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Human Leukocyte Antigen Profile Predicts Severity of Autoimmune Liver Disease in Children of European Ancestry

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BACKGROUND AND AIMS: Genetic predisposition to autoimmune hepatitis (AIH) in adults is associated with possession of human leukocyte antigen (HLA) class I (*A*01*, *B*08*) and class II (*DRB1*03*, *-04*, *-07*, or *-13*) alleles, depending on geographic region. Juvenile autoimmune liver disease (AILD) comprises AIH-1, AIH-2, and autoimmune sclerosing cholangitis (ASC), which are phenotypically different from their adult counterparts. We aimed to define the relationship between HLA profile and disease course, severity, and outcome in juvenile AILD.

APPROACH AND RESULTS: We studied 236 children of European ancestry (152 female [64%], median age 11.15 years, range 0.8-17), including 100 with AIH-1, 59 with AIH-2, and 77 with ASC. The follow-up period was from 1977 to June 2019 (median 14.5 years). Class I and II HLA genotyping was performed using PCR/sequence-specific primers. HLA *B*08*, *-DRB1*03*, and the *A1-B8-DR3* haplotype impart predisposition to all three forms of AILD. Homozygosity for *DRB1*03* represented the strongest risk factor (8.8). HLA *DRB1*04*, which independently confers susceptibility to AIH in adults, was infrequent in AIH-1 and ASC, suggesting protection; and *DRB1*15* (DR15) was protective against all forms of AILD. Distinct HLA class II alleles predispose to the different subgroups of juvenile AILD: *DRB1*03* to AIH-1, *DRB1*13* to ASC, and *DRB1*07* to AIH-2. Possession of homozygous *DRB1*03* or of *DRB1*13* is associated with fibrosis at disease onset, and possession of these two genes in addition to *DRB1*07* is associated with a more severe disease in all three subgroups.

CONCLUSIONS: Unique HLA profiles are seen in each subgroup of juvenile AILD. HLA genotype might be useful in predicting responsiveness to immunosuppressive treatment and course. (Hepatology 2021;0:1-15).

Genetic predisposition to autoimmune hepatitis (AIH), a progressive inflammatory liver disease with a female preponderance, has been associated with alleles of the major histocompatibility complex (MHC) class I and II genes in adult patients. Susceptibility to type 1 AIH (AIH-1), which is the most common form of the disease, characterized by antinuclear antibodies (ANA) and/or anti–smooth muscle antibodies (SMA), has been linked to MHC class II human leukocyte antigen (HLA) *DRB1* alleles encoding the similar amino acid sequences LLEQKR and LLEQRR at positions 67-72 of the DRβ polypeptide. These motifs are encoded by the *DRB1*0301* and *DRB1*0401* alleles, which

Abbreviations: AIH, autoimmune hepatitis; AILD, autoimmune liver disease; ANA, anti-nuclear antibodies; AP, alkaline phosphatase; ASC, autoimmune sclerosing cholangitis; AST, aspartate aminotransferase; CD, cluster of differentiation; ESLD, end-stage liver disease; ESPGHAN, European Society for Paediatric Gastroenterology, Hepatology and Nutrition; GGT, gamma-glutamyltransferase; GR, good responder; HAI, histology activity index; HC, healthy controls; HLA, human leukocyte antigen; IBD, inflammatory bowel disease; KCH, King's College Hospital; LC1, liver cytosol type 1; LKM1, liver kidney microsomal antibody type 1; LT, liver transplant; MHC, major histocompatibility complex; PSC, primary sclerosing cholangitis; RR, relative risk; SMA, anti–smooth muscle antibodies; SR, suboptimal responder.

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predispose adults of European ancestry from northern Europe, North America, and Iran to AIH-1[1-3]; by *DRB1*0405*, the susceptibility allele in Japan and Argentina[4,5]; and by *DRB1*0404*, the AIH-1 predisposing allele in Mexico,[6] although a recent investigation has questioned its role in susceptibility.[7] A recent paper suggests the impact of specific killer cell Ig-like receptor/HLA pairs in conferring susceptibility and influencing disease progression in Japanese patients with AIH-1.[8] *DRB1*1501*, which is associated with protection toward AIH-1, encodes alanine (A) at position 71, suggesting that the amino acid at this position is a primary determinant of disease susceptibility or resistance.[9-13]

Childhood AIH differs from its adult counterpart in two key aspects: firstly, a third of patients are affected by AIH type 2 (AIH-2), characterized by positivity for anti–liver kidney microsomal type 1 (anti-LKM1) and/or anti–liver cytosol type 1 (anti-LC1) antibodies; secondly, some 50% of children with laboratory and histological features of AIH-1 (i.e. ANA and/or SMA positivity and interface hepatitis) have bile duct damage on cholangiography at disease onset and are diagnosed as having autoimmune sclerosing cholangitis (ASC).[14]

Reports of MHC-encoded disease susceptibility in pediatric autoimmune liver disease (AILD) have been limited to either small numbers of patients or AIH subgroups[1,15-19] and have not differentiated AIH-1 from ASC. Reported HLA associations in childhood AIH are summarized in Supporting Table S1. Of note, in contrast to adult patients, the *DRB1*0401* allele is not a predisposing factor and can even exert a protective role,[20,21] and the *DRB1*1301* allele reported to predispose to AIH-1 in Argentinian and Venezuelan children does not conform to the shared motif model mentioned above, harboring the sequence LIEDER at positions 67-72.[11,13,22]

The aim of the present study was to investigate the HLA profile and its influence on disease predisposition, course, severity, and outcome in a large series of children with AILD, including AIH and ASC, followed up at a single center, King's College Hospital (KCH), London.

This paper is dedicated to Dr. James Underhill (J.U.), PhD, who performed over the years the HLA typing of our patients with autoimmune liver disease and who died untimely in 2015.

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Potential conflict of interest: Dr. Thompson consults for Mirum, Albireo, Sana, EVOX, and Alnylam. He consults for and owns stock in Generation Bio and Rectify Therapeutics. Dr. Hadzic consults for Arrowhead, Alnylam, and Albireo. He advises Takeda.

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Patients and Methods

PATIENTS

A total of 236 unrelated children of European ancestry (152 female, 64%) fulfilling the criteria for the diagnosis of AILD according to the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) scoring system for juvenile AILD,[23] were referred to our center between 1977 and 2016 and followed up to 2019. Retrospective data were collected from 1977 to December 1999 for 116 patients. From January 2000 until June 2016, 120 patients were recruited prospectively, and their blood was collected with full consent for research, including HLA typing. The study was approved by the Ethics Committee of KCH. The informed consent in writing was obtained from each patient or their guardians since January 2000 and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Ethics Committee of KCH. For retrospective data where informed consent was not possible, the requirement for informed consent was waived by the review committee of KCH.

