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**Director
Prof. C. Pignata**

PhD Thesis

***PATHOPHYSIOLOGY, CLINICAL FEATURES, AND
MANAGEMENT OF CHILDREN WITH CHRONIC LIVER
DISEASES***

**Tutor
Prof. R. Iorio**

**Student
Dr. F. Cirillo**

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^a Pediatric Hepatology and Liver Transplantation, ^b Pneumology, ^c Abdominal Surgery, ISMETT, University of Pittsburgh Medical Center Italy, Palermo. ^d Infectious Diseases, Università dell'Insubria, Varese. ^ePathology, Ospedali Riuniti, Bergamo. ^f Pediatrics, Università di Pisa. Early detection of lymphoproliferative disorders (PTLD) in paucisymptomatic pediatric liver transplant recipients by adenotonsillar histology. EASL Special Conference "Liver Transplantation" 2011, December 15-17. Lisbon, Portugal

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***PATHOPHYSIOLOGY, CLINICAL FEATURES, AND MANAGEMENT
OF CHILDREN WITH CHRONIC LIVER DISEASES***

Introduction.

Modern hepatology has only taken off in the last 50 years. Developments have depended not only on specialist hepatologists, but on developments in other related disciplines of medicine such as virology, immunology, biochemistry, and now, molecular medicine. A huge literature is available describing liver disease in adults, but pediatrics has lagged behind.

Nevertheless, pediatric liver disease represent a significant cause of morbidity and mortality worldwide.

The etiology of liver disease in childhood is wide and varies significantly from the adult population encompassing structural, genetic, infectious, metabolic, iatrogenic, and unknown causes; in addition, differently from adults, signs and symptoms of liver disease in pediatric population are often non specific and can vary greatly from child to child among the different liver diseases, providing challenges to the primary care provider.

More recent advances in diagnosis and treatment, particularly the successful development of transplantation, have dramatically improved the outcome for infants and children with liver disease, so that many can now expect to grow into adult life. The gratifying survival of increasing numbers of young people with liver disease into adult life means that it is essential for adult practitioners to be cognizant of pediatric liver disease.

Unfortunately, the investigation and management of significant pediatric liver disease rightly remains within the remit of specialist or transplant units.

To date, the same multidisciplinary approach that provided so many advances in clinical and research aspects of adult hepatology is still lacking in the pediatric experience, so recognition of pathogenesis of specific pediatric liver diseases, implications of new therapies, characterization of new pathological entities is still delayed by necessity for multidisciplinary working.

Therefore, our research starts from the assumption that only knowledge of unexplored aspects can be an important tool for advancing into the management of hepatic disorders in children. It should be a contribute to both provide a framework to understand pathophysiology of some hepatobiliary disorders and offer analyses of their clinical-laboratory manifestations and the strategies for managing them. This project might be also useful to create specific competences related to a integrated and multidisciplinary approach, as required in pediatric liver disease. Our study concerns four areas that still present several either pathogenetic or diagnostic uncertainties, focusing on the following aspects:

1. Role of cellular immunity in the pathogenesis of Biliary Atresia.

- In Biliary Atresia inflammatory obstruction of the extrahepatic bile ducts is accompanied by a characteristic intrahepatic mixed cellular infiltrate composed of lymphocytes, macrophages, and eosinophils; do cellular immune mechanisms play a role in pathogenesis of Biliary Atresia? If so, how effector and regulatory Tcells are distributed in the liver tissue and bile duct remnant of children with Biliary Atresia?

2. New clinical and therapeutic aspects in pediatric autoimmune liver disease.

- Immunoglobulin G subclass 4 (IgG4)-related liver disease is well described in adults and is associated with multisystemic symptoms and a particularly good response to corticosteroid treatment. prevalence and clinical significance of raised IgG4 levels in children with autoimmune are still unknown.

- Cyclosporin has been shown to allow recovery from liver failure and to induce and safely maintain remission in children with autoimmune liver disease, but is usually used as a bridge to conventional treatment and its long-term safety and efficacy are unknown in pediatric patients.

3. Pediatric liver transplant: immunological features and complication of calcineurin inhibitors treatment.

- Transplant immunological response is a balance between the alloimmune effectors T cells and the immunological tolerance; calcineurin inhibitors may represent a barrier to immune tolerance in organ transplantation by decreasing the percentage of Treg in the peripheral blood in liver transplant recipients. However contradictory results have been reported. Indeed there are some evidence indicating that tacrolimus promotes in vitro expansion of Treg cells especially in humans. To better understand the effect of tacrolimus in maintaining of immune tolerance versus donor alloantigens, we evaluated the immunephenotype and the amount of Treg cells in tacrolimus treated patients.

- PTLD is a severe complication of transplantation linked in most cases to EBV infection. Prevalence in pediatric liver transplant recipients is 5-7%, mortality over 50%. PTLD is often recognized at the stage of lymphoma; in order to early detect PTLDs in liver

transplanted children, presenting with mild symptoms we evaluate adenotonsillar histology, obtained with a mini-invasive technique, as “sentinel tissue”.

4. Broadening the spectrum of UDCA indications.

Ursodeoxycholic acid is increasingly used for the treatment of cholestatic liver diseases but experimental evidence suggests several mechanisms of action other than stimulation of hepatobiliary secretion, such as cytoprotective, antiapoptotic, antioxidative, immunomodulatory, and altering cell-signaling properties. We report the first case of a patient with primary NRHL associated with portal hypertension in whom UDCA therapy was followed by a prompt reduction and sustained normalization of liver enzymes and no progression of portal hypertension throughout 30 months of follow-up.

Chapter 1. Role of cellular immunity in the pathogenesis of Biliary Atresia.

1.2. Immunohistochemical characterization of T-cells in the liver and bile duct remnant of children affected by Biliary Atresia.

Introduction.

Biliary Atresia (BA) is an enigmatic cause of neonatal cholestasis, occurring in 1 in 8,000 to 1 in 18,000 live births and presenting typically within the first months of life with jaundice, acholic stools, and hepatomegaly in an otherwise apparently healthy infant (1). Inflammatory obstruction of the extrahepatic bile ducts is accompanied by a characteristic intrahepatic portal lesion. If diagnosed in the first 2–3 mo of life, hepatic portoenterostomy can restore bile flow from the liver into the intestinal tract in 30 – 80% of patients (1–3). Despite successful surgery, progressive inflammation and fibrosis of intrahepatic bile ducts develops to varying degrees in all patients, leading to biliary cirrhosis in the majority of patients. Consequently, 70 – 80% of BA patients will eventually require liver transplantation, approximately half in the first 2 y of life (4 – 6). Thus, BA is the most common indication for liver transplantation in children, responsible for almost 50% of all pediatric liver transplants. It should be emphasized that it is the intrahepatic biliary lesion that determines overall prognosis and outcome, thus, this disease is no longer called “extrahepatic” BA. Without a better understanding of the etiology and pathogenesis of this intrahepatic sclerosing cholangitic process in BA, little progress can be expected in improving the nontransplantation outcome of patients. Therefore, there has been a renewed interest in recent years in understanding the underlying pathogenetic mechanisms of BA. It is now apparent that BA is a phenotype resulting from several pathogenic processes that culminate in obstruction of the biliary tree (1,7).

Cellular immune mechanisms are central to the pathogenesis of experimental BA.

(ABSTR) Biliary innate immunity is involved in the pathogenesis of cholangiopathies in patients with with primary biliary cirrhosis (PBC) and BA. Biliary epithelial cells possess an innate immune system consisting of the Toll-like receptor (TLR) family and recognize

pathogen-associated molecular patterns (PAMPs). Tolerance to bacterial PAMPs such as lipopolysaccharides is also important to maintain homeostasis in the biliary tree. In PBC, CD4-positive Th17 cells characterized by the secretion of IL-17 are implicated in the chronic inflammation of bile ducts and the presence of Th17 cells around bile ducts is causally associated with the biliary innate immune responses to PAMPs (8).

At the time of diagnosis of BA, the extrahepatic duct remnant is composed of fibrous tissue and scattered lymphocytes (9) whereas intrahepatic ducts are surrounded by a mixed cellular infiltrate composed of lymphocytes, macrophages, and eosinophils (36; 10). The role of the immune system in bile duct injury and obstruction is poorly understood at present and is the focus of intense investigation.

Aim

To characterize immunohistochemically effector and regulatory T cells in the liver tissue and bile duct remnant of children with BA, to investigate for the first time the presence of Th17 cells in liver and biliary remnants of BA patients.

Patients and Methods.

Archived paired percutaneous liver biopsies (LB) and biliary remnants (BR) from 7 children with BA were studied. Bile duct tissue from 6 children with choledochal cyst (CC) were also evaluated as control. Avidin-Biotin immunoperoxidase technique was used to investigate the following lineage-specific markers: transcription factor T-bet (Th1), GATA-3 (Th2), FOXP3 (regulatory T cells, Tregs) and IL-17 (Th17).

Briefly, antigen was demasked by placing the biopsies in plastic jar containing citrate buffer saline (pH: 6.1) and micro waved at 600 W (2 x 5 minutes). After cooling for 15 to 30 minutes, biopsies were washed and placed in phosphate-buffered saline for 5 minutes. Endogenous peroxidase was neutralized using Peroxidase Block for 30 minutes, then washed and placed in PBS for 5 minutes. Non-specific background staining was inhibited by 10 minutes incubation of the biopsies with 10 % horse serum. (Vector Laboratories) After washing avidin -biotin blocking reagent was applied for 30 min to block endogenous biotin. After washing the sections were then incubated with a polyclonal goat

anti-human IL-17 antibody for one hour at room temperature. Here we used three dilutions of goat anti-human IL-17 antibody at 1/10,1/30,1/100 in PBS. Optimal dilution of the primary antibodies used in this detecting system was defined to give a specific staining with no or minimal background. After washing, the sections were then incubated with biotin conjugated secondary antibody anti-goat (Horse anti-goat IgG, Vector Laboratories, diluted 1/50) for 30 minutes. Immune reactivity was visualized by avidin-biotin- peroxidase complex (ABC complex, Vector Laboratories) kit reagents. Peroxidase activity was developed by using mixture of DAB substrate solution (as mentioned above) for 8 minutes then washed and placed in PBS. Finally biopsies were counterstained with hematoxylin and prepared for microscopic examination by placing a cover slip on the liver biopsies on the slide. All tissues used were also tested as negative controls (omitting the primary antibody) with each detection to detect background or any false positivity caused by the detection system.

A semi-quantitative scoring system (0 to 3+) was used for evaluation of results.

Results.

Main results of our study are summarized in Table.

Table. Immunohistochemical characterisation of T-cells in 7 children affected by BA (Pt) and in 6 children affected by CC (Control).

Biliary atresia	T-bet	GATA-3	FOXP3	IL-17
PT 1 LB BR	++ +	+/- neg	+++ neg	+++ +++
PT 2 LB BR	++ ++	neg neg	+ neg	+++ ++
PT 3 LB BR	++ neg	neg neg	+++ +	+++ +
PT 4 LB BR	+++ +	neg neg	+ neg	+++ ++
PT 5 LB BR	+++ neg	neg neg	+ neg	++ +
PT 6 LB BR	+++ +++	neg neg	neg neg	+++ ++
PT 7 LB BR	++ neg	neg neg	neg neg	++ ++
Choledocal				
Control 1	+	neg	+	++
Control 2	neg	neg	neg	++
Control 3	+	neg	neg	+
Control 4	neg	neg	+	++
Control 5	neg	neg	neg	Neg
Control 6	neg	neg	neg	Neg

LB = liver biopsy; BR = biliary remnants

In BA, Th1 and Th17 effector cells are abundant, with a particularly high frequency of Th17 cells in both liver tissue and bile duct remnant (Figure 1). Th2 cells are negligible and none of our patients showed clear immunohistochemical positivity for GATA-3; also all choledocal cyst controls were negative for GATA-3.

Tregs are variably represented, being particularly abundant in the liver biopsies of patients 1 and 3.

Both Th1 and Th17 cells are well represented both in LB and BR, but in 5/7 patients it is present a greater positivity in LB than in BR that could be explained by the presence of more abundant fibrotic tissue in BR samples. In CC, all cell lineages are markedly less represented than in BA apart from Th17 (Figure 3) that is present in 4 patients, though less abundantly than in BA.

Discussion.

T-cell activation requires the interaction of the T cell with an APC-bearing antigen in the context of MHC class I (for cytotoxic CD8+ T cells) or MHC class II (for helper CD4+ T cells). In the inflammatory environment, naïve CD4+ T cells differentiate to either Th1 effector cells (driven by IL-12 and producing IFN- γ , IL-2, TNF- β , and TNF- α) or Th2 effector cells (driven by IL-4 and producing IL-4, IL-5, and IL-10).

Previous studies showed that in BA patients, T-cell infiltrates surround and invade intrahepatic bile ducts (11); this infiltrates are characterized predominantly by CD4+ T cells (12-14), CD8+ T cells (15), or a mixture of both (16). Activated effector T cells produce cytokines that can directly damage epithelial cells or indirectly damage them through stimulation of other immune cells.

Recent investigation has revealed that BA is associated with Th1-cell-mediated portal tract inflammation. Mack CL et al. have shown that the periductular immune cells in BA produce IL-12, IFN- γ , IL-2, and TNF- α ; in their study this localized inflammatory profile was unique to BA and was not found in other neonatal cholestatic diseases (16). Bezerra et al. (17 44) used gene expression microarray techniques to analyze BA liver biopsies and observed up-regulation of a number of pro-inflammatory genes (including the Th1 cytokines IFN- γ and osteopontin) and down-regulation of Ig genes suggesting inhibition of Th2 pathway.

Confirming these previous results, our study showed a considerable presence of Th1 cells infiltration both in liver parenchyma and in biliary remnants of children affected by BA and the absence of Th2 cells.

However, main result of our study was to demonstrate for the first time in literature the presence of an abundant infiltrate of Th17 cells in BA patients; specifically our data show that Th17 is the most prevalent type of effector T-cell in BA, a condition hitherto considered to be mainly Th1 induced.

It is known that the recently discovered Th17 cells characterized by the secretion of IL-17 have a pro-inflammatory property. Specifically, it has been reported that Th17 and Treg have reciprocal developmental pathways (18).

A fine balance between Th17 and Treg may be crucial for the stability of immune homeostasis. Once the equilibrium is broken, the destabilization may lead to chronic inflammation and autoimmunity.

Intriguingly, it has been demonstrated that murine helper T cell (Th) differentiates towards Th17 and Treg in a mutually exclusive manner (18).

Overall, in our study Tregs cells were consistently less represented than Th17 cells and this finding was specifically evident in biliary remnants where, at time of sampling, the inflammatory damage was more advanced than in liver parenchyma.

Nevertheless, how cells of the Th17 lineage may be involved in pathogenesis of BA remain still unknown and further investigations are needed to clarify the role of these cellular immunity effectors in bile duct damage.

