Immunity against post-translationally modified proteins in autoimmune diseases

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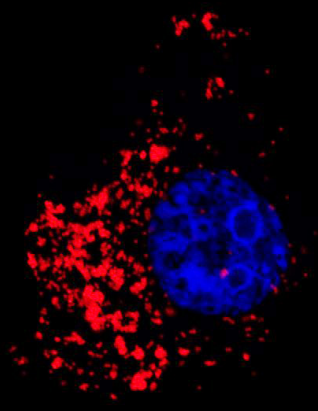

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Antibodies against multiple post-translationally modified proteins aid in diagnosis of autoimmune hepatitis and associate with complete biochemical response to treatment.

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Abstract

Background

(Auto)immune mediated and cholestatic liver disease (AILD) includes autoimmune hepatitis (AIH), primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC). Especially AIH is characterized by the presence of autoantibodies and elevated serum immunoglobulins. In rheumatoid arthritis, autoantibodies against post-translational modifications (PTMs) such as citrullination (Cit) and carbamylation (CarP) are used as diagnostic and prognostic markers, respectively. We studied the presence of six anti-PTM antibodies in patients with the three AILDs and non-AILD.

Methods

Antibodies against six PTMs (malondialdehyde–acetaldehyde adducts (MAA), advanced glycation end-products (AGE), CarP, acetylation (AL), Cit, and nitration (NT)) were tested in sera of patients with AILD (n=106), non-AILD (n=101) and compared with healthy controls (HC) (n=100). Levels and positivity were correlated with clinical and biochemical features in a well-defined cohort of untreated AIH patients.

Results

Anti-PTM antibodies were more often detectable in sera from AILD patients compared with HCs (anti-MAA: 67.9% vs 2.0%, anti-AGE: 36.8% vs 4.0%, anti-CarP: 47.2% vs 5.0% and anti-AL: 18.9% vs 5.0%). In untreated AIH, time to complete biochemical response (CBR) was associated with anti-MAA, anti-AGE, anti-CarP and anti-AL antibodies. Significantly more patients with at least three anti-PTM antibodies attained CBR at 12 months of treatment (13 vs 3 p=0.01).

Conclusions

Anti-PTM antibodies are frequently present in AILD. The presence of anti-MAA, anti-AGE and anti-CarP antibodies correlates with the presence of AIH within this cohort. In AIH, harboring at least three anti-PTM antibody responses is positively associated with CBR. Determination of anti-PTM antibodies in liver disease may have diagnostic and prognostic value.

Introduction

(Auto)immune mediated and cholestatic liver disease (AILD) is a heterogeneous group of both cholestatic and hepatocellular diseases, consisting of primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), autoimmune hepatitis (AIH) and overlap variants. AIH and PBC are characterized by the presence of autoantibodies and elevated total immunoglobulin (Ig) G and IgM, respectively (1). The presence of autoantibodies against for example smooth muscle (SMA) and mitochondria (AMA) play an important role in the diagnostic scoring of AIH and PBC, respectively (2, 30). Although testing for different autoantibodies is implemented in the standard diagnostic work-up for liver disease with an unknown origin, they are not disease specific (4).

In another autoimmune disease, namely rheumatoid arthritis (RA), autoantibodies are also present, but in this disease antibodies frequently target proteins that have undergone post-translational modifications (PTM) (5). In particular, antibodies that target citrullination (anti-citrullinated antibodies: ACPA) and anti-carbamylated protein (anti-CarP) antibodies are used as diagnostic and prognostic markers in RA, respectively (6, 7). During inflammation, peptidyl arginine deiminases and cyanate are formed resulting in extracellular citrullination of arginine and carbamylation of lysine amino acids, respectively (8, 9). More recently, we have discovered antibody responses against the modifications malondialdehyde-acetaldehyde adducts (MAA) and advanced glycation end-products (AGE) in patients with systemic lupus erythematosus (SLE), defining a group of patients with neuropsychiatric manifestations (10). Both MAA and AGE are a result of oxidative stress and modify lysine amino acids (11, 12). Additionally, under oxidative stress nitration (NT) of the tyrosine amino acids and acetylation of lysines occur as a result of a reaction with peroxynitrite species and dysregulation of acetylation and deacetylation pathways, respectively (13, 14).

Inflammation occurs in both AIH and cholestatic liver disease, albeit at different sites (hepatocytes versus biliary tract). Oxidative stress occurs more frequently in patients with AIH compared to patients with cholestatic liver disease(15, 16). PTMs that are the result of oxidative stress have been reported to be highly immunogenic which could therefore result in anti-PTM antibody production, also in the context of AILD (17-19). However, studies assessing anti-PTM antibody responses in AILD are limited. Antibodies against cyclic citrullinated peptide (CCP) have been studied and were found in 9-11% of patients with type 1 AIH (20, 21), commonly in the absence of RA (21). Additionally, MAA modifications have shown to induce liver damage and to cause an autoimmune like pathophysiology in mice (22).

Since AIH, PBC and PSC are often considered (auto)immune mediated diseases that, like RA and SLE, display a variety of autoantibodies, we hypothesized that anti-PTM antibodies may be present in AILD and could have diagnostic or prognostic associations. Here we report that anti-PTM antibodies are present in AILD, allow discrimination between subgroups of AILD and are related to treatment response in AIH.

Materials and methods

Study design and population

Patients visiting the Department of Gastroenterology and Hepatology of the Leiden University Medical Centre (LUMC) between 1996 and 2020 who signed informed consent for the Biobanking facility were eligible for inclusion. Patients visiting the Department of Gastroenterology and Hepatology of Erasmus Medical Centre, Rotterdam, with no objection against the use of residual material, were also included. The biobank protocol (B21.032) was prospectively approved by the Medical Ethical Committee of the LUMC. For the purpose of this study, patients were divided into three groups: AILD, (i.e. AIH, PBC or PSC), miscellaneous chronic liver diseases (non-AILD) and healthy controls (HC). HC were preselected from a biobank containing serum from healthy individuals. They were matched based on sex and age to the AILD cohort. No data on medical history of medication use was available, mimicking the general population. Although clinical, biochemical and histological overlap can occur, patients with overlap variants (AIH-PBC or AIH-PSC) were not included in the AILD cohort. AIH was diagnosed using the *revised original* or *simplified* criteria for the diagnosis for AIH (2, 23, 24). Patients with AIH were included at diagnosis. Of all AILD patients, 66 were diagnosed with AIH. Of these patients 8 patients already started treatment before inclusion. PBC and PSC were diagnosed according to the diagnostic criteria in the European guidelines and were included during follow-up (25). As the AIH cohort was the largest cohort with complete data, this was the cohort in which the final analyses were done.

Patient characteristics

Demographics and patient characteristics were collected from electronic patient files at the time of visit to the outpatient clinic. This included: age, sex, comorbidities, disease duration, presence of liver cirrhosis, simplified criteria for the diagnosis of AIH (24), revised original criteria for AIH (23), presence of self-reported arthralgia (i.e., extrahepatic manifestation of AIH) and medication use. Follow-up data (i.e., time to complete biochemical response (CBR), treatment response, mortality and liver transplantation) was also collected. CBR was defined as normalization of aminotransferases and IgG below the upper limit of normal (26). Time to CBR was defined as the time from treatment initiation until the first time CBR was reached.

In addition to routine laboratory assessments (aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), IgG, gamma-glutamyl transferase (GGT), alkaline phosphatase (AP) and presence of autoantibodies), serum samples from each patient were collected (*Supplementary Table 1*). For patients with cholestatic liver disease, data regarding cholangiographic findings and laboratory assessments (GGT, AP and autoantibodies) were also collected (*Supplementary Table 1*).

Generation of PTMs

Modified proteins and their corresponding control non-modified protein were produced by either enzymatic or chemical reactions as previously described [10).

Assessment of anti-PTM antibodies

Anti-PTM antibodies were detected using an in-house enzyme-linked immunosorbent assay (ELISA), based on modified fetal calf serum (FCS) as described previously (10). Briefly, modified and non-modified FCS were coated to a Nunc Maxisorp ELISA plate (430341, Thermofisher). In between each sequential step plates were washed three times using Phosphate Buffered Saline (PBS)/0.05%Tween (Sigma, P1379). After blocking (PBS/1% Bovine Serum Albumin (BSA)) for 6 hours at 4°C, plates were incubated overnight at 4°C with 1/50, 1/100 or 1/1000 diluted serum. Each plate contained a standard of anti-PTM antibody positive serum to calculate arbitrary units. After incubation, IgG levels were detected using horseradish peroxidase (HRP) labelled Rabbit-anti-Human IgG (Dako, P0214). Plates were developed by incubating with 2,2'-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS)/0.015% H_2O_2 (A1888 and 7722-84-1, both from Merck) and absorbance at 415nm was measured using a microplate reader (Bio-Rad iMark). The cut-off for positivity was set as the mean arbitrary units plus two times the standard deviation of 100 HCs, excluding values higher than 10x the mean.

Statistical analysis

Statistical analyses were performed using IBM SPSS 25.0 (IBM, Armonk, NY). Baseline characteristics were evaluated using descriptive statistics. Differences in levels of anti-PTM antibodies between HCs, AILD and non-AILD were assessed using Kruskall-Wallis Test and Chi-2 test. Analyses of correlation between anti-PTM antibody levels and clinical variables were done using Spearman rank analyses for continuous clinical variables and point biserial correlation (i.e. mathematical equivalent of Pearson correlation) for dichotomous clinical variables. The anti-PTM antibody levels were transformed to natural logarithms to perform point-biserial correlations. Wilcoxon signed-rank test was used to compare anti-PTM antibody levels at baseline versus levels at the second visit.