To confirm the AILD diagnosis, all patients were scored retrospectively using the ESPGHAN diagnostic criteria.[23]

Four patients from abroad were lost to follow-up soon after diagnosis, while 232 patients were followed up until June 2019 (median 14.5 years, range 20 days-42 years, 3,489.5 person-years). Cholangiograms by endoscopic retrograde cholangiopancreatography or MR cholangiopancreatography to assess possible bile duct damage were performed in all patients, at or soon after presentation. One hundred patients had AIH-1 (median age at diagnosis 12 years, range 2.4-17), 77 had ASC (median age at diagnosis 11.3 years, range 2.1-15.7), and 59 had AIH-2 (median age 7.4 years, range 0.8-16). Viral hepatitis, Wilson disease, NASH, and other causes of liver disease were excluded by appropriate investigations. Laboratory, clinical, and histological indices were recorded in a dedicated database, those at the time of diagnosis being shown in Table 1. Two hundred and nine local subjects of European ancestry (110 female, 53%, median age 31 years, range 23-62) served as healthy controls (HCs), after providing informed consent. Two national and one London-based population controls (predominantly of European ancestry), comprising 1,798, 2,041, and 935 subjects, respectively, served as references to our local HCs (Supporting Table S2).[24-26]

HLA DETERMINATION

HLA class I genotyping for 14 HLA A and 17 HLA B antigens and class II genotyping for 37 HLA DRB, 8 DQA, and 15 DQB antigens was performed by PCR/sequence-specific primers using kits obtained from Biotest (Dreiech, Germany).[15,27] Alleles were assigned to broad antigens. HLA typing was carried out in the same laboratory, under similar conditions, and by the same investigator (J.U. who died untimely before the writing up of the paper and to whom the paper is dedicated), who was unaware of the diagnosis and the details of the patients.

DETECTION OF AUTOANTIBODIES

ANA, SMA, anti-LKM1, and anti-LC1 were tested by indirect immunofluorescence on a composite substrate including rat liver, kidney, and stomach at the initial dilution of 1/10 in phosphate-buffered saline according to the recommendations of the International Autoimmune Hepatitis Group.[28,29] Positive sera were double-diluted to extinction. Titers of ≥1/20 were considered positive.[28,29]

STATISTICAL ANALYSIS

c^2 and one-tailed Fischer exact tests were used to compare HLA frequencies in different groups. In order to control family-wise error rate for multiple testing, the Holm-Bonferroni correction was applied.[30,31] Relative risk (RR) was calculated as ORs.[31] The normality of variable distributions was tested using the Kolmogorov-Smirnov goodness-of-fit test. Differences in aspartate aminotransferase (AST), IgG, and bilirubin levels and autoantibody titers among patients with different HLA alleles were analyzed either by *t* test for parametric data or the Wilcoxon rank sum test for nonparametric data. Transplant-free survival in years from diagnosis was calculated by a Kaplan-Meier survival plot. $P < 0.05$ was considered significant.

TABLE 1. Demographic, clinical, laboratory, and histologic data at diagnosis of 236 children with AILD

Features Median (Range)	AIH-1, n = 100; follow-up, n = 98	*P* AIH-1 vs. ASC	ASC, n = 77; follow-up, n = 77	*P* ASC vs. AIH-2	AIH-2, n = 59; follow up, n = 57	*P* AIH-2 vs AIH-1
Age	12 (2.4-17)	NS	11.3 (2.1-15.7)	0.005	7.4 (0.8-16)	0.019
Female, n (%)	66 (66)	0.017	37 (48)	0.000038	49 (83)	NS
Acute presentation, n (%)	60 (60)	NS	38 (49)	NS	37 (63)	NS
AST, IU/L (nv ≤ 50)	591 (60-4,830)	NS	258 (62-3,344)	0.0045	548 (51-6,600)	0.0015
Total bilirubin, μMol/L (nv ≤ 20)	58 (4-329)	0.0001	21 (6-400)	0.0001	91 (6-445)	0.0198
AP, IU/L (nv ≤ 130)	288 (66-1,587)	0.02	323 (72-1,243)	0.004	221 (73-573)	NS
GGT, IU/L (nv ≤ 55)	85 (20-656)	0.0002	198 (13-623)	0.0003	81 (6-225)	NS
IgG, g/L (nv 5-18)	26.5 (9.55-68.9)	0.03	20.5 (7.81-63.7)	NS	16.9 (4.65-67.4)	0.03
ANA titer*	160 (neg-10,240)	NS	160 (neg-5,120)	NS	160 in one case	NS
SMA titer*	160 (neg-2,560)	NS	160 (neg-1,280)	NS	20 in one case	NS
LKM1 titer*	Negative in all	NS	Negative in all	NS	1,280 (neg†-20480)	NS
HAI	8 (1-17) (in 75 patients)	NS	Not applicable	NS	6 (1-17) (in 41 patients)	0.05
Fibrosis score	4 (0-6)	NS	Not applicable	NS	3 (0-6)	0.05
Diagnostic score	11 (7-14)	0.01	8 (7-14)	NS	10 (7-14)	0.01
GRs, n (%)	64/98 (65)	NS	43/77 (56)	NS	30/57 (53)	NS
SRs, n (%)	21/98 (21)	NS	14/77 (18)	NS	10/57 (18)	NS
ESLD (LT and/or death), n (%)	13/98 (13)	NS	20/77 (26)	NS	17/57 (30)	NS
Associated autoimmune disorders	28 (28)	3.92E7	51 (66)	8.3E5	19 (32)	NS
Ulcerative colitis	10		37		1	
Crohn disease	2		6		1	
Indeterminate colitis	1		1		0	
Systemic lupus erythematosus	4		2		1	
Diabetes mellitus type 1	5		2		9	
Autoimmune pancreatitis	1		0		0	
Celiac disease	2		1		1	
Sjogren syndrome	1		0		0	
Juvenile arthritis	1		1		2	
Autoimmune thyroiditis	1		1		2	
Autoimmune polyendocrine syndrome type 1	0		0		2	

Transaminase, bilirubin, IgG values and autoantibody titers of 28 patients treated with immunosuppression longer than 3 months before referral are not included in this table. HAI of 20 patients who did not undergo a liver biopsy before or shortly after starting immunosuppressive treatment are also not included in the table.
*Titers are shown as reciprocal values.
†Two cases being anti-LKM1-negative but anti-LC1-positive.
Abbreviations: neg, negative; NS, not significant; nv, normal value.

Results

CLINICAL DATA

Eight of 100 patients with AIH-1, 14/77 patients with ASC, and 6/59 patients with AIH-2 were already on immunosuppression for 3 months to 3 years before referral to KCH. Liver biopsy at presentation was performed in 216 patients (92%), before starting immunosuppressive treatment in 194 and within 6 months from starting treatment in 22 with coagulopathy, after prothrombin time normalization. In the remaining 20 patients, liver biopsies were performed ≥8 months after referral while on immunosuppression or in their local hospitals before referral and reported as compatible with AILD, but these were not available for review. Four out of 20 patients had complications of portal hypertension (hematemesis/melena from esophageal/gastric varices, bleeding diathesis, splenomegaly), and 16 had evidence of severe fibrosis by noninvasive tests, including ultrasound, showing nodular liver parenchyma and splenomegaly, FibroScan,

or MR elastography. All 216 patients biopsied at or within 6 months from diagnosis had histological features of interface hepatitis. At diagnosis, 170 patients had moderate to severe and 46 mild necroinflammation. For patients with AIH (75 AIH-1 and 41 AIH-2), severity of liver damage and degree of fibrosis were reviewed independently for the purpose of this study by two histopathologists (A.Q. and Y.Z.) and reported as histological activity index (HAI) and fibrosis score according to the Desmet and Ishak grading systems,[21,28] which record the degree of periportal or periseptal interface hepatitis, confluent necrosis, lytic necrosis, focal inflammation, portal inflammation, and fibrosis. These scoring systems were not applied to patients with ASC as they have not been validated for this condition. Ninety-eight patients (42%) had concurrent immune-mediated diseases, including type 1 diabetes, inflammatory bowel disease (IBD; ulcerative colitis, indeterminate colitis, or Crohn disease) or celiac disease (Table 1).

