

# Autoimmune Hepatitis: A Childhood Disease

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**Abstract:** Autoimmune hepatitis is a severe and unresolving inflammatory disease of the liver of unknown etiology carrying high morbidity and mortality. All ages and genders are concerned with a peak of incidence in girls in prepubertal age, even if the disease has been diagnosed as early as 6 months. Autoimmune hepatitis may be classified in two major subgroups on a presence of a specific set of autoantibodies: anti-smooth muscle mostly with anti-actin specificity and/or by antinuclear antibody in type 1 and anti-liver-kidney microsome and/or the anti-liver cytosol in type 2. The histological hallmark is "interface hepatitis", with a mononuclear cell infiltrate in the portal tracts, variable degrees of necrosis, and progressive fibrosis. The disease follows a chronic but fluctuating course usually progressing to cirrhosis and liver failure. The most frequent type onset is similar to that of an acute viral hepatitis with acute liver failure in some patients; about a third of patients have an insidious onset with progressive fatigue and jaundice while 10-15% are asymptomatic and are accidentally discovered by the finding of hepatomegaly and/or an increase of serum aminotransferase activity. Corticosteroids alone or in conjunction with azathioprine are the treatment of choice inducing remission in over 90% of patients. An alternative therapeutic strategy is cyclosporine. Withdrawal of immunosuppression is associated with high risk of relapse. Liver transplantation manages patients with decompensated liver disease unresponsive to "rescue" medical treatment.

**Keywords:** Chronic liver disease, Autoimmune hepatitis, Interface hepatitis, Immunosuppressive therapy, Cyclosporine, Liver transplantation.

Autoimmune hepatitis (AIH) is a severe and unresolving inflammatory disease of the liver of unknown etiology carrying a high morbidity and mortality [1]. All ages and genders are concerned with a peak of incidence in prepubertal girls even if the disease has been diagnosed as early as age 6 months [2-4].

Waldenström first described this disease entity about 50 years ago in a young woman presenting with a chronic inflammatory liver disease, rapidly evolving to cirrhosis, associated with jaundice, elevated gamma globulins and amenorrhea [5]. The disease was defined as "lupoid hepatitis" by Mackay *et al.* [6] because of the presence of antinuclear antibodies [ANA] and of lupus erythematosus [LE] cells. Further progresses in the identification and characterization of the autoantibodies typically present in AIH patients, led to a consensus on definition and classification of this disease [7-8].

## DEFINITION

Autoimmune hepatitis is a heterogeneous clinical entity. It is characterized by a cryptogenic and unresolving inflammation of the liver leading to a progressive and irreversible architectural change. The histological hallmark is a picture of "interface hepatitis", with a dense mononuclear cell infiltrate in the portal tracts, variable degrees of necrosis, and progressive fibrosis. The disease follows a chronic but fluctuating course [1-3], usually progressing to cirrhosis and

liver failure, even though the rapidity of progression is highly variable [4, 9].

Other major features of autoimmune hepatitis include [1-4, 9]:

1. serological findings of activation and deregulation of the immune system such as hypergammaglobulinemia, with a wide panel of circulating autoantibodies, low levels of Complement factor 4 and partial or complete IgA deficiency;
2. association with different types of autoimmune disorders;
3. Response to immunosuppressive treatment even in case of severe liver function impairment [10].

## EPIDEMIOLOGY

Autoimmune hepatitis is a rare disease, but with a wide range of prevalence in different populations. While in Northern Europe, its prevalence is of 17 cases per 100.000, with an incidence of 1, 9 per 100.000 per year [11, 12]; it is only of 0.015 per 100.000 per year in Japan [13]. In Western Europe, AIH accounts for about 20% of chronic hepatitis, while in Brazil it represents no more than 5-10% [12]. Incidence of AIH is apparently increasing in childhood; it can be estimated that it accounts for at least 5 to 7 new diagnosis/year, for a referral pediatric liver unit in Europe, affecting up to 10 % of patients followed in the same center and representing more than 50% of all cases of chronic hepatitis [14].

Female gender is concerned more than male with an F to M ratio up to 9:1[2]. All races and ages are concerned even if

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**Table 1. Main features of the two subtypes of AIH in children.** ANA= antinuclear antibodies, SMA= anti-smooth muscle antibodies; LKM= anti-liver-kidney microsome autoantibodies type 1; LC1= anti-liver cytosol antibodies type 1; p-ANCA= anti-neutrophil cytoplasmic antibodies with a perinuclear pattern; SLA= anti-soluble liver antigen antibodies; ASGP-R= anti-asialoglycoprotein receptor antibodies.

	AIH-1	AIH-2
<b>Age</b>	Usually adolescents	More frequent in infancy and early childhood
<b>Most frequent type of onset</b>	"Chronic" type of onset	Acute hepatitis
<b>Cirrhosis at onset</b>	Frequent	Less frequent
<b>Hypergammaglobulinemia</b>	++++	+ / ++
<b>Biliary lesions</b>	Possible	Absent
<b>Autoantibodies</b>	ANA, SMA, p-ANCA, SLA, ASGP-R	LKM-1, LC-1, ASGP-R
<b>Typical extrahepatic manifestations</b>	Chronic juvenile arthritis Hypergammaglobulinemic purpura	Grave's disease Autoimmune Thyroiditis Vitiligo Alopecia

AIH is mainly a pediatric disease since about 40% of diagnosis of type 1 and 80% of type 2 AIH are made during childhood and adolescence.

### DIFFERENT SUBTYPES: A CONTROVERSIAL ISSUE

AIH is heterogeneous in nature and it is widely, even if not universally, accepted that it may be classified in two major subgroups [2, 15]. These subtypes of AIH are currently recognized according to the pattern of the autoantibody panel detected at the time of diagnosis:

- AIH type 1 by the presence of anti-smooth muscle antibody (SMA) and/or antinuclear antibody (ANA) [1, 15, and 17].
- AIH type 2 by anti-liver-kidney microsome antibody (LKM-1) and/or the anti-liver cytosol type 1 antibody (LC-1) [1, 2, 16, and 18].

The ratio of incidence of the two subtypes in Europe is 2:1 while it seems to be 6-7:1 or greater in North and South America and in Japan [12, 13].

Differences between these two subtypes consist in epidemiological distribution, genetic markers and clinical presentation which might underlies different pathogenetic mechanisms (Table 1) [19, 20]. Despite this heterogeneity, available data suggest similar outcome and similar response to treatment [21-23].

AIH type 1 shows two peaks of incidence: in adolescents and in adults over 30 years where this form is widely prevalent. The onset of AIH type 2 is significantly earlier,

even in the first year of life, often associated to an acute/severe onset, mimicking a fulminant hepatitis [2, 3, and 24]. In Europe AIH type 2 represents about 20% of new diagnosis, while in USA his prevalence is lower than 5% [13].

Hypergammaglobulinemia, which is typical of type 1, is moderate and occasionally absent in type 2 [1, 2, 4, 14, and 15]. AIH type 2, progresses through "flares" of necrosis this can explain why it is sometimes characterized by transitory mild histological activity and as well as by multiacinar collapse in acute/fulminant onset. Typically it is almost never associated to bile duct lesions [2, 17] at difference of type 1 where different degrees of bile duct lesion are common [1,2,4,14-17].

Extrahepatic manifestations differ slightly in the two subtypes, with prevalence of autoimmune thyroid (Grave's and Hashimoto diseases) and skin (vitiligo and alopecia) diseases in type 2 [2, 4, 14, and 15].

### CLINICAL FEATURES AND NATURAL HISTORY

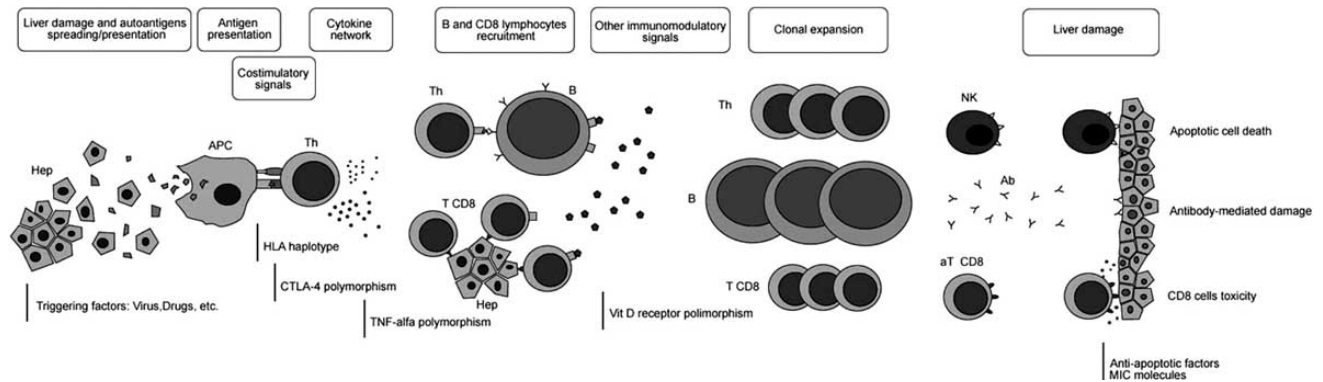
In children, the mean age at diagnosis is about 10 years for type 1 AIH and 7 years for type 2. The clinical presentation is quite different than in adults. The most frequent onset is similar to that of an acute viral hepatitis, with jaundice, dark urine and pale stools, malaise and anorexia and associated with nausea/vomiting and abdominal pain [2, 4, 15-17]. Raised serum aminotransferase activity is constant. In some cases patients develop acute liver failure with encephalopathy. These patients, with fulminant hepatitis, are typically younger and more frequently affected by type 2 AIH [24].

