similarities with

rejection. This reflects the sharing of specific pathways of tissue injury between rejection and other inflammatory insults, and is in keeping with what has been described in kidney transplantation [25]. However, the degree of overlap observed between TCMR and HCV-induced chronic hepatitis was significantly smaller than between TCMR and the subset of protocol biopsies exhibiting high expression of the two selected modules.

Although our study lacked paired longitudinal liver biopsies and thus was not designed to evaluate the histological progression of allograft damage, the analysis of the blood tests collected over time revealed that patients with high expression of the two selected gene modules exhibited a mild but significant worsening in non-invasive markers of fibrosis, liver function and portal hypertension. This was consistent with the over-expression of a distinct set of genes known to be specific for stellate cells and whose expression in the liver tissue of non-transplant patients with chronic liver disease has been reported to be associated with liver failure and patient survival [18]. Taken together, these data suggest that idiopathic post-transplant hepatitis is likely to constitute a form of unrecognized alloimmune injury that, at least in a subset of patients, results in progressive allograft damage.

Previous studies have reported an association between circulating donor-specific anti-HLA antibodies (DSA) and subclinical liver allograft inflammatory lesions [9,26–29]. The influence of DSA on the liver tissue gene expression profile could not be explored in our study due to the lack of donor HLA information. The fact that the gene modules correlated with portal inflammation and interface activity closely resembled the transcriptome of liver TCMR would argue against a significant role for antibody-mediated rejection

(ABMR) in this setting. It should be acknowledged, however, that in kidney transplantation the transcriptional profile of TCMR and ABMR are known to significantly overlap [25], and that transcripts specific for ABMR in liver transplantation have not been defined yet. Hence, our study cannot unambiguously elucidate the relative roles of cellular and humoral alloimmunity. This will require the performance of transcriptional studies of large numbers of accurately classified TCMR and ABMR cases.

The majority of patients enrolled in the current study were on very low dose immunosuppression. This reflects the standard practice at Hospital Clinic Barcelona and was not the result of an intentional drug minimization protocol. Our findings could be interpreted as indicating that long-term surviving liver transplant recipients are insufficiently immunosuppressed. This interpretation needs to be balanced against the significant morbidity and mortality caused by immunosuppressive medications and the fact that evidences of progressive allograft damage were only noted in a minority of the patients enrolled in our study. Hence, we would caution against the use of more aggressive immunosuppressive regimens in this patient population until randomized controlled trials investigating the risk/benefit of these regimens are conducted. In the meantime, the main practical lesson to be drawn from our data is the need to recognize the possibility of subclinical rejection in long-term surviving liver recipients with normal liver function tests, and to consider sequential surveillance liver biopsies as a tool that could potentially optimize graft and patient survival.

REFERENCES

[1]   Sebagh M, Samuel D, Antonini TM, Coilly A, Degli Esposti D, Roche B, et al. Twenty-year protocol liver biopsies: Invasive but useful for the management of liver recipients. J Hepatol 2012;56:840–7. doi:10.1016/j.jhep.2011.11.016.

[2]   Hübscher SG. What is the long-term outcome of the liver allograft? J Hepatol 2011;55:702–17. doi:10.1016/j.jhep.2011.03.005.

[3]   Demetris AJ, Bellamy C, Hübscher SG, O'Leary J, Randhawa PS, Feng S, et al. 2016 Comprehensive Update of the Banff Working Group on Liver Allograft Pathology: Introduction of Antibody-Mediated Rejection. Am J Transplant 2016;16:2816–35. doi:10.1111/ajt.13909.

[4]   Feng S, Bucuvalas J. Tolerance after liver transplantation: Where are we? Liver Transplant 2017;23:1601–14. doi:10.1002/lt.24845.

[5]   Demetris A. Importance of liver biopsy findings in immunosuppression management: Biopsy monitoring and working criteria for patients with operational tolerance. Liver Transplant 2012;18:1154–70. doi:10.1002/lt.23481.

[6]   Evans HM, Kelly DA, McKiernan PJ, Hübscher S. Progressive histological damage in liver allografts following pediatric liver transplantation. Hepatology 2006;43:1109–17. doi:10.1002/hep.21152.

[7]   Ekong UD, Melin-Aldana H, Seshadri R, Lokar J, Harris D, Whitington PF, et al. Graft histology characteristics in long-term survivors of pediatric liver transplantation. Liver Transplant 2008;14:1582–7. doi:10.1002/lt.21549.

