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Review

Not only a small liver - The pathologist's perspective in the pediatric liver transplant setting

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Summary

Pediatric liver transplantation represents a safe and long-lasting treatment option for various disease types, requiring the pathologist's input. Indeed, an accurate and timely diagnosis is crucial in reporting and grading native liver diseases, evaluating donor liver eligibility and identifying signs of organ injury in the post-transplant follow-up. However, as the procedure is more frequently and widely performed, deceptive and unexplored histopathologic features have emerged with relevant consequences on patient management, particularly when dealing with long-term treatment and weaning of immunosuppression.

In this complex and challenging scenario, this review aims to depict the most relevant histopathologic conditions which could be encountered in pediatric liver transplantation. We will tackle the conditions representing the main indications for transplantation in childhood as well as the complications burdening the post-transplant phases, either immunologically (*i.e.,* rejection) or non-immunologically mediated. Lastly, we hope to provide concise, yet significant, suggestions related to innovative pathology techniques in pediatric liver transplantation.

Key words: pediatric liver transplantation; histopathology; acute complication; chronic complication; next-generation pathology

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Introduction

Pediatric liver transplantation (PLTx) has made crucial improvements since the first surgical interventions by Dr. Starzl in the late '60 in Pittsburgh [1,2], and now represents a relevant part of the annual liver transplant rate worldwide: in 2020, 5.2% of liver transplantations performed in Italy involved the pediatric population (0 to 17-year-old patients) and similar percentages are reported in Europe and North America [3,4].

Thanks to improved technical procedures and patient management (*i.e.,* donor-recipient matching strategies, surgical approaches and immunosuppressive protocols), PLTx now shows a 10-year and 20-year survival rate of more than 80% of transplanted children and young adults and represents an appropriate long-term therapeutic option for several end-stage/terminal hepatic conditions [5-7]. Similar to adult liver transplantation, the successful rate of PLTx is burdened by donor shortage, requiring (1) accurate recipient selection and stratification, (2) innovative surgical and organ preservation techniques, (3) early and specific recognition of graft disease, and (4) optimization of postoperative care. However, differently from the adult setting, PLTx presents peculiar features related to indications, immunosuppression and life-long postoperative follow-up [8]. Indeed, acute complications requiring biopsy assessment are now uncommon but not negligible (and, subtle), while histologic diagnosis of long-term conditions is more frequently required and may be challenging.

To this end, a multidisciplinary approach to PLTx is essential, and pathologists can notably contribute in almost every step of the procedure [9,10]. In particular, pathologists are involved in diagnosing and grading native diseases, evaluating donor organ status and performance, identifying early signs of organ injury and differentiating transplant-related conditions [*e.g.,* acute and chronic rejection, post-transplant lymphoproliferative disorder (PTLD)] from liver native disease recurrence and *de novo diseases*. Supporting our role in this challenging scenario, newly introduced next-generation pathology procedures (*e.g.,* multiplex immunohistochemistry, tissue-tethered digital morphometrics, single-cell molecular analysis) have allowed us to extract innovative data from tissue biopsy and thoroughly advance our understanding of transplant-related conditions, particularly regarding immune activation and regulation [11,12].

In this evolving and challenging panorama for pathologists approaching pediatric liver transplant pathology, this review will tackle the most significant aspects of PLTx, providing a pictural essay of the main histopathologic features, an Introduction. to the most innovative procedures of next-generation pathology, and, eventually, highlighting the role that pathologists should fulfill within the multidisciplinary management of pediatric transplantation.

Setting the stage of PLTx

At first sight, the pre-transplant setting could be considered somehow extraneous to the pathologist's commitment: clinical- and laboratory-based organ allocation systems have been developed, namely the Pediatric End-Stage Liver Disease (PELD) and the Model of End-Stage Liver Disease (MELD), and are continuously updated [13,14], whereas native liver disease diagnostic procedures tend to be as less invasive as possible, particularly in the neonatal setting [15]. However, liver biopsy still maintains a relevant role in numerous pediatric hepatic conditions, particularly in diseases with atypical clinical pictures either to assess the diagnosis or to define the stage of the disease and potentially underestimated concurrent disease. Conversely, the histomorphologic evaluation of donor liver represents a crucial step of the whole procedure, especially considering the overall shortage of donor organs [15].

Indications and contraindications of PLTx

Pediatric conditions leading to PLTx are quite vast and heterogenous but classically distinguished in cholestatic diseases, metabolic and genetic disorders (either hepato-specific or systemic), acute liver failure scenarios (including drug induced liver injury) [16] and primary liver neoplasms (Tab. I, Fig. 1A) [10,17-19].

Adult type conditions such as autoimmune hepatitis

Table I. Categories and specific entities of potential pediatric liver transplantation - leading liver diseases.

Cholestatic disease	Acute liver failure	Metabolic disorder	Neoplastic disease	Other
Biliary atresia*	Drug toxicity (acetaminophen)	A1AD*	Benign tumor	Polycystic liver*
Alagille syndrome	Autoimmune liver disease	Tyrosinemia	Malignancy*	Hemochromatosis
PFIC	Viral hepatitis	Wilson disease		Budd-Chiari syndrome
Cystic fibrosis	Wilson disease*	Galactosemia		Trauma
Caroli syndrome	Poisoning	GSD		Re-transplantation
Congenital hepatic fibrosis		UCD		Cryptogenic cirrhosis
PSC		Crigler-Najjar Syndrome		
		Primary hyperoxaluria		
		LSD		
		Methyl malonic acidemia		
		MSUD		
		Mitochondrial hepatopathies		

PFIC: progressive familiar intrahepatic cholestasis; PSC: primary sclerosing cholangitis; A1AD: alfa-1 antitrypsin deficiency; GSD: glycogen storage disorders; UCD: urea cycle disorders; LSD: lysosomal storage disorders; MSUD: maple syrup urine disease; *: representative images available in Figure 1.