Correlations between the difference in anti-PTM antibody levels at baseline versus the second visit and the change in levels of ALAT, ASAT and IgG were done using Spearman's

rho (r_s). Landmark analysis was used for the evaluation of CBR, with pre-determined timepoints at 3, 6 and 12 months, to prevent immortal time bias. P-values <0.05 were considered statistically significant.

Results

Study cohort

We studied 207 patients with liver disease comprising an AILD cohort (n=106) and a non-AILD cohort (n=101). The AILD cohort consisted of patients with AIH (n=66), PBC (n=10) and PSC (n=30) and was subsequently divided into two separate cohorts: AIH and cholestatic liver disease (CLD) (i.e. PBC and PSC). The non-AILD cohort consisted of patients with alcoholic liver disease (ALD) (n=29), chronic hepatis B (HBV) (n=4), chronic hepatitis C (HCV) (n=22), non-alcoholic fatty liver disease (NAFLD) (n=30), non-alcoholic steatohepatitis (NASH) (n=1), or a combination of these (n=15) (*Table 1*). In the AILD and non-AILD cohort 63.2% and 33.7% of the patients were female (p <0.001) with a mean age of 48.2±16.6 years and 54.0±11.0 years, respectively (p = 0.003) (*Table 1*). Cirrhosis was present in 39.6% of the AILD cohort and in 56.4% of non-AILD patients (p= 0.035). In the AILD cohort, 96.7% of patients with PSC had large duct PSC on cholangiographic imaging. Eighty percent of PBC patients was AMA positive. The mean age of the HCs was 50.2±10.5 years and 49% were female.

Anti-MAA, anti-AGE, anti-CarP and anti-AL antibodies are more prevalent in AILD compared to HC and non-AILD and are more likely to be positive for more than one anti-PTM antibody

Anti-PTM IgG antibody levels directed against 6 PTMs were measured in 207 patients with liver disease and 100 HCs (*Figure 1 and Supplementary Table 2*). Anti-MAA, anti-AGE, anti-CarP and anti-AL antibody levels differed significantly between AILD and HCs (1036.0, 234.5, 352.5 and 13.3 aU/mL vs 266.9, 88.9, 74.0 and 0.0 aU/mL respectively, all p<0.01). Only anti-MAA and anti-CarP antibodies were significantly increased comparing non-AILD compared to HCs (495.8 and 241.0 aU/mL vs 266.9 and 74.0 aU/mL, respectively, both p<0.01). Additionally, AILD showed significantly higher median levels of anti-MAA, anti-AGE, anti-CarP and anti-AL antibodies compared to non-AILD (anti-MAA, anti-AGE and anti-AL 1036.0, 234.5, 13.3 aU/mL vs 495.8, 130.0, 6.4 aU/mL, respectively, p<0.01 and anti-CarP 352.5aU/mL vs 241.0 aU/mL, p<0.05). Median levels of anti-NT and anti-Cit differed significantly between AILD and HCs (269 and 3.1 aU/mL vs 108 and 1.3 aU/mL, p<0.01 and p<0.05, respectively) but did not differ significantly between non-AILD and HCs.

Table 1: Characteristics of study population with autoimmune mediated and cholestatic liver disease (AILD) and non-AILD at time of inclusion. Results are presented as n (%), mean ±SD or median (IQR). P<0.05 is considered statistically significant (*). *Abbreviations: AIH, autoimmune hepatitis; AILD, autoimmune liver disease; ALD, alcoholic liver disease; HBV, chronic hepatis B; HCV, chronic hepatitis C; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.*

Patient characteristics	Auto-immune liver disease (AILD) (n=106)	Non-autoimmune liver disease (non-AILD) (n=101)	p-value
Primary diagnosis			
AIH	66 (62.3)	-	
PBC	10 (9.4)	-	
PSC	30 (28.3)	-	
NAFLD	-	30 (29.7)	
ALD	-	29 (28.7)	
HCV	-	22 (21.8)	
HBV	-	4 (4.0)	
NASH	-	1 (1.0)	
Hemochromatosis	-	0 (0.0)	
Combination†	-	15 (14.9)	
Female sex	67 (63.2)	34 (33.7)	<0.001*
Age sample (years)	48.2±16.6	54.0±11.00	0.003*
Cirrhosis	42 (39.6)	57 (56.4)	0.035*
Yes, compensated	28 (26.4)	27 (26.7)	-
Yes, decompensated	14 (13.2)	30 (29.7)	-
No cirrhosis	59 (55.7)	44 (43.6)	-
Unknown	5 (4.7)	0 (0.0)	-

† Combinations: ALD + HBV (N=1), ALD + HCV + HBV (N=1), ALD + HCV (N=6), ALD and hemochromatosis (N=2), ALD + NASH (N=2), ALD + PSC (N=1), HBV + HDV (N=1), HCV + HIV (N=1)

Comparing the frequency of positivity, AILD patients showed significantly increased positivity of anti-MAA, anti-AGE, anti-CarP and anti-AL antibodies compared to HCs (67.9, 36.8, 47.2 and 18.9% vs 2.0, 4.0, 5.0 and 5.0%, all p<0.01). Increased positivity for anti-MAA, anti-AGE and anti-CarP antibodies (28.7, 17.8 and 27.7% vs 2.0, 4.0 and 5.0%, respectively, all p<0.01) was observed when comparing non-AILD and HCs. Additionally, increased positivity between non-AILD and AILD was observed for anti-MAA, anti-AGE and anti-CarP antibodies (67.9, 36.8 and 47.2% vs 26.7, 17.8 and 27.7%, respectively, all p<0.01). Also when the non-AILD control group is limited to a more stringent set of conditions, excluding HBV, NASH and hemochromatosis, all statistical associations remain intact (data not shown). Anti-PTM antibody positivity for different anti-PTM antibodies were combined to calculate positivity for multiple anti-PTM antibodies (*Figure 2*). Patients with AILD more frequently harbored at least one type of anti-PTM antibody compared to non-AILD and HCs (AILD: 81.2%, non-AILD: 58.4% and HCs: 20%). The data in Figure 2 also indicate that AILD patients are more likely to be positive for multiple

anti-PTM antibodies. Overall, these data indicate that anti-PTM antibodies are especially present in patients with AILD.

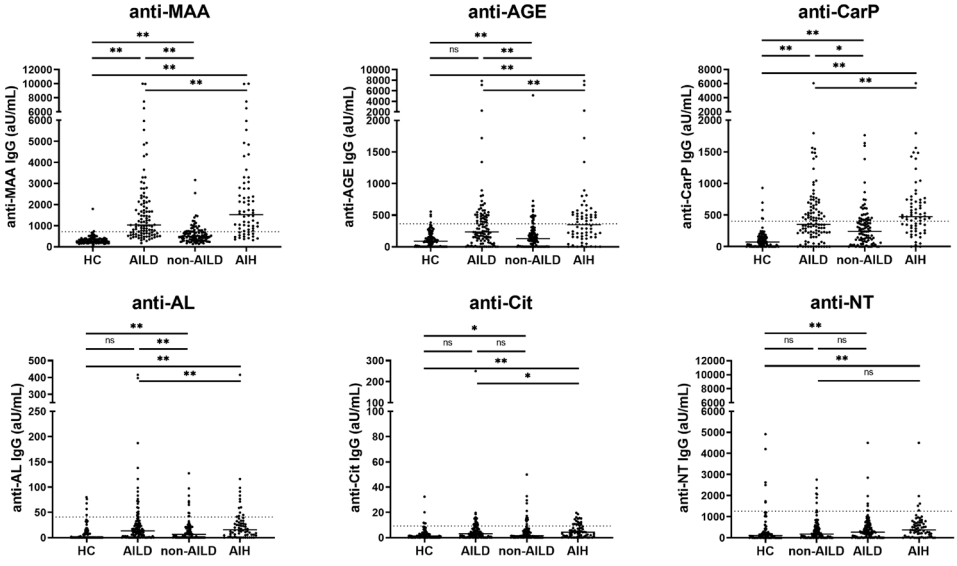

Figure 1: Anti-MAA, anti-AGE, anti-CarP, and anti-AL antibodies are increased in patients with AILD, and especially in patients with AIH. IgG antibody levels are presented as arbitrary units per milliliter (aU/mL) and cut-off for each PTM is indicated by the dashed line. *p<0.05, **p<0.01. *Abbreviations: Autoimmune Liver Disease: AIH, PBC and PSC; non-Autoimmune Liver Disease: NAFLD, HCV, HBV, ALD, Combination, NASH. AGE, advanced glycation end-product; AIH, autoimmune hepatitis; AL, acetylated protein; CarP, carbamylated protein; Cit, citrullinated protein; MAA, malondialdehyde–acetaldehyde adduct; ns, not significant; NT, nitrated protein.*

