Study of **L**iver allograft - **I**mmune mediated **F**ibrosis post-**T**ransplant (**LIFT**), predicting fibrogenesis and digital quantification of histological characteristics

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Promotor: Professor Etienne Sokal

The pictures seen on the cover represents a screenshot of the digital quantification of liver fibrosis in the portal tract process module functioning.

I, Prof. Etienne Sokal, MD, PhD certify that Dr. Sharat Varma has made this study under my mentorship and supervision. After having read and revised the contents of this thesis, I consider it meets the appropriate scientific requirements. It is suitable to be presented and defended as a PhD thesis.

Prof Etienne Sokal

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To

my mentor, colleagues, friends and family for their inspiration, belief and encouragement.

Deepam – my best buddy, for all that is

Monaal – my baby friend, for your smile and time

Mom's Dad's and didi – for showing me "karma" by example

Prof Sokal – for opening up the world to me

"Dream – Explore – Inspire"

Acknowledgements

This research and PhD would have never been possible without the collaboration, support and encouragement from many colleagues, friends and family members. These years have been period of incredible fun, enjoyment, learning and professional growth. An incidental meeting with Prof Etienne Sokal at Taipei was the defining moment for this journey, where he became my mentor and guide. It's his unflinching support, quest for results, ethics and risk taking - which been the driving force behind this project.

I carried forth the work whose foundations were laid by Carla Venturi and Prof Raymond Reding a few years earlier.

The passion for histology and seeing beyond the evident on the microscope with Prof Mina Komuta was always intellectually stimulating. Over the years we saw innumerable slides together, learnt a lot and created a fertile ground for ideas to seed and germinate. From the deep archives to requesting Yves Guiot for always a bit more, I always found support in the pathology department. Prof Tania Roskams provided an in-depth understanding of the fibrosis mechanisms and extended the valuable support of her lab.

When we started our work on immune mediated allograft fibrosis in children, we were walking on an unexplored path. A major aspect of our study was based on the HLA antibody mediated responses. This novel approach could be done only by the dedicated help from Prof Dominique Latinne and her team. She individually went through each report to identify the pathogenic variants etc and made sense of the huge amounts of data that were generated.

Discussing portal tracts and fibrosis as we ran, Jerome made statistical sense of all the observations we had. His infectious sense of perfectionism and teaching skills ensured that finally even I could start to understand the intriguing world of numbers and statistics. In our endeavor to improve the histological evaluation, Caroline Bouzin opened a door each time we hit a blind end. Digital histology from our imagination was translated to reality by her dedicated problem solving.

The service of pediatric gastroenterology and Unit 92 made me feel welcome and comfortable. Prof Xavier Stephenne motivated me to start a PhD when I was a fellow and subsequently did not let me quit, when I wanted to. Prof Francoise Smets and Isabelle Scheers always ensured an enriching academic discussion. Catherine and the team of infirmiers were my best teachers of French and audience of my language practice sessions. The numerous stagiares and assistants that I had opportunity to work with, taught me a lot and some became good friends.

Prof Raymond Reding incisively dissected my proposals, work and provided valuable insights. He together with Catherine de Magnee made pediatric liver transplantation seem like the most routine pediatric surgical procedure. Roberto explained me the surgical aspects and we had many excellent discussions.

Julia Vanessa and Sonia helped enormously for study protocols, sample collection among other things. Without the support from Magdha "Wikipedia of pediatric liver transplant at St Luc" this study could have never been started. Ilse with her impeccable planning ensured a smooth administrative sailing course.

At PEDI lab the multinational – multicultural environment was empowering and stimulating. Catherine is the keystone of the lab and had the most calming effect on frayed nerves. Mustapha helped with his “out of the box” approach. The energy and expertise of Joachim, Florianne, Jonathan and Charlotte ensured that technical details are not overlooked and don’t impede progress. Pierre and Tanguy completed the trio in the loudest office which had most fun. Work, run, swim and fun seamlessly merged in our daily routines. Louise added some sanity and perspective.

This project could not have been completed without the effort of Pauline, Elise and Nour. They came in as students and did one important portion independently. Sandrine Wollanders provided prompt administrative support which made it an effortless exercise.

Contents

Summary

Protocol biopsies (PB) from stable liver transplant (LT) recipient children frequently exhibit idiopathic fibrosis. We have limited understanding about the natural history of allograft fibrosis progression, its predisposing factors, role of humoral immune response and possibility to predict future fibrogenesis. The aim of this PhD study was to analyze these aspects in stable pediatric LT recipients using serial PB's and to develop methodology for digital fibrosis quantification.

The study was structured to first analyze the natural history of allograft fibrosis progression, then evaluating the predisposing factors, defining clinically valid cut off values for these factors and finally devising methods to predict the fibrogenesis. Simultaneously we developed novel digital histological evaluation protocols for the liver.

At first, we analyzed 281 serial PB from 89 children to understand the evolution of allograft histology. The PBs were taken as per a uniform methodology, at 1–2, 2–3, 3–5, 5–7, and 7–10 years post-LT. Thee were evaluated for inflammation and fibrosis using liver allograft fibrosis score (LAFSc). We used mixed model statistical tools for analyzing repeated observations from the same patients i.e. longitudinal progression in the cohort for histological features of the allograft. Further as the outcomes of fibrosis and inflammation were ordinal, we factored that in the statistical tools used. Our findings of this study were contradictory to previously published literature. We observed that allograft inflammation and fibrosis evolution trend were patient specific characteristics and no general trend could be identified, while it's a common thread in the published reports that allograft fibrosis is progressive, universal and correlated to post LT duration. These contradictions were explicable based on serial protocol biopsies and appropriate statistical tools used in our study. Further, portal fibrosis was seen to be dynamic, with frequent increase and decrease; and not being a one way irreversible process.

Evaluation of the predisposing factors for allograft fibrosis revealed that presence of portal inflammation, HLA-DRB1*03/04 allele and post-LT class II donor specific HLA antibodies (DSA) (p =

0.03; p=0.03, p=0.02 respectively) were the significant factors. In contrast to the portal fibrosis, centrilobular fibrosis was persistent and was higher in recipients who received a deceased donor graft.

Among the predisposing factors the donor specific HLA antibodies attracted our maximal interest. There are many reports on the role of HLA antibodies among adult LT recipients but few among pediatric LT recipients. The current understanding is limited as we yet do not understand if all the subtypes are equally involved and if there exist any specific cut off values (MFI) beyond which these antibodies assume significance. We addressed these aspects in our second study. The HLA antibody detection and specificity typing was performed in 102 LT recipient children using the Luminex platform. We found that among all the subtypes of HLA antibodies pre or post LT, only the post LT class II DSA of the DQ subtype could be considered as predisposing to portal inflammation and consequent fibrosis. Further using the MFI cutoff at 5000, had a sensitivity and specificity of 83.3% and 71.4% to predict portal inflammation. These findings are significant as it facilitates the applicability of these detection tests on routine clinical practice. We suggest that if on screening (which is an economical test) we find MFI >5000, then we should proceed for the expensive analysis to confirm the subtypes and establish if they are donor specific. This algorithm makes it cost effective and increases the possibility for universal application.

After determining the predisposing factors for allograft fibrosis, we focused on predicting the course of fibrogenesis i.e. the possibility to predict the change in fibrosis on basis of the current biopsy findings. Activated hepatic stellate cells express cytoplasmic ASMA prior to secreting collagen, accordingly we hypothesized that quantifying ASMA could predict future fibrogenesis. The rationale being that activated HSC's need to secrete significant quantity of collagen to result in histological fibrosis. This time lag between activation and hepatic fibrosis would provide us the window, to quantify the ASMA positivity ad predict the soon to be deposited collagen and resulting fibrosis. For this, 32 pairs of serial protocol biopsies i.e. "baseline" and "follow-up" biopsies taken at 1-2 year

intervals from stable pediatric LT recipients were selected. Morphometric quantification of “ASMA positive area percentage” was performed on the baseline biopsy and correlated to the difference of fibrosis severity between the “baseline” and “follow-up”; which was termed “prospective change in fibrosis”. Significant association was observed using Metavir (p value = 0.02), cumulative LAFSc (p value= 0.02), and portal LAFSc (p value = 0.01) values. ASMA positive area percentage >1.05 predicted increased fibrosis on next biopsy with 90.0% specificity. Additionally, an association was observed between extent of ASMA positivity and concomitant ductular reaction (DR) (p=0.06). Hence we confirmed that ASMA quantification can predict the future course of fibrosis. The observation of DR being associated with ASMA quantification, provoked us to evaluate the role of DR in fibrogenesis as well.

DR contains proliferating hepatic progenitor cells, cholangiocytes and is assessed by CK7 staining. In cases where there is no biliary obstruction, its suggested that DR is the outcome of hepatocyte replicative arrest and stimulation of the progenitor cells. The DR has been correlated to the severity of fibrosis in HCV, NASH and hemochromatosis. But what remains unclear is whether DR is the cause or effect of fibrosis. To study this we designed the following study. In 72 paired serial biopsies, we stained the baseline biopsy for CK7 and did morphometric quantification of “CK7 positive area percentage”. This was correlated to the difference of fibrosis severity between the “baseline” and “follow-up”; which was termed “prospective change in fibrosis”. We observed no correlation between these parameters, but in contrast we observed that CK7 quantification correlated to the severity of concurrent fibrosis i.e. CK7 quantification on baseline biopsy correlated to fibrosis on baseline biopsy. This implied that CK7 and DR were not predictive of fibrosis but could be considered as co-associated. Hence DR is not fibrogenic but could be the outcome of fibrosis. This is also supported by experimental data that show that changes in the extra-cellular matrix is a strong promoter of DR.

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Digital evaluation and quantification of histological characteristics has the potential to address the unmet need of finding an objective, reproducible and accurate tool to quantify liver fibrosis. A digital image analysis tool to quantify the fibrosis in the complete biopsy, termed as collagen proportionate area (CPA) and in portal, sinusoidal and centrilobular regions was developed by us. Automated region localization on basis of density, texture and quantification of histological characteristics based on pixel counting was the underlying principle. This was optimized and validated in the pediatric LT and non-LT scenario by us. The CPA total and regional showed a strong correlation to the visually assigned Metavir and LAFSc scores. The strength of this modality was much increased capability to discriminate between different fibrosis severities and quantify the fibrotic tissue distribution in a biopsy rather than identification of fibrosis patterns.

Overall, in these studies we have could address allograft fibrosis as a complete entity by digital quantification of fibrosis, identification of predisposing factors, determining the relative importance DQ subclass of HLA antibodies and MFI levels, and finding methods to predict the future fibrogenesis course.

Abbreviations

LT: Liver transplantation

LB: Liver biopsy

PSR: PicoSirrus Red

LAFSc: Liver allograft fibrosis scoring system

AIH: Autoimmune hepatitis

CPA: Collagen proportionate area

SE: Strain elastography

SWE: Shear wave elastography

MRE: Magnetic resonance elastography

miRNAs: MicroRNAs

RNA: Ribonucleic acid

PB: Protocol biopsy

AIH: Autoimmune hepatitis

PSC: Primary sclerosing cholangitis

ANA: Anti-nuclear antibody

SMA: Smooth muscle antibody

LKM: Liver kidney microsomal antibody

HLA: Human leukocyte antigen

DSA: Donor specific antibody

ASMA: Alpha smooth muscle actin

CK7: cytokeratin 7

HSC: Human stellate cell

IHC: immunohistochemistry

AUROC: Area under receiver operating characteristic

CPA: collagen proportionate area

APP: Image analysis protocol

DR: Ductular reaction

Introduction

Liver transplantation (LT) is one of the most remarkable healthcare achievements of the last three decades. This is attributable to the unique characteristics of the liver as an organ, enhanced surgical, medical skills and the innovation of new immune-suppressive drugs (1;2). Currently the 5 year survival among children having undergone LT is in excess of 90% though the histological examination on liver biopsy (LB) reveals significant changes (fibrosis and inflammation) in 90% of the recipients within one year post-LT (3-10). These findings are a source of concern as the mechanism of this fibrogenesis is not well understood making it impossible to take steps to mitigate this process, as of now (6;10;11). The lack of objective methods to estimate hepatic fibrosis on the "gold standard" of liver biopsy further add another dimension to the problem of understanding allograft fibrosis(6;12-16). In our endeavor to advance towards the goal of maintaining a healthy liver allograft the following research was performed, focusing on allograft fibrosis – estimation, mechanisms and prediction.

Background

Chapter 1: Liver fibrosis quantification

In this chapter are described the various methods of liver fibrosis estimation, their rationale, advantages and drawbacks. The ideal method of quantifying liver fibrosis should be precise, sensitive and reproducible. It should discriminate between adjacent fibrosis stages, progression or regression and give clues to relevant etiological mechanisms. For such a methodology to be clinically applicable it should also be reasonably easy to perform.

Liver fibrosis estimation can be done on histological specimens obtained on performing LB i.e. *invasive fibrosis estimation*. Thereafter quantification of fibrosis is done by microscopic examination using various observer dependent semi-quantitative scores or by computer assisted image analysis i.e. morphometry. Hepatic fibrosis can also be assessed without access to histological tissue .i.e. without performing a LB, called *non-invasive fibrosis estimation.* This is done using specialized radiology modules e.g. Fibroscan or certain biomarkers eg Fibrotest, present in the blood that correlate to fibrosis severity (14-22).

Invasive fibrosis estimation methods

The liver tissue obtained by LB is processed prior to examination. The processing involves fixation in paraffin blocks, obtaining 5 micron thick sections on glass slides and specifically staining the fibrotic tissue. This enables the visualization of the fibrosis pattern and severity on the specimen, which can be done either by microscopic examination or by digital pathology (computerized methods)

Visual examination based semi-quantitative scoring systems for liver fibrosis assessment:

Most commonly used methodology of staging liver fibrosis is by applying various semi-quantitative ordinal scores e.g. METAVIR, Scheuer and Ishak systems. These systems intend to represent categories of increasing severity based on a combination of patterns and

quantity of collagen deposition. These were developed for adult patients having chronic viral hepatitis, hence their applicability to pediatric LT recipients is sub-optimal (27-30). This concern has been addressed by the development and validation of the liver allograft fibrosis scoring system (LAFSc) (9;10).

However, all the visual based scoring systems have similar inherent flaws. Firstly, the grading of severity is based on identification of various patterns of fibrous but not on degree of fibrosis or amount of fibrosis, hence have limited applicability in evaluating fibrosis evolution. Secondly as the outcome of these scoring systems is ordinal and not continuous, this makes the statistical interpretation of these scores difficult and often inaccurate e.g. use of "mean" Metavir or LAFSc is misleading, inappropriate. Thirdly, being observer dependent and semi-quantitative scores they have a very high inter and intra-observer variability, which does not permit for standardization of fibrosis estimation (31-33). Fourthly, these systems were established in the era where fibrosis was considered as a one-way street and never thought to regress. As they do not include any pattern of fibrosis regression that can be identified or included in the score, they do not accurately evaluate the fibrosis in a case of regressing liver disease. Lastly as cirrhosis is assigned to a single category, it does not fulfill our current need to discriminate between severities of cirrhotic patterns. This is clinically needed as discrimination of cirrhosis sub-stages can predict the clinical outcome and guide accurate therapeutic intervention (14;34-36).

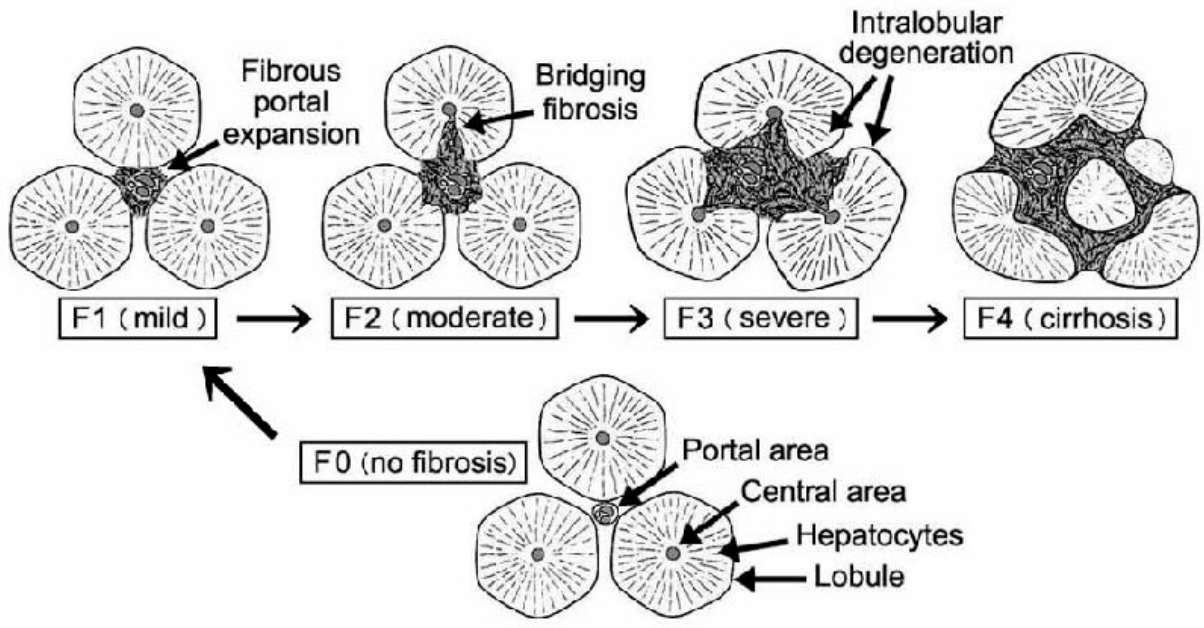
The most commonly used scores are described below:

i. METAVIR system: It was developed in 1994 for chronic hepatitis C infection (30). It grades the severity of fibrosis and inflammatory activity seen on a LB specimen. Fibrosis is graded on a scale from 0 to 4, as described in table1 & figure 1. The system is rather simplified and the scoring is primarily focused on the fibrosis pattern seen in the portal regions.

Table 1: METAVIR system of fibrosis grading

Histological pattern	METAVIR fibrosis grade
No fibrosis	F0
Fibrous portal expansion	F1
Few bridges or septa	F2
Numerous bridges or septa	F3
Cirrhosis	F4

Figure 1: Diagrammatic representation of METAVIR fibrosis scoring scheme



ii. Ishak et al system: This was developed in 1995 and tailored for autoimmune hepatitis (AIH) (28;29). Fibrosis is graded on a scale from 0 to 6, as described in table 2 (12). Expectedly as this system was developed for AIH, it also focused on the portal region.

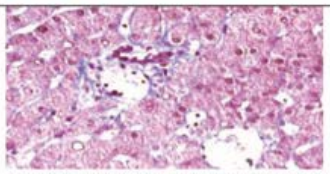
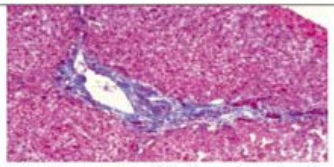
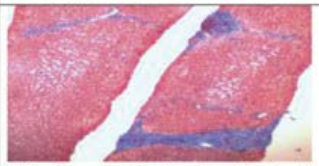
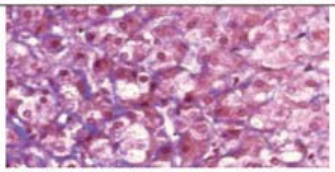
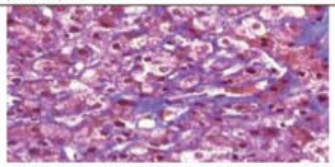
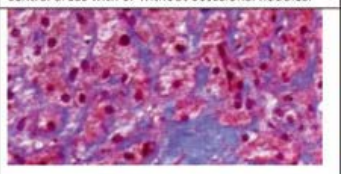
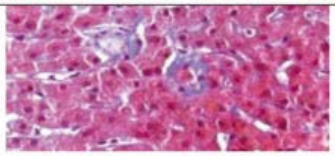
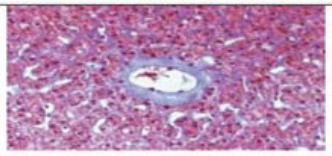
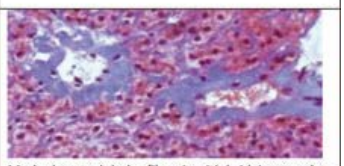
Table 2 ISHAK scoring system

Histological pattern	ISHAK stage
No fibrosis (normal)	0

Fibrosis expansion of some portal areas +/- short fibrous septa	1
Fibrosis expansion of most portal areas +/- short fibrous septa	2
Fibrosis expansion of most portal areas with occasional P-P bridging	3
Fibrosis expansion of portal areas with marked P-P , P-C bridging	4
Marked bridging (P-P and / or P-C) with occasional nodule (incomplete cirrhosis)	5
Cirrhosis	6

iii. LAFSc: This was developed in 2012 by Venturi et al in our unit, to assess liver allograft fibrosis and was specifically designed for children (9). The main advantage of the LAFSc is the regional assessment of fibrosis in the portal tracts, sinusoids and centrilobular vein area. Each area is assigned equal weight in this scoring system as described in table 3. This system has been subsequently validated and adopted by most pediatric LT centers in the world.

Table 3: Histologic features and staging definitions of the liver allograft fibrosis semiquantitative scoring system (LAFSc)

Structure	0	I	II	III
Portal Tract	No Fibrosis	 Non-expanding fibrosis in less than 50% of portal tracts.	 Fibrosis in more than 50% of portal tracts and/or expansion into short fibrous septa into periportal parenchyma.	 Marked expansion of most or all portal tracts with bridging fibrosis expanding to other portal tracts or central areas with or without occasional nodules.
Sinusoids (zones 1, 2)	No Fibrosis	 Little fibrosis with thin focal collagen deposits involving less than 50% of sinusoids.	 Little fibrosis with thin diffuse collagen deposits involving more than 50% of sinusoids, or thicker but focal fibrosis in less than 50% of sinusoids.	 Thick, marked, diffuse sinusoidal fibrosis.
Centrolobular Vein (zone 3)	No Fibrosis	 Circular perivenular fibrosis involving less than 50% of central veins without invasion into the perivenular parenchyma.	 Circular perivenular fibrosis in more than 50% of central areas and/or expansion into short fibrous septa into perivenular parenchyma.	 Marked centrolobular fibrosis with bridging to other central areas and/or portal tracts.

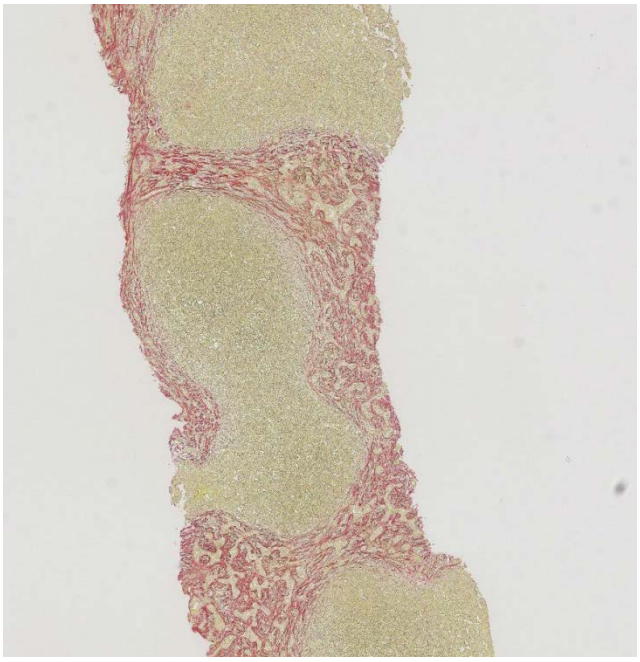
Computer assisted image (morphometric) analysis for quantitative assessment of liver fibrosis: To overcome the limitations of semi-quantitative scoring systems, researchers have been trying to develop a morphometric tool for assessment of fibrosis. Herein digital scanning of a PSR slide is done and the area that is stained red i.e. collagen is quantified using pixel counting. This is compared against the total area of the tissue. On doing a proportion of the two we arrive at the index of collagen proportionate area (CPA) (37-40). The process is demonstrated figure 2. The CPA is a continuous variable that measures collagen presence in the complete biopsy sample. Additionally, it considers the fibrosis quantification on the whole biopsy and not only on certain fields that are being visualized under the microscope.

