Immunologic Research (2019) 67:39–47
https://doi.org/10.1007/s12026-019-9068-1

ORIGINAL ARTICLE

Tryptophan-kynurenine profile in pediatric autoimmune hepatitis

Simon D. Lytton[1] · Marcin Osiecki[2,3] · MałgorzataWoźniak[2,3] · Bożena Cukrowska[2,3] · Aldona Wierzbicka[2,3] · Maria Goliszek[2,3] · Piotr Socha[2,3] · Wojciech Janczyk[2,3] · Dilek Dayanakli[3] · Dieter Abendroth[4] · Sabine Kramp[5] · Kai Fechner[5] · Thomas Scheper[5] · Michael Mahler[6] · Chelsea Bentow[5] · Dimietrios Bogdanos[6] · Dietmar Fuchs[7] · Marek Woynarowski[2,3]

Published online: 21 January 2019

Abstract

The impairment of regulatory T cells (Tregs) is a characteristic feature of autoimmune hepatitis (AIH), and the degradation of tryptophan (Trp) to kynurenine (Kyn), by gamma interferon-induced indoleamine-2,3-dioxygenase-1 (IDO-1), is a central metabolomics check point in the differentiation of Tregs. For this reason, we investigate whether or not Kyn and IDO activity is potentially useful biomarkers in pediatric AIH.

Between January 2016 and January 2017, children of AIH type-1 (AIH-1, $n = 37$), AIH type-2 with liver kidney microsome-1 autoantibodies (AIH-2-LKM-1, $n = 8$), and autoantibody-negative Wilsons Disease (WD, $n = 8$) and alpha-1 anti-trypsin deficiency (AATD, $n = 10$), were enrolled in a cross-sectional survey of Kyn and Trp levels and Kyn/Trp ratios (IDO activity) by HPLC, and neopterin levels by ELISA.

The mean Kyn and mean Kyn/Trp ratios of AIH-1 with smooth muscle antigen (SMA) 1.85 μM and 27 μmole/mmole, and AIH-2-LKM-1; 1.7 μM and 28.6 μmole/mmole were lower than that of the WD; 2.2 μM $p = 0.03$ and 33 μmole/mmole $p = 0.02$ and of AATD; 2.3 μM, $p = 0.02$ and 55 μM, $p = 0.001$. Kyn/Trp ratios of AIH relapse; 23.6 μmole/mmole were lower than Kyn/Trp ratios of AIH remission; 27.6 μmole/mmole ($p < 0.05$). The stage of liver disease and grade of liver biopsies in AIH-1 patients negatively correlated with the Kyn/Trp ratios.

The serum Kyn levels and Kyn/Trp ratio of AIH patients, within or below the normal range, indicate a trend of IDO activity lower than non-autoimmune WD or AATD. Prospective monitoring of serum tryptophan metabolomics in larger cohorts of pediatric AIH patients is required to confirm the apparent paradigm of weak IDO activity contributing to the Treg deficit and pathogenesis of pediatric AIH.

Keywords Pediatric autoimmune hepatitis · Autoantibodies · Tryptophan · Kynurenine · Treg cells · Serum IDO activity

✉ Simon D. Lytton
simon.lytton@t-online.de

[1] SeraDiaLogistics, Benediktenwandstr 7, 81545 Munich, Germany

[2] Children's Memorial Health Institute, Warsaw, Poland

[3] Zentrum für Chirurgie Universitätsklinikum Universität Ulm, Parkstr 11, 89073 ULM, Germany

[4] Division of Biological Chemistry, Biocenter Innsbruck Medical University, Center for Chemistry and Biomedicine, Innrain 80, 4th Floor, Room M04-313, A-6020 Innsbruck, Austria

[5] Euroimmun AG, Seekamp 31, D-23560 Lübeck, Germany

[6] Department of Rheumatology and Clinical Immunology, University General Hospital of Larissa, Faculty of Medicine, School of Health Sciences, University of Thessaly, Viopolis, 40500 Larissa, Greece

[7] Inova Diagnostics, 9900 Old Grove Road, San Diego, CA 92131, USA

Abbreviations

AIH-1	type 1 autoimmune hepatitis
AIH-2	type II autoimmune hepatitis
ALT	alanine aminotransferase
IDO	indoleamine-2,3-dioxygenase-1
Tregs	T-regulatory cells
IFNγ	interferon gamma
IFA	immune fluorescence assay
ANA	anti-nuclear antigen
AST	aspartate aminotransferase
AZA	azathioprine
GGTP	gamma-glutamyltranspeptidase
LC1	anti-liver cytosol antigen 1
LKM-1	anti-liver kidney microsome 1
SMA	anti-smooth muscle antigen
AATD	anti-alpha trypsin deficiency

WD	Wilsons Disease
Kyn	kynurenine
Trp	tryptophan

Introduction

Autoimmune hepatitis (AIH), incidence of 1–3 per 100,000, is a progressive inflammatory liver disorder attributed to interactions between individual immune genetics and environmental exposures. The numerical and functional impairment of Treg cells is considered imperative for the development and the progression of the disease [1–5]. The established AIH international guidelines (IAIHG) provide the best and most probable assessment but not definitive diagnosis [6–10].

The strict classification of pediatric AIH according to autoantibody profile, mainly the smooth muscle antigen (SMA) and F-actin of type 1 AIH, and the liver kidney microsome 1 (LKM-1) and liver cytosol antigen 1 (LC1) of type 2 AIH, is important at initial diagnosis [11–15, 17] to distinguish between forms of AIH and to rule out liver inflammation due to inborn errors of metabolism, viral infection, and drug hepatotoxicity. Autoantibodies in AIH-1 have limited clinical relevance for the follow-up of patients [12, 18, 19] as evident by their low titer or seroconversion to negative SMA/F actin autoantibodies after treatments with steroids and/or immune suppressive drugs [11, 12]. Children or young adults that show loss of anti-LKM-1 expression may retain elevated serum transaminase and eventually require liver transplantation even though they test LKM-1 negative [13–15]. Autoantibodies to soluble liver antigen (SLA), liver protein (LP) develop in more severe autoimmune liver disease [13, 18] and autoantibodies to pyruvate dehydrogenase complex, multi-enzyme complexes within the mitochondria inner membrane (AMA) and rare autoantibodies associate with primary biliary cholangitis and overlap syndromes [19–23].