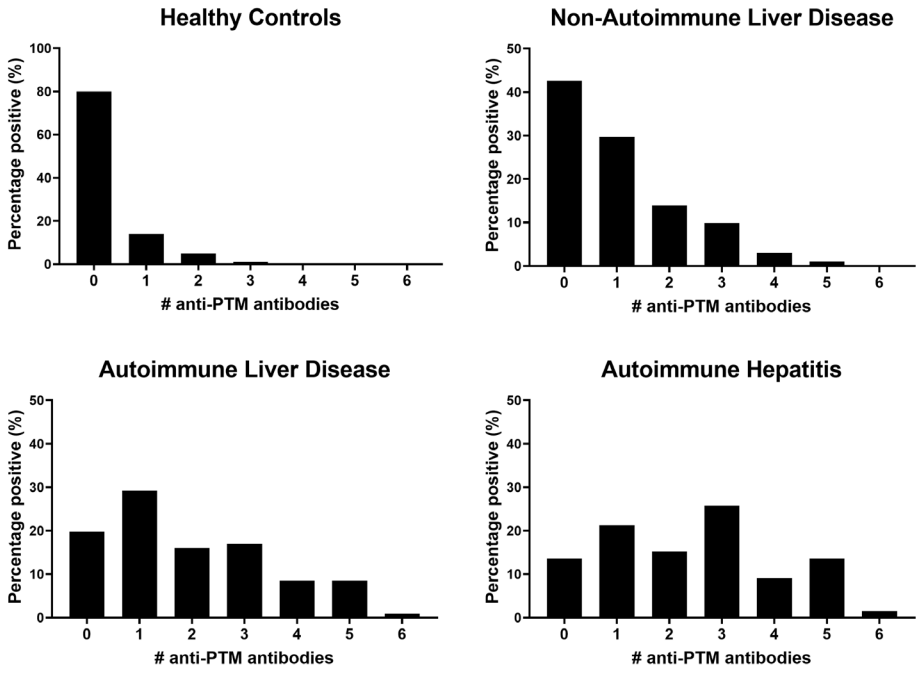

Figure 2: Patients with AILD, and especially AIH, are more likely to be positive for more than one anti-PTM antibody. Data is presented as percentage positive (%) patients for a number of anti-PTM antibodies in (from left to right) healthy controls, non-autoimmune liver disease autoimmune liver disease and autoimmune hepatitis.

Within AILD, patients with AIH harbor anti-PTM antibodies more often and present with specific combinations of anti-PTM antibodies

Next, AILD was dissected into the three major immune liver disease subgroups, namely AIH, PBC and PSC. Presence of anti-MAA, anti-AGE, anti-CarP and anti-AL antibodies were assessed in these subgroups, or AIH alone, and compared to non-AILD *(Figure 1 and 2, and Supplementary Table 3*). Interestingly, patients with AIH harbored significantly more of these antibodies compared to non-AILD patients (anti-MAA: 77.3% vs 26.7%, anti-AGE: 48.5% vs 17.5% and anti-CarP: 63.6% vs 27.7%, all $p<0.001$, respectively). Within the AILD cohort, predominantly patients with AIH harbored anti-PTM antibodies. We did however also see some patients with CLD who were positive for some. Subsequently, the AILD was divided into two cohorts: AIH and CLD. Patients with AIH were significantly more often positive for anti-MAA, anti-AGE, anti-CarP and anti-Cit (77.3, 48.5, 63.6 and 25.8% vs 52.2, 17.5, 20.0 and 2.5% respectively, all $p<0.01$) compared to patients with CLD (*Table 2*).

Table 2: The association between the presence of anti-PTM antibodies in HC, non-AILD, AIH and cholestatic liver disease.

	Healthy Controls *n=100*				**Non-Autoimmune Liver Disease** *n=101*				**AIH** *n=66*				**Cholestatic liver disease** *n=40*			
	aU/mL [IQR]		**n (% positive)**		**aU/mL [IQR]**		**n (% positive)**		**aU/mL [IQR]**		**n (% positive)**		**aU/mL [IQR]**		**n (% positive)**	
Anti-MAA	266.9	[200.4 – 370.2]	2	(2.0)	495.8	[315.2– 726.8]	27	(26.7)	1519.5	(760.0 – 2775.3)	51	(77.3)	771.5	538.3 – 1247.7)**,#	21	(52.5)**,#, +
Anti-AGE	88.9	[0.0 – 182.5]	4	(4.0)	130.0	[4.2 – 261.2]	18	(17.8)	349.0	(156.0 – 537.0)	32	(48.5)	143.5	(27.0 – 304.0)+	7	(17.5)*,+
Anti-CarP	74.0	[1.5 – 157.9]	5	(5.0)	241.0	[83.5 – 422.0]	28	(27.7)	475.5	(293.2 – 741.8)	42	(63.6)	226.5	(9.8 – 328.5)**,++	8	(20.0)*,++
Anti-AL	0.0	[0.0 – 9.8]	5	(5.0)	6.4	[0.0 – 10.0]	10	(9.9)	15.4	(4.7 – 33.4)	13	(19.7)	10.8	(0.5 – 25.4)*	7	(17.5)*
Anti-Cit	1.3	[0.0 – 3.2]	6	(6.0)	1.6	[0.0 – 5.9]	15	(14.9)	4.3	(1.2 – 9.7)	17	(25.8)	1.8	(0.0 – 4.2)	1	(2.5)#,+
Anti-NT	108.0	[0.0 – 250]	6	(6.0)	179	[0.0 – 501.5]	7	(6.9)	369.0	(65.8 – 732.5)	5	(7.6)	212.0	(0.0 – 400.8)	2	(5.0)

Results are presented as median (IQR) of n (%). Chi-2-tests were used to assess the difference between the presence of the specific manifestations and non-AILD patients. HC versus cholestatic liver disease *p<0.005, **p<0.001; non-AILD versus cholestatic liver disease #p<0.005, ##p<0.001; and AIH versus Cholestatic liver disease +p<0.005, ++p<0.001. *AGE, advanced glycation end-product; AIH, autoimmune hepatitis; AL, acetylated protein; aU/mL, arbitrary units per milliliter; CarP, carbamylated protein; Cit, citrullinated protein; IQR, interquartile range; MAA, malondialdehyde–acetaldehyde adduct; NT, nitrated protein; non-AILD, non-autoimmune liver disease; PBC, Primary Biliary Cirrhosis; PSC, Primary Sclerosing Cholangitis*

Analysis of different anti-PTM antibody combinations showed that AILD patients mostly harbored a combination of anti-MAA, anti-AGE and anti-CarP antibodies (15/85 = 17.6%) or anti-MAA and anti-CarP antibodies (7/85 = 8.2%) compared to non-AILD (anti-MAA/-AGE /-CarP: 5/58 = 8.6% and anti-MAA/-CarP: 3/58 = 5.2%) (*Supplementary Figure 1A and B*). Strikingly, comparing AIH patients with total AILD, all double (anti-MAA/-CarP), almost all (except 1) triple (anti-MAA/-AGE /-CarP) and all quintuple (anti-MAA/-AGE /-CarP /-AL/-Cit) positive patients from the AILD group belonged to the AIH group (*Supplementary Figure 1C*). Taken together, patients with AIH harbored anti-PTM antibodies more often compared to other subgroups of AILD.

There are no significant associations between anti-PTM antibody positivity, presence of ANA and SMA, cirrhosis and sex in AIH patients

In AIH several other antibodies have been described such as ANA and SMA. We have analyzed to what extent these antibodies occur together with the anti-PTM antibody responses or to what degree detectable anti-PTM antibody responses differ depending on the positivity status for ANA or SMA. We did not observe a significant difference in the presence of anti-PTM antibodies in patients positive or negative for ANA or SMA, with the exception of anti-MAA positivity and ANA positivity in patients with AIH (Chi-2 (1)> = 4.687, p=0.030). We further analyzed the positivity for anti-PTM antibodies in patients with AIH who were negative for both ANA and SMA. Despite it being a small cohort (n=11), we observed that the absolute percentages for positivity of anti-MAA, anti-AGE, anti-CarP, anti-AL, anti-Cit and anti-NT was in general higher in patients who were both ANA and SMA negative compared to patients who were either ANA negative of SMA negative (*Table 3*). This further supports the idea that anti-PTM antibodies provide different information compared to the already known antibodies ANA and SMA. Additionally, in the AIH cohort positivity for any of the anti-PTM antibodies did not show significant differences between patients when stratifying for cirrhosis. Furthermore, in the AILD cohort, we observed a significant association between anti-CarP and female sex (Chi-2 (1)> = 4.740, p=0.029). In the AIH cohort however, none of the anti-PTM antibodies showed significant associations with sex.

Anti-MAA and anti-CarP antibodies significantly correlate with measures of biochemical treatment response

We investigated if increased anti-PTM antibodies correlated with commonly used serological and clinical markers in patients with AIH (*Figure 3A-D and Supplementary Table 4*). As treatment for AIH consists of immunomodulatory treatment, and might therefore influence biochemical markers, only patients with treatment naïve AIH were included in these analyses (*Table 4*). Both anti-MAA and anti-CarP correlated positively with serum IgG (p<0.000/p=0.001) and antinuclear antibodies (ANA) (p=0.001). Anti-CarP correlated positively with ASAT (p=0.009). We demonstrated correlations between anti-MAA,

self-reported arthralgia and antibodies against soluble liver antigen (SLA) approaching statistical significance (p=0.082 and 0.059 respectively). No significant correlations were found for anti-AGE and anti-AL.