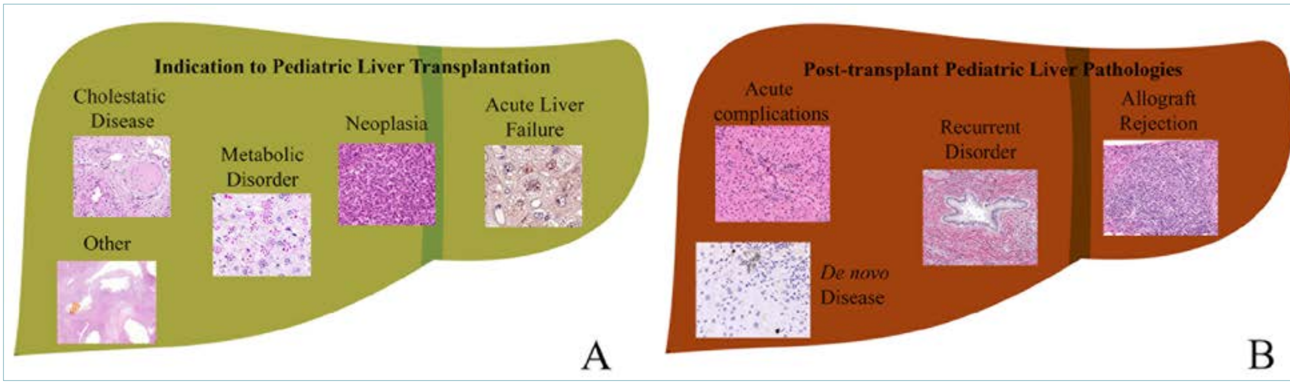

Figure 1. Overall representation of the main indications to PLTx (A) and the main pathologies affecting the transplanted liver (B). (A) Main indications to PLTx are usually grouped in cholestatic disease [*e.g.,* biliary atresia (hematoxylin and eosin stain showing bile duct injury in a portal tract)], metabolic disorder [*e.g.,* alpha-1 antitrypsin deficiency (PAS stain after diastase digestion highlighting intracytoplasmic globules accumulation in periportal hepatocytes)], neoplasia [*e.g.,* hepatoblastoma (hematoxylin and eosin stain of an embryonal subtype)], acute liver failure [*e.g.,* Wilson disease (rhodamine stain highlighting granular coarse deposits in hepatocytes)], and "Other" categories [*e.g.,* polycystic liver (hematoxylin and eosin stain showing a cluster of intrahepatic cysts)], the latter containing heterogeneous conditions that could not be otherwise classified. (B) Post-transplant liver diseases could be sorted in acute complications [ischemic-reperfusion injury (hematoxylin and eosin stain showing parenchymal necrosis and neutrophils aggregates)], allograft rejection [T-cell mediated rejection (hematoxylin and eosin stain showing a portal tract with severe inflammatory infiltration and injury)], recurrent disorders [primary biliary sclerosis (hematoxylin and eosin stain showing periductal concentric fibrosis)], and *de novo* disease [cytomegalovirus hepatitis (immunohistochemical staining for cytomegalovirus highlighting positive nuclei of infected hepatocytes)].

(AIH) [20,21] may also impact the pediatric population, while a pathologic condition that is rapidly increasing and concerning pediatric hepatologists is fatty liver disease[22,23]. Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) are well-known conditions affecting adults in the Western world [24], although this "fatty liver pandemic" is now moving from the adult to the pediatric population. Although NASH and NAFLD do not represent actually a leading indication of PLTx, we should expect an increasing rate in the next decades [22,23].
Contraindications to PLTx are mainly represented by poor patient clinical conditions before transplantation and general contraindication to surgical procedure (*e.g.,* septic status, overlapping multiorgan failure/life-threatening defects in other organs, irreversible and severe neurologic dysfunction) or presence of extrahepatic malignancy [9,25,26]. However, in this context the pathologist's evaluation has a limited influence.

Non-neoplastic disease as PLTx indication

An in-depth treatise on all causes of liver injury in the pediatric population is beyond the scope of this manuscript, although some important conditions will be briefly touched upon.

Cholestatic disorders

Although geographic and demographic variables could influence specific disease incidence, biliary atresia is globally reported as the leading cause of liver failure in the pediatric population, thus representing the primary indication for PLTx [27]. Apart from biliary atresia, other cholestatic diseases which may require PLTx include: Alagille syndrome, progressive familial intrahepatic cholestasis (PFIC), ductal plate abnormalities including Caroli syndrome and congenital hepatic fibrosis, autoimmune sclerosing cholangitis, bile acid synthesis defects and cystic fibrosis related disease. Some of these conditions are described in Table II.
Rare genetic and metabolic disorders may lead to PLTx for different reasons: 1) Disorders which affect the liver and PLTx is performed for end stage liver disease and complications such as tyrosinemia, alpha1 anti-trypsin deficiency, Wilson's disease, glycogen storage disorders etc.; 2) Disorders in which enzymes are produced in the liver but manifestations are extrahepatic (rare end stage liver damage) and PLTx is performed for extrahepatic organ involvement such as urea cycle deficits, primary hyperoxaluria, etc.; 3) Disorders in which enzymes are produced in the liver and in extrahepatic tissues for which PLTx only partially corrects enzyme deficiency and alleviates extrahe-

Table II. Main pediatric cholestatic disorders.

Disease	Epidemiology	Pathogenesis	Clinical characteristics	Principal Pathologic features	Transplant rate
Biliary Atresia (BA) [84,85]	Onset within the first 3 months of life	Uncertain (role of single nucleotide polymorphisms (e.g., CFC1 and ADD3 genes) and extrinsic factors (e.g., viruses and toxins) as susceptibility and/or triggering factors that target bile ducts	Progressive disorder leading to end stage liver disease. Four phenotypes: isolated BA, BA associated with laterality defects, BA associated with other major congenital malformations, BA associated with a bile duct cyst	Duct/ductular bile plugs, generalized moderate to marked ductular reaction and bile duct proliferation, portal stromal edema, higher stages of portal fibrosis (stages 3 and 4), prominent pseudorosette formation, moderate to marked peribiliary neutrophilic infiltrates and interlobular bile duct injury	80% of patients will require PTLx
Alagille Syndrome [86,87]	2/3 of patients present before 4 months	Mutisystem autosomal dominant condition caused by deletion or duplication in a single gene (JAG1 or NOTCH2) in the Notch signaling pathways	Variable clinical manifestations (due to variable penetrance), including hepatic (cholestasis,), cardiac, renal, skeletal, ophthalmologic and facial abnormalities ranging from subclinical to a life-threatening condition (mortality - 10%)	Intrahepatic bile duct paucity, early onset biopsies show biliary obstructive picture, cholestasis, extramedullary hematopoiesis, giant cell change and early copper accumulation.	20-30% of patients will require PTLx
Progressive Intrahepatic Cholestasis (PFIC) [88]	Variable depending on mutation; may present as neonatal hepatitis and progression to cirrohosis or as bland cholestatic aspects in adults	Heterogeneous group of autosomal recessive diseases, due to specific deficiency of bile transporter secondary to mutations in the encoding genes (eg ATP8B1, ABCB11, or ABCB4 etc)	Variable clinical patterns as homozygous or compound heterozygous mutations with marked loss of activity result in early and severe cholestatic disease that can progress to fibrosis and cirrhosis while heterozygosity or mutations may cause a milder phenotype	Range of cholestatic disorders, including progressive disease, benign recurrent intrahepatic cholestasis (BRIC), cholestasis precipitated by external factors, (eg pregnancy or drugs). Histologic aspects are variable including bland cholestatsis, neonatal hepatitis which may progress rapidly to cirrhosis, obstructive biliary pattern.	Variable depending on severity and type of PFIC
Cystic fibrosis [89]	Onset of liver damage is before the age of 10 and developes in about a third of patients	Mutation in the gene encoding CTFR, an ATP-dependent chloride channel promoting chloride/bicarbonate exchange leading to altered biliary transport of bile acids, duct plugging by inspissated secretions and toxicity to cholangiocytes and hepatocytes,	Portal hypertension develops about 10% of patients with liver disease; it may be the result of focal biliary fibrosis to multilobular cirrhosis (children) or due to non-cirrhotic portal hypertension as a result of porto-sinusoidal vascular disease (young adults).	Inspissated eosinophilic mucin in the lumen of small bile ducts, steatosis, obstructive pattern with ductular reaction, portal inflammation and portal fibrosis with bridging fibrous septa, with an uneven distribution within the liver, progression to biliary cirrhosis.	5-10% of patients may require PTLx
Autoimmune sclerosing cholangitis [90,91]	Median age of onset 12 years; Association with autoimmune disorders and IBD; Positive autoantibodies, ANA and SMA, hypergammaglobulinemia	Autoimmune condition with associated genetic predisposition, within the spectrum of juvanile autoimmune hepatitis	Acute onset, compliations of chronic liver disease or insidious onset. Frequent overlap with autoimmune hepatitis but cholangiographic abnormalities are present	Florid autoimmune features, interface hepatitis, only 50% show bile duct changes characteristic of sclerosing cholangitis.	27% of patients will require PTLx. High recurrence rates

