Published in final edited form as:

Hepatol Int. 2012 January ; 6(1): 379–385. doi:10.1007/s12072-011-9277-8.

Clinical Significance of Serum Autoantibodies in Patients With NAFLD: Results From The Nonalcoholic Steatohepatitis Clinical Research Network (NASH CRN)

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Abstract

Purpose—Some studies have suggested that autoantibodies might define a subcategory and phenotype of NAFLD associated with advanced histological features. We evaluated the relationship between the presence of serum autoantibodies and liver histology in a large cohort of well-characterized patients with NAFLD.

Methods—A total of 864 NAFLD patients participating in two prospective multicenter clinical studies underwent testing for serum autoantibodies within 24 months of a liver biopsy. Liver histology was compared between patients with and without ANA 1:160 or ASMA 1:40 or both.

Results—Autoantibodies were present in 182 patients (21%). There was no difference in age, gender, race, BMI, HOMA-IR or history of diabetes between the two groups. Biopsies in subjects with autoantibodies were less likely to have moderate-to-severe steatosis (i.e. >33%) compared to controls (57.1% vs. 43.0%, p-value = 0.0006). Lobular inflammation (46.7% vs. 47.5%), ballooning degeneration (38.5% vs. 42.5%), and advanced fibrosis (33.2% vs. 29.3%) were not different between the two groups. Histologic evidence of ‘definite’ NASH did not differ significantly between the two groups (55.5% vs. 58.9%). After adjusting for age, gender, BMI, race and diabetes, the presence of autoantibodies was independently associated with lower prevalence of moderate-to-severe steatosis (odds ratio [OR], 0.58; 95% confidence interval [CI], 0.41–0.82; p=0.01).

Conclusion—Autoantibodies are frequently positive in NAFLD in the absence of autoimmune hepatitis and their occurrence is not associated with more advanced histologic features.

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The authors declare that they do not have anything to disclose regarding funding from industries or conflicts of interest with respect to this manuscript.

Keywords

Autoantibodies; NASH; NAFLD; liver histology

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a common cause of chronic liver disease in the Western world and is estimated to be present in one third of the U.S. population [1, 2]. A certain subset (up to 15%) of NAFLD patients have nonalcoholic steatohepatitis (NASH) that is characterized by progressive inflammation and hepatic fibrosis leading to cirrhosis, hepatocellular cancer, liver failure and premature mortality [3]. Due to lack of a reliable noninvasive test, NASH remains a histological diagnosis requiring liver biopsy [4]. Efforts to identify the small subset of patients with NASH from the larger NAFLD patient population in order to avoid unnecessary liver biopsy have led to the identification of various clinical and biochemical variables predictive of advanced fibrosis associated with NASH [4–8]. Few investigators have also proposed complex scores based on a combination of these variables [7, 9, 10]. The presence of serum autoantibodies such as antinuclear antibody (ANA) and smooth muscle antibody (SMA) have been reported to be associated with a higher inflammatory grade and advanced fibrosis, prompting some experts to recommend liver biopsy in NAFLD patients with positive autoantibodies [11–13].

Previous studies examining the prevalence of serum autoantibodies (ANA and SMA) in patients with NAFLD reported a wide range of prevalence rates depending on the threshold values used for an abnormal titer [11, 12, 14–17]. However, these studies have been limited by heterogeneity with regard to study design, sample size and referral bias. Adams et al. reported an association between the presence of autoantibodies (ANA > 1:40) with significantly higher fibrosis stage and inflammatory grade in 225 patients with liver biopsy proven NAFLD [11]. Nina et al. reported a significantly greater degree of portal inflammation, hepatocellular ballooning and advanced histological features of NASH in 35 ANA-positive NASH patients compared to 36 ANA-negative NASH patients in a case-control study [13]. Interestingly, the degree of steatosis was lower in the high-titer (1:320) ANA group ($p=0.01$). Lorie et al. also showed that the autoantibody positive subjects exhibited severe steatosis less frequently (1 of 7) than the autoantibody-negative (50%) group [18]. An association between patients with high titer autoantibodies and insulin resistance in this cohort led the investigators to hypothesize that autoantibodies may represent an epiphenomenon of insulin resistance leading to the progression of NAFLD [19]. However, this finding was not confirmed by Adams et al. in their cohort [11, 20]. Thus, studies examining the relationship between disease severity and the presence of autoantibodies have reported conflicting results. The goal of the current study was to determine the prevalence of significant autoantibodies (defined as ANA 1:160 or SMA 1:40 or both) in a large cohort of well-characterized NAFLD patients. A second aim of the study was to systematically evaluate the relationship between the presence of these autoantibodies, presence of diabetes/insulin resistance, and the histologic severity of NAFLD.