Transaminases, bilirubin, and immunoglobulins, routinely used for monitoring the hepatic inflammation of AIH [25, 26] and WD [27], revert to normal range after steroids with or without azathioprine and immune suppressive drugs in approximately 80% of AIH cases [28]. The infiltrates of intrahepatic self-reactive lymphocytes which lead to cholestasis and fibrosis [2, 4, 5] coexist in the inflamed human liver with foxp3+ Treg cells and CD4+ Th17 [1, 2, 5]. Although immune suppressive treatments diminish the interferon gamma production and effect PD-1 T cell subsets of pediatric AIH, the clinical relevance of immune modulation remains speculative [3]. Thus, the identification of new biochemical and immunological markers which correlate with liver histopathology and predict sustained remission in AIH would vastly improve clinical practice [29–32].

Dysregulated tryptophan (Trp) catabolism and the activity of IFNγ/IL-1-induced rate limiting enzyme indoleamine2,3-dioxygenase-1 (IDO-1), major pathways of immune activation and inflammation, have proven useful indicators of IFN-gamma and Treg cell responses. Increased IDO and PD1-Tregs are implicated in the promotion of tumor growth whereas decreased IDO activity is linked to deficit of functional Treg cells in autoimmunity [33–40]. The Trp degradation metabolite kynurenine (Kyn), generated via IDO in the lymphoid cells, is a key checkpoint of the Th17/Treg balance in graft rejection [41, 42], tumor immunity [33, 34], and autoimmunity [35–40]. Serum Kyn levels are elevated in chronic viral infections [43–47] and neurological inflammation [34, 48].

Neopterin, a byproduct of purine metabolism, is produced by monocytes and macrophages upon stimulation with IFN gamma through the enzyme GTP cyclohydrolase and is an established biomarker for monitoring IFN gamma immune activation. In cases where neopterin levels positively correlate with Kyn/Trp ratio, the gamma interferon-induced IDO activity is assessed [40, 49, 50]. Despite the widespread use of serum IDO activity and neopterin for the assessment of inflammation and immune activation, there are so far no reports of tryptophan degradation and neopterin in AIH.

For this reason, we investigate whether or not the serum Kyn levels and IDO activity, indicated by Kyn/Trp ratios would represent a practical and non-invasive approach to stratify pediatric AIH according to tryptophan metabolism.

The aim of this study is to investigate tryptophan degradation in autoantibody-positive pediatric AIH compared with pediatric non-autoimmune genetic metabolic liver disease and to determine if the levels of serum Kyn and the Kyn/Trp ratio of AIH patients associate with the liver pathology scores, serum transaminases, or neopterin.

Patients and methods

Patient population and clinical examinations

Pediatric patients ($n = 63$), AATD (Group I, $n = 10$), WD (Group II, $n = 8$), AIH-1 (Group III, $n = 37$), and AIH-2 (Group IV, $n = 8$) were enrolled in the study between January 2016 and January 2017.

The AATD patients included 10 patients homozygous for the mutant Z allele (PiZZ); all developed liver failure and underwent liver transplantation within mean 9 years (range 3–15 years); 5 of them were tested before orthotopic liver transplantation, the remaining after transplantation at various times points. All patients developed liver failure and underwent liver transplantation within mean 9 years (range 3–15 years) after the date of serum sample collected at initial diagnosis. The diagnosis of WD with EASL score 3.3 ± 0.82 had serum ceruloplasmin 113 ± 50 mg/dL by nephelometry (Siemens BN Prospec) and urinary copper 112 ± 57 μg/24 h by atomic absorption spectrometry. Mutations of ATPase

copper transporter ATP7B were found in either one allele (n = 5) or both alleles (n = 8) by PCR [27]. The diagnosis of AIH was based on typical laboratory and liver histology abnormalities and IAHG score [6, 9]. All biopsies were assessed according to Batts and Ludwig classification [25] by single pathologists in order to avoid inter-observer error [26]. Liver biopsies were performed in all but three AIH cases; one with AIH-2 and two with AIH-1. The three pediatric AIH cases were cirrhotic and liver biopsy was contraindicated (1 case) or the parents refused their child to undergo liver biopsy (2 cases). The AIH patients newly diagnosed (n = 2) and in relapse (n = 14) were assessed according to active liver disease with increased ALT values and immunoglobulins. The AIH patients in remission (n = 29) were assessed according to the normal ALT levels and inactive liver disease after steroid and immune suppressive treatments.

The mean disease duration of WD was 4 years (range 1–8); AIH-1, 2 years (range 0.2–6); and AIH-2, 5 years (range 0.4–11). At the initial workup, viral liver disease (HAV, HBV, and HCV) was excluded in all patients. *Alpha-1 anti-trypsin and ceruloplasmin* were normal in all AIH subjects, 170 ± 33 mg/dl and 36 ± 15 mg/dl respectively. The WD and all groups of AIH patients enrolled in the study had laboratory hematological values within normal range. During outpatient care, clinic liver function tests were evaluated.

Abdominal Doppler ultrasound examination was performed if indicated to assess the portal blood flow and to witness portal venous hypertension as a complication of cirrhosis [51]. AIH therapy consisted of prednisone dosage initially 2 mg/kg/day with azathioprine dosage 1–2 mg/kg/day. The remission of the disease was defined as full normalization of ALT. When remission was achieved, prednisone was tapered to 5–15 mg each second day, while azathioprine was continued under blood level control. Relapse of AIH was diagnosed when ALT flare appeared noted and thus the doses of steroids have to be increased. UDCA, 15 mg/kg/day, was introduced in case of concomitant biochemical cholestasis. The main comorbidity in the cohort was ulcerative colitis, diagnosed in 4 patients. Single patients were diagnosed with inflammatory bowel disease, Crohn disease, coeliac disease, and diabetes mellitus type I with concomitant autoimmune thyroiditis. The encephalopathy was assessed according to West-Haven scale.