[8]   Venturi C, Sempoux C, Bueno J, Ferreres Pinas JC, Bourdeaux C, Abarca-Quinones J, et al. Novel Histologic Scoring System for Long-Term Allograft Fibrosis After Liver Transplantation in Children. Am J Transplant 2012;12:2986–96. doi:10.1111/j.1600-6143.2012.04210.x.

[9]   Kelly D, Verkade HJ, Rajanayagam J, McKiernan P, Mazariegos G, Hübscher S. Late graft hepatitis and fibrosis in pediatric liver allograft recipients: Current concepts and future developments. Liver Transplant 2016;22:1593–602. doi:10.1002/lt.24616.

[10]   Bohne F, Martínez-Llordella M, Lozano J-J, Miquel R, Benítez C, Londoño M-C, et al. Intra-graft expression of genes involved in iron homeostasis predicts the development of operational tolerance in human liver transplantation. J Clin Invest 2012;122:368–82. doi:10.1172/JCI59411.

[11]   Bonaccorsi-Riani E, Pennycuick A, Londoño M-C, Lozano J-J, Benítez C, Sawitzki B, et al. Molecular Characterization of Acute Cellular Rejection Occurring During Intentional Immunosuppression Withdrawal in Liver Transplantation. Am J Transplant 2016;16:484–96. doi:10.1111/ajt.13488.

[12]   Chou R, Wasson N. Blood Tests to Diagnose Fibrosis or Cirrhosis in Patients With Chronic Hepatitis C Virus Infection. Ann Intern Med 2013;158:807. doi:10.7326/0003-4819-158-11-201306040-00005.

[13]   Sterling RK, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J, et al. Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. Hepatology 2006;43:1317–25. doi:10.1002/hep.21178.

[14] Hatem A, Bozdağ D, Toland AE, Çatalyürek Ü V. Benchmarking short sequence mapping tools. BMC Bioinformatics 2013;14:184. doi:10.1186/1471-2105-14-184.

[15] Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 2015;43:e47–e47. doi:10.1093/nar/gkv007.

[16] Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics 2008;9:559. doi:10.1186/1471-2105-9-559.

[17] Yaari G, Bolen CR, Thakar J, Kleinstein SH. Quantitative set analysis for gene expression: a method to quantify gene set differential expression including gene-gene correlations. Nucleic Acids Res 2013;41:e170. doi:10.1093/nar/gkt660.

[18] Zhang DY, Goossens N, Guo J, Tsai M-C, Chou H-I, Altunkaynak C, et al. A hepatic stellate cell gene expression signature associated with outcomes in hepatitis C cirrhosis and hepatocellular carcinoma after curative resection. Gut 2016;65:1754–64. doi:10.1136/gutjnl-2015-309655.

[19] Mells G, Mann C, Hubscher S, Neuberger J. Late protocol liver biopsies in the liver allograft: A neglected investigation? Liver Transplant 2009;15:931–8. doi:10.1002/lt.21781.

[20] Abraham SC, Poterucha JJ, Rosen CB, Demetris AJ, Krasinskas AM. Histologic Abnormalities are Common in Protocol Liver Allograft Biopsies From Patients With Normal Liver Function Tests. Am J Surg

Pathol 2008;32:965–73. doi:10.1097/PAS.0b013e3181622490.

[21] Herzog D, Soglio DB-D, Fournet J-C, Martin S, Marleau D, Alvarez F. Interface hepatitis is associated with a high incidence of late graft fibrosis in a group of tightly monitored pediatric orthotopic liver transplantation patients. Liver Transplant 2008;14:946–55. doi:10.1002/lt.21444.

[22] Briem-Richter A, Ganschow R, Sornsakrin M, Brinkert F, Schirmer J, Schaefer H, et al. Liver allograft pathology in healthy pediatric liver transplant recipients. Pediatr Transplant 2013;17:543–9. doi:10.1111/petr.12119.

[23] Fouquet V, Alves A, Branchereau S, Grabar S, Debray D, Jacquemin E, et al. Long-term outcome of pediatric liver transplantation for biliary atresia: a 10-year follow-up in a single center. Liver Transpl 2005;11:152–60. doi:10.1002/lt.20358.

[24] Sanada Y, Matsumoto K, Urahashi T, Ihara Y, Wakiya T, Okada N, et al. Protocol liver biopsy is the only examination that can detect mid-term graft fibrosis after pediatric liver transplantation. World J Gastroenterol 2014;20:6638. doi:10.3748/wjg.v20.i21.6638.

[25] Halloran PF, Venner JM, Famulski KS. Comprehensive Analysis of Transcript Changes Associated With Allograft Rejection: Combining Universal and Selective Features. Am J Transplant 2017;17:1754–69. doi:10.1111/ajt.14200.