All patients were treated with prednisolone at a starting dose of 2 mg/kg/day (maximum 60 mg/day), weaned within 6-8 weeks to a maintenance dose of 2.5-5 mg/day according to child's age and weight; azathioprine (1-2 mg/kg/day) was added in the absence of gradual improvement of transaminase levels on prednisolone alone. Patients with ASC were treated equally with addition of ursodeoxycholic acid (15 mg/kg/day). Response to treatment was assessed according to ESPGHAN criteria.[23] Mycophenolate mofetil or a calcineurin inhibitor was used as second-line treatment when standard immunosuppression failed. The 232 patients followed for up to 42 years were divided in three groups: good responders (GRs; 137, 59%) achieved remission (normal transaminase and IgG levels) and maintained it long term, with or without minor, easily treatable, transaminase flares (less than twice the upper limit of normal); suboptimal responders (SRs; 45, 19.4%), comprising 21 who did not achieve complete remission despite response to treatment and 24 frequent relapsers (up to 4 relapses per decade with transaminase levels more than twice the upper limit of normal), experienced liver disease progression not requiring liver transplant (LT);[29] and patients with end-stage liver disease (ESLD; 50, 21.5%), including 44 who required LT and 6 who died before LT. Transplant-free survival tended to be higher in AIH-1 than in AIH-2 (P = 0.06) (Fig. 1A).

HLA ALLELES CONFERRING SUSCEPTIBILITY TO AILD

The frequencies of 5 HLA class I alleles, 9 HLA class II alleles, and two haplotypes conferring susceptibility or resistance to AILD, chosen from 20 published reports since 1991 (Supporting Table S1), were compared to local controls (Table 2). Frequencies significantly different from local controls are presented in Fig. 2. The authenticity of the local controls was confirmed by comparing their HLA profiles to those of the three national control groups (Supporting Table S2), though the frequencies of five individual HLA alleles and the two haplotypes were not available in the national control groups. The significance of the frequency of HLA alleles was confirmed after Bonferroni correction.

Children with AIH-1 possessed more frequently HLA *A*01* and *B*8* than HCs. *DRB1*03* was more frequent in patients with AIH-1 than in those with ASC and AIH-2. The frequency of homozygous *DRB1*03* was higher in patients with AIH-1 than in HCs.

Possession of HLA *B*08* and homozygosity for *DRB1*03* were more frequent in patients with ASC than in HCs. Frequencies of *DRB1*13* and of *DRB1*13* in *DRB1*03*-negative patients were higher in patients with ASC than in HCs and patients with AIH-2.

Patients with AIH-2 were more frequently positive for *DRB1*03* or homozygous for *DRB1*03* than HCs. There was an overrepresentation of HLA *DRB1*07* in patients with AIH-2 compared to HCs and patients with AIH-1 or ASC. The frequency of *DRB1*07* in *DRB1*03*-negative patients was significantly higher in patients with AIH-2 than in HCs and patients with AIH-1 or ASC.

*DRB3*0101*, which encodes *DR52a*, was more frequent in patients with AIH-1 and ASC than in HCs. *DQB1*0201* was more frequent in patients with AIH-1, ASC, and AIH-2 than in HCs.

The *A*01-B*08-DRB1*03* haplotype was more frequent in patients with AIH-1, ASC, and AIH-2 than in HCs. The frequency of *B*08-DRB1*03-DQB1*0201* (*DQ2*) tended to be higher in patients with AIH-1 (37%) than in HCs (22%, P = 0.04 without Bonferroni correction).

Homozygosity for *DRB1*03* conferred the highest risk for patients with AIH-1, ASC, and AIH-2 (RR,