This method though is theoretically objective, suffers greatly from variances of staining quality, staining procedure, threshold cut off taken for pixel counting and software optimization. It also does not consider the relative fibrosis deposition in different regions of the liver. Although many studies have shown that CPA corresponds well to other histological scoring systems and clinical outcomes, unless it provides a spatial distribution pattern of fibrosis distribution it would not be useful (10;41-

44) . The further refinement of this technique to provide topographical CPA information would make it more intuitive and suitable for widespread clinical practice.

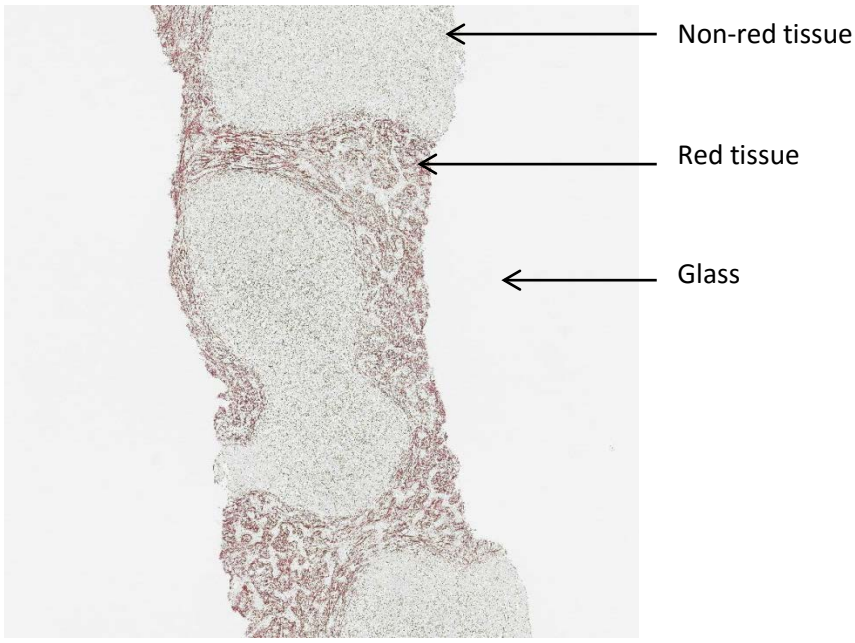
Figure 2: Steps involved in calculation of collagen proportionate area (CPA)

2(a) Scanned image of pico-Sirrus Red staining for liver fibrosis at 10x magnification



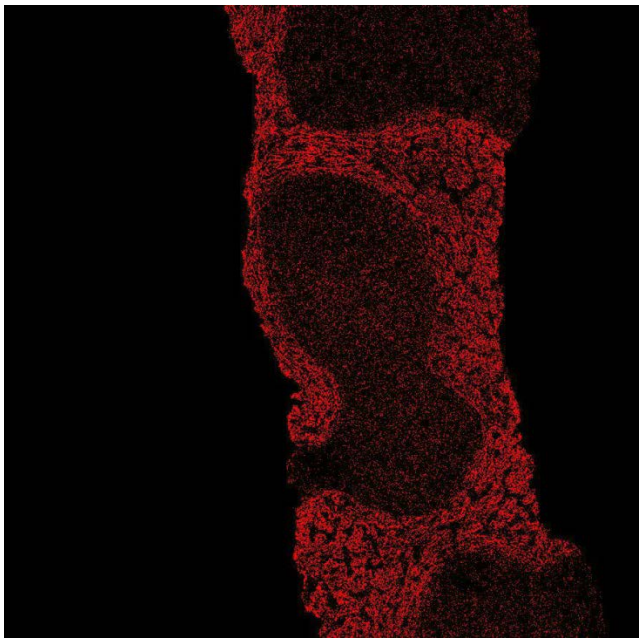
(Personal picture – Sharat Varma)

2(b) Identification of areas below and above cut-off threshold for positive staining and tissue. Ie tissue is separated from glass and within the tissue there is separation of the two staining characteristics based on pixel color



(Personal picture – Sharat Varma)

2(c) Application of complete tissue mask. The red area shows fibrosis and the black is represented by non-fibrosed tissue. To calculate the CPA = (red area/black tissue + red area) *100



(Personal picture – Sharat Varma)

2. Non-Invasive liver fibrosis estimation methods

The principle of using blood tests to quantify hepatic fibrosis is based on the principle that changes in the extracellular matrix (ECM) – either secretion or degradation result in the release of certain biomarkers that can be quantified in the blood.

The ECM secretion would result in increase of the liver stiffness, which can have assessed by radiological methods for noninvasive estimation of liver fibrosis.

a. Biomarkers of liver fibrosis estimation: Many of the serum biomarkers are enzymes that are measured in routine laboratory tests but are not specific to the liver and can be released upon inflammation of other tissues. These markers are not of much use by themselves but useful when combined in marker panel (15;45;46) which have been established for clinical use in the recent years. The most common ones are summarized in table 4(46;47). They are increasingly useful in the exclusion of advanced fibrosis and cirrhosis but do not distinguish well between early and intermediate stages of fibrosis. The other drawback being that although some of these markers are now established in clinical use, their prognostic value is not clearly established.

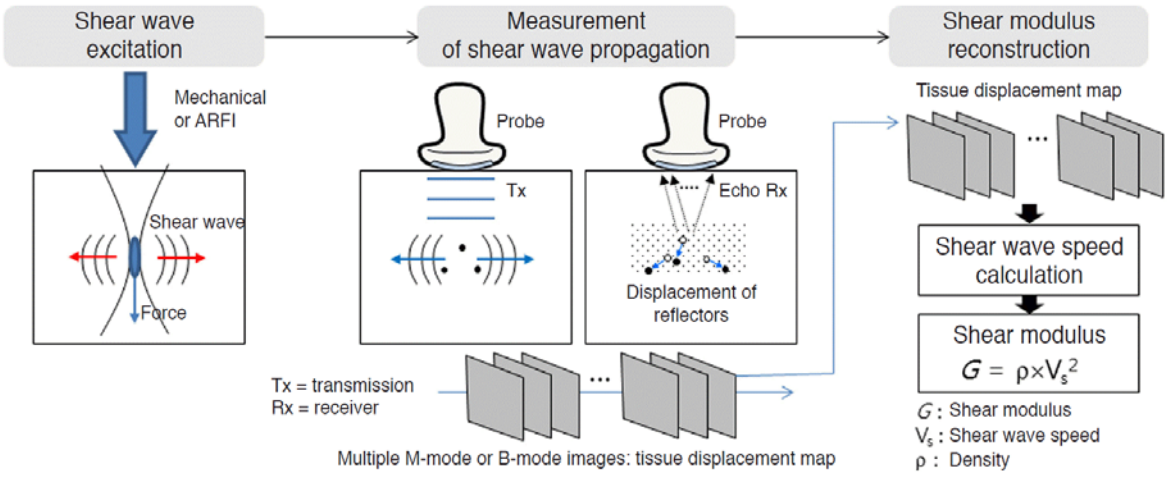
Table 4: Non-invasive fibrosis estimation markers and their diagnostic accuracy

Test	Parameters	Prognosis	Sensitivity	Specificity	AUROC
APRI	AST, platelet count	Significant fibrosis	81	55	0.77
FIB-4	Platelet count, AST, ALT, Age	Significant fibrosis	64	68	0.74
Fibrotest	Haptoglobin, alpha2-macroglobulin, apolipoprotein A1, GGT, bilirubin	Significant fibrosis	92	38	0.79
Forn's index	Age, platelet count, GGT, cholesterol	Significant fibrosis	88	52	0.76

HA	Hyaluronan	Significant fibrosis	-	-	0.75
HepaScore	Bilirubin, GGT, hyaluronan, alpha2-macroglobulin, age, gender	Significant fibrosis	66	79	0.79

b. Radiology based techniques for liver stiffness estimation: A wave once stimulated in a medium, propagates with a velocity and wavelength which depends on the stiffness of the medium. Application of this principle is the basis of ultrasound elastography and MR Elastography. As in liver fibrosis the ECM deposition increases the liver stiffness; the velocity and wavelength of the wave propagating in the liver increases as the stiffness or fibrosis increases (20;48-50). The production of the wave, assessment of the propagation characteristics and the correlation to the liver fibrosis is done by specific software and hardware which are required to perform the elastography tests. The principle of elastography is outlined in figure 3.

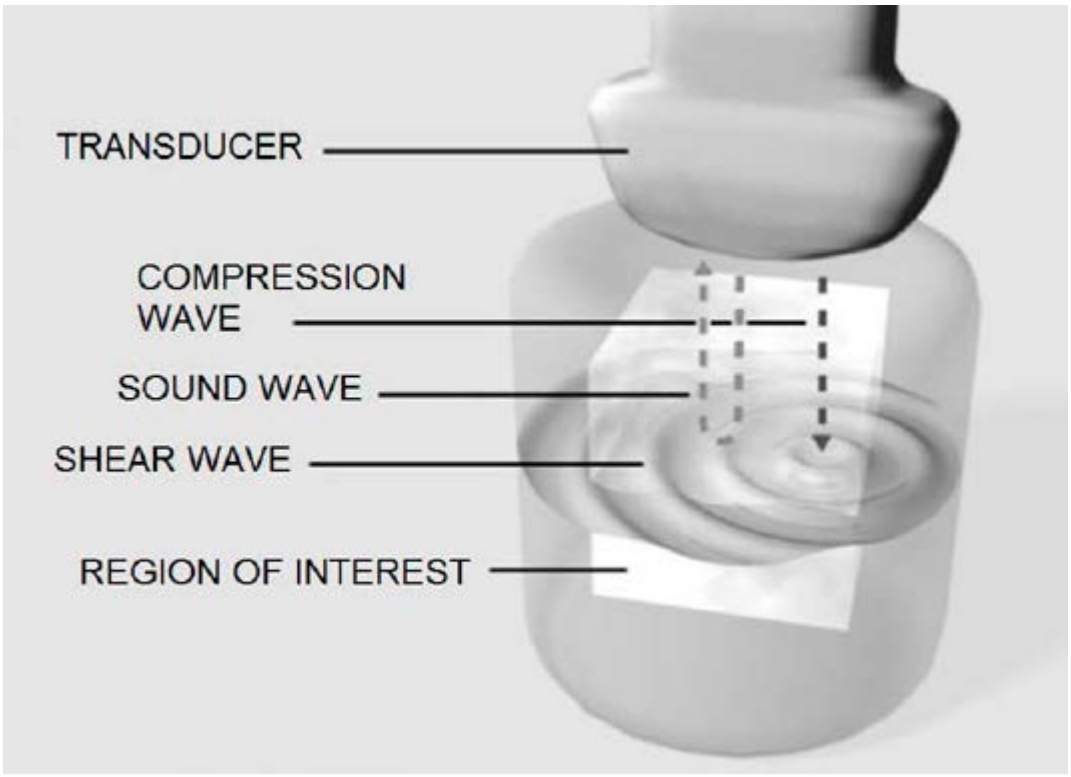
Figure 3: Basic principle of shear wave, compression wave elastography - The motion of the compression or shear wave is detected by the sensor in the transducer probe. The speed of the wave propagation is dependent on the stiffness of the tissue and consequently indicative of the hepatic fibrosis.



(Image from brochure of ARFI)

i. <u>Ultrasound elastography:</u> There are two types of US elastography, strain elastography (SE), also named real time elastography and shear wave elastography (SWE), as shown in Figure 4 (20;48;50-53). SE is a qualitative technique and evaluation of the tissue stiffness is obtained after manual compression. SWE is a technique that provides a quantitative measure of stiffness, expressed in meters per second (the shear wave speed) or in kilopascals (Young's Modulus) after an acoustic/ mechanical pulse induced by the machine itself. Among SWE methods Fibroscan is the only non-imaging method, while Acoustic Radiation Force Impulse (ARFI) (Siemens, Erlangen, Germany and Philips) and 2D-Real Time Shear Waves Elastography (2D-SWE) (Aixplorer system, Supersonic Imagine, Aix-en-Provence, France) are both methods implanted in ultrasound machines

Figure 4: Schematic diagram showing compression and shear waves travelling through a medium. The compression waves move particles in the medium along the direction of motion, while shear waves result in particles oscillating perpendicular to the direction of motion.



(Image from brochure of ARFI)

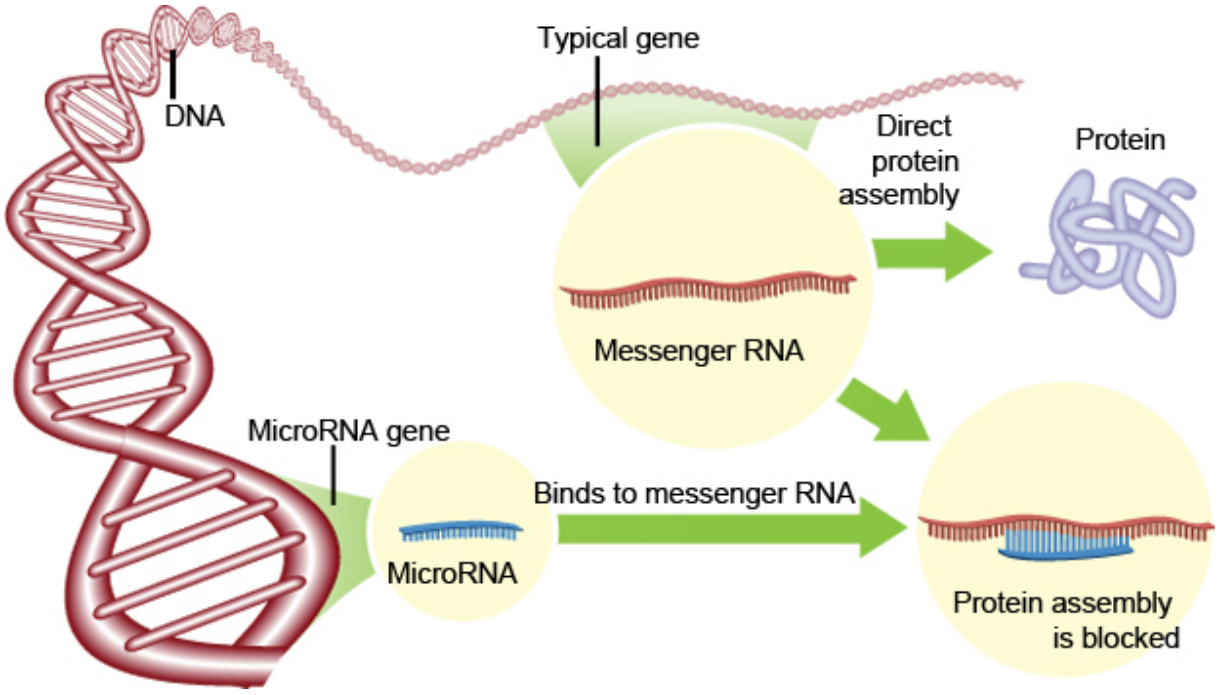
ii. MR Elastography: Like ultrasound elastography, MR Elastography (MRE) is based on the fact that the velocity and wavelength of the wave propagating in the tissue increases as the stiffness of the medium increases, e.g., the fibrotic liver. A device generates acoustic pressure waves at 40-120 Hz which are transmitted to the liver, where shear waves are created. The software that captures the images depicting the propagation characteristics of the mechanical wave generate quantitative stiffness map. The quantitative assessment of fibrosis assessed by MRE correlated significantly with the histological stage of fibrosis. It has also much higher inter-observer agreement. As opposed to ultrasonography, MRE is not affected by lack of acoustic window, obesity or presence of ascites and it is not operator dependent (20;54). In a meta-analysis of 12 studies done by Singh et al (55) using LB as a standard, MR elastography was found to be highly accurate for the diagnosis of advanced fibrosis independent age, sex, BMI, inflammation, and etiology of the liver disease. Limitations of MRE are its cost and the fact that it is time consuming. Liver stiffness may be affected also from hepatic iron overload, steatosis, vascular congestion, cholestasis, and portal hypertension. In these cases, the accuracy of MRE may be altered (54-59).

3. Newer investigational tests

MicroRNAs (miRNAs): The non-coding RNA molecules that perform functions in RNA silencing and post-transcriptional regulation of gene expression are called miRNAs as shown in Figure 5. The hepatic miRNAs have been seen to predict inflammation and rejection (11;60). miR-122 specifically has been the focus of attention lately, and has been explored as a potential biomarker for alcohol induced liver disease and HCV (61-63). The expression of miR-122 and miR-148a were found to be elevated post-LT in

patients with liver injury, acute rejection and positively correlated with aminotransferase levels. They seem to be promising candidates as early, stable, and sensitive biomarkers of hepatic injury after liver transplantation, though their role is being explored (64).

Figure 5: The micro-RNA molecules perform functions in RNA silencing and post-transcriptional regulation of gene expression



Chapter 2: Liver allograft fibrosis after liver transplantation

LT has been established as a successful modality and most pediatric liver diseases are potentially curable with LT (1;2). In contrast to the adult LT recipients where primary disease recurrence is common, among children the liver diseases are most often non-recurrent and cured by the LT. In this scenario, it's expected that the children would have a normal life expectancy and the graft survival would parallel the life expectancy. Unfortunately, it has been reported from centers that perform protocol biopsies that in a majority of the recipients, there would be unexpected graft inflammation or fibrosis even though the child would be clinically stable (4;8;10).

1. Incidence of allograft fibrosis and inflammation among pediatric LT recipients

Since 2005 until 2014 there have been 12 major studies focused on pediatric allograft changes (studying histological inflammation and fibrosis) after LT, the details of which are mentioned in table 5 (3-5;7;8;10;65-70). These studies show that majority of the recipients have abnormal histology in the long term with evidence of chronic inflammation or fibrosis seen in 42 – 94%. Earlier in our unit, Venturi et al had shown in 2012 that 94% children at 7 years' post LT had abnormal histological findings. Surprisingly the 1-year post-LT LB also showed significant histological changes in 32% and 34% of the children studied at Birmingham and Groningen respectively (4;8;10). The studies done at Birmingham, Groningen and Brussels demonstrated that allograft fibrosis was progressively increasing with post LT duration. *On extrapolation, it would imply that in the long term i.e. by when these infant LT recipients would be adults – a vast number of them would eventually re-develop cirrhosis and probably be re-listed for the second LT.*

Table 5: Histological findings in twelve studies using LB from children post-LT

Center	N° of LB	Timing of LB	Abnormal histology	Main histological findings
Paris*	67	>10 yrs	73%	Chronic rejection (42%),

				centrilobular fibrosis (22%), biliary cirrhosis (4%), other (4%)
Birmingham*	64	10yrs	69%	Chronic hepatitis +/- fibrosis (64%), biliary fibrosis (2%), recurrent PSC (2%), other (2%)
Chicago*	63	>3 yrs	97%	Fibrosis (97%), inflammation (70%)
Groningen*	55	10 yrs	69%	Fibrosis (69%)
Osaka	24	>1 yrs	71%	Fibrosis (71%) inflammation (58%)
Kyoto	67	>5 yrs	84%	Fibrosis (84%) inflammation (58%)
Brussels	38	7 yrs	94%	Fibrosis (94%), inflammation (74%), ductal proliferation (26%), steatosis (26%)
Tokyo	59	0-15 yrs	86%	Fibrosis (86%), inflammation (39%), steatosis (10%)
Helsinki	54	>3 yrs	43%	Steatosis (43%), ductular reaction (43%), fibrosis (39%), inflammation (22%)
Hanburg	60	>1 yrs	40%	Fibrosis (33%), mild acute rejection (20%),

				steatosis (17%), early chronic rejection (3%)
London*	56	>1 yrs	84%	Hepatitis (41%), bridging fibrosis/cirrhosis (27%), NRH (16%), biliary problem (12.5%), rejection (4%0, other (11%)
Tochigi	55	5 yrs	42%	Inflammation (42%), fibrosis (34.5%)

* Centers that studies protocol biopsies

2. Critical analysis of existing evidence

Undoubtedly the above-mentioned studies made us aware of the problem concerning allograft fibrosis and inflammation but on critical analysis we can identify many severe shortcomings in the study design and methodology. It's important to re-analyze these studies to have a correct assessment about the magnitude of the problem. The shortcomings that we could observe have been summarized below

a. <u>Inclusion criteria of patients:</u>

Vascular or biliary complications, chronic rejection are known factors to predispose to fibrogenesis. Also, there are high chances of recurrence of primary disease post-LT in case of AIH or PSC, which are also pro-fibrogenic diseases. In the above-mentioned studies which evaluated the allograft fibrosis prevalence, all LT recipient children were included irrespective of whether they had these pro-fibrogenic predispositions. Additionally, in studies from Groningen, Birmingham and Osaka the immune suppression protocol was not the same during the entire study duration (4;8;65).

Hence a diverse study population was included with a significant number of participants having known pro-fibrogenic conditions which would have biased the results towards a more fibrogenic trend. This population would also not correctly depict the natural history of the allograft evolution or address the main concern regarding "idiopathic" fibrosis.

b. Inclusion criteria of biopsies:

Less than half of the biopsies included in the above studies were protocol biopsies, the remaining being "indication" based (3-5;8;69). This would imply that majority of the children were not stable and had ongoing clinical issue that mandated a biopsy. To assess these samples would also not reflect the natural course of the allograft in any remote manner.

c. Lack of serial biopsies:

Other than the studies from Birmingham and Groningen, serial biopsies have not been analyzed and single cross-sectional biopsies from patients have been considered. To comment on cross-sectional samples of different patient groups cannot be considered as representative of histological evolution. Additionally, there would be a longitudinal evolution of inflammation or fibrosis which would be completely ignored on analysis of the cross-sectional samples.

d. Disregard of patient specific predispositions for fibrogenesis:

As highlighted in the section above concerning the inclusion criteria of patients, certain patients have higher predisposition for fibrogenesis. This could be partially explained by the primary liver disease e.g. HCV, AIH PSC etc., LT related complications, and other additional unexplored factors. For example - presence of HLA DRB1*03/04 allele has been found to predispose to higher fibrogenesis in patients having AIH due to altered antigen presenting characteristics. This has not been studied in LT scenario yet (71;72). Thus, to factor in a "patient effect" which

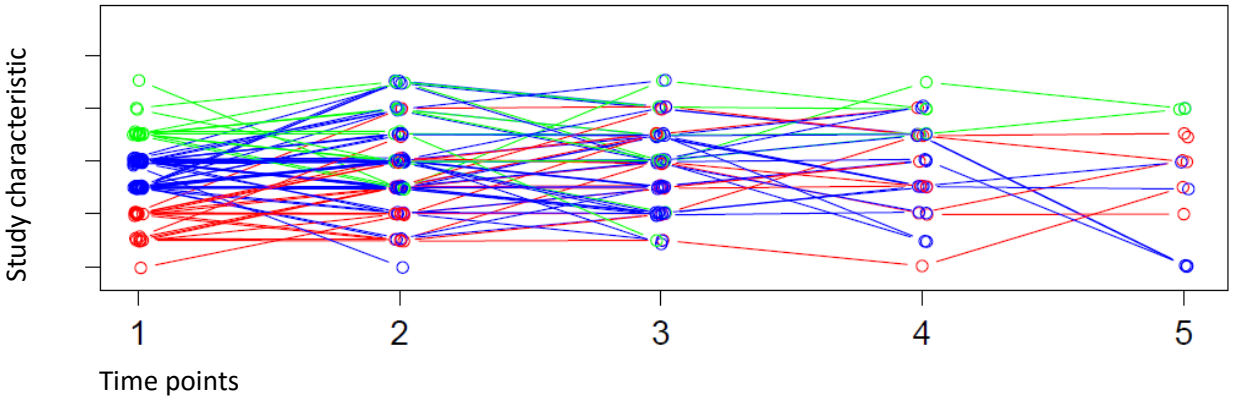
would attribute to the individual fibrogenic potential, would be essential to study the longitudinal progression of the histological characteristics and this would be statistically possible only in case of longitudinal serial PB's.

e. Inappropriate statistical models:

The fibrosis and inflammation scores used in these studies are ordinal and categorical variables. Hence the described "mean fibrosis or inflammation" is an inappropriate statistical analysis making the interpretation of such results inaccurate and rather worthless.