[26] Wozniak LJ, Hickey MJ, Venick RS, Vargas JH, Farmer DG, Busuttil RW, et al. Donor-specific HLA Antibodies Are Associated With Late Allograft Dysfunction After Pediatric Liver Transplantation.

Transplantation 2015;99:1416–22. doi:10.1097/TP.0000000000000796.

[27] Curry MP, Forns X, Chung RT, Terrault NA, Brown R, Fenkel JM, et al. Sofosbuvir and Ribavirin Prevent Recurrence of HCV Infection After Liver Transplantation: An Open-Label Study. Gastroenterology 2015;148:100–107.e1. doi:10.1053/j.gastro.2014.09.023.

[28] O'Leary JG, Demetris AJ, Friedman LS, Gebel HM, Halloran PF, Kirk AD, et al. The Role of Donor-Specific HLA Alloantibodies in Liver Transplantation. Am J Transplant 2014;14:779–87. doi:10.1111/ajt.12667.

[29] Kaneku H, O'Leary JG, Banuelos N, Jennings LW, Susskind BM, Klintmalm GB, et al. *De Novo* Donor-Specific HLA Antibodies Decrease Patient and Graft Survival in Liver Transplant Recipients. Am J Transplant 2013;13:n/a-n/a. doi:10.1002/ajt.12212.

FIGURE LEGENDS

Figure 1: Study participant flow chart.

Figure 2: Identification of gene co-expression modules. Heatmap of the 23 gene modules (y-axis) identified by weighted gene co-expression network analysis of the liver tissue transcriptiome. The clinical and histological variables correlated with the gene modules are shown in the x-axis. The colour scale in the heatmap corresponds to the magnitude of the Person correlation coefficients. Correlations with P value ≤0.01 are denoted by asterisks. The CYAN and PINK modules, which showed the most significant correlation with the most prevalent histological abnormalities, were selected for further analysis.

Figure 3: Transcriptional signature of T cell mediated rejection.
A) Expression profiles of the 13-gene signature identified by Predictive Analysis of Microarrays (PAM) that showed the highest accuracy in classifying T cell mediated rejection taking place during immunosuppression withdrawal. The analysis was performed by comparing the expression profile of liver biopsies collected before initiation of drug weaning (baseline) with liver biopsies collected at the time of rejection. Results are expressed as a matrix view of gene expression data where rows represent genes and columns represent hybridized samples. The intensity of each colour denotes the standardized ratio between each value and the average expression of each gene across all samples. Red coloured pixels correspond to an increased

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abundance of the mRNA in the indicated blood sample, whereas green pixels indicate decreased mRNA levels. **B)** Receiver operating characteristic (ROC) curves displaying the overall diagnostic performance of the 13-gene transcriptional signature in classifying liver biopsies as rejecting or non-rejecting. The dotted line curve corresponds to the 4-fold cross-validated data. **C)** Correlation of the CYAN and PINK gene modules with the 13-gene classifier of T cell mediated rejection. To reduce the multi-dimensionality, consensus eigengenes from each of the 3 sets of genes were first generated. Correlation was analyzed using Person's coefficient.

Figure 1: Study participant flow chart

654 Liver Transplants in
589 Patients

Dead (n=312)
Lost to follow-up (n=38)
Recurrent liver disease (n=112)
Chronic rejection (n=6)

121 evaluable patients

No liver biopsy
Refused consent (n=37)
Dialysis (n=6)
Neoplasms (n=5)
Morbid obesity (n=2)
Anticoagulation (n=3)
Extra-hepatic severe disease (n=1)