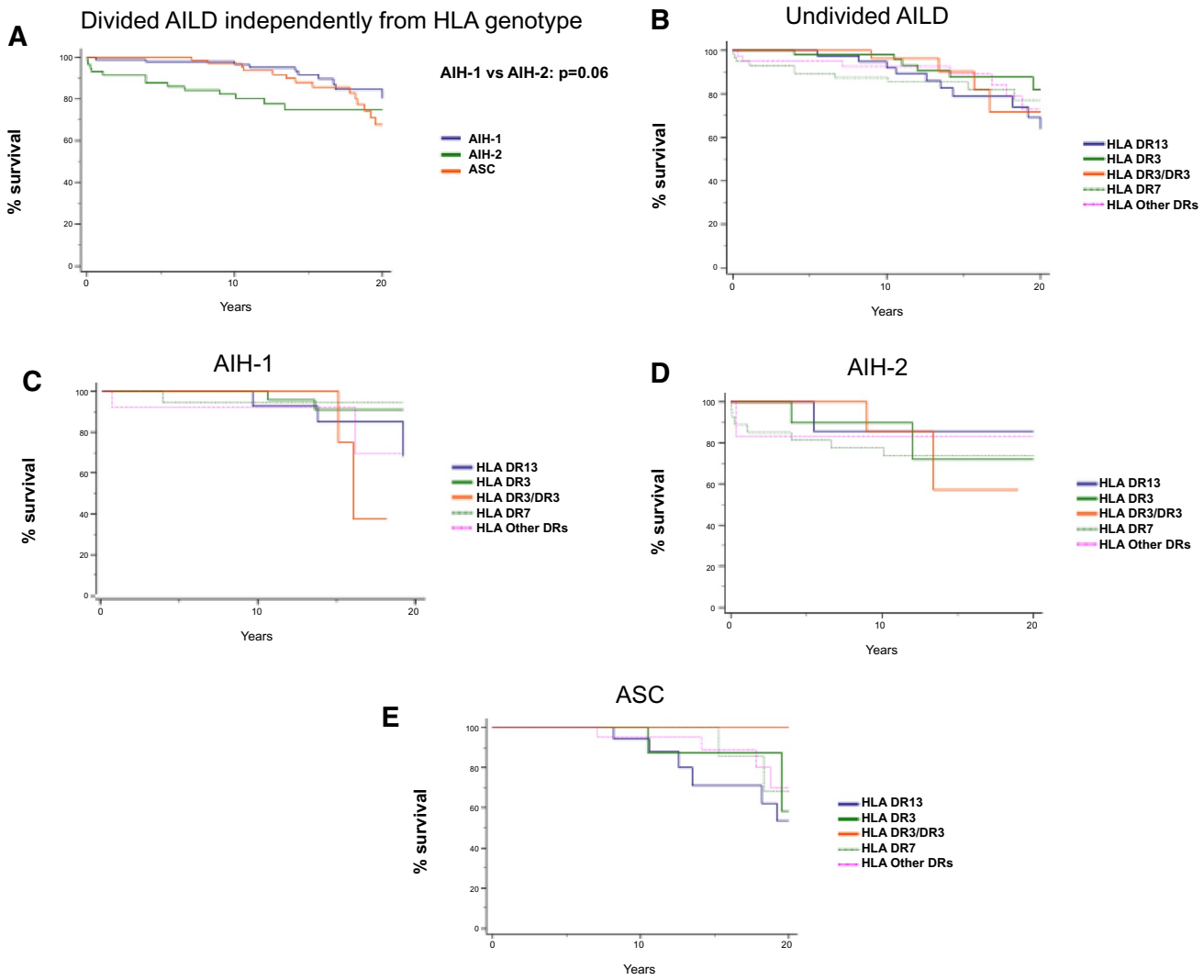

FIG. 1. Transplant-free survival curves of children with AILD with different HLA genotypes. The association of *HLA DRB1** genotypes and transplant-free survival was calculated by Kaplan-Meier survival plot. The *x*-axis shows the year of survival, defined as the years from diagnosis until the time of LT or death, cutoff at year 20; and the *y*-axis shows the percentage of survival. (A) Overall survival in the different subgroups of AILD independently from HLA genotype. The survival rate in AIH-1 tended to be higher than in AIH-2 ($P = 0.06$). (B-E) Survival according to *HLA DRB1** genotypes in AIH-1, AIH-2, and ASC, respectively. No significant difference in survival was observed among subgroups of patients with AILD.

8.88, 7.46, and 5.31, respectively). Among patients negative for *DRB1*03*, the possession of *DRB1*07* conferred the highest risk for AIH-2 (RR, 3.72), and possession of *DRB1*13* conferred a risk for ASC (RR, 2.58). Possession of the *A1-B8-DR3* haplotype conferred RRs of 3.28, 2.13, and 2.53 for AIH-1, ASC, and AIH-2, respectively.

For HLA genes conferring resistance to AILD (Fig. 2), *DRB1*04*, an AIH-susceptibility allele in adult AIH-1, was less frequent in patients with AIH-1 and ASC than in HCs. Among HLA *DRB1*03*-negative patients, the frequency of *DRB1*04* in AIH-1 was comparable to that in HCs, while it was lower in patients with ASC than in HCs.

Two genes, *DRB1*15* and *DQB1*0302* (DQ8), previously reported to confer resistance to AIH in adults, were also found less frequently in children with AILD than in HCs. Patients with AIH-2 had a DQ8 frequency similar to that of HCs.

No significant differences in the HLA profile were detected in patients with AIH-1 or ASC divided according to presence or absence of IBD (Supporting Table S3).

TABLE 2. Frequencies of HLA class I and II alleles and haplotypes in 236 patients with AILD and HCs

HLA Allele Allotypes	AIH-1, n = 100, n (%)	*P** (*P*$_c$)	RR	ASC, n = 77, n (%)	*P** (*P* after BC)	RR	AIH-2, n = 59, n (%)	*P** (*P*$_c$)	RR	Local HCs, n = 209 (%)
*A*01*	**55 (55)**	*0.00013 (0.0026)*	1.72	37 (48)	*0.01 (NS)*	1.50	26 (44)	*NS*	1.38	67 (32)
A*02	**41 (41)**	***NS***	**0.82**	**32 (42)**	***NS***	**0.84**	**24 (41)**	***NS***	**0.82**	104 (50)
*B*07*	***15 (15)***	*0.03 (NS)*	0.58	20 (26)	*NS*	1.01	11 (19)	*NS*	0.72	54 (26)
*B*08*	**63 (63)**[A]	*7.9E-11(1.58E-9)*	**2.80**	**36 (47)**	*6.06E-5 (0.0012)*	**2.08**	**25 (42)**	*0.0013 (0.037)*	1.88	47 (22)
*B*14*	5 (5)[B]	*NS*	0.55	5 (7)	*NS*	0.71	9 (15)	*NS*	1.68	19 (9)
*DRB1*03*	**70 (70)**[C]	*7.6E-11 (1.52E-9)*	**2.61**	**32 (42)**	*0.016 (NS)*	1.55	**25 (42)**	*0.013 (NS)*	1.58	56 (27)
*DRB1*03/*03*	**17 (17)**	*8.23E-7 (1.65E-5)*	**8.88**	**11 (14)**	*3.14E-5 (6.28E-4)*	**7.46**	**6 (10)**	*0.0024 (0.048)*	**5.31**	4 (2)
*DRB1*04*	***22 (22)***	*0.021 (NS)*	0.63	***10 (13)***	*0.00029 (0.006)*	***0.37***	14 (24)	*NS*	0.68	73 (35)
*DRB1*04 in DRB1*03 –ve*	11/30 (37)[D]	*NS*	1.06	***5/45 (11)***	*0.0023 (0.046)*	***0.32***	8/34 (24)	*NS*	0.68	53/153 (35)
*DRB1*07*	21 (21)[E]	*NS*	1.19	12 (16)	*NS*	0.88	**31 (53)**	*1.84E-8(3.67E-7)*	**2.97**	37 (18)
*DRB1*07 in DRB1*03 –ve*	9/30 (30)[F]	*NS*	1.58	8/45 (18)	*NS*	0.94	**24/34 (71)**	*1.59E-9 (3.2E-8)*	**3.72**	29/153 (19)
*DRB1*13*	19 (19)[G]	*NS*	1.02	**24 (31)**	*0.02 (NS)*	1.67	9 (15)	*NS*	0.82	39 (19)
*DRB1*13 in DRB1*03 –ve*	8/30 (27)	*NS*	1.63	**19/45 (42)**[H]	*0.0005 (0.01)*	**2.58**	7/34 (21)	*NS*	1.26	25/153 (16)
*DRB1*15*	***7 (7)***	*0.0018 (0.036)*	**0.33**	***9 (12)***	*0.065 (NS)*	0.55	***5 (9)***	*0.027 (NS)*	**0.4**	44 (21)
*DRB3*0101 (DR52a)*	**15/19 (79)**[I]	*1.79E-6 (3.5E-5)*	**3.21**	**9/14 (64)**	*1.74E-3 (0.035)*	**2.61**	7/20 (35)	*NS*	1.42	33/134 (25)
*DQB1*0201 (DQ2)*	**22/30 (73)**[J]	*0.0023 (0.046)*	1.70	**27/41 (66)**	*0.0092 (NS)*	1.53	**19/27 (70)**	*0.0085 (NS)*	1.64	68/158 (43)
*DQB1*0302 (DQ8)*	***2/31(7)***[K]	*0.00146 (0.029)*	**0.18**	***1/41 (2)***	*2.4E-5 (0.00028)*	**0.068**	7/27 (26)	*NS*	0.72	57/158 (36)
Haplotypes										
A1-B8-DR3	**44 (44)**[L]	*2.7E-9 (5.4E-8)*	**3.28**	22 (29)	*0.0027 (0.05)*	**2.13**	**20 (34)**	*2.87E-4 (0.0057)*	**2.53**	28 (13)
B8-DR3-DQ2	**11/30 (37)**	*0.04 (NS)*	1.7	11/41 (27)	*NS*	1.25	**9/25 (36)**	*NS*	1.67	34/158 (22)