Methods

The Nonalcoholic Steatohepatitis Clinical Research Network (NASH CRN) has successfully conducted two prospective clinical trials in adult patients with NAFLD and NASH [21, 22]. The baseline demographic, laboratory and histological data from all adult patients from two prospective studies (NAFLD Database study and the PIVENS Trial, Clinical Trial No. NCT00063622) were considered for this study. The NAFLD Database Study is an observational study of patients with definite NAFLD, suspected NAFLD, and cryptogenic cirrhosis [21]. The PIVENS trial is a randomized, multicenter, double masked, placebo controlled trial that evaluated pioglitazone or vitamin E in the treatment of biopsy-proven NASH [22]. These studies were conducted across eight clinical centers with a central Data Coordinating Center (see Appendix for roster). Study protocols were approved by all participating center institutional review boards. Inclusion criteria for this study were patients in whom autoantibodies were detected within 2 years of their liver biopsy. The titers of autoantibodies were considered significant if ANA 1:160 or SMA 1:40 or both. Among patients who had antibodies detected by an indirect immunofluorescence method, ANA 3 U and SMA 30 U was considered clinically significant. These “cut-off values” for autoantibody titers were chosen a priori to increase specificity and to improve the distinction between clinically-significant and positive ANA and SMA titers that can occur in normal individuals [23]. Liver biopsies were reviewed centrally in a systematic blinded fashion by the Pathology subcommittee and scored for various histologic features including steatosis, lobular inflammation, hepatocellular injury (ballooning), fibrosis, NAFLD activity score (NAS) and steatohepatitis diagnosis (i.e., “not steatohepatitis”, “borderline”, “definite steatohepatitis”) [24]. NAS is a composite score taking into consideration the degree of steatosis, inflammation and hepatocyte ballooning [24].

Statistical analysis

Histological features were compared between those with and without significant titers of autoantibodies. Sample size calculation for this study were based on fibrosis ranging from 0–6 (continuous response variable) as per the standard protocol used by the NASH CRN Pathology subcommittee [24] with 1:1 ratio between NAFLD patients with and without autoimmune antibodies. We assumed that the fibrosis within each subject group was normally distributed with standard deviation 1. To detect a difference of at least 2 points (33%) in fibrosis score, we would need to study 145 subjects with autoantibodies and 145 control subjects to be able to reject the null hypothesis that the population means of the autoantibody positive and control groups are equal with probability (power) 0.8. The Type I error probability associated with this test of this null hypothesis is 0.05. P-values were determined by a chi-square test for categorical variables and by the Wilcoxon rank-sum test for continuous variables. Nominal, two-sided P-values were used and were considered statistically significant if $P < 0.05$. Multiple logistic regression analyses were performed to examine the relationship between the presence of significant ANA/ASMA and histological variables. Separate models were fit for each histologic variable as the outcome and all models were adjusted for age, gender, race, BMI, and diabetes. Analyses were performed using SAS statistical software (version 9; SAS Institute Inc., Carey, NC).