67 patients were enrolled
and underwent protocol liver
biopsies

49 liver biopsies were
employed for transcriptional
studies

Figure 2: Weighted gene co-expression network analysis of liver tissue transcriptome
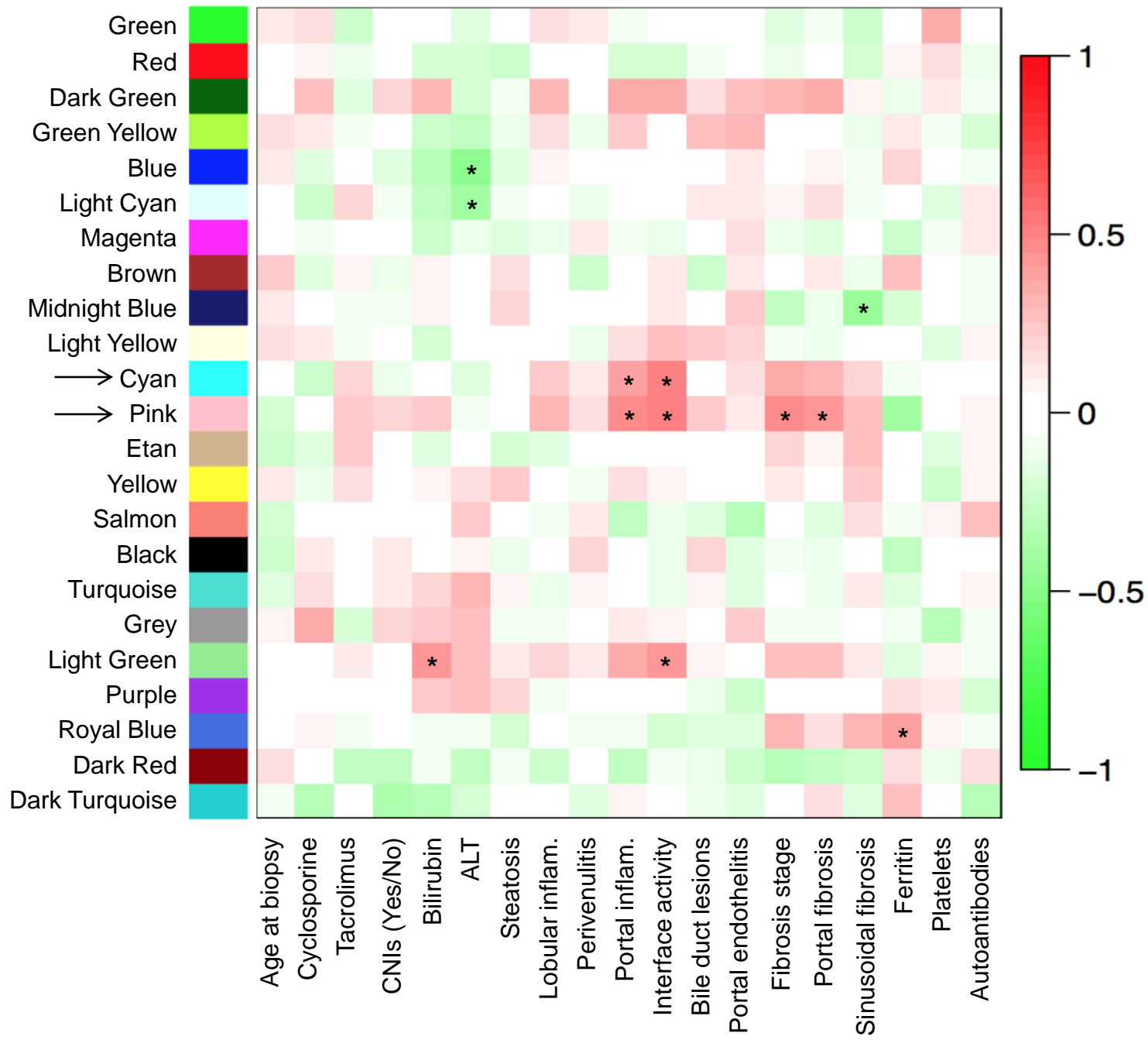
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Figure 3