PTLx: pediatric liver transplantation; IBD: inflammatory bowel disease; ANA: anti-nuclear antibody; SMA: smooth muscle antibody patic manifestations (but does not entirely cure them) such as methylmalonic acidemia, maple syrup urine disorder, etc.

Neoplastic lesions as PLTx indication

Between 5 and 10% of PLTx are performed for neoplastic lesions, the most frequent being hepatoblastoma, which is also the most frequent primary liver cancer in children [28].

Hepatoblastoma

The pathologic aspects of hepatoblastoma (HB) have been extensively described elsewhere in this issue. In the US, between 17 and 20% of surgically treated HB patients receive PLTx [29] and 5-year overall survival is approximately 80%. In HB, PLTx is offered to children who cannot be safely and radically resected due to large tumor size or surgical/anatomic characteristics [30] after pre-operative chemotherapy.

Hepatocellular carcinoma

Pediatric HCC is a rare malignancy more often seen in adolescents. Differences exist between adult and pediatric HCCs including [31,32]: 1) approximately 70% of pediatric HCC develop on normal liver background compared to 85% of adult HCCs developing in chronic liver disease; 2) pediatric underlying liver diseases include perinatal acquired hepatitis B virus, tyrosinemia, glycogen storage disease, Alagille syndrome, progressive familial intrahepatic cholestasis, and congenital portosystemic shunts; 3) differences at the molecular level between adult and pediatric HCCs have been identified; 4) pediatric HCCs are more often sensitive to chemotherapy compared to adult HCCs; 5) outcomes are better in pediatric HCC even at more advanced stages.

Approximately a quarter of pediatric HCCs (late childhood and adolescence) are of the fibrolamellar variant and show similar morphologic aspects as adult fibrolamellar HCC, together with the DNAJB1-PRKCA fusion transcript [33]. This variant occurs in normal liver background, expanding treatment options, nonetheless relatively recent studies show that it does not have better survival compared to conventional HCC [34]. Pooled data have shown that outcomes of PLTx for HCC reach 70-80% 5-year survival rates [35].

Sarcomas and Vascular Tumors

Embryonal Sarcoma has been discussed elsewhere and it represents a rare indication for PLTx as tumors are chemo-sensitive and can usually be cured with chemotherapy and surgery alone. Data on PLTx are scarce, but show excellent outcomes.

Malignant Rhabdoid Tumor (MRT) of the liver is a rare, aggressive malignancy of infancy characterized by round to polygonal cells with abundant dense eosinophilic cytoplasm with inclusions, large vesicular and eccentric nuclei, numerous nucleoli and loss of expression of INI1 in the nuclei of the tumor cells (but present in the nuclei of all normal cells) [36]. Few children have undergone PLTx with generally negative results.

The understanding of *Vascular Lesions of the liver* has expanded over the years through more accurate histologic description and use of immunohistochemistry, as well as molecular advancements. The International Society for the Study of Vascular Anomalies (ISSVA) in 2018, classified pediatric vascular tumors based on their behavior into benign (hepatic congenital hemangioma and hepatic infantile hemangioma), locally aggressive, and malignant neoplasms (hepatic hemangioendothelioma and hepatic angiosarcoma) The differential diagnosis requires immunostaining and (in case of EHE) molecular characterization [37]. PLTx for benign liver neoplasms, such as hepatic infantile hemangiomas, is rarely considered (and only in life threatening conditions as medical treatment is available) as most lesions are asymptomatic and, after rapid post-natal proliferation, tend to spontaneous involution. Malignant vascular tumors, such as epithelioid hemangioendothelioma and hepatic angiosarcoma, were historically not considered for transplantation, however few cases have been treated with PLTx with variable results [38-40].

Assessment of transplantable liver

Pretransplant evaluation is often considered as the recipient assessment of disease severity and transplant urgency [17]. However, we would like to shift the focus of the pretransplant evaluation from the recipient to the donor.

The shortage of donor organs is a well-known "side effect" of the successful therapeutic rate of transplantation and its resultant expansion of suitable indications (*e.g.,* liver transplant oncology)[41]. As a consequence, the donor pool has been increased by implementing expanded criteria for donors [*e.g.,* steatotic liver, cold ischemia time > 12 hours, partial allograft, and donation after circulatory death (DCD)] [42] and *ex vivo* machine perfusion techniques.

Among the expanded criteria, the most relevant for the pathologist approaching donor evaluation is the percentage of steatosis. Pre-transplant steatosis assessment is focused on reporting the percentage of large droplet macrovesicular steatosis that is defined as lipid vacuoles larger than a non steatotic hepatocyte and pushing the nucleus peripherally [43]. Lipid vacuoles not fulfilling these criteria should be considered as small droplet macrovesicular steatosis, whereas microvesic-