About a third of patients, with higher frequency for type 1, have an insidious onset with progressive fatigue, anorexia and weight loss and intermittent jaundice. Firm hepatomegaly, splenomegaly and signs of liver function impairment are however frequent, in these patients and cirrhosis or severe fibrosis is often presents.

Moreover, 10 to 15% of patients are completely asymptomatic and the underlying process is accidentally discovered by the finding of hepatomegaly or by an increase of aminotransferase activity. Rarely it may reveal with the sings of portal hypertension such as bleeding from esophageal varices or by symptoms related to an associated extrahepatic autoimmune disorder.

Extrahepatic autoimmune diseases are in fact reported in 10-20% of patients and in 20-40% of first degree relatives. Interestingly, distribution of autoimmune processes in other family's members does not differ in type 1 or 2 AIH. Presence of another case of AIH in first degree relatives is anecdotic.

Children and adolescents thus often present as an acute disease sometimes with sign and symptoms of severe liver failure that need urgent concern and treatment. In adults, AIH is usually scarcely symptomatic with a mild disease activity and with scarce fibrosis at liver biopsy, leading sometimes to a decision of not to treat [25]. Moreover, a very slow progression of fibrosis is reported in adult patients with a disease incompletely controlled by treatment [26].



**Fig. (1).** Hypothetical model of pathogenesis of AIH. Hep=hepatocytes; APC=Antigen presenting cell; Th=T helper lymphocyte; T CD8=T cytotoxic lymphocyte; B=B lymphocyte; NK=Natural Killer lymphocyte; aT CD8= activated cytotoxic lymphocyte.

## LABORATORY FEATURES

Besides the presence of autoantibodies characterizing the two subgroups of AIH, serum aminotransferases activity is increased in all untreated patients, sometimes markedly, up to 50 times the upper normal limit. Gamma glutamyl transferase (GT) activity may be normal or slightly elevated; a significant increase of GT should lead to a suspicion of bile duct damage as in case of overlap syndrome or autoimmune cholangitis. Immunoglobulins G are characteristically elevated in about 80% of patients, sometimes severely. This feature is typical of type 1 AIH, but may be absent in younger patients with type 2 AIH. Serum albumin may be normal in the early phases, but it may be reduced in case of cirrhosis with liver insufficiency or in presence of ascitis. Reduction of prothrombin activity reflects the severity of liver function impairment. Partial serum immunoglobulin A deficiency is quite common in type 2 AIH and may be associated with inherited reduced levels of C4 [27-29].

The histological hallmark of AIH is “interface hepatitis” with mononuclear cell, lymphocytes, mainly T helper and Natural Killer, plasma cells and activated macrophages infiltrating the portal tract, invading the adjacent parenchyma and surrounding apoptotic hepatocytes [30].

## PATHOGENESIS

The mechanisms leading to autoimmune injury of the hepatocytes are not known, but several observations suggest that AIH is a multifactor disease. A complex genetic background is probably required to confer ability to react against liver autoantigens and to configure a heightened immune responsiveness against both environmental and self antigens. High titers of antibodies against different microbial antigens have been found in patients with chronic active hepatitis in several studies [31, 32]. This non-antigen specific defect, present also in some first-degree relatives is corrigible both *in vivo* and *in vitro* by pharmacological doses of corticosteroids and was generically related to a defective “T-cell suppression” [33, 34]. Indeed the immune system is involved *in toto* and all steps leading to develop an immune response are in fact concerned (Figure 1).

## Genetic Background

### Autoantigen Presentation

The first compulsory step in triggering an immune reaction is the activation of T lymphocytes by professional antigen presenting cells (APC) that present, on the cell surface, an antigenic peptide within the binding groove of class II Human Leukocytes Antigens (HLA) molecules [35]. These HLA class II molecules are encoded in the chromosome 6, among the 6p21.3 band, in close proximity to the genes for HLA class I and III, configuring a number of ancestral haplotypes due to strong linkage disequilibrium among the HLA *loci*. HLA class II molecules, expressed on the membrane of specialized APC cells, but inducible also in different cells lines, are able to hold only short peptides of 13-23 amino acid residues which are the final product of the internalization and partial digestion by the APC cells of extra-cellular proteins. The recognition of the complex “HLA class II-exogenous peptide” is restricted by the specificity of the T helper cell receptor as well as by CD4 co-recognition. Consequently HLA class II molecules play a central role in T helper activity, but also in B cell activation [36].

The functional site of the HLA II molecules, the peptide binding groove, is hosted mainly within the DR polypeptidic chain which composes, together with the DR $\beta$ , the DR heterodimerous [37]. Alleles of the DRB locus are highly polymorphous, as shown by X-ray crystallographic studies, and present three hypervariable regions (HVR) encoding amino acid motifs [38, 39]. Moreover, some DRB haplotypes include, in strong linkage disequilibrium, a second *locus* encoding for a different DR chains. The consequence of this complex polymorphism is that each individual carries two to four different DR molecules with different binding properties and affinities. Consequently, the nature and the structure of the class II HLA polypeptidic chains, especially of the DR molecules, critically affects both the nature and the alignment of the antigenic peptide and the linkage affinity and avidity between the antigen-MHC II complex and the T cell receptor [40]. If this binding process is not effective the T-helper cell remains uncommitted. Consequently, the suitability of a certain autoantigens to

trigger a CD4 cells immune response and, probably, the effectiveness of immunocytes activation, is genetically determined and depends strictly by the genotypic asset of HLA class II [41].

### **AIH Type 1 and MHC Class II Molecules**

In Caucasian adult population, early investigations focused on the A1-B8-DR3 (also called A1-B8-Dw3) ancestral 8.1 haplotype showing a significative association with type 1 AIH as well as with several autoimmune disorders [42-44]. The HLA A1 B8 DR3 haplotype and the HLA DR4 were identified as independent risk factors for AIH whereas the HLA DR2 was accounted to have a protective role [45].

With the availability of high resolution, PCR-based, HLA typing methods, the molecular basis of this association became investigable [46]. The principal susceptibility alleles for AIH type 1 are DRB1\*0301, DRB3\*0101 and DRB1\*0401 as a part of DR3 and DR4 linked alleles [47-49]. Odd ratio [OR] for DRB1\*0301 is 3.39 rising to 7.6 in case of DRB1\*0301 homozygosity. DRB1\*0401 homozygosity does not carry an increased risk for AIH while homozygosity of DRB3\*0101 raises the relative risk to develop AIH from 4.2 to 14.7 and decreases significantly the 10-yr survival [50].

The presence of these haplotypes not only confers increased risk to AIH, but seems also to influence some clinical features of the disease. Patients with HLA A1-B8 and/or DRB1\*0301 present at significant younger age, respond less favorable to corticosteroid treatment, carry an higher risk of relapse and require liver transplantation more frequently for end stage untreatable liver failure [45,48,50,51]. The DRB1\*0301 genotype was also associated with the detection of anti Soluble Liver Antigen (SLA) autoantibodies [52], while DRB1\*401 patients present preferentially anti-smooth muscle [SMA] and high titers antinuclear [ANA] autoantibodies [53].

A strong feature of genetic predisposition to AIH type 1 is the regional variation of HLA disease-associated alleles depending on the population studied. Several studies outside Northern Europe and Northern America have found different susceptibility alleles at DRB1 locus. In Japan, the prevalence of A1-B8-DR3 haplotype in general population is very low and DR4 is the principal risk factor for AIH type 1, occurring in 90% of Japanese patients and in 39% of healthy controls [54, 55]. In Japanese patients with AIH, HLA B54-DR4-DR53-DQ4 is the most common haplotype and DR2 is common among DR4-negative patients. Among the DR4 patients the prevalent allele is DRB1\*0405 and it was associated with a later onset of the disease, milder activity, slower progression to cirrhosis and with a better response to immunosuppressive therapy [54, 55]. These clinical feature are similar to that of Caucasoid patients carrying the DR4/DRB1\*0401 allele [50, 53].