Anti-MAA, anti-AGE and anti-CarP antibodies positively correlate with CBR

Time to CBR negatively correlated with the presence of anti-PTM antibodies in patients with AIH, reaching significance for anti-AGE (p=0.042) (*Figure 3A-D*). In line with these findings, anti-MAA and anti-AGE correlated positively with CBR at 3 months (p=0.015 and 0.036, respectively). In addition, anti-MAA, anti-AGE, and anti-CarP positively correlated with CBR at 12 months (p=0.014, 0.005, and 0.012, respectively) (*Figure 3A-D*). A trend towards significance was found for anti-AL and CBR at 12 months. No association between the presence of anti-PTM antibodies and long-term follow-up (i.e. liver transplantation or mortality) was found. A logistic regression was performed to analyze the effects of positivity for all six individual anti-PTM antibodies on the likelihood of reaching CBR at 3, 6 and 12 months. Positivity for any individual anti-PTM antibody was not independently associated with an increased or decreased likelihood of reaching CBR at 3, 6 or 12 months (data not shown).

Table 3: Percentage of positivity for anti-PTM antibodies in ANA positive, ANA negative, SMA positive, SMA negative and double negative (ANA and SMA) patients with AIH.

	AIH (n=66)				
	ANA positive (n=42)	**ANA negative (n=24)**	**SMA positive (n=35)**	**SMA negative (n=31)**	**ANA negative / SMA negative (n=11)**
Anti-MAA positive	85.7%	62.5%	71.4%	83.9%	81.8%
Anti-AGE positive	54.8%	37.5%	40.0%	58.1%	63.6%
Anti-CarP positive	69.0%	54.2%	62.9%	64.5%	72.7%
Anti-AL positive	23.8%	12.5%	20.0%	19.4%	27.3%
Anti-Cit positive	31.0%	16.7%	22.9%	29.0%	27.3%
Anti-NT positive	4.8%	12.5%	8.6%	6.5%	18.2%

AGE, advanced glycation end-product; AL, acetylated protein; ANA, anti-nuclear antibodies; CarP, carbamylated protein;Cit, citrullinated protein; MAA, malondialdehyde–acetaldehyde adduct; NT, nitrated protein; SMA, smooth muscle antibody

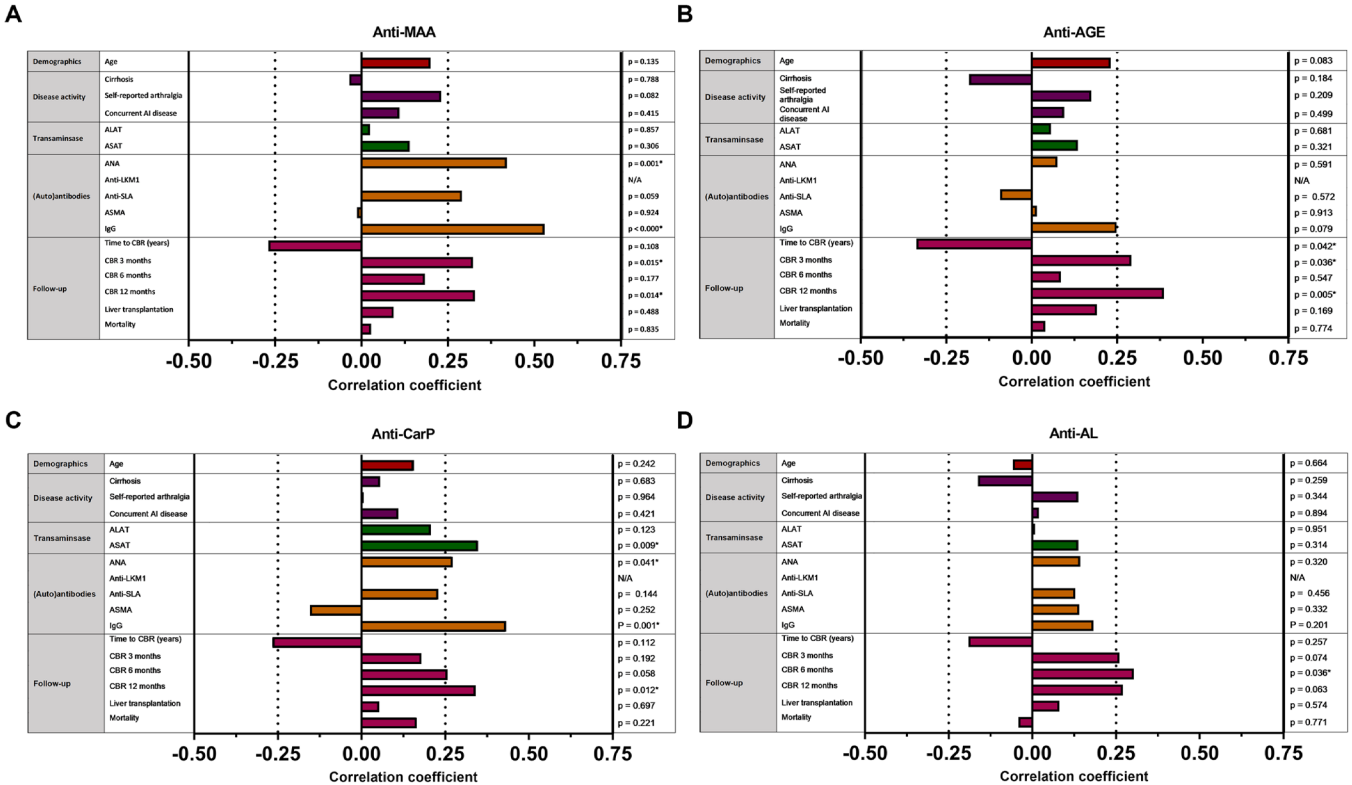

Figure 3: Correlation between (A) anti-MAA IgG, (B) anti-AGE IgG, (C) anti-CarP IgG and (D) anti-AL IgG antibodies and clinical and serological markers in patients with untreated auto-immune hepatitis (n=58). Correlation analyses are done using Spearman's rho correlation analysis and point-biserial correlation analysis. P<0.05 is considered statistically significant (*). *AGE, advanced glycation end-product; ALAT, alanine aminotransferase; ANA, anti-nuclear antibodies; ASAT, aspartate aminotransferase; SMA, smooth muscle antibody; CBR, complete biochemical response; IgG, immunoglobulin gamma; LKM, Liver Kidney microsomal antibody; SLA, soluble liver antigen;*

Table 4: Characteristics of untreated AIH patients in the AILD cohort (n=58).

Patient characteristics	Autoimmune hepatitis (n=58)
Female sex	43 (74.1)
Age diagnosis (years)	46.4±19.4
Simplified criteria for the diagnosis of AIH	8 (6 – 8)
Original revised criteria for AIH	16.7±3.3
Positive antibodies	
ANA (n=58)	38 (65.5)
SMA (n=58)	29 (50.0)
Anti-LKM (n=43)	0 (0.0)
Anti-SLA	2 (3.4)
*Others**	8 (13.8)
IgG (n=57)	24.80 (19.85-32.65)
Histology	
Typical	45 (77.6)
Compatible	9 (15.5)
Atypical/biopsy not done	3 (5.2)
Negative viral hepatitis serology	57 (98.3)
Cirrhosis	23 (39.7)
Yes, compensated	14 (24.1)
Yes, decompensated	9 (15.5)
No cirrhosis	35 (60.3)
Unknown	0 (0.0)
Self-reported arthralgia	12 (20.7)
(More than one) concomitant auto immune disease**	17 (29.3)

Results are presented as n(%), mean ±SD or median (IQR). *AIH, autoimmune hepatitis; ANA, anti-nuclear antibodies; SMA, smooth muscle antibody; IgG, immunoglobulin gamma; IQR, interquartile range; LKM, Liver Kidney microsomal antibody; SD, standard deviation; SLA, soluble liver antigen. *Others: pANCA (n=8) **Other: auto-immune hemolysis (n=1), celiac disease (n=1), diabetes mellitus type 1 (n=2), granulomatosis with polyangiitis (n=1), Henloch-Schönlein purpura (n=1), Hyperthyroidism (n=3), hypothyroidism (n=6), myastenia gravis (n=1), sclerodermia (n=2) ulcerative colitis (n=1)*

Patients with AIH and positive for at least three anti-PTM antibodies reach CBR quicker after initiating treatment

Based on the discovery of multiple anti-PTM antibody positivity in patients with AIH, we attempted to discover the clinical relevance of harboring these multiple anti-PTM antibodies. The median follow-up was 8.7 years (4.6 - 15.3) (*Supplementary Table 5)*. Patients with at least three anti-PTM antibodies scored significantly higher on the revised original score for AIH and had significantly higher levels of IgG at time of diagnosis (*Supplementary Table 5)*. Aminotransferase levels were higher in the group with at least three positive anti-PTM antibodies, albeit not significant. Anti-MAA and anti-CarP correlated positively with ASAT at baseline in the group with less than three anti-PTM antibodies present (r_s=0.37 and r_s=0.45, p=0.037 and p=0.009 respectively), but

not in AIH patients with at least three anti-PTM antibodies. After 3 months treatment, significantly more AIH patients with at least three anti-PTM antibodies had reached CBR (p=0.03). After 12 months of treatment, the difference was still significant (p=0.01). Overall, a trend towards significance for time to CBR (in years) was found in favor of multiple anti-PTM antibody positivity.