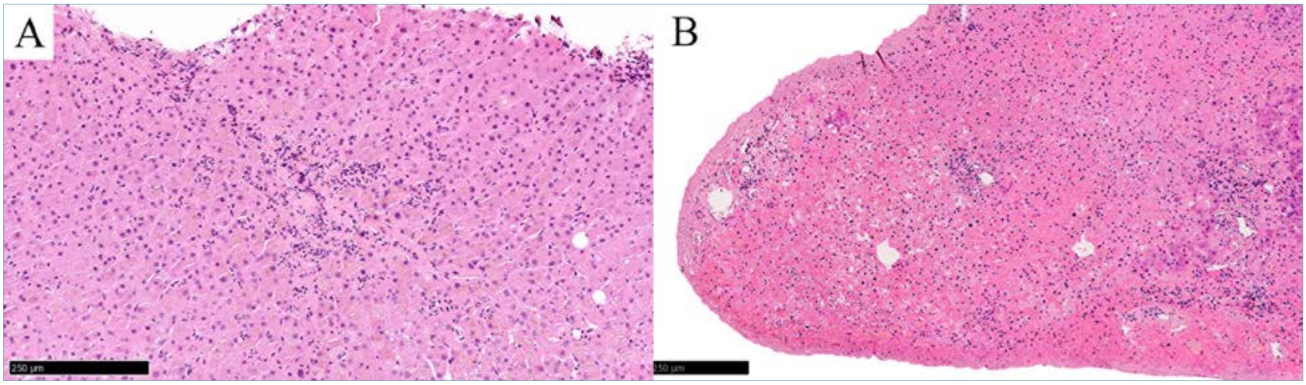

Figure 2. Non-immunological acute complications. (A) Terminal hepatic vein-centered pattern of injury in a case of ischemia-reperfusion injury (hematoxylin and eosin); (B) Several erythrocytes located underneath the rim of the liver capsule in a case of subcapsular hemorrhage.

ular steatosis represents a diffuse "foamy" cytoplasmic appearance of hepatocytes occurring in specific pathological subset (*e.g.,* Reye syndrome) [43]. Recently, the recommendation of the Banff Working Group on Liver Allograft Pathology, introduced a detailed definition and diagnostic algorithm to specifically and accurately evaluate steatosis in the donor liver [43]. These consensus recommendations are helpful in standardizing the assessment of a variable with relevant consequences on organ management but burdened by relevant interobserver rates. Livers suitable for transplantation should present at most less than 60% (preferably less than 30%) of large droplet macrovesicular steatosis to prevent graft primary non-function and acute failure, but no consensus has been published regarding a specific acceptance cut-off.

In the adult setting, machine perfusion is demonstrating remarkable success, now introducing the opportunity to specifically and directly treat retrieved organs [44,45]. Nevertheless, limited evidence of machine perfusion implementation in the PLTx setting is available to date. The first report of a successful application of machine perfusion in the pediatric setting is dated 2019 and reported by Werner et al. [46], followed by the experience reported by the Turin group[47]. Indeed, PLTx requires a cautious approach, but once the benefits of machine perfusion will be ensured by multicenter studies, this technique would represent a valid support and eventually increase the PLTx donor pool.

Pathologic evaluation of the pediatric transplanted liver

Post-transplant liver biopsy evaluation represents the main core of the pathologist's role in the PLTx setting. Indeed, features of acute injury and chronic evolution have to be promptly identified to guide clinical management, establish transplanted liver fitness, and predict functional decline and graft loss (Fig. 1B). Additionally, inflammatory and fibrotic findings have been identified in clinically-silent follow-up protocol biopsies, but the consequences of these features on patient management and long-term organ survival still need to be further explored.

Ischemia/reperfusion injury and other non-immunologically mediated acute complications

During organ procurement and transplantation phases, the liver is first exposed to metabolic stress due to oxygen deprivation and then to inflammatory and ROS effect, leading to so-called ischemia/reperfusion injury (IRI). Indeed, the abrupt vascularization interruption and subsequent replenishment characterizing organ transplant can cause endothelial cell swelling (particularly affecting the sinusoids) and terminal hepatic vein-based parenchymal injury (hepatocyte ballooning and apoptosis, neutrophil aggregates and parenchymal necrosis), and cholestatic features (Fig. 2) [48]. These signs of injury usually last for the first two-three weeks after transplantation, are relatively common and usually mild, but IRI may, rarely, evolve as a severe event leading to graft primary non-function and organ loss [48]. Additional conditions related to inadequate vascularization/parenchymal perfusion are represented by subcapsular hemorrhage [48,49] (Fig. 2) and small-for-size syndrome. The former is an effect caused by poorly vascularized peripheric parenchyma, particular evident in wedge biopsy sample where subcapsular parenchyma is more represented (Fig. 2) [48,49]. The lat-

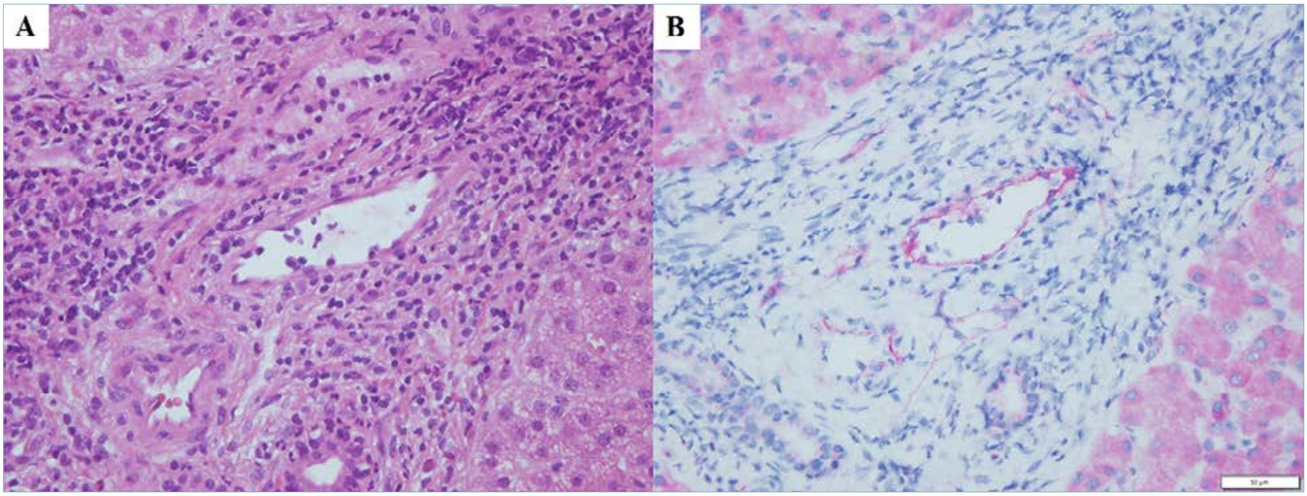

Figure 3. Pathologic features of antibody-mediated rejection (AMR) 14 days post-transplant. (A) Portal tract showing inflammation and endotheliitis in small portal vessels. (B) Strong staining in the portal vein with C4d staining (used to detect complement deposition).

ter is a condition of graft size mismatch in the context of elevated metabolic demands (*i.e.,* elevated MELD score) and portal hypertension [50], and is characterized by a portal-centered pattern of injury (portal/periportal hemorrhage, arterial occlusion and ischemic bile duct injury) due to portal hypoperfusion/hypertension and reflex arterial vasospasm [50-52].

The biliary tree can also incur into early dysfunction, generally related to anastomotic complications and secondary bile duct obstruction. Histopathological changes are consistent with a biliary obstructive etiopathogenesis, showing features of cholangitis (peri- and intraductal neutrophills infiltration), ductular injury and proliferation, and bile leakage (portal edema) [48].