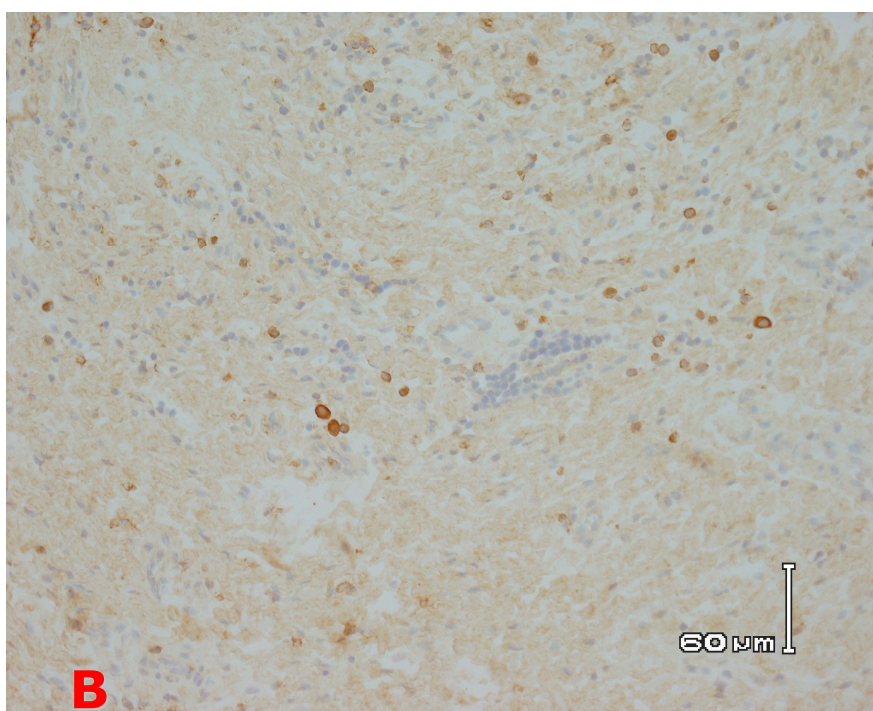
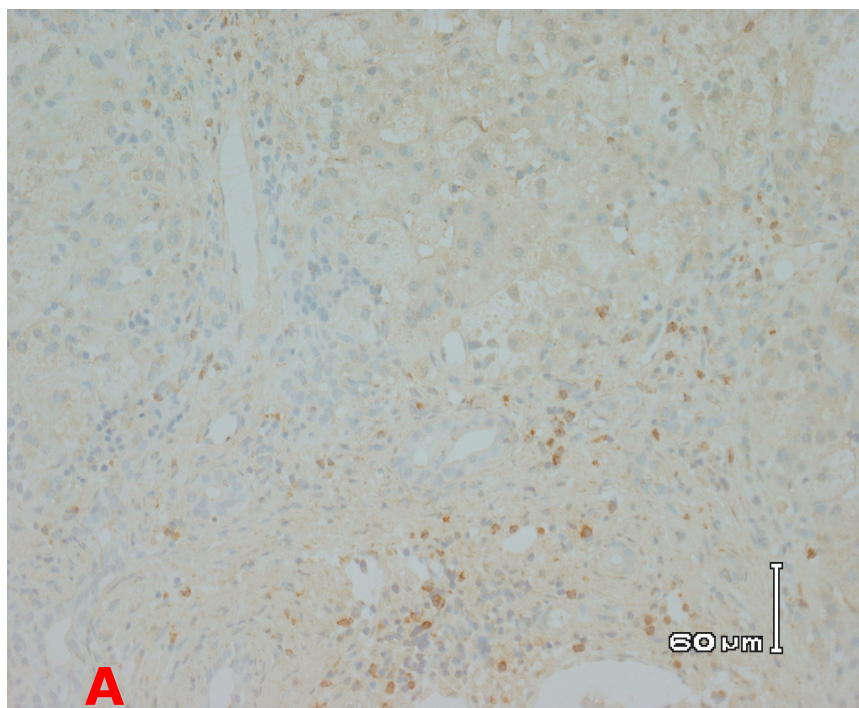


Figure 1.

Th17 positivity in liver tissue (A) and biliary remnants (B) in Pt 1
Th17 positivity in liver tissue (A) and biliary remnants (B) in Pt 1

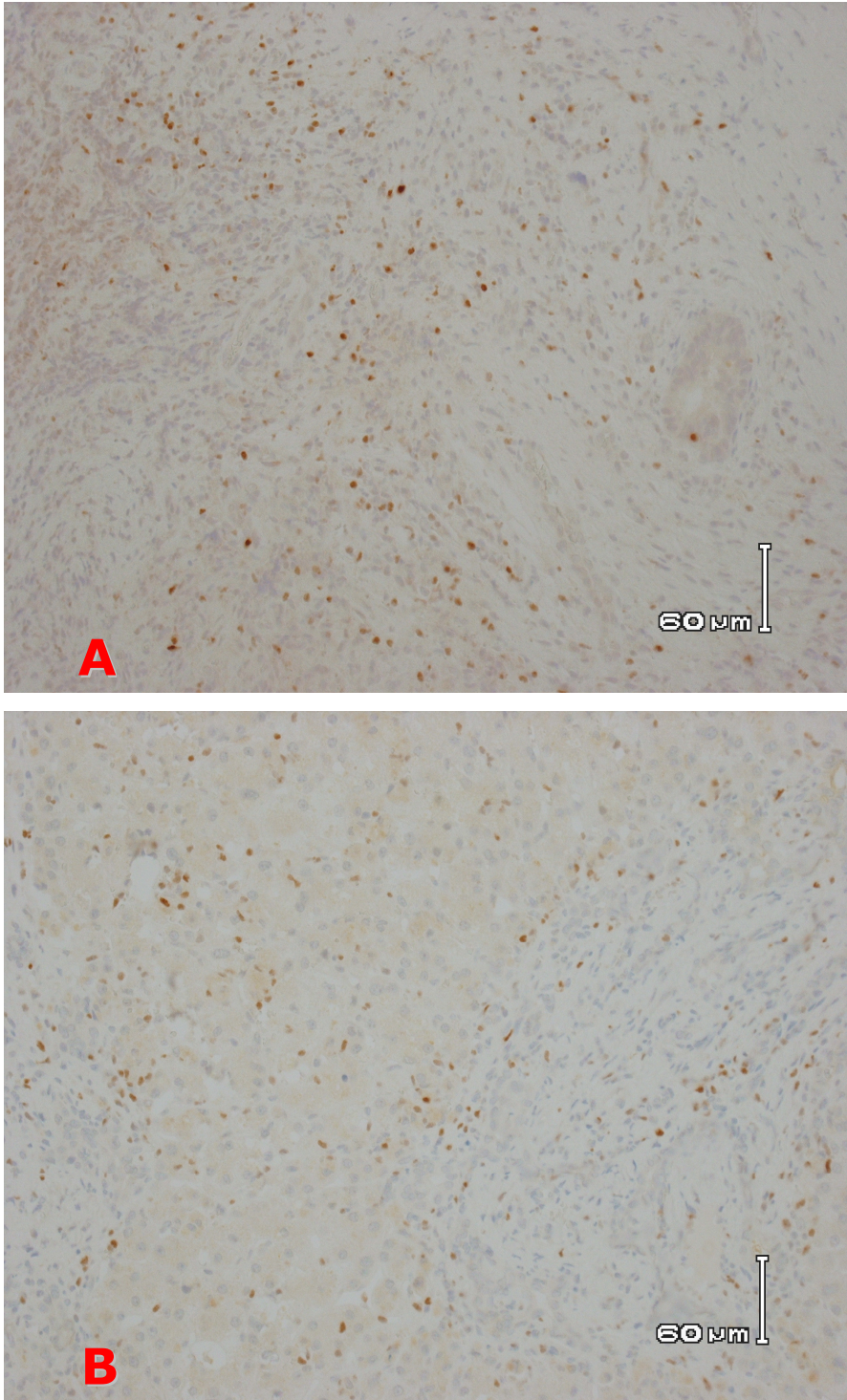


Figure 2.

Th1 positivity in liver tissue (A) and biliary remnants (B) in Pt 6
Th1 positivity in liver tissue (A) and biliary remnants (B) in Pt 6

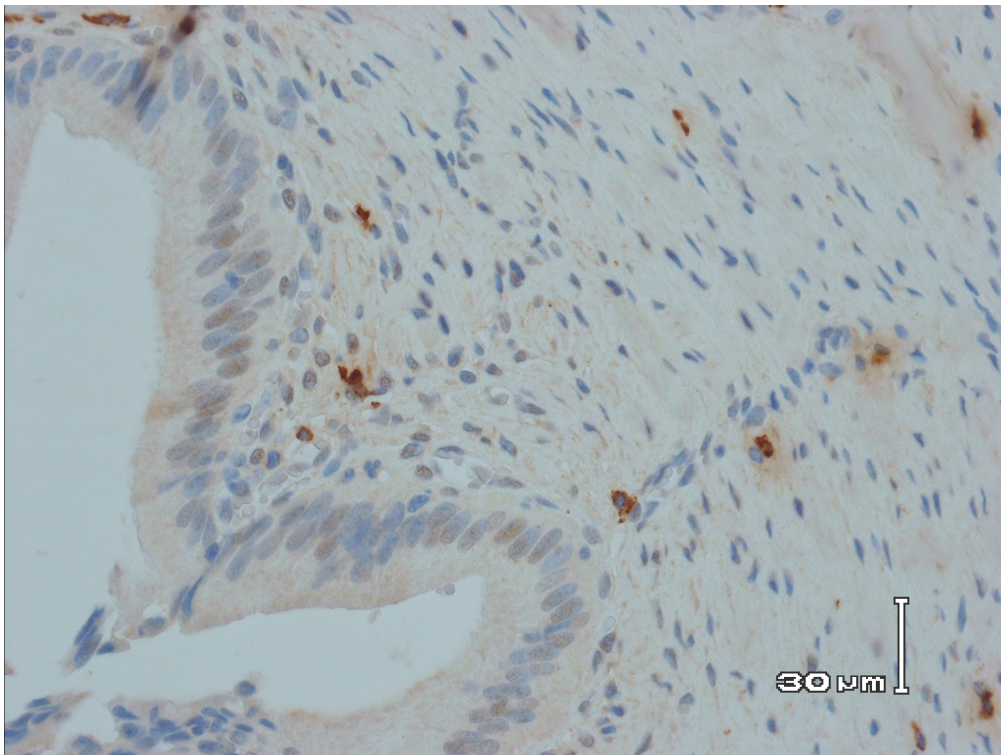
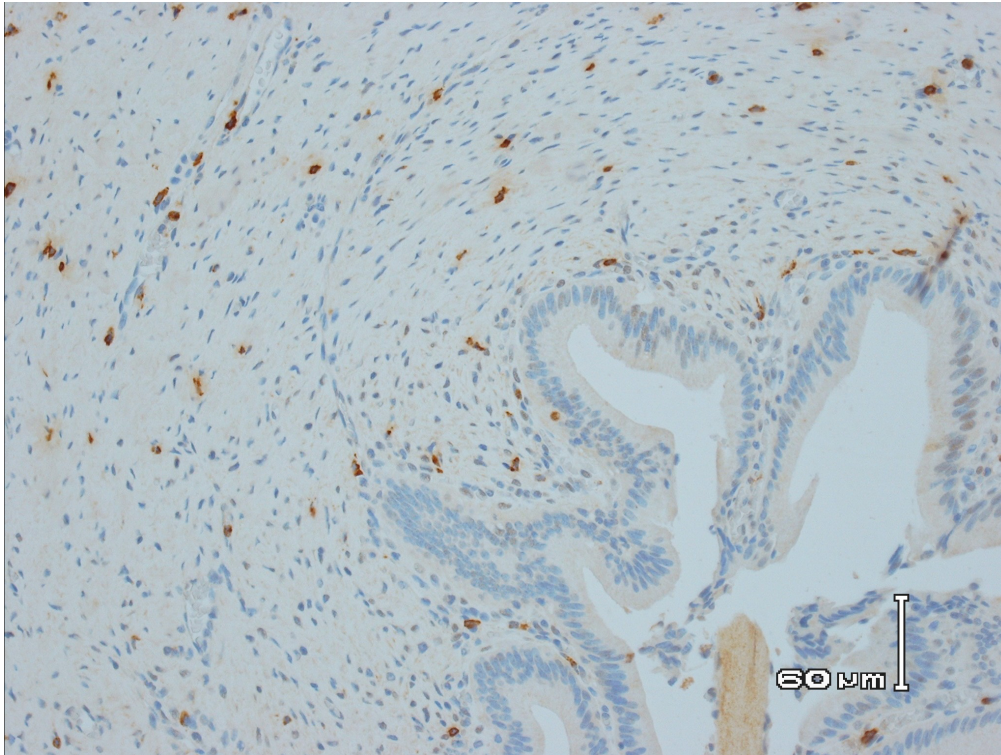


Figure 2.
Th17 positivity in Control 1 at different magnifications.

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IMMUNOHISTOCHEMICAL CHARACTERISATION OF EFFECTOR AND REGULATORY T-CELLS IN THE LIVER TISSUE AND BILE DUCT REMNANT OF CHILDREN WITH BA SHOWS A HIGH PREVALENCE OF TH17

Francesco Cirillo, Munther J. Hussain, Giorgina Mieli-Vergani, Mark Davenport, Diego Vergani, Nedim D. Hadzic; King's College Hospital, London, United Kingdom

Background: Biliary atresia (BA) results from inflammatory destruction of the bile ducts, often leading to end stage liver disease despite corrective surgery. Cellular immune mechanisms are central to the pathogenesis of experimental BA. **Aim:** To characterise immunohistochemically effector and regulatory T-cells in the liver tissue and bile duct remnant of children with BA. **Methods:** Archived paired percutaneous liver biopsies (LB) and biliary remnants (BR) from 7 children with BA were studied. Bile duct tissue from 6 children with choledochal cyst (CC) served as control. Avidin-Biotin immunoperoxidase technique was used to investigate the following lineage-specific markers: transcription factor T-bet (Th1), GATA-3 (Th2), FOXP3 (regulatory T cells, Tregs) and IL-17 (Th17). A semi-quantitative scoring system (0 to 3+) was used. **Results:** In BA, Th1 and Th17 effector cells are abundant, with a particularly high frequency of Th17 cells in both liver tissue and bile duct remnant; Th2 cells are negligible, while Tregs are variably represented, being particularly abundant in the liver biopsies of patients 1 and 3. Both Th1 and Th17 cells are more numerous in LB than BR. In CC, all cell lineages are markedly less represented than in BA apart from Th17 that is present in 4 patients, though less abundantly than in BA. **Conclusions:** Our data show that Th17 is the most prevalent type of effector T-cell in BA, a condition hitherto considered to be mainly Th1 induced. Cells of the Th17 lineage may be involved in bile duct damage.