Autoantibody and biochemical tests

The initial screening was 1:40 sera dilution detected by commercial indirect immunofluorescent tests using HEp-2 cells for ANA, and rat panel substrates (stomach, liver, and kidney) for smooth muscle antigen (SMA) (Mosaic Basic Profile, Euroimmun, Germany). The anti-mitochondrial antigen subunit 2 (AMA-M2) and liver kidney microsomal 1 cytochrome P450-2D6 (LKM-1) at cut-off of 1/10 was considered positive (Mosaic Basic Profile, Euroimmun, Germany). Further serum dilutions were performed to investigate the F-actin specific reactivity on VSM47 cells, the patterns on rat liver, stomach, and kidney substrates and HEp-2 (Biochip Mosaic, Euroimmun, Germany) and the ANA immune fluorescence patterns; homogenous, granular, chromosomal, nuclear, and cytosolic (Liver Mosaic 8, Euroimmun, Germany). Autoantibodies were tested by different ELISA systems as follows: AMA-M2-IgG, AMA-M2-IgM, liver cytosol 1 formiminotransferase cyclodeaminase (LC1), LKM-1 and soluble liver antigen and liver-pancreas antigen (SLA/LP) ELISA (Euroimmun, Germany); F-Actin, LC-1 and LKM-1 QUANTA Lite® ELISA (Inova Diagnostics, USA) and ASGP-R ELISA (Generic Assays Medipan, Germany) [16]. Kelch-like 12; KELCH and hexokinase; HK-1 autoantibodies were assessed in non-immunoblots and ELISA (Inova Diagnostics) [22]. The differential IgG immunoblots were performed on Euroline autoimmune liver disease recombinant proteins (Euroimmun, Germany); 74Kd pyruvate dehydrogenase full-length AMA-M2 and subunit M2-3E; speckled protein (sp100) and glycoprotein (gp210), promyelocytic leukemia protein (PML), LKM-1, LC1, SLA/LP, 60Kd soluble substance A (SSA), 52 Kd interferon-inducible protein (Ro52) belonging to the tripartite motif family of proteins, topoisomerase 70Kd (Scl70), centromere A (CA), centromere B (CB), and phosphoglycerate dehydrogenase (PGDH). The signal band intensities in EUROLine scan of 6–10 and > 11 were reported as borderline and positive respectively.

Biochemical markers (ALT, AST, and GGTP) were assessed by enzymatic-colorimetric method using Roche COBAS p 512 analyzer. Total IgG was measured using nephelometric method [24]. All samples were tested with the Siemens BNII analyzer utilizing Siemens (total IgG) reagents and capillary zone electrophoresis of serum proteins was performed on a MiniCap instrument (Sebia) to measure gamma globulin concentration.

Kynurenine and neopterin measurements

All serum samples were in frozen storage (− 20 to − 30 °C) for longitudinal *en bloc* Kyn measurements. The concentrations of Trp and Kyn were measured by reverse-phase HPLC method, using a Varian ProStar HPLC system equipped with a solvent delivery module (model 210), an auto sampler (model 400, both Varian ProStar), an UV-spectrometric detector (SPD-6A, Shimadzu), and a fluorescence detector (model 360, Varian ProStar) [49, 50]. In brief, 200 μL of serum, 200 μL of internal standard, 50 μmol/L 3-nitro-L-tyrosine, and 50 μL of 2 mol/L trichloroacetic acid were vortexed and centrifuged to precipitate proteins and generate the supernatants that were measured. The IDO-1 activity was estimated by the calculated Kyn/Trp ratio expressed as μmol Kyn per mmol Trp. The concentrations of neopterin, 6-(D-erythro-1′,2′,3′-trihydroxypropyl)-pterin, were determined by ELISA (BRAHMS, Hennigsdorf, Germany).

Table 1 Pediatric patient characteristics

	AATD $n = 10$	WD $n = 8$	AIH-1 $n = 37$	AIH-2 $n = 8$
Characteristic				
Age, yrs.	6 (2–10)	8 (2–17)	15 (8.75–18)	13 (6.5–16.5)
Gender (f/m)	3/7	4/4	19/18	5/3
Disease duration yr	NA	4 (1–8)	2 (0.2–6)	5 (0.4–11)
ALT, U/L	NA	60 (22–184)	24 (6–226)	18 (9–398)
AST, U/L	65 (17–109)	37 (23–104)	27 (14–554)	22 (17–51)
GGTP, U/L	308 (14–1129)	24 (12–115)	27 (7–637)	13 (8–53)
γ-Globulins, g/L		12 (8–14)	14 (4.7–29)	13 (7.9–15)
Bilir.Direct, mg/dL	0.6 (0.1–1.4)	0.21 (0.1–0.38)	0.23 (0.1–0.39)	0.32 (0.16–0.58)
Bilir.Total, mg/dL		0.56 (0.19–1.02)	0.54 (0.21–5.9)	0.6(0.36–2.2)
INR	1.2 (0.9–10)	1.08 (0.9–1.2)	1.09 (0.87–1.4)	1.1 (0.95–1.2)
Portal flow cm/s	NA	26 (14–35)	21 (13–45)	21 (12–30)
Echo E		5/8	2/37	2/8
Echo H		0/8	6/37	1/8
IAHG score	NA	NA	7 (4–8)	7 (5–8)
Liver grade	NA	1 (0–3)	3 (0–4)	1 (0–3)
Liver stage	NA	2 (0–3)	2 (0–4)	1 (0–3)
Therapy				
Steroids	0/10	0/8	30/37	3/8
Az	0/10	0/8	36/37	4/8
UCDA	0/10	0/8	36/37	1/8

Median (range) of anti-alpha trypsin deficiency (Group I, AATD) Wilsons Disease (Group II, WD),AIH-1 (Group III) and AIH-2 (Group IV)

Data not available or not applicable (NA). Elevated (E) or heterogeneous (H) echogenicity (Echo)

Normal ranges: ALT and AST 10–31 U/L, GGTP 10–60 U/L, γ-globulins 4.6–16.7 g/L, Bilirubin (Bilir.) total 0.2–1.3 mg/dL and direct < 0.3 mg/d L, INR 0.9–1.2, portal flow rate 18–26 cm/s

Statistical analysis

Statistical analysis was done with MedCalc version 14 for Windows (MedCalc Software, Mariakerke, Belgium). Comparisons of the values between patient groups were assessed by non-parametric Wilcoxon sum rank test. A p value of 0.05 was considered to be statistically significant with differences in values between independent groups. Linear-log transformation and regression analyses were calculated according to the formula $f(x) = a + b \log(x)$. The correlation coefficients of $r > 0.5$ or $r < -0.5$ with significance at $p < 0.05$ were considered strong positive or strong negative associations, respectively.