Of note, all these conditions are not strictly related to immune system activation or rejection processes, and therefore it is mandatory to properly recognize their features to avoid diagnostic misinterpretation and overtreatment.

Allograft rejection

Allograft rejection still represents a major threat to PLTx despite the liver's tolerogenic immune environment and efficient immunosuppression protocols that dampen the graft directed immune response. Indeed, PLTx aims to represent a life-long treatment, thus requiring long-standing graft survival even though it is characterized by complicated immunosuppression management with a balance between the need to prevent rejection and to avoid infection and drug side effects [9,53]. To this end, the recognition of precocious signs of rejection is of pivotal interest to guide subsequent therapeutic management and prevent severe consequences (*i.e.,* graft loss). Diagnostic criteria and grading schemes related to organ rejection pathology are continuously discussed and subsequently updated by The Banff Foundation for the Allograft Pathology [54-56]. Transplant rejection is classically differentiated into antibody and T-cell mediated rejection, although mechanistically different, considerable overlap exists between these two conditions and mixed episodes could occur.

Antibody-mediated rejection (AMR) is an immune-mediated condition triggered by donor sensitization and was especially described in cases of ABO incompatible transplantation. Differently from other transplanted organs (kidneys above all), AMR rarely occur in the liver due to its overall immunological resistance to AMR mechanism of injury [57]. Nevertheless, the 2016 Banff Working Group on Liver Allograft Pathology distinguishes two forms of AMR, namely acute and chronic AMR [55,58].

Acute AMR usually occurs days to weeks after PLTx and presents histopathological features of an immune related micro/small vascular pathology (*e.g.,* capillary and inlet venule hypertrophy, dilation and endotheliitis) affecting both portal/periportal small vessels and centrilobular veins associated with features of biliary compartment involvement (cholestasis, ductular reaction, portal edema). In addition to the histopathology picture of an acute injury, the complement fragment 4d (C4d) linear and granular vascular deposition (demonstrated through specific immunohistochemical or immunofluorescence stain) (Fig. 3) and positive

serum Donor-Specific Antibodies (DSA) are required criteria to obtain a definitive diagnosis [48,55,59,60]
Chronic AMR still involves the vascular compartment, but presents also more non-specific inflammatory (portal inflammation, interface activity) and chronic (portal, sinusoidal, or perivenular moderate fibrosis) features, thus showing subtle morphologic features that could be easily misinterpreted and blurred by overlapping (and more frequent) conditions such as recurrent diseases [55]. In this regard, PLTx represents an ideal setting to evaluate putative chronic AMR features thanks to the low-rate of disease recurrence that could disguise diagnostic interpretation [55]. Similar to the acute form, a definitive diagnosis of chronic AMR requires C4d (portal microvascular) positivity and the correct clinical setting (DSA positivity) [55].
Detailed acute and chronic AMR features are reported in Table III.
T-cell mediated rejection (TCMR) is the most frequent immune-mediated form of injury following transplantation. In its typical form, acute TCMR (a-TCMR) manifests as an inflammatory process mainly involving portal tracts, bile ducts, and venous endothelia usually occurring within the first trimester after transplantation and becomes less common as time pass. Accordingly, the Banff Working Group proposes diagnostic and grading criteria providing an overall evaluation and a more specific semi-quantitative index, namely the Rejection Activity Index (RAI), which specifically addresses the grades of involvement of the targeted structures. Typical a-TCMR presents portal tracts expansion by an inflammatory infiltrate mainly composed of lymphocytes, but other cell types (*e.g.,* eosinophils, neutrophils, macrophages) are often involved, as well as immature and activated immune cells (*e.g.,* lymphoblasts) (Fig. 4). Bile ducts are directly injured by the host's immune activation, presenting various degrees of intraductal inflammatory infiltrates and morphologic signs of cell injury (*e.g.,* apoptosis, nuclear overlapping/stratification) (Fig. 4). Similarly, vascular endothelia show lymphocyte infiltration and cell injury and detachment (*i.e.,* endotheliitis) (Fig. 4). As a general rule, the more the inflammation and involvement of anatomical structures, the higher the RAI. Specific descriptors and grades are reported in Table IV. It is worth mentioning that RAI is not a diagnostic but grading index and it should be applied once the diagnosis of rejection has already established.
Chronic T-cell mediated rejection (c-TCMR) is described as an irreversible event occurring as a consequence of recurrent rejection episodes and patient inadequate immunosuppression compliance. Therefore, due to its temporal development and reiterated acute injury requirement, it is very unlikely that it could occur before the first six months after transplantation. A well-known and described caveat of c-TCMR diagnosis is related to the structures involved, particularly the larger hepatic arteries as an obliterative arteriopathy [48]. Indeed, these structures are usually not sampled with the (relatively) small biopsy performed, thus reducing the possibility of recognizing this event. Together with the larger arteries, c-TCMR also affects the bile ducts

Table III. Diagnostic criteria of acute and chronic antibody mediated rejection (AMR).

	Main histopathological features	C4d	DSA	Other criteria
Acute AMR	Portal edema, neutrophil-rich portal inflammation, ductular reaction, and microvascular injury (dilation, endothelial hypertrophy, vasculitis). Neutrophils may also be observed within sinusoids and vessel lumen. Centrilobular swelling, hepatocanalicular cholestasis, and features of acute TCMR may also occur. Fulminant forms developing within hours after PLTx (hyperacute rejection) show diffuse hemorrhagic necrosis but are rarely observed and lack associated features of acute TCMR.	Strong and diffuse C4d expression in portal/periportal microvascular structures (*i.e.,* C4d positive in > 50% microvessels of > 50% portal tracts) is required.	Recent circulating DSA required for diagnosis. De novo DSA against HLA class II antigens (HLA-DQ) emerged as particularly associated with chronic AMR pathogenesis.	Exclusion of mimicking (and more frequent) conditions required in both acute and chronic scenarios.
Chronic AMR	Lympho-plasma cellular portal/periportal (interface hepatitis) and lobular inflammatory infiltrates, portal vein obliteration and portal tract collagenization. Features of microvessel involvement are less frequently observed and less prominent. Pathological fibrosis reported as subsinusoidal and centrilobular.	Although required for the diagnosis, C4d usually presents a focal and mild positivity.		

AMR: antibody mediated rejection; TCMR: T-cell mediated rejection; DSA: donor specific antibodies.