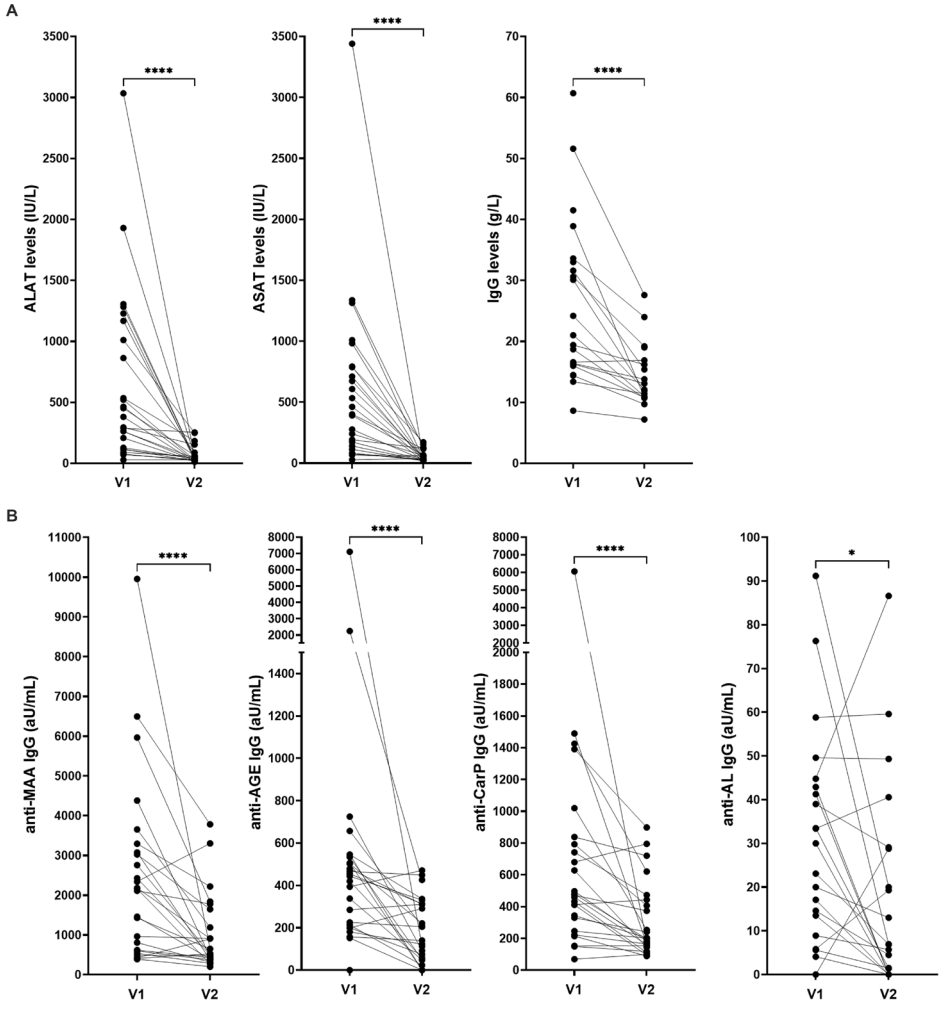

Figure 4: Levels of (A) ALAT, ASAT, IgG and (B) anti-PTM antibodies over time in patients with AIH (n=25). ALAT, ASAT and IgG levels were determined as standard procedure after inclusion. V1: before commencing treatment, V2: during treatment. Median time interval: 65 months (6 – 138). Reactivity towards anti-PTM antibodies was determined using ELISA and is depicted as arbitrary units per milliliter (aU/mL). *p=0.0244 and ****p≤0.0001. *AGE, advanced glycation end-product; AL, acetylated protein; ALAT, Alanine aminotransferase; ASAT, Aspartate aminotransferase; CarP, carbamylated protein; IgG, immunoglobulin gamma; MAA, malondialdehyde–acetaldehyde adduct; V1, first visit; V2, second visit.*

Anti-PTM antibody responses decrease over time and show distinct associations with ALAT, ASAT or total IgG levels.

Clinical data of two different timepoints were available of 25 AIH patients and antibody responses over time was investigated *(Figure 4)*. The first sample was taken before commencing treatment, the second sample during treatment. The median time interval between visit one and two was 65 months (6 – 138). Median ΔALAT, ΔASAT and ΔIgG were 352IU/L (951 – 79) , 324IU/L (722 – 83) and 8g/L (14 – 2) respectively. Levels of all four anti-PTM antibody responses decreased significantly over time (anti-MAA, anti-CarP, and anti-AL (p≤0.0001) and anti-AGE (p=0.024)). Change in anti-AL antibody titers associated significantly with change in ASAT and ALAT (r_s: 0.46 and 0.40 p=0.02 and 0.05 respectively) but did not associate with change in total IgG (r_s: 0.17 p=0.53) (*Supplementary Table 6*). Change in anti-AGE antibody titers significantly associated with change in IgG (r_s: 0.63 p=0.007). Change in anti-MAA and anti-CarP antibody levels was not associated with decrease in ALAT, ASAT and IgG. However, change in anti-CarP antibody levels did show a positive trend towards significant association with decrease of IgG (r_s: 0.48 p=0.052) (*Supplementary Table 6*).

Discussion

To the best of our knowledge, this is the first report on the presence of anti-PTM antibodies in AILD. The presence of anti-PTM antibodies has been described in several other autoimmune diseases where they can serve as diagnostic or prognostic markers (6, 7, 27). Based on these results, we hypothesized that anti-PTM antibodies are also generated in patients with AILD. Additionally, we speculated that patterns in the presence of anti-PTM antibodies might serve diagnostic or prognostic purposes in AILD. In this study there were five significant findings: First, four anti-PTM antibodies were more prevalent in patients with AILD compared to HCs and to non-AILD: anti-MAA, anti-AGE, anti-CarP, and anti-AL. Second, patients with AILD and particularly patients with AIH often harbored multiple types of anti-PTM antibodies. Third, anti-MAA and anti-CarP antibody positivity significantly correlated with markers for biochemical response in AIH. Fourth, AIH patients with at least three types of anti-PTM antibodies reached CBR at 12 months after initiating treatment more frequently. Lastly, after initiating immunosuppressive treatment next to aminotransferases and IgG also anti-AGE and anti-AL antibody titers decreased. These findings confirmed that anti-PTM antibodies are present in AILD and moreover multiple anti-PTM antibodies identify a group of AIH patients in which these anti-PTM antibodies associate with CBR. Interestingly, we observed that several anti-PTM antibodies are present and even more prevalent in AIH patients who were 'sero-negative' for the classical autoantibodies at diagnosis, compared to patients who were positive for either ANA or SMA. This is particularly captivating since conventional antibodies

are not disease specific and may be expressed at a later stage of the disease in 'sero-negative' AIH patients. We suggest that anti-PTM antibodies may be present in patients with AIH before conventional antibodies can be detected. Therefore anti-PTM antibody assessment could especially be interesting in the diagnostic work-up for 'sero-negative' AIH patients. Future studies should further determine the possible implementation of anti-MAA, anti-AGE or anti-CarP assessment, all associated with CBR at 12 months, in the diagnostic algorithm for AIH.

The clinical presentation of AIH is very heterogeneous and can vary from asymptomatic disease to acute (on chronic) liver failure. Occasionally, polyarthralgia without arthritis is present in patients with AIH (1, 28), and is considered an extra hepatic manifestation of AIH. However, this is often not recognized and is underreported. In clinical practice, reoccurrence of arthralgia is often seen during corticosteroid withdrawal (28). Next to arthralgia, RA is sometimes seen in AIH. We have previously reported that, in the context of RA, anti-CarP (29) and anti-Cit (30) antibodies in arthralgia predict development of RA. Additionally, anti-PTM antibodies have been described in the context of rheumatic disease (10, 31, 32). In this study only a trend was found for the correlation between self-reported arthralgia and anti-MAA antibodies in AIH. This could be a result of the small cohort size and would require further investigation.

Previous research showed that IgG levels are not associated with long-term outcomes in AIH, whereas normalization of aminotransferases is the main treatment goal in AIH, as this positively associates with survival in the first 12 months after diagnosis (33). Additionally, Hartl *et al*. found that patients with normal IgG levels showed a comparable treatment response to patients with elevated IgG (34). On the contrary, CBR is defined as normalization of ALAT, ASAT and IgG (26). The role of IgG remains a pivoting point in disease progression in AIH. The results of this study suggest that specific subsets of anti-PTM antibodies are associated with treatment response.

In this study, patients with AIH positive for at least three types of anti-PTM antibodies had significantly higher IgG levels at diagnosis and tended to reach CBR more often at 12 months of treatment than patients with AIH with less than three anti-PTM antibodies. By choosing more than three anti-PTM reactivities as a cut-off in this analysis we achieved an equal number of AIH patients in each group (26 with less and 32 with at least three anti-PTM antibodies). Larger studies could determine whether combinations of anti-PTM antibodies, also combined with serum levels of IgG, ALAT, and ASAT at baseline could be better predictors for the likelihood of treatment response. The anti-PTM antibody response is an IgG mediated response and is part of the significantly elevated IgG in this specific group of patients. Positivity for multiple autoantibodies has been reported to provide more reliable information than single biomarkers in for example diabetes (35) and pre-RA (36).