In the studies that have used serial biopsies, unfortunately "mixed model" which is specifically for longitudinal repeated measures has not been used. This model considers the evolution of each patient as a separate entity and analyzes repeatedly measured observations over time for the same patient (73). By doing so it incorporates the effect of previous histological findings on the current one e.g. the influence of previous inflammation or fibrosis at time point "t-1" on the current fibrosis at time point "t" (Figure 6). In contrast by pooling the information of all patients at different time points, the information of individual patient is lost. By doing so we have average fibrosis at different time points but do not understand the evolution trend. Hence inappropriate statistical models would have given us confounding conclusions.

Figure 6: Mixed effect model-Each patient is represented by a different color and the evolution of the study characteristic is evaluated at 5 time points (represented as circles) connected by the solid lines. This shows evolution of each patient separately and not a cumulative mean at each cross-section of time i.e. the 5 time points.



(Personal picture – Sharat Varma)

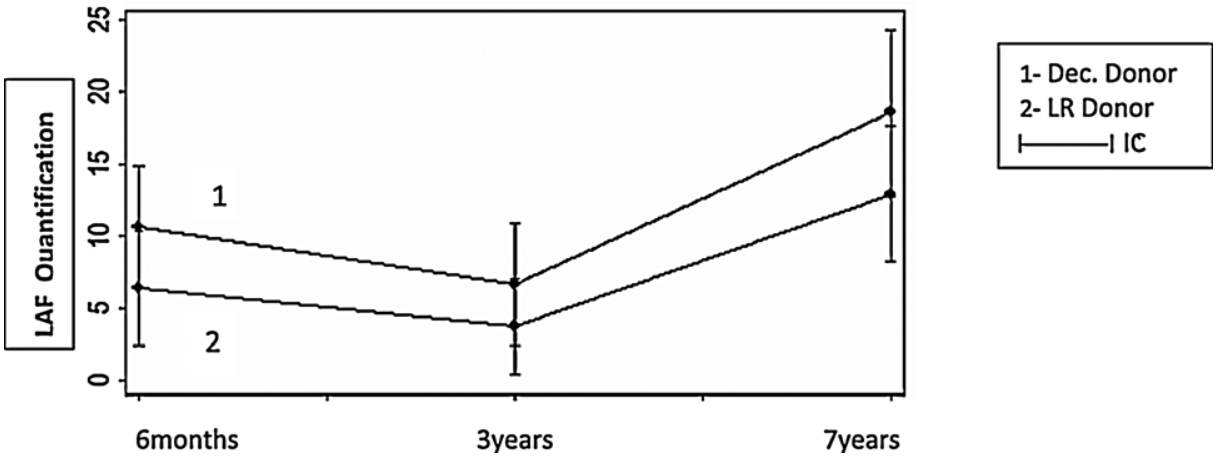
f. Non-uniform and poorly reproducible histological parameters used to evaluate outcomes:

Studying the table 5 shows that each study has used a different terminology and histological characteristics, scoring systems. The lack of uniform and reproducible criteria for inflammation or fibrosis makes it difficult, if not impossible to pool this data to form a global picture. This aspect was previously discussed in the fibrosis quantification section and perhaps digital quantification of histology would solve this problem to have reproducible, objective and consistent information about graft fibrosis.

3. Dynamics of allograft histological evolution

The study from Venturi et al showed for the first time in the setting of pediatric LT - the dynamic nature of allograft fibrosis as seen in Figure 7 (10). They showed that fibrosis could increase, remain stable or also decrease over time. 26% of the included patients had shown a regression of fibrosis in the 7-year study duration. These results are in concordance with multiple studies done in adult HCV as outlined in table 6, where reversal of fibrosis was seen on successful clearance of the virus (74-82).

Figure 7: LAF quantification using morphometric analysis (PSR %) along the three evaluation periods showing the dynamic nature of the fibrosis.



This observation presents us with a window of opportunity, that if we can identify the mechanism of fibrogenesis and correct them like in the case of HCV eradication, then we have the possibility to salvage the liver that is becoming fibrotic. Extrapolating this to our pediatric LT recipients, we can then envisage very-long term healthy graft survival.

Table 6: Changes in fibrosis stage in HCV-infected liver transplant recipients after successful treatment

Author (ref)	Improvement of fibrosis in (%) of patients
Rodriguez-Luna et al.	60%
Neff et al.	46%
Biselli et al.	44%
Berenguer et al.	20%
Mukherjee et al.	9%
Mukherjee et al.	31%
Angelico et al.	20%
Carrion et al.	61%
Roche et al.	26%

Chapter 3: Predisposing factors for allograft hepatic fibrogenesis.

Various clinical studies have analyzed and identified certain factors that predispose to fibrogenesis in the allograft. Yet in a significant proportion of patients' fibrosis is seen in the absence of these factors and is labelled "idiopathic allograft fibrosis".

Known predisposing factors for allograft fibrosis

a. Recurrence of disease: Recurrence of primary liver disease post-LT can cause re-appearance of fibrosis. This is undeniably the biggest factor for allograft fibrosis in the adult LT scenario where HCV related chronic liver disease is common. Within 5 years after LT, histological recurrence of HCV is reported in 80% of patients and development of cirrhosis in 30% (83). HCV related liver disease is uncommon in children but a significant number of children receive LT for autoimmune liver disease. AIH accounts for 2-5% of all pediatric LT and 19-55% of children with AIH finally need a LT (84). Although, children transplanted for AIH generally have a good outcome, it may recur in 5-11% post LT, table 7 (85-87). The risk factors associated with AIH recurrence include presence of HLA DR3 or DR4 genotype of the recipient. A higher rate of fibrosis progression to cirrhosis has been seen in these children (88;89). Similarly, recurrence post LT of primary sclerosing cholangitis (PSC) is seen in 10-50% pediatric recipients (85;90-93) wherein an accelerated course of fibrous cholangitis, fibro-obliterative lesions and cirrhosis are frequently seen.

Table 7: Reports on recurrence of AIH after pediatric LT

Author (Ref)	N° of LT for AIH	Recurrent AIH post LT n (%)
Birbaum et al (26)	6	5 (83)
Bahar et al (18)	28	11 (39)
Chai et al (17)	13	5 (38)

b. Vascular or biliary complications: Presence of vascular or biliary complications, duration of ischemia time have been seen to have a strong pro-fibrogenic role, as demonstrated in many studies (8;10;65;70). The close correlation of the co-existence of vascular and biliary complications is understandable, due to the delicate blood supply of the bile ducts and their limited regenerative potential.

Factors "needing evaluation" for fibrogenesis

a. Antibody mediated rejection: Though the liver is relatively resistant to antibody mediated rejection in case of ABO compatible LT, there has been increasing evidence to suggest the contrary. Histological features suggestive of immune mediated hepatitis can be seen in 10-50% of patients undergoing a PB >1 year post-LT and up to 60% of children at 10 years (6). The presence of auto-antibodies (ANA, SMA, LKM) have been associated with higher prevalence of allograft inflammation and fibrosis (4;6;9;10). It remains to be understood if these antibodies are the "cause or effect" of hepatic insult.

The presence of donor specific HLA antibodies (DSA), specifically of subclass II has been linked to allograft fibrosis and inflammation in many recent studies (7;94-97). The presence of the HLA class II antigens in the immunologically active portal zones, makes these antibodies likely candidates to afflict a persistent sub-clinical immune mediated injury. Though our understanding of the role of these antibodies is evolving, there is lack of evidence to determine appropriate cut off titres - mean fluorescence index (MFI). Also unknown is if all subtypes are equally important. The proportion of patients with DSA's post-LT has been reported to vary between 8 and 67%, depending on the MFI cut-off that has been used, which ranges from 1000 to 5000 (7;94-100). In earlier studies done in adult LT recipients MFI of 5000 was found to correlate to increased incidence of chronic rejection and poorer graft outcomes (99;100). In contrast the landmark study by Miyagawa et al, showed that allograft fibrosis in children was associated with presence

of class II DSA > 1000 MFI (7). This wide range of MFI selection highlights the lack of information on identifying clinically valid cut offs. The class II antibodies comprise of DP, DQ and DR subclasses and though the antibodies against the HLA-DQ antigens are more frequently seen post-LT and been implicated in de-novo autoimmune hepatitis (97;99), there is no clear consensus on the relative importance of the different subclasses, especially among children.

b. Histological inflammation:

While continuity between uncontrolled inflammation and hepatic fibrosis has been documented in hepatitis C and auto-immune hepatitis (AIH), this is 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. This crucial bit of information is missing in the context of pediatric LT where previously done studies have evaluated biopsies cross-sectionally studying the co-existing inflammation and fibrosis but not sequential development (3;5;66-70)

The study from Birmingham on PBs at 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 (4). The authors evaluated fibrosis and inflammation cross-sectionally at three time-points over 10 years.

c. HLA DRB1*03/04:

In AIH, the HLA-DRB1*03/04 allele is considered an independent predictor of portal fibrosis (71;72;101). 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 repopulate the allograft (102), any host antigen-presenting cell defects would be evident in the new graft. Additionally, as AIH is the prototype of immune dysregulation-

mediated hepatic disease, examining its role in LT recipients is the next logical step, which has yet not been done.

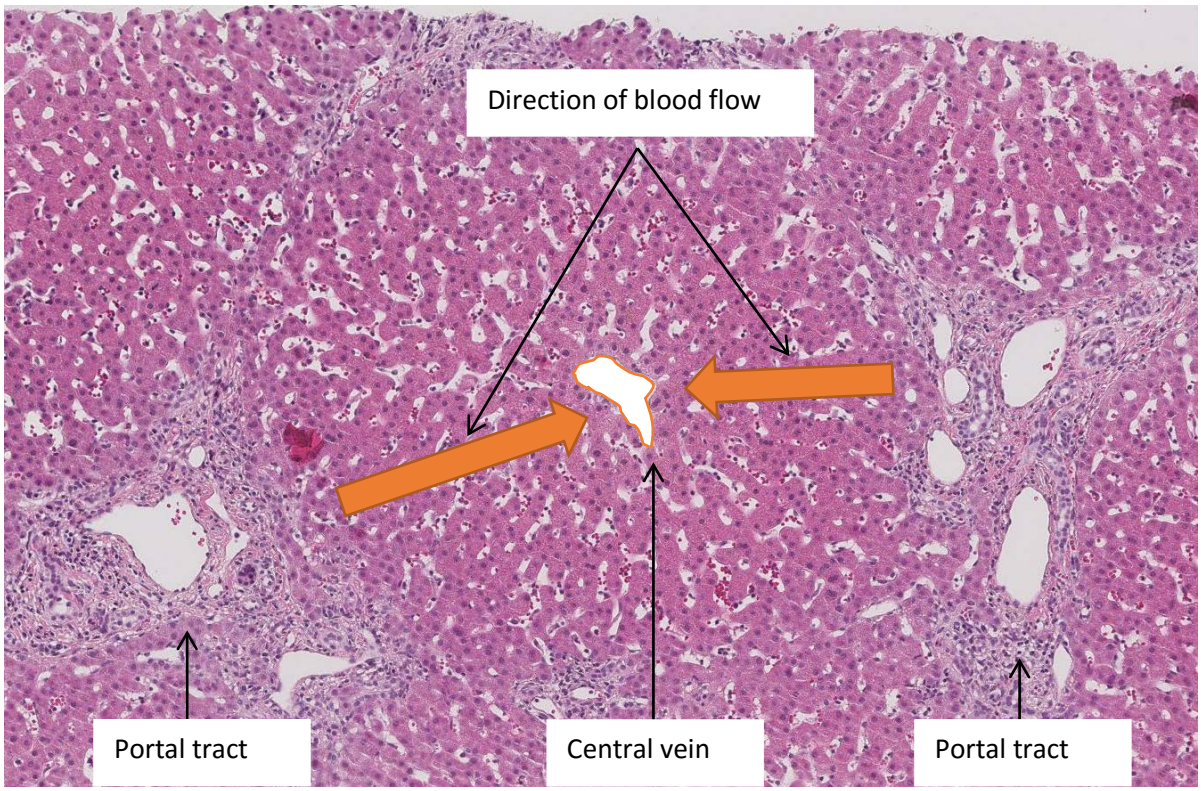
d. Combined effect of non-HLA and HLA antibodies:

Both HLA and non-HLA antibodies has been considered as predisposing factors for fibrogenesis but no study has tried to elucidate whether they have a synergistic or additive effect. This is a strong possibility given the multifactorial involvement in fibrosis.

Regional effect of certain predisposing factors:

Venturi et al on describing topographic distribution of the fibrosis patterns could observe that deceased donors, longer ischemia time contributed to portal fibrosis while biliary complications were associated with sinusoidal fibrosis. Centrilobular fibrosis was seen to be associated with gamma globulin levels and presence of auto-antibodies (10). As the liver functionally and by perfusion can be divided into three zones, it's apparent that different types of insult i.e. vascular, biliary, immune mediated, etc. would have different targets and zones of fibrogenesis predisposition (Figure 8). Better description of fibrosis in these zones and understanding the region-specific predisposing factors would make the analysis of the PB's more intuitive and effective.

Figure 8: Regional predisposition of fibrosis – The blood flows from the portal area to the central making the central area cells most sensitive to vascular complications as they are least oxygenated physiologically. The bile ducts are in portal tracts hence biliary complications result in portal fibrosis.



(Personal picture – Sharat Varma)

Chapter 4: Mechanism and predictive factors of fibrogenesis:

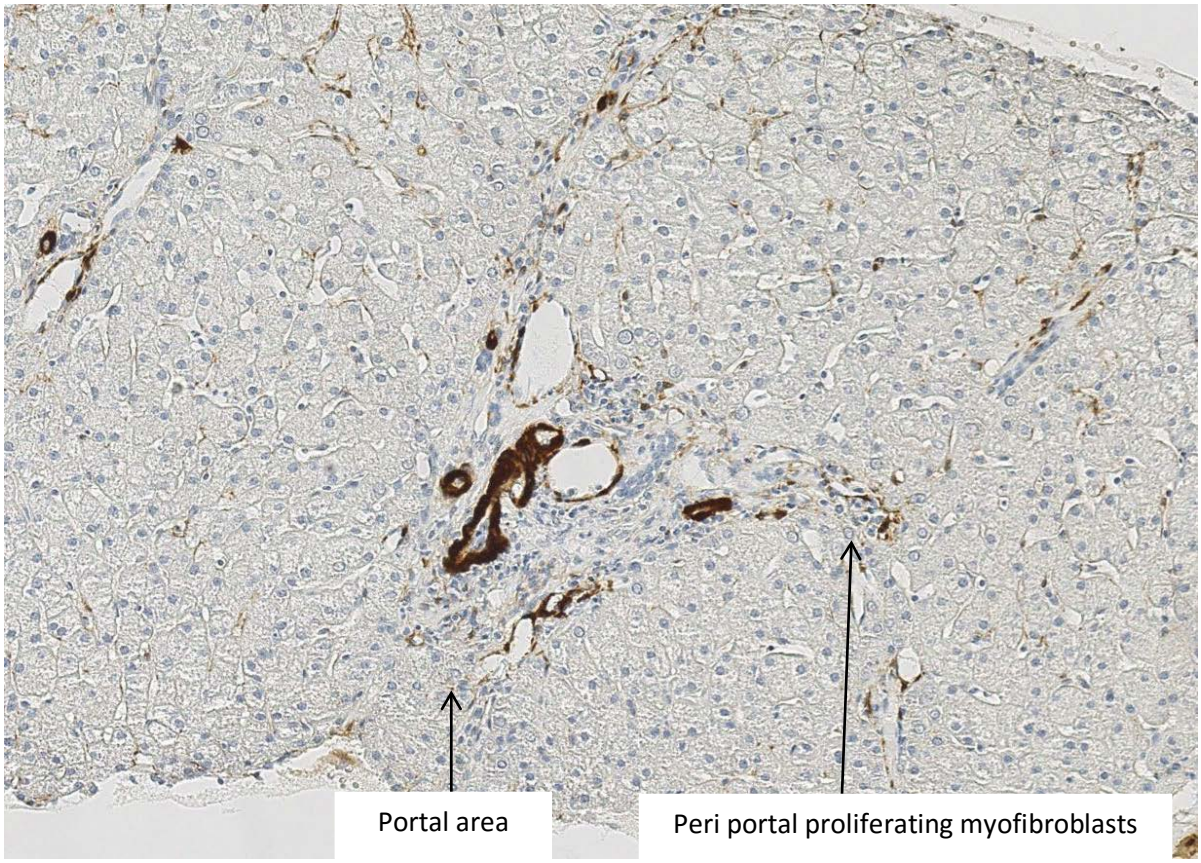
Our current understanding of the cellular and molecular mechanisms involved in hepatic fibrosis have paved the way for mechanism based anti-fibrotic strategies, which are now in clinical trials. This is a significant step forward for individuals with established fibrosis. But ideally in context of LT recipient children we should be able to predict the evolution of future fibrosis and take timely preventive action rather than search for anti-fibrotic drugs. Fortunately, these children routinely undergo PB giving us access to study directly the involved tissue in all stages of fibrogenesis. After achieving the ability to predict fibrosis and perform risk stratification, we can try to mitigate this process by reversing the culprit factors.

Among the histological characteristics considered as predictive of hepatic fibrosis – alpha smooth muscle actin (ASMA) and cytokeratin 7 (CK7) are most accessible, relevant, evaluated and have been discussed below.

1. **ASMA:**

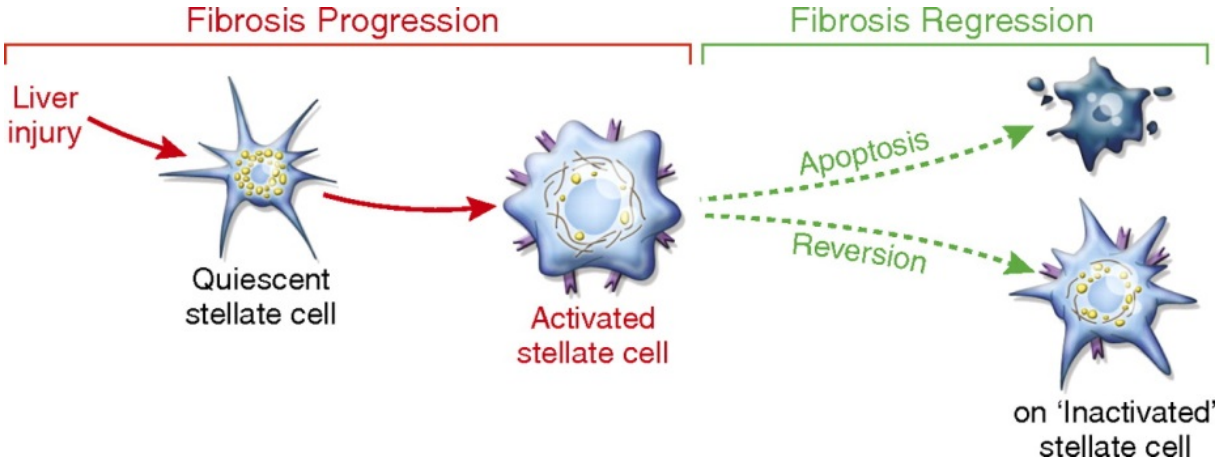
   The stellate cells (HSC) when become activated from a quicent vitamin A storing cell to a proliferative myofibroblast – exhibit a cytoplasmic protein called ASMA (103;104). This can be stained by immunohistochemistry (IHC) of the liver tissue as shown in Figure 11 and subsequently quantified by computerized morphometric quantification. The activated HSC then secrete collagen into the extracellular matrix. After the stimuli for HSC activation have ceased they will either become inactivated, undergo apoptosis or senescence as shown in Figure 12 (103;104). With the reversion of these activated cells the stage is primed for the fibrinolytic pathways to act and the macroscopic fibrosis diminishes.

Figure 9: ASMA staining pattern-The myofibroblasts at the margin of the portal tract are stained, especially closer to the ductular reaction. The endothelial tissue also takes the ASMA stain.



(Personal picture – Sharat Varma

figure 10: HSC activation and deactivation related to fibrosis evolution.



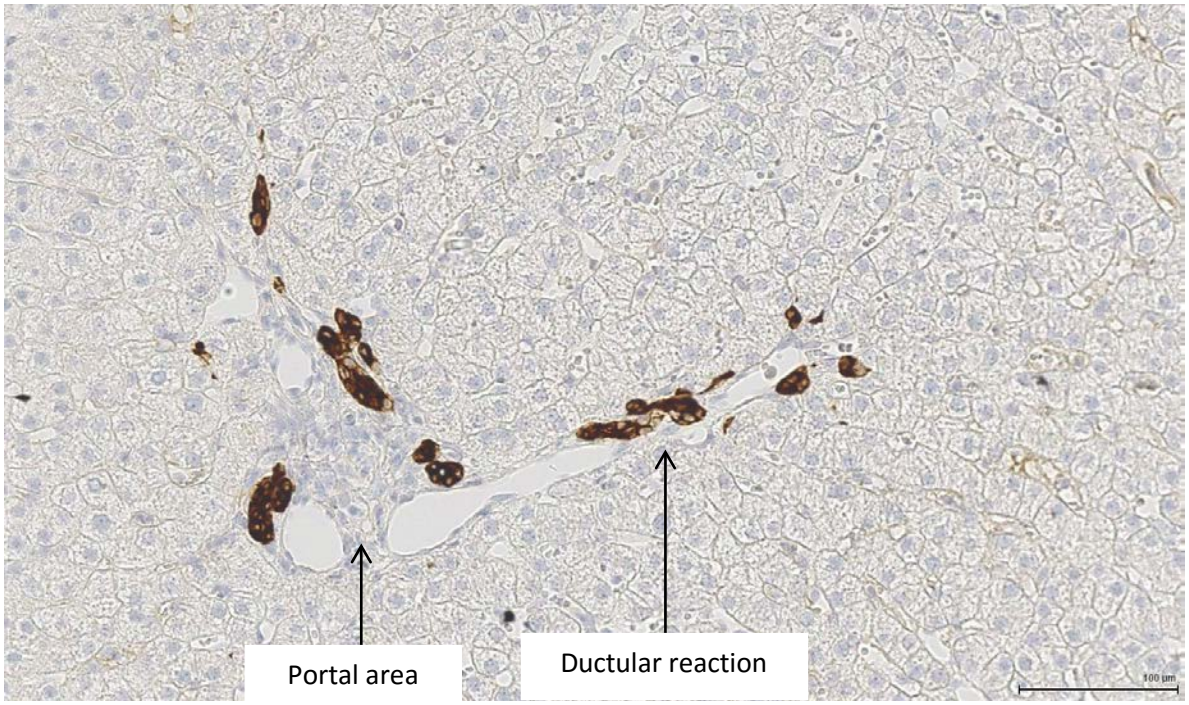
With this understanding it's not difficult to imagine the quantification of ASMA can predict the future fibrosis and give us a "timely" clue to prognosis of near future. Two independent

studies performed on adult LT-recipients infected with hepatitis C, have demonstrated the predictive ability of ASMA quantification for fibrosis in the two years post-LT (105;106). Though these results are corroborative, the methods of ASMA quantification differed between the studies. For validating this hypothesis we need to standardize staining and quantification procedures. Data about its applicability in the pediatric cohort and specifically LT recipients is lacking.

2. **CK7:**

CK7 is a marker of the cholangiocytes (Figure 11(a)) and also hepatic progenitor cells in the niche of Canal of Herring as seen in Figure 11(b)(107).

Figure 11(a): Cytokeratin 7 staining pattern of the cholangiocytes and the ductular reaction.



(Personal picture – Sharat Varma)

Figure 11 (b) Schematic diagram of a normal branching of the biliary tree. Note: the ductule segment may only extend to the limiting plate and no further, in which case there would be no intralobular portion.