RRs higher than 2 and lower than 0.5 are shown in bold. Overrepresented alleles are shown in bold. Underrepresented alleles are shown in italic bold.
**P* values compare patients and local HCs. A-L: *P* values comparing among AIH-1, ASC, and AIH-2. A: AIH-1 vs. ASC, *P* = 0.03 (NS) and vs. AIH-2, *P* = 0.01 (NS); B: AIH-1 vs. AIH-2, *P* = 0.027 (NS); C: AIH-1 vs. ASC, *P* = 1.47E-5 (0.00029) and vs. AIH-2, *P* = 5.99E-4 (0.012); D: AIH-1 vs. ASC, *P* = 0.0081 (NS); E: AIH-2 vs. AIH-1, *P* = 4.2E-5 (0.00084) and AIH-2 vs. ASC, *P* = 4.35E-6 (8.7E-5); F: AIH-2 vs. AIH-1, *P* = 0.0019 (0.038) and vs. ASC, *P* = 2.2E-6 (4.4E-5); G: ASC vs. AIH-1, *P* = 0.06 (NS) and vs. AIH-2, *P* = 0.03 (NS); H: ASC vs. AIH-2, *P* = 0.04 (NS); I: AIH-1 vs. AIH-2, *P* = 0.00567 (NS); ASC vs. AIH-2, *P* = 0.09; J: AIH-1 vs. ASC, *P* = 0.02 (NS), AIH-2 vs. ASC, *P* = 0.064; K:AIH-1 vs. AIH-2, *P* = 0.04 (NS); ASC vs. AIH-2, *P* = 0.0037 (0.074); L: AIH-1 vs. ASC, *P* = 0.03 (NS).
Abbreviations: BC, Bonferroni correction; NS, not significant.

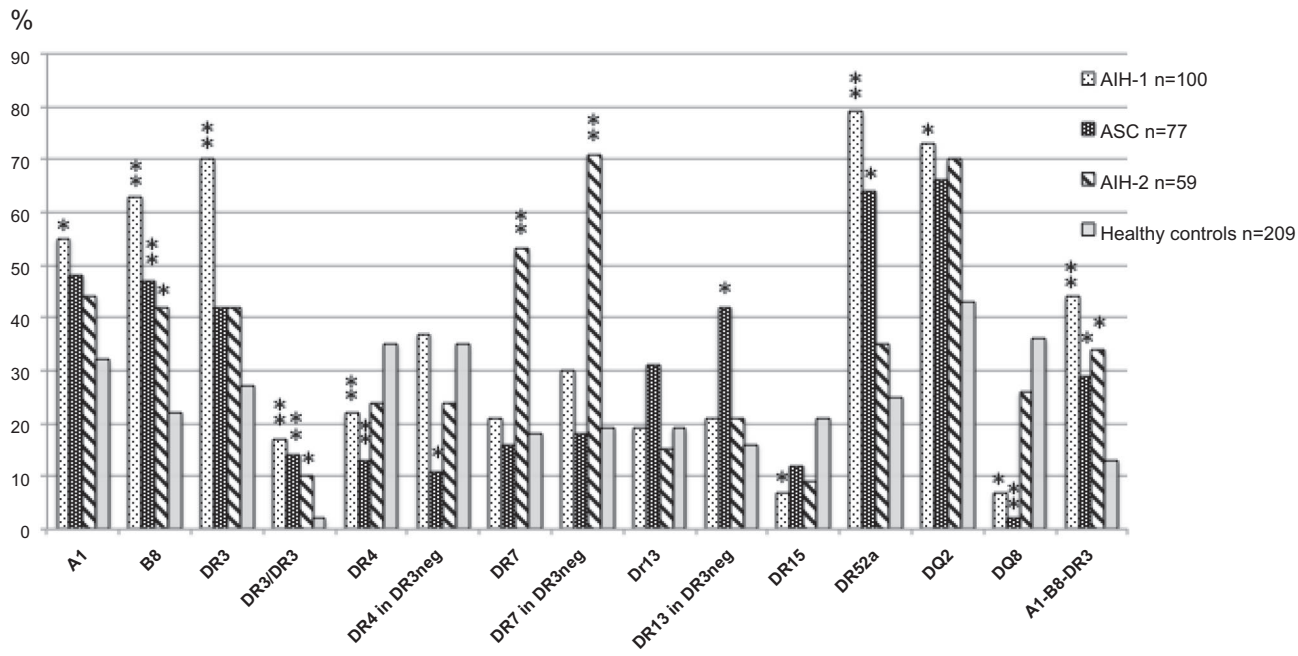

FIG. 2. Frequencies of HLA class I and class II alleles and haplotypes in 236 patients with AILD and 209 HCs. Light dotted bar, AIH-1; dark dotted bar, ASC; hatched bar, AIH-2; gray bar, HCs. $^{*}P < 0.05$, $^{**}P \leq 0.001$ compared to HCs.

ASSOCIATIONS BETWEEN HLA DRB1* GENOTYPES AND CLINICAL FEATURES

To address the impact of the HLA susceptibility *DRB1** allotypes on clinical features, response to treatment, and outcome, patients with AILD were divided according to positivity for *DRB1*03*, homozygosity for *DRB1*03*, positivity for *DRB1*07* or *DRB1*13*, or "other DRs," including *DRB1*01, -04, -09, -10, -11, -12, -14*, and *-15*, which could not be analyzed individually owing to their low frequency (Table 2 and Fig. 2; Supporting Tables S4-S6).