Our study has some limitations: the cohort is heterogeneous and has a limited size. We found that forty percent of AIH patients identify with specific combinations of anti-PTM antibodies (anti-MAA/anti-CarP; anti-MAA/-AGE /-CarP; anti-MAA /-AGE /-CarP /-AL/-Cit) within the AILD group. These specific combinations might aid in the diagnostic work-up for AIH. Noteworthy is that anti-PTM antibodies are not solely found in AIH, but are also found in other liver diseases possibly as a result of breach in tolerance against PTMs that are formed during inflammation. For several autoimmune diseases it is well known that certain autoantibodies are already present many years before the patients develop clinically overt disease for example anti-Cit and anti-CarP Ab in the context of RA (6,7). Whether this is also the case in these autoimmune liver diseases is currently unknown. PTMs formed as a consequence of inflammation together with impaired liver function may well accumulate and mediate a breach in tolerance, and in this setting anti-PTM antibodies can be formed as a consequence of liver disease. The same set of six anti-PTMs were studied in SLE, and anti-MAA, anti-AGE and anti-CarP antibodies were also most frequently found in patients with SLE compared to healthy controls (10). Interestingly, anti-MAA and anti-CarP associated with neuropsychiatric manifestations of SLE, a manifestation that lacked a biomarker. These findings are in the same range as anti-PTM antibody responses found in AILD. Anti-CarP and anti-Cit are well studied anti-PTM antibodies in RA and are found in approximately 50% of RA patients (6,7). Discovery of new anti-PTM antibodies in RA helped in diagnosis and in following disease progression, and can potentially help to distinguish groups within so-called seronegative RA (5). In order to further validate these findings and prove the sensitivity and specificity of these anti-PTM antibody combinations in the diagnostic work-up of AIH, anti-PTM antibodies need to be assessed extensively in a larger cohort. This could provide the opportunity to set a cut-off titer level and perhaps even distinguish AIH from other liver diseases. In this limited cohort it was not possible to evaluate the prognostic value of anti-PTM antibodies for disease progression as 40% of patients already had cirrhosis at diagnosis. A larger cohort study should be conducted in patients with AILD and no cirrhosis at diagnosis with set follow-up timepoints.

Different clinical parameters are measured to monitor disease activity for AIH, PBC and PSC. As a result, the three groups within the AILD cohort are incomparable. However, in the AILD cohort we have included PBC and PSC, which are not pure auto-immune diseases (where immune injury results in cholestasis) (25, 37-43). We hoped to evaluate possible differences in anti-PTM antibodies patterns in AIH, PBC and PSC. When analyzing AIH patients, only untreated patients were included to prevent impact of treatment. One of the strengths of this study is that the group of interest, AILD, is well-defined according to simplified or original revised score for AIH. Therefore, we can state that the results found for these subgroups are representative.

Combining the prevalence data of all 6 anti-PTM antibodies tested we observe that approximately 20% of healthy controls harbored at least one anti-PTM antibody (*Figure 2*). PTM of proteins occurs in all individuals, these PTMs may represent neo-epitopes towards which antibodies can be formed. Interestingly, this is apparently often not associated with disease, but is known to predispose to disease.

The standard therapy for patients with AIH consists of a combination of glucocorticoids and azathioprine (2). Most patients with AIH in this cohort were initially treated with this preferred treatment. Pape *et al.* demonstrated that a higher or lower initial predniso(lo)ne dose does not have impact on reaching CBR. In our study, stratification of the results by the initial steroid dose was not possible, as 42 patients (85.7%) received an initial predniso(lo)ne dose above 30mg/day (44). When patients do not reach CBR, the treating physician may decide to intensify or adjust treatment regimens. The nature of the disease, characterized by intermittent loss of remission and flares, may give reason to frequently adjust therapy. The size of the studied cohort limited us to correct for change in therapy over time. Since it was not possible to obtain the necessary data we were not able to correct the correlation analyses regarding CBR for duration of steroid treatment, duration of tapering schemes, dose modification or drug withdrawal during follow-up. The median ASAT and ALAT did not differ between the patients who were prescribed budesonide compared to predniso(lo)ne, although this has been previously reported (45).

However, according to the guidelines and Delphi consensus on treatment response, treatment effect is first evaluated 6 months after commencing treatment (2, 26). We additionally did see more patients reaching CBR at 12 months of treatment if they had at least three positive anti-PTM antibodies. This may imply that having anti-PTM antibodies for at least three PTMs may be prognostically favorable regarding treatment response. Despite higher ALAT and ASAT levels at baseline in the AIH patients with at least three anti-PTM antibodies present, no association between transaminase levels and multiple positivity could be found. Only in patients with less than three anti-PTM antibodies present, a positive correlation between anti-MAA, anti-CarP and ASAT was found. This strengthens the implication that multiple positivity for at least three anti-PTM antibodies may be beneficial for treatment response and may guide treating physicians to earlier treatment intensification.

In conclusion: anti-PTM antibodies are present in patients with AILD. Some patients are positive for multiple anti-PTM antibodies. Having three or more anti-PTM antibody responses is associated with a favorable response to treatment in AIH.

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Supplementary files

Supplementary Table 1: Characteristics of AIH, PBC and PSC patients in the AILD cohort. Results are presented as n (%), or median (IQR).

	AIH (n=66)	PBC (n=10)	PSC (n=30)
Laboratory assessments			
ALAT	338.0 (125.0 - 1057.5)	37.5 (28.0 – 76.8)	54.0 (37.0 – 114.0)
ASAT	420.0 (142.8 - 935.3)	50.5 (34.5 – 53.8)	48.0 (36.0 – 99.0)
IgG	24.8 (19.4-33.2)	-	-
ALP	178.5 (123.0 – 279.0)	277.0 (158.0 – 433.0)	229.0 (132.0 – 405.0)
GGT	212.0 (116.0 – 371.5)	147.0 (58.0 – 571.0)	166.0 (96.5 – 278.5)
Positive antibodies			Insufficient data
ANA	42 (63.6)	3 (33.3)	-
SMA	35 (53.0)	-	-
Anti-LKM	1 (1.5)	-	-
Anti-SLA	2 (3.0)	-	-
AMA	-	8 (80.0)	-
*Other**	9 (13.6)	-	-
Large duct anomalies **	N/A	N/A	29 (96.70)

AIH, autoimmune hepatitis; ALAT, alanine aminotransferase; ALP, alkaline phosphatase; AMA, anti-mitochondrial antibodies; ANA, anti-nuclear antibodies; ASAT, aspartate aminotransferase; SMA, smooth muscle antibody; CBR, complete biochemical response; GGT, gamma-glutamyl transferase; IgG, immunoglobulin gamma; LKM, Liver Kidney microsomal antibody; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; SLA, soluble liver antigen. * pANCA (n=8), anti-parietal cell (n=1) ** based on cholangiographic findings with magnetic resonance/endoscopic retrograde cholangiopancreatography.

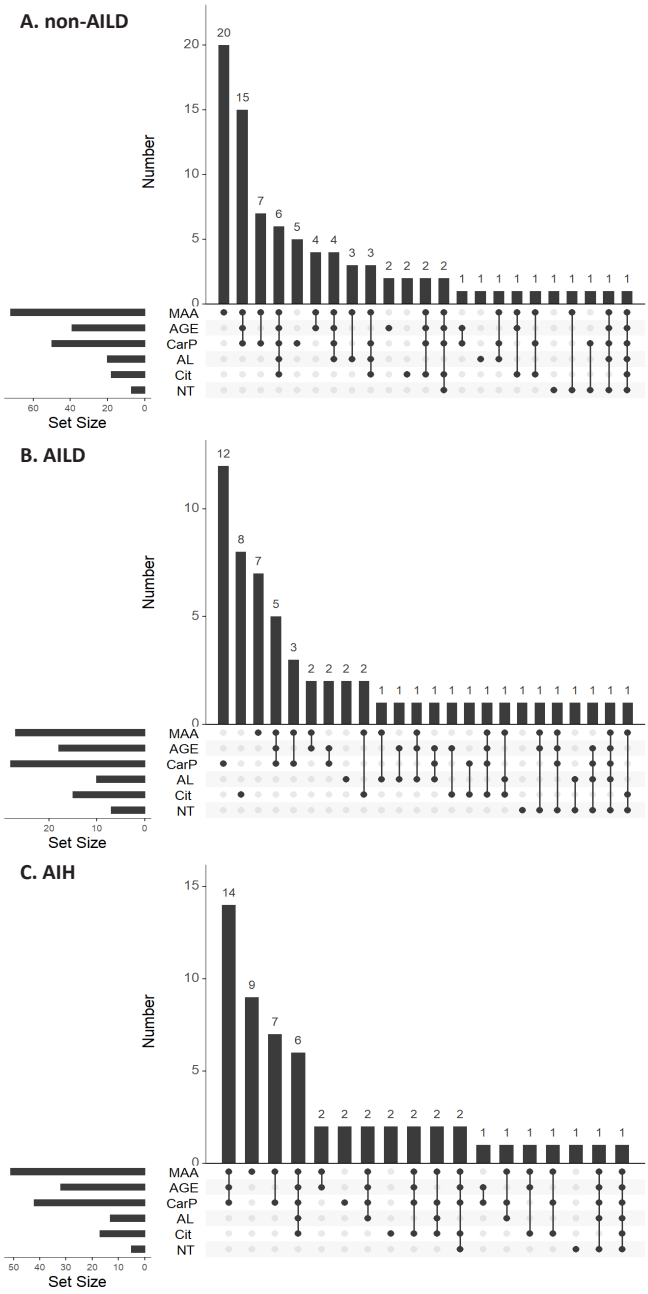
Supplementary Table 2: Prevalence of antibodies against post-translationally modified proteins in patients with autoimmune (n=106) or non-autoimmune liver disease (n=101) and healthy controls (n=100).