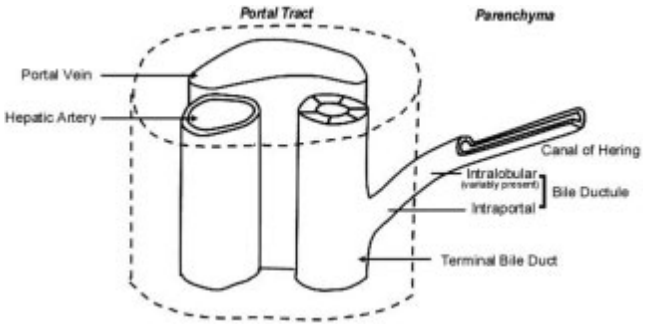


Diagram credit: Tania Roskams et al. HEPATOLOGY 2004; 39:1739 –1745

Ductular reaction (DR) is identified by using CK7 staining and defined as “reaction of a ductular phenotype, possibly but not necessarily of ductular origin – as it may arise from proliferation of cholangiocytes, progenitor cells or rarely hepatocytes”. It is frequently seen on PB’s, significance of which has not yet been deciphered in cases with no biliary damage. It’s controversial wether it’s a “cause or an effect” of hepatic fibrosis as clinical observations and in-vivo, in-vitro evidence exists on both sides of the argument, though conclusive proof is lacking (103;104;108;109). These aspects make it an attractive marker that needs to be studied further.

DR has been linked to hepatic fibrosis progression in animal studies (103;104) and recently also with HCV and hemochromatosis in humans (110;111). As all these human studies, have been cross-sectional, conclusive evidence is lacking to confirm whether DR i.e. CK7 increased quantification increases before increased fibrogenesis, as is expected.

Aims and objectives

1. To understand the natural history of allograft histology in a stable and complication free cohort.
2. To identify the predisposing factors for “idiopathic allograft fibrosis”.
3. To understand the role and significance of HLA antibodies in allograft dysfunction.
4. To assess the possibility of predicting future allograft fibrosis progression.
5. To develop a digital quantification tool which can perform regional quantification of histological characteristics

Overall study design

As our aims were to study evolution and factors involved in the natural history of allograft we selected a stable (complication free) population of LT recipients that have been under regular follow up with serial PB's. By doing so we were able to study the sequential evolution of histological characteristics in allografts free from pro-fibrogenic confounding factors.

As we changed our immune suppression regime in 2003, all (n=235) LT recipients between 2004 and 2012 were screened for inclusion. The rationale being that this would ensure a minimum of 3 years of follow up and 2 PB's. We used the same study cohort to evaluate the aims 1, 2 and 3. A smaller subgroup of patients from this cohort was analyzed for aim 4. For aim 5 the quantification tool was developed and optimized over a wide range of samples and tested for validation on a different set of children as a technology demonstrator.

Methods and results

Below are the main findings followed by the details of the methods and results in the respective articles.

1. **Understanding natural history of allograft histology, predisposing factors for "idiopathic" allograft fibrosis and the role of HLA antibodies (Aims 1, 2 & 3) – (Details in attached article 1, 2 & 3 at the end of the section)**

   On studying 281 serial PB's we observed that

   a) Allograft inflammation and fibrosis evolution patterns were patient-specific and no generalizable patterns can be described. There is no universal progression of allograft fibrosis as we could observe.

   b) In the portal area:

      a. Fibrosis was correlated with preceding portal Inflammation presence and severity, presence of Class II DSA, and recipient HLA-DRB1 status. Implying that allograft inflammation is a precursor to allograft fibrosis.
      b. Fibrosis was not persistent with time i.e. portal fibrosis is dynamic – can show progression or regression with time.
      c. Inflammation correlated with pre-existing Inflammation, Class II DSA, and non-HLA antibodies.

   c) In the non-portal area

      a. Centrilobular area fibrosis correlated with pre-existing fibrosis and deceased-donor.
      b. Lobular Inflammation Correlated with Pre-existing Lobular Inflammation and Non-HLA Antibodies

   d) HLA-DRB1*03/04 Allele in Recipients Was Associated with Allograft Portal and Sinusoidal FibrosisPost-LT

e) Class II DSA was determined that older donor age decreases the risk of developing class II DSA while older recipient age increases this risk.

2. **<u>Assessing possibility to predict future fibrosis (Aim 4) – (Details in attached article 4 & 5, at the end of the section)</u>**

We attempted to predict future fibrogenesis using two histological markers – ASMA and CK7. Our hypothesis being that quantification of ASMA and / or CK7 on the baseline biopsy would be able to predict the change in fibrosis in the next biopsy.

The depictive steps in ASMA quantification are shown in Figure 14 (a-d)

a) ASMA based prediction:

a. There was a significant association between the ASMA quantification on the baseline biopsy and the "prospective change in fibrosis on the next PB" as assessed by the Metavir score, cumulative LAFSc and LAFSc-P.

b. Area under receiver operating characteristic (AUROC) curve analysis demonstrated that a cut-off of ASMA positive area percentage >1.05 predicted an in the Metavir score and cumulative LAFSc on the next biopsy, performed within two years, with a sensitivity of 60.0% and specificity of 90.0%.

c. ASMA positive area percentage was associated with extent of ductular reaction and, to a lesser extent, with severity of lobular inflammation and portal tract inflammation.

d. ASMA positive area percentage was not related to Tacrolimus level, gamma-globulin level, recipient age or donor age, HLA antibody development post-LT or recipient gender or donor gender.

b) CK7 based prediction

a. CK7 quantificaion was correlated to the extent of concurrent fibrosis and portal inflammation

b. CK7 quantification was not predictive of the future course of fibroisas assessed using paired serial biopsies.

3. Development of digital quantification (morphometry) tool to perform regional quantification of fibrosis (Aim 5)

Methods

Development and design of the morphometry tool

Liver fibrosis was quantified on pSR - stained paraffin sections, digitalized using a SCN400 slide scanner (Leica Biosystems, Wetzlar, Germany) at 20x magnification, with software applications using the image analysis tool Author version 6.0.0 (Visiopharm, Hørsholm, Denmark). To implement this software based calculations, image analysis protocols are used – called as APPS (image analysis protocol). We designed a series of APPS to calculate the CPA total or CPA regional (portal, sinusoidal and centrilobular). Below is described the general workflow of an APP and subsequently the specific APPS that were developed.

a. The general workflow of an APP is as follows:

- **i.** Step 1: Setup – Defining magnification and regions in which the analysis will be performed.
- **ii.** Step 2: Preprocessing – Specification of the features in the image which highlight the characteristics to be detected.
- **iii.** Step 3: Classification - Based on the above selected features, each pixel is then classified into a "label" (e.g. tissue, background, pSR staining, etc.) using a thresholding method.
- **iv.** Step 4: Post process - Refining and quality control of the above classification.
- **v.** Step 5: Calculation

b. The specific APP for CPA total are as below and snapshots of the sequential steps are as per Figure 18:

- **i.** Step 1& 2: Tissue was first detected from the background at a low digital magnification (4x) using a thresholding classification method based on the RGB-blue image feature. Tissue borders, sometimes aspecifically stained, were eroded and excluded from the analysis, as were irrelevant small tissue areas.
- **ii.** Step 3: Pixels classified as tissue or as glass (background)
- **iii.** Step 4: The post-processing included
    1. Elimination of small tissue areas
    2. Elimination of peripheral area of tissue (erosion) as they take aspecific staining.
- **iv.** Step 5: Calculation
    1. Pixels classified as pSR + or pSR – depending on their staining color
    2. Elimination of artifact staining
    3. Calculation of CPA total as = (Pixels sPR+ / Total pixels in tissue) *100

As it measured pSR + proportionate area in all the biopsy area it can be adapted to measure the CPA in certain predefined regions i.e. portal, centrilobular and sinusoidal. For this application, prior APP's (APP delineation 1 and 2, as described below) would be needed to delineate these zones of interest and subsequently CPA could be measured in them by the APP-quantification as described above.

c. The specific APP's for quantifications of CPA regional portal/sinusoidal/centrilobular) are as below and the snapshots of sequential steps are as per Figure 19:

- **i.** Process 1: APP delineation 1:

1. Central veins and portal tracts were first delineated with the “ROI” tool.
2. These delineations were automatically extended inside the tissue in two concentric regions of 62.5 µm each (corresponding to 50 pixels), defined as peri-portal and peri-centrilobular areas.

ii. Process 2: APP delineation 2:

1. Designed to detect the sinusoidal tissue based on preprocessing steps highlighting the RGB-blue image feature.
2. Post processing steps were applied to remove truncated pieces of tissue (< 5000 µm2) and large collagen areas (>1000 µm2) corresponding to bridging fibrosis or liver capsule.

iii. Process 3: APP quantification:

Designed in a similar way as the CPA total APP to detect and quantify sirius red-positive pixels. Results were expressed as percentage of pSR+ area on tissue area within the analyzed zones.

Statistics:

We assessed the concordance between the visual scoring performed by the pathologist (i.e. Metavir and LAFSc score) and the digital quantification using the CPA module. Considering that the visual score is an ordinal variable while the digital quantification produces a continuous variable, Polyserial correlation coefficients were used. Statistical analyses were performed using the polycor packages of the R version 3.2.3 statistical software.

Results

High concordance between CPA and visual scoring

When the digital score (CPA total) and the Metavir score obtained from the same biopsy were compared, a high correlation was observed (PCC = 0.857) (Figure 20), traducing a high concordance between both methods. The same was observed with the LAFSc-T with a PCC = 0.871 (Figure 21).

Correlation analysis between regional CPA and regional LAFSc showed a higher concordance for the Portal area (PCC = 0.75) than for the Sinusoidal and the Central area (PCC = 0.21 and PCC= 0.053, respectively) (Figure 22 (a-c))

High concordance between CPA and visual scoring to assess prospective change in fibrosis

When the difference of fibrosis severity between both biopsies from the same patient (PB2 – PB1) were computed using the CPA module and the visual score (Metavir, LAFSc) and compared together, a high concordance was also observed (PCC = 0.735 for Metavir and PCC = 0.94 for LAFSc-T) (Figure 23)

Despite these high concordances, wide ranges of CPA percentage were observed within the same category of the visual score. This was seen both for Metavir and LAFSc scores (Figure 20&21). For example, in a patient categorized with a Metavir score of 2, the CPA percentage could range between 18% and 40%

From figure 23, it can also be observed that a patient without any evolution of Metavir (or LAFSc) score between the paired biopsies could nevertheless show significant change is fibrosis severity using CPA. For example for a Metavir score change (PB2-PB1) equal to 0, the CPA percentage could range between -27% and 34%

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

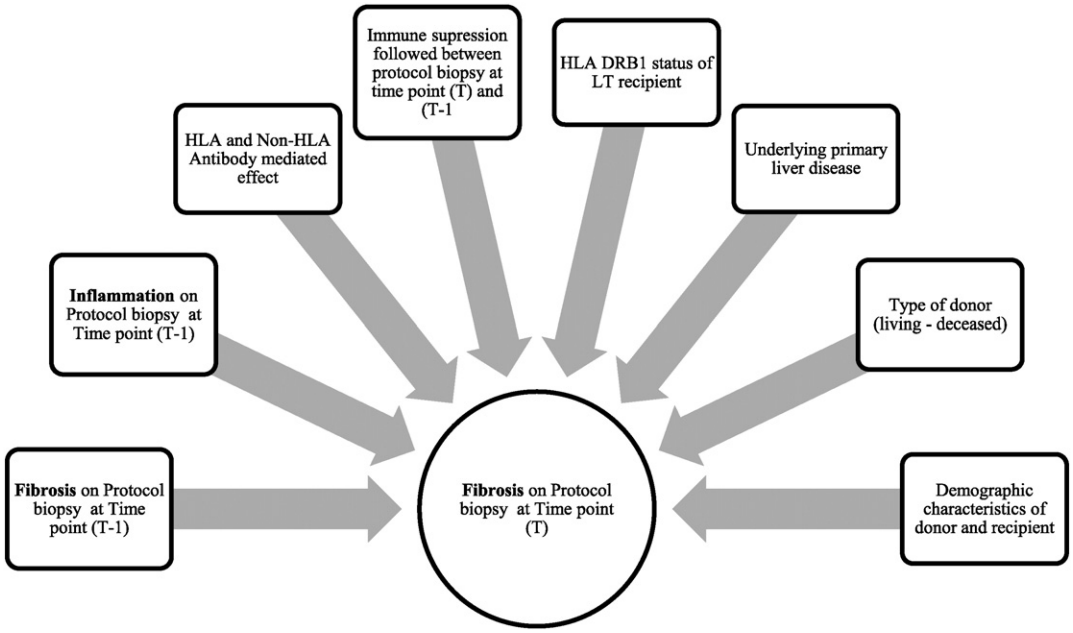

Fig. 1. Hypothesis to test, for development of allograft fibrosis (Our hypothesis for allograft inflammation had same variables other than Fibrosis on Protocol biopsy at time point ($t - 1$)).

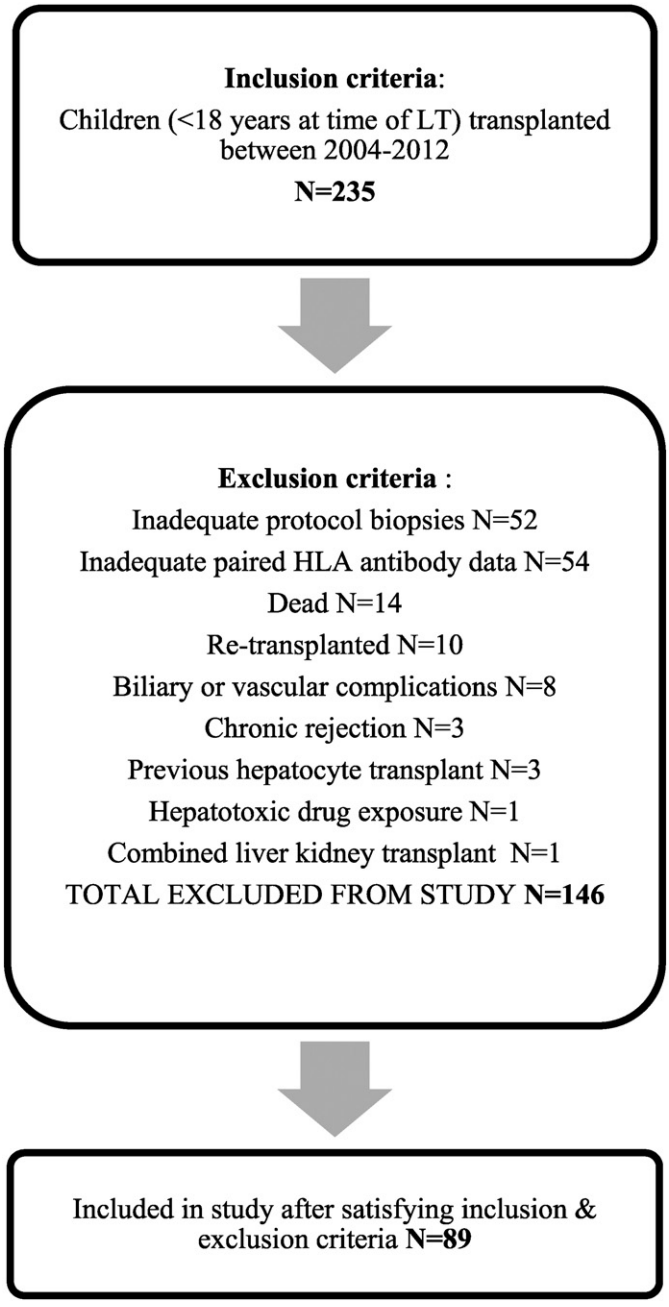

Fig. 2. Screening, inclusion and exclusion steps followed in the study.

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

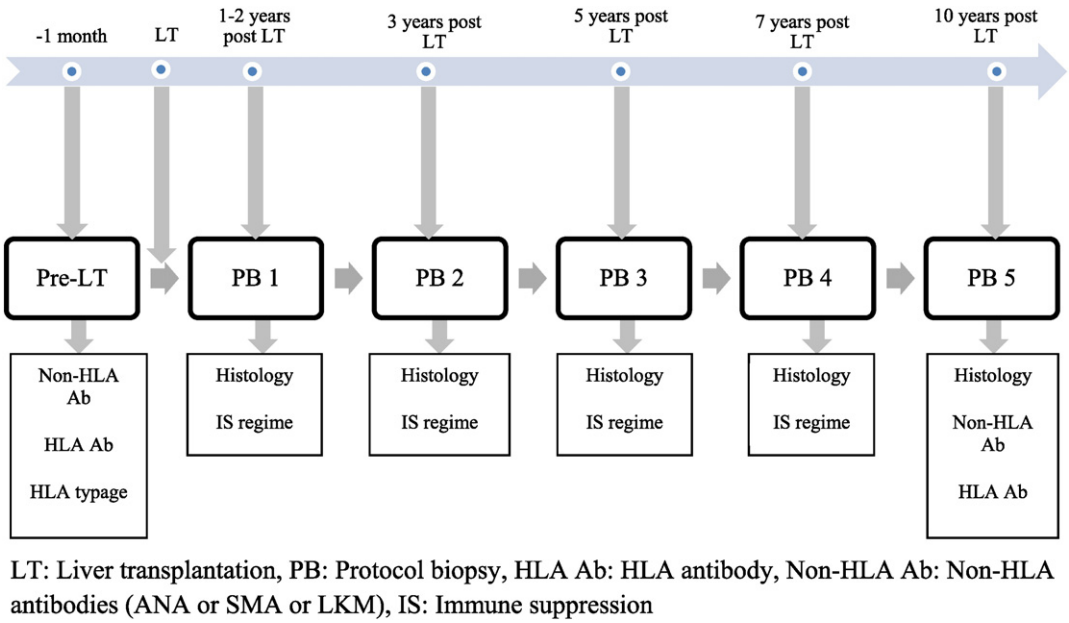

Fig. 3. Data collection time points and schematic, representative of a patient followed up for >10 years. LT: Liver transplantation, PB: Protocol biopsy, HLA Ab: HLA antibody, Non-HLA Ab: Non-HLA antibodies (ANA or SMA or LKM), IS: Immune suppression.

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

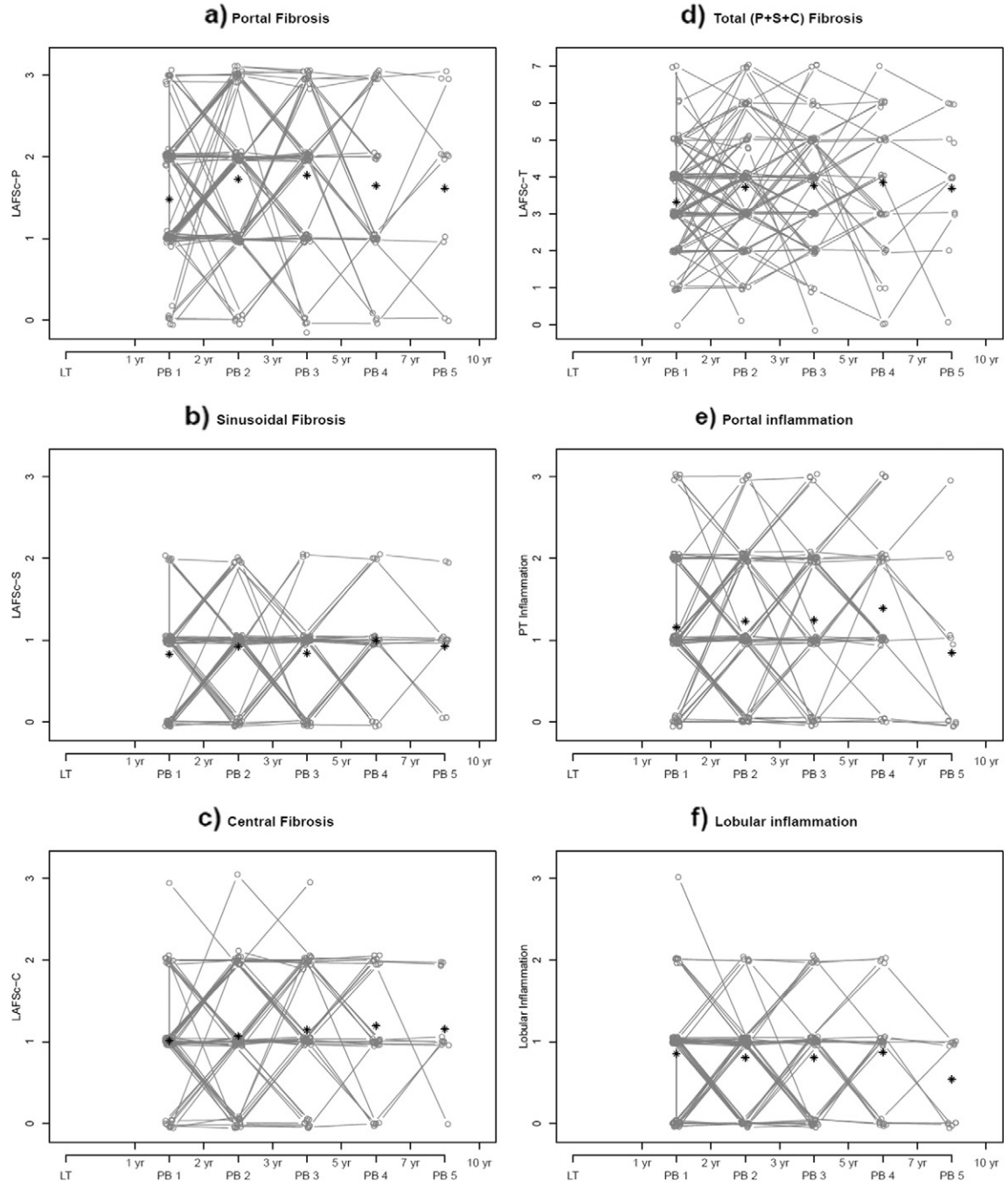

Fig. 4. Individual (gray circles) and averaged (black stars) evolutions of fibrosis across serial protocol biopsies are shown. Longitudinal evolution of each patient is plotted independently using the gray circles at different time-points and connected via solid lines. Longitudinal evolution of fibrosis (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

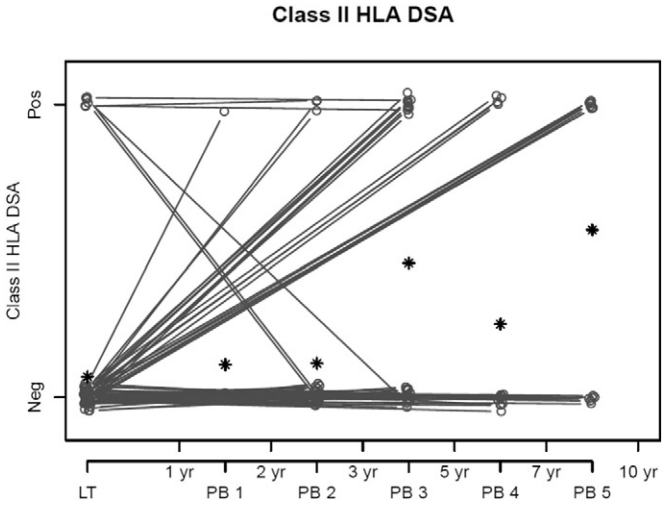

Fig. 5. Demonstrates the fate of pre-LT class II DSA and the development of "de-novo" antibodies post LT in all cases except two. Evolution of Class II HLA DSA status recorded 1 month pre-LT and at the last PB. Individual evolution of each patient is reported with connected gray circles while averaged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

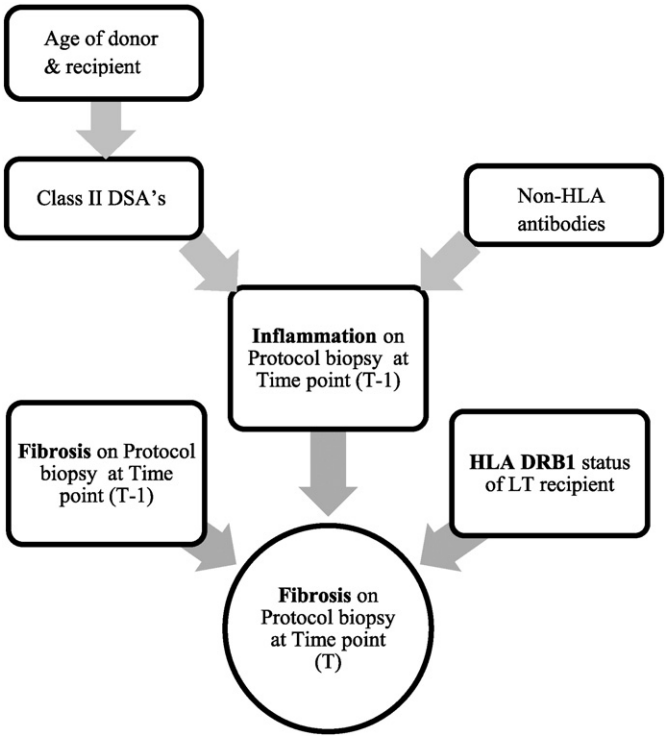

Fig. 6. Mechanism of "idiopathic" allograft histological changes.