Ethical consideration

This study did not require any other than standard of care physical examination and blood sampling for laboratory evaluations procedures. Informed guardian and/or patient consent was obtained from each child included into the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by Ethics Committee of the Children's Memorial Health Institute no 192/KBE/2015 and 231/KBE/2015.

Results

Biochemical, serological, and liver function characteristics were compared in non-autoimmune WD versus different groups of pediatric AIH (Table 1). The 8 WD patients diagnosed according to EASL guidelines gave scores of 3–4, indicative of either possible or established disease. Corneal Kayser-Fleischer (KF) rings were absent in all WD patients. None of the patients presented with end stage of liver disease related encephalopathy. Concentration and attention disturbances were more frequently reported among the AIH-1 (15/40, 37%) and AIH-2 (4/8, 50%) patients than among the WD (0/8, 0%). The simplified criteria of the International Autoimmune Hepatitis Group (IAHG) were applied to all AIH patients [6, 9]. The IAHG cumulative scores of AIH-1 and AIH-2 gave a median of 7 (range 4–8) and 7 (range 5–8) respectively indicative of a probable diagnosis. The median values of

Table 2 Autoantibody profile of pediatric patients

	WD $N=8$	AIH-1 $N=37$	AIH-2 $N=8$
IIF			
Total	4/8 (50%)	36/37 (97%)	8/8 (100%)
ANA	4/8 (50%)	27/37 (73%)	3/8 (38%)
SMA	0/8 (0%)	25/37 (67%)	0/8 (0%)
LKM	0/8 (0%)	0/37 (0%)	5/8 (63%)
SLA	0/8 (0%)	4/37 (11%)	0/8 (0%)
ELISA/IB			
Total	0/8 (0%)	31/37 (84%)	8/8 (100%)
F-actin	0/8 (0%)	20/37 (54%)	0/8 (0%)
LKM-1	0/8 (0%)	0/37 (0%)	8/8 (100%)
SLA/LP	0/8 (0%)	5/37 (14%)	0/8 (0%)
LC-1	0/8 (0%)	3/37 (8%)	3/8 (38%)
AMA	0/8 (0%)	2/37 (5%)	2/8 (24%)
Gp210	0/8 (0%)	1/37 (3%)	1/8 (12%)
Sp100	0/8 (0%)	2/37 (5%)	1/8 (12%)
*Other	0/8 (0%)	3(CENP), 2(PML), 1(Ro52), 1(KELCH)	2(PGDH), 1(KELCH)

The number of autoantibody positive out of total evaluable samples is indicated by percent. IIF = indirect immune fluorescence ELISA and/or immunoblot

immunoglobulin, bilirubin, and INR prothrombin clotting were within normal range in all groups of pediatric patients (Table 1). The median serum transaminases of WD were significantly higher than that of the AIH-2 (Table 2). One of the 8 LKM-1 positive AIH-2 patients at 3 years of age had acute liver failure with elevated transaminases; ALT 398 U/L, AST 51 U/L, and GGTP 53 U/L.

Liver biopsies of WD patient median grade 1 and median stage 1 were similar to that of the AIH-2 patients, and significantly lower than the mean ranked values of liver biopsy grades of AIH-1 patients, median grade 3 and median stage 2. No significant differences between groups were observed for portal flow rates. The ultrasound examinations revealed elevated echogenicity in 5 of 8 WD patients, and in 2 of 8 AIH-2 patients, compared with 3 of 37 AIH-1 patients, suggesting greater steatosis and peri-hepatic fat tissue in the liver of WD and AIH-2 patients (Table 1).

Autoantibody profile of pediatric liver disease

WD patients were devoid of autoantibodies; 0 of 8 (0%) ELISA and/or IB. The 4 of 8 (50%) ANA positive (Table 2) had non-specific weak granular immune fluorescence < 1:320 (results not shown). ANA lack antigen specificity while SMA in AIH are highly specific targeting F-actin; their titres, especially in children, may decrease during immunosuppression-induced remission leading to seroconversion. Thus the autoantibodies of the pediatric AIH were also ranked according to percent positivity in ELISA and/or immunoblot; AIH-1 < AIH-2 (Table 2). As it was expected that all AIH-2 patients tested positive for antibodies to LKM-1 in ELISA of both Euroimmun and Quanta lite and were confirmed positive by IIF (5/8, 63%) and by Euroimmun and Quanta lite immunoblots (7/8, 88%). SLA/LP autoantibodies in AIH-1 were confirmed positive by ELISA and immunoblots of both Euroimmun and Quanta lite in 2 of the 5 and by IIF in 4 of 5 patients (Table 2).

The two patients with KELCH autoantibodies (Table 2) gave high ELISA reactivity > 1000 AU (not shown) and were associated with co-morbidities and elevated aminotransferases; 1 Crohn's disease AIH-1 (ALT 97 U/L, AST 114 U/L, and GGTP 286 U/L) and 1 acute liver failure of the AIH-2

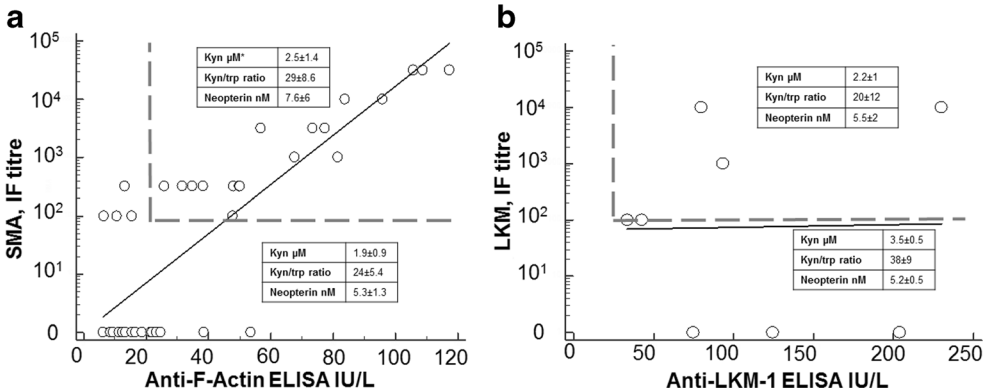

Fig. 1 Autoantibody levels of AIH in ELISA versus immune fluorescence. **a** Linear regression analysis of scatter plots of F-actin Quanta lite ELISA versus SMA IIF of AIH-1 patients; $r = 0.81$, $p < 0.0001$, and weighted kappa inter rate agreement of 0.125 ± 0.07 and **b** LKM-1 Euroimmun ELISA versus LKM IIF of AIH-2 patients; $r = 0.0004$, $p = 0.96$. The autoantibody levels at or above the manufacturer cut-offs 1:100 IIF and 20 U/L ELISA (within dotted lines) and of patients having autoantibody levels below the cut-off in one or both methods (outside of dotted lines)