	T-bet	GATA-3	FOXP3	IL-17
BA				
1: LB	++	+/-	+++	+++
BR	+	neg	neg	+++
2: LB	++	neg	+	+++
BR	++	neg	neg	++
3: LB	++	neg	+++	+++
BR	neg	neg	+	+
4: LB	+++	neg	+	+++
BR	+	neg	neg	++
5: LB	+++	neg	+	++
BR	neg	neg	neg	+
6: LB	+++	neg	neg	+++
BR	+++	neg	neg	++
7: LB	++	neg	neg	++
BR	neg	neg	neg	++
chol.cyst				
1	+	neg	+	++
2	neg	neg	neg	++
3	+	neg	neg	+
4	neg	neg	+	++
5	neg	neg	neg	neg
6	neg	neg	neg	neg

Disclosures:

The following people have nothing to disclose: Francesco Cirillo, Munther J. Hussain, Giorgina Mieli-Vergani, Mark Davenport, Diego Vergani, Nedim D. Hadzic

IMMUNOHISTOCHEMICAL CHARACTERISATION OF T-CELLS IN THE LIVER AND BILE DUCT REMNANT IN BILIARY ATRESIA SHOWS A HIGH PREVALENCE OF Th17

Cirillo F[†], Hussain MJ, Quaglia A, Mieli-Vergani G, Davenport M^{*}, Vergani D, Hadzic N.

[†]Department of Paediatrics, University of Naples "Federico II", Naples, Italy
^{*}Institute of Liver Studies and ^{*}Department of Paediatric Surgery,
King's London School of Medicine at King's College Hospital
London, United Kingdom

Biliary atresia (BA) results from inflammatory destruction of the bile ducts, often leading to end stage liver disease despite corrective surgery. Cellular immune mechanisms are central to the pathogenesis of experimental BA.

AIM: To characterise immunohistochemically effectors and regulatory T-cells in the liver tissue and bile duct remnant of children with BA.

METHODS: Archived percutaneous liver biopsies (LB) and biliary remnants (BR) from 7 children with BA were studied. Bile duct tissue from 6 children with choledochal cyst (CC) served as control. Avidin-Biotin immunoperoxidase technique was used to investigate the following lineage-specific markers: transcription factor T-bet (Th1), GATA-3 (Th2), FOXP3 (regulatory T cells, Tregs) and IL-17 (Th17). A semi-quantitative scoring system (0 to 3+) was used.

RESULTS: Main results of our study are summarized in **Table**. In BA, Th1 and Th17 effector cells are abundant, with a particularly high frequency of Th17 cells in both liver tissue and bile duct remnant (**Fig. 1**); Th2 cells are negligible, while Tregs are variably represented, being particularly abundant in the liver biopsies of patients 1 and 3. Th1 (**Fig. 2**) and Th17 cells are well represented both in LB and BR. In CC, all cell lineages are markedly less represented than in BA apart from Th17 (**Fig. 3**) that is present in 4 patients, though less abundantly than in BA.

CONCLUSION: Immunohistological phenotypes are similar in LB and in BR. Our data show that Th17 is the most prevalent type of effector T-cell in BA, a condition hitherto considered to be mainly Th1 induced. Cells of the Th17 lineage may be involved in bile duct damage.

Biliary atresia	T-bet	GATA-3	FOXP3	IL-17
Pt 1 LB	++	+/-	+++	+++
BR	+	neg	neg	+++
Pt 2 LB	++	neg	+	+++
BR	++	neg	neg	++
Pt 3 LB	++	neg	+++	+++
BR	neg	neg	+	+
Pt 4 LB	+++	neg	+	+++
BR	+	neg	neg	++
Pt 5 LB	+++	neg	+	++
BR	neg	neg	neg	+
Pt 6 LB	+++	neg	neg	+++
BR	+++	neg	neg	++
Pt 7 LB	++	neg	neg	++
BR	neg	neg	neg	++
Choledochal cyst				
Control 1	+	neg	+	++
Control 2	neg	neg	neg	++
Control 3	+	neg	neg	+
Control 4	neg	neg	+	++
Control 5	neg	neg	neg	neg
Control 6	neg	neg	neg	neg

Table. Immunohistochemical characterisation of T-cells in 7 children affected by BA (Pt) and in 6 children affected by CC (Control).



Fig. 1 Th17 positivity in liver tissue (A) and biliary remnants (B) in Pt 1

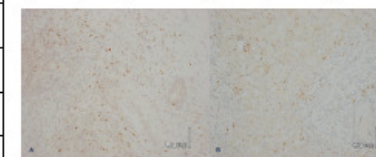


Fig. 2 Th1 positivity in liver tissue (A) and biliary remnants (B) in Pt 6

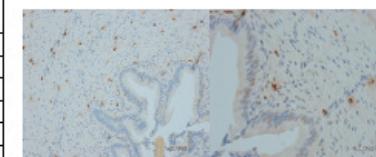


Fig. 3. Th17 positivity in Control 1

Chapter 2. New clinical and therapeutic aspects in pediatric autoimmune liver diseases.

2.1 Prevalence and significance of raised IgG4 in paediatric autoimmune liver disease: a retrospective study.

Introduction.

Autoimmune hepatitis (AIH) is an organ-specific autoimmune disease characterized by chronic inflammation of the liver, elevated transaminase levels, hypergammaglobulinemia, serum autoantibodies, histological evidence of interface hepatitis, and a favorable response to immunosuppressive treatment (1-3). A working model for its pathogenesis postulates that environmental triggers, a failure of immune tolerance mechanisms, and a genetic predisposition collaborate to induce a T cell-mediated immune attack upon liver antigens, leading to a progressive necroinflammatory and fibrotic process in the liver (4,5). The diagnosis is based on histologic abnormalities, characteristic clinical and laboratory findings, abnormal levels of serum globulins, and the presence of one or more characteristic autoantibodies (6 -10).

IgG4 is a minor component of the four subclasses of IgG in serum; little attention has been paid to this minor component of IgG since Hamano et al. found elevated serum IgG4 level in patients with autoimmune pancreatitis (AIP) (11).

Thereafter, many studies of AIP were reported, mainly by Japanese investigators. The histopathological findings of AIP are characterized by the periductal localization of predominantly CD4-positive T cells, IgG4-positive plasma cells, storiform fibrosis with

acinar cell atrophy frequently resulting in stenosis of the main pancreatic duct, and obliterative fibrosis (12-14), which is also called lymphoplasmacytic sclerosing pancreatitis (LPSP) (15). In 2003, Kamisawa et al. (16) suggested that AIP is a systemic sclerosing disease, based on the findings that the pancreas and other involved organs have fibrosis with abundant infiltration of IgG4-positive plasma cells, which is similar to the concept of multifocal fibrosclerosis proposed by Comings et al. (17) a systemic disease characterized by extensive IgG4-positive plasma cell and T lymphocyte infiltration of various organs. Clinical manifestations are apparent in organs such as the pancreas, bile duct, gallbladder, salivary gland, retroperitoneum, and others where tissue fibrosis with obliterative phlebitis is present on pathology.

Immunoglobulin G subclass 4-associated cholangitis (IAC) is a biliary disease of unknown immunopathogenesis, with cholangiographic features indistinguishable from PSC, but distinct histological findings and a dramatic response to corticosteroids (18). In the largest cohort of 53 IAC patients from the Mayo clinic, median age at diagnosis was 60 years in a predominantly male-patient group (85%), frequently presenting with obstructive jaundice (19). IBD is less frequently associated with IAC than PSC, described predominantly in case reports and small series, although IgG4 positive plasma cells have been detected in colonic specimens in patients with autoimmune pancreatitis and IgG4-related colitis may mimic IBD (20,21). Diagnostic criteria have been proposed for IAC based on characteristic histological and cholangiographic findings, elevations in serum IgG4, systemic organ involvement and response to corticosteroids (19). These are still to be cross-validated in an independent cohort of patients.

Recently, Umemura T et al. reported a case suggesting the existence of a new disease entity termed “immunoglobulin G4 (IgG4)-associated AIH”; although the revised IAIHG disease score of this patient was 18 and representative of definite AIH, a high serum IgG4 concentration was detected before the administration of corticosteroid therapy. Moreover, immunostaining of liver tissues prior to treatment showed abundant plasma cells with strong immunohistochemical reactivity to IgG4 (22).

Immunoglobulin G subclass 4 (IgG4)-related liver disease is well described in adults and is associated with multisystemic symptoms and a particularly good response to corticosteroid treatment. The significance of raised serum IgG4 in paediatric liver disease is unclear.

Aim.

Aim of present study was to investigate prevalence and clinical significance of raised IgG4 levels in a large cohort of children with autoimmune liver disease.

Methods.

Retrospective review of medical records, clinical, biochemical, radiological and histological data of 75 children with autoimmune liver disease (42 autoimmune hepatitis [AIH] and 33 autoimmune sclerosing cholangitis [ASC]) diagnosed from 2005–08. IgG4 levels were tested retrospectively in stored serum (-80°C) collected at or close to diagnosis by immunoenzymatic assay (ELISA). Levels >1.35 g/L were considered abnormal, based on published data in autoimmune pancreatitis. Patients were divided into

two groups: Group 1 (high IgG4) and Group 2 (normal IgG4). In 63 children with available liver biopsy at presentation, histological activity, staging, and number of IgG4⁺ plasma cells per high power field were also investigated.

Results.

Group 1 comprised 25 children (33%), 12 with AIH and 13 with ASC; group 2 comprised 50 children, 30 with AIH and 20 with ASC. Inflammatory bowel disease was diagnosed in 28% of both groups. Group 1 had significantly higher IgG levels (median 29.4 g/L, range 14.5–63.8, $P < 0.001$) and IgG4/IgG ratio (9.9 [2.3–27.7], $P < 0.001$) compared to Group 2. Within patients with AIH, those in Group 1 had lower C3 levels (0.86 g/L, [0.41–1.35] $P = 0.019$) than those in Group 2. There was no difference in liver function tests and histological activity/staging at presentation between the 2 groups. IgG4⁺ cells (≥ 5 cells) within the liver tissue were more commonly seen in Group 1 than Group 2 (27% vs. 2%), but ≥ 10 IgG4 positive cells, as described in adult IgG4-related disease, were not identified. Granulocyte epithelial lesions were seen in only one case of ASC within Group 2. After a median follow up of 3 years [2–5] normal AST, immunoglobulins and negative auto-antibodies were recorded in 62% in Group 1 and 51% in Group 2.

Conclusion.

Thirty-three percent of children with autoimmune liver disease evaluated in our study have high IgG4 levels at diagnosis with no difference between AIH and ASC. This proportion is higher than that reported in adults with AIH (3%) or primary sclerosing cholangitis (9%). No difference in disease severity or response to treatment was observed medium-term between patients with low or high IgG4, but longer follow up is necessary to determine whether children with high IgG4 represent a specific subtype of paediatric autoimmune liver disease.

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Objectives and Study: Blue rubber bleb naevus syndrome (BRBNS) is an unusual form of haemangioma, characterised by multiple cutaneous and visceral venous malformations. Intestinal lesions, often in the small bowel, are common and presents with haematemesis, haematochezia and transfusion-dependant haemorrhage. Assessment and treatment of BRBNS can be challenging, especially in the small bowel. Systemic agents such as corticosteroids, octreotide, vincristine, tranexamic acid and interferon have been tried with varying success. Reports of endoscopic interventions are few and limited surgical experience exists in this condition.

We proposed a review of the management of all patients presenting to our centre with BRBNS.

Methods: A review of all patients with BRBNS who presented to a paediatric tertiary gastrointestinal centre between 1999 and 2010, with particular emphasis on medical, endoscopic and surgical treatments.

Results:

Patient	Treatment
Patient 1 Age 16	Multiple colonoscopy and sclerotherapy. Laparotomy with resection of 80 lesions, resulting in symptom resolution for more than 1 year. Previously partially steroid-responsive. Recurrence of lesions now treated with a combination of medications, endoscopic and enteroscopic sclerotherapy with good result (2 blood transfusions in the last year).
Patient 2 Age 12	Multiple endoscopies and 7 single-balloon enteroscopies combined with sclerotherapy and Thalidomide, resulting in good bleeding control and 6-monthly blood transfusions. Thalidomide will be discontinued due to the onset of peripheral neuropathy. Previously steroid-responsive but suffered from side effects. An intra-operative enteroscopy with resection of lesions is planned.
Patient 3 Age 14	Endoscopic surveillance and resection of mesenteric lesion. All medical therapies had failed. Intestinal lesions have resolved but now suffer from large porto-systemic shunt with high-output congestive cardiac failure and pulmonary lesions.
Patient 4 Age 4	Currently asymptomatic despite evidence of lesions throughout the small bowel on Video Capsule Endoscopy. Annual monitoring is expected. Prophylactic tranexemic acid has been started.

Conclusion: Thalidomide, an inhibitor of angiogenesis by suppression of vascular endothelial growth factor, is very effective at controlling bleeding but appears to be more effective when used in combination with other therapies. Its use is limited by its side effects. Other medications, with the exception of corticosteroids, were ineffective in our group. Endoscopic and enteroscopic treatments perhaps limit the need for surgery, but possibly delay rather than avoid surgery. Endoscopic sclerotherapy and surgery are useful in the management of BRBNS but does not prevent recurrences. Despite these limitations, improved care and management have been achieved with these new developments.

Disclosure of Interest: None declared.

PA-H-0051

Hepatology

PREVALENCE AND SIGNIFICANCE OF RAISED IGG4 IN PAEDIATRIC AUTOIMMUNE LIVER DISEASE: A RETROSPECTIVE STUDY

T. Grammatikopoulos^{1,2,*}, F. Cirillo¹, Y. Zen², E. Davies³, D. Vergani², G. Mieli-Vergani^{1,2}. ¹Paediatric Liver, GI & Nutrition Centre, ²Institute of Liver Studies, ³Department of Immunology, King's College Hospital, London, United Kingdom.

Objectives and Study: Immunoglobulin G subclass 4 (IgG4)-related liver disease is well described in adults and is associated with multisystemic symptoms and a particularly good response to corticosteroid treatment. The significance of raised serum IgG4 in paediatric liver disease is unclear. We have investigated prevalence and clinical significance of raised IgG4 levels in a large cohort of children with autoimmune liver disease.

Methods: Retrospective review of medical records, clinical, biochemical, radiological and histological data of 75 children with autoimmune liver disease (42 autoimmune hepatitis [AIH] and 33 autoimmune sclerosing cholangitis [ASC]) diagnosed from 2005–08. IgG4 levels were tested retrospectively in stored serum (–80°C) collected at or close to diagnosis by immunoenzymatic assay (ELISA). Levels >1.35 g/L were considered abnormal, based on published data in autoimmune pancreatitis. Patients were divided into two groups: Group 1 (high IgG4) and Group 2 (normal IgG4). In 63 children with available liver biopsy at presentation, histological activity, staging, and number of IgG4+ plasma cells per high power field were also investigated.