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

Author Contributions

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Baldin Pamela: Histological evaluation.

Reding Raymond: Manuscript preparation.

Smets Francoise: Study design, manuscript preparation.

Stephenne Xavier: Concept, study design, manuscript preparation.

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Financial support

None.

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Late Graft Hepatitis and Fibrosis in Pediatric Liver Allograft Recipients: Current Concepts and Future Developments

TO THE EDITOR:

In a recent review by Kelly et al.,[1] the authors conclude that allograft fibrosis is progressive with time in pediatric liver transplant recipients. We are opposed to this conclusion, which for us is a misleading message that may have an impact on the medical and patient community.

In an important single-center series that we published early in 2016,[2] we evaluated 281 serial protocol biopsies from 89 longterm stable recipients, with concomitant information of their human leukocyte antigen (HLA) and non-HLA antibodies. These are precisely the aspects covered in the review, and we consider that our results deserve to be mentioned because the outcomes were contradictory.

We did not find allograft fibrosis being progressive with time; rather it was dynamic and could also decrease between serial biopsies from individual patients. Fibrosis evolution was observed to be an individually driven trend with no generalized patterns. This could be influenced by certain genetic and acquired characteristics. To study this, we used a mixed model of statistics that considers the temporal evolution of a characteristic in an individual as opposed to in the article, which included studies where cross-sectionally pooled data were used to determine "mean fibrosis." That in and of itself is questionable because it is staged using an ordinal scoring system. Hence, we oppose the conclusion made by using this methodology, of allograft fibrosis being progressive with time, because we think that this message may be misleading. Additionally, the studies that have been included in the review suggest that these observations are not based on only stable recipients, nor on protocol biopsies, and expectedly, they do not reflect the natural history of an allograft in an uncomplicated recipient.

The authors describe allograft hepatitis and infer that "in majority of patients with idiopathic hepatitis no etiology was found." This is based on studies that did not evaluate the contribution of the HLA antibodies. The role of HLA antibodies in allograft inflammation is well documented as also mentioned in the review. Hence, class II HLA antibodies have to be accounted for. The review mentions the role of DQ subtype of HLA class II antibodies and that they are detected in 50%-60% of children. In our series, we have found that only donor-specific DQ antibodies with mean fluorescence intensity (MFI) > 5000 were associated with allograft inflammation and fibrosis. Using a lower cutoff of 1500 MFI, we detected class II donor-specific antibodies in 15.6%, and 11.7% had DQ subclass of >5000. Also, the HLA and non-HLA antibodies had an additive effect on causing allograft inflammation and fibrosis.

In Table 1 (second line) of the review, 164 biopsies are said to be evaluated, whereas in the original article by these authors, 64 biopsies were said to be included.

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Received November 22, 2016; accepted December 5, 2016.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/lt.24707

Potential conflict of interest: Nothing to report.

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Accepted: 21 September 2016

DOI: 10.1111/petr.12834

ORIGINAL ARTICLE

WILEY

The histological quantification of alpha-smooth muscle actin predicts future graft fibrosis in pediatric liver transplant recipients

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Abstract

Activated hepatic stellate cells express cytoplasmic ASMA prior to secreting collagen and consequent liver fibrosis. We hypothesized that quantifying ASMA could predict severity of future fibrosis after LT. For this, 32 pairs of protocol biopsies, that is, "baseline" and "follow-up" biopsies taken at 1- to 2-year intervals from 18 stable pediatric LT recipients, transplanted between 2006 and 2012 were selected. Morphometric quantification of "ASMA-positive area percentage" was performed on the baseline biopsy. Histological and fibrosis assessment using Metavir and LAFSc was performed on all biopsies. The difference of fibrosis severity between the "baseline" and "follow-up" was termed "prospective change in fibrosis." Significant association was seen between extent of ASMA positivity on baseline biopsy and "prospective change in fibrosis" using Metavir (P=.02), cumulative LAFSc (P=.02), and portal LAFSc (P=.01) values. ASMA-positive area percentage >1.05 predicted increased fibrosis on next biopsy with 90.0% specificity. Additionally, an association was observed between extent of ASMA positivity and concomitant ductular reaction (P=.06), but not with histological inflammation in the portal tract or lobular area. Hence, ASMA quantification can predict the future course of fibrosis.

KEYWORDS

alpha-smooth muscle actin, fibrosis prediction, hepatic fibrosis, hepatic stellate cells, liver biopsy, pediatric liver transplantation

1 | INTRODUCTION

Since its humble beginnings in 1963, pediatric LT has achieved consistently improved outcomes, with current 10-year survival rates exceeding 85%.[1] Increased long-term survival has however revealed increasing graft fibrosis and other histological changes which progressively increase over time following LT.[2-5] The factors involved in late allograft fibrosis and potential treatments that can reverse or delay its development are a subject of current research.[6,7] To monitor allograft health and treatment efficacy, finding an efficient predictive marker of fibrosis development proves to be a top priority.

Fibrosis in the liver is primarily attributed to the activation of HSCs, which results in secretion of collagen types 1 and 3. HSCs can be activated in the presence of inflammation, and the expression of cytoplasmic ASMA is a consistent marker of this activation.[8,9] On removal of the activation-inducing factors, the HSCs subsequently either de-activate without expressing ASMA or undergo apoptosis.[10,11] Hence, we hypothesized that ASMA expression should mirror the later

Abbreviations: ANA, antinuclear; ASMA, alpha-smooth muscle actin; AU, area under; AUROC, AU receiver operating characteristic; HLA, human leukocyte antigen; HSC, hepatic stellate cell; LAFSc, liver allograft fibrosis score; LKM, liver–kidney microsomal; LT, liver transplantation; MT, Masson's trichrome; ROC, receiver operator characteristic; SMA, smooth muscle actin.

changes in fibrosis and be able to predict its severity. To avoid the influence of early post-transplantation events, we analyzed the biopsies taken after 1 year following LT in stable patients who exhibited no surgical or medical complications. Our study sought to evaluate whether ASMA expression, measured after 1 year post-LT in stable patients, could be considered a predictor of change in fibrosis severity in the future 2 years among LT recipients.

1.1 | Patients

This retrospective study sought to evaluate only successive protocol biopsies, which were taken at ≥1 year of LT and had an interval of 1-2 years between each other. Children who underwent LT from 2006 to 2012 and had ≥2 such biopsies were included. Exclusion criteria were death of LT recipient, re-transplantation, biliary or vascular complications, chronic rejection, acute rejections in the preceding 2 years, hepatitis B or C infection, autoimmune hepatitis, or hepatotoxic drug exposure. All included children were maintained on the same immunosuppression drugs between studied biopsies.

1.2 | Data collection

Data were collected as outlined in Figure 1.

2 | METHODS

Protocol biopsy details: Only paired protocol biopsies were included (Figure 1), wherein the first biopsy was taken ≥1 year post-LT and labeled as "baseline biopsy." The successive protocol biopsy which was taken within 2 years of the "baseline biopsy" was labeled as "follow-up biopsy." In cases where a child had >1 such pair of protocol biopsies, that is, pairs of biopsies within 1-2 years of each other, all such pairs were included. The change of fibrosis severity between the "baseline" and "follow-up" biopsy was termed as "prospective change in fibrosis."

Histological evaluation: Each protocol biopsy was evaluated for inflammation and fibrosis by three independent observers (MK, SV, XS), with a consensus made for categorization differences. Fibrosis was assessed using the Metavir system and LAFSc on Masson's trichrome-stained slides, with scores assigned to portal, sinusoidal, and central areas, as reported earlier.[3,12] Histological inflammation was assessed on hematoxylin–eosin-stained slides. Severity of lobular inflammation, portal tract inflammation, and ductular reaction was graded as none, mild, moderate, or severe, as detailed in Table 1 and Fig. S1A-C.

ASMA staining and quantification: Immunostainings for ASMA were performed by iVIEW DAB Detection Kit for Ventana BenchMark XT (Ventana Medical Systems, Tucson, AZ, USA) automated slide stainer.[13] Primary antibodies for ASMA (Clone 1A4, NeoMarkers) were diluted 1:500 and incubated for 60 minutes. Positive and negative controls were run concurrently, which were appendix and in the absence of primary antibody, respectively. Stained slides were digitalized using an SCN400 slide scanner (Leica Biosystems, Wetzlar, Germany) at 20× magnification. These were analyzed using Tissue IA software (Leica Biosystems, Dublin, Ireland). Color deconvolution was applied to each pixel using hematoxylin and DAB matrices of the software. On the DAB matrices, a threshold was adjusted for DAB detection according

TABLE 1 Severity grading criteria of histological characteristics which were made on representative area affecting >50% of the biopsy

	Mild	Moderate	Severe
Portal tract inflammation	Discreet infiltration of lympho-plasmocytes or lymphoid aggregates	Lympho-plasmacytic infiltrates but no interface hepatitis	Presence of interface hepatitis
Lobular inflammation	Discreet infiltration of lympho-plasmocytes or lymphoid aggregates	Lympho-plasmacytic infiltrates but no lobular necrosis	Presence of lobular necrosis
Ductular reaction	Focal increased ductular reaction involving <30% of portal circumference (Fig. S1A)	Increased ductular reaction involving 30-60% of portal circumference (Fig. S1B)	Increased ductular reaction involving >60% of portal circumference (Fig. S1C)

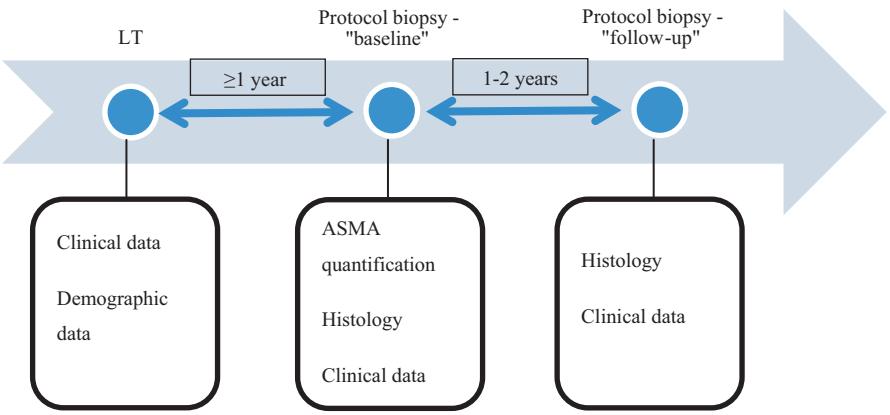

FIGURE 1 Schematic of data collection that was followed

to intensity (gray values from 0 to 255) on representative stained vs not stained tissue areas. In a similar way, a threshold was also adjusted for tissue detection. The results were expressed as ASMA-positive area percentages and calculated as (ASMA-stained area/total tissue area) ×100. The threshold parameters were kept constant for all slides, with the analysis performed on complete tissue sections.

This study was accorded approval by the hospital's ethics committee.

2.1 | Statistical analyses

The ASMA-stained area expressed as percentage of the total biopsy area was labeled the "ASMA-positive area percentage."

The association between ASMA-positive area percentage and each continuous variable (tacrolimus level, gamma-globulin level, as well as recipient and donor ages) was assessed using Pearson's product-moment correlation (Pearson's correlation) coefficient. The association between ASMA area percentage and each binary variable (recipient and donor genders and HLA antibody presence post-LT) was assessed using Student's *t* test.

Associations between ASMA-positive area percentage and baseline fibrosis score, graded by the Metavir (ordinal scale from 0 to 4) and cumulative LAFSc (ordinal scale from 0 to 9) systems, were assessed using ordinal logistic regression models. The same regression models were also used to evaluate the association between ASMA-positive area percentage and ductular reaction (ordinal scale from 0 to 3), lobular inflammation (ordinal scale from 0 to 3), and portal tract inflammation (ordinal scale from 0 to 3).

The differences in fibrosis severity between baseline and follow-up fibrosis as graded by the Metavir and cumulative LAFSc scores were labeled as "prospective change in fibrosis" and discretized into three categories (decrease, stable, or increase). The association between ASMA-positive area percentage and "prospective change in fibrosis" scores from both scoring systems was assessed using ordinal logistic regression models.

The ability of ASMA-positive area percentage to predict increases in fibrosis severity (ie, a positive prospective change in fibrosis) was determined for both fibrogenic potential scores (Metavir and cumulative LAFSc) using ROC curve analyses.

P values <.05 were considered statistically significant. Ordinal regression models were built using the "ordinal" package from the R.3.2.1 software (http://www.r-project.org), while ROC curve analyses were performed using the "ROCR" package.

3 | RESULTS

Our study included 32 pairs of "baseline" and "follow-up" biopsies from 18 children, wherein we included 10 children with one pair, two children with two pairs, and six children with three pairs of biopsies in the study. Median age at LT was 1.3 years (0.41-14.60) for the recipients and 29.17 years (4.49-43.61) for the donors. The clinical and histological details are listed in Table 1a and 1b. The protocol biopsy specimens stained for ASMA, that is "baseline biopsy," were taken at a mean of 2.89 years (1-7.46 years) post-LT and had a size of 23555780±11831897 μm² (mean±SD), and the average staining intensity of ASMA was 153±1.9 units (mean±SD) as measured by the digital morphometric software. The time interval between the protocol ASMA-stained biopsy and the next biopsy taken for "prospective fibrosis score" was 1.43 years (0.6-2.0 years). The mean duration of follow-up was 6.34 years (2.04-8.87 years).

ASMA-positive area percentage on the initial biopsy was not related to tacrolimus level (r=.29, P=.12), gamma-globulin level (r=.15, P=.22), recipient age (r=.07, P=.69) or donor age (r=−.08, P=.67), HLA antibody development post-LT (P=.48), or recipient gender (P=.42) or donor gender (P=.63).

Ordinal logistic regression models revealed ASMA-positive area percentage to display a non-significant impact on baseline fibrosis score assessed by the Metavir score (P=.66, Table 2, Figure 2 – left panel) and the cumulative LAFSc (P=.88, Table 2, Figure 2 – right panel).

ASMA-positive area percentage was associated with extent of ductular reaction (P=.06, Table 3, Figure 3 – left panel) and, to a lesser extent, with severity of lobular inflammation (P=.27, Table 3, Figure 3 – central panel) and portal tract inflammation (P=.44, Table 3, Figure 3 – right panel).

There was a significant association between the ASMA-positive area percentage of the initial biopsy and the "prospective change in fibrosis" as assessed by the Metavir score (P=.02, Table 4, Figure 4 – left panel) and cumulative LAFSc (P=.02, Table 4, Figure 4 – right panel). Within the LAFSc components, the strongest association was observed with prospective change in fibrosis in the portal tract (P=.01) (Tables S3 and S4).

TABLE 2 ASMA-positive area percentage association with baseline biopsy fibrosis score (Metavir and LAFSc)

Baseline fibrosis	ASMA-positive area percentage		Odds ratio* (*P*-value)
	Mean	SD	
Metavir			0.73 (.66)
0 (N=5)	0.55	.42	
1 (N=20)	0.72	.54	
2 (N=6)	0.47	.51	
3 (N=1)	0.35	–	
LAFSCc cumulative			1.10 (.88)
0 (N=1)	0.27	–	
1 (N=5)	0.50	.48	
2 (N=1)	–	–	
3 (N=10)	0.71	.63	
4 (N=10)	0.78	.47	
5 (N=3)	0.59	.44	
6 (N=1)	0.35	–	
7 (N=1)	0.06	–	

*Odds ratio and *P*-value were computed using an ordinal logistic regression model.

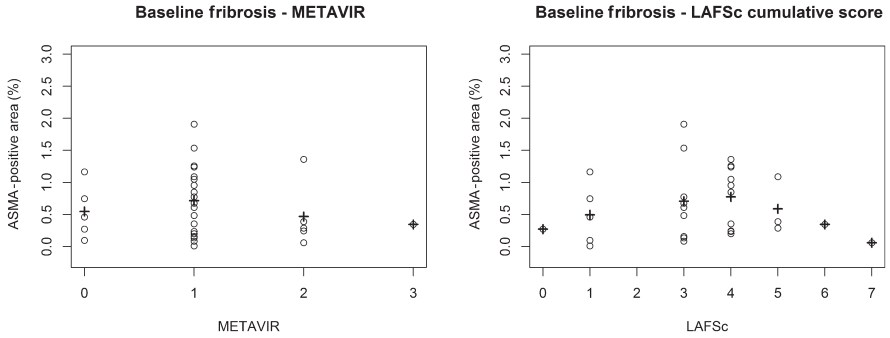

FIGURE 2 ASMA-positive area percentage mean as per the current fibrosis score expressed with Metavir (left panel) and LAFSc cumulative score (right panel)

TABLE 3 ASMA-positive area percentage association with histological characteristics on baseline biopsy

Histological characteristics	ASMA-positive area percentage		Odds ratio* (*P*-value)
	Mean	SD	
Ductular reaction			4.35 (.06)
Absent (N=8)	0.32	.41	
Mild (N=14)	0.74	.53	
Moderate (N=6)	0.82	.55	
Lobular inflammation			2.32 (.27)
Absent (N=10)	0.50	.49	
Mild (N=15)	0.74	.56	
Moderate (N=2)	0.77	.67	
Portal tract infiltration			1.70 (.44)
Absent (N=7)	0.45	.52	
Mild (N=11)	0.73	.61	
Moderate (N=6)	0.57	.37	
Severe (N=4)	0.77	.61	

*Odds ratio and *P*-value were computed using an ordinal logistic regression model.

AUROC curve analysis demonstrated that a cutoff of ASMA-positive area percentage >1.05 predicted an increase (vs stabilization or decrease) in the Metavir score and cumulative LAFSc on the next biopsy, performed within 2 years of the first, with a sensitivity of 60.0% and specificity of 90.0% (Figure 5).

4 | DISCUSSION

We demonstrated that ASMA expression is predictive of decreased, increased, or unchanged graft fibrosis over the next 2 years. Cutoff was identified, and the AUROC analysis revealed that an ASMA-positive area percentage >1.05 indicated with a 90% specificity that the fibrosis would increase over the next 2 years. In this study, ASMA quantification was conducted on biopsies obtained when the patients were stable in the post-LT period in order to avoid any early post-transplant events known to influence early graft fibrosis.[3] In addition, the computerized ASMA quantification of complete biopsy specimens, along with our selection of a clinically stable study population exhibiting no known factors that cause HSC activation, adds significance and excludes bias in the interpretation of our results. Unpublished results from our center using a different subset of population and methodology show that ASMA quantification at 6 months post-LT can also predict long-term allograft fibrosis.

Two independent studies, both very similar in design and performed on adult LT recipients infected with hepatitis C, have also demonstrated the predictive ability of ASMA quantification for fibrosis in the 2 years post-LT.[14,15] These authors reported that ASMA quantification performed 4 months post-LT correlated with the extent

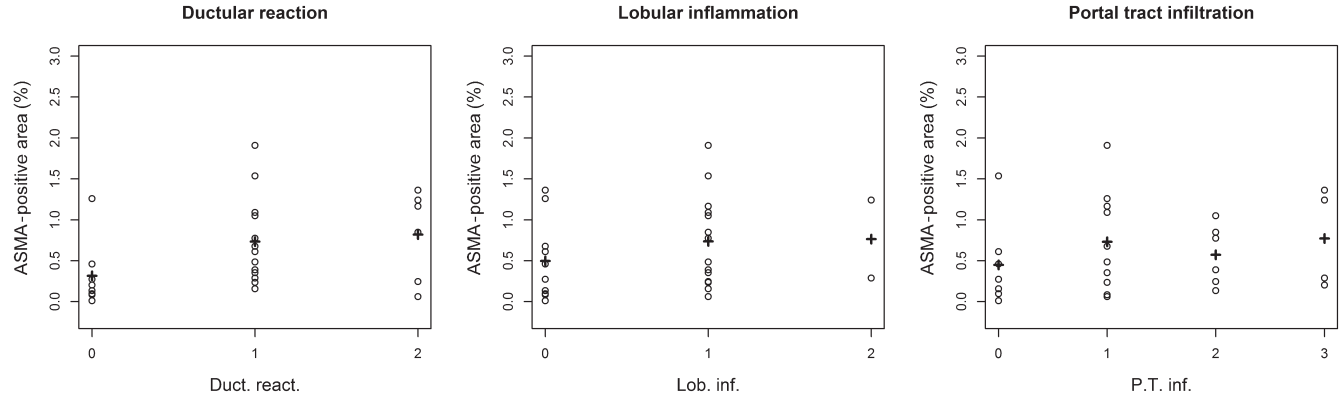

FIGURE 3 ASMA-positive area percentage mean as per the biliary duct proliferation (left panel), lobular inflammation (central panel), and portal tract infiltration (right panel)

of fibrosis observed 2 years post-LT. Although these results are corroborative, the authors' methods of ASMA quantification differed between the studies, thus highlighting the need to standardize both ASMA staining and quantification procedures if it is to be used as standard biomarker of future fibrosis. To address this issue, we used an automated ASMA staining protocol and a non-user-dependent, easily reproducible method for quantifying ASMA. The aforementioned studies both used a time interval of 2 years between ASMA quantification and fibrosis evaluation, which is a step forward as compared to the previous cross-sectional studies that showed an association between ASMA quantification and baseline fibrosis.[16-19]

TABLE 4 ASMA-positive area percentage association with Prospective change in fibrosis (Metavir and LAFSc)

Prospective change in fibrosis	ASMA-positive area percentage		Odds ratio* (*P*-value)
	Mean	SD	
Metavir			7.41 (.02)
Decrease (N=3)	0.15	.21	
Stable (N=18)	0.56	.52	
Increase (N=11)	0.90	.40	
LAFSc (cumulative)			6.77 (.02)
Decrease (N=6)	0.44	.32	
Stable (N=14)	0.46	.45	
Increase (N=12)	0.99	.52	

*Odds ratio and *P*-value were computed using an ordinal logistic regression model.

In contrast, we did not find ASMA quantification to be correlated with the severity of the concomitant fibrosis in the baseline biopsy. This is understandable as HSC activation would precede fibrosis and is not a marker of coexisting fibrosis. Fibrosis is, in fact, a dynamic process, and experimental models have demonstrated that clearance of activated HSCs is a key issue in fibrosis regression.[20] A longitudinal evaluation leaving an adequate time difference between the two biopsies was therefore essential for evaluating the predictive effect of HSC activation or deactivation. Activated HSCs remain so until the eradication of the activation stimulus, at which point they undergo either de-activation or apoptosis, in both cases losing ASMA positivity.[10,11] These aspects further justify that ASMA quantification should predict the consequent fibrosis and not its association with coexisting fibrosis, which if seen could be accidental. Our study is in line with observations made in experimental models that clearance of activated HSCs precedes the decrease in fibrosis.