In South America a number of studies have been performed, but in a very heterogeneous population characterized by a large predominance of children, so they deserve a separate discussion. In a white Argentinean adults HLA A11 and DR4 were reported in AIH type 1- with a

strong synergistic effect [56, 57]. A secondary association, once excluded the pediatric subgroup, was found with the DRB1\*0405 allele (RR=10, 4;  $p<0,005$ ) which is identical to that of Japanese population [57]. In the adult subset of a Brazilian mixed population with different percentage of white, black and Amerindian, a weak association with DRB1\*1301 (DR6) was found with a relative risk of 2.2 and a secondary association with DRB1\*03 carries a relative risk of 3.8. However patients with DR6 were significantly younger than their DR3 counterparts [58, 59]. In Mestizo Mexicans the primary HLA association is between AIH type 1 and DRB1\*0404 as a part of the DR4 serologic subset [60]. A summary of HLA class II alleles associated with AIH type 1 is shown in Table 2.

The issue of unifying all these different observations was addressed to the analysis of the HLA-D gene sequences and translation to the amino acidic structure of different alleles. It is well known that the panel of potential binding peptides and the hierarchy of affinity are defined, for each MHC class II allele, by the specific three-dimensional structure and electrostatic configuration of the functional portion, the binding groove, with special attention to the  $\alpha$ -elical region and to the floor of the cleft [35-41]. Different alleles with similar stereo-electrical alignment are likely to have similar binding properties.

These considerations focused the attention to the small epitope LLEQKR at position 67-72 of the DR polypeptide, a part of the hypervariable region 3 of DR  $\alpha$  chain, located at the edge of the  $\alpha$ -helix region. The two major susceptibility alleles DRB\*0301 and DRB1\*0401 in white Caucasian share this same sequence [48, 49]. Moreover, the presence of Lysine at position 71 is accounted to play a big influence, based on the observation that the DRB\*1501 allele, thought to have a protective role in the same population, differ substantially only at position 71 [48, 49]. In addition the DRB1\*0405 and DRB1\*0404 alleles found in Japan, Argentina and Mexico could be included within this model since they present Arginine, an amino acid with electric properties comparable to that of Lysine, at position 71 [50].

A second hypothesis is based on the Japanese experience. It highlights the role of position 13 of the DR polypeptide as critical spot for susceptibility. In fact, all patients studied in Japan carries a Histidine in position 13 as a part of DRB1\*04 alleles [50, 54, 55]. Unfortunately, DRB1\*03 alleles, the primary susceptibility markers outside Japan, encode Serine in position 13, that is a very different, small, non polar amino acid. Far from exhausting the issue, the "shared motif theory" offers a good molecular approach integrated with a functional, biochemical point of view in elucidate genetic mechanisms of susceptibility in AIH type 1 patients and it offer the chance to deduce the nature of autoantigens involved in type 1 AIH.

Data regarding HLA typing in large series of children with type 1 AIH are scanty but some of them very interesting. First data from Northern European children showed the typical pattern for AIH type 1 in Caucasoid patients with a significative prevalence of DR3, DR52a (DRB3\*0101), A1-B8-DR3 and A1-B8-DR3-DR52a (68%, 70%, 57%, 53% vs. 25%, 25%, 14%, 14% of controls: respectively,  $p<0,0001$ ). Curiously, there was a lower

**Table 2. HLA DRB alleles implicated in the pathogenesis of autoimmune hepatitis.**

Allele (Broad Antigen)	Allele (High Resolution)	HLA DRB allele Usually Associated,	Disease	Comment
DR3 (Dw3)	DRB1*0301	DRB3(DR52) DRB3*0101 (DR52a)	AIH 1 AIH 2	Primary risk factor for AIH 1 in adult Caucasian population. Both alleles involved. Dose effect: homozygous patients have increased risk. Associated with SLA and more severe disease. Secondary risk factor for AIH 1 in European, and South American children. Association with AIH 2 also reported in adults and in children.
DR4	DRB1*0401	DRB4	AIH 1	Secondary risk factor for AIH 1 in adult Caucasian population. Only primary allele involved. Dose effect not present. Associated to older age and to concurrent other autoimmune diseases.
	DRB1*0405	DRB4	AIH 1	Primary risk factor in Japanese adult patients. Secondary risk factor in Argentinean adult patients.
	DRB1*0404	DRB4	AIH 1	Primary risk factor in Mestizo Mexican adult patients.
DR6	DRB1*1301	DRB3	AIH 1	Secondary association in Brazilian young adults. Primary risk factor in Caucasian children. Associated with a protracted immune response to HAV
	DRB1*1302	DRB3	AIH 1	Weak protective role in Argentinean children
DR2	DRB1*1501	DRB5	AIH 1	Protective role in Caucasian adults especially in homozygosity. Secondary risk factor in Japanese adults? (very common in such population)
DR7	DRB1*07	DRB4	AIH 2	Associated with AIH 2 in adult population. Both alleles involved.

prevalence of DR4 in patients (13% vs. 40% in controls;  $p < 0,004$ ) [4].

In a large Argentinean series, HLA DRB1\*1301 (DR6) was the primary susceptibility allele (66, 4% vs. 10, 6% in controls;  $RR=16, 3$ ) and HLA DRB1\*1302, which differs for only one amino acid, showed a weak protective role. A secondary association with DRB1\*0301 was also observed, but DRB1\*1301 was associated with a lower response rate to treatment [57]. This data substantially match that of a previous report in a different Argentinean pediatric population [61].

A study concerning a mixed Brazilian population with a great (67%) prevalence of patients < 16 years old reported comparable results: DRB1\*1301 was observed more frequently both in children (84% vs. 26% in controls;  $RR=12,8$ ) than in adults (43% vs. 26% in controls;  $RR=2,2$ ), but was clearly more significant in pediatric AIH type 1 patients. A secondary association with DRB1\*0301 was recorded in all age groups, but DRB1\*1301 patients were significantly younger than the DRB\*0301 counterpart (11 vs. 17 yr,  $p < 0, 01$ ) [58, 59].

Recently an array of 50 families from France and Quebec (Canada) was studied using the transmission disequilibrium test attempting to follow the segregation of the HLA class II alleles in affected versus unaffected offspring [62]. Once more the DRB1\*1301 allele resulted as the primary genetic risk factor for AIH type 1 in children (90% in patients vs. 40% in unaffected offspring). The DRB\*03 allele family confirmed its role (81% vs. 42%) and, interestingly, it shows

a strong transmission distortion also in families of type 2 AIH-affected children (100% vs. 42, 8).

Tempting to speculate on this data in comparison with the patterns of susceptibility showed by adult patients, three major considerations are likely to be done:

1. DRB1\*1301 is a relevant risk factor peculiar of pediatric age. DRB1\*0301 confirmed his role in children and, interestingly, among the adult patient population it is associated with a younger age [43, 46, 48]. The DR4 family of alleles seems not to be implicated in children, whereas in adults they have been accounted as a marker of a mild, late onset disease [45, 47, and 53]. This might explain the peculiar epidemiology of AIH type 1 in Japan where HLA DR3 is very low prevalent in general population and pediatric cases of AIH seem to be rare [54,55].
2. A deeper statistical approach was tempted, as it is was done with adult form susceptibility alleles, to associate single amino acid residues with disease occurrence. A number of positions were highlighted such as Glutamic Acid at DR 9 or Tyrosine at DR 10, but the stronger association was found with Valine at position DR 86, carried both by DRB1\*1301 and DRB1\*0301 [57].
3. Most of all, data exists showing that children in South America carrying DRB1\*1301 have a major susceptibility to persistent HAV infection with a protracted immune response to HAV [63]. This virus, highly endemic in South America, was environmental

pattern of microbial or toxic agents and therefore they could differ in different geographical areas is fascinating indeed because it could easily explain heterogeneity in susceptibility markers between adults and children and between different populations.

### AIH type 2 and MHC class II Molecules

Genetic background of AIH type 2 has been poorly investigated to date, partially in reason of the low prevalence of this form in adults. Two reports one from Spain [65] and the other from Italy/United Kingdom [66] focused on the DRB1\*03 and DQB1\*02 alleles whereas another study conducted in a German population reported an increased frequency of DRB1\*07, DRB4\*01 and DQB1\*06 [67]. The significance of these data is however hampered by the small sample size and by the lack of a racially-matched group of control. In a population from Brazil, composed for the large majority by pediatric patients, a significant increase of DRB1\*07, DRB4 and DQB1\*02 was observed when compared with healthy controls. The last two alleles were in strong linkage disequilibrium with DRB1\*07. A secondary association with DRB1\*03 was also present: 93% of patients vs. 44% of controls carried the DRB1\*07 or the DRB1\*03 allele ( $p < 0,0001$ ) and, even if it not reached statistical significance, patients with DRB1\*07 tended to be younger [58].

### MHC Class III Molecules

MHC class III genes encode a heterogeneous group of proteins involved, at various degrees, in the immune pathway including, among others, tumor necrosis factor and , complement proteins like C2, C4, Bf, and the MIC [MHC-class I chain related proteins] and . In almost all cases, they are not strictly functionally related with MHC class I and II proteins, but their encoding *loci* are close to those of MHC class I and II therefore segregating as a unique entity in Mendelian way. Hence several linkage disequilibrium exists among MHC region loci and a number of haplotypes including MHC class III region.