Results

A total of 864 patients who participated in the two NASH CRN studies were included in this study. The mean age at enrollment for these studies was 49 ± 12 years and the mean BMI was 34 ± 6 kg/m² in the entire cohort. Sixty-four percent were women. Eighty-four percent of the cohort was Caucasian. Diabetes (type 1 or 2) was present in 28% and in those who did not have a diagnosis of diabetes, the median homeostasis model assessment of insulin resistance score (HOMA-IR) score of 3.9 (inter-quartile range: 2.5–6.1) was indicative of their underlying insulin resistance. One hundred and eighty-two patients (21%) had significant titers of autoantibodies. There were no statistically significant differences between the two groups with regard to gender, BMI, race, presence of diabetes, or HOMA-IR score (Table 1). Analysis of histological features showed that patients with positive autoantibodies were less likely to have moderate-to-severe steatosis (33%) compared to those who did not have autoantibodies (57.1% vs. 42.9%, $p=0.0006$). The mean NAS was also lower in patients with significant autoantibodies compared to those without (4.1 ± 1.8 vs. 4.4 ± 1.7 , $p=0.04$). However, lobular inflammation with a threshold of >2 foci under 20X magnification (46.7% vs. 47.5%), ballooning degeneration with a threshold of many cells (38.5% vs. 42.5%), and advanced fibrosis defined by the presence of bridging fibrosis or cirrhosis (33.2% vs. 29.3%) were not significantly different between the two groups. Histologic evidence of 'definite' NASH also did not differ significantly between the two groups (55.5% vs. 58.9%, $p=0.41$) (Table 2). Patients with both positive ANA and SMA were separately categorized and additional analysis of detailed histological category sub groups are shown (Table 2). Chronic portal inflammation (more than mild) was significantly higher (31.6% vs. 22.0%, $p=0.03$) compared to patients without autoantibodies. Subgroup analysis of histological features in patients with either ANA or SMA titer 1:640 positivity showed similar demographic and histological features compared to patients without autoantibodies (Supplementary Table 1). Multiple regression analysis after adjusting for age, gender, BMI, race and diabetes, the presence of autoantibodies in significant titer was independently associated with lower prevalence of moderate-to-severe steatosis (odds ratio [OR], 0.58; 95% confidence interval [CI], 0.41–0.82; $p=0.01$) on liver histology.

Discussion

Autoantibodies such as ANA and ASMA are often discovered during the routine evaluation of patients with suspected NAFLD and their presence leads to concerns about autoimmune hepatitis; the standard treatment for autoimmune hepatitis includes corticosteroids which may lead to unnecessary complications in patients with NAFLD. In the current multicenter study from across 8 centers in United States, we observed that significant titers of autoantibodies were detectable in 21% of the study cohort, demonstrating that presence of autoantibodies is common in this population. This prevalence rate is similar to the two previous studies with much smaller cohorts of liver biopsy-proven NAFLD patients [11, 18].

Prior studies evaluating the relationship between the presence of these antibodies with insulin resistance, severity of liver histology and diagnosis of NASH have yielded conflicting results [11, 12, 18]. We observed that the prevalence of diabetes (type 1 and 2) and the median HOMA-IR score were not significantly different between NAFLD patients

with and without significant autoantibodies. Our results indicate that the presence of autoantibodies may be an epiphenomenon and may not indicate underlying insulin resistance. This is consistent with the earlier findings by Adams et al. in a smaller cohort of patients [20]. Our study does not confirm the previously reported observation that the presence of autoantibodies in significant titer is associated with advanced fibrosis as systematic evaluation of various histological features such as hepatocyte ballooning, lobular inflammation, and advanced fibrosis did not reveal any statistically significant differences between the two groups. Similarly, histologic evidence of 'definite' NASH did not differ significantly between the two groups. NAS was lower in those with significant autoantibodies compared to those without, likely due to lesser degree of hepatic steatosis in this group. Overall, systematic comparison of various histological features did not reveal any significant difference between NAFLD patients with and without significant autoantibodies with the exception of the amount of steatosis. The finding of a significantly lower grade of steatosis in patients with significant autoantibodies is intriguing and is consistent with the previous two studies [13, 18].

Previous reports describing the presence of autoantibodies in patients with NAFLD and NASH have not yielded insights into the possible mechanisms for this phenomenon. Recently, Syn et al. reported that hepatic natural killer T (NKT) cell accumulation was associated with more advanced NAFLD [25]. Concomitant activation of autoantibody production with activation of NKT T cells has been previously reported [26, 27]. We speculate that autoantibody production in NAFLD subjects may be a consequence of hepatic NKT cell accumulation. In support of this hypothesis, a significant increase (31.6% vs. 22.0 %) in chronic portal inflammation (more than mild) was present in patients with autoantibodies compared to patients without autoantibodies (Table 2). Loria et al. previously proposed the existence of an "autoimmune hepatitis – NAFLD" overlap syndrome [18]. However, treatment with corticosteroids in this subgroup of patients was not associated with biochemical or histological improvement.