Our study is also in accordance with a previous report on two immunotolerant pediatric LT recipients, weaned from immune suppression, who exhibited an increased percentage of activated HSCs following immunosuppression withdrawal, leading to subsequent fibrosis.[21] HSCs play a role in maintaining hepatic immune homeostasis as antigen-presenting cells, as well as in promoting tolerance. The increased HSC activation observed in these two cases could thus be due

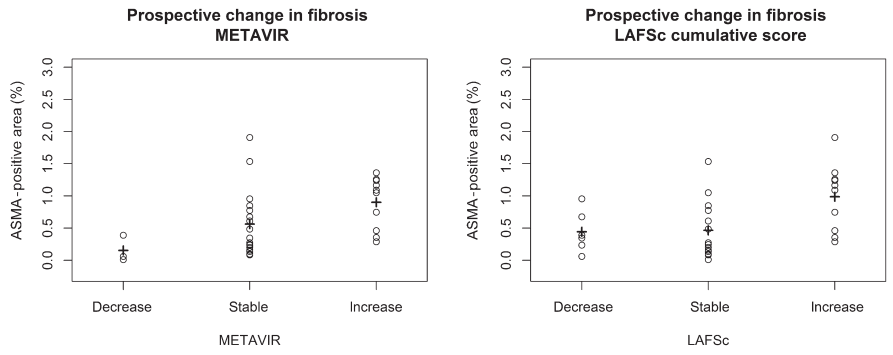

FIGURE 4 ASMA-positive area percentage mean as per the prospective change in fibrosis assessed by the Metavir score (left panel) and by the LAFSc cumulative score (right panel)

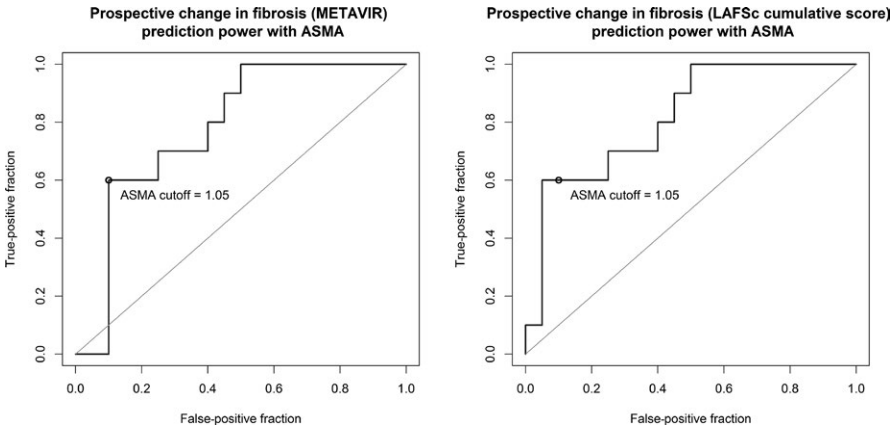

FIGURE 5 Receiver-operator characteristic curves for ASMA for the prediction of prospective change in fibrosis (increase vs stabilization or decrease) expressed with METAVIR (left panel) and with LAFSc cumulative score (right panel)

to the cumulative effect accounted for by the fibrogenic potential and lack of immune suppression. This aspect was also highlighted when children with autoimmune hepatitis exhibited fewer ASMA-positive HSCs following treatment as compared to prior to treatment.[22,23] ASMA quantification could thus perhaps be a surrogate marker of immune dynamics, although in our study we observed no relation to the tacrolimus level, gamma-globulin level, or antibody (HLA, ANA, LKM) status, nor any evidence of post-LT alloimmunity [2]).

Protocol biopsies on pediatric LT recipients remain a matter of debate as the majority present non-specific histological changes, and the clinical significance of any findings is unclear.[4,5] The importance of protocol biopsy and their interpretation was recently studied using successive protocol biopsies from stable pediatric LT recipients. It was observed that allograft inflammation preceded fibrosis and the severity of inflammation was related the magnitude of the consequent fibrosis.[7] This study establishes the link between the allograft inflammation and fibrosis and probably opens up an avenue for intervention. In the current study, we found that ductular reaction tended to be associated with ASMA quantification and fibrosis severity, although this association needs to be confirmed in a prospective study. These findings are in concordance with previously published data, wherein ductular reaction has been shown to correlate closely with fibrosis severity in chronic hepatitis C infection,[24] alcoholic liver disease, NASH,[25] and hemochromatosis.[26] Although controversial, the origin of this ductular reaction could also be linked to the epithelial mesenchymal transition process during hepatic fibrosis.[9,27] This process could explain the acquisition of ASMA positivity in these cells prior to and also during fibrogenesis. Although portal inflammation is known to predict portal fibrosis and we observed ASMA expression to precede fibrosis, no correlation was observed between the severity of portal inflammation and ASMA expression. This could be attributed to the fact that we quantified ASMA on the whole biopsy specimen and not independently in peri-portal, sinusoidal, and centrilobular areas. With improving morphometric technology, automated quantification in these regions would be possible and this aspect would gain more clarity. Another interesting yet concerning development reported in a recent publication, which reported that reverted HSCs in culture from activated to quiescent state displayed an enhanced susceptibility to subsequent activation by TGF-B1 or mitogens.[10,11,20] De-activated HSCs retain an intermediate activation state, and the matrix stiffness and components like collagen 1 and integrin are thought to maintain HSCs in an activated state.[28,29] The implication of these findings if extended to the clinical spectrum could be immense, as a decrease in fibrosis severity would thus not necessarily mean monitoring could be scaled back, but that it should rather be enhanced to ensure sustained good prognosis.

Although our study has provided significant results, we included and evaluated only a limited number of children from our large transplant cohort, as we selected a very homogeneous population with evaluable criteria. A prospective multicenter study should now be planned and implemented for validating this concept, as well as the cutoffs for ASMA quantification and pediatric allograft-specific fibrosis quantification.

CONFLICT OF INTEREST

None.

AUTHORS' CONTRIBUTIONS

Sharat Varma (SV): Participated in study design and concept, staining, digital quantification, data interpretation, and manuscript preparation; Xavier Stéphenne (XS): Participated in study design, data interpretation, and manuscript preparation; Mina Komuta (MK): Participated in histopathological evaluation; Caroline Bouzin (CB): Participated in digital quantification; Jerome Ambroise (JA): Participated in statistical analysis; Francoise Smets (FS): Participated in data review and manuscript review; Raymond Reding (RR): Participated in data review and manuscript review; Etienne Sokal (ES): Participated in study design and concept, data interpretation, and manuscript preparation.

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Accepted: 21 September 2016

DOI: 10.1111/petr.12834

ORIGINAL ARTICLE

WILEY

The histological quantification of alpha-smooth muscle actin predicts future graft fibrosis in pediatric liver transplant recipients

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Abstract

Activated hepatic stellate cells express cytoplasmic ASMA prior to secreting collagen and consequent liver fibrosis. We hypothesized that quantifying ASMA could predict severity of future fibrosis after LT. For this, 32 pairs of protocol biopsies, that is, "baseline" and "follow-up" biopsies taken at 1- to 2-year intervals from 18 stable pediatric LT recipients, transplanted between 2006 and 2012 were selected. Morphometric quantification of "ASMA-positive area percentage" was performed on the baseline biopsy. Histological and fibrosis assessment using Metavir and LAFSc was performed on all biopsies. The difference of fibrosis severity between the "baseline" and "follow-up" was termed "prospective change in fibrosis." Significant association was seen between extent of ASMA positivity on baseline biopsy and "prospective change in fibrosis" using Metavir (P=.02), cumulative LAFSc (P=.02), and portal LAFSc (P=.01) values. ASMA-positive area percentage >1.05 predicted increased fibrosis on next biopsy with 90.0% specificity. Additionally, an association was observed between extent of ASMA positivity and concomitant ductular reaction (P=.06), but not with histological inflammation in the portal tract or lobular area. Hence, ASMA quantification can predict the future course of fibrosis.

KEYWORDS

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1 | INTRODUCTION

Since its humble beginnings in 1963, pediatric LT has achieved consistently improved outcomes, with current 10-year survival rates exceeding 85%.[1] Increased long-term survival has however revealed increasing graft fibrosis and other histological changes which progressively increase over time following LT.[2-5] The factors involved in late allograft fibrosis and potential treatments that can reverse or delay its development are a subject of current research.[6,7] To monitor allograft health and treatment efficacy, finding an efficient predictive marker of fibrosis development proves to be a top priority.

Fibrosis in the liver is primarily attributed to the activation of HSCs, which results in secretion of collagen types 1 and 3. HSCs can be activated in the presence of inflammation, and the expression of cytoplasmic ASMA is a consistent marker of this activation.[8,9] On removal of the activation-inducing factors, the HSCs subsequently either de-activate without expressing ASMA or undergo apoptosis.[10,11] Hence, we hypothesized that ASMA expression should mirror the later

Abbreviations: ANA, antinuclear; ASMA, alpha-smooth muscle actin; AU, area under; AUROC, AU receiver operating characteristic; HLA, human leukocyte antigen; HSC, hepatic stellate cell; LAFSc, liver allograft fibrosis score; LKM, liver–kidney microsomal; LT, liver transplantation; MT, Masson's trichrome; ROC, receiver operator characteristic; SMA, smooth muscle actin.

changes in fibrosis and be able to predict its severity. To avoid the influence of early post-transplantation events, we analyzed the biopsies taken after 1 year following LT in stable patients who exhibited no surgical or medical complications. Our study sought to evaluate whether ASMA expression, measured after 1 year post-LT in stable patients, could be considered a predictor of change in fibrosis severity in the future 2 years among LT recipients.

1.1 | Patients

This retrospective study sought to evaluate only successive protocol biopsies, which were taken at ≥1 year of LT and had an interval of 1-2 years between each other. Children who underwent LT from 2006 to 2012 and had ≥2 such biopsies were included. Exclusion criteria were death of LT recipient, re-transplantation, biliary or vascular complications, chronic rejection, acute rejections in the preceding 2 years, hepatitis B or C infection, autoimmune hepatitis, or hepatotoxic drug exposure. All included children were maintained on the same immunosuppression drugs between studied biopsies.

1.2 | Data collection

Data were collected as outlined in Figure 1.

2 | METHODS

Protocol biopsy details: Only paired protocol biopsies were included (Figure 1), wherein the first biopsy was taken ≥1 year post-LT and labeled as "baseline biopsy." The successive protocol biopsy which was taken within 2 years of the "baseline biopsy" was labeled as "follow-up biopsy." In cases where a child had >1 such pair of protocol biopsies, that is, pairs of biopsies within 1-2 years of each other, all such pairs were included. The change of fibrosis severity between the "baseline" and "follow-up" biopsy was termed as "prospective change in fibrosis."

Histological evaluation: Each protocol biopsy was evaluated for inflammation and fibrosis by three independent observers (MK, SV, XS), with a consensus made for categorization differences. Fibrosis was assessed using the Metavir system and LAFSc on Masson's trichrome-stained slides, with scores assigned to portal, sinusoidal, and central areas, as reported earlier.[3,12] Histological inflammation was assessed on hematoxylin–eosin-stained slides. Severity of lobular inflammation, portal tract inflammation, and ductular reaction was graded as none, mild, moderate, or severe, as detailed in Table 1 and Fig. S1A-C.

ASMA staining and quantification: Immunostainings for ASMA were performed by iVIEW DAB Detection Kit for Ventana BenchMark XT (Ventana Medical Systems, Tucson, AZ, USA) automated slide stainer.[13] Primary antibodies for ASMA (Clone 1A4, NeoMarkers) were diluted 1:500 and incubated for 60 minutes. Positive and negative controls were run concurrently, which were appendix and in the absence of primary antibody, respectively. Stained slides were digitalized using an SCN400 slide scanner (Leica Biosystems, Wetzlar, Germany) at 20× magnification. These were analyzed using Tissue IA software (Leica Biosystems, Dublin, Ireland). Color deconvolution was applied to each pixel using hematoxylin and DAB matrices of the software. On the DAB matrices, a threshold was adjusted for DAB detection according

TABLE 1 Severity grading criteria of histological characteristics which were made on representative area affecting >50% of the biopsy

	Mild	Moderate	Severe
Portal tract inflammation	Discreet infiltration of lympho-plasmocytes or lymphoid aggregates	Lympho-plasmacytic infiltrates but no interface hepatitis	Presence of interface hepatitis
Lobular inflammation	Discreet infiltration of lympho-plasmocytes or lymphoid aggregates	Lympho-plasmacytic infiltrates but no lobular necrosis	Presence of lobular necrosis
Ductular reaction	Focal increased ductular reaction involving <30% of portal circumference (Fig. S1A)	Increased ductular reaction involving 30-60% of portal circumference (Fig. S1B)	Increased ductular reaction involving >60% of portal circumference (Fig. S1C)

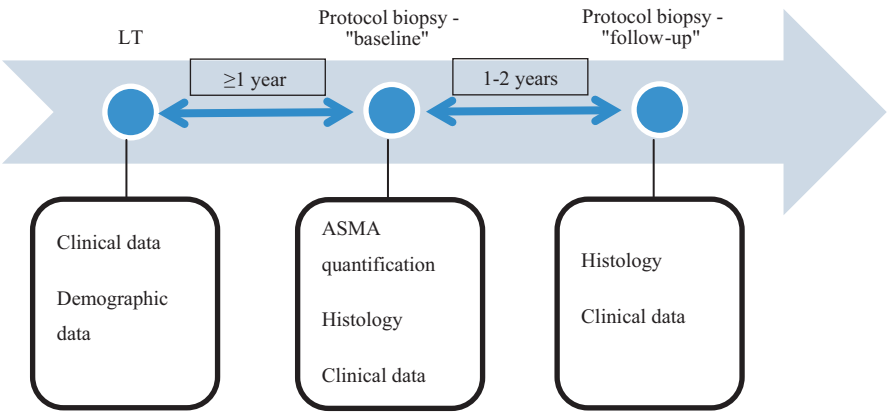

FIGURE 1 Schematic of data collection that was followed

to intensity (gray values from 0 to 255) on representative stained vs not stained tissue areas. In a similar way, a threshold was also adjusted for tissue detection. The results were expressed as ASMA-positive area percentages and calculated as (ASMA-stained area/total tissue area) ×100. The threshold parameters were kept constant for all slides, with the analysis performed on complete tissue sections.

This study was accorded approval by the hospital's ethics committee.

2.1 | Statistical analyses

The ASMA-stained area expressed as percentage of the total biopsy area was labeled the "ASMA-positive area percentage."

The association between ASMA-positive area percentage and each continuous variable (tacrolimus level, gamma-globulin level, as well as recipient and donor ages) was assessed using Pearson's product-moment correlation (Pearson's correlation) coefficient. The association between ASMA area percentage and each binary variable (recipient and donor genders and HLA antibody presence post-LT) was assessed using Student's *t* test.

Associations between ASMA-positive area percentage and baseline fibrosis score, graded by the Metavir (ordinal scale from 0 to 4) and cumulative LAFSc (ordinal scale from 0 to 9) systems, were assessed using ordinal logistic regression models. The same regression models were also used to evaluate the association between ASMA-positive area percentage and ductular reaction (ordinal scale from 0 to 3), lobular inflammation (ordinal scale from 0 to 3), and portal tract inflammation (ordinal scale from 0 to 3).

The differences in fibrosis severity between baseline and follow-up fibrosis as graded by the Metavir and cumulative LAFSc scores were labeled as "prospective change in fibrosis" and discretized into three categories (decrease, stable, or increase). The association between ASMA-positive area percentage and "prospective change in fibrosis" scores from both scoring systems was assessed using ordinal logistic regression models.

The ability of ASMA-positive area percentage to predict increases in fibrosis severity (ie, a positive prospective change in fibrosis) was determined for both fibrogenic potential scores (Metavir and cumulative LAFSc) using ROC curve analyses.

P values <.05 were considered statistically significant. Ordinal regression models were built using the "ordinal" package from the R.3.2.1 software (http://www.r-project.org), while ROC curve analyses were performed using the "ROCR" package.

3 | RESULTS

Our study included 32 pairs of "baseline" and "follow-up" biopsies from 18 children, wherein we included 10 children with one pair, two children with two pairs, and six children with three pairs of biopsies in the study. Median age at LT was 1.3 years (0.41-14.60) for the recipients and 29.17 years (4.49-43.61) for the donors. The clinical and histological details are listed in Table 1a and 1b. The protocol biopsy specimens stained for ASMA, that is "baseline biopsy," were taken at a mean of 2.89 years (1-7.46 years) post-LT and had a size of 23555780±11831897 μm² (mean±SD), and the average staining intensity of ASMA was 153±1.9 units (mean±SD) as measured by the digital morphometric software. The time interval between the protocol ASMA-stained biopsy and the next biopsy taken for "prospective fibrosis score" was 1.43 years (0.6-2.0 years). The mean duration of follow-up was 6.34 years (2.04-8.87 years).

ASMA-positive area percentage on the initial biopsy was not related to tacrolimus level (r=.29, P=.12), gamma-globulin level (r=.15, P=.22), recipient age (r=.07, P=.69) or donor age (r=−.08, P=.67), HLA antibody development post-LT (P=.48), or recipient gender (P=.42) or donor gender (P=.63).

Ordinal logistic regression models revealed ASMA-positive area percentage to display a non-significant impact on baseline fibrosis score assessed by the Metavir score (P=.66, Table 2, Figure 2 – left panel) and the cumulative LAFSc (P=.88, Table 2, Figure 2 – right panel).

ASMA-positive area percentage was associated with extent of ductular reaction (P=.06, Table 3, Figure 3 – left panel) and, to a lesser extent, with severity of lobular inflammation (P=.27, Table 3, Figure 3 – central panel) and portal tract inflammation (P=.44, Table 3, Figure 3 – right panel).

There was a significant association between the ASMA-positive area percentage of the initial biopsy and the "prospective change in fibrosis" as assessed by the Metavir score (P=.02, Table 4, Figure 4 – left panel) and cumulative LAFSc (P=.02, Table 4, Figure 4 – right panel). Within the LAFSc components, the strongest association was observed with prospective change in fibrosis in the portal tract (P=.01) (Tables S3 and S4).

TABLE 2 ASMA-positive area percentage association with baseline biopsy fibrosis score (Metavir and LAFSc)

Baseline fibrosis	ASMA-positive area percentage		Odds ratio* (*P*-value)
	Mean	SD	
Metavir			0.73 (.66)
0 (N=5)	0.55	.42	
1 (N=20)	0.72	.54	
2 (N=6)	0.47	.51	
3 (N=1)	0.35	–	
LAFSCc cumulative			1.10 (.88)
0 (N=1)	0.27	–	
1 (N=5)	0.50	.48	
2 (N=1)	–	–	
3 (N=10)	0.71	.63	
4 (N=10)	0.78	.47	
5 (N=3)	0.59	.44	
6 (N=1)	0.35	–	
7 (N=1)	0.06	–	

*Odds ratio and *P*-value were computed using an ordinal logistic regression model.

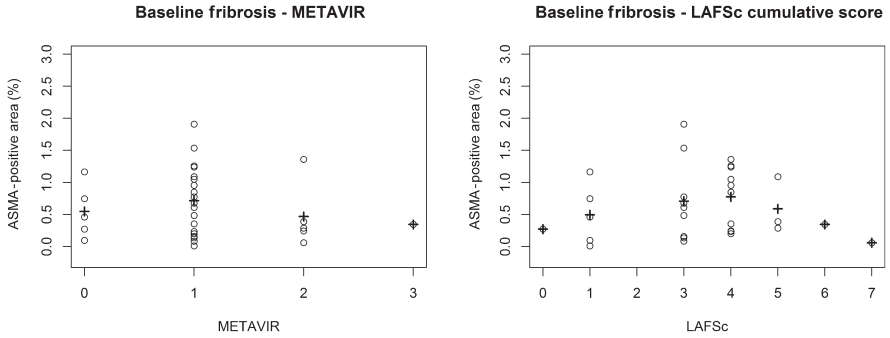

FIGURE 2 ASMA-positive area percentage mean as per the current fibrosis score expressed with Metavir (left panel) and LAFSc cumulative score (right panel)

TABLE 3 ASMA-positive area percentage association with histological characteristics on baseline biopsy

Histological characteristics	ASMA-positive area percentage		Odds ratio* (*P*-value)
	Mean	SD	
Ductular reaction			4.35 (.06)
Absent (N=8)	0.32	.41	
Mild (N=14)	0.74	.53	
Moderate (N=6)	0.82	.55	
Lobular inflammation			2.32 (.27)
Absent (N=10)	0.50	.49	
Mild (N=15)	0.74	.56	
Moderate (N=2)	0.77	.67	
Portal tract infiltration			1.70 (.44)
Absent (N=7)	0.45	.52	
Mild (N=11)	0.73	.61	
Moderate (N=6)	0.57	.37	
Severe (N=4)	0.77	.61	

*Odds ratio and *P*-value were computed using an ordinal logistic regression model.

AUROC curve analysis demonstrated that a cutoff of ASMA-positive area percentage >1.05 predicted an increase (vs stabilization or decrease) in the Metavir score and cumulative LAFSc on the next biopsy, performed within 2 years of the first, with a sensitivity of 60.0% and specificity of 90.0% (Figure 5).

4 | DISCUSSION

We demonstrated that ASMA expression is predictive of decreased, increased, or unchanged graft fibrosis over the next 2 years. Cutoff was identified, and the AUROC analysis revealed that an ASMA-positive area percentage >1.05 indicated with a 90% specificity that the fibrosis would increase over the next 2 years. In this study, ASMA quantification was conducted on biopsies obtained when the patients were stable in the post-LT period in order to avoid any early post-transplant events known to influence early graft fibrosis.[3] In addition, the computerized ASMA quantification of complete biopsy specimens, along with our selection of a clinically stable study population exhibiting no known factors that cause HSC activation, adds significance and excludes bias in the interpretation of our results. Unpublished results from our center using a different subset of population and methodology show that ASMA quantification at 6 months post-LT can also predict long-term allograft fibrosis.

Two independent studies, both very similar in design and performed on adult LT recipients infected with hepatitis C, have also demonstrated the predictive ability of ASMA quantification for fibrosis in the 2 years post-LT.[14,15] These authors reported that ASMA quantification performed 4 months post-LT correlated with the extent

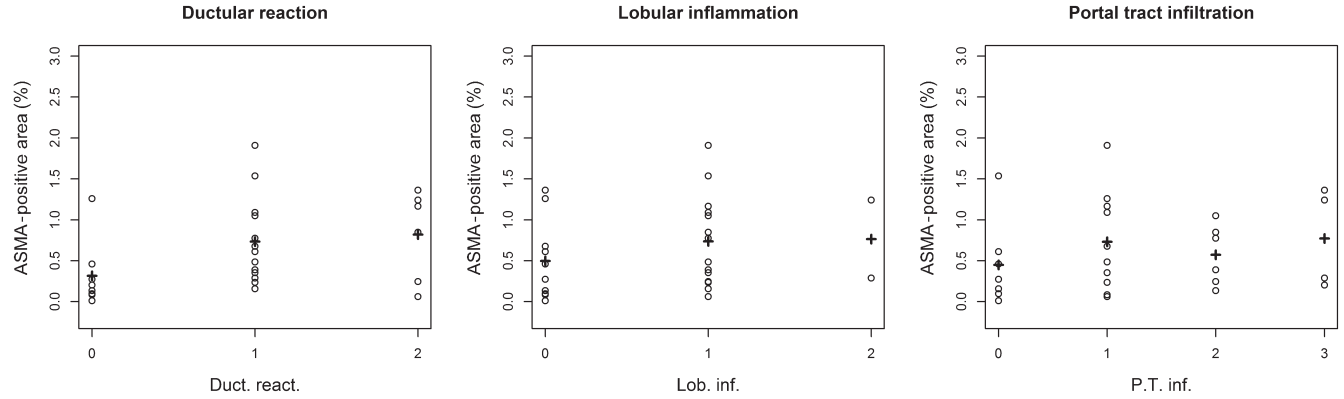

FIGURE 3 ASMA-positive area percentage mean as per the biliary duct proliferation (left panel), lobular inflammation (central panel), and portal tract infiltration (right panel)

of fibrosis observed 2 years post-LT. Although these results are corroborative, the authors' methods of ASMA quantification differed between the studies, thus highlighting the need to standardize both ASMA staining and quantification procedures if it is to be used as standard biomarker of future fibrosis. To address this issue, we used an automated ASMA staining protocol and a non-user-dependent, easily reproducible method for quantifying ASMA. The aforementioned studies both used a time interval of 2 years between ASMA quantification and fibrosis evaluation, which is a step forward as compared to the previous cross-sectional studies that showed an association between ASMA quantification and baseline fibrosis.[16-19]

TABLE 4 ASMA-positive area percentage association with Prospective change in fibrosis (Metavir and LAFSc)

Prospective change in fibrosis	ASMA-positive area percentage		Odds ratio* (*P*-value)
	Mean	SD	
Metavir			7.41 (.02)
Decrease (N=3)	0.15	.21	
Stable (N=18)	0.56	.52	
Increase (N=11)	0.90	.40	
LAFSc (cumulative)			6.77 (.02)
Decrease (N=6)	0.44	.32	
Stable (N=14)	0.46	.45	
Increase (N=12)	0.99	.52	

*Odds ratio and *P*-value were computed using an ordinal logistic regression model.