	Healthy Controls n=100			Autoimmune Liver Disease n=106				Non-Autoimmune Liver Disease n=101			
	aU/mL [IQR]		n (% positive)	aU/mL [IQR]		n (% positive)		aU/mL [IQR]		n (% positive)	
Anti-MAA	266,9	[200,4 – 370,2]	2 (2,0)	1036,0	[625,5 – 2119,6]**	72	(67,9)**	495,8	[315,2– 726,8]$^{\#\#,++}$	27	(26,7)$^{\#\#,++}$
Anti-AGE	88,9	[0,0 – 182,5]	4 (4,0)	234,5	[95,5 – 480,5]**	39	(36,8)**	130,0	[4,2 – 261,2]$^{++}$	18	(17,8)$^{\#\#,++}$
Anti-CarP	74,0	[1,5 – 157,9]	5 (5,0)	352,5	[213,5 – 633,0]**	50	(47,2)**	241,0	[83,5 – 422,0]$^{\#\#,+}$	28	(27,7)$^{\#\#,++}$
Anti-AL	0,0	[0,0 – 9,8]	5 (5,0)	13,3	[2,8 – 30,7]**	20	(18,9)**	6,4	[0,0 – 10,0]$^{++}$	10	(9,9)
Anti-Cit	1,3	[0,0 – 3,2]	6 (6,0)	3,1	[0,5 – 7,2]*	18	(17,0)*	1,6	[0,0 – 5,9]	15	(14,9)$^{\#}$
Anti-NT	108,0	[0,0 – 250]	6 (6,0)	269,0	[33,8 – 602,5]**	7	(6,6)	179	[0,0 – 501,5]	7	(6,9)

Results are presented as median [IQR] or n (%). *p<0.05, **<0.01 between HC and AILD #p<0.05, ##<0.01 between HC and non-AILD +p<0.05, ++<0.01 between AILD and non-AILD Autoimmune Liver Disease: AIH, PBC and PSC; non-Autoimmune Liver Disease: NAFLD, HCV, HBV, ALD, Combination, NASH. *AGE, advanced glycation end-product; AL, acetylated protein; aU/mL, arbitrary units per milliliter; CarP, carbamylated protein; Cit, citrullinated protein; IQR, interquartile range; MAA, malondialdehyde–acetaldehyde adduct; NT, nitrated protein;*

Supplementary Table 3: The association between the presence of anti-PTM antibodies and the three major autoimmune liver diseases. Results are presented as median (IQR) of n (%). Chi-2-tests were used to assess the difference between the presence of the specific manifestations and non-AILD patients. *p=0.006, **p<0.001. *AGE, advanced glycation end-product; AIH, autoimmune hepatitis; AL, acetylated protein; aU/mL, arbitrary units per milliliter; CarP, carbamylated protein; IQR, interquartile range; MAA, malondialdehyde–acetaldehyde adduct; non-AILD, non-autoimmune liver disease; PBC, Primary Biliary Cirrhosis; PSC, Primary Sclerosing Cholangitis*

	AIH		**PBC**		**PSC**		**Non-AILD (reference)**	
	Yes, n=66		**Yes, n=10**		**Yes, n=30**		**No, n=101**	
Anti-MAA								
aU/mL	1519.5	(760.0 – 2775.3)	787.6	(436.3 – 1669.7)	771.5	(545.9 – 1223.0)	495.8	(315.2– 726.8)
Positive	**51**	**(77.3)** **	5	(50.0)	**16**	**(53.3)** *	27	(26.7)
Anti-AGE								
aU/mL	349.0	(156.0 – 537.0)	76.5	(0.0 – 177.9)	172.5	(59.3 – 359.3)	130.0	(4.2 – 261.2)
Positive	**32**	**(48.5)** **	0	(0.0)	7	(23.3)	18	(17.8)
Anti-CarP								
aU/mL	475.5	(293.2 – 741.8)	148.7	(0.0 – 376.0)	230.5	(41.3 – 336.0)	241.0	(83.5 – 422.0)
Positive	**42**	**(63.6)** **	2	(20.0)	6	(20.0)	28	(27.7)
Anti-AL								
aU/mL	15.4	(4.7 – 33.4)	15.9	(0.8 – 24.8)	9.9	(0.2 – 28.0)	6.4	(0.0 – 10.0)
Positive	13	(19.7)	1	(10.0)	6	(20.0)	10	(9.9)



Supplementary Figure 1: Patients with AILD present with double or triple positivity for different anti-PTM antibodies compared to non-AILD and define a group of AIH patients harboring three anti-PTM antibodies. Upset plot indicating the frequency of combinations of anti-PTM antibodies in (A) non-AILD, (B) AILD, and (C) AIH. Horizontal bars represent the number of individuals in each of the anti-PTM antibodies of interest with a table on the right indicating single or combinations of anti-PTM antibodies. Vertical bars represent the number of patients positive for that anti-PTM antibody or combinations thereof.

Supplementary Table 4: Detailed overview of correlation coefficients (including 95% confidence intervals) as provided in Figure 5.

	Anti-MAA		Anti-AGE		Anti-CarP		Anti-AL	
	corr	95% CI*	corr	95% CI*	corr	95% CI*	corr	95% CI*
Demographics								
Age	0.199	-0.06 – 0.43	0.230	-0.03 – 0.46	0.156	-0.11 – 0.40	-0.058	-0.31 – 0.20
Disease activity								
Cirrhosis	-0.36	-0.29 – 0.22	-0.183	-0.43 – 0.09	0.055	-0.21 – 0.31	-0.163	-0.42 – 0.12
Self-reported arthralgia	0.230	-0.03 -0.46	0.174	-0.10 – 0.42	0.006	-0.25 – 0.26	0.137	-0.15 – 0.40
Concurrent AI disease	0.110	-0.15 – 0.36	0.095	-0.18 – 0.35	0.110	-0.16 – 0.36	0.019	-0.26 – 0.30
Transaminases								
ALAT	0.024	-0.24 – 0.28	0.056	-0.21 – 0.31	0.207	-0.06 – 0.44	0.008	-0.25 – 0.27
ASAT	0.139	-0.13 – 0.38	0.135	-0.13 – 0.38	0.348	0.09 – 0.56	0.137	-0.13 – 0.38
(Auto) antibodies								
ANA	0.420	0.18 – 0.61	0.075	-0.19 – 0.33	0.272	0.01 – 0.49	0.143	-0.14 – 0.40
Anti-LKM1	-	-	-	-	-		-	
Anti-SLA	0.290	-0.03 – 0.51	-0.093	-0.39 – 0.23	0.229	-0.08 – 0.49	0.128	-0.21 – 0.43
SMA	-0.013	-0.31 – 0.29	0.015	-0.25 – 0.28	-0.154	-0.40 – 0.11	0.140	-0.14 – 0.40
IgG	0.529	0.29 – 0.70	0.248	-0.03 – 0.49	0.435	0.18 – 0.63	0.182	-0.10 – 0.43
Follow-up								
Time to CBR (years)	-0.269	-0.54 – 0.06	-0.337	-0.59 – 0.01	-0.266	-0.54 – 0.06	-0.191	-0.48 – 0.14
CBR 3 months	0.322	0.06 – 0.54	0.291	0.02 – 0.52	0.179	-0.09 – 0.42	0.260	-0.03 – 0.50
CBR 6 months	0.183	-0.08 – 0.42	0.085	-0.19 – 0.35	0.257	-0.01 – 0.49	0.303	0.02 – 0.54
CBR 12 months	0.328	0.07 – 0.54	0.386	0.12 – 0.59	0.341	0.08 – 0.55	0.270	-0.02 – 0.51
Liver transplantation	0.093	-0.17 – 0.34	0.190	-0.08 – 0.43	0.053	-0.21 – 0.31	0.081	-0.20 – 0.35
Mortality	0.028	-0.23 – 0.28	0.040	-0.23 – 0.30	0.165	-0.10 – 0.41	-0.042	-0.31– 0.24

All confidence intervals were calculated using Fisher's transformation. *AGE, advanced glycation end-product; AI, autoimmune*; *AL, acetylated protein; ALAT, alanine aminotransferase; ALP, alkaline phosphatase; AMA, anti-mitochondrial antibodies; ANA, anti-nuclear antibodies; ASAT, aspartate aminotransferase; SMA, anti-smooth muscle antibody; CarP, carbamylated protein; CBR, complete biochemical response; CI, confidence interval; corr, correlation coefficient; IgG, immunoglobulin gamma; MAA, malondialdehyde–acetaldehyde adduct; LKM, Liver Kidney microsomal antibody; SLA, soluble liver antigen.*

Supplementary Table 5: Baseline characteristics of patients with AIH and at least 3 positive anti-PTM antibodies versus less than 3.