Table 3 Tryptophan metabolism of pediatric patients

Parameter	I. I. WD $N = 8$	II. II. AIH-1 $N = 37$	III. III. AIH-2 $N = 8$	*p* value	
				s I vs II	I vs III
Kyn, μM	2.2 (1.7–2.7)	1.85 (1.7–1.97)	1.66 (1.1–2)	*0.03*	0.1
Trp, mM	0.068 (0.06–0.076)	0.07 (0.067–0.073)	0.07 (0.06–0.08)	0.7	0.6
Kyn/Trp ratio	33 (26–39)	27 (25–28)	28.6 (21–36)	*0.02*	0.38
Neopterin, nM	5.8 (3.7–7.8)	5.6 (5.1–6.0)	6 (4.5–7.5)	0.7	0.86

The mean (95% CI). The *p* values of unpaired equal variance Student's *t* test between the WD versus pediatric AIH

Italic values are statistically significant

Normal ranges: kynurenine (Kyn) 1.4–2.1 μM, tryptophan (Trp) 54–74 μM, Kyn/Trp ratio 20–33 μmol/mmol, neopterin 4.3–7.5 nM (ref [40])

patient (ALT 398 U/L, AST 51 U/L, and GGTP 53 U/L). The PML autoantibody positive AIH-1 patient and the KELCH positive AIH-1 patient (Table 2) show strong nuclear membrane IIF; 1:32000 and 1:3200 respectively (not shown).

The patients with rare autoantibodies, asialoglycoprotein receptor (ASGP-R) and 52 Kd nuclear antigen (Ro52) in AIH-1 and phosphoglycerate dehydrogenase (PGDH) in AIH-2, showed serum aminotransferases within normal range without co-morbidities. The five AIH-1 patients with positive autoantibodies, two AMA-M2, two sp100, and one gp210 positive, all had elevated aminotransferases, but bilirubin levels were within normal range. The two cases of AIH-2 having sp100/AMA-M2 positive and the one case of gp210/AMA-M2 positive, showed elevated bilirubin, but their serum aminotransferase values were within normal range without obstruction of liver function and without requirement for UDCA treatment.

The F-actin ELISA positively correlated with the SMA IIF; (Fig. 1a), whereas AIH-2 LKM-1 ELISA activity did not correlate with LKM IIF (Fig. 1b).

Clinical features and kynurenine-tryptophan profile

The mean Kyn concentration and Kyn/Trp ratio of AIH-1 patients were significantly lower than that of WD (Table 3, Fig. 2a, b). The 14 AIH patients with active disease show consistent trend of lower Kyn levels and lower Kyn/Trp ratios than the 29 AIH patients in drug-induced remission with inactive disease (Fig. 2c, d). No differences were found in the levels of neopterin levels between groups (Table 3). The Kyn/Trp ratios positively correlate with duration of disease, (Fig. 3a) but show no association with the levels of serum neopterin (Fig. 3b).

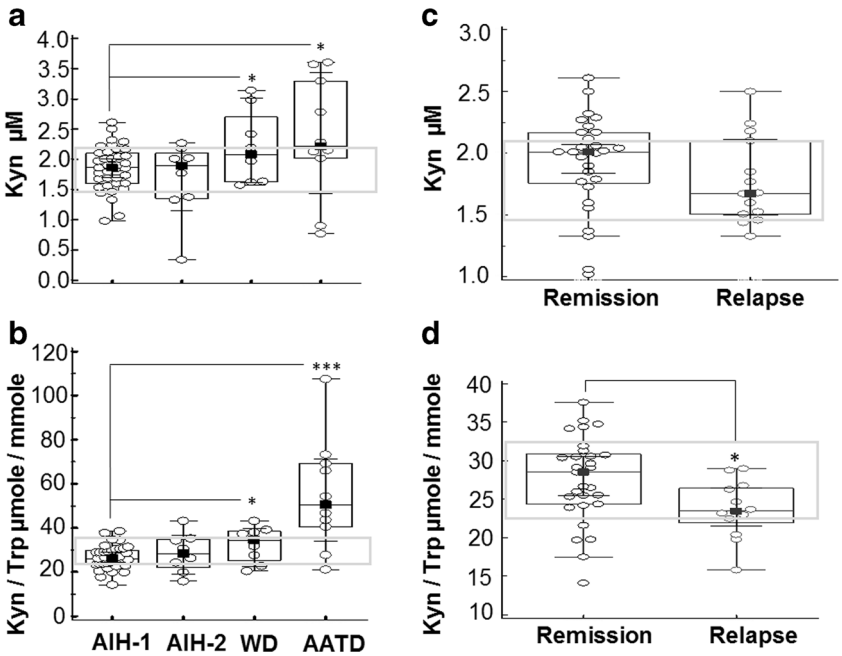

Fig. 2 Tryptophan degradation in AIH versus inborn metabolic liver disease. The serum **a** Kyn and **b** Kyn/Trp ratios of the AIH-1 AIH-2 and the inborn metabolic liver disease of genetic predisposition; WD and AATD. The serum **c** Kyn and **d** Kyn/Trp ratios of AIH patients in remission versus AIH patients in relapse. Mean (horizontal line), 95% CI (box) and standard deviation (error bars) and limits of normal range values (gray rectangle). *$p < 0.05$ unpaired Student's *t* test between mean of AIH group and mean of WD