Results: Group 1 comprised 25 children (33%), 12 with AIH and 13 with ASC; group 2 comprised 50 children, 30 with AIH and 20 with ASC. Inflammatory bowel disease was diagnosed in 28% of both groups. Group 1 had significantly higher IgG levels (median 29.4 g/L, range 14.5–63.8, $P < 0.001$) and IgG4/IgG ratio (9.9 [2.3–27.7], $P < 0.001$) compared to Group 2. Within patients with AIH, those in Group 1 had lower C3 levels (0.86 g/L, [0.41–1.35] $P = 0.019$) than those in Group 2. There was no difference in liver function tests and histological activity/staging at presentation between the 2 groups. IgG4+ cells (≥ 5 cells) within the liver tissue were more commonly seen in Group 1 than Group 2 (27% vs. 2%), but ≥ 10 IgG4 positive cells, as described in adult IgG4-related disease, were not identified. Granulocyte epithelial lesions were seen in only one case of ASC within Group 2. After a median follow up of 3 years [2–5] normal AST, immunoglobulins and negative auto-antibodies were recorded in 62% in Group 1 and 51% in Group 2. **Conclusion:** 33% of children with autoimmune liver disease have high IgG4 levels at diagnosis with no difference between AIH and ASC. This proportion is higher than that reported in adults with AIH (3%) or primary sclerosing cholangitis (9%). No difference in disease severity or response to treatment was observed medium-term between patients with low or high IgG4, but longer follow up is necessary to determine whether children with high IgG4

represent a specific subtype of paediatric autoimmune liver disease.

Disclosure of Interest: None declared.

PA-H-0052

Hepatology

LIMITATIONS OF TRANSIENT LIVER ELASTOGRAPHY IN CHILDREN

I. Goldschmidt^{1,*}, C. Dingemann², S. Bockisch¹, E. D. Pfister¹, S. Rauschenfels¹, U. Baumann¹. ¹Paediatric Hepatology and Gastroenterology, ²Paediatric Surgery, Hannover Medical School, Hannover, Germany.

Objectives and Study: Transient elastography (Fibroscan) is increasingly recommended for the non-invasive diagnosis of liver fibrosis in children. This study aims at examining technical issues and limitations in transient elastography raised by the varying age and size of children.

Methods: Transient elastography was performed in 78 children aged 0.2–17 (median 5.9) years (33 f, 45 m; 38 liver patients, 40 healthy volunteers). Fibroscan results were accepted if the ratio of interquartile range and median of 10 successive readings was <30%. Fibroscan examinations were performed at up to 4 different sites in each patient. The position of the liver was determined by percussion, and transient elastography first attempted in the highest possible intercostal space (ICS) in the anterior axillary line (AAL1), then one ICS below the first (AAL2). A 3rd reading was attempted in the mid-clavicular line (MCL1), and 1 ICS below (MCL2). Success of the examination and diagnostic acceptability were recorded. After completion of the Fibroscan examination, position and thickness of the liver were verified by B-mode ultrasound. Quantitative variables are given as median (range). Results of Fibroscan examinations are compared by signed Wilcoxon rank test.

Results: Fibroscan examination was technically possible in AAL1/AAL2/MCL1/MCL2 in 92%/60%/53%/33% of cases and acceptable with IQR/Median <30% in 80%/45%/46%/27%, respectively. The number of acceptable readings correlated with age of the child ($r=0.62$). Total failure rate (No reading obtainable at all) was 3.8%. In 14%, no reading was acceptable. Failure to obtain any acceptable reading was associated with low patient age. In children below 24 months of age ($n=28$), total failure rate increased to 11%, and no acceptable reading was obtained in 39%. Examinations performed under general anaesthesia did not have significantly higher success rate in this age group. Liver stiffness values in MCL were significantly lower than in AAL of the same patients (5.3 (3.3–7.5) kPa vs. 6.5 (2.4–67.8) kPa, $P<0.05$). The recommended cut-offs for S1 probe choice (chest circumference (CC) <45 cm/age <6 years) did not correspond in our patients ($n=15$, age 0.2–6 (3.5) years, CC 31–60.5 (51) cm). S1 measurements were significantly higher than S2 at AAL1 in $n=8$ patients aged 4.8–8.3 years ($P<0.05$). In contrast, S2/M measurements did not differ significantly in $n=9$ patients aged 10–17 years.

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Conclusion: Transient elastography using the Fibroscan is feasible in children. Success rate is limited in children below 24 months of age both for anatomical and behavioural reasons. Site of examination and probe choice significantly influence results and should be taken into account when interpreting results.

Disclosure of Interest: None declared.

PA-H-0053

Hepatology

CLAUDIN-1, A TIGHT JUNCTION PROTEIN INVOLVED IN NISCH SYNDROME, PLAYS A ROLE IN HEPATIC PARACELLULAR PERMEABILITY: EVIDENCE IN HEPATOCELLULAR AND CHOLANGIOCELLULAR POLARIZED LINES

E. Gonzales^{1,2,*}, B. Grosse², N. Yousef^{2,3}, D. Cassio², E. Jaquemin^{1,2}. ¹Pediatric Hepatology, Bicêtre Hospital, AP-HP-University Paris Sud 11, Le Kremlin-Bicêtre, ²UMR-S 757, INSERM-University Paris Sud 11, Orsay, ³Pediatric Intensive Care Unit, Bicêtre Hospital, AP-HP-University Paris Sud 11, Le Kremlin-Bicêtre, France.

Objectives and Study: Neonatal ichthyosis and sclerosing cholangitis (NISCH) syndrome is a rare recessive autosomal liver disease caused by truncating mutations of the *CLDN1* gene encoding claudin-1, a tight junctions (TJ) protein. In this syndrome it is speculated that cholestasis is due to absence of claudin-1, leading to increased paracellular permeability and to hepatocyte and bile duct injuries secondary to paracellular bile regurgitation. The present work was performed to test the role of claudin-1 in the maintenance of hepatic paracellular permeability.

Methods: Two polarized rat cell lines, the hepatocellular Can 10 line and the cholangiocellular NRC line, were used. Both of them form TJ. However, in contrast to NRC, Can 10 cells do not express claudin-1. Therefore, Can 10 cells were first transfected with a plasmid encoding the normal sequence of human claudin-1. Claudin-1 expression was evaluated (qPCR, western blotting, immunolocalisation) in stable transfected clones and paracellular permeability of these clones was assessed by FITC-dextran passage. Then, in these transfected clones claudin-1 expression was inhibited by siRNA and the impact of this inhibition on paracellular permeability was evaluated. A similar approach of claudin-1 expression evaluation and inhibition was used for NRC cells. In this latter case, the effect on paracellular permeability was assessed, by measuring transepithelial resistance.

Results: Stable Can 10 clones expressing different levels of claudin-1 were isolated. In all of them, claudin-1 was colocalized with zonula occludens-1 at the TJ. Paracellular permeability was significantly ($P<0.01$) decreased compared to parental Can 10 cells and negatively correlated to claudin-1 expression level. The silencing of claudin-1 in these clones led to a significant decrease in claudin-1 expression and to a significant increase in paracellular permeability to a level similar to that of parental Can 10 cells.

E29

2.2 Steroid Responsive Autoimmune Sclerosing Cholangitis with Liver Granulocytic Epithelial Lesions.

Introduction.

During a retrospective review to determine the long-term outcome of patients with autoimmune liver disease referred to our tertiary Centre, we identified a boy with autoimmune sclerosing cholangitis (ASC), inflammatory bowel disease (IBD), and a dramatic response to steroid treatment, whose original liver biopsy showed granulocytic epithelial lesions identical to those described in autoimmune pancreatitis type 2.

Case report

A 13-year-old boy was referred to our Centre in March 1985 with a 5-month history of abdominal pain, bloody diarrhoea, abnormal liver function tests (LFTs) and weight loss. Positive findings were splenomegaly, leg and buttock erythema nodosum and pruritus. Paternal grandmother had rheumatoid arthritis and maternal grandmother Reynaud's syndrome and ankylosing spondylitis.

In December 1984, admitted locally with pyrexia and abdominal pain, he had abnormal LFTs, raised white cell count and erythrocyte sedimentation rate. A month later, a barium follow-through suggested Crohn's disease, prompting referral to a gastroenterology unit, where abdominal ultrasound (US) showed diffuse liver parenchymal changes and moderate splenomegaly. An endoscopic retrograde cholangiopancreatography (ERCP) demonstrated normal pancreatogram, but intrahepatic cholangiopathy. Aspartate aminotransferase (AST) levels were 6-10 times the upper limit of normal (ULN), gamma glutamyltransferase (GGT) levels 5 times/ULN, alkaline phosphatase (ALP) levels at ULN for age. An exploratory laparotomy to exclude tuberculosis or lymphoma, was negative. A wedge liver biopsy showed active cholangitis. He was treated with salazapyrin 2g/day, with improvement of bowel symptoms, and was referred to our Centre for further evaluation/management.

On admission, AST was 180 IU/l, ALP 160 IU/l (n.v. <200IU/l), GGT 87 IU/l, immunoglobulin G (IgG) 20.9g/l (n.v. 7-18.6g/l), serum amylase 270 IU/L (n.v. <100

IU/L), antinuclear antibody positive (1/160). Serum IgG4, tested retrospectively in a sample stored at -80°C from his first admission, were 0.64 g/l (n.v. <1.35g/l).

A colonic biopsy demonstrated active colitis. Review of the liver wedge biopsy performed in the referring hospital showed slightly distorted lobular architecture with periportal fibrosis (Fig. 1A); enlarged portal tracts with moderate lymphocyte and plasma cell infiltrate and interface activity; inflamed bile ducts with epithelial damage and focal periductal concentric fibrosis (Fig. 1B). Current review of the same biopsy additionally reveals bile duct injury characterised by irregular epithelial configuration with intraepithelial neutrophilic infiltration (Fig. 1C, D), and obliterative phlebitis of portal vein branches (Fig. 1E).

ASC with IBD was diagnosed and treated with sulphasalazine 2 g/day and prednisolone 10 mg qds for 2 weeks, gradually reduced to 5 mg od. Over six months the patient became asymptomatic and normalised LFTs, amylase and IgG. A year later, after a surveillance liver biopsy showed mild periportal fibrosis without portal inflammation or bile duct damage (Fig. 1F), prednisolone was decreased to 5 mg/alternate days. Two years later a repeat liver biopsy showed minimal non-specific changes with well preserved bile ducts.

A follow-up ERCP in 1989 evidenced cholangiopathy of the second, third and fourth order bile ducts (Fig 2). In 1990, a further ERCP showed no progression of cholangiopathy.

Between 2000 and 2008 three episodes of IBD exacerbation responded to a temporary increase of steroid dose and mesalazine 800 mg tds. Maintenance prednisolone was stopped in 2008.

At the age of 37 he underwent cholecystectomy for symptomatic gallstones. At last follow-up in 2010, he had normal LFTs and no bowel symptoms while on mesalazine 800 mg tds and ursodeoxycholic acid 500 mg bd (started in 2005).

Discussion

Autoimmune pancreatitis (AIP) is an idiopathic disorder characterised by positive autoantibodies, association with other autoimmune pathologies and excellent response to steroids (1). AIP affects mostly adults, though a paediatric case is described (2), and is classified into type 1 (AIP-1) and type 2 (AIP-2).

AIP-1 is the pancreatic manifestation of an IgG4-related systemic disease characterized by high serum IgG4, IgG4-positive plasma cell infiltration of the pancreas, proximal bile ducts, retroperitoneal, renal, and salivary tissue, (3). AIP-1 is associated to bile duct damage (5) (6). A 3-year-old girl with steroid responsive? IgG4-associated cholangitis (elevated serum IgG4 and hepatic infiltration with IgG4-positive plasma cells), but without pancreatic involvement, has been recently reported (7).

AIP-2 has no specific serological markers, but is characterised on histology by a neutrophilic pancreatic duct damage known as granulocytic epithelial lesion (GEL) (4). Cholangiopathy is not a feature of AIP-2. A recent paediatric case report, however, describes a 10-year-old boy with severe abdominal pain, normal IgG4 levels, borderline raised serum amylase, negative autoantibodies, multiple stenosis of the pancreatic duct, dilatation of the bile ducts, and good response to steroids, who appears to have an AIP-2 related cholangiopathy, though no histological description of the liver or pancreas is provided (2).

The patient with ASC described in this case report is unusual because of his dramatic response to steroid treatment with a sustained long-term remission of liver disease, even on alternate day low-dose prednisolone and after complete withdrawal. Such a benign course is uncommon in ASC, where progressive liver damage is observed in most cases at medium/long-term follow-up (8). At presentation, he had histological liver lesions characterised by periductal and intraepithelial neutrophilic infiltration identical to those described in the pancreatic duct in AIP-2. Though GEL in our patient may have been due to a superimposed suppurative cholangitis, the histological findings do not support this, since suppurative cholangitis is characterised by numerous neutrophils in the ductal lumen with abscess formation and attenuation or disruption of the epithelial layer, while our patient had predominantly periductal and intraepithelial neutrophilic infiltration with irregular configuration of the bile duct epithelium. Despite a mild elevation of serum

amylase at disease onset, he had no radiological evidence of pancreatitis. It is conceivable that, as raised IgG4 levels characterise a systemic disorder encompassing AIP-1, cholangitis, autoimmune hepatitis, salivary gland disease etc (4), GEL may be the common denominator of a spectrum of clinical manifestations including pancreatic and/or bile duct disease of variable severity. ASC with histological similarities to AIP-2 may account for a subgroup of juvenile sclerosing cholangitis with excellent response to steroids and favourable long-term outcome. Whether GEL on liver histology predicts steroid responsiveness in children with sclerosing cholangitis should be evaluated prospectively.