Among the HLA class III genes, persistently low serum levels of C4 have been found in 69% of children with AIH [27]. C4 genotyping in adults revealed an association with the silent allele C4AQ0 at the C4A locus [28, 29]. This allele results from a large deletion (up to 6 Kb) in the MHC class III encoding region. About 50% of Caucasoid adult patients carry a null C4A allele and 16% are homozygous vs. 1% of age and race matched controls [29]. The homozygous form of the C4AQ0 allele has been associated also with a more severe form of AIH at younger age [28]. The C4AQ0 allele is in linkage disequilibrium with the HLA A1, B8 and DR3 haplotypes and it is part of the 8.1 ancestral haplotype and, like HLA A1-B8-DR3, it has been reported in association with other autoimmune disorders [29, 68]. It is not clear whether C4 deficiency plays an active role in inducing disease either its statistical strong association is due exclusively to other loci of the 8.1 haplotype as suggested by recent models. It is however possible that a defective clearance of immune complex or viruses as well as cellular debris mediated by a less active complement activity might

influence inflammation severity or increase the risk of autoimmunity.

MIC molecules are stress-inducible protein expressed exclusively in epithelial cells of the thymus and gastrointestinal tract [69]. They function as a ligand for subclasses of T cells as the T cells and normal liver has a large resident population of T cells [69]. MIC A-B loci are highly polymorphic and the allele MICA\*008 have been associated with susceptibility for primary sclerosing cholangitis [70]. Even if this polymorphism has not been yet studied in AIH, individuals homozygous for MICA\*008 might express an aberrant molecule leading to a perturbed activation of T and NK lymphocytes. The MICA\*008 allele is a part of the 8.1 ancestral haplotype and his frequency in AIH type 1 patients is expected to be higher than general population.

### Co-Stimulatory Signals and Cytokine Network

Although the large majority of studies focused on the HLA genes polymorphisms, the binding of the complex antigen/MHC with the TCR on lymphocyte surface is only the first step of the complex stream of events leading to immunocytes recruitment, activation, proliferation and chemotaxis. Moreover, a number of anti-inflammatory factors, such as anti-inflammatory cytokines and the activation induced cell death (AICD) mechanisms, acts as a counterpart to hamper immune stimulation, to prevent excessive tissue damage and, in a finalistic point of view, autoimmunity triggering.

The recent improvement of knowledge concerning the human genome has evidenced the enormous number of inherited variations in gene sequences, most of all as single nucleotide polymorphisms (SNPs), but also as repeat sequences as mini- and microsatellite DNA. It is estimated that more of 1.42 million of SNPs exists both in coding and non coding regions [71]. Obviously, only a minority of these leads to protein variation but all of them has a potential role as flags of an association to other *loci* for linkage disequilibrium.

First of all, after HLA-peptide complex-TCR interaction, a second signal is required to achieve cellular activation. It involves the B7 molecule on the APC surface. CD4 and CD8 cells express the CD28 protein that is the positive, second signal ligand of the B7 molecule. Activated CD4 and CD8 cells also express the cytotoxic T lymphocyte antigen-4 (CTLA-4) which has 20 to 50 fold higher affinity for the B7 molecule [72]. CTLA-4 is accounted to prevent an excessive stimulation of immunocytes by competing with the CD28 and to have a down regulating effect by delivering an apoptosis signal [73]. Three different SNPs were found on the 2q33 region encoding for the CTLA-4: one of them, the Guanine-Adenine substitution in position 49, leads to an Alanine-Threonine shift in the position 17 of the main peptide [74]. The CTLA-4\*G allele has been previously associated to susceptibility for several autoimmune disorders; in a case-control study in an adult population with AIH type, the patients group had a higher frequency (54% vs. 37%) of CTLA-4\*AG genotype of controls group [75]. Interestingly CTLA4\*GG genotype was equally found in patients and controls, but it was significantly more common

in HLADRB1\*0301 patients representing a case of potential synergy between functionally related genes located on different chromosomes. As mentioned above, it is possible that other *loci* located nearby the CTLA4 encoding region 2q32, as the CD28 locus, might play a role in producing this association.

On the other hand, a remarkable multicentric family study was performed on a Caucasian pediatric population with AIH type 1 and 2 involving a total of 32 families with 32 affected offspring and 34 unaffected offspring [76]. They were assessed for CTLA-4\*AG and for the third intron CD28 polymorphisms. In addition, three different repeat regions within these loci were genotyped. A significantly increased transmission rate of the CTLA-4/A allele (affected/unaffected 87,5/12,5;  $p=0.002$ ) and of the eight copies allele of the dinucleotide repeat [AT] $_n$  [AT-8] in the 3'-untranslated region of the CTLA-4 gene (affected/unaffected 83,5/16,5;  $p=0.005$ ) was highlighted in AIH type 1 affected offspring. These alleles are spaced only by 5.3 kb and they are in strong linkage disequilibrium.

This study interestingly remarks that differences in genetic background of disease susceptibility between adults and children are not limited to HLA genes and suggests the potential role of microsatellite region of the CTLA-4 gene as a primary susceptibility marker by modifying stability of RNA and proteins expression [76].

The sub-sequential steps that follow T-cells recruitment and activation involve several cell lines as B-cells, non helper T-cells, macrophagic elements, and thrombocytes, located in different districts. Communications between all these subjects is predominantly humoral and cytokines are likely to have the crucial task to coordinate the immune reaction. Cytokines could be roughly classified as pro-inflammatory [TNF- $\alpha$ , IL-1, IL-6] or as negative modulator [IL-4, IL-5, and IL-10] either they could be distinguished on the basis of the effect on the predominant immune response: cellular or humoral.

The differentiation of T-helper lymphocytes to Th-1 or Th-2 phenotypes is under control of a different cytokines profile. Interleukin (IL)-2, interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) constitute the Th-1 cytokine response while IL-4, IL-5, IL-6, IL-8, IL-10 and IL-13 are responsible of Th-2 response, facilitating humoral immunity.

It is known that children with type 1 and type 2 AIH have different cytokines profiles [20] and adults with AIH type 1 have lower serum levels of IL-2 and IL-4 than normal subjects [77]. Cytokine profile of AIH varies according to the disease phase, with a Th-1 predominance in the liver during the acute phase and a Th-2 predominance in peripheral blood at remission [77,78]. Moreover, evidence exists that cytokines profile differs in AIH type 1 and AIH type 2 and versus controls, both in peripheral blood and, most interestingly, on liver samples [20,79]. These observations could represent either the marker of an unpaired immune response, or an epiphenomenon of the normal course of a severe tissue inflammation, but it is possible that a particular combination of SNPs in cytokine genes might influence severity and duration of inflammation and disease activity and presentation.

The first cytokine encoding locus to be investigated has been the TNF gene cluster which is located within the HLA region and includes genes for TNF- $\alpha$  and TNF- $\beta$ . TNF- $\alpha$  is one of the cytokines acting in the early phase of the inflammatory cascade and its action includes amplification of the immune process and fibrogenesis. Sixteen different SNPs are described in the TNF gene cluster [80]. An important polymorphism consisting in substitution of an Adenine for a Guanine has been described in the position 308 of the TNF- $\alpha$  gene [81].

In Caucasian adult patient with AIH type 1 the allele TNFA\*2 is associated with high basal and inducible level of TNF- $\alpha$  enhancing a Th-1 response. This allele is also carried preferentially by young patients with AIH type 1 and by subjects that poorly responds to immunosuppressive therapy [82]. These data overrun the consideration that a strong association of the allele TNFA\*2 and AIH type 1 is largely expected in Caucasian patients because of the belonging of this allele to the 8.1 ancestral haplotype [81]. However in adult patients with primary sclerosing cholangitis the association between TNFA\*2 and disease is stronger than between DRB1\*0301 and disease [83]. This polymorphism, however, does not seem to act as a risk factor among Brazilian patients [84].

#### Other immunomodulatory Mechanisms

A number of complementary signals, especially from endocrine and nervous systems, interact with the immune system. Recent data indicated the 1, 25-dihydroxy-vitamin D $_3$  as an important immunomodulatory factor [85-87]. The activation of the vitamin D receptor [VDR], which is expressed by several immunocompetent cells such as peripheral blood monocytes or activated T lymphocytes, elicits a complex immunoregulatory role. Several effects were described: activation of monocytes/macrophages, inhibition of activated lymphocytes proliferation and prevention of the maturation of dendritic cells into active antigen-presenting cells and administration of 1,25-dihydroxy-vitamin D $_3$  is able to prevent the development of some autoimmune diseases in experimental animal models [88].