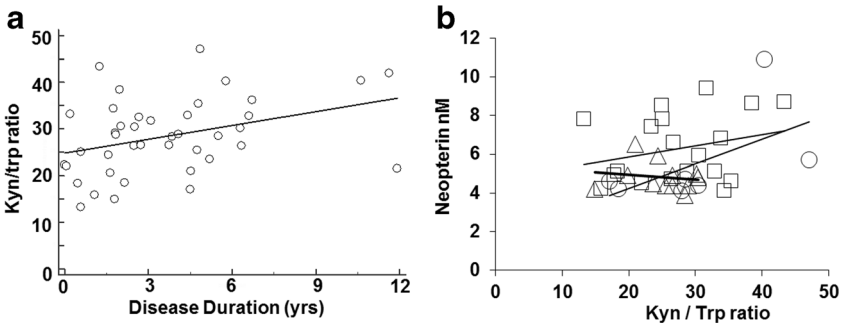

Fig. 3 Tryptophan degradation during the course of AIH. **a** The fitted regression (solid line), $r = 0.36$ (95% CI 0.06–0.6), $p < 0.02$, shows 34 of 40 observations during the first 6 years of disease. **b** Kyn/Trp ratio versus neopterin without association

Correlations of Kyn/Trp ratios with type and severity of AIH

Tryptophan degradation and liver histology scores of the patient groups revealed striking differences in their trend to either associate or not associate with liver disease grade or stage of liver pathology (Fig. 4). The Kyn/Trp ratios of AIH-1 negatively correlate with the scores of liver disease grade and liver stage (Fig. 4a) whereas no associations were found in AIH-2 (Fig. 4b) and WD (Fig. 4c).

Discussion

Pediatric AIH is a rare disease and the undertaking of large studies are difficult. Our comprehensive analysis of tryptophan degradation in children with AIH demonstrated that the serum Kyn and Kyn/Trp ratios of AIH-1 and AIH-2 were within or below the normal range and were consistently lower than that of pediatric non-autoimmune WD and AATD. Taking into account that significant results were obtained in a relatively small cohort of pediatric AIH, our data clearly indicate a disease-specific effect which may bear a pathophysiological meaning rather than a liver damage-related epiphenomenon. The mean neopterin levels of all groups were < 7.5 μM, without correlation to Kyn/Trp ratios. This indicates weak IDO activity and an absence of gamma interferon immune activation. The modest trend of increased Kyn and Kyn/Trp ratios during the duration of disease is consistent with the report of altered kinetics of IFNγ and PD1 expression in AIH after exposures to immune suppressive drugs [4]. Corticosteroids and stress have been reported to induce expression of liver tryptophan deoxygenase (TDO) [33] and thus the measurements of Kyn/Trp ratios in AIH may in part be attributed to TDO activity. The low Kyn levels and null associations between Kyn/Trp ratio and neopterin levels observed in this study are in striking contrast to the accelerated tryptophan catabolism widely reported during acute viral infection and liver disease involving strong IFNγ signaling especially the increased Kyn and/or Kyn/Trp ratios observed in HIV and HBV hepatitis relative to control levels [43–47].

Previous reports of low Kyn/Trp ratios in autoimmune patients are likely explained by reduced expression of IDO-1, IDO-2, and/or TDO or a defect in

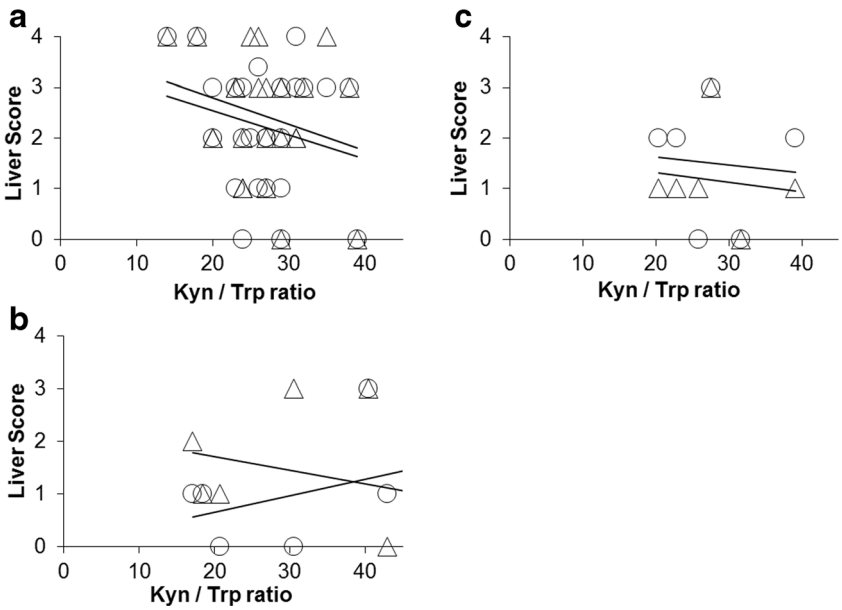

Fig. 4 IDO activity and liver pathology in pediatric AIH. **a** AIH-1 $n = 25$, **b** AIH-2; $n = 7$, and **c** WD; $n = 6$. The fitted regression lines of Kyn/Trp ratios versus the matched scores of biopsy grade (triangles) and liver stage (circles). Negative correlations were of no significance; AIH-1 grade $r = -0.05$, $p = 0.2$ and liver stage $r = -0.0.048$, $p = 0.27$. No associations AIH-2 and WD

activation of the Kyn pathway [35–39]. Although this study does not distinguish between hepatic and extrahepatic tryptophan degradation, the measurements of serum Kyn and Kyn/Trp ratios are the best estimate of overall enzymatic activity. Our findings of Kyn levels and Kyn/Trp ratios similar across age and gender of AIH and WD are consistent with the report of normal ranges of Kyn and Kyn/Trp ratio valid for all ages and gender [49, 50].

The pronounced differences in the association of Kyn/Trp ratios with liver pathology suggest a trend of poor tryptophan breakdown associated with worsening immune pathology. The grade of liver disease and stage of AIH-1 negatively correlated with Kyn/Trp ratios over a narrow range of 13–43 μmole/mmole, whereas the AIH-2 and WD liver grade and stage show no associations with Kyn or Kyn/Trp ratios over the same range. The trend of lower values of Kyn and Kyn (Trp ratios among the AIH patients in relapse than that of the Kyn and Kyn/Trp ratios of the AIH patients in remission) is of borderline significance and requires confirmation with larger numbers of patients. Never the less, the findings imply that a deficit of IDO activity is associated with worsening liver function and lower capacity to respond to steroid and immune suppressive treatments.