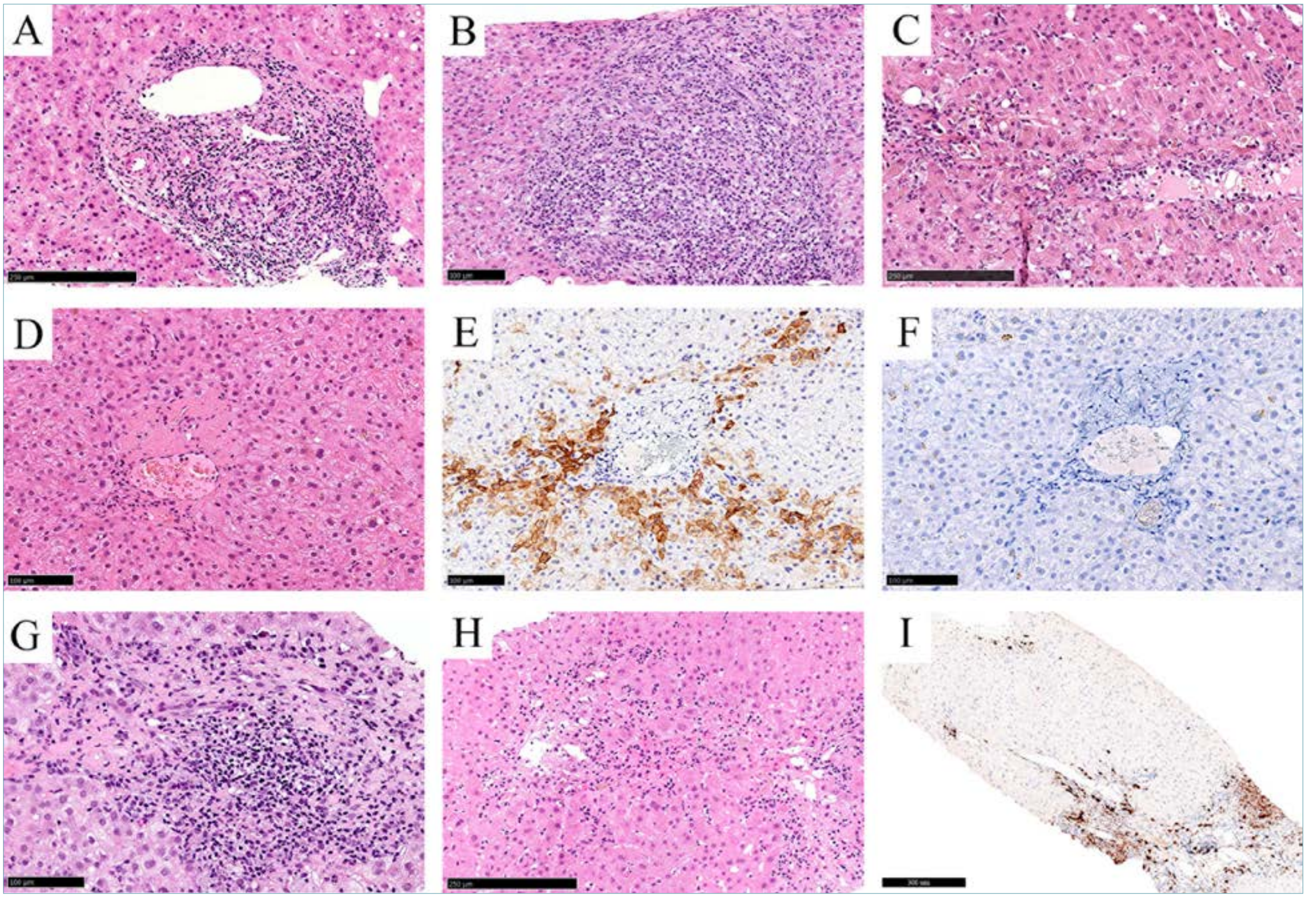

Figure 4. Pathologic features of rejection. (A) Mixed rejection infiltrate (lymphocytes, plasma cells, eosinophils) expanding a portal tract, presenting features of endotheliitis and bile duct injury. (B) Severe rejection infiltrate with conspicuous eosinophils, lymphoblasts, and plasma cells "obscuring" portal structures; notice how the infiltrate, although severe, is strictly confined to the portal tract, showing minimal parenchymal spillover only. (C) Severe centrilobular vein endotheliitis, showing endothelial cells swelling and detachment as well as immune cells aggressive behavior. (D) Bile duct loss in a portal tract of a liver affected by chronic rejection. (E) The absence of bile duct and ductular reaction are suggestive features of chronic rejection, whereas periportal hepatocyte ductular metaplasia is common and diffuse (cytokeratin 7 immunohistochemical staining). (F) Cytokeratin 19 further highlights the absence of bile duct and ductular reaction, similarly to cytokeratin 7, although it is not expressed by metaplastic hepatocytes (please, notice that tiles D, E, and F represent the same portal tract). (G) The plasma cell rich variant of rejection presents an inflammatory infiltrate with conspicuous plasma cells and interface hepatitis. (H) Centrilobular-based injury with hepatocyte apoptosis and necrosis and neutrophilic aggregates, is an additional characteristic feature of plasma cell rich rejection. (I) Immunohistochemical staining highlights plasma cells (here represented by MUM-1) and proves useful in evaluating the quantity of plasma cells and their location when dealing with plasma cell rich rejection,

showing lesions ranging from initial cell injury (cytoplasmic eosinophilia and cell atrophy/nuclear loss) of early-phase disease to complete bile duct extinction and ductopenia characterizing the long-standing phase [48]. Diagnosing ductopenia can be challenging considering that portal tracts could physiologically lack bile ducts on biopsy (up to 7% of evaluable portal tracts, approximatively) [61]. Additionally, liver biopsy can present an overall low number or partially sampled portal tracts. Thus, Banff guidelines recommend to perform the diagnosis of ductopenia (and therefore of c-TCMR) if the biopsy presents at least 10 portal tracts with at least > 50% showing clear ductopenia. A useful hint to the diagnosis is represented by the (almost) complete absence of ductular reaction that characterizes c-TCMR and differs it from other condi-

Table IV. Banff grading system for acute T-cell mediated rejection (TCMR) (Reject Activity Index – RAI).

	Mild	Moderate	Severe
Portal tract	Lymphocytic prevalent inflammation; few portal tracts involved **(RAI = 1).**	Mixed (lymphocytes, neutrophils, eosinophils, and few lymphoblasts) inflammation; most/all portal tracts involved **(RAI = 2).**	As for moderate with increased blasts and eosinophils. Although infiltrates tend to be portal-centered, inflammatory spillover in the periportal parenchyma may occur **(RAI = 3)**.
Bile ducts	Cuffed and infiltrated by mixed inflammatory cells; few bile ducts are involved, showing mild signs of injury **(RAI = 1).**	Most/all bile ducts involved showing moderate signs of injury (pyknosis, basement membrane loss) **(RAI = 2).**	Increased signs of bile ducts injury such as cell disruption **(RAI = 3).**
Venous structures (portal and centrilobular)	Subendothelial lymphocytic prevalent inflammation; few venules involved **(RAI = 1).**	Focal feature of confluent centrilobular necrosis; most/all venules involved by the inflammatory infiltrates **(RAI = 2).**	Increased severity of venular inflammation with parenchymal extension and associated perivenular parenchymal necrosis **(RAI = 3).**
The final score is obtained by combining the three pattern and adding the relative points, thus ranging from 0 to 9.			

tions affecting bile ducts (Fig. 4). Additionally, portal inflammation is usually mild/minimal, as well as endothelial injury. Similar to a-TCMR, the Banff Working Group on Liver Allograft Pathology published a grading system for c-TCMR, differentiating early-phase to late c-TCMR.