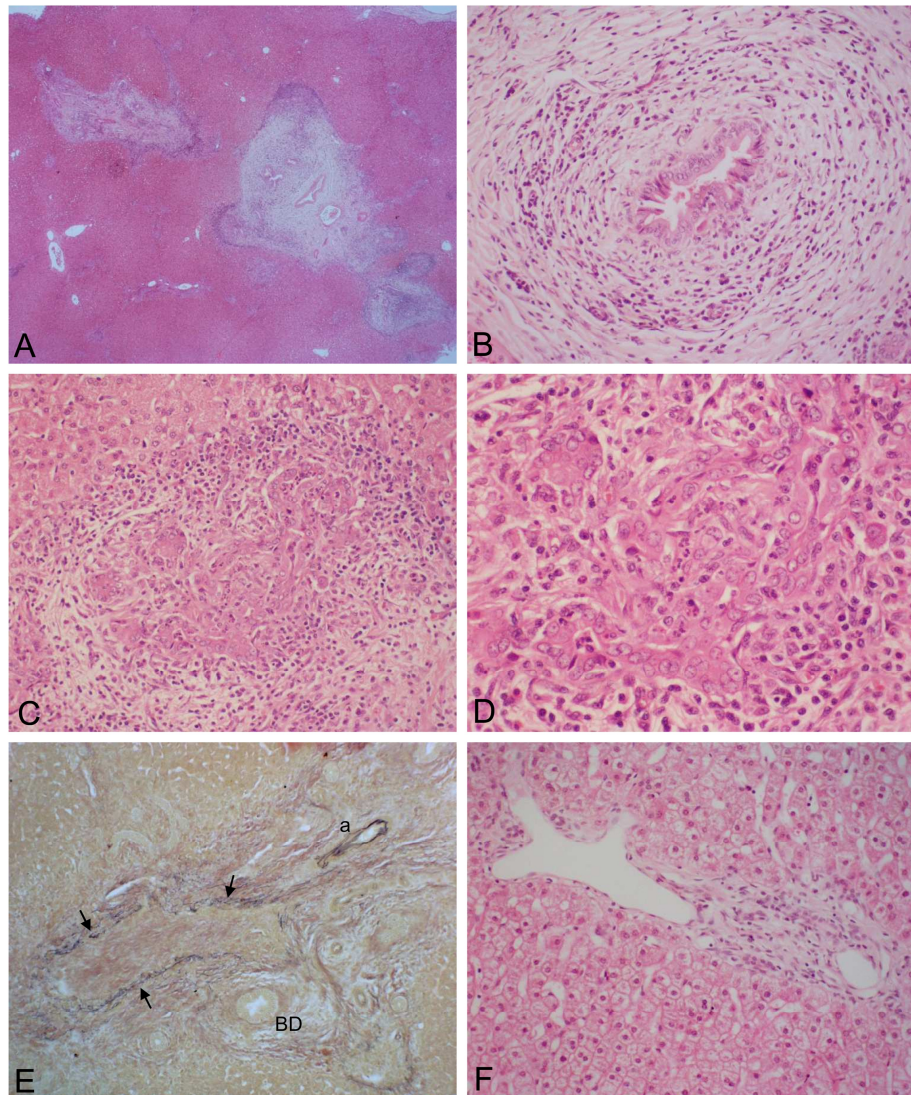


Figure 1

Figure 1. A to E. Wedge liver biopsy specimen before steroid therapy. (A) There is fibrous enlargement of portal tracts. (B) Periductal concentric fibrosis with inflammatory cell infiltration is noted. (C and D) A bile duct shows a convoluted appearance with irregular epithelial configuration and intraepithelial neutrophilic infiltration. (E) Obliterative phlebitis with elastic fibres of an obliterated portal venule indicated by arrows (a, artery; BD, bile duct). (F) Needle biopsy specimen taken after 1 year on steroid therapy shows only mild periportal fibrosis with a few reactive bile ductules. A-D and F: Haematoxylin and eosin; E: Elastica van Gieson; Magnification: $\times 20$ (A), $\times 100$ (B, E), and $\times 200$ (C, D, F).

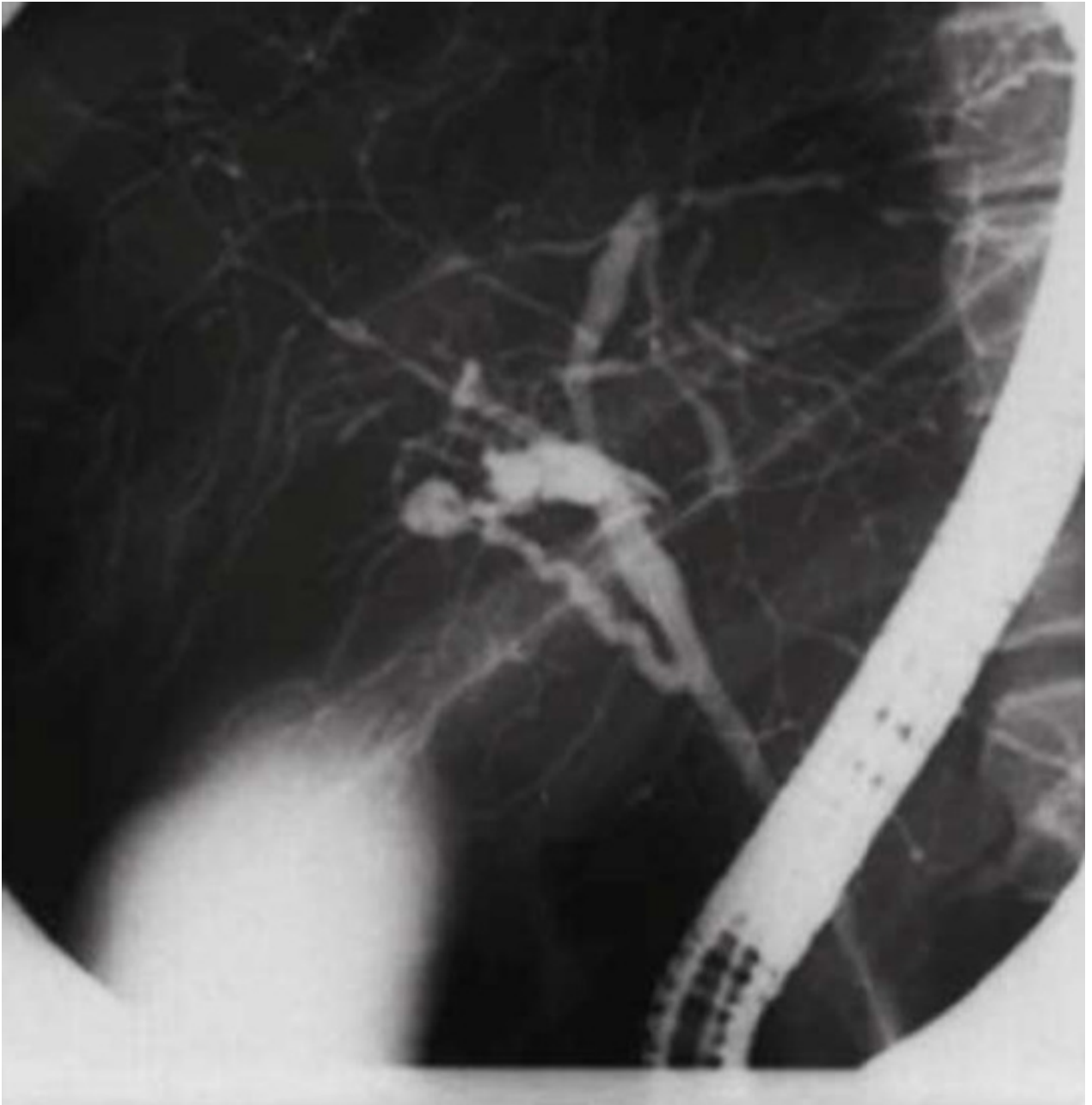


Figure 2. Endoscopic retrograde cholangio pancreatography (ERCP). There is localised stricturing, dilatation and irregularity of the second, third and fourth order intrahepatic ducts with minimal changes of the first order main left and right ducts. The extrahepatic common hepatic and common bile ducts are spared.

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2.3 Autoimmune liver diseases (AILD) treatment: cyclosporin is safe and efficacious in the long term.

Objectives and Study.

Cyclosporin (CS) has been shown to allow recovery from liver failure and to induce and safely maintain remission in children with AILD. However CS is generally used as a bridge to conventional treatment and its long-term safety and efficacy are unknown. This study reviewed patient data to evaluate the long-term safety and efficacy of CS treatment of AILD.

Methods.

We reviewed records of 30 patients (23 females) treated with CS for more than 12 months in the last 20 years: 22 had autoimmune hepatitis (12 type 2, 6 type 1 and 4 seronegative) and 8 an overlap syndrome (5 with autoimmune sclerosing cholangitis, 3 with autoimmune cholangitis). 19 of them were CS first-line treated while in 11 CS was a secondline option. Indications for CS as a second-line option were treatment failure and/or contraindications to/refusal of steroids. In 17 of the patients, CS was the only immunosuppressant drug administered. As for the remaining 13, CS was administered with azathioprine and/or prednisone in 12 cases, with prednisone and mycophenolate mofetil in 1. Patients with overlap syndrome also received ursodeoxycholic acid. Treatment was started at a median age of 116 mo. (26-253) and the median treatment duration was 48 mo. (12-192). 18 patients were shifted to another treatment (17) or withdrawn from therapy (1) after a median of 16 mo. (1-54), while 12 are currently in treatment with CS.

Results.

The median follow-up was 4.8 yrs (1.3-18). All 19 patients first-line treated with CS achieved complete remission in a median period of 8 wks (3-20). 4 of them required a combination with conventional therapy due to severely impaired liver function. In all but one patient who shifted to CS after conventional treatment during a relapse, CS allowed maintenance of remission with minimal to no dosages of steroids. In 1 patient with autoimmune sclerosing cholangitis, CS was not clearly beneficial. Tolerance to CS was excellent. Mild to moderate hypertrichosis occurred in 11 and gingival hypertrophy in 13. These side effects improved after dose tapering, or disappeared at the end of treatment and were well tolerated. In 1 patient the occurrence of painful gingival ulcers lead to withdrawal of CS. No signs of renal function deterioration were recorded: none presented hypertension and no statistically significant changes in median glomerular filtration rate were observed between the beginning and the end of treatment. A transient serum creatinine elevation up to 55% occurred in 4 cases. In all it returned to baseline after a reduction of dose.

Conclusion.

CS may be considered a safe treatment and an effective alternative for front-line therapy in AILD. Even after prolonged treatment and long-term follow-up renal function remains normal, however accurate renal function monitoring is mandatory.

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AUTOIMMUNE LIVER DISEASES (AILD) TREATMENT: CYCLOSPORIN IS SAFE AND EFFICACIOUS IN THE LONG TERM

S. Nastasio^{1*}, M. Sciveres², S. Riva², F. Cirillo², G. Maggiore^{1,2}

¹Department of Pediatrics, University of Pisa, Pisa, ²Pediatric Hepatology, ISMETT UPMC, Palermo, Italy

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Conclusion: CS may be considered a safe treatment and an effective alternative for front-line therapy in AILD. Even after prolonged treatment and long-term follow-up renal function remains normal, however accurate renal function monitoring is mandatory.

Disclosure of Interest: None Declared

Chapter 3. Pediatric liver transplant: immunological features and complication of calcineurin inhibitors treatment.

3.1 Tacrolimus regulates the proliferation of Regulatory T cells in liver transplant recipients

Introduction

Tacrolimus (TAC) is a T cell immunosuppressive drug also known as calcineurin inhibitor commonly used to treat transplant recipients. Tacrolimus binds to FKBP and acts inhibiting calcineurin .[1] The inhibition of the phosphatase calcineurin and, in consequence, of the transcription factor NFAT leads to reduction of the expression of several cytokine genes, including IL-2, which is an important growth factor for T cells, and this inhibits T cell activation. [2] Since T lymphocyte have a key role in the acute or chronic rejection in organ transplant, TAC is largely used to treat transplant recipients and in particular in liver transplants to prevent alloantigen reaction that affect the long term life span of transplant patients. It is clear that the transplant immunological response is a balance between the alloimmune effectors T cells and the immunological tolerance. Recent evidences indicate that Regulatory T cells (Treg) have crucial roles in the mechanism of transplantation tolerance determining the tolerance of the graft. [3] Treg cells are a subset of T lymphocyte that represents about 5-10% of CD4+ T cells. They constitutively express the high affinity IL-2 receptor (CD25) and the forkhead family transcription factor, Foxp3. This cellular subset inhibits the activation and the proliferation of effectors T cells in vivo and in vitro and has a crucial role in several contests such as the prevention of immunoinflammatory and autoimmune diseases. [4] There are several data indicating that Treg cells have an important role also in controlling alloresponses and in induction of immunological tolerance to allografts.[5] Previous studies have shown that immunosuppressive therapy may have a role in tolerance control among liver transplant recipients. In particular calcineurin inhibitors may represent a barrier to immune tolerance in organ transplantation by decreasing the percentage of Treg

in the peripheral blood in liver transplant recipients. [6,7] However some contradictory results have been reported. Indeed there are some evidence indicating that TAC promotes in vitro expansion of Treg cells especially in humans. [8] Despite Treg cells are anergic to in vitro stimulation, they are highly proliferative in vivo. [9] To better understand the effect of TAC in maintaining of immune tolerance versus donor alloantigens in liver transplant recipients, we evaluated the immunophenotype and the amount of Treg cells in TAC treated patients. Since the amount of Treg is correlated with the proliferative capacity of these cells in vivo.

Aim

To evaluate the role of TAC in the control of the in vivo proliferation of Treg cells in pediatric liver transplant recipients.

Materials and Methods

Patients

A total of 10 stable liver transplant recipients and 13 controls were enrolled after informed consent. Liver transplant recipient receiving TAC based immunosuppression. All patients showed stable liver function. All blood samples from patients and controls were collected at 9.00 am in heparinised vacutainers (BD Biosciences, Milan, Italy) and processed within the following 3 h.

Immunophenotypic Analysis

Immunophenotypic analysis of peripheral blood from TAC treated patients and controls was performed with an EPICS XL flow cytometer (Beckman Coulter) using the Beckman Coulter software program XL system II. Triple combinations of different anti-human mAbs, e.g., FITC- and phycoerythrin (PE)-anti-CD3, PE- and PC-5-anti-CD4, PC5-anti-CD8, PE-anti-CD16, PC5-anti-CD19, PE-anti-CD25, FITC-anti-CD45, and PE-anti-CD56 (Coulter Immunotech, Marseille, France), were used for immunofluorescence staining.

Flow cytometry analysis of Foxp3 and Ki67 expression

All cytometry analysis were performed on PBMCs isolated by Ficoll Hypaque gradient centrifugation from TAC treated patients and controls. The anti-human Foxp3 staining set was from eBiosciences (San Diego, CA). FITC-anti-human-Ki67 and PeCy5.5-anti-human-CD4 were from BD Biosciences.

Fluorescent bead-based immunoassay

All serum samples from patients and controls were collected and stored at -80°C before the analysis. Human obesity kit (Bender MedSystems GmbH, Vienna, Austria) was used to perform the bead based Analyte Detection Assay for quantitative detection of soluble MPO, ICAM-1, CD-40L, TNF-R, MCP-1, IL-6, Resistin and Leptin by Flow Cytometry. Preparation of the samples was performed according to manufacturer's instructions. A 96-well plate format was used, including two eight-point standard curves (inclusive blank). Concentrations of analytes were proportional to fluorescent intensity measured on a BD FACSCanto (BD Biosciences). Data were acquired using a BD FACSCanto (BDBiosciences) and analysed using FlowCytomixPro 2.2 Software (Bender MedSystems GmbH, Vienna, Austria).

Statistical analysis

Data are presented as mean (SD). We used an unpaired t test with Welch's correction. The comparisons was performed using StatView® (SAS Institute). A p value ≤ 0.05 was considered statistically significant.

Results.