The precise interpretation of Kyn levels for Th17/Treg "gate keeping" and the effective use of steroids, immunosuppressive drugs, and T cell targeting therapies in autoimmunity is of key importance. The ideal assessment of inflammatory biomarkers should be made relative to the baseline values of newly diagnosed and untreated patients [31, 32]. Since such cases are not common in clinical practice, our findings of low Kyn levels and low Kyn/Trp ratios in pediatric AIH relative to AATD and WD require confirmation by prospective studies which monitor Kyn/Trp ratios in larger AIH cohorts at presentation and at follow-up visits during steroid and immunosuppressive treatments. The paradigm of low IDO activity linked to deficit of functional Treg cells, increased inflammation, and worsening liver function is intriguing and should be considered in future studies of the Treg cells in pediatric AIH.

Acknowledgements We thank all the patients who kindly consented to participate in this study and the cooperation of nursing staff for the collection and storage of serum.

Financial support The research expenses were incurred in part by SeraDiaLogistics and EB Group Sp. All material support is identified in the acknowledgements section.

Compliance with ethical standards The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by Ethics Committee of the Children's Memorial Health Institute no 192/KBE/2015 and 231/KBE/2015.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Doherty DG. Immunity, tolerance and autoimmunity in the liver: a comprehensive review. J Autoimmun. 2018;66:60–75.
2. Wawman RE, Bartlett H, Oo YH. Regulatory T cell metabolism in the hepatic microenvironment. Front Immunol. 2018;8:1889. https://doi.org/10.3389/fimmu.2017.01889.
3. Grant CR, Holder BS, Liberal R, Heneghan MA, Ma Y, Mieli-Vergani G, et al. Immunosuppressive drugs affect interferon (IFN)-γ and programmed cell death 1 (PD-1) kinetics in patients with newly diagnosed autoimmune hepatitis. Clin Exp Immunol. 2017;189:71–82.
4. Behairy BE, El-Araby HA, Abd El Kader HH, et al. Assessment of intrahepatic regulatory T cells in children with autoimmune hepatitis. Ann Hepatol. 2016;15:682–90.
5. Xue Y, Michalopoulos G. Tregs: a therapeutic target for the treatment of portal fibrosis? Dig Dis Sci. 2015;60:1878–80.
6. Alvarez F, Berg PA, Bianchi FP. International autoimmune hepatitis group report: review of criteria for diagnosis of autoimmune hepatitis. J Hepatol. 1999;31:929e938.
7. Manns MP, Czaja AJ, Gorham JD, Krawitt EL, Mieli-Vergani G, Vergani D, et al. Diagnosis and management of autoimmune hepatitis. Hepatology. 2010;51:2193–213.
8. Hennes EM, Zeniya M, Czaja AI, Pares A, Dalekos GN, et al. International autoimmune hepatitis group simplified criteria for the diagnosis of autoimmune hepatitis. Hepatology. 2008;48:169–76.
9. EASL Clinical Practice Guidelines. Autoimmune hepatitis European association for the study of the liver. J Hepatol. 2015;63:971–1004.
10. Mieli-Vergani G, Vergani D, Baumann U, Czubkowski P, Debray D, Dezsofi A, et al. Diagnosis and management of paediatric autoimmune liver disease: ESPGHAN hepatology committee position statement. J Pediatr Gastroenterol Nutr. 2017;66(2):345–60.
11. Floreani A, Liberal R, Vergani D, Mieli-Vergani G. Autoimmune hepatitis: contrast and comparison in children and adults-a comprehensive review. J Autoimmun. 2013;46:7–16.
12. Healey R, Corless L, Gordin P, Holding S. Do anti-smooth muscle antibodies predict development of autoimmune hepatitis in patients with normal liver function? A retrospective cohort review. Autoimmun Rev. 2016;15:668–72.
13. Liberal R, Vergani D, Mieli-Vergani G. Update on autoimmune hepatitis. J Clin Trans Hepatol. 2015;3:42–52.
14. Woynarowski M, Woźniak M, Cukrowska B, Cukrowska B, Wierzbicka A, Lytton SD. Autoantibody profile of adult patients with childhood onset type 2 autoimmune hepatitis. J Clin Lab Anal. 2016;30(5):590–6.
15. Duchini A, McHutchison JG, Pockros PJ. LKM-positive autoimmune hepatitis in the western United States: a case series. Am J Gastroenterol. 2000;95:3238–41.
16. Gregorio GV, Portmann B, Reid F, Donaldson PT, Doherty DG, McCartney M, et al. Autoimmune hepatitis in childhood: a 20-year experience. Paedatic J Clin Transl Hepatol. 2015;42–52(43):3.
17. Gatselis NK, Zachou K, Koukoulis GK, Dalekos GN. Autoimmune hepatitis, one disease with many faces: etiopathogenetic, clinicolaboratory and histological characteristics. World J Gastroenterol. 2015;21:60–83.
18. Bogdanos DP, Invernizzi P, Mackay IR, Vergani D. Autoimmune liver serology: current diagnostic and clinical challenges. World J Gastroenterol. 2008;14:3374–87.