In contrast, we did not find ASMA quantification to be correlated with the severity of the concomitant fibrosis in the baseline biopsy. This is understandable as HSC activation would precede fibrosis and is not a marker of coexisting fibrosis. Fibrosis is, in fact, a dynamic process, and experimental models have demonstrated that clearance of activated HSCs is a key issue in fibrosis regression.[20] A longitudinal evaluation leaving an adequate time difference between the two biopsies was therefore essential for evaluating the predictive effect of HSC activation or deactivation. Activated HSCs remain so until the eradication of the activation stimulus, at which point they undergo either de-activation or apoptosis, in both cases losing ASMA positivity.[10,11] These aspects further justify that ASMA quantification should predict the consequent fibrosis and not its association with coexisting fibrosis, which if seen could be accidental. Our study is in line with observations made in experimental models that clearance of activated HSCs precedes the decrease in fibrosis.

Our study is also in accordance with a previous report on two immunotolerant pediatric LT recipients, weaned from immune suppression, who exhibited an increased percentage of activated HSCs following immunosuppression withdrawal, leading to subsequent fibrosis.[21] HSCs play a role in maintaining hepatic immune homeostasis as antigen-presenting cells, as well as in promoting tolerance. The increased HSC activation observed in these two cases could thus be due

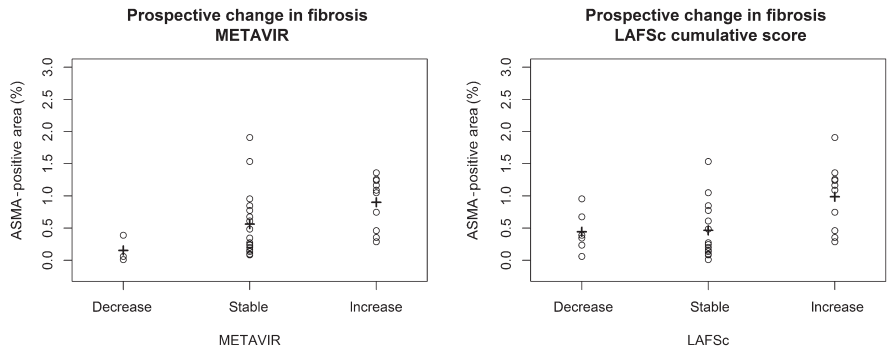

FIGURE 4 ASMA-positive area percentage mean as per the prospective change in fibrosis assessed by the Metavir score (left panel) and by the LAFSc cumulative score (right panel)

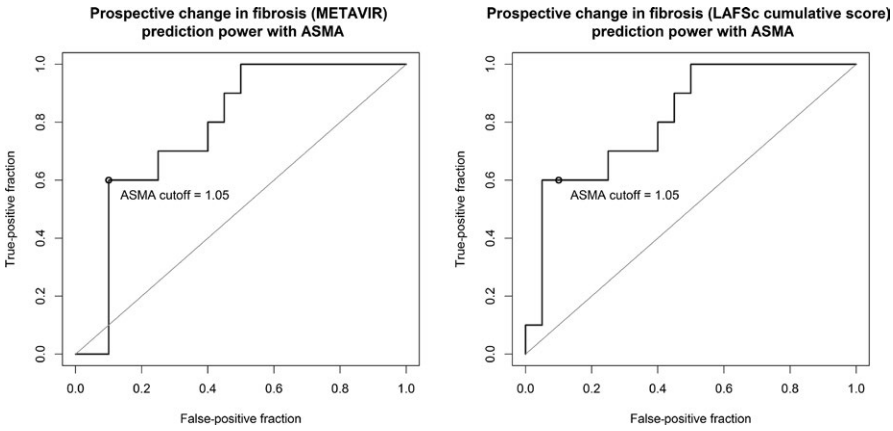

FIGURE 5 Receiver-operator characteristic curves for ASMA for the prediction of prospective change in fibrosis (increase vs stabilization or decrease) expressed with METAVIR (left panel) and with LAFSc cumulative score (right panel)

to the cumulative effect accounted for by the fibrogenic potential and lack of immune suppression. This aspect was also highlighted when children with autoimmune hepatitis exhibited fewer ASMA-positive HSCs following treatment as compared to prior to treatment.[22,23] ASMA quantification could thus perhaps be a surrogate marker of immune dynamics, although in our study we observed no relation to the tacrolimus level, gamma-globulin level, or antibody (HLA, ANA, LKM) status, nor any evidence of post-LT alloimmunity [2]).

Protocol biopsies on pediatric LT recipients remain a matter of debate as the majority present non-specific histological changes, and the clinical significance of any findings is unclear.[4,5] The importance of protocol biopsy and their interpretation was recently studied using successive protocol biopsies from stable pediatric LT recipients. It was observed that allograft inflammation preceded fibrosis and the severity of inflammation was related the magnitude of the consequent fibrosis.[7] This study establishes the link between the allograft inflammation and fibrosis and probably opens up an avenue for intervention. In the current study, we found that ductular reaction tended to be associated with ASMA quantification and fibrosis severity, although this association needs to be confirmed in a prospective study. These findings are in concordance with previously published data, wherein ductular reaction has been shown to correlate closely with fibrosis severity in chronic hepatitis C infection,[24] alcoholic liver disease, NASH,[25] and hemochromatosis.[26] Although controversial, the origin of this ductular reaction could also be linked to the epithelial mesenchymal transition process during hepatic fibrosis.[9,27] This process could explain the acquisition of ASMA positivity in these cells prior to and also during fibrogenesis. Although portal inflammation is known to predict portal fibrosis and we observed ASMA expression to precede fibrosis, no correlation was observed between the severity of portal inflammation and ASMA expression. This could be attributed to the fact that we quantified ASMA on the whole biopsy specimen and not independently in peri-portal, sinusoidal, and centrilobular areas. With improving morphometric technology, automated quantification in these regions would be possible and this aspect would gain more clarity. Another interesting yet concerning development reported in a recent publication, which reported that reverted HSCs in culture from activated to quiescent state displayed an enhanced susceptibility to subsequent activation by TGF-B1 or mitogens.[10,11,20] De-activated HSCs retain an intermediate activation state, and the matrix stiffness and components like collagen 1 and integrin are thought to maintain HSCs in an activated state.[28,29] The implication of these findings if extended to the clinical spectrum could be immense, as a decrease in fibrosis severity would thus not necessarily mean monitoring could be scaled back, but that it should rather be enhanced to ensure sustained good prognosis.

Although our study has provided significant results, we included and evaluated only a limited number of children from our large transplant cohort, as we selected a very homogeneous population with evaluable criteria. A prospective multicenter study should now be planned and implemented for validating this concept, as well as the cutoffs for ASMA quantification and pediatric allograft-specific fibrosis quantification.

CONFLICT OF INTEREST

None.

AUTHORS' CONTRIBUTIONS

Sharat Varma (SV): Participated in study design and concept, staining, digital quantification, data interpretation, and manuscript preparation; Xavier Stéphenne (XS): Participated in study design, data interpretation, and manuscript preparation; Mina Komuta (MK): Participated in histopathological evaluation; Caroline Bouzin (CB): Participated in digital quantification; Jerome Ambroise (JA): Participated in statistical analysis; Francoise Smets (FS): Participated in data review and manuscript review; Raymond Reding (RR): Participated in data review and manuscript review; Etienne Sokal (ES): Participated in study design and concept, data interpretation, and manuscript preparation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Varma S, Stéphenne X, Komuta M, et al. The histological quantification of alpha-smooth muscle actin predicts future graft fibrosis in pediatric liver transplant recipients. *Pediatr Transplant*. 2017;21:e12834. doi: 10.1111/petr.12834

Article 5

Title

Ductular reaction on protocol biopsy, post liver transplant corresponds to concurrent fibrosis and does not predict fibrogenesis

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Abbreviations:

Liver transplantation (LT)

Protocol biopsy (PB)

Ductular reaction (DR)

Cytokeratin-7 (CK7)

Liver allograft fibrosis score (LAFSc)

Masson's trichrome (MT)

Keywords:

1. Cytokeratin 7
2. Hepatic progenitor cells
3. Portal inflammation
4. Pediatric liver transplantation
5. Fibrosis prediction

Conflict of interest: None

Financial support: None

Author contributions:

Varma Sharat (SV): Study design and concept, Staining, Digital quantification, Data interpretation, Manuscript preparation

Ambroise Jerome (JA): Statistical analysis

Komuta Mina (MK): Histo-pathological evaluation

Bouzin.Caroline (CB): Digital quantification

Reding Raymond (RR): Data review, Manuscript review

Sokal Etienne (ES): Study design and concept, Data interpretation, Manuscript preparation

Abstract

Background and aims:

Liver transplantation among children has been established to be a successful modality with excellent long term survival. Recently, studies assessing protocol biopsies have shown that allograft fibrosis and other histological changes are frequently present even among stable LT recipients. Ductular reaction is frequently seen on PB and is considered as a predictor of increasing fibrogenesis. This hypothesis is controversial and there is lack of clarity whether fibrosis a cause or effect of DR development. We analyzed the impact of ductular reaction on fibrosis, using paired protocol biopsies of stable liver transplant recipient children.

Methods:

Children who underwent LT from 2012 to 2014 and had ≥2 PB at an interval of 1-2 years between each other were included. The first PB was labeled as "baseline biopsy" and the successive biopsy as "follow-up biopsy." The change of fibrosis severity between the "baseline" and "follow-up" biopsy was termed as "prospective change in fibrosis." Each biopsy was evaluated for inflammation and fibrosis using the Metavir and LAFSc system. The baseline biopsy was stained with CK7 and digitally evaluated to obtain "CK7 – positive area percentage" i.e. CK7-stained area expressed as percentage of the total biopsy area. The association between CK7-positive area percentage on baseline biopsy and "prospective change in fibrosis" scores, ductular reaction, lobular inflammation, and portal tract inflammation was assessed using ordinal logistic regression models.

Results

Our study included 64 pared PB from 32 children. The baseline PB stained for CK7, were taken at a mean of 2.89 years post-LT. The time interval between the two paired PB's was 1.41 years. CK7-positive area percentage was significantly associated with baseline fibrosis, extent of ductular reaction (p=.006) and portal tract inflammation (p=.01). There was a no significant association between the CK7-positive area percentage of the baseline biopsy and the "prospective change in

fibrosis" as assessed by the Metavir score (p=.36) or cumulative LAFSc (p=.25) or with recipient or donor age, donor type, HLA antibodies, or recipient or donor gender.

Conclusion:

Ductular reaction, CK7 expression is significantly associated with extent of portal tract inflammation and concurrent severity of fibrosis but does not predict the future course of fibrogenesis.

Introduction

Liver transplantation (LT) among children has been established to be a successful modality with excellent long term survival. With this success, our expectations have evolved towards maintaining normal hepatic allograft function and histology. Recently, studies assessing protocol biopsies (PB) have shown that allograft fibrosis and other histological changes are frequently present even among stable LT recipients. The etio-pathological understanding of allograft fibrosis would enable us to devise potential treatments that can reverse or delay its development.

Ductular reaction (DR) which is identified as proliferation of the most terminal branches of the biliary tree at the interface of the portal tracts, is frequently seen on PB. Histologically it's evident on cytokeratin 7 (CK7) staining. In chronic liver diseases like Hepatitis C infection, non-alcoholic steatohepatitis and haemochromatosis, DR has been shown to correlate to extent of fibrosis and severity of portal inflammation. It's been suggested that replicative arrest of hepatocyte stimulates the DR which includes proliferation of the bi-potential progenitor cells in the canal of Herring. This results in progressive collagen deposition and fibrosis; hence DR is considered as a marker for increasing fibrogenesis. This hypothesis is controversial as there is experimental data demonstrating that altered extracellular matrix and fibrosis predispose to DR development. As there exists no conclusive human study to verify either hypothesis, we decided to analyze the impact of DR if it is a "cause or effect" of fibrosis, using paired PB's.

Methods

Patients: We did a retrospective study sought to evaluate only successive protocol biopsies, which were taken at ≥1 year of LT and had an interval of 1-2 years between each other. Children who underwent LT from 2012 to 2014 and had ≥2 such biopsies were included.

Protocol biopsy details: Only paired protocol biopsies were included, wherein the first biopsy was taken ≥ 1 year post-LT and labeled as "baseline biopsy." The successive protocol biopsy which was taken within 2 years of the "baseline biopsy" was labeled as "follow-up biopsy." The change of fibrosis severity between the "baseline" and "follow-up" biopsy was termed as "prospective change in fibrosis."

Histological evaluation: Each protocol biopsy was evaluated for inflammation and fibrosis by three independent observers, with a consensus made for categorization differences. Fibrosis was assessed using the Metavir system and Liver allograft fibrosis score (LAFSc) on Masson's trichrome (MT) stained slides. Histological inflammation was assessed on hematoxylin–eosin-stained slides. Severity of lobular inflammation and portal tract inflammation was graded as none, mild, moderate, or severe.

CK7 staining and quantification: Immunostainings for CK7 were performed by iVIEW DAB Detection Kit for Ventana BenchMark XT (Ventana Medical Systems, Tucson, AZ, USA) automated slide stainer (112). Primary antibodies for CK7 were diluted 1:500 and incubated for 60 minutes. Positive and negative controls were run concurrently, which were appendix and in the absence of primary antibody, respectively. Stained slides were digitalized using an SCN400 slide scanner (Leica Biosystems, Wetzlar, Germany) at 20× magnification. These were analyzed using the image analysis tool Author version 6.0.0 (Visiopharm, Hørsholm, Denmark).

Tissue was first detected from the background at a low digital magnification (5x) using a thresholding classification method based on the intensity (IHS-I) image feature. Tissue borders, sometimes aspecifically stained, were eroded and excluded from the analysis, as were irrelevant small tissue areas.

CK7-positive pixels were then detected within the previously delineated tissue at high resolution (20x) using a thresholding classification method based on preprocessing steps highlighting the DAB staining (HDAB-DAB matrice of the software). Threshold was adjusted on representative stained vs not stained tissue areas. The results were expressed as CK7-positive area percentages and calculated as (CK7-stained area/total tissue area) ×100. The parameters were kept constant for all slides, with the analysis performed on complete tissue sections.

Statistical analyses: The CK7-stained area expressed as percentage of the total biopsy area was labeled the "CK7 – positive area percentage." The association between CK7-positive area percentage and each continuous variable (tacrolimus level, gamma-globulin level, as well as recipient and donor ages) was assessed using Pearson's product-moment correlation (Pearson's correlation) coefficient. The association between CK7 area percentage and each binary variable (recipient and donor genders and HLA antibody presence post-LT) was assessed using Student's t test. Associations between CK7-positive area percentage and concurrent fibrosis score, graded by the Metavir (ordinal scale from 0 to 4) and cumulative LAFSc (ordinal scale from 0 to 9) systems were assessed using ordinal logistic regression models. The same regression models were also used to evaluate the association between CK7-positive area percentage and ductular reaction (ordinal scale from 0 to 3), lobular inflammation (ordinal scale from 0 to 3), and portal tract inflammation (ordinal scale from 0 to 3). The differences in fibrosis severity between baseline and follow-up fibrosis as graded by the Metavir and cumulative LAFSc scores were labeled as "prospective change in fibrosis" and discretized into three categories (decrease, stable, or increase). The association between CK7-positive area percentage on baseline biopsy and "prospective change in fibrosis" scores from both scoring systems was assessed using ordinal logistic regression models. P values <.05 were considered statistically significant. Ordinal regression models were built using the "ordinal" package from the R.3.2.1 software (http://www.r-project.org), while ROC curve analyses were performed using the "ROCR" package.

Results

Our study included 64 PB from 32 children with “baseline” and “follow-up” biopsies. Median age at LT was 1.2 years (0.9-15.5) for the recipients and 32.1 years (23.8-52.2) for the living donors. The PB specimens stained for CK7, that is “baseline biopsy,” were taken at a mean of 2.89 years (1-7.46 years) post-LT. The time interval between the two protocol biopsy taken for “prospective fibrosis score” was 1.41 years (0.5-2.0 years). The mean duration of follow-up was 5.84 years (1.07-20.24 years).

CK7-positive area percentage on the initial biopsy was not related to recipient age (P=.26) or donor age (P=.28), donor type (P=0.23), HLA antibody development post-LT (P=.33), or recipient gender (P=.42) or donor gender (P=.59).

Ordinal logistic regression models revealed CK7-positive area percentage to display a significant impact on baseline fibrosis score assessed by the Metavir score (P=.02, Table 1, Figure - left panel) and the cumulative LAFSc (P=.01, Table 1, Figure 1 – right panel). CK7-positive area percentage was significantly associated with extent of ductular reaction (P=.006, Table 2, Figure 2 – left panel) and, portal tract inflammation (P=.01, Table 2, Figure 2– right panel) but to a lesser (insignificant) extent, with severity of lobular inflammation (P=.17, Table 2, Figure 2 – central panel). There was a no significant association between the CK7-positive area percentage of the baseline biopsy and the “prospective change in fibrosis” as assessed by the Metavir score (P=.36, Table 3, Figure 3 – left panel) and cumulative LAFSc (P=.25, Table 3, Figure 3 – right panel).

Figure 1: “CK7 positive area percentage mean as per the current fibrosis score expressed with Metavir (left panel) and LAFSc cumulative score (right panel)

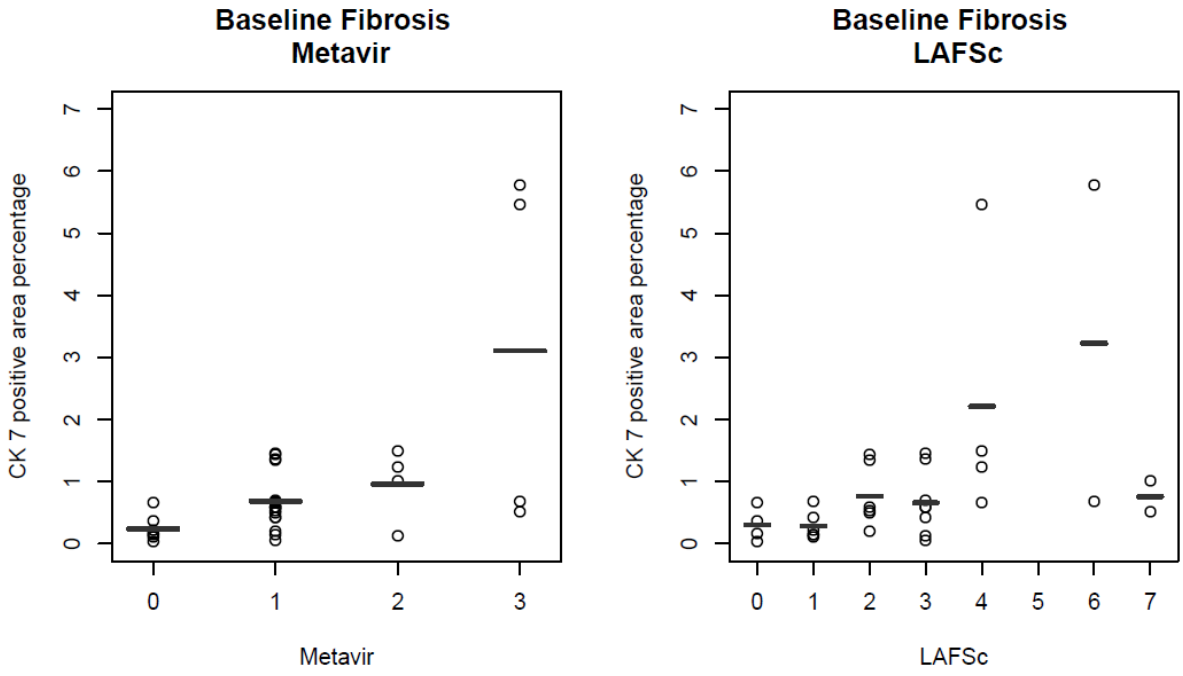

Table 1 CK7-positive area percentage association with baseline biopsy fibrosis score (Metavir and LAFSc)

	CK7-positive area Percentage		
Baseline fibrosis	**mean**	**sd**	**Odds-ratio (P-value)**
Metavir			7.43 (0.02)
0 (N=7)	0.24	0.22	
1 (N=17)	0.69	0.45	
2 (N=4)	0.96	0.6	
3 (N=4)	3.11	2.9	
LAFSc cumulative			1.86 (0.01)
0 (N=4)	0.3	0.28	
1 (N=6)	0.29	0.23	
2 (N=6)	0.76	0.5	
3 (N=8)	0.66	0.52	
4 (N=4)	2.21	2.19	
5 (N=0)	-	-	
6 (N=2)	3.23	3.59	
7 (N=2)	0.76	0.35	

* Odds ratio and p-value were computed using a ordinal logistic regression model

Figure 2: CK7 positive area percentage mean as per the biliary duct proliferation (left panel), Lobular inflammation (central panel) and Portal tract infiltration (right panel).

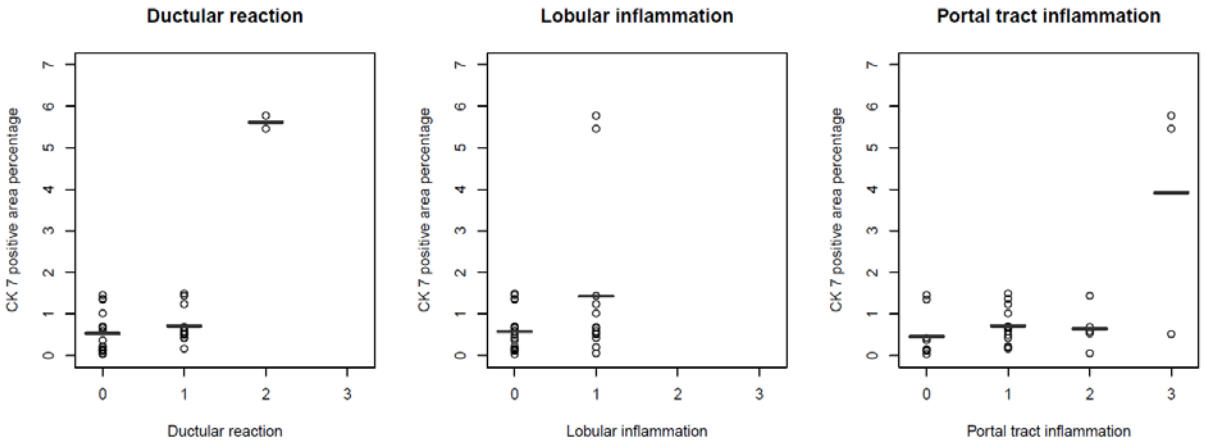

Table 2 CK7-positive area percentage association with histological characteristics on baseline biopsy

	CK7-positive area Percentage		
Histological characteristics	**mean**	**sd**	**Odds-ratio (P-value)**
Ductular reaction			4.62 (0.006)
0 (N=16)	0.53	0.51	
1 (N=14)	0.71	0.39	
2 (N=2)	5.61	0.22	
Lobular inflammation			2.06 (0.17)
0 (N=19)	0.58	0.49	
1 (N=13)	1.43	1.90	
2 (N=0)	-	-	
Portal tract infiltration			3.13 (0.01)
0 (N=9)	0.46	0.55	
1 (N=14)	0.71	0.43	
2 (N=6)	0.64	0.45	
3 (N=3)	3.91	2.95	

* Odds ratio and p-value were computed using a ordinal logistic regression model

Figure 3: CK7 positive area percentage mean as per the prospective change in fibrosis assessed by the Metavir score (left panel) and by the LAFSc cumulative score (central panel) and by the CPA total (right panel)

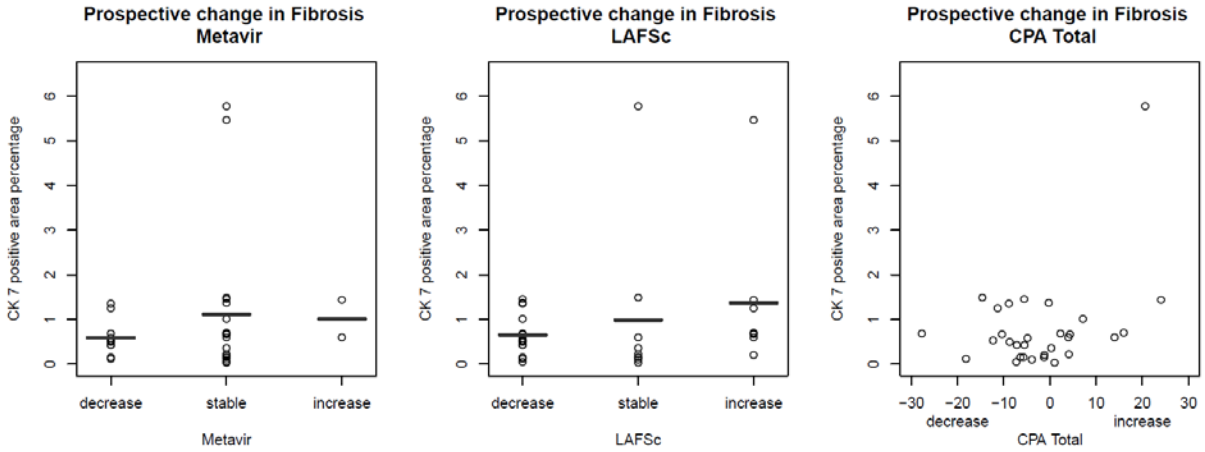

Table 3 CK7-positive area percentage association with prospective change in fibrosis (Metavir and LAFSc)

	CK7-positive area Percentage		
Baseline fibrosis	**mean**	**sd**	**Odds-ratio (P-value)**
Metavir			1.29 (0.36)
Decrease (N=11)	0.59	0.39	
Stable (N=19)	1.11	1.66	
Increase (N=2)	1.01	0.6	
LAFSc cumulative			1.32 (0.25)
Decrease (N=15)	0.65	0.45	
Stable (N=9)	0.98	1.85	
Increase (N=8)	1.37	1.7	

* Odds ratio and p-value were computed using a ordinal logistic regression model

Discussion:

Our study using paired biopsies from stable LT recipients demonstrated that DR correlated to the severity of concurrent portal inflammation and fibrosis but was not predictive of the prospective change in fibrosis i.e. fibrogenesis. The aspect that two serial biopsies, at one year interval were evaluated from each patient brings forth these findings.