Tacrolimus treated patients show a “memory” immune-phenotype

We performed a full lymphocyte immunophenotyping of liver transplant recipients who did receive TAC treatment and of controls. Overall TAC treated patients showed a lower number of total lymphocytes, CD3+ and CD4+ cells/mm³ compared with controls. Of interest in TAC treated patients there was a trend toward a “memory phenotype”. Indeed

the absolute numbers of CD3+ CD45RA+ and CD4+CD45RA+ cells were lower in the TAC treated patients (793±332 and 332±177, respectively) compared with the controls group (1515±831 and 843±482) (p=0.01 and 0.002). Moreover the percentage of CD3+CD45RO+ cells trended toward being higher in the TAC treated patients (28±7) compared with the controls group (18±10) (p=0.01). In agreements with these findings TAC treated patients had a significant lower percentage of CD4+CD45RA+ cells compared with controls group (17±7 vs 29±9, p=0.001). In addition patients showed a significant lower number of CD4+CD28+ and CD8+CD11b+ cells (595±255 and 22±16, respectively) compared with controls (1067±559 and 50±28). No differences were seen among TAC treated liver transplant recipients and controls in other cellular population analyzed.

Tacrolimus treated patients show a lower proliferation of Regulatory T cells compared with controls.

To evaluate the immunological tolerance state of TAC treated patients we analyzed the absolute number and the percentage of CD4+CD25+Foxp3+ Regulatory T cells in peripheral blood of TAC treated patients and controls. The percentage and number of Regulatory T cells was decreased in TAC treated patients compared with controls. Human regulatory T cells are anergic to in vitro anti-CD3 and anti-CD28 stimulation but are high proliferative in vivo. We evaluated the in vivo proliferation of regulatory T cells through the evaluation of the expression of the proliferative marker ki67. We observed that TAC treated patients showed a lower number of proliferating Treg cells testified by the lower number of Ki67+ Treg cells.

Tacrolimus treated patients shown an higher circulating level of sTNF-R compared with controls.

To assess the role of tacrolimus in the metabolic complications of liver transplant recipients we measured the serum level of Leptin, sTNF-R, sICAM-1, IL-6, sCD40-L, Resistin, MPO and MCP-1 that represent a panel of markers involved in the mechanism and consequences of obesity and in maintaining of inflammatory state correlated with this pathology. We observed that TAC treated patients showed an higher circulating level of

sTNF-R compared with controls. Conversely, we did not observe any differences between liver transplant recipients and controls when we evaluated the others markers. However a more detailed analysis have shown that tacrolimus treated patients could be divided into two groups characterized by different levels of serum leptin. Interestingly the group with the higher level of leptin present a more severe clinical score.

Conclusion.

In recent years much evidence has been published on the beneficial effect of Tregs in the induction of allotolerance. Treg cells are involved in maintaining of alloantigen immune tolerance through the suppression of the effectors T cells against donor antigen. Immunosuppressive therapy may differently impact memory and regulatory T-cell subsets in liver transplant children. We analyzed the level of Tregs in liver transplant recipients who received TAC immunosuppressive therapy and in controls. Our data showed that full lymphocyte immunophenotyping of liver transplant recipients of tacrolimus treated patients was characterized by a lower number of total lymphocytes when compared with age matched controls. It is interesting to note that in tacrolimus treated patients there was a trend toward a “memory phenotype” even if the absolute numbers of CD3+ CD45RA+ and CD4+CD45RA+ cells were lower than in controls.

We also observed that TAC treated patients showed a lower number of proliferating Treg cells testified by the lower number of Ki67+ Treg cells.

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3.2 Early detection of lymphoproliferative disorders (PTLD) in paucisymptomatic pediatric liver transplant recipients by adenotonsillar histology.

Background and aims: PTLT is a severe complication of transplantation linked in most cases to EBV infection. Prevalence in pediatric liver transplant recipients is 5-7%, mortality over 50%. PTLT is often recognized at the stage of lymphoma; less aggressive variants are a/paucisymptomatic.

Methods: We prospectively evaluated for PTLT all liver transplanted children with symptoms of nasal obstruction and/or intermittent diarrhoea and/or unexplained failure to thrive. Adenotonsillar tissue was obtained by rhinofibroscopy and biopsy or by adenotonsillectomy.

Results: Among 120 liver transplant pediatric recipients 25 (16 males) were symptomatic. Median age was 4,6 years (2,5-15,6), median age at liver transplant (OLT) was 1,1 years (0,3-11,25), median time from OLT was 3,25 years (0,75-6,5). 5 children underwent adenotonsillectomy and 20 adenotonsillar biopsy. PTLT was diagnosed in 22 (88%), in 12 identified as polymorphic, in 10 as "early lesion". Age at diagnosis or time from OLT weren't significantly different between these two groups. 11 patients underwent gastrointestinal endoscopy. In all, PTLT involved also gastrointestinal tract. 21/22 patients with PTLT were EBV naïve at the time of OLT. At diagnosis of PTLT EBV DNA on peripheral blood mononuclear cells (PBMC) was positive in 13 patients: median value 100/10⁵PBMC (15-1950) but EBER RNA was detected in lymphoid tissue in 20. Treatment consisted in decreasing doses of tacrolimus in PTLT "early lesions" and in shift from tacrolimus to rapamycin in the others. After a median follow-up of 14 months (9-60) all are alive without signs of progression to aggressive variants of PTLT.

Conclusion: Waldeyer ring hypertrophy is associated to low grade variants of PTLT in liver transplanted children. EBV naïve status is a common feature. At diagnosis EBV DNA in PBMC is highly variable and even absent. Adenotonsillar biopsy is an easy and cost effective procedure to achieve diagnosis. Histological picture is consistent with gastrointestinal lymphoid tissue.

Chapter 4. Broadning the spectrum of UDCA indications.

4.1 Successful Use of Ursodeoxycholic Acid in Nodular Regenerative Hyperplasia of the Liver

Introduction.

Nodular regenerative hyperplasia of the liver (NRHL) is a benign uncommon condition characterized by the diffuse transformation of normal hepatic parenchyma into regenerative nodules typically without fibrous septa.¹ Rather than a “primitive entity”, nodular transformation in NRHL seems to be an aspecific adaptive response to alterations in blood flow secondary to a wide range of insults. Indeed, autopsy studies and case series have shown that the atrophic regions between nodules are associated with obliterative changes in the portal veins, leading to decreased blood flow in the supplied acini, while the nodular areas are believed to be a hypertrophic response to normal or slightly increased blood flow.² NRHL is probably underdiagnosed since it is often found accidentally or at autopsy, and imaging studies have a poor accuracy in establishing diagnosis.³

The diagnosis of NRHL is made by liver biopsy and histological criteria include the presence of hepatocellular nodules less than 3 mm in diameter not surrounded by fibrosis. 1 This condition is more frequent in adults than in children ⁴ and in most instances is diagnosed in association with long-standing systemic diseases (vasculitis, collagen diseases, haematological or cardiovascular disorders, neoplasm and metabolic disease).¹ Some cases of NRHL have been attributed to toxic liver injury (i.e., azathioprine and 6-thioguanine).¹ NRHL is defined primary when occurs without underlying or associated disease.

NRHL may remain clinically asymptomatic for many years. Common presenting features are abnormal liver function tests (predominantly cholestatic) and portal hypertension.¹ Since no specific treatment is available, the current management of patients with a primary form of NRHL consists exclusively in treating the complications of portal hypertension.¹ In NRHL patients with associated conditions (secondary form), it is

unclear whether treatment of the latter affects the course of NRHL. In patients with NRHL and inflammatory bowel disease it has been observed that azathioprine withdrawal was followed by progressive normalization of liver enzymes and platelets.⁴ Here we report the case of a patient with primary NRHL associated to portal hypertension in whom ursodeoxycholic acid (UDCA) therapy had a favourable effect on liver enzymes and was associated to non-progression of portal hypertension.

Case report.

A symptom-free 13-year-old boy was hospitalized for splenomegaly and thrombocytopenia identified during a routine check-up. Infectious, autoimmune and neoplastic causes were ruled out. In particular, bone marrow aspiration was unremarkable. The patient remained symptom-free for two years. When he was 15-year-old, various laboratory parameters were deranged: white blood cells 1,960/mm³, platelets 54,000/mm³, aspartate aminotransferase (AST) 67 IU/L, alanine aminotransferase (ALT) 176 IU/L, and gamma-glutamyltransferase (GGT) 102 IU/L. Bilirubin, albumin and prothrombin time were in the normal range. Ultrasound scanning of the abdomen revealed an enlarged liver, with a sonolucent, homogeneous echopattern, and an enlarged spleen. The diameter of the portal vein (P) was increased (14.5 mm) and mean portal vein flow velocity (PV_{mean}) was decreased (12.5 cm/sec) at ultrasonographic pulsed Doppler. The portal vein congestion index (CI) (cm²/[cm/sec]) was 0.132.5 Abdominal computed tomography showed homogeneous density of the liver, an ectasic portal vein and portosystemic collaterals (gastric, splenic and umbilical collaterals). The patient was referred to our pediatric liver unit for assessment of liver disease and related portal hypertension.

On admission, the patient looked well; physical examination showed hepatosplenomegaly. The clinical history of the patient was reviewed for drug intake and parenteral exposure. Neither drugs or supplements were given within the past 6 months nor there was drug abuse history. There was no history of pruritus or clinical signs of genetic disorders. Umbilical vein catheterization was not reported, and growth and neurologic development were normal. The following serological tests were performed and all tested negative: hepatitis A, B, C, Epstein-Barr, cytomegalovirus, HIV,

toxoplasmosis and Brucella. Autoimmune hepatitis and celiac disease were excluded. To exclude progressive familial intrahepatic cholestasis type 3, we carried out a molecular analysis of the MDR3 gene and the result was negative. Thyroid hormones, iron, ferritin, alpha-1 antitrypsin, lactate dehydrogenase, and creatine phosphokinase values were normal. Ceruloplasmin serum concentration was borderline (20 mg/dl) and thus we evaluated liver copper metabolism in detail. Basal urinary copper excretion was just above the upper normal limit value (43 μ g/24 h), whereas post-penicillamine urinary copper excretion was 1165 μ g/24h. Corneal Kayser-Fleisher rings were absent. Since, based on the patient's Ferenci score,⁶ a diagnosis of Wilson's disease was probable, we carried out a molecular genetic study of the ATP7B gene with negative result. Similarly, the most common genetic disorders with hepatic involvement, such as cystic fibrosis, were ruled out. Evaluation of the biliary tree structure by magnetic resonance was performed to exclude anatomic disorders and primary sclerosing cholangitis (PSC). The liver was enlarged; there were no focal lesions of liver signal intensity but only a slightly inhomogeneous liver structure on T1 and T2 weighted images. Magnetic resonance did not show any sign of PSC. Spleen size was 27 cm. Angiographic sequence of umbilical vein system refilling revealed signs of portal hypertension; hepatic veins were normal; hepatic artery was stretched with a filiform calibre (1 mm). Esophagogastrosocopy showed an esophageal varicose vein (type 1, grade 1-2), without red spots; erosive not congestive antral gastropathy was diagnosed.

A liver biopsy was obtained and, because of the low platelet count, a laparoscopic procedure was preferred to control eventual bleeding. Laparoscopy showed macronodules on the liver surface. The two surgical liver biopsy specimens showed irregular and confluent white areas with multinodular pattern, without fibrotic or cirrhotic aspect. Histological evaluation showed diagnostic features of NRHL. There were no histological features of cholangitis.

Given the biochemical evidence of cholestasis, UDCA was administered at a dosage of 10 mg/kg/day that was progressively increased to 20 mg/kg/day (1800 mg/day). First GGT and then AST and ALT normalized after a few months of treatment and remained normal in the following months (Figure 1a). Platelet count slightly increased on therapy (Figure 1b), while white blood cells remained unchanged (Figure 1c).

After 15 months of UDCA therapy, the esophageal varicose vein was not evident on esophagogastrosocopy. Ultrasound confirmed the enlarged liver with a homogeneous echopattern and a hypoechoic lesion. The latter was studied with ultrasound contrast medium, which showed a non-enhancing nodule on arterial-phase and signal enhancement similar to the surrounding parenchyma on late-phase imaging. At ultrasonographic pulsed Doppler, P was 16 mm and PVmean was regular (24 cm/sec). The portal CI was reduced to 0.084 cm²/[cm/sec]. The magnetic resonance pattern, angiographic sequence and spleen size were unchanged.

The patient after 30 months of therapy, during the summer holidays, arbitrarily suspended UDCA treatment and a rapid increase in transaminases was observed 1 month after suspension. The prompt reinstatement of UDCA was followed by sustained normalization of liver enzyme (Fig. 1a). No drug other than UDCA was given throughout the entire observation period. This was documented by detailed interviews performed at each visit. In this way, a close chronological relationship between the short period of UDCA withdrawal and the transient liver enzymes flare was observed.

No systemic disease linked to NRHL was identified in our patient. Serum homocysteine, plasma factor II, V, IX and X activities, plasma protein C, protein S, and antithrombin III levels, lupus anticoagulant, anticardiolipin antibodies, anti-nuclear antibodies, serum complement levels, cytoplasmic-staining anti-neutrophil cytoplasmic antibodies, and perinuclear-staining antineutrophil cytoplasmic antibodies were within normal limits.

Discussion.

The clinical course of patients with NRHL is very variable and tends to be more severe in subjects presenting with haematemesis due to esophageal variceal bleeding. Since the natural history of NRHL is not well defined, management of patients is directed at treating the underlying disorder, if identified, and treating the complications of portal hypertension.¹

To the best of our knowledge, this is the first case of a patient with primary NRHL associated to portal hypertension in whom UDCA therapy was followed by a prompt reduction and sustained normalization of liver enzymes and no progression of portal hypertension throughout the follow-up.

Although the observation period was short to draw definitive conclusions about the therapeutic effect of UDCA administration on portal hypertension, it is to note that before the administration of UDCA, laboratory and sonographic signs of portal hypertension progressively worsened, whereas these parameters tended to improve during UDCA therapy. Previously, few cases of NRHL treated with UDCA^{7,8} have been reported but the impact of the drug on portal hypertension progression has never been documented.

UDCA is a hydrophilic, tertiary bile acid widely used to treat patients with chronic cholestatic liver diseases. Given its cytoprotective, anti-apoptotic, anti-oxidative, choleric, immunomodulatory and altering cell signaling properties, UDCA may affect various pathogenetic mechanisms involved in NRHL.⁹ UDCA prevents apoptosis in preclinical models of human diseases by inhibiting the mitochondrial pathway and disrupting the apoptotic cascade.¹⁰ It is conceivable that in NRHL an apoptotic mechanism, induced by ischemic injury, determines atrophy in the central areas (zone III), which are more vulnerable to ischemia. Central atrophy, in turn, leads to proliferation of neighbouring hepatocytes, which form regenerative nodules.¹ These nodules compress intrahepatic portal radicles and intraparenchymal venules and/or sinusoids and promote portal hypertension. Moreover, as suggested by Wanless et al, structural obstruction of the portal venules due to recurrent embolization of the portal vein radicles by platelet aggregates as well as by larger thrombi generated in the portal venous system contribute to the progression of portal hypertension.² UDCA therapy in patients affected by NRHL could disrupt key signalling pathways involved in apoptotic and necrotic cell death and so prevent hepatocyte proliferation and indirectly impede the development of portal hypertension. It is noteworthy that UDCA reduce the portohepatic gradient in rats with portal hypertension associated with biliary cirrhosis thereby suppressing hepatic thromboxane A₂ production and lipid peroxidation, and increasing the antioxidative defence mechanism.¹¹ UDCA may prevent the development of portal hypertension also by improving endothelial function.¹² In fact, in human vascular endothelial cells, bile acids increase nitric oxide production by increasing the intracellular concentration of Ca²⁺, and inhibit production of endothelin-1. The latter is directly involved in increasing intrahepatic vascular resistance in patients with hepatobiliary diseases.