UNDIVIDED AILD

Among undivided AILD, age at diagnosis was higher in patients possessing heterozygous or homozygous *DRB1*03* or *DRB1*13* than in patients with *DRB1*07* or "other DRs" (Table 3). Female gender was more frequent in the *DRB1*07*-positive group than in patients possessing other HLA types. Acute presentation was more frequent in *DRB1*07*-positive than in *DRB1*13*-positive patients. Higher baseline AST levels were seen in patients possessing *DRB1*03* (heterozygous or homozygous) or *DRB1*07* compared to *DRB1*13*-positive patients and in patients heterozygous for *DRB1*03* compared to those with "other DRs." Higher bilirubin levels were found in patients possessing heterozygous *DRB1*03* compared to *DRB1*13*-positive patients; bilirubin levels were also higher in *DRB1*07*-positive patients than in those with homozygosity for *DRB1*03* or with *DRB1*13*. The highest alkaline phosphatase (AP) levels were seen in patients heterozygous for *DRB1*03* or with *DRB1*13* compared to "other DRs." Gamma-glutamyltransferase (GGT) levels were higher in *DRB1*13*-positive patients than in homozygous *DRB1*03*, in *DRB1*07*, or in "other DR" patients. IgG levels in patients heterozygous/homozygous for *DRB1*03* were higher than in those with other HLA types. ANA titers in patients homozygous for *DRB1*03* or positive for *DRB1*07* were higher than in those with "other DRs." The highest titers of SMA were seen in patients possessing *DRB1*07* and *DRB1*13*. In patients with AIH, the median HAI was similar among those possessing different HLA class II alleles. However, the median fibrosis score was higher in patients homozygous for *DRB1*03* or

TABLE 3. Demographic data of 236, clinical data at diagnosis of 208,* and outcome of 232 patients with AILD according to HLA *DRB1 allotypes**

Features Median (Range)	*DRB1*03* Heterozygous (*DR3*), n = 57	*DRB1*03* Homozygous (*DR3/DR3*), n = 33	*DRB1*07* (*DR7*), n = 58	*DRB1*13* (*DR13*), n = 45	Other *DRB1** Allotypes,‡ n = 43	Comparisons	*P*
Age (years)	10.76 (4.3-16)	11.2 (3.8-17)	9.11 (2.2-16)	11.7 (2.33-15.4)	8.49 (2.1-15)	*DR3 vs. DR7*	0.009
						DR3 vs. other DRs	0.05
						DR3/DR3 vs. DR7	0.0056
						DR3/DR3 vs. other DRs	0.01
						DR13 vs. other DRs	0.002
Female (%)	60	61	79	53	58	*DR7 vs. DR3*	0.022
						DR7 vs. DR3/DR3	0.05
						DR7 vs. DR13	0.005
						DR7 vs. other DRs	0.02
Acute presentation (%)	56	61	67	44	56	*DR7 vs. DR13*	0.00025
AST (IU/L; nv ≤ 50)	603 (53-6,600)	664 (60-2,725)	549 (51-2,642)	475 (84-2,950)	270 (62-4,830)	*DR3 vs. DR13*	0.03
						DR3 vs. other DRs	0.05
						DR3/DR3 vs. DR13	0.04
						DR7 vs. DR13	0.053
Total bilirubin (μMol/L; nv ≤ 20)	61 (5-367)	43 (6-268)	62 (9-445)	49 (9-382)	38 (4-379)	*DR3 vs. DR13*	0.05
						DR7 vs. DR3/DR3	0.045
						DR7 vs. DR13	0.02
AP (IU/L; nv ≤ 130)	323 (66-859)	295 (64-1,000)	227 (33-1,578)	335 (77-1,115)	218 (73-1,243)	*DR3 vs. other DRs*	0.03
						DR13 vs. other DRs	0.02
GGT (IU/L; nv ≤ 55)	99 (13-656)	83 (13-469)	65 (6-623)	102 (23-516)	72 (20-591)	*DR3/DR3 vs. DR13*	0.003
						DR7 vs. DR13	0.025
						Other DRs vs. DR13	0.03
IgG (g/L; nv 5-18)	23 (9.13-68.9)	26.2 (9.3-68.58)	20.4 (4.65-58)	19.9 (9.4-53.6)	17.3 (7.81-40.4)	*DR3 vs. DR7 , DR13, other DRs*	0.035, 0.037, 0.002
						DR3/DR3 vs. DR7, DR13, other DRs	0.016, 0.014, 0.0017
ANA titer‡	80 (neg-2,560)	120 (neg-2,560)	160 (neg-10,240)	40 (neg-2,560)	40 (neg-1,280)	*DR3/DR3 vs. other DRs*	0.03
						Other DRs vs. DR7	0.03
SMA titer‡	40 (neg-2,560)	40 (neg-640)	80 (neg-2,560)	160 (neg-1,280)	20 (neg-640)	*DR3 vs. DR7*	0.03
						Other DRs vs. DR7	0.006
						Other DRs vs. DR13	0.03
Diagnostic score	11 (7-14)	11 (7-14)	10 (7-14)	10 (7-14)	9 (7-14)	*DR3 vs. other DRs*	0.01
						DR3/DR3 vs. other DRs	0.01
HAI (for AIH-1 and AIH-2 only)	7 (1-17)	8 (2-17)	8 (2-17)	7 (1-16)	8 (2-18)		NS

TABLE 3. *Continued*

Features Median (Range)	*DRB1*03* Heterozygous (*DR3*), n = 57	*DRB1*03* Homozygous (*DR3/DR3*), n = 33	*DRB1*07* (*DR7*), n = 58	*DRB1*13* (*DR13*), n = 45	Other *DRB1** Allotypes,‡ n = 43	Comparisons	*P*
Fibrosis score (for AIH-1 and AIH-2 only)	3 (0-6)	5 (2-6)	3 (1-5)	5 (0-6)	3 (0-6)	*DR3/DR3 vs. DR3, DR7, other DRs*	0.01, 0.01, 0.017
						DR13 vs. DR3, DR7, other DRs	0.06, 0.01, 0.02
Time to achieve biochemical remission (months)	3.8 (1-12)	4 (1-14)	5 (0.5-36)	6.5 (1-48)	3 (1-12)	*DR3 vs. DR7, DR13*	0.0015, 0.0019
						DR3/DR3 vs. DR7, DR13, other DRs	0.035, 0.014, 0.034
						DR7 vs. other DRs	0.0008
						Other DRs vs. DR13	0.001
GRs, n (%)	43 (78)	16 (50)	27 (47)	24 (49)	27 (69)	*DR3 vs. DR3/DR3*	0.0066
Total = 137						*DR3 vs. DR7*	0.000758
						DR3 vs. DR13	0.0019
						Other DRs vs. DR13	0.0559
SR+FR,	2 (4)	11 (34)	18 (32)	12 (25)	2 (5)	*DR3 vs. DR3/DR3*	0.0001
n (%)	(SR = 1, FR = 1)	(SR = 3, FR = 8)	(SR = 6, FR = 12)	(SR = 9, FR = 3)	(SR = 2, FR = 0)	*DR3 vs. DR7*	0.0001
Total = 45						*DR3 vs. DR13*	0.00187
						Other DRs vs. DR3/DR3	0.0015
						DR7 vs. other DRs	0.0017
						DR13 vs. other DRs	0.0136
ESLD n (%)	10 (19)	5 (16)	12 (21)	13 (27)	10 (26)		NS
Total = 50							
SR + ESLD, n (%)	12 (22)	16 (50)	30 (53)	25 (51)	12 (31)	*DR3 vs. DR3/DR3*	0.0066
Total = 95						*DR3 vs. DR7*	0.00076
						DR3 vs. DR13	0.0019
						Other DRs vs. DR3/DR3	0.099
						Other DRs vs. DR7	0.034
						Other DRs vs. DR13	0.056

*Transaminase, bilirubin, and IgG values and autoantibody titers of 28 patients treated with immunosuppression longer than 3 months before referral are not included in this table.
†Titers are shown as reciprocal values.
‡Patients with *DRB1*01*, *04*, *09*, *10*, *11*, *12*, *14*, and *15*.
Abbreviations: FR, frequent relapsers; neg, negative; NS, not significant; nv, normal value.

positive for *DRB1*13* than in those possessing heterozygous *DRB1*03*, *DRB1*07*, or "other DRs."