It is possible to speculate that a malfunction of VDR might play a role in triggering an inappropriate immune response facilitating autoimmune diseases. Several polymorphisms of the VDR gene have been described by studying the property of altering restriction endonuclease sites of ApaI, BsmI, Fok and TaqI enzymes [89]. These polymorphisms were associated to several autoimmune disorders such as multiple sclerosis or Graves' disease [90,91]. In German adult patients with AIH type 1 and 2, two polymorphism of VDR receptor showed a significative linkage with disease [92]. TaqI is a polymorphism restriction site in the exon 9 of the VDR gene, if present, it results in a silent mutation. It is quite improbable a biological effect of this polymorphic site, but it could act as mutation marker. Patients with AIH carries this allele in homozygous fashion with decreased frequency than in unaffected subjects [ $p=0.01$ , OR=0.53]. In contrast, the Fok site, situated in the exon 2, results, if present, in a protein with 3 additional amino acids. This allele is also negatively linked with AIH

patients ( $p=0,004$ ,  $OR= 0.44$ ). Early reports indicate a biological action of this polymorphism but it is not possible yet to explain the role of this mutation on the pathogenesis of AIH [93].

Another interesting research field is to elucidate mechanisms through apoptosis preserves self-tolerance inducing deletion of auto reactive immunocytes clones through the activation induced cell death (AICD). Programmed cell death, accomplished mainly by the interaction of Fas receptor with the Fas ligand [94,95], but also through the TNF-receptor I with a complex reciprocal influence [96], is under control of several anti-apoptotic factor such as IL-4, IL-7 and IL-15 through the up-regulation of the anti-apoptotic protein Bcl-2. It may contribute to preserve the high tolerogenic environment normally present in the liver which is exposed to several xeno-antigens carried by the portal blood. Liver cells express at high levels the Fas receptor while Bcl-2 is present at low levels [94]. Few studies had explored the role of Fas gene as immunomodulatory factor in autoimmune liver disease focusing on the A670G polymorphism and disclosing a positive association with disease severity [97]. Moreover recent data shows that the Fas protein is highly expressed in hepatocytes of patients affected by type 1 autoimmune hepatitis and, typically, the morphologic picture of the Fas protein expression reproduces the histological picture of liver damage [98]. These preliminary data suggest that apoptosis, eventually induced by T cytotoxic cells, might have a prominent role in mediating liver damage. This field represents a hot spot for future investigations.

### Environmental Factors

There is circumstantial evidence that the several known and unknown genetic predisposing factors need an environmental triggering factor to cause fully clinically developed disease. Indeed, even if many speculations on the pathway of the pathological immune response and of the effectors of damage recently aroused, the answer to the following question is still missing: why a small group of the genetically predisposed population develops a self-perpetuating autoimmune disease?

The ideal candidate to represent the triggering factor must be able to cause primitive liver damage and dispersion of cellular debris and cryptic liver antigens.

Many drugs cause liver damage, from mild and transient cell damage to fulminant hepatic failure. Apart from a toxic, predictable, dose-dependant, liver damage caused by drugs like acetaminophen most drug-induced adverse reactions are unpredictable. They occur in 0.01 to 1% of exposed individuals and have an intermediate or long latency onset [99]. Hypersensitivity and immune-mediated reactions appears with 1 to 8 weeks of latency, usually disappears with drug withdrawal and rapidly relapse on rechallenge [99,100]. They can be hardly distinguished from AIH and are associated with immunopathological events as rash, eosinophilia and autoantibodies production and some of these autoantibodies are similar to those present in AIH as ANA, SMA and LKM. The pathogenesis of drug-induced immune-mediated liver diseases is focused on the phase I of drug metabolism by the cytochromes P450 (CYPs) involved

in drugs detoxification where high reactive intermediates are generated. It is currently believed that the macromolecular adducts of these products can be targeted by immune system leading to autoantibodies production [101]. The most studied drugs supposed to cause immune-mediated liver damage are Halothane, Tienilic acid, Anticonvulsivants, Dihydralazine and Minocycline. Typically all these drugs are associated to the LKM or LM autoantibodies (staining rat liver but not kidney microsomes) and, curiously, the molecular target of these autoantibodies are the CYPs involved in that particular drug metabolism (i.e. CYP 2E1 for halothane, CYP 2C9 for Tienilic acid, CYP 1A2 for Dihydralazine and CYP 3A6 and CYP 2C4 with a less clear mechanism for Minocycline) [102-105]. Despite to these evidences, the pathogenic mechanism is still quite unclear. A number of drugs cause covalent binding of macromolecules but rarely or never cause immune-mediated disease. Moreover, several individual shows transient LKM or LM reactivity after exposition to drugs without signs of disease. The complete pathogenesis is alike to be more complex and, curiously, the genetic background of these patients is poorly known and it could be similar to that of AIH patients.

Drug-related, immune-mediated liver diseases are entities distinct from AIH, although clinical features are similar, they have different molecular specification of autoantibodies and the disease spontaneously resolves after drug discontinuation. The triggering of an idiopathic, self-perpetuating AIH is alike to be exceptional even if recently reported [106].

Infectious agents, with special regard to viruses, are commonly cited as potential triggers for autoimmune disease. Even if the exact mechanism by which they could cause the pathological immune reaction is unknown, some theoretical models of interaction were formulated on the basis of several observations [107].

First of all, a virus may specifically trigger an autoimmune reaction through the molecular mimicry phenomenon. In other words, it can display some fragments of structural or enzymatic polypeptides that are similar to that of the host and capable of cross-reacting with the effectors of immune system [i.e. antibody or cytotoxic cells]. This process may concern both linear and conformational epitopes. Distinct but structurally related exogenous peptides could be recognized by specific, auto-reactive cells like T-cells specific for myelin basic protein in patients with multiple sclerosis [108]. Consequently a sequence-comparing approach may fail on recognizing the cross-reaction of structurally related peptides as well as it may indicate as candidate identical peptides without any clinical relevance *in vivo*. Despite the great effort to demonstrate the role of molecular mimicry as a trigger of autoimmune diseases, to date no conclusive prove had been shown and no definite animal model exist [109]. Even the homology between the GAD65 enzyme and peptides by Cocksackie B4 virus, thought to be one of the sharpest examples of molecular mimicry in humans in the pathogenesis of diabetes mellitus type 1, has been challenged and bystander activation of T-cells by Cocksackie virus infection is also invoked as main pathogenic factor [110]. The so-called bystander effect consists in a non specific up regulation or deregulation of

immune reactivity which occurs in a specific target tissue. It is mediated by several factors as local expression of proinflammatory cytokines and/or activation of formerly silent auto reactive immunocytes and even facilitation of super antigen mediated T-cell proliferation. In this way common viral infections localized in a specific organ can produce an organ-specific autoimmune disease in a genetically susceptible host [111,112].

Several viruses had been proposed as triggering factor in the pathogenesis of AIH such as Measles Virus, Epstein - Barr virus, HAV or HSV. Molecular mimicry had been equally invoked between CYP2D6 and the IE 175 protein of HSV [113-116], but presently none of these viruses is considered as a specific cause of AIH in genetically susceptible individuals.

### Autoantigens

The immune response, independently of the trigger, develops against one or more autoantigens. Recognition of these autoantigens might be the key factor to develop an etiologic-based therapy. Unfortunately most of the antigens recognized by autoantibodies detected in AIH are either non-specific or intracellular molecules, unlikely involved in breaking self-tolerance and in provoking the emergence of liver-infiltrating immunocytes. The most likely candidate autoantigens seems to be the asialoglycoproteins receptor (ASGP-R) for type 1 and the cytochrome P450 2D6 for type 2 AIH.

The ASGP-R is an organ specific molecule located in the membrane and with a prevalent periportal expression [117,118]. Both peripheral and infiltrating lymphocytes taken from adults and children as well with AIH show a proliferative response to purified human ASGP-R and can induce autologous B-lymphocytes to produce anti-ASGP-R autoantibodies *in vitro* [119-122]. A lack in T-suppressing function specific for the ASGP-R has been also reported both in patients and in their healthy relatives. This defect seems to reside in a subpopulation of CD4+ve T-cells and it is inherited as an autosomal, non-HLA linked trait and it is corrigible by immunosuppressive therapy [123]. Unfortunately, autoreactivity against ASGP-R is not AIH-specific and its pathogenetic role it's far from to be defined.