Furthermore, additional, non-canonical patterns of rejection have also been reported in the literature. They usually occur later after transplantation (starting from six months, approximatively) and prove to be particularly difficult to identify and relevant in PLTx.

1) *Centrilobular rejection*: refers to the isolated injury of the terminal hepatic vein (inflammation, endotheliitis and hepatocyte extinction) with no portal tract/bile duct involvement. It was described as an early sign of rejection in the PLTx setting [48,62].

2) *Plasma cell rich-rejection*: previously reported as *de novo* AIH, the plasma cell rich mediated liver injury is now fully considered a form of rejection by the Banff guidelines [55]. Histopathologically, it does not differ greatly from usual AIH (plasma cell rich portal inflammation, interface hepatitis, lobular/bridging necrosis), but presents some additional peculiar features such as a more frequent severe bile ducts involvement (lymphocytic cholangitis) and a prevalent IgG4 positive plasma cell inflammation (Fig. 4) [55,62]. Typical features of both AMR (C4d positivity) and TCMR could also coexist. As a form of rejection, although atypical, it responds adequately to immunosuppression, but it occurs later (> 6 months) after transplantation [55]. Of note, morphological distinction between recurrent AIH and plasma cell rich-rejection represents, to date, a challenging scenario based on slight differences only.

3) *Hepatitis-like rejection*: a form of rejection presenting portal inflammation together with interface and lobular features mimicking chronic hepatitic processes [62,63]. It has emerged as a relatively common pattern of injury observed in clinically-silent protocol follow-up biopsy and currently represents a "hot-topic" of research studies trying to describe its temporal evolution (it is probably related to complicated therapeutic compliance from adolescent patients) and identify precocious signs of injury [64-66].

De novo and recurrent diseases

Curiously, the diagnostic routine of a pathologist approaching PLTx biopsy can greatly vary depending on the follow-up protocols adopted by the specific transplant center. In particular, pathologists practicing in Institutions performing protocol liver follow-up biopsies will probably face a relatively high number of cases showing clinically silent (*i.e.,* without associated clinical-serological signs and symptoms) signs of inflammation and fibrosis [17,67-70].

On the other hand, if liver biopsies are performed following clinical indications only, the main features that we can encounter will be related to rejection processes, as described in the previous paragraph, or *de novo* and recurrent disease, both of them further addressed in this paragraph.

Recurrence disorders are particularly rare in the pediatric setting. Indeed, the main indications to PLTx are of metabolic and inherited nature (*e.g.,* biliary atresia), thus harboring almost no recurrence potential. Additionally, disease with post-PLTx recurring capability (primitive sclerosing cholangitis, HBV and HCV hepatitis) do not present peculiar morphologic findings, and therefore we direct the readers to the specific literature addressing these conditions [24,48,71].

On the other hand, *de novo* diseases (defined as the occurrence of a disease not experienced by the patient before transplantation) represent a relevant issue in long-term PLTx, particularly if considering infection-related conditions. Indeed, pediatric patients usually reach transplantation in an infection "naïve" condition and immunosuppression protocols to prevent rejection constitute a major risk factor for infection outbreak. In particular, infective agents can determine direct liver parenchymal injury (*i.e.,* CMV, EBV and HSV hepatitis) or cause proliferative/neoplastic disorders (*i.e.,* EBV and HHV8). The former group of infectious causes are mostly represented by hepatitic features (portal and lobular inflammation) with peculiar pathogen-specific characteristics. If suspected, ancillary techniques (immunohistochemical and RNA *in situ* hybridization) and laboratory tests are essential to perform a definitive diagnosis and prevent misinterpretation. Specific patterns of infective-related post-transplant relevant diseases are summarized in Table V.

Regarding infectious (viral) induced neoplastic disorders, two pathogens are particularly noteworthy: HHV8 and EBV. HHV8 leads to the development of Kaposi sarcoma, a malignant vascular tumor, fortunately only rarely reported in the PLTx setting [72]. Similarly, EBV has been associated with induction of uncontrolled cell proliferation, particularly of B-cells, leading to the development of posttransplant lymphoproliferative disorder (PTLD) [73]. Indeed, PTLD is a group of multifaceted B-cell proliferative disorders affecting transplanted patients. Several patterns are described, grouped by the WHO in four categories, namely (1) non-destructive PTLD, (2) polymorphic PTLD, (3) monomorphic PTLD, and (4) classical Hodgkin's lymphoma PTLD. Although PTLD rarely affects the graft liver of PLTx patients [74-76], more in depth discussion is beyond the aim of this manuscript. Conversely, we would like to mention and briefly illustrate the features of another EBV-related condition, that is EBV related-smooth muscle tumor (EBV-SMT). EBV-SMT is a rare (and frequently unrecognized) neoplastic disorder affecting immunosuppressed patients (transplanted and immunodeficient patients) mainly developing in the liver (both native and transplanted). It is particularly frequent in the pediatric population presenting features of a benign-looking (no/minimal cytological atypia, no necrosis, low proliferative index/mitotic count) soft-tissue neoplasm with morphological (spindle cells arranged in fascicles) and immunohistochemical (smooth muscle actin and h-caldesmon diffuse expression, desmin focal expression) features of smooth muscle differentiation (Fig. 5) [77-79]. Neoplastic cell EBV expression is a required criterion to differentiate this entity from similar conditions (Fig. 5).

Additional neoplastic conditions that can newly develop with increased frequency in the PLTx population are represented by skin, lung, liver, kidney and anogenital cancer [80].

Back to the future: next-generation pathology to revamp the benefit of the liver biopsy

Proclaiming a Liver Biopsy *Manifesto* is beyond the aim of this Review, but we believe that we are at the edge of a new era for liver histomorphologic evaluation, particularly in the PLTx setting. Indeed, liver biopsy is

Table V. Most frequent opportunistic viral infections affecting the transplanted liver.