Certain aspects of our study deserve further discussion. Since there are multiple causes for ANA positivity (medications, chronic liver disease, seropositive arthritis and advanced age) [23, 28], a concomitant evaluation of ANA positivity with hyperglobulinemia could potentially indicate a phenotype or a co-existing autoimmune hepatitis or more advanced disease than an isolated ANA. However, we raised the negative cut-off titer from 1:40 to 1:160 in order to improve the distinction between clinically significant ANA and ANA occurring in normal individuals from other causes. In addition, our study cohort consisted of adult NAFLD patients (with or without significant autoantibodies) who were selected for inclusion in the study based on the absence of another concomitant liver disease. Therefore, patients with clinical evidence of autoimmune hepatitis were excluded. In contrast to the present study, a prior study from NASH CRN evaluating the significance of autoimmune serology in pediatric NAFLD reported a statistically significant increased prevalence of SMA positivity with increasing NAS [29]. Therefore our findings may not be applicable to the pediatric NASH/NAFLD population. We also analyzed the study cohort after categorization into three study groups: no antibody detected (ANA<1:40) vs. nonsignificant titer antibody (ANA 1:40 – 1:80) and significant titer antibody (ANA >1:160) in order to examine any gradient of features that were important and could lead to a more criteria-based

definition. This analysis yielded similar conclusions (data not shown). Additional analysis included limiting the study cohort to those who had histology and labs within 6 months yielded a sample size of 678 (antibody positive: 148, antibody negative: 530). This ratio is very similar to the cohort reported in this study (antibody positive: 182, antibody negative: 682). Since the ratio is very similar (0.26), we do not anticipate a different conclusion either with 6 months or 2 years cut off for study entry criteria. Alternative inclusion criteria with limitation of the time interval between liver biopsy and autoantibody to 3 months along with the presence of ANA titer 1:40 or ASMA titer 1:40 or both, yielded a prevalence of 12.6%. Analysis of this cohort yielded similar conclusion with autoantibody positive group having a lower prevalence of severe steatosis (>33%) in (44.9% vs.58.7%, $p=0.004$).

In summary, the current study found that presence of a positive ANA or SMA was not associated with advanced histological features and presence of “definite NASH” among patients with NAFLD. However, we did identify an association between a lower grade of steatosis and possible increased portal inflammation in NAFLD patients with significant titers of autoantibodies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Source of funding

The Nonalcoholic Steatohepatitis Clinical Research Network (NASH CRN) is supported by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) (grants U01DK061718, U01DK061728, U01DK061731, U01DK061732, U01DK061734, U01DK061737, U01DK061738, U01DK061730, U01DK061713), and the National Institute of Child Health and Human Development (NICHD).

Several clinical centers use support from General Clinical Research Centers or Clinical and Translational Science Awards in conduct of NASH CRN Studies (grants UL1RR024989, M01RR000750, M01RR00188, UL1RR02413101, M01RR000827, UL1RR02501401, M01RR000065, M01RR020359, DK-02957-KK).

This work is supported by the National Institute of Diabetes & Digestive & Kidney Diseases and the National Institute of Child health and Human Development. A list of members of the Nonalcoholic Steatohepatitis Clinical Research Network is located in Appendix.

Abbreviations

NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
PIVENS	Pioglitazone vs. Vitamin E vs. Placebo for the treatment of non diabetic patients with NASH

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Appendix: Members of the Non-alcoholic Steatohepatitis Clinical Research Network

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Table 1

Select demographics and co-morbidities of the study cohort.

	Significant autoantibody titers <u>present</u> (N=182)	Significant autoantibody titers <u>absent</u> (N=682)	P value
Age (Years, mean (95%CI))	49.0 (19.0–67.6)	49.2 (25.5–69.3)	0.87
Female gender (%)	115 (63.2)	441 (64.7)	0.71
BMI (kg/m ² , mean (95%CI))	34.3 (24.5–46.5)	34.3 (24.2–48.8)	0.92
Race (Caucasian)	139 (83.2)	560 (84.6)	0.67
Diabetes (Type I or II)	47 (25.8)	193 (28.3)	0.58
HOMA-IR (non diabetic) [‡]	3.8 (2.4–6.8)	4.0 (2.5–6.0)	0.70

Values are N (%) or mean (95% confidence interval). HOMA-IR presented as median (IQR).