Seven isoforms of cytochrome P450 are highly expressed in human liver; 2 major: CYP2C, CYP3A, that account for 50% of the total CYP expression, and 5 minor isoforms, CYP1A2 (13%), CYP2E1 (7%), CYP2A6 (4%), CYP2D6 (2%) and CYP2B6 (0,4%) [124]. All these isoforms have been identified as LKM target specificities in different types of liver diseases as autoimmune, viral and drug induced liver diseases [125]. The cytochrome P450 2D6 (CYP2D6), the likely molecular target of autoimmune hepatitis type 2, is an intracellular enzyme active in phase I detoxification of several drugs. The CYP2D6 was extensively studied to map the most frequent epitopes in LKM-1. Several short sequences were identified and, commonly, each of them was labeled, *via* Gene bank searches, as cross-reacting with viral proteins or human proteins involved in other autoimmune disorders. The 257-269 region of the CYP2D6 is similar to a sequence of the IE 175 protein of the HSV-1 [126]; the 321-351 region, a dominant epitope, cross-react with the 31-51

region of carboxypeptidase H and the 307-325 region of 21-hydroxylase, putative autoantigens in IDDM and Addison's disease. The sequence 193-212 of CYP2D6 is recognized by 93% of the AIH-2 sera and 50% of the LKM-1 positive hepatitis C sera and presents extensive cross-reaction with HCV and CMV peptides [127]. Furthermore, inhibition studies of the CYP2D6 enzymatic activity showed clearly that conformational epitopes exist and are functionally prevalent [128].

By effect of some cytokines, CYP2D6 can be expressed on hepatocytes surface becoming a potential target for auto reactive T-cells [129,130]. It is able to induce proliferation of peripheral blood lymphocytes mainly of Th-1 phenotype [131] and it is also accounted to trigger an antibody-mediated cytotoxicity *in vitro*.

### Autoantibodies

Determination of autoantibodies is very helpful when diagnosing autoimmune hepatitis. Historically, the autoantibodies positively associated with autoimmune hepatitis are antinuclear antibodies (ANA), smooth muscle antibodies (SMA) and liver-kidney microsome autoantibodies type 1 (LKM-1) [2,4,17]. Their assessment is performed by immunofluorescence techniques, easy and widely available.

ANA and SMA are the hallmarks of autoimmune hepatitis type 1. They are usually present at high ( 1:100) titer. Usually patients with AIH type 1 present with significant titer of isolated SMA, associated to ANA in about half of cases. Only 5% of patients, or less, show an isolated ANA reactivity [4,17]. The presence of LKM-1 autoantibodies defines the AIH type 2 [2,4]. In children these classic autoantibodies are present almost in all patients [4]. In patients who does not carry these autoantibodies anti-liver cytosol type 1 autoantibody [LC1], as recently described, can be detected [19]. Twenty to 30 % of adult patients lack ANA/SMA/LKM-1 autoantibodies [132] and diagnosis can be suggested by the finding of anti-soluble liver antigen autoantibodies (SLA) [132,133] or by the presence of anti-neutrophils cytoplasm autoantibodies with perinuclear staining pattern (p-ANCA) [134] or of the anti-liver-specific asialoglycoprotein receptor (ASGPR) [135]. None of these autoantibodies is specific for AIH since they can be found either in several systemic autoimmune diseases or in liver diseases of different etiology such as, typically, the hepatitis C virus infection, when associated with immunological abnormalities [136]. Although some reports claim a good specificity for SLA [137], the identification of the target antigen is still debated [138]. Moreover there is not a wide experience with control populations [132].

Once diagnosis is made, the autoantibodies assessment has not a great clinical relevance. Autoantibody reactivity is fluctuating during treatment, reducing in titer in case of remission, but also independently to the remission [139]. Autoantibody status is not predictive of laboratory and histological features; moreover, high serum titers at presentation do not identify patients with more aggressive disease or different treatment outcomes. Finally disappearance of autoantibodies is not predictive of low risk of relapse during treatment or of sustained remission in case of stopping the treatment [140].

**Table 3. Characterization of autoantibodies present in autoimmune hepatitis in children.**

Antibody	Target antigen	Disease	Percentage
ANA	Various	AIH-1 Autoimmune sclerosing cholangitis	Up to 60%
SMA	F-actin	AIH-1 Autoimmune sclerosing cholangitis	Virtually 100%
LKM-1	Cytocrome P4502D6	AIH-2	Virtually 100%
LKM-3	Uridine-diphosphate-glucuronosyl-transferase	AIH-2	Rare
SLA	UGA-serine t-RNA-protein complex (UGA suppressor tRNA-associated protein)	AIH-1 AIH-2 Autoimmune sclerosing cholangitis	Up to 42% in both.
p-ANCA	Various	AIH-1 Autoimmune sclerosing cholangitis	Unclear
LC-1	Forminotransferase cyclodeaminase	AIH-2	Up to 30%
ASGP-R	asialoglycoprotein receptor	AIH-1 AIH-2	75% (AIH-1) 40% (AIH-2)

It must be remarked that, although all these antibody specificities can present isolated in AIH patients, specific association patterns exist. They are ANA/SMA/ANCA/ASGPR in type 1 AIH and LKM/LC1 in type 2 AIH. These are rigid associations, both in adults as in children, supporting the idea that the two subtypes of AIH are distinct in pathogenesis.

Here follows a brief description of all these autoantibodies [Table 3].

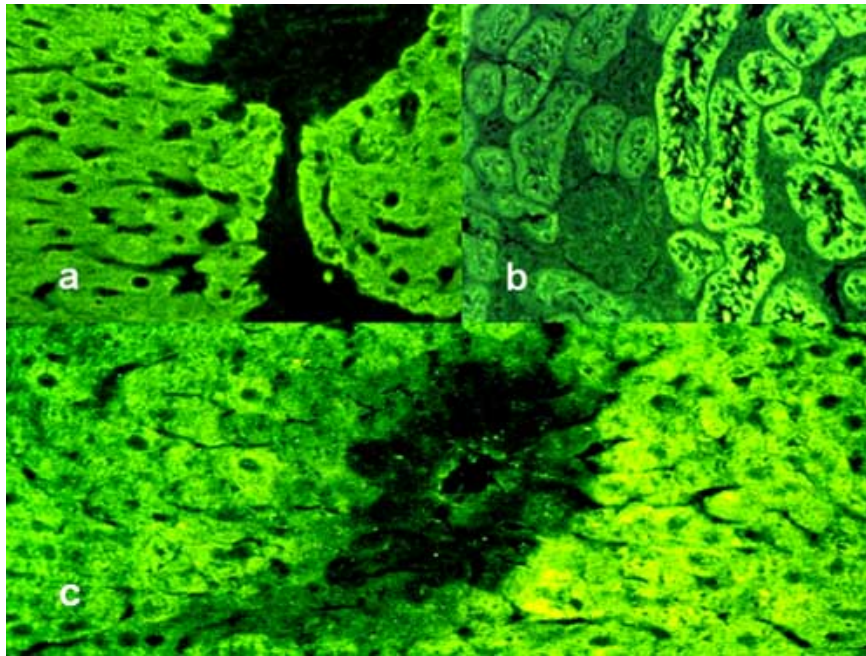
**Anti-nuclear antibody (ANA).** Up to 60% of children affected by AIH type 1 carry antinuclear autoantibodies. Rarely they are an isolated finding (4-8%), more commonly they are associated with SMA [4]. Similar data are reported in adults [139,140]. Usually, detection of ANA is made by immunofluorescence techniques on Hep-2 cells, less commonly on sections of rat liver. Various patterns of immunofluorescence were observed: homogeneous (50-60%), speckled (15-25%) mixed homogeneous/speckled (10%), centromeric (5-10%), homogeneous-perinuclear (3-6%) [141,142].

Several nuclear antigens, with a wide range of molecular weight, were identified as a target of ANA reactivity, with predominance of some of them for each pattern of immunofluorescence. ANA are a very heterogeneous group of autoantibodies and almost all subtypes that occur in rheumatologic disorders can be identified in AIH patients. Commonly, antigens like ss-DNA, ds-DNA, sn-RNP, t-RNA, or intranuclear proteins such as laminine A and C or cycline A are concerned [142,143]. Recently nuclear ribonucleoprotein A2/B1 was identified as a target antigen in AIH type 1 [144]. The ANA positive AIH patients have no peculiar features and, unlike to rheumatologic disease, there is no evidence of association of a particular antigen with AIH, with clinical manifestations of AIH or with the treatment outcome [139,140]. The pattern of immunofluorescence is not accounted to have clinical importance both in adults and children and it could vary in the same patient, during follow up [139,140].

The cut-off titer for the positivity of ANA is commonly indicated to be 1/40 in children [145]. Our experience suggest, in clinical practice, to raise the cut-point to 1/100 to avoid over diagnosis due to low specificity of such autoantibody.