	≥3 anti-PTM positive	<3 anti-PTM positive	p-value
Demographics			
Patients	32 (55.2)	26 (44.8)	-
Female sex	24 (75.0)	19 (73.1)	0.87
Age diagnosis (years)	47.7 (±20.0)	44.8 (±18.9)	0.571
Simplified criteria for the diagnosis of AIH	8.0 (6.0 – 8.0)	7.0 (5.8 – 8.0)	0.19
Revised original criteria for AIH	17.9 (±2.6)	15.3 (±3.6)	<0.05*
Cirrhosis	12 (37.5)	11 (42.3)	0.71
Yes, compensated	8 (25.0)	6 (23.1)	-
Yes, decompensated	4 (12.5)	5 (19.2)	-
No cirrhosis	20 (62.5)	15 (57.7)	-
Self-reported arthralgia	7 (21.9)	5 (19.2)	0.81
Auto immune comorbidities	11 (34.4)	6 (23.1)	0.31
Laboratory			
ALAT	535.0 (186.0 – 1028.0)	333.0 (118.3 – 1136.0)	0.65
ASAT	580.0 (190.8 – 989.3)	304.0 (111.3 – 827.8)	0.29
IgG	28.8 (22.2 – 39.6)	21.0 (14.0 – 31.1)	0.02*
Treatment started			0.47
Azathioprine	1 (3.1)	0 (0.0)	-
Budesonide	0 (0.0)	2 (7.7)	-
Budesonide + azathioprine	2 (6.3)	1 (3.8)	-
Prednisolone	2 (6.3)	1 (3.8)	-
Prednisolone + azathioprine	25 (78.1)	20 (76.9)	-
Prednisolone + thioguanine	1 (3.1)	0 (0.0)	-
Thioguanine	0 (0.0)	1 (3.8)	-
Unknown	1 (3.1)	0 (0.0)	-
No treatment started	0 (0.0)	1 (3.8)	-
Follow-up			
Duration of follow-up	9.8 (4.9 – 18.2)	7.01 (4.0 – 12.26)	0.15
Time to CBR (years)	0.8 (0.3 – 2.3)	2.0 (0.8 – 4.1)	0.06
CBR 3 months	8 (25.0)	1 (3.8)	0.03*
CBR 6 months	9 (28.1)	3 (11.5)	0.12
CBR 12 months	13 (40.6)	3 (11.5)	0.01*
Liver transplantation	2 (6.3)	1 (3.8)	0.68
Mortality during follow-up	8 (25.8)	5 (19.2)	0.60
Switch in medication	15 (46.9)	10 (38.5)	0.36

Results are presented as n (%), mean ±SD or median (IQR). *AIH, autoimmune hepatitis; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; CBR, complete biochemical response; IgG, immunoglobulin gamma.*

Supplementary Table 6: Correlations between change in anti-PTM antibody levels and changes in aminotransferases and immunoglobulin gamma (IgG). All confidence intervals were calculated using Fisher's transformation. *AGE, advanced glycation end-product; AL, acetylated protein; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; CarP, carbamylated protein; CI, confidence interval; corr, correlation coefficient; IgG, immunoglobulin gamma; MAA, malondialdehyde–acetaldehyde adduct.*

	Δ Anti-MAA			**Δ Anti-AGE**			**Δ Anti-CarP**			**Δ Anti-AL**		
	corr	p-value	95% CI*	corr	p-value	95% CI*	corr	p-value	95% CI*	corr	p-value	95% CI*
Δ ALAT	0.1	0.6	-0.3 – 0.5	0.28	0.2	-0.1 – 0.6	0.2	0.4	-0.2 -0.5	0.4	0.0*	0.0 – 0.78
Δ ASAT	0.2	0.4	-0.2 – 0.5	0.29	0.2	-0.1 – 0.6	0.3	0.1	-0.1 – 0.6	0.5	0.0*	0.18 – 0.7
Δ IgG	0.3	0.3	-0.2 – 0.7	0.6	0.007*	0.2 – 0.8	0.5	0.1	0.0 – 0.8	0.2	0.5	-0.3 – 0.6

Response to commentary on

Antibodies against multiple post-translationally modified proteins aid in diagnosis of autoimmune hepatitis and associate with complete biochemical response to treatment

We read with great interest the commentary of Taubert and colleagues (1) on our article "Antibodies against multiple post-translationally modified proteins aid in diagnosis of autoimmune hepatitis and associate with complete biochemical response to treatment (2)". In their kind commentary the authors bring up the very important and relevant subject of polyreactive IgG (pIgG) as they have described to occur in autoimmune hepatitis (AIH) (3). The team of Taubert have identified such pIgG using an experimental set up roughly similar to the enzyme-linked immunosorbent assay (ELISA) set up as we have used for the detection of the antibodies against post-translationally modified proteins (anti-PTM). In their commentary they raise the concern that part of the antibodies identified in our assays as anti-PTM antibodies may in fact be pIgG. We can reassure the authors and readers that we are specifically detecting anti-PTM antibodies in our assay. Importantly, this is because of the setup of our ELISA system. Ever since the identification of antibodies binding to carbamylated antigens (anti-CarP) (4) we have used both carbamylated fetal calf serum (Ca-FCS) and unmodified FCS as control antigens for the coating of the ELISA plates. In practice one half of the ELISA plate is coated with Ca-FCS and the other with unmodified, control FCS. The entire plate is blocked with bovine serum albumin (BSA). Each serum sample is tested on both the Ca-FCS and the control FCS. The levels of antibody binding are calculated from absorbance values into arbitrary units per milliliter based on a standard line on the same plate. Next, the level of carbamylation specific antibodies is defined as the level of antibody binding to the Ca-FCS minus the level of antibodies binding to the control FCS. Hence, we report the PTM-specific response. In many of the analyses that we have run for rheumatoid arthritis (RA) and for systemic lupus erythematosus (SLE) (5) the reactivity of the control protein is very low. Indeed, we have observed that in AIH this was somewhat higher, but importantly we have subtracted this from the anti-PTM response, allowing us to conclude on the PTM-specific antibodies and avoiding undesired interference from pIgG. We realize that we may not have stressed this to the greatest extend in our manuscript and thank the authors for bringing up this point and for the opportunity to clarify this.

In our manuscript we have used six different PTMs. To make the best comparisons, we have not used the same control FCS for all the PTMs but have actually generated a separate control FCS for each of the conditions. For example, the control for carbamylation is an aliquot of the same FCS, incubated at the same time point, for the same duration at

the same temperature and dialysis steps as the carbamylated FCS, but only without the addition of the KOCN, the carbamylating chemical. For the modification with Advanced Glycation End-products, we have performed the incubations of the control FCS also for 10 days at 37° C, all to ensure that we make the best possible comparisons.

In the original paper we already reported that each of the anti-PTM reactivities has clearly different sensitivities, while all of the assays are based on FCS coating and bovine serum albumin (BSA) blocking, indicating that the assays do not detect pIgG. We have tested if there was any correlation between the signals observed on PTM-FCS versus control FCS. For the four anti-PTMs with the highest percentage of positive samples we did not find any correlation, again indicating that the anti-PTM antibodies are specifically binding to the PTM. The authors raise interesting questions regarding the nature of the antibody response to the PTM proteins. As can be seen in Supplementary Figure 1 of the manuscript (2), we studied how often the different anti-PTM antibodies can be found together in the same patients, as this may be an indication of either co-induction or cross-reactivity. We clearly observe different patterns with some individuals positive for one anti-PTM and other positive for several others (2), again indicating that the different assays are clearly identifying different antibodies. Additionally, while between some anti-PTM responses we do observe a correlation (as observed before), for other anti-PTM responses we do not detect any correlations. Importantly, some patients can be highly positive for one anti-PTM reactivity and simply negative for the other.

We did find that overall levels of some anti-PTM antibodies (weakly) associate with levels of IgG, but this may simply reflect that a polyclonal B cell stimulation (6) will stimulate the anti-PTM reactive B cells as well as other B cells, but it will only result in positivity in individuals that actually have anti-PTM reactivity. In the context of RA, we have observed that many of the anti-PTM antibodies are isotype switched but are of low-avidity (7, 8) indicating that there has been T-cell help, but lack of avidity maturation. The authors finally raise the point of serum storage time. This is an important issue and difficult to address experimentally. We have previously studied this in detail for our cohort in the context of our previous paper on AIH, focused on other biomarkers (9), where we concluded that the quality of the samples was good, as there was no difference in the sensitivity of the markers in the samples that were stored for a long time (i.e., ≥10 years) versus the samples that were stored more recently (i.e., <10 years) , suggesting that the storage was not a major factor in these analyses. Also for the current study on anti-PTM antibodies we have now carefully plotted the levels of all the 6 anti-PTM reactivities versus the time of storage of the sample and observed that positivity for the anti-PTM antibodies is not influenced by storage time (data not shown).

Importantly, for the anti-PTM responses in AIH we do observe associations with response to treatment while in the work of Taubert *et al.* (3) no such association is observed for pIgG, again indicating that the anti-PTM detection does measure different antibodies. For a subset of patients we have analyzed changes in anti-PTM antibody levels over time, and we observed that upon treatment the levels decrease. The data obtained from these two time points does not reveal if the anti-PTM positivity will completely seroconvert.

In conclusion, we agree with the authors of the commentary that unintentional detection of pIgG is an important factor to consider when running ELISA experiments on sera of patients with AIH. However, we are convinced that the careful set up of our experiments excluded the detection of pIgG and specifically measures anti-PTM antibodies.

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