Huet et al. recently reported worsening of portal hypertension in patients with primary biliary cirrhosis not treated with UDCA, and stabilization or improvement of portal hypertension in an encouraging percentage of UDCA-treated patients.¹³ Huet and colleagues monitored portal hypertension using the portohepatic gradient, whereas we used laboratory and sonographic indices and the esophagogastroscopy pattern. ¹³

On the basis of our experience, UDCA administration could be considered as specific therapy for primary NRHL. Furthermore, it would be interesting to evaluate the role of UDCA in larger series of patients affected by NRHL and to analyze the potential impact of treatment on the natural history of disease and prevention of its complications. Lastly, because UDCA is effective in the prevention of drug-induced hepatotoxicity,¹⁰ studies should be conducted to evaluate the effect of UDCA in NRHL induced by chronic treatment with drugs such as azathioprine or 6-thioguanine.

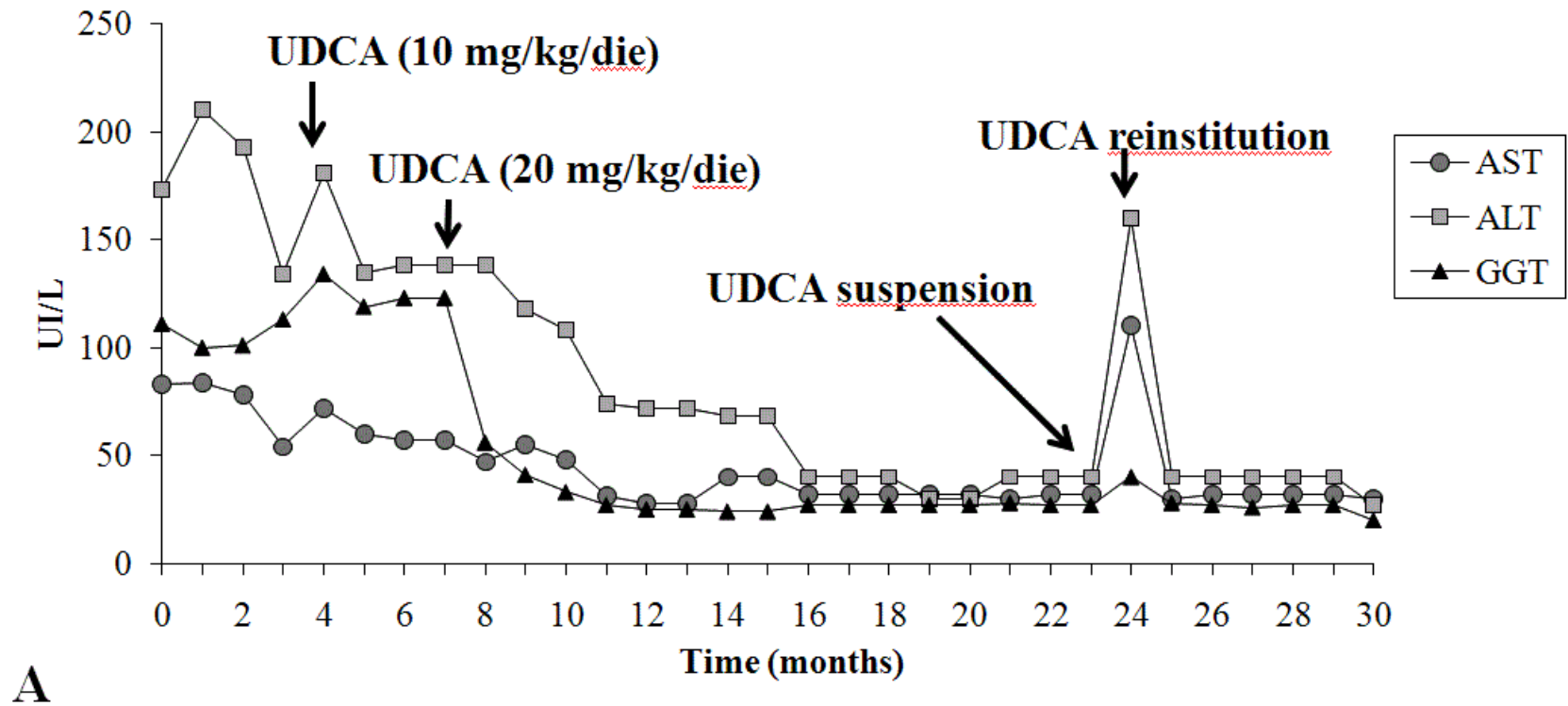


Figure 1A. Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT) profiles after ursodeoxycholic acid (UDCA) treatment. (b) Platelet count and white blood cells (c) profiles over time.

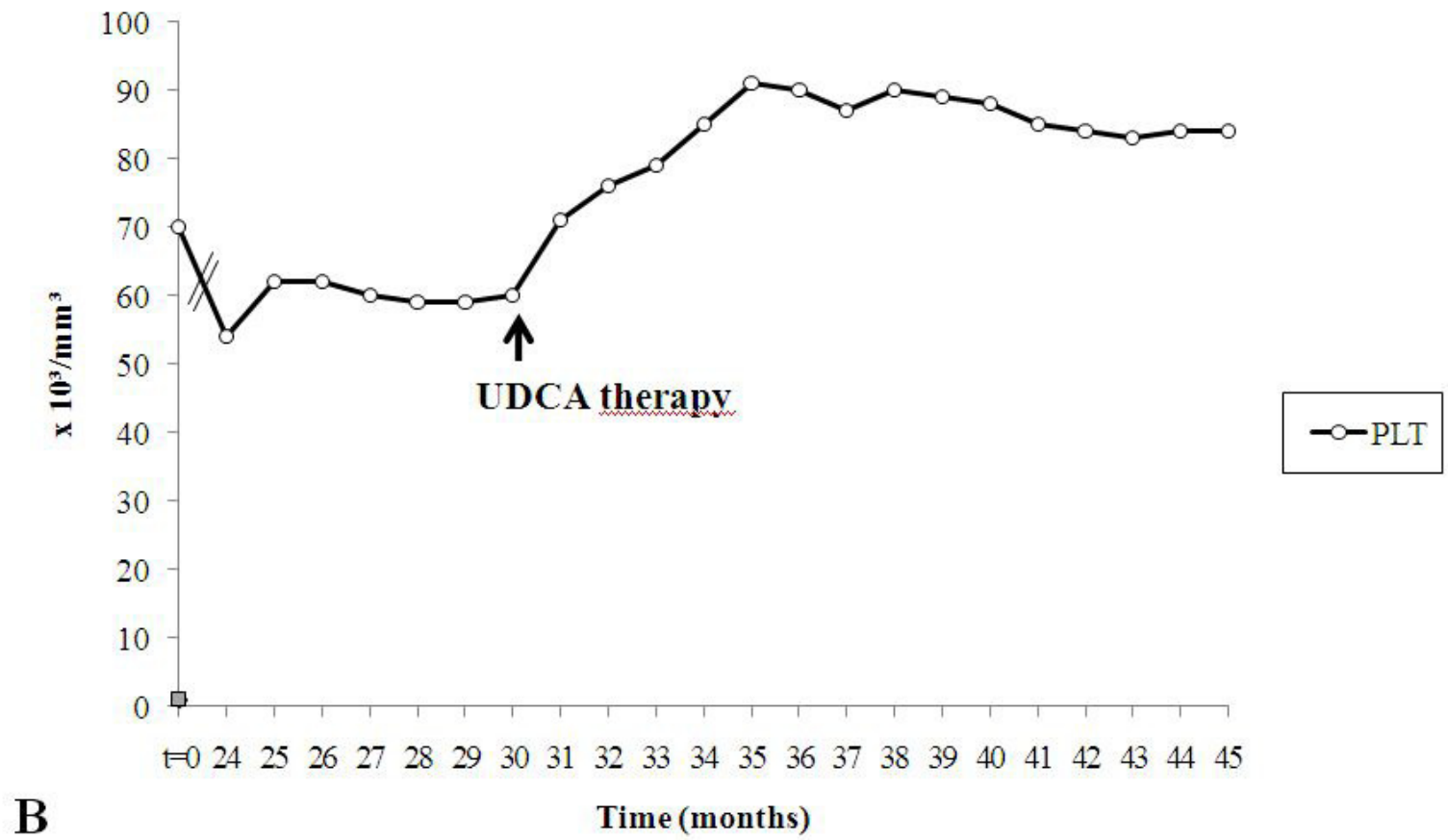


Figure 1B. Platelet count profiles over time.

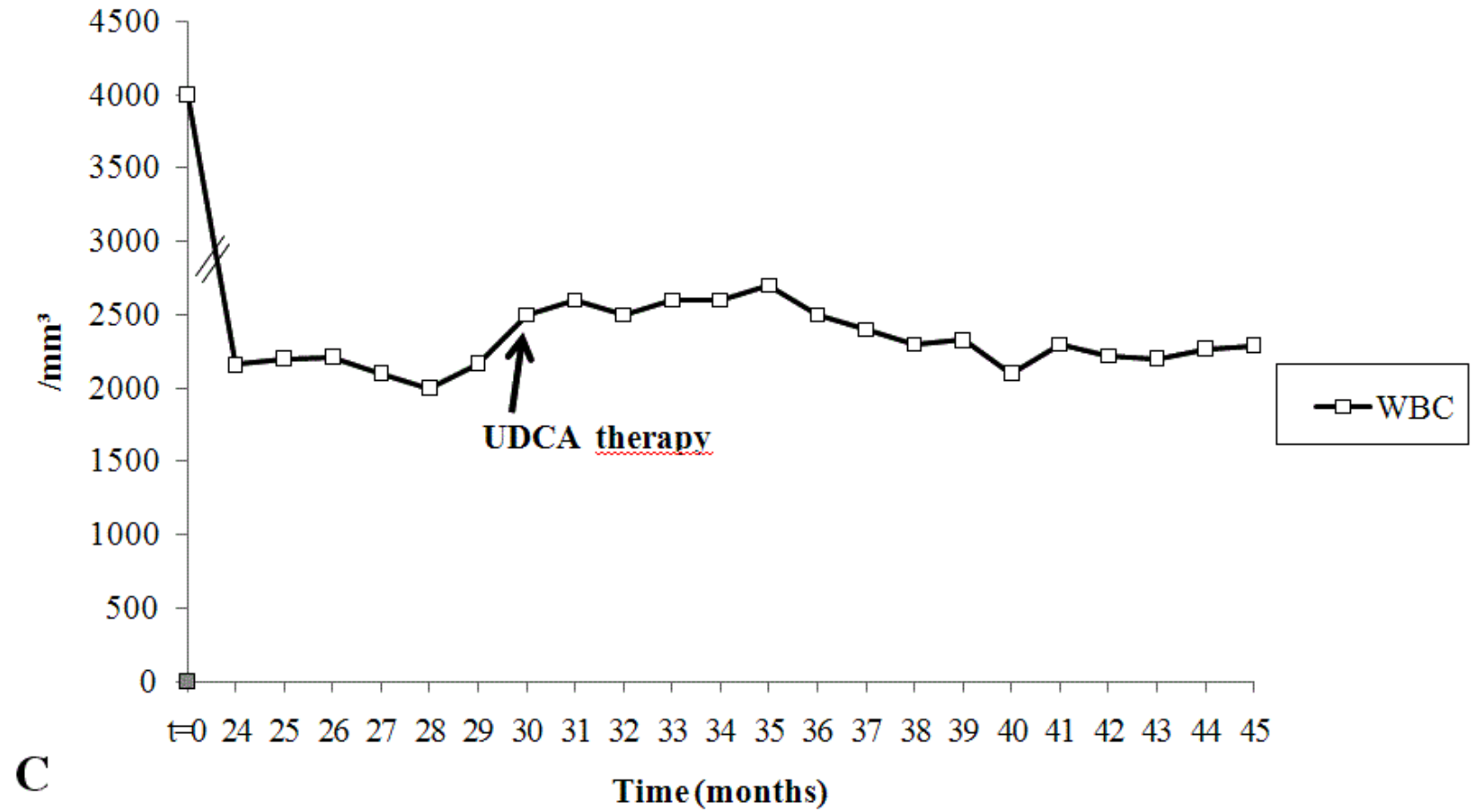


Figure 1C. White blood cells count profile over time.

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Successful Use of Ursodeoxycholic Acid in Nodular Regenerative Hyperplasia of the Liver

Giusy Ranucci, Francesco Cirillo, Claudia Della Corte, Raffaella Vecchione, Gianfranco Vallone, and Raffaele Iorio

Nodular regenerative hyperplasia of the liver (NRHL) is a benign, uncommon condition characterized by diffuse transformation of normal hepatic parenchyma into regenerative nodules, typically without fibrous septa.¹ Rather than a primary entity, nodular transformation in NRHL seems to be an aspecific adaptive response to alterations in blood flow secondary to a wide range of insults. Indeed, autopsy studies and case series have shown that the atrophic regions between nodules are associated with obliterative changes in the portal veins, leading to decreased blood flow in the supplied acini, while the nodular areas are believed to be a hypertrophic response to normal or slightly increased blood flow.² NRHL is probably underdiagnosed since it is often found accidentally or at autopsy, and imaging studies have a poor accuracy in establishing diagnosis.³

The diagnosis of NRHL is made by liver biopsy, and histologic criteria include the presence of hepatocellular nodules less than 3 mm in diameter that are not surrounded by fibrosis.¹ This condition occurs more frequently in adults than in children⁴ and, in most instances, is diagnosed in association with long-standing systemic diseases (vasculitis,

OBJECTIVE: To report, to our knowledge, the first case of a patient with nodular regenerative hyperplasia of the liver (NRHL) associated with portal hypertension in whom ursodeoxycholic acid (UDCA) therapy had a therapeutic effect on liver enzymes and was associated with nonprogression of portal hypertension.