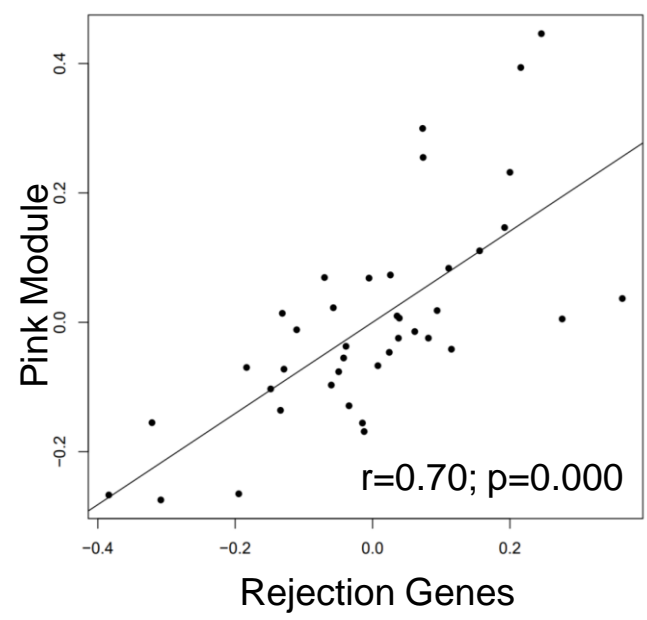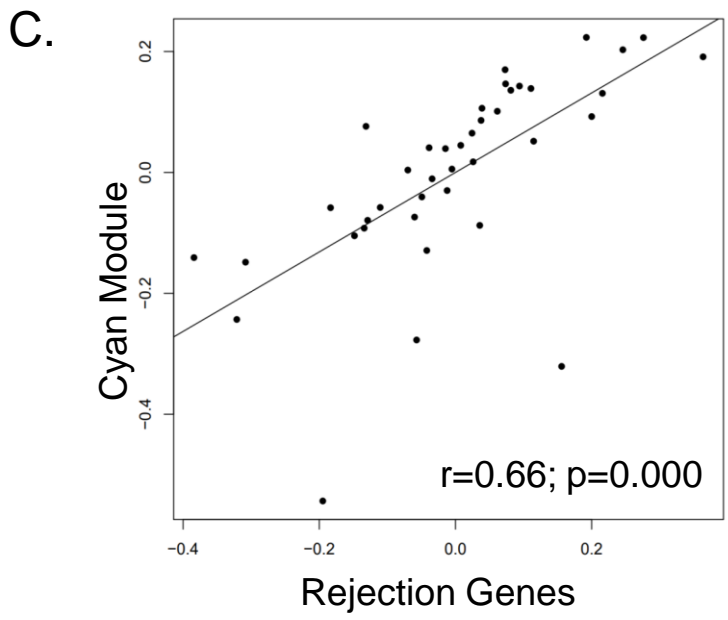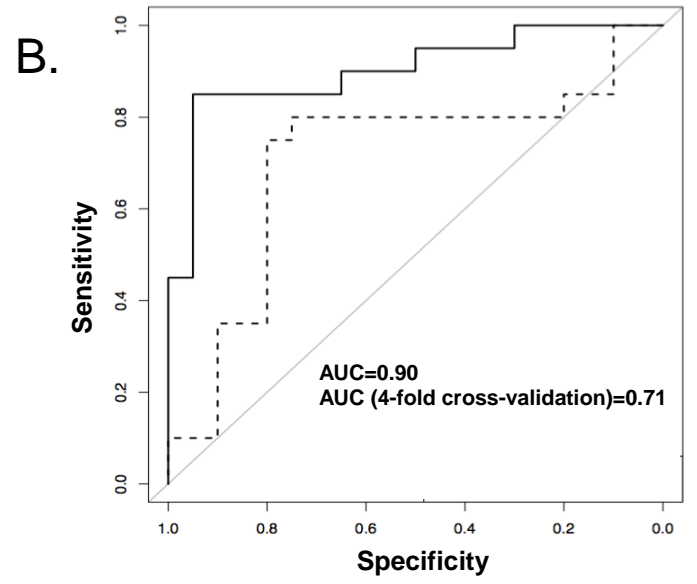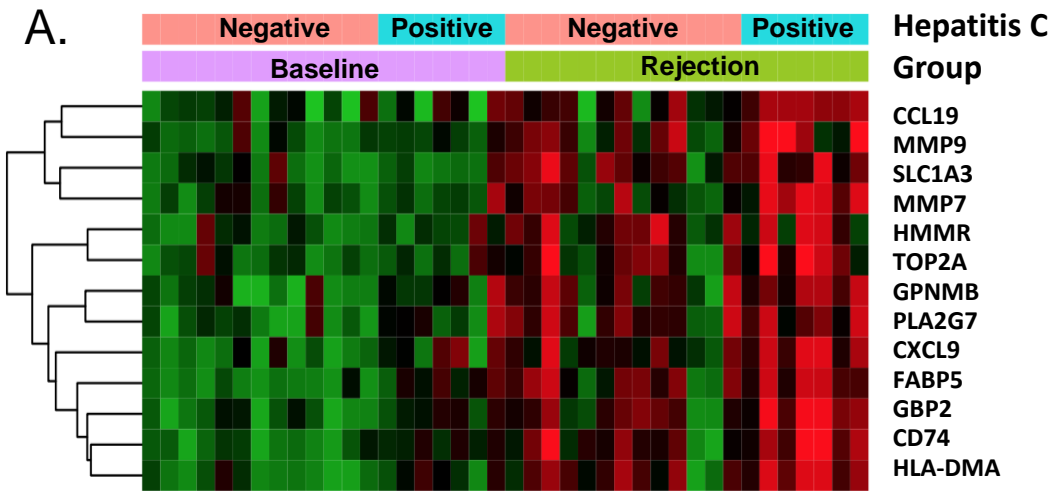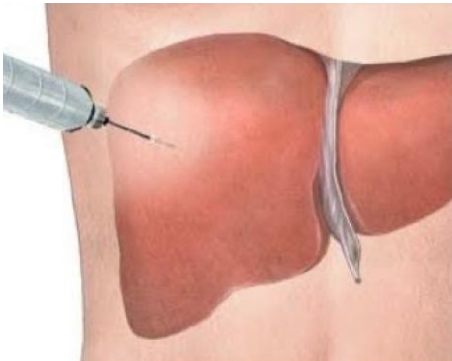
Stable liver transplant recipients >10 years after LT (n=67)