**Anti-smooth muscle antibody (SMA)** is directed against structural components of the cytoskeleton such as actin, desmin and troponin. Antibody anti-smooth-muscle is present in 50-55% of children with AIH (up to 90% in AIH type 1) [4]. Equally, up to 80-85% of adults with AIH type 1 present SMA, isolated or associated with ANA [139,140]. Indirect immunofluorescence assay using rat stomach as substrate is commonly used to detect SMA. A specific staining of the muscularis mucosa in a uniform pattern is characteristic as well as the staining of the blood vessels walls and of the parietal cell. If a rat kidney tissue is used, a faint staining of the mesangial area of glomeruli and of the brush border of proximal renal tubular cells is also present. Also Hep-2 cells can be used as substrate and, if present, SMA are evidenced in "cable like" cytoplasmic staining [146]. Although SMA are present in different liver diseases, in autoimmune liver diseases are directed mostly against F-actin, present in hepatocytes as a part of the cytoskeleton in close proximity with the plasma membrane. Anti-actin autoantibodies are specific for autoimmune hepatitis [15,147], and their predictive value for AIH is very high, especially in children. Anti-actin positivity strongly correlates with the presence of the haplotype HLA A1 B8 DR3. However the presence of SMA is not specific of an autoimmune liver disease since they are also present in the serum of patients with infectious diseases or rheumatic disorders. SMA. With F-actin specificity can be detected using fibroblasts cultured in presence of colchicine or on Hep-2 cells as mentioned above [146]. Interestingly, adults with actin-positive autoantibodies are younger, more commonly DR3 positive and have a more severe disease than other patients with ANA and without actin reactivity [148].

**Anti-liver/kidney microsome antibody (LKM1).** Antimicrosome antibodies are a heterogeneous group,



**Fig. (2). Immunofluorescence pattern of autoantibodies in type 2 autoimmune hepatitis.** Anti-LKM1 reactivity staining rat hepatocytes homogeneously (a) and proximal renal tubules distally (b). Anti-LC1 reactivity staining rat hepatocytes sparing the centrolobular zone (c).

associated to a number of immunomediated hepatic diseases like drug-induced hepatitis, chronic viral hepatitis (HCV and HDV) and autoimmune polyendocrine syndrome type 1 [149]. The distinctive immunofluorescence pattern of the anti-liver/kidney microsome antibody type 1 [LKM-1] on rodent liver and kidney sections is a diffuse cytoplasmic staining of microsomes of hepatocytes and of the proximal renal tubular cells [146,150] (Figure 2). A weak staining of the distal tubules is occasionally present (10%) and could generate confusion with anti-mitochondrial autoantibodies. LKM-1 autoantibodies target a 50 kilodalton antigen identified as cytochrome P450 2D6 [CYP2D6] [151-154] and inhibits *in vitro* but not *in vivo* CYP2D6 activity [155]. LKM-1 are the hallmark of AIH type 2 and they are present in 6-10% of HCV-related hepatitis [156] even if the target epitopes are different also between adults and children [157,158].

The role of this antibody in the pathogenesis of liver cell injury is debated. The recent demonstration of the expression of CYP2D6 on liver cell surface [129,130] and the finding that liver damage in mice is induced by immunization with human CYP2D6 [159] suggests that LKM1 antibodies may play a role in inducing liver cell damage in AIH.

Finally, a different type of LKM autoantibodies, called LKM-3 is present rarely in AIH type 2 and in 10% of HDV-related hepatitis. They target the C-terminus of uridine diphosphate-glucuronosyl transferase 1 a 55 Kd liver microsomal protein and their significance is not known [160,161].

**Anti-liver cell cytosol antibody (LC-1).** This is an organ-specific autoantibody and its presence characterizes AIH type 2 [162,163]. LC1 was identified both by indirect

immunofluorescence, immunodiffusion and immunoblotting techniques. LC1 stains characteristically the cytoplasm of the rat hepatocytes in a homogeneous pattern sparing the cellular layer around lobular central veins without staining of the proximal renal tubules [162,164] (Figure 2). LC1 antibodies recognize a 58-62 Kd, liver specific, antigen [163]. Recently, the target antigen was identified and the cloned proteins had strong homologies with forminotransferase cyclodeaminase (FTCD) [165,166].

It can be found in association with LKM1 reactivity in about 50% of AIH type 2 [162,163,167,168] and occasionally it has also been described associated with anti-SMA and in adults with HCV-related chronic hepatitis. In some patients with autoimmune hepatitis anti-LC1 can be the sole autoantibody found [16].

**Anti-soluble Liver Antigen antibody (SLA).** Is a non specie-specific, non organ-specific antibody that reacts to the cytosolic fraction of different cellular lines but the highest reactivity is found on human liver homogenates [133]. The SLA assessment is currently performed by immunoenzymatic or radioimmunological assay. The target antigen is likely to be a 50 Kd, 422-aminoacids protein, as recently described by several authors [138-169], and it is currently acknowledged that it is identical to the *liver pancreas antigen*, the target of the liver/pancreas (LP) antibodies [170]. This protein, identified as an UGA-serine t-RNA-protein complex may belong to the super family of piridoxal phosphate dependent transferases and it could act as a serine-hydroxy-methyl-transferase [171]. Similarly *Gelpi et al.* identified this protein as a UGA suppressor tRNA-associated protein [172, 173].

SLA antibodies are present in 10-20% of patients with ANA/SMA positive AIH type 1 and, remarkably, in at least 20% of patients with cryptogenic chronic hepatitis, as unique marker of self-immunity [133,174, 175] even if in some recent reports, using a different assay in pediatric population, SLA were found in a remarkable percentage of patients with AIH type 1, AIH type 2, autoimmune sclerosing cholangitis and in approximately 10% of HCV infected patients [176-178]. SLA antibody might recognize a cohort of patients with more severe outcome and poor response to therapy [179].

**p-ANCA.** Antineutrophil cytoplasmic antibodies, with a perinuclear pattern, have been described in patients with AIH type 1, primary sclerosing cholangitis and/or ulcerative colitis patients [180-182]. It is well known that those antigens targeted by p-ANCA are quite heterogeneous, in some cases of AIH, lactoferrin (20%), catalase (19%), and enolase (16%) were identified as target antigens [183]. It was also reported that patients with a longer duration of disease or patients experiencing a relapse have more probably p-ANCA positivity [184]. The clinical significance of p-ANCA autoantibodies in AIH patients is really unclear and their routine determination is not recommended especially in children.

**Autoantibodies to asialoglycoprotein receptor (ASGP-R).** Since 70s, a lot of interest rose around autoantigens present on the hepatocyte membrane surface, since they were accessible to immune system and not segregated inside the cell, like nuclear antigens or actin do. A liver specific membrane lipoprotein (LSP) was identified [185] and the molecular analysis found that ASGP-R (asialoglycoprotein receptor) was a fundamental constituent [186].

Presently, the assessment of anti-ASGP-R antibodies is based on the availability of the purified protein. Two techniques had been standardized, a liquid-phase radioimmunoassay with ASGP-R from rabbit liver [187] and a solid-phase enzyme immunoassay with human ASGP-R [186]. ASGP-R is a liver specific glycoprotein located in the hepatocytes membrane and autoimmune reactivity directed against this antigen is present in the majority of untreated patients with AIH independently from the presence of LKM1 or SMA antibodies [188,189] however its titer was correlated to disease activity [190,191]. Anti-ASGP-R seems not to be correlated with age of onset, female gender or aminotransferase activity, but only with hypergammaglobulinemia [189]. These antibodies however are not specific of AIH since they can be found in HBV chronic hepatitis, in alcoholic liver disease and in primary biliary cirrhosis and have to be considered as a generic marker of an immune-mediated liver damage. The disease-specificity is fair, but it seems to rise significantly using the human antigen suggesting that some epitopes of human ASGP-R may be immunogenic only in AIH [188,187].

### Animal Model for Autoimmune Hepatitis

Several murine models of AIH have been developed by immunization with liver subcellular fractions or by over expression of proinflammatory cytokines such as interferon gamma in the liver, but none of them has been completely satisfactory. A new murine model has been recently

generated by DNA immunization using a plasmid constructed to produce a chimeric fusion protein containing the antigenic region of human CYP2D6 and of human forminotransferase cyclodeaminase (FCTD) that characterize the 2 self antigens of type 2 AIH together with the extracellular region of mouse CTLA-4 as an immunological modulator. Immunized mice showed elevated levels of alanine aminotransferase activity and an interface hepatitis on liver biopsy constituted mainly by CD4 positive T-helper cells, CD8 positive lymphocytes and B lymphocytes. Moreover mice developed anti-LKM1 and anti-LC1 antibodies. This model demonstrates how a break of tolerance against self-antigens through molecular mimicry can occur [159]. On the other hand, another new murine model for AIH hepatitis was created inducing the mouse liver to express osteopontin, a crucial factor for Th1 immune response [192]. This could suggest that an aspecific, enhanced immunocytes activation in liver may lead to damage and autoimmune phenomena as proposed by several authors invoking the "bystander effect" [110,111].

### DIAGNOSIS

The diagnosis of autoimmune hepatitis in children can be easy when all the typical hallmarks of the disease are present, such as the presence of another autoimmune disease in the same patient, hypergammaglobulinemia of IgG type with the presence of specific autoantibodies (LKM-1, LC-1) even at low titer. However in some patient the diagnosis may become a challenge and in this case it is usually made by a combination of clinical, serological and histological criteria and by the exclusion of other possible known causes of severe hepatic disease [145].