CASE SUMMARY: A symptom-free 13-year-old boy was hospitalized for splenomegaly and thrombocytopenia identified during a routine check-up. Infectious, autoimmune, and neoplastic causes were ruled out. Two years later, laboratory findings revealed high levels of aminotransferases and γ -glutamyltransferase (GGT), thrombocytopenia, and neutropenia. Ultrasound scanning of the abdomen confirmed portal hypertension. Results of liver pathology studies showed diagnostic features of NRHL. Given the biochemical evidence of cholestasis, UDCA was administered, with an initial dosage of 10 mg/kg/day that was progressively increased to 20 mg/kg/day (1800 mg/day). After 5 months of treatment, GGT and then aminotransferase levels normalized and remained within normal limits in the following months. With arbitrary withdrawal of UDCA after 30 months of therapy, a rapid increase in transaminase levels was observed. Prompt reinstitution of UDCA was followed by sustained normalization of liver enzymes. Laboratory and sonographic signs of portal hypertension remained stable and tended to improve during UDCA therapy, as demonstrated by regularization of the mean portal vein flow velocity, reduction of the congestion index, progressive increase of the platelet count, and improvement of the esophagogastroscopy pattern.

DISCUSSION: NRHL is a rare disease that is characterized by multiple regenerative nodules in the hepatic parenchyma that may lead to noncirrhotic portal hypertension. No specific treatment is available, and management of patients with a primary form of NRHL consists mainly of treating the complications of portal hypertension. In our patient, UDCA therapy was followed by a prompt reduction and sustained normalization of liver enzyme levels and no progression of portal hypertension throughout the follow-up period.

CONCLUSIONS: Since in this patient with primary NRHL, ongoing UDCA administration resulted in improved biochemical and portal hypertension markers, this therapy can be considered in cases of NRHL associated with abnormalities of liver enzymes.

KEY WORDS: liver function tests, nodular regenerative hyperplasia of the liver, portal hypertension, ursodeoxycholic acid.

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collagen diseases, hematologic or cardiovascular disorders, neoplasm, and metabolic disease).¹ Some cases of NRHL have been attributed to toxic liver injury (ie, associated with azathioprine and 6-thioguanine).¹ NRHL is defined as primary when it occurs without underlying or associated disease.

Individuals with NRHL may remain asymptomatic for many years. Common presenting features are abnormal results of liver function tests (predominantly cholestatic) and portal hypertension.¹ Since no specific treatment is available, current management of patients with the primary form of NRHL consists of treating the complications of portal hypertension.¹ In patients with NRHL and associated conditions (secondary form), it is unclear whether treatment of the latter affects the course of NRHL. In patients with NRHL and inflammatory bowel disease, azathioprine withdrawal has been followed by progressive normalization of liver enzyme and platelet levels.⁴

We report the case of a patient with primary NRHL associated with portal hypertension in whom ursodeoxycholic acid (UDCA) therapy had a beneficial effect on liver enzymes and was associated with no progression of portal hypertension.

Case Report

A symptom-free 13-year-old boy was hospitalized for splenomegaly and thrombocytopenia identified during a routine check-up. Infectious, autoimmune, and neoplastic causes were ruled out. In particular, findings of bone marrow aspiration were unremarkable. The patient remained symptom-free for 2 years. When the patient was 15 years old, various laboratory parameters became abnormal: white blood cell count 1960/ μ L, platelets $54 \times 10^3/\mu$ L, aspartate aminotransferase (AST) 67 IU/L, alanine aminotransferase (ALT) 176 IU/L, and γ -glutamyltransferase (GGT) 102 IU/L. Bilirubin, albumin, and prothrombin time were within the reference range. Ultrasound scanning of the abdomen revealed an enlarged liver, with a sonolucent, homogeneous echopattern and an enlarged spleen. The diameter of the portal vein was increased (14.5 mm) and mean portal vein flow velocity was decreased (12.5 cm/sec) at ultrasonographic pulsed Doppler. The portal vein congestion index was 0.132 $\text{cm}^2/(\text{cm}/\text{sec})$.⁵ Abdominal computed tomography showed homogeneous density of the liver, an ectatic portal vein and portosystemic collaterals (gastric, splenic, and umbilical collaterals). The patient was referred to our pediatric liver unit for assessment of liver disease and related portal hypertension.

On admission, the patient looked well; physical examination showed hepatosplenomegaly. The clinical history of the patient was reviewed for drug intake and parenteral exposure to blood products or illegal substances. Neither drugs nor supplements had been administered within the

past 6 months and there was no history of drug abuse. There also was no history of pruritus or clinical signs of genetic disorders. Umbilical vein catheterization was not reported, and growth and neurologic development were normal. The results of the following serologic tests were negative: hepatitis A, B, and C; Epstein-Barr virus; cytomegalovirus; HIV; toxoplasmosis; and *Brucella* spp. Autoimmune hepatitis and celiac disease were excluded. To exclude progressive familial intrahepatic cholestasis type 3, we carried out a molecular analysis of the *MDR3* gene, and the result was negative. Results of thyroid hormone, iron, ferritin, α -1 antitrypsin, lactate dehydrogenase, and creatine kinase tests were normal. Ceruloplasmin serum concentration was borderline (20 mg/dL); thus, we evaluated liver copper metabolism in detail. Basal urinary copper excretion was just above the upper normal limit value (43 $\mu\text{g}/24$ h), whereas post-penicillamine urinary copper excretion was 1165 $\mu\text{g}/24$ h. Corneal Kayser-Fleischer rings were absent. Because, based on the patient's Ferenci score,⁶ a diagnosis of Wilson's disease was probable, we carried out a molecular genetic study of the *ATP7B* gene; the result was negative. Similarly, the most common genetic disorders with hepatic involvement, such as cystic fibrosis, were ruled out. Evaluation of the biliary tree structure by magnetic resonance was performed to exclude anatomical disorders and primary sclerosing cholangitis (PSC). The liver was enlarged; there were no focal lesions of liver signal intensity, but only a slightly inhomogeneous liver structure on T1- and T2-weighted images. Magnetic resonance imaging did not show any sign of PSC. Spleen size was 27 cm. Angiographic sequence of the umbilical vein system refilling revealed signs of portal hypertension; the hepatic veins were normal, but the hepatic artery was stretched with a filiform caliber (1 mm). Esophagogastroscopy showed an esophageal varicose vein (type 1, grade 1-2) without red spots; erosive, not congestive, antral gastropathy was diagnosed.

A liver biopsy was performed and, because of the low platelet count, a laparoscopic procedure was preferred to control bleeding. Laparoscopy showed macronodules on the liver surface. The 2 liver biopsy specimens showed irregular and confluent white areas with a multinodular pattern, without fibrotic or cirrhotic aspects. Histologic evaluation showed diagnostic features of NRHL; there were no histologic features of cholangitis.

Given the biochemical evidence of cholestasis, UDCA was administered at a dosage of 10 mg/kg/day, which was progressively increased to 20 mg/kg/day (1800 mg/day). Following this therapy, a progressive reduction in liver enzyme levels was observed, with normalization of GGT, AST, and ALT after 5, 7, and 12 months, respectively (Figure 1A). The platelet count slightly increased during therapy (Figure 1B), while the white blood cell count remained stable (Figure 1C).

After 15 months of UDCA therapy, the esophageal varicose vein was not evident on esophagogastrosocopy. Ultrasound confirmed the enlarged liver with a homogeneous echopattern and a hypochoic lesion. The latter was studied with ultrasound contrast medium, which showed a nonenhancing nodule on arterial-phase imaging and signal enhancement similar to the surrounding parenchyma on late-phase imaging. At ultrasonographic pulsed Doppler,

the portal vein was 16 mm and mean portal vein flow velocity was regular (24 cm/sec). The portal congestion index was reduced to 0.084 cm²/(cm/sec). The magnetic resonance pattern, angiographic sequence, and spleen size were unchanged.

After 30 months of therapy, during the summer holidays, the patient arbitrarily suspended UDCA treatment and a rapid increase in transaminase levels was observed 1

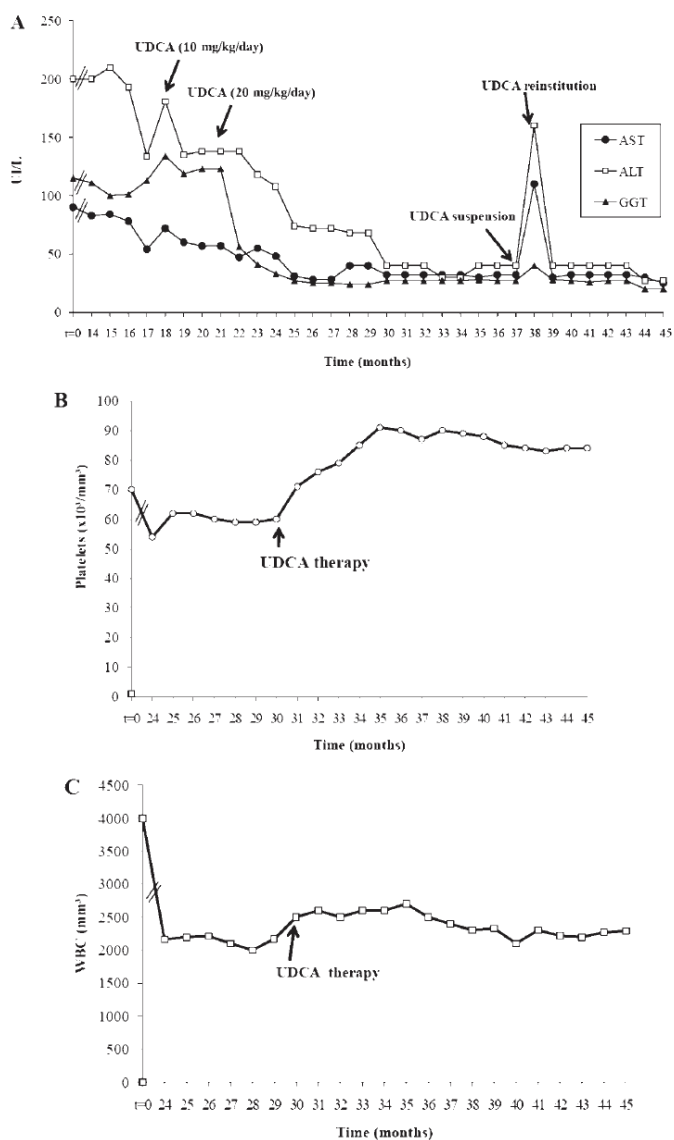


Figure 1. (A) Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ -glutamyltransferase (GGT) profiles after ursodeoxycholic acid (UDCA) treatment. Platelet count (B) and white blood cell count (C) over time.

month later. Prompt reinstatement of UDCA was followed by sustained normalization of liver enzyme levels (Figure 1A). No drug other than UDCA was given throughout the observation period. This was documented by detailed interviews performed at each visit. In this way, a close chronological relationship between the short period of UDCA withdrawal and the transient liver enzyme flare was observed.

No systemic disease linked to NRHL was identified in our patient. Serum homocysteine level; plasma factor II, V, IX, and X activities; plasma protein C, protein S, and antithrombin III levels; lupus anticoagulant; anticardiolipin antibodies; antinuclear antibodies; serum complement levels; cytoplasmic-staining antineutrophil cytoplasmic antibodies; and perinuclear-staining antineutrophil cytoplasmic antibodies were within normal limits.

Discussion

The clinical course of patients with NRHL is variable and tends to be more severe in those presenting with hematemesis due to esophageal variceal bleeding. Since the natural history of NRHL is not well defined, management of patients is directed at treating the underlying disorder and the complications of portal hypertension.¹

To our knowledge, this is the first case of a patient with primary NRHL associated with portal hypertension in whom UDCA therapy was followed by a prompt reduction and sustained normalization of liver enzymes and no progression of portal hypertension throughout 30 months of follow-up.

Although the observation period was too short to draw definitive conclusions about the therapeutic effect of UDCA administration on portal hypertension, it is significant that, before the administration of UDCA, laboratory and sonographic signs of portal hypertension progressively worsened, whereas those parameters tended to improve during UDCA therapy. Few cases of NRHL treated with UDCA^{7,8} have been reported, but the impact of the drug on portal hypertension progression has not been documented.

UDCA is a hydrophilic, tertiary bile acid widely used for treatment of chronic cholestatic liver diseases. Given its cytoprotective, antiapoptotic, antioxidative, choleric, immunomodulatory, and altering cell-signaling properties, UDCA may affect various pathogenetic mechanisms involved in NRHL.⁹ UDCA prevents apoptosis in preclinical models of human diseases by inhibiting the mitochondrial pathway and disrupting the apoptotic cascade.¹⁰ It is conceivable that in NRHL an apoptotic mechanism, induced by ischemic injury, produces atrophy in the central areas (zone III), which are more vulnerable to ischemia. Central atrophy, in turn, leads to proliferation of neighboring hepatocytes, which form regenerative nodules.¹ These nodules

compress intrahepatic portal radicles and intraparenchymal venules and/or sinusoids and promote portal hypertension. Moreover, as suggested by Wanless, structural obstruction of the portal venules due to recurrent embolization of the portal vein radicles by platelet aggregates, as well as by larger thrombi generated in the portal venous system, contributes to the progression of portal hypertension.² UDCA therapy in patients affected by NRHL could disrupt key signaling pathways involved in apoptotic and necrotic cell death and therefore prevent hepatocyte proliferation and indirectly impede the development of portal hypertension. It is noteworthy that UDCA reduces the portohepatic gradient in rats with portal hypertension associated with biliary cirrhosis, thereby suppressing hepatic thromboxane A₂ production and lipid peroxidation and increasing the antioxidative defense mechanism.¹¹ UDCA may prevent the development of portal hypertension also by improving endothelial function.¹²

Huet et al. recently reported worsening of portal hypertension in patients with primary biliary cirrhosis not treated with UDCA and stabilization or improvement of portal hypertension in an encouraging percentage of UDCA-treated patients.¹³ In the study, portal hypertension was monitored using the portohepatic gradient, whereas we used laboratory and sonographic indices and the esophagogastrosopy pattern.¹³

On the basis of our experience, UDCA administration could be considered as specific therapy for primary NRHL. Furthermore, it would be interesting to evaluate the role of UDCA in a larger series of patients affected by NRHL and to analyze the potential impact of treatment on the natural history of the disease and prevention of its complications. Lastly, because UDCA is effective in the prevention of drug-induced hepatotoxicity,¹⁰ studies should be conducted to evaluate the effect of UDCA in NRHL induced by chronic treatment with drugs such as azathioprine or 6-thioguanine.

Giusy Ranucci MD, Department of Pediatrics, University of Naples, Federico II, Naples, Italy

Francesco Cirillo MD, Department of Pediatrics, University of Naples, Federico II

Claudia Della Corte MD, Department of Pediatrics, University of Naples, Federico II

Raffaella Vecchione MD, Department of Biomorphological Science and Functional Sciences, University of Naples, Federico II

Gianfranco Vallone MD, Department of Biomorphological Science and Functional Sciences, University of Naples, Federico II

Raffaele Iorio MD, Department of Pediatrics, University of Naples, Federico II

Correspondence: Dr. Iorio, riorio@unina.it

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