76% had histological abnormalities
-Portal inflammation: 67%
-Interface activity: 27 %
-Fibrosis: 43%

* Correlation p-value <0.01

Gene co-expression modules

Age at biopsy
Cyclosporine
Tacrolimus
CNIs (Yes/No)
Bilirubin
ALT
Steatosis
Lobular inflm.
Perivenulitis
Portal inflam.
Interface activity
Bile duct lesions
Portal endothelitis
Fibrosis stage
Portal fibrosis
Sinusoidal fibrosis
Ferritin
Platelets
Autoantibodies
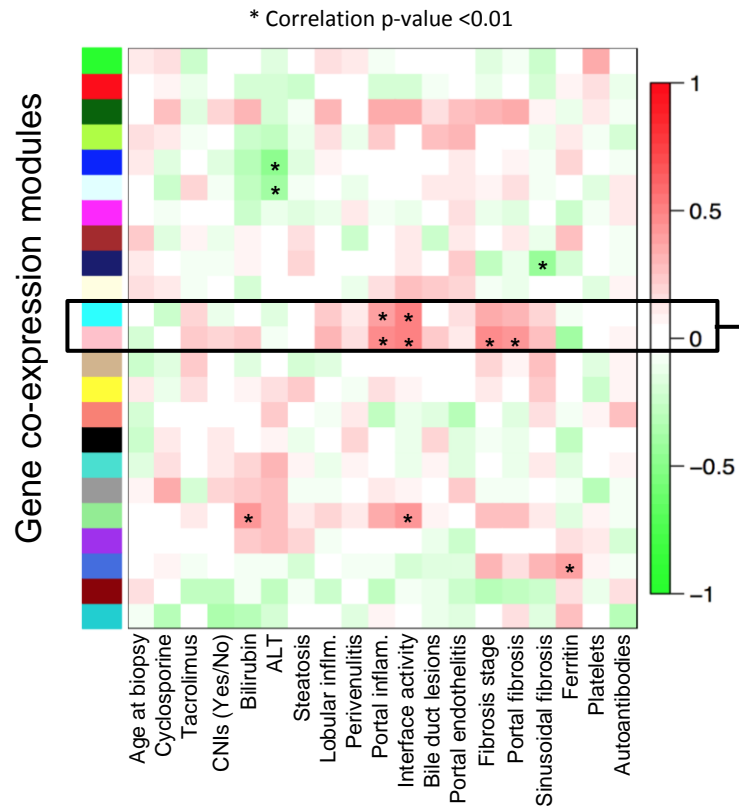
Enrichment in molecular pathways involved in T cell mediated rejection and extracellular matrix remodelling

Table 1. Characteristics of the patients included in the study (n=67)

Characteristic	
Age (years)	60.4 (29.5-75.5)
Male gender (n,%)	52 (77.6%)
Indication for LT (n,%)	
Alcoholic cirrhosis	23 (34.3%)
Hepatitis B-related cirrhosis	15 (22.4%)
Cryptogenic	9 (13.4%)
Fulminant hepatitis B	5 (7.5%)
Unknown etiology fulminant hepatitis	5 (7.5%)
Other	10 (14.9%)
Time from LT to liver biopsy (years)	13 (10-22)
Comorbidities (n,%)	
Diabetes Mellitus	9 (13.4%)
Arterial hypertension	25 (37.3%)
Dylipemia	14 (20.9%)
Cardiovascular disease	3 (4.5%)
Immunosuppression (n,%)	
Cyclosporine (± MMF)	36 (53.8%)
Tacrolimus (± MMF)	11 (16.4%)
mTOR inhibitor (monotherapy)	3 (4.5%)
MMF (monotherapy)	17 (25.3%)
Cyclosporine trough levels (ng/mL)	32 (0-143)
Tacrolimus trough levels (ng/mL)	1.6 (0-7.4)
Sirolimus trough levels (ng/mL)	3.6
Everolimus trough levels (ng/mL)	3.3 (2.5-4.1)
AST/ALT (IU/mL)	26 (11-53)/25 (3-74)
AP/GGT (IU/mL)	173 (24-494)/40 (3-434)
Biochemistry	
Bilirubin (mg/dL)	0.7 (0.3-2.9)
Prothrombin time (%)	94 (59-100)
Albumin (g/dL)	42 (35-68)
White blood cell count (x 10^3/mm^3)	6.15 (2-9.6)
Platelet count (x 10^3/mm^3)	182 (64-355)
Transient elastography (Fibroscan)	4.95 (3.4-33.8)
APRI score	0.34 (0.12-1.48)
FIB4 score	1.61 (0.62-9.20)