Time to remission was shorter in patients heterozygous or homozygous for *DRB1*03* and in those with "other DRs" than in those with *DRB1*07* or *DRB1*13*. The percentage of GRs was similar among those heterozygous for *DRB1*03* and "other DRs," being higher than in patients homozygous for *DRB1*03*, *DRB1*07*, or *DRB1*13*. The percentage of SRs was lower among patients heterozygous for *DRB1*03* or with "other DRs" than in those homozygous for *DRB1*03*, *DRB1*07*, or *DRB1*13*. The percentage of patients with ESLD was similar among the groups, but when SRs and patients with ESLD were considered together their percentage was lower among those heterozygous for *DRB1*03* or "other DRs" than in those homozygous for *DRB1*03*, *DRB1*07*, or *DRB1*13*. Transplant-free survival was similar among patients possessing different HLA genotypes (Fig. 1B).

AIH-1

In patients with AIH-1, mode of presentation, frequency of female gender, baseline transaminase and bilirubin levels, and HAI were similar among those possessing different HLA DRB1 alleles (Supporting Table S4). Patients with heterozygous or homozygous *DRB1*03* and *DRB1*13* were older and had higher IgG levels and median SMA titers at presentation than those with "other DRs." In heterozygous *DRB1*03*-positive patients, levels of AP were higher than in those possessing "other DRs"; GGT levels were higher in heterozygous *DRB1*03*-positive patients than in those with *DRB1*07* or with "other DRs" and in homozygous *DRB1*03*-positive patients than in those with "other DRs." The highest ANA titers were seen in patients with homozygous *DRB1*03*.

Time to remission was shorter in patients heterozygous for *DRB1*03* and "other DRs" than in patients homozygous for *DRB1*03*, *DRB1*07*, or *DRB1*13*. The median fibrosis score was higher in patients with AIH homozygous for *DRB1*03* and in those positive for *DRB1*13* than in those possessing heterozygous *DRB1*03*, *DRB1*07*, or "other DRs."

The percentage of GRs was similar in patients with heterozygous and homozygous *DRB1*03* or "other DRs," being higher than in patients with *DRB1*07* or *DRB1*13*. The percentage of SR+ESLD was lower in those heterozygous for *DRB1*03* than in those with *DRB1*07* or *DRB1*13*. The highest percentage of patients requiring transplant or dying was seen among those possessing *DRB1*13*. Transplant-free survival was similar among patients possessing different HLA genotypes (Fig. 1C).

AIH-2

In patients with AIH-2, the proportion of females, mode of presentation, levels of IgG, and LKM-1 titers were similar among those with different HLA allotypes (Supporting Table S5). Patients with *DRB1*13* were older than patients possessing other allotypes. The highest AST levels were seen in patients heterozygous for *DRB1*03* and in patients with *DRB1*07* or "other DRs," reaching statistical significance when patients with *DRB1*07* or "other DRs" were compared to homozygous *DRB1*03*. Baseline bilirubin levels were higher in patients with *DRB1*07* and "other DRs" compared to homozygous DRB1*03. Baseline GGT levels were higher in *DRB1*13* patients compared to those with all other HLA allotypes. The highest HAI was seen in patients with "other DRs" and the lowest with *DRB1*13*.

*DRB1*13*-positive patients required longer time to achieve remission than patients heterozygous or homozygous for *DRB1*03* with *DRB1*07* or "other DRs." The median fibrosis score was higher in patients with AIH-2 homozygous for *DRB1*03* or positive for *DRB1*13* than in those possessing heterozygous *DRB1*03*, *DRB1*07*, or "other DRs."

The lowest proportion of GRs was seen among patients with homozygous *DRB1*03* or positive for *DRB1*13*. Most patients with heterozygous *DRB1*03* or "other DRs" responded well to treatment. The percentage of SR+ESLD was lower in those heterozygous for DRB1*03 (30%) or with "other DRs" (33%) than in those with homozygous *DRB1*03* (58%), *DRB1*07* (52%), or *DRB1*13* (58%); but the difference was not statistically significant due to the small case number. Transplant-free survival was similar among patients possessing different HLA genotypes (Fig. 1D).

ASC

In patients with ASC, mode of presentation and titers of SMA antibodies were similar among the different HLA allotypes (Supporting Table S6). Patients with "other DRs" or with *DRB1*07* were younger

than patients with heterozygous *DRB1*03*. There were more female patients among those possessing *DRB1*07* than among those with all other HLA allotypes. Baseline AST levels were higher in patients homozygous for *DRB1*03* or with *DRB1*07* than in those with "other DRs." The highest levels of AP and GGT were seen in patients possessing *DRB1*07*. IgG levels in patients homozygous for *DRB1*03* and in those with *DRB1*07* were similar, being higher than in those possessing other HLA alleles. ANA titers in patients heterozygous for *DRB1*03* or with *DRB1*07* were higher than in those with *DRB1*13* or "other DRs."

Time to remission in patients heterozygous or homozygous for *DRB1*03* or with "other DRs" was shorter than in those with *DRB1*07* or *DRB1*13*. The percentage of GRs was similar among patients with heterozygous *DRB1*03* or with "other DRs," being higher than in patients with homozygous *DRB1*03* or with *DRB1*07*. All patients with heterozygous *DRB1*03* or "other DRs" responded well to treatment. The percentage of SR+ESLD was similar among heterozygous *DRB1*03* and "other DRs," being lower than in those homozygous for *DRB1*03* or positive for *DRB1*07*. Transplant-free survival was similar among patients possessing different HLA genotypes (Fig. 1E).

Discussion

We report the largest study of HLA association in juvenile AILD, with the longest follow-up. We included only children of European ancestry, and owing to the single-center nature of the study, all were rigorously phenotyped and divided into the three main groups of juvenile autoimmune liver disease: AIH-1, AIH-2, and ASC.