Even if histopathological features pathognomonic of autoimmune hepatitis are lacking, liver histology should be always performed at diagnosis if hemostasis allow it. The characteristic lesion is an *interface hepatitis* characterized by a predominantly lymphoplasmatic necro-inflammatory periportal infiltrate, with or without lobular involvement and by portal-portal or central-portal bridging necrosis, often with the formation of liver cell rosettes and nodular regeneration, even in the early stages, in the most severe cases. Usually features of biliary damage are absent and storage of metals like iron, copper or intracellular proteins are excluded by appropriate histochemical techniques.

In case of doubt, for example in absence of typical serum autoantibodies, it is mandatory to send a serum sample to a reference laboratory to investigate the presence of autoantibodies not routinely assessed such as SLA or LC-1. If the doubt persists, in case of severe cryptogenic inflammatory disease, once Wilson's disease is excluded, it should be advisable to attempt an immunosuppressive treatment for at least 6 weeks, to evaluate the sensitivity of the disease to an immunosuppressive therapy.

To help diagnosis of autoimmune hepatitis a panel of physicians and pathologists have published a descriptive set of criteria to classify patients as having either "definite" or "probable" autoimmune hepatitis [8] (Table 4). This scoring system has been used in a large number of studies concerning 983 patients and it has shown a good sensibility (89,9%), but a low specificity (60,8 %), particularly in case

**Table 4. Diagnostic criteria for autoimmune hepatitis established by the International Association for the Study of Autoimmune Hepatitis [145]. A “score” > 15 before treatment and > 17 after treatment corresponds to a diagnosis of definite AIH. A score between 10 and 15 before treatment and between 12 and 17 after treatment correspond to a diagnosis of probable AIH.**

Parameter	Score
<b>Gender</b>	
Female	+ 2
Male	0
<b>Biochemistry</b>	
Ratio alkaline phosphatase/aminotransferase	
3.0	- 2
1.5 - 3	0
< 1.5	+ 2
<b>Gamma globulins or serum IgG</b> [times x upper normal limit]	
2	+ 3
1.5-2	+ 2
1 -1.5	+ 1
< 1	0
<b>Auto-antibodies [titers by immunofluorescence]</b>	
ANA, SMA, anti-LKM1	
> 1:80	+ 3
1:40	+ 2
< 1:40	+ 1
<b>Anti-mitochondrial antibody</b>	0
present	- 4
absent	+ 4
<b>Others auto antibodies</b> [ anti-LC1, anti-SLA]	+ 2
<b>Hepatotropic viruses infection markers</b>	
presents	- 3
absents	+ 3
<b>History of drug usage</b>	
present	- 4
absent	+ 1
<b>Alcohol [average consumption]</b>	
< 25 g/day	+ 2
> 60 g/day	- 2
<b>Association with other autoimmune diseases</b>	+ 2
<b>Association with HLA DR3 o DR4</b>	+ 1
<b>Response to therapy</b>	+ 2
<b>Relapse</b>	+ 3
<b>Liver histology</b>	
Interface hepatitis	+ 3
Predominant lymphoplasmacytic infiltrate	+ 1
Rosetting of hepatocytes	+ 1
None of the above issues	- 5
Biliary lesions	- 3
Others changes	- 3

of immune mediated biliary diseases like sclerosing/autoimmune cholangitis or primary biliary cirrhosis, who often score as a “probable” autoimmune hepatitis. In 1999 the score has been reviewed [145]. However this scoring system still remains more adapted to adulthood than to childhood, since some items, for example concerning the

of alkaline phosphatase in exploring the alkaline phosphatase/aminotransferase ratio or alcohol consumption, are not easily adaptable to children.

## TREATMENT

Currently, the most effective therapy for AIH is immunosuppression. The standard initial treatment includes prednisone as monotherapy or a combination of prednisone and azathioprine [193]. Prednisone or prednisolone is used at the dose of 2 mg/kg/day with a maximal daily dose of 60 mg in the adolescent, and Azathioprine is prescribed from 1.5 to 2 mg/kg/day. Combination therapy is generally preferred because of the significant reduction of the severe side effects due to high dose steroids. Teratogenicity and oncogenicity issues of azathioprine in humans have not been conclusively demonstrated. However pregnancy should be ruled out in the adolescent girl before starting treatment, since azathioprine therapy during pregnancy cannot be recommended.

**Efficacy of the treatment.** The first goal of the treatment is to induce a remission of the clinical (jaundice, hepato/splenomegaly) and biochemical “activity” of the disease (aminotransferases, gamma globulins). Treatment is generally able to induce clinical, laboratory and histological improvement in most patients with AIH and it may decrease hepatic fibrosis by suppressing the inflammatory activity and the immune mechanisms of liver injury. Clinical and laboratory remission can be achieved in 6 to 10 weeks and it is generally followed by restoration of liver function as demonstrated by the normalization of prothrombin activity [21]. Few cases, particularly in case of severe liver function impairment at onset, however deteriorates despite compliance with therapy. In case of treatment failure a “rescue” immunosuppressive therapy including cyclosporine, as a third immunosuppressant agent, should be assayed. Treatment failure should, in the same time, lead to discuss the opportunity of a liver transplantation.

**Sustained response.** Once remission achieved, the goals of treatment become to maintain remission and to prevent a relapse. The dose of prednisone should then be progressively reduced, by 2.5 mg, to 1 mg/kg/day or to 2 mg/kg/every other day. Different therapeutic schedules exist, however discontinuation of therapy should be tailored in individual patients in relation to the characteristics of onset. The shift to every other day use of corticosteroid has been associated with a lower incidence of side effect particularly concerning growth [21]. Every other day dose of prednisone is inversely related to patient’s age with the necessity of lower doses to maintain remission [0.2 to 0.4 mg/kg/every other day] in the adolescent [21]. Thereafter the dose of corticosteroids should be reduced to the lowest dose compatible to maintain a clinical and biochemical remission (strictly normal aminotransferases and gamma globulins levels). A bad control of the disease, once the remission achieved, is in most cases related to an inappropriate patient’s compliance to the treatment prescribed.

To demonstrate a histological remission by performing a liver biopsy in a patient with long standing, complete, biochemical remission is a question of debate. Histological remission lags 3 to 6 months behind normalization of liver biochemistry and complete histological remission is not

predictive of absence of relapse [21]. For these reasons we doubt that biopsy-proven remission is required for deciding the modality of discontinuation of treatment.

**Progression of fibrosis.** Hepatic fibrosis progress only in a minority of patients who are compliant to treatment and who maintain a persistent remission. In some case fibrosis can even diminish during treatment. Patients with progressive fibrosis more commonly carry HLA DR3/DR4 [26].

**Duration of treatment.** No evidence-based data exist on the optimal duration of immunosuppressive treatment. Relapse is frequent if treatment is withdrawal within the first two years. We believe that sustained remission should be maintained for at least five years, then in case of treatment combining prednisone and azathioprine, prednisone should be stopped during the sixth year maintaining azathioprine for at least another year. Azathioprine maintenance has been show to reduce the likelihood of relapse [194]. Absence of serum autoantibodies or of inflammatory activity in the liver biopsy are not absolutely predictive of an absence of relapse. However a sharp increase of the titer of autoantibodies suggests caution in reducing the immunosuppression.

**Side effects of treatment.** Combination therapy is associated with side effects mostly caused by an inappropriately high dose of prednisone producing severe cosmetic changes such as obesity, growth failure, dorsal hump formation and cutaneous *striae*. Severe side effects are less frequent and include vertebral collapse, secondary diabetes cataracts responsible of severe visual loss and psychosis. Rarely azathioprine is responsible of severe side effects such as severe cytopenia necessitating a dosage reduction.

**Alternative treatments.** Failure to respond to conventional treatment or severe side effects of corticosteroids are an indication to the use of cyclosporine A. Cyclosporine therapy is effective in inducing remission in patients with AIH [195-197]. Side effects of cyclosporine treatment were few, well tolerated and disappeared after reduction of the dose [195-197]. Once the remission achieved treatment may be continued at low dose or patient may be shifted to conventional treatment [197].

Mycophenolate-mofetil was recently successfully employed in addition to steroids in patients who either did not tolerate azathioprine or did not respond to standard therapy suggesting that it may represent another alternative strategy of treatment [198].

Liver transplantation is the treatment option of choice in end stage AIH or in patients with acute severe/fulminant onset who do not respond to rescue immunosuppression [51].

**Long term results.** Long term prognosis of children with AIH remains uncertain. A sustained remission can be maintained in most patients without notable side effects with low-dose immunosuppression. A limited number of patients maintain a sustained remission once the treatment stopped. Some patients with cirrhosis, in absence of an evident relapse of the disease, may develop a progressive liver insufficiency and need liver transplantation.

In conclusion autoimmune hepatitis is a rare, but life-threatening liver disorder. This disease should well kept in mind by the pediatrician because this disease frequently start in childhood or within adolescence before young adulthood and also because its frequency is increasing. The disease can, in most cases, be efficaciously controlled by an appropriate immunosuppressive treatment and an early diagnosis may prevent its natural evolution toward cirrhosis and liver failure.

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