Continuous data are expressed as median (range).
Abbreviations: LT (liver transplantation), MMF (mofetil mycophenolate), mTOR (mammalian target of rapamycin).

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Table 2. Histological characteristics of baseline protocol liver biopsies

Histological Evaluation	Median (range)	0	1	2	3	4
Number of complete portal tracts	8 (1-18)					
Number of central veins	6 (0-13)					
Architectural abnormalities	0 (0-2)	No 45 (67%)	Mild 12 (18%)	Moderate-marked 10 (15%)		
Lobular inflammation	0 (0-2)	No 35 (52%)	Mild 27 (40%)	Moderate 5 (8%)	Marked 0	
Central perivenulitis	0 (0-2)	No 56 (84%)	Mild 8 (12%)	Moderate 3 (4%)	Marked 0	
Portal inflammation	1 (0-3)	No 22 (33%)	Mild 29 (43%)	Moderate 14 (21%)	Marked 2 (3%)	
Interface hepatitis	0 (0-2)	No 49 (73%)	Mild 12 (18%)	Moderate 6 (9%)	Marked 0	
Bile duct lesions	0 (0-1)	No 51 (76%)	Mild 16 (24%)	Moderate 0	Marked 0	
Bile duct loss	0 (0-2)	No 62 (92%)	<50% 4 (6%)	>50% 1 (2%)		
Portal vein branches	0 (0-2)	Present in all 42 (63%)	Absence in a minority 11 (16%)	Absence in a majority 2 (3%)		
Portal vein endothelitis	0 (0-1)	No 49 (73%)	Mild 18 (27%)	Moderate 0	Marked 0	
Portal fibrosis (METAVIR score)	0 (0-4)	No 38 (57%)	Minimal 13 (19%)	Moderate 9 (13%)	Bridging 1 (2%)	Cirrhosis 6 (9%)
Perisinusoidal fibrosis	0 (0-2)	No 40 (60%)	Focal 20 (30%)	Marked 7 (10%)		
Esteatosis	0 (0-3)	No 38 (57%)	Mild 20 (30%)	Moderate 6 (9%)	Severe 2 (3%)	

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Highlights

- Histological abnormalities are frequently observed in long-term liver transplant recipients despite the presence of normal or near normal liver tests,

- Recipients with portal inflammation with/without fibrosis exhibit a liver tissue transcriptional profile closely resembling what is observed in the setting of T-cell mediated rejection.

- Recipients exhibiting subclinical lesions and high expression of rejection-associated transcripts are at risk of developing progressive liver damage.

Table 3. Enrichment of rejection and stellate cell associated gene sets in study groups employing Quantitative Set Analysis for Gene Expression (QuSAGE).