We found that HLA *B*08*, *-DRB1*03*, and the A1-B8-DR3 haplotype, known to predispose to adult AIH-1, also predispose to all three types of childhood AILD. Because in populations of European ancestry the A1-B8-DR3 haplotype does almost invariably include *DRB3*0101* (DR52a) and *DQB1*0201* (DQ2), not surprisingly we found that DR52a and DQ2 also predispose to juvenile AILD. Genes that were underrepresented in children with AILD, indicating their protective role, were *DRB1*04* (DR4), a susceptibility gene in adult AIH-1[32]; *DRB1*1501* (DR2), which protects against AIH also in adults[1]; and *DQB1*0302* (DQ8), a gene reported to predispose to autoimmunity when associated to DR4.[33] A genome-wide association study in a large number of adult patients from northern Europe[33] reported an association between AIH-1 and possession of both *DRB1*03* and *DRB1*04*. However, the latter was associated with later-onset disease, confirming previous reports[34] and in keeping with the lack of association, indeed protection, we observed between possession of DR4 and the juvenile form of the disease. Interestingly, DR4 has been reported to be associated with AIH susceptibility only in DR3-negative adult patients[1] and to predispose to a later age at onset and less severe disease. This observation has led to the suggestion that AIH in adults may consist of two distinct diseases with distinct HLA associations. In support of this hypothesis, DR3/DR4 heterozygosity in adults was not associated with susceptibility. Similarly, we found no evidence that possession of DR3/DR4 predisposes to AIH in children: only a small number of our patients possess DR3/DR4 (12% of AIH-1 and 10% of AIH-2), and clinical features and outcomes of these patients are similar to those who are DR3/DR4-negative. If DR4 does predispose to a distinct form of late-onset AIH, it is not surprising that it does not predispose to juvenile AILD. DR4 protection against juvenile AILD could derive either from an unrelated HLA association with a disease distinct from adult AIH or from DR4 binding an autoantigenic peptide and preventing it from binding to DR3 or another allotype containing the shared epitope, such as DR52a.

We have also defined individual disease risk alleles for each subgroup of AILD—HLA *DRB1*03* for AIH-1, *DRB1*13* for ASC, and *DRB1*07* for AIH-2—indicating that the three conditions are nosologically distinct entities. These results are in partial agreement with previous studies in smaller pediatric cohorts.

Pediatric AIH-1 has been associated to HLA *DRB1*0301*,[20,21] pediatric AIH-2 to possession of HLA *DRB1*0701*,[35-37] and in DR7-negative patients, *DRB1*0301*.[38] In Egypt, AIH-2 has been associated also with the presence of HLA *DRB1*15*.[36] In Brazil and in Egypt, the primary susceptibility allele for AIH-1 appears to be *DRB1*1301*, the allele that we find in association with ASC, though a secondary association with *DRB1*0301* has also been reported.[36,39] Importantly, however, not all patients in

these reports had undergone cholangiographic studies to exclude bile duct disease. Intriguingly, in South America, HLA *DRB1*1301* has been reported to predispose to childhood AIH-1 as well as to persistent HAV infection, suggesting a possible link between the two conditions.[11,40]

A follow-up extending to 42 years has allowed us to define HLA alleles associated to disease severity: possession of heterozygous *DRB1*03* or "other DRs" predicts a form of AILD better controlled by immunosuppressive treatment than *DRB1*03* homozygosity or possession of *DRB1*07* or *DRB1*13*. *DRB1*03* homozygosity and possession of *DRB1*13* are associated with established liver fibrosis at disease onset and a more severe disease. Possession of these allotypes might account for the third of patients who have established cirrhosis at disease onset.[20,41] Although DR3-positive patients appear to have a more active liver disease, as judged by higher autoantibody titers and higher IgG levels at disease onset, akin to the report by de Boer et al.[42] in adult patients with AIH-1, they had a better initial response to immunosuppression with a shorter time to achieve biochemical remission and tended to maintain remission long term and relapse less frequently than those possessing *DRB1*07* and *DRB1*13* alleles. Interestingly, patients possessing heterozygous *DRB1*03* genes progressed less frequently to ESLD than those with *DRB1*07* or *DRB1*13*. At variance with our findings, Junge et al.[43] suggested that DR3-positive children with AILD are less likely to reach remission. However, no accepted criteria for remission were used in their small cohort of ethnically mixed patients. In a large Dutch cohort of adult patients with AIH-1, HLA-*DRB1*03:01* was also found to be the strongest genetic modifier of disease severity, while HLA *DRB1*13* was not investigated[3] because it had not been previously found to be associated with AIH-1 in the Netherlands, although the population studied was similar to ours, being of European ancestry.

We do show that possession of homozygous *DRB1*03* influences disease severity and response to treatment, the frequency of GRs being lower in homozygous than heterozygous *DRB1*03* patients. Relevantly, in a family study, we noted that the strongest predisposition to juvenile AILD is homozygosity for HLA *DRB1*03* because it was more frequent in affected children than in their first-degree relatives and in HCs, the only first-degree relative affected by AILD (primary sclerosing cholangitis [PSC]) in the whole cohort sharing HLA *DRB1*03* homozygosity with his proband brother, who had ASC.[44]

Most patients with AIH-2 responded well to immunosuppressive treatment initially, but fewer of them could stop immunosuppression compared to AIH-1. This may be due to the frequent possession of *DRB1*07* that was present in 71% of patients with AIH-2 when those who were *DRB1*03*-positive were excluded. *DRB1*07*-positive patients with AIH-2 presented with the highest AST and bilirubin levels at diagnosis and responded to immunosuppression less frequently compared to patients possessing other types of HLA genes. A similarly lower response rate was observed in *DRB1*07*-positive patients with AIH-1 or ASC. Preliminary data from our laboratory show that *DRB1*07*-positive patients have an impairment of cluster of differentiation 4–positive ($CD4^{pos}$) $CD25^{high}CD127^{low}$forkhead box P3–positive regulatory T cells, both at disease onset and during remission, when compared to patients possessing *DRB1*03*, *DRB1*13*, or "other DRs," suggesting that possession of *DRB1*07* lessens control over autoimmunity.

The relationship between juvenile ASC, which has overlapping features with AIH-1, and adult PSC, both strongly associated with IBD, remains unclear. The HLA profile of ASC, while partially overlapping with AIH-1, also shows dissimilarities as up to one third of patients with ASC are positive for *DRB1*13*, this proportion being even higher among those negative for *DRB1*03*, indicating that *DRB1*13* is a risk gene (RR, 2.6) for ASC. The similarities between juvenile ASC and adult PSC extend to HLA because both conditions share predisposing HLA genes, like *B*08*, *DRB1*03*, *DRB1*13*, and A1-B8-DR3 haplotype,[45] suggesting that at least some adult patients with PSC might have an immunologically burnt-out end-stage form of juvenile ASC. Possession of *DRB1*13* in adult PSC is associated with more severe liver disease, a greater need for LT, and decreased graft survival.[46,47] In our cohort, *DRB1*13*-positive patients with ASC responded to immunosuppression less well than those possessing *DRB1*03* genes.

An external validation cohort of carefully phenotyped (i.e., with cholangiographic studies to differentiate between AIH-1 and ASC) patients with AIH-1, AIH-2, and ASC recruited prospectively would be desirable to confirm our data. It would, however, require decades to recruit patients with these

rare conditions and obtain the relevant follow-up information.

In summary, we have defined both HLA class I and II profiles for each subgroup of childhood AILD: *DRB1*03* for AIH-1, *DRB1*03* plus *DRB1*07* for AIH-2, and *DRB1*13* for ASC. *DRB1*03* and the A1-B8-DR3 haplotype are disease-predisposing genes for all three subgroups. The influence of HLA class II genes on disease severity is strong, *DRB1*03* homozygosity and possession of *DRB1*13* being associated to histologically more advanced disease from onset, while *DRB1*07* is linked to the least optimal response to immunosuppression. The influence of the distinct HLA genes on effector and regulatory immune responses in the different AILD subgroups should be further explored.

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