Pathogen	Histopathological features
Cytomegalovirus (CMV)	Scattered neutrophilic microabscesses, usually surrounding hepatocytes with nuclear eosinophilic inclusion/nuclear atypia. Additional features are represented by unspecific portal inflammation also affecting bile ducts and an increased hepatocytes mitotic index. Immunohistochemical confirmation of hepatocyte infection (nuclear staining) is useful to confirm the diagnosis.
Adenovirus	Usually occurring within 6 months after transplant, adenovirus liver infection presents a CMV-like hepatitis condition presenting concurrent confluent parenchymal necrosis and hemorrhage, particular in severe cases. Bile ducts could also be directly and severely injured (necrosis), whereas hepatocytes may show irregular nuclear atypia. Immunohistochemical confirmation of hepatocyte infection (nuclear staining) is useful to confirm the diagnosis.
Herpes virus	Foci of well-delimited parenchymal necrosis that may be confluent in severe condition. Hepatocellular nuclear inclusion, although evident, are rarely observed. Immunohistochemical confirmation of hepatocyte infection (nuclear staining) is useful to confirm the diagnosis.
Epstein Barr virus (EBV)	Diffuse portal and lobular B-cell rich inflammation. B-cell may also be observed within sinusoids and infiltrating portal and central vein endothelium. Confirmation of diffuse B-cell EBV expression (either LMP-1 immunohistochemistry or EBER in situ hybridization) is useful to confirm the diagnosis.

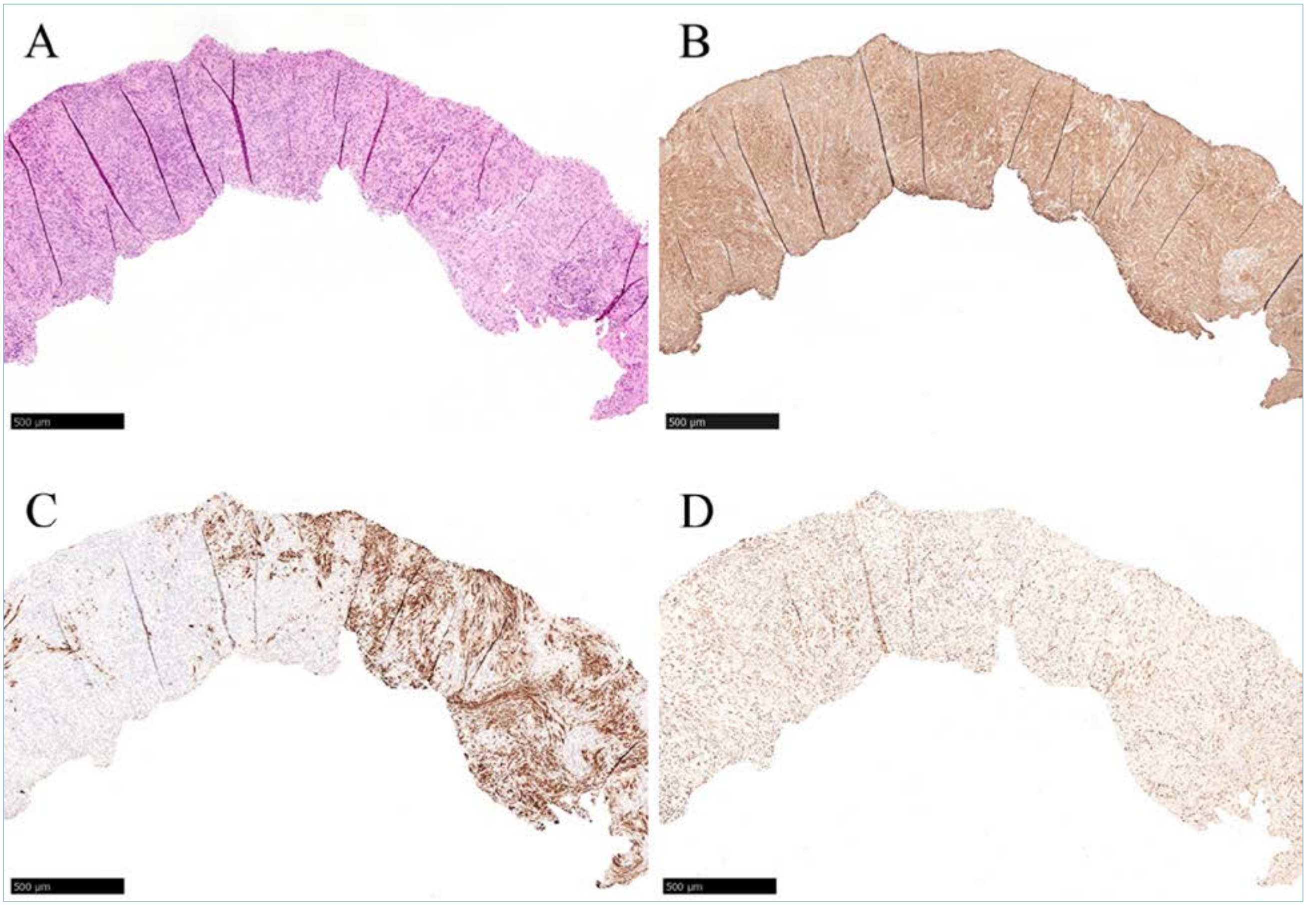

Figure 5. Pathological features of EBV-SMT. (A) Spindle cells with none/minimal cytological atypia organized in fascicles, no/minimal necrosis, and low mitotic index are characteristic features of EBV-SMT (A; hematoxylin and eosin). (B) Diffuse expression of smooth-muscle actin helps confirm the nature of EBV-SMT. (B) EBV-SMT usually presents a focal/heterogeneous positivity to desmin. (C) Proof of diffuse positivity to EBV [here represented by Epstein-Barr encoding region (EBER) in situ hybridization] is a required criterion to perform the diagnosis of EBV-SMT.

burdened by well-known and potentially severe complications, but next-generation pathology, namely the combination of multiplex immunohistochemistry and tissue-tethered digitally assisted analysis, harbor the potential to extract specific single-cell morphometric, phenotypic, and spatial characterization from the biopsy, thus obtaining a brand-new type of data enriched by the correlation with tissue architecture [11,12,81,82]. In particular, multiplex immunohistochemistry allows to contemporary evaluate multiple antigens within the same slide with cell detail and has proved particularly helpful in defining the specific phenotype of immune cells, whereas digital analysis sheds light on morphologic and spatial data that would have been otherwise remained unexplored. We believe that next-generation pathology will considerably renew liver biopsy indications in the next future, especially considering that it has already efficiently identified (pediatric) patients that would benefit from immunosuppression weaning, thus preserving them from long-term treatment side effects [56,65,66,83].

Conclusions

PLTx is a life-saving treatment for several terminal conditions. Due to the life-long persistence of the transplanted organ, every step of PLTx needs to be thoroughly approached and analyzed, thus frequently requiring liver tissue evaluation. As pathologists, our role is to render the most specific and reliable yet early diagnosis. Pathologists are involved in every crucial step of PLTx including definition of liver graft function, immediate-post transplant injury recognition, rejection and long-term complications identification. Innovative next-generation pathology procedures are expanding our knowledge of graft pathology with unexplored data, allowing us to identify precocious signs of tissue injury, and renew the histological assessment of transplanted organs.

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