Gene Set	Description	*Cyan&Pink High* (FDR)	T-cell mediated rejection (FDR)	Late Rejection (FDR)	Hepatitis C infection (FDR)
QCAT*	Transcripts associated with cytotoxic T lymphocytes defined in purified cell lines; associated with TCMR in renal transplants with expression levels correlating with T cell infiltration.	**0.0006**	0.1300	**0.0143**	0.7020
QCMAT*	Macrophage associated transcripts defined in purified cell lines, associated with TCMR in kidney patients.	**0.0027**	**3e-06**	**0.0241**	0.1060
GRIT1*	Human orthologs of IFN-γ dependent, rejection-associated transcripts defined in mice; expressed in TCMR, especially in association with AMAT1.	**0.0025**	**3e-06**	**0.0143**	**0.0027**
DSAST*	DSA-positive-specific transcripts derived from comparative analysis of DSA with or without renal biopsies; observed both in ABMR and TCMR with higher levels in ABMR.	0.4540	**0.0010**	0.4090	0.5470
AMAT1*	Alternative Macrophage Associated Transcript 1; human orthologs of mouse data; high GRIT plus AMAT1 scores correlate with TCMR correlate with TCMR.	**0.0400**	**0.0203**	**0.0721**	0.1450
IGT*	Immunoglobulin associated transcripts, observed both in ABMR and TCMR and associated with allograft fibrosis.	0.2510	0.8270	0.7760	0.3120
ENDAT*	Endothelial cell associated transcripts derived from purified cell lines; increased in ABMR and TCMR with higher levels in ABMR.	**0.0040**	0.9070	0.2610	0.1450
IRRAT*	Injury-repair response associated with transcripts, defined in early renal transplants with no rejection, derived as a model for acute kidney injury.	0.2570	**0.0064**	0.2610	**0.0917**
NKB*	Natural killer cell-specific transcripts derived from purified cells lines; identified in early TCMR and late ABMR in renal patients.	0.9090	**0.0387**	0.9720	0.1450
TCB*	T cell-specific transcripts based on purified cell lines.	**0.0448**	0.2930	0.5080	0.9430
BAT*	B cell-associated transcripts derived from purified B cells; upregulated in both ABMR and TCMR.	**0.0005**	0.6090	**0.0143**	**0.0354**
MCAT*	Mast cell associated transcripts, associated with scarring and poor survival in renal transplants.	**0.0875**	**0.0266**	0.9250	0.6300
IRITD3*	Injury and rejection induced transcripts upregulated 3 days after isograft transplant (humanized results from mouse model).	**0.0025**	**0.0013**	**0.0421**	0.1450
IRITD5*	Same as for IRITD3 but measured on day 5.	**0.0008**	**0.0203**	**0.0355**	0.1060
Stellate Cell	122-gene set preferentially expressed in hepatic stellate cells and that correlates with the extent of liver fibrosis and is associated with patients survival in cirrhosis.	**0.0880**	0.1890	0.7760	0.6400

* Pathogenesis-Based Transcript gene sets from the Alberta Transplant Applied Genomics Centre.
Abbreviations: TCMR (T cell mediated rejection), ABMR (antibody mediated rejection), DSA (donor specific antibodies), FDR (False Discovery Rate).

Table 4. Characteristics of patients grouped according to their baseline liver tissue module gene expression profile

Variable	Cyan&Pink_High (n=12)		Cyan&Pink_Low (n=13)		p value (change in High vs Low groups)
	Baseline	Last Follow-up	Baseline	Last Follow-up	
Bilirubin (mg/dL)	0.8 (0.6-2.7)	1 (0.4-45)	0.6 (0.3-1.9)	0.6 (0.2-1.7)	
mean change#	0.15 (-0.06 , 0.41)		-0.10 (-0.22 , 0.09)		0.0475
AST (IU/mL)	29 (17-44)	30 (25-60)	22 (13-53)	21 (13-41)	
mean change#	3.90 (-0.17 , 8.39)		-0.44 (-4.92 , 4.43)		0.1005
ALT (IU/mL)	25 (16-45)	26 (18-76)	17 (3-61)	18 (8-61)	
mean change#	2.47 (-1.923 ,8.81)		-4.90 (-9.00 , 0.55)		0.0124
GGT (IU/mL)	24 (13-345)	29 (15-662)	51 (10-118)	47 (3-303)	
mean change#	28.05 (-1.85 , 80.35)		4.70 (-13.04 , 36.30)		0.2360
INR	1.1 (1-1.3)	1.0 (1-2.4)	1.0 (1-1.1)	1.0 (0.8-3.6)	
mean change#	0.02 (-0.03, 0.08)		0.04 (-0.01, 0.10)		0.2371
Platelet count (x10^9)	155 (76-262)	145 (15-662)	219 (116-333)	262 (97-370)	
mean change#	-19 (-51 , 12)		51 (7, 100)		0.0025
APRI	0.44 (0.2-1.45)	0.51 (0.3-4.6)	0.268 (0.12-0.96)	0.25 (0.09-0.89)	
mean change#	0.11 (0.02, 0.25)		0.01 (-0.07, 0.09)		0.0277
FIB4	1.8 (0.9-7.1)	2.47 (1.2-23)	1.61 (0.6-2.4)	1.24 (1-3.48)	
mean change#	1.23 (0.26, 3.24)		0.35 (-0.16, 1.34)		0.1109

Baseline and follow-up data are expressed as median (range).Variables were compared by a non-parametric ANCOVA. P-values correspond to the comparisons of the changes observed in the two groups, adjusted by the baseline values and the time of follow-up. # Estimated mean change (with 95% CI), adjusted for mean baseline values and mean length of follow-up