19. Villata D, Girolami E, Alessio MG, et al. Autoantibody profiling in a cohort of pediatric and adult patients with autoimmune hepatitis. J Clin Lab Anal. 2016;30:41–6.
20. Paolo M, Cumali E, Luigi M, Ersan O Schiano T, Yoshida EM, Heurgué-Berlot, et al. Clinical implications of antimitochondrial antibody seropositivity in autoimmune hepatitis: a multicentre study. Eur J Gastroenterol Hepatol. 2017;29:777–80.
21. Roggenbruck D, Mytilnaiou MG, Lapin SV, Rheinhold D, Conrad K. Asialoglycoprotein receptor (ASGPR): a peculiar target of liver-specific autoimmunity. Autoimmun Highlights. 2012;3:119–25.
22. Norman GL, Yang CY, Ostendorff HP, Lim MJ, Wang J, et al. Anti-Kelch-like 12 and anti-hexokinase 1: novel autoantibodies in primary biliary cirrhosis. Liver Int. 2015;35:642–51.
23. Lytton SD, Berg U, Nemeth A, Ingelman-Sundberg M. Autoantibodies against cytochrome P450s in sera of children treated with immunosuppressive drugs. Clin Exp Immunol. 2012;127: 293–302.
24. Woynarowski M, Nemeth A, Baruch Y. Budesonide versus prednisone with azathioprine for the treatment of autoimmune hepatitis in children and adolescents. J Pediatr. 2013;163:1347–53.
25. Batts KP, Ludwig J. Chronic hepatitis. An update on terminology and reporting. Am J Surg Pathol. 1995;19:1409–17.
26. Goodman GD. Grading and staging systems for inflammation and fibrosis in chronic liver diseases. J Hepatol. 2007;47:598–607.
27. Roberts EA, Schilsky ML. Diagnosis and treatment of Wilson Disease: an update. Hepatology. 2008;47:2090–111.
28. Lauletta G, Russi S, Pavone F, Marzullo A, Tampoia A, Sansonno D, et al. Autoimmune hepatitis: factors involved in initiation and methods of diagnosis and treatment. Crit Rev Immunol. 2016;36: 407.428.
29. Dhaliwal HK, Hoeroldt BS, Dube AK, McFarlane E, Underwood JC, Karajeh MA, et al. Long-term prognostic significance of persisting histological activity despite biochemical remission in autoimmune hepatitis. Am J Gastroenterol. 2015;110:993–9.
30. Aizawaa Y, Abea H, Sugitaa T, Sekia N, Chuganjic Y, Furumotoc Y, et al. Centrilobular zonal necrosis as a hallmark of a distinctive subtype of autoimmune hepatitis. Eur J Gastroenterol Hepatol. 2016;28:391–7.
31. Abdel-Razik A, Mousa N, Zakaria S, Elhelaly R, Elzehery R, Zalata K, et al. New predictive factors of poor response to therapy in autoimmune hepatitis: role of mean platelet volume. Eur J Gastroenterol Hepatol. 2017;29:1373–9.
32. Diestelhorst J, Junge N, Jonigk D, Schlue J, Falk CS, Manns MP, et al. Baseline IL-2 and the AIH score can predict the response to standard therapy in paediatric autoimmune hepatitis. Sci Rep. 2018;8:419.
33. Dounay AB, Tuttle JB, Verhoest PR. Challenges and opportunities in the discovery of new therapeutics targeting the kynurenine pathway. J Med Chem. 2015;58:8762–82.
34. Zuo H, Ueland PM, Ulvik A, Eussen S, et al. Plasma biomarkers of inflammation, the kynurenine pathway, and risks of all-cause, cancer, and cardiovascular disease mortality: the Hordaland health study. Am J Epidemiol. 2016;183:249–58.
35. Lippens C, Duraes FV, Dubrot J, Brighouse D, Lacroix M, Irla M, et al. IDO-orchestrated crosstalk between pDCs and Tregs inhibits autoimmunity. J Autoimmun. 2016;75:39–49.
36. Mancuso R, Hernis A, Agostini S, Rovaris M, Caputo D, Fuchs D, et al. Indoleamine 2,3 dioxygenase (IDO) expression and activity in relapsing-remitting multiple sclerosis. PlosOne. 2015;10: e0130715. https://doi.org/10.1371/journal.pone.0130715.
37. Llamas-Velasco M, Bonay P, José Concha-Garzón M, Corvo-Villén L, Cibrián D, Sanguino-Pascual A, et al. Immune cells from patients with psoriasis are defective in inducing indoleamine 2,3-dioxygenase expression in response to inflammatory stimuli. Br J Dermatol. 2017;176:695–704.
38. Bernard NJ. Rheumatoid arthritis: who knows why regulatory T cells are defective in RA. IDO Nat Rev Rheumatol. 2014;10:381–96.
39. Konstantia-Maria C, Shukla D, Keteepe-Arachi T, Sekel JA, Fuchs D, Pussey CD, et al. Regulation of myeloperoxidase-specific T cell responses during disease remission in anti-neutrophil cytoplasmic antibody–associated vasculitis: the role of Treg cells and tryptophan degradation. Arthritis Rheum. 2010;62:1539–48.
40. Palabiyik SS, Keles S, Girgin G, Arpali-Tanas E, Topdagi E, Baydar T. Neopterin release and tryptophan degradation in patients with uveitis. Curr Eye Res. 2016;41:1513–7.
41. Kaden J, Abendroth DE, Völp M, Marzinzig M, Wesslau C. Causes and prognostic value of pre-transplant elevated kynurenine level in kidney allograft recipients. Ann Transplant. 2014;19:51–9.
42. Kaden J, Abendroth D, Völp A, Marzinzig M. Dynamics and diagnostic relevance of kynurenine serum level after kidney transplantation. Ann Transpl. 2015;20:327–37.
43. Yoshio S, Sugiyama M, Shoji H, et al. Indoleamine-2,3-dioxygenase as an effector and an indicator of protective immune responses in patients with acute hepatitis B. Hepatol. 2016;63:3–94.
44. Jenabian MA, Patel M, Kema I, Kanagaratham C, Radzioch D, Thébault P, et al. Distinct tryptophan catabolism and Th17/Treg balance in HIV progressors and elite controllers. PLoS One. 2013;8:e78146. https://doi.org/10.1371/journal.pone.0078146.
45. Bipath P, Levay PF, Viljoen M. The kynurenine pathway activities in a sub-Saharan HIV/AIDS population. BMC Infect Dis. 2015;15: 346–61.
46. Mehraj J, Routy JP. Tryptophan catabolism in chronic viral infections: handling uninvited guests. Int J Tryptophan Res. 2015;8:41–6.
47. Zoller H, Jenal A, Staettermayer AF, Schroecksnadel S, Ferenci P, Fuchs D. Tryptophan breakdown in patients with HCV infection is influenced by IL28B polymorphism. Pharmaceuticals. 2015;8: 337–50.
48. Danikowski KM, Jayaraman S, Prabhaka BS. Regulatory T cells in multiple sclerosis and myasthenia gravis. J Neuroinflammation. 2017;14:117–33.
49. Geisler S, Mayersbach P, Becker K, Schennach H, Fuchs D, Gostner JM. Serum tryptophan, kynurenine, phenylalanine, tyrosine and neopterin concentrations in 100 healthy blood donors. Pteridines. 2015;26:31–6.
50. Schröcksnadel K, Wirleitner B, Winkler C, Fuchs D. Monitoring tryptophan metabolism in chronic immune activation. Clin Chim Acta. 2016;364:82–90.
51. Gibson RN, Donlan JD, Ditchfield MR, Bhathal PS. Duplex Doppler ultrasound of the ligamentum teres and portal vein: a clinically useful adjunct in the evaluation of patients with known or suspected chronic liver disease or portal hypertension. J Gastroenterol Hepatol. 1991;6:61–5.