DR is frequently observed on PB's from LT recipients without biliary complications and in this scenario, it was thought to be an innocuous observation. In patients with hemochromatosis, NASH and HCV it was shown that replicative arrest of hepatocytes and severity of portal inflammation was

correlated to the extent of DR. This is explicable as the DR contains the bipolar hepatic progenitor cells (HPC), which get stimulated due to replicative arrest of the hepatocytes and the portal inflammation provides further stimuli. The HPC's have dual potential i.e. can form cholangiocytes or hepatocytes depending on the chemokine milieu, presence of surrounding myofibroblasts and the composition of the extracellular matrix.

Various models have been used to analyze if fibrosis is stimulatory for the DR or a by-product of the DR and there exists evidence in support of both the hypothesis. BALB/c mice fed on CDE diet have shown that decreased HPC stimulation results in decreased fibrosis. Also, increased HPC's by interferon stimulation resulted in increased fibrosis. These findings are supported by the profibrogenic role of HPC stimulatory chemokine – TWEAK, which could be link between these histological characteristics. These in-vivo models form the basis for the hypothesis that histological DR is predictive of increased fibrogenesis. Approached from a different perspective, studies to assess if changes in extracellular matrix resulted in HPC proliferation demonstrated that collagen deposition occurred prior to HPC activation. Cell cultures on various matrix components showed that Laminin is integral to HPC status. Both sides of the argument have supportive evidence but all these experimental designs have a big limitation - the inflammatory signaling pathways and fibrogenesis mechanisms of humans are vastly different from these animal models.

The unique feature of this study is the inclusion of paired serial biopsies from each patient. We had earlier shown that quantifying activated stellate cells on a PB was predictive of fibrogenesis over the next 1-2 years. In the current study the interval between the serial PB was 1-2 years as well, hence an adequate time for change in fibrosis severity if there was an underlying pro or anti-fibrogenic basis on the baseline PB. We had considered that DR, housing the HPC's would be pro-fibrogenic, given the observations from other earlier studies. Our findings were on the contrary and we presume that DR is perhaps an effect of the collagen deposition and change in the extracellular matrix composition. All DR are not equal and when co-localised with HSC, they are pro-fibrogenic, as was shown in a study

on hemochromatosis patients. When our findings are seen in this context, it seems likely that DR is not a homogenous entity but has variants which are either a cause or an effect of fibrosis. Until we can histologically differentiate between the two, finding of DR on a PB in absence of biliary complications would not be considered as a harbinger of any significant clinical correlate.

Conclusion:

CK7-positive area percentage was significantly associated with extent of ductular reaction, portal tract inflammation and severity of fibrosis on the same PB. CK7-positive area percentage does not predict the future course of fibrogenesis.

Discussion

In a large cohort of prospectively collected serial protocol biopsies we demonstrated that development of allograft fibrosis followed a patient specific pattern and was not universally progressive with time. Fibrosis progression and predisposing factors for fibrogenesis are loco-regional. The portal fibrosis is dynamic in progression and mostly related to immune mediated factors, while the centrilobular fibrosis is irreversible and related to vascular issues. We could develop predictive model of fibrogenesis using ASMA quantification and rule out the utility of CK7 quantification for the same. Further, digital quantification of fibrosis was refined in the study and the developed module was validated in a pediatric cohort.

Allograft histology – natural history and predisposing factor for "idiopathic" allograft fibrosis

To unravel the natural history of allograft histology, we had a unique study design by evaluating an uncomplicated patient cohort and only serial PBs. This allowed us to observe the longitudinal course of "idiopathic" inflammation and fibrosis. Allograft fibrosis was observed NOT to be time dependent i.e. duration since LT was not found to be a significant predictive factor for fibrosis. Rather it was a patient specific pattern of histological evolution that could be observed. This finding contrasts with what has been published in many recent articles from different centers (3-5;7;8;10;11;65;70). The notable differences between our study and the others before that explain the conflicting results are two-fold. First, our selective study cohort which was specifically designed to elucidate the "natural history" i.e. the uncomplicated histological evolution that can be considered as the baseline or normal evolution pattern. Secondly, application of mixed model statistics that are specifically designed to consider repeated measures of the same individual in longitudinal time course. Unlike the mean fibrosis that have been used previously, which disregard the ordinal variable characteristics of the fibrosis scoring and pool the data at each time point without consideration of individual evolution patterns.

Transplant related factors, namely cold ischemia time, preservation solution, biliary or vascular complications, surgical complications and CMV infection have been linked to allograft fibrosis (1;2;6;9-11;88;103). In our study evaluating zone-specific fibrosis evolution as outlined by LAFSc score we observed that portal fibrosis was dynamic while the central fibrosis was persistent, as was earlier reported by Venturi et al (9;10). The dynamic portal fibrosis is because portal tracts are immunologically-active zones with many hepatic stellate cell (HSC) (113). HSCs can be either fibrogenic or fibrinolytic which explains the dynamic nature of the portal fibrosis. The fibrogenic state of HSC are when activated by persistent inflammation and assume the fibrinolytic state in the quiescent state (114-116). This aspect that portal inflammation was seen to be persistent over successive PB's presumably provided the stimuli for HSC activation. This portal inflammation persistence is not unexpected as we did not change the immunosuppression regime based on histological inflammation characteristics, as until now they were of doubtful significance. Hence the progression of persistent portal inflammation, resulting in HSC activation and finally collagen deposition seems to be the attractive pathogenesis hypothesis.

Despite the well-documented link between inflammation and fibrosis in hepatitis C, this link has remained inconclusive in pediatric LT. In the study by Evans et al on PBs 1, 5, and 10 years post-LT, they demonstrated greater fibrosis in biopsies with co-existing chronic hepatitis, inflammatory activity being not being predictive of consequent fibrosis (4). The authors had evaluated fibrosis and inflammation cross-sectionally at three time-points over 10 years. While other studies had similar findings as Evans (117), we tried to evaluate the explanation for our contradictory findings. To mimic the methodology as used by Evans et al. we correlated fibrosis and preceding inflammation with PB intervals like their study. On doing so we did not find any correlation between preceding inflammation and consequent fibrosis, either. Hence, the discrepancies were produced by taking different PB intervals rather than between-cohort differences. We concluded that to assess the outcome of inflammation a reasonable time-frame of 1-2 years between the paired biopsies is

needed whereby there is sufficient time for a histological consequence to develop but short enough to not let the characteristics of inflammation change.

Portal fibrosis was also associated with presence of post-LT class II DSA as has been reported in previous studies. As we used regional fibrosis assessment, we could pin-point that the presence of these antibodies was related the portal fibrosis. Our further work on this aspect, which has filled some lacunae in our understanding, has been discussed subsequently.

The central area fibrosis to be predisposed by deceased donor grafts seen by us was previously described by Venturi et al. This correlates to the cold ischemia time associated with a deceased donor. Further as described in the natural history above, this fibrosis in this zone is not dynamic and is persistent. This is explicable by the low concentration of the myofibroblasts that are involved in fibrinolysis, in this zone (103;104).

Among the genetic factors HLA-DRB1*03/04 allele in LT recipients was an independent risk factor for portal fibrosis, with no significant association with inflammation and this corroborates with the AIH scenario (101;118). These alleles are believed to result in faulty antigen processing, with antigenic mimicry resulting in hepatic injury (71). Given that, within 4 weeks, the recipient's Kuffer cells were shown to completely repopulate the allograft (119), any host -antigen presenting cell defects would be evident in the new graft, explaining our findings.

Role of HLA antibodies – zonal significance, subtypes and MFI.

Portal inflammation and fibrosis were associated with presence of post LT class II DSA specifically the DQ subclass when present with MFI higher than 5000. The correlation of class II DSA and portal inflammation or fibrosis is not unexpected as portal tracts are the most immunologically active zones in the liver and contain MHC class II antigens (major histocompatibility complex) expressing cells (113;114). The MHC II is expressed by the portal resident antigen presenting cells and by peri-portal

hepatocytes facing persistent inflammatory insult (120). Thus, the presence of class II antibodies would maximally affect this zone.

The proportion of patients with DSA's post-LT has been reported to vary between 8 and 67%, depending on the MFI cut-off that has been used, which range from 1000 to 5000 (7;94;95;97-100). In earlier studies done in adult LT recipients, MFI of 5000 was found to correlate to increased incidence of chronic rejection and poorer graft outcomes (99;100). In contrast the landmark study by Miyagawa et al showed that allograft fibrosis in children was associated with presence of class II DSA > 1000 MFI. This wide range of MFI selection highlights the lack of information on identifying clinically valid cut offs (7). Our finding of specific subclass and MFI is based on an evidence regarding a clinically valid outcome of allograft inflammation and fibrosis.

The class II antibodies comprise of DP, DQ and DR subclasses and though the antibodies against the HLA-DQ antigens are more frequently seen post-LT and been implicated in de-novo autoimmune hepatitis (97;99), there is no clear consensus on the relative importance of the different subclasses, especially among children. In our cohort we clearly could observe that DQ subclass was much more correlated to the histological changes of inflammation and fibrosis, than the DP or the DR DSA antibodies and more so when the cut off was set at 5000 MFI.

Predicting allograft fibrogenesis

Predicting fibrogenesis on protocol biopsy, would serve as a marker for effectuating fibrosis prophylaxis and allow us to make preventive changes in the treatment regime. For this purpose, we selected two candidates – ASMA and CK7.

We demonstrated that ASMA expression is predictive of decreasing, increasing, or unchanged graft fibrosis over the next 2 years. Cutoff MFI, identified using AUROC analysis showed that an ASMA-positive area percentage >1.05 indicated with a 90% specificity that the fibrosis would increase over the next 2 years. This is understandable in the context that fibrosis is a dynamic process, and experimental models have demonstrated that clearance of activated HSCs in fibrosis regression

(115). Activated HSCs remain so until the eradication of the activation stimulus, at which point they undergo either de-activation or apoptosis, in either scenario ASMA becomes non-detectable (114;116). Our study is in line with observations made in experimental models wherein clearance of activated HSCs preceded the decrease in fibrosis. Similar findings were reported in two clinical studies, which had similar methodology and were performed on adult LT recipients infected with hepatitis C (106;121). Both these studies used non-standardized and observer dependent methods of ASMA staining and quantification. Our work addresses this issue by using automated, reproducible, standard procedure for staining and quantification, allowing ASMA to be used as standard biomarker of future fibrosis in any other center.

In the ASMA study, we found that ductular reaction was associated with ASMA quantification and fibrosis severity. These findings are in concordance with previously published data, wherein ductular reaction has been shown to correlate closely with fibrosis severity in chronic hepatitis C infection, alcoholic liver disease, NASH, and hemochromatosis (122-124).

Ductular reaction (DR) as assessed by CK7 staining, contain both hepatic progenitor cells and extra-cellular matrix are seen in response to acute severe and chronic liver injury. The DR in humans and animal models is closely associated with collagen and laminin deposition in the liver, though there is an ongoing debate about “cause or effect” relationship. We observed CK7 quantification to correlate with severity of fibrosis in the same biopsy, as reported by Clouston et al (122-124). As we had evaluated serial paired biopsies in our study, impact of the CK7 quantification on baseline biopsy on the fibrosis evolution in follow up biopsy was also evaluated. Herein we observed that unlike the ASMA, the CK7 at baseline had no predictive potential on the course of fibrosis evolution. On the face of these results it seems that DR is co-existing with fibrosis but not a precursor to the fibrosis. We need to be cautious before drawing such conclusions, as there is a possibility that the DR may have different variants and with different outcomes. It maybe hypothesized that proliferative phase of DR maybe stimulatory for the myofibroblasts to become fibrogenic while the regression phase of DR maybe non-fibrogenic. In this scenario the CK7 quantification would not be a true marker of the

activation potential of the DR and would necessitate another co-marker to differentiate between the two DR variants.

Digital quantification of fibrosis and other histological characteristics

To overcome the limitations of existing visual based scoring systems we developed a module of morphometric assessment of fibrosis to estimate total CPA and regional CPA in the portal, sinusoidal and centrilobular area. It was validated to be reproducible and on a large cohort and operated by an independent and blinded user.

Computerized CPA estimation methods have been existing for many years but have not been adopted outside the realm of research as it was not intelligent enough to exclude areas of artifacts, capsule, large vessels which would result in gross over estimation of fibrosis. These systems are not free from user intervention either and time intensive. These inherent shortcomings have been addressed by our module, most importantly used in the clinical validation study it showed good discrimination between minor changes in fibrosis severity which were not detected by visual based scoring systems. Using the LAFSc or Metavir the actual change in fibrosis severity was over or under-estimated in majority. Our module had many novel aspects, high level of automation, rapid processing speed, inbuilt quality control steps and the power to quantify regional fibrosis distribution as well. These aspects address many of the shortcomings of the previous systems and the module is ready for clinical use.

Perspective

Hepatic fibrosis is a focus of research and perhaps the next “big breakthrough” for science wherein it would be reversible with treatment. Current generation anti-virals for HCV have shown that it’s no more a distant dream to reverse cirrhosis. The implications of this would be potentially avoiding liver cirrhosis related morbidity – mortality, transplant in a significant number of patients. Understandably the industry interest in this domain is immense, given the huge burden of liver cirrhosis which is increasing each year due to global NASH epidemic and alcohol consumption. Herein lies the caveat of liver fibrosis research where the emphasis of mechanistic understanding and drug development should not be towards fibrinolysis but equally to fibrogenesis.

Identifying markers to predict increasing fibrogenesis would provide key information about timing to start anti-fibrogenic treatment or increasing immune-suppression where immune dysregulation is the culprit. The clinical adoption of such tools would be only when they are non-invasive and need at most, only a blood sampling. Progression in the realm of – liquid biopsy, should be close-tracked with biomarkers of liver fibrosis progression. Detection and quantification of circulating micro-particles and exosomes of ASMA activation seem to be the most logical step forward. HSC activation would release certain micro-RNA entrapped in exosomes or microparticles which can be detected by using PCR and sequencing platforms in conjunction with ultracentrifugation. Once such techniques are homed, then the blood sample based “liquid biopsy” would guide the treatment. An equally exciting spin-off from this research would be possibly better immune-modulation in the post LT scenario. The HSC are immunologically active and the endpoint of the chain where immune-dysregulation results in fibrosis. Hence assessing their activity status could be a better marker of immune activity rather than dosing the Tacrolimus level, as is the order of the day.

Fibrogenesis, progenitor cells and ductular reaction are closely intertwined as has been seen. Better delineation and categorization of ductular reaction as pro and anti-fibrogenic variants would determine the role of stem cells in hepatic fibrosis treatment. The adult derived hepatic stem cells

(ADHSC), closely mimic the hepatic progenitor cells in the canal of Herring and have immune-regulatory function, making them an exciting modality in altering the course of hepatic fibrosis.

The digital quantification of fibrosis is a technology of today, not tomorrow and must be adopted to have better fibrosis estimation. This standardization would be the bedrock of all trials and techniques to alter the hepatic fibrosis course. Without accurate mapping, free of subjectivity liver fibrosis would remain a self-constructed maze for researchers. Additionally, as zonal fibrosis mapping must be more widely adopted given the important etiological clue that it holds.

Fibrosis research is undoubtedly progressing at a lightning speed but academic researchers hold the moral responsibility that we address the preventive and predictive dimension as much as the low hanging attractive fruit of fibrinolysis.

Publications during PhD

Published

1. Late graft hepatitis and fibrosis in pediatric liver allograft recipients: Current concepts and future developments

   Varma S1, Sokal EM1.

   Liver Transpl. 2017 Mar;23(3):403-404. doi: 10.1002/lt.24707.

2. The histological quantification of alpha-smooth muscle actin predicts future graft fibrosis in pediatric liver transplant recipients

   Varma S, Stéphenne X, Komuta M, Bouzin.C, Ambroise J, Smets F, Reding R and **Sokal EM**

   Pediatr Transplant. 2016 Oct 23. doi: 10.1111/petr.12834

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

   S. Varma, J. Ambroise, M. Komuta, D. Latinne, P. Baldin, R. Reding,F. Smets, F. Stephenne, **E.M. Sokal,**

   EBioMedicine (2016), http://dx.doi.org/10.1016/j.ebiom.2016.05.040

4. Retargeting of bile salt export pump and favorable outcome in children with progressive familial intrahepatic cholestasis type 2.

   Varma S, Revencu N, Stephenne X, Scheers I, Smets F, Beleza-Meireles A, Reding R, Roskams T, **Sokal EM.**

   Hepatology. 2015 Jul;62(1):198-206.

5. Pnematosis Intestinalis and Portal Venous Gas in Pediatric Liver Transplant Recipients.

   Varma S, Dumitriu D, Stephenne X, Smets F, Clapuyt P, **Sokal E**.

   J Pediatr Gastroenterol Nutr. 2016 Feb;62(2):e14. doi: 10.1097

6. Atypical presentation and treatment response in a child with familial hypercholesterolemia having a novel LDLR mutation

   Varma S, McIntyre AD, Hegele RA

   JIMD 2016 October – accepted

7. Sofosbuvir/ledipasvir and ribavirin tolerability and efficacy in pediatric liver transplant recipients.

   Huysentruyt K, Stephenne X, **Varma S**, Scheers I, Smets F, Sokal E

   Liver Transplantation

Submitted and under review:

Ductular reaction does not predict the allograft fibrogenesis

Liver Transplantation

Post-liver transplant class II donor specific HLA antibodies of DQ subtype with MFI>5000 are predictive of allograft dysfunction.

Varma S, *, Ambroise J, *, Houvet E, Komuta M,, Latinne D, Reding R, Smets F, Stephenne X, and Sokal EM

Journal of Hepatology

Antibiotics in pediatric PSC-AIH overlap syndrome facilitate remission and corticosteroid-free therapy

Varma S, Sambon P, Komuta M , Clapuyt P and **Sokal EM**

Hepatology

Post-liver transplantation follow-up over 17 years for mild Zellweger spectrum disorder and additional cases.

T. Demaret*, S. Varma*, X. Stephenne, F. Smets, I. Scheers, L. Van Maldergem, R. Reding, and **E. Sokal**

JIMD 2016 October

Predicting development of hepatocellular carcinoma among patients with progressive familial intrahepatic cholestasis type2

Varma S, Emannuel G, Rapahel F, Revencu N, Davit-Spraul A, Charlotte M , Xavier S and **Sokal EM**

Presentations

2017

1. Annual meeting of ESPGHAN at Prague, Czech republic: Oral presentations titled –
    a. Ductular reaction does not correlate to future fibrogenesis in allograft recipient children
    b. HLA DQ with MFI > 5000 correlaated to allograft histological changes
    c. Predicting development of HCCin PFIC 2 children.
2. Annual liver meeting of EASL – Amsterdam, 3 posters titles same as above.

2016

3. Annual meeting of ESPGHAN at Athens, Greece: Oral presentation titled - The histological quantification of alpha-smooth muscle actin predicts future graft fibrosis in pediatric liver transplant recipients
4. EASL Monothematic Conference: “Liver Fibrosis: the next goal of targeted therapy?” at Porto, Portugal: Oral presentation titled -Progressive fibrosis is driven by genetic predisposition, allo-immunity, and inflammation in paediatric LT recipients
5. “The liver meeting” organized by AASLD at Boston, USA: Co-host of early morning workshop on “Primary sclerosing cholangitis and autoimmune cholangitis”.
6. “International liver congress” organized by EASL at Barcelona, Spain: Co-host of early morning workshop on “Beyond UDCA in cholestatic diseases”.
7. 1001 Liver transplant meeting organized by Service of pediatric hepatology and gastroenterology at Brussels, Belgium: Oral presentation.
8. “International liver congress” organized by EASL at Barcelona, Spain: 2 x Posters
    a. Allograft inflammation and fibrosis among pediatric LT recipients – genetic predisposition and antibodies, connecting the missing links.

b. The histological quantification of alpha-smooth muscle actin predicts future graft fibrosis in pediatric liver transplant recipients.

2015

1. Annual meeting of ESPGHAN at Amsterdam, Netherlands: Oral presentation titled - Retargeting of bile salt export pump and favorable outcome in children with progressive familial intrahepatic cholestasis type 2
2. "The liver meeting" organized by AASLD at San-Francisco, USA: Poster titled - Dynamics of pediatric liver allograft histological changes and the role of HLA, non-HLA antibodies.
3. BASL annual meeting at Brussels, Belgium: Oral presentation titled - Retargeting of Bile Salt Export Pump (BSEP) and criteria of favourable outcome in children with Progressive Familial Intrahepatic Cholestasis type II (PFIC-II)
4. Annual Conference of Indian National Association for Study of the Liver at Delhi, India: Oral presentation titled – Future of familial cholestasis
5. EURYPA annual meeting at Istanbul, Turkey: Oral presentation tiltled – Challenges in pediatric liver transplantation

2014

Annual meeting of ESPGHAN at Jerusalem, Israel: Poster titled – To evaluate non-transplant treatment modalities for familial cholestasis type II

Awards

2016

1. NASPGHAN – ESPGHAN exchange award in collaboration with Prof Rohit Kohli for transplant hepatology at children's hospital - Los angeles, USA.
2. Mentor-mentee programme award by European association for liver (EASL), in collaboration with Prof U.Beuers at Amsterdam Medical Centre (AMC).
3. Young investigator award by European association for liver (EASL) for ILC at Barcelona, Spain
4. Young investigator award by European association for liver (EASL) for monothematic fibrosis meeting at Porto, Portugal

2015,

1. Young investigator award by European association for liver (EASL) for ILC at Vienna, Austria
2. Young investigator award ESPGHAN for annual meeting at Amsterdam, Netherlands

2014

Young investigator award ESPGHAN for annual meeting at Jerusalem, Israel

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