[‡]HOMA-IR: Homeostasis model assessment method for insulin resistance, calculated as (fasting insulin (μU/mL)*fasting glucose (mmol/L))/22.5.

Table 2

Histologic features from liver biopsies in patients with significant titers of autoantibodies compared to those patients without significant titers of autoantibodies.

	ANA or SMA or both titers present (N=182)	Significant auto antibody titers absent (N=682)	ANA and SMA present (N=19)	P	
				ANA or SMA titers present vs. absent [*]	ANA and SMA present vs. absent [†]
Steatosis				0.007	0.38
<5%	16 (8.8)	39 (5.7)	0 (0.0)		
5–33%	88 (48.4)	254 (37.2)	11 (57.9)		
34–66%	43 (23.6)	226 (33.1)	5 (26.3)		
>66%	35 (19.2)	163 (23.9)	3 (15.8)		
Lobular inflammation				0.93	0.52
0	1 (0.6)	3 (0.4)	0 (0.0)		
<2 under 20× mag	96 (52.8)	355 (52.1)	13 (68.4)		
2–4 under 20× mag	64 (35.2)	254 (37.2)	5 (26.3)		
>4 under 20× mag	21 (11.5)	70 (10.3)	1 (5.3)		
Portal chronic inflammation				0.88	0.03
None	29 (15.9)	100 (14.7)	6 (31.6)		
Mild	112 (61.5)	432 (63.3)	7 (36.8)		
More than mild	41 (22.5)	150 (22.0)	6 (31.6)		
Ballooning				0.60	0.06
None	64 (35.2)	220 (32.3)	11 (57.9)		
Few	48 (26.4)	172 (25.2)	4 (21.1)		
Many	70 (38.5)	290 (42.5)	4 (21.1)		
Fibrosis				0.89	0.22
None	43 (23.8)	167 (24.7)	7 (36.8)		
Mild, zone 3	23 (12.7)	97 (14.3)	3 (15.8)		
Moderate, zone 3	15 (8.3)	73 (10.8)	0 (0.0)		
Portal/periportal only	7 (3.9)	20 (3.0)	2 (10.5)		
Zone 3 & periportal	33 (18.2)	122 (18.0)	1 (5.3)		
Bridging	40 (22.1)	130 (19.2)	4 (21.1)		

	ANA or SMA or both titers present (N=182)	Significant auto antibody titers absent (N=682)	ANA and SMA present (N=19)	P	
				ANA or SMA titers present vs. absent [*]	ANA and SMA present vs. absent [†]
Cirrhosis	20 (11.1)	68 (10.0)	2 (10.5)		
NAFLD Activity Score (range 0–8)	4.1 ± 1.8	4.4 ± 1.7	3.6 ± 1.0	0.04	0.02
Presence of NASH					
No	44 (24.2)	147 (21.6)	9 (47.4)		
Borderline	37 (20.3)	133 (19.5)	2 (10.5)		
Definite	101 (55.5)	401 (58.9)	8 (42.1)	0.68	0.05

* P-values derived from chi-square test for categorical variables and Wilcoxon rank sum test for NAFLD Activity Score

† P-values derived from Fisher's Exact test for categorical variables and Wilcoxon rank sum test for NAFLD Activity Score

Table 3

Results of multiple regression analysis examining the relationship between the presence of significant antibodies (ANA and/or ASMA) with various histological features (N=828).

	OR (95% CI)	P
Steatosis (34% vs. <34%)	0.58 (0.41–0.82)	<0.01
Lobular inflammation (2 vs. <2 under 20× mag)	0.90 (0.63–1.27)	0.53
Portal chronic inflammation (Mild/more than mild vs. none)	0.98 (0.60–1.61)	0.95
Ballooning (Few/many vs. none)	0.88 (0.62–1.27)	0.51
Fibrosis (Any vs. none)	1.08 (0.71–1.63)	0.73
Fibrosis (Advanced vs. mild/moderate/none)	1.30 (0.88–1.91)	0.19
NAFLD Activity Score (5 vs. 4)	0.76 (0.54–1.08)	0.13
NASH (Definite/borderline vs. none)	0.87 (0.58–1.30)	0.48

Separate models were fit for each histology variable as the outcome and all models were adjusted for age, gender, race, BMI, and diabetes.