PRESENCE OF SERUM ANTINUCLEAR ANTIBODIES DOES NOT IMPACT LONG-TERM OUTCOME IN NON-ALCOHOLIC FATTY LIVER DISEASE

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Abbreviations:

NAFLD non-alcoholic fatty liver disease, NASH non-alcoholic steatohepatitis, ANA antinuclear antibodies, AIH autoimmune hepatitis, MetS metabolic syndrome, T2DM type 2 diabetes mellitus, BMI body mass index, ALT alanine aminotransferase, AST aspartate aminotransferase, GGT gamma-glutamyl-transpeptidase, ALP alkaline phosphatase, aSMA anti-smooth muscle antibodies.

Word count: 961

Abstract

Background & aims: We investigated the longitudinal impact of Antinuclear Antibody (ANA) on clinical outcomes and survival in NAFLD.

Approach & Results: ANA were found in 16.9% of 923 biopsy-proven NAFLD patients, but none of them had histologic AIH or developed AIH after a mean follow up of 106±50 months. Although ANA-positive cases had a higher prevalence of NASH at baseline, the occurrence of liver-related events, HCC, cardiovascular events, extra-hepatic malignancy as well as overall survival were similar to ANA-negative.

Conclusions: Once AIH has been ruled out, the long-term outcomes and survival are unaffected by the presence of ANA in NAFLD patients.

Word count: 100 (without headings)

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is rapidly becoming the leading cause of chronic liver disease worldwide, in parallel with the pandemic of metabolic syndrome (MetS) and obesity, although it may also affect non-obese individuals[1,2]. Antinuclear antibodies (ANA) are routinely investigated in patients with NAFLD suspicion in order to exclude autoimmune disorders[3,4]. Overall, 7-52% of patients with chronic liver disease of different aetiologies are positive for serum autoantibodies[5]. Likewise, previous studies reported that the prevalence of positive ANA in biopsy-proven NAFLD ranges from 16 to 34%, but the clinical significance and the risk of developing autoimmune hepatitis (AIH) are incompletely explored [6-10]. The aim of this study was to investigate the cross-sectional associations and long-term impact of baseline ANA positivity on clinical outcomes and survival in NAFLD patients.

MATERIAL AND METHODS

Between 1990 and 2017, a total of 923 adult Caucasian, prospectively recruited, non-cirrhotic patients underwent liver biopsy for clinical suspicion of NAFLD in four GI tertiary centres in Italy (Turin, n=271; Milan, n=52; Rome, n=198; Palermo, n=134) and in the United Kingdom (Newcastle Upon Tyne, n=268). Investigation for ANA positivity was part of the routine screening with a dilution titre of 1:40 and above considered positive. AIH was ruled out according to standard criteria[4,11]. Patients attended regular follow-up visits at the GI outpatient clinics and staff researchers recorded liver events (end-stage cirrhosis, cirrhosis decompensation including ascites, hepatic encephalopathy and oesophageal bleeding), hepatocellular carcinoma occurrence[12], cardiovascular events, autoimmune disease occurrence, non-liver related cancers and patient deaths. Liver biopsies were analysed by expert liver pathologists and graded and staged according to Kleiner et al.[13]. The study was approved by the local ethics committees of the enrolling centres. Statistical analysis was performed with SPSS version 25 (SPSS Inc, Chicago, Illinois).

RESULTS

At baseline, 156 NAFLD patients (16.9%) were ANA-positive (ANA+ve), but they did not show significant higher titres of aSMA compared to ANA-ve patients (p=0.22). These patients were significantly older and twice as likely to be female (**Table 1**). High ANA titres (≥ 1:160) were found in 63 ANA+ve patients (40.4%), with 26 of them (41.3%) having a titre ≥ 1:320. No patient had histological features of AIH or met the criteria for “definite” AIH, hence no one received treatment for AIH. At liver histology, NASH was more often diagnosed in ANA+ve,

due to a significantly higher prevalence of hepatocyte ballooning (79.5% vs 69.8%, p=0.014) (**Table 2**). Mild/moderate fibrosis (F1/F2/F3), but not cirrhosis (F4), was more prevalent in ANA+ve (**Table 2**) and was independent of age, gender and BMI (OR 1.547, CI: 1.025-2.334).

The longitudinal analysis was performed after a mean follow up of 106 months (± 50 months), during which no patients had been diagnosed with AIH. No significant differences between NAFLD patients with or without ANA positivity was found in the occurrence of liver events (ANA+ve 8.6% vs ANA-ve 9.4%, p=0.742), hepatocellular carcinoma (ANA+ve 2% vs ANA-ve 2.8%, p=0.580), cardiovascular events (ANA+ve 11.2% vs ANA-ve 11.9%, p=0.813) or extra-hepatic malignancy (ANA+ve 12% vs ANA-ve 8.5%, p=0.175). As shown in **Figure 1**, after nearly 9 years, survival was similar in ANA+ve and ANA-ve patients (log-rank 0.899, p=0.343); total deaths were 50/9239, 9 (5.8%) in ANA+ve vs 41 (5.3%) in ANA-ve (p=0.340). Finally, to ensure that low-titre ANA cases were not skewing the data, we performed a sub-analysis in NAFLD patients with a high ANA titre (≥ 1:160). Once again, compared with ANA-ve subjects, no significant difference was found in terms of events occurrence, including onset of AIH, or overall survival (p=0.961 at the Kaplan-Meyer survival analysis).

DISCUSSION

This study provides new insights into the clinical implications of ANA positivity in NAFLD patients. The cross-sectional analysis confirms that incidental findings of ANA positivity in patients with NAFLD are relatively common. However, ANA+ve subjects did not exhibit histological features of AIH at index biopsy and none were subsequently diagnosed with AIH during lengthy specialist follow-up. Whether ANA positivity is simply an epiphenomenon or directly related to the underlying pathogenesis of NAFLD/NASH remains unclear. In a large US study[9], Vuppalachi et al. speculated that autoantibody production in NAFLD may be a consequence of hepatic NKT cell accumulation, supported by a significant increase in chronic moderate-severe portal inflammation[9]. In our cohort, ANA+ve subjects had a similar inflammatory grade but a higher prevalence of histological NASH. A key finding of this study is that although ANA+ve NAFLD subjects reported greater mild/moderate fibrosis, they did not show a more aggressive disease course or a worse long-term outcome than those without. This is consistent with previous reports in NAFLD[8,9], although the present study benefits from a greater duration of follow-up. These findings imply it remains necessary to exercise due clinical judgement and perform a liver biopsy to confirm or exclude AIH in NAFLD patients with autoimmunity features (i.e., ANA positivity), especially in patients without sufficient elements characterizing AIH phenotype (e.g. aSMA/LKM/SLA positivity, elevated aminotransferases and/or IgG, history of autoimmune disorders), as this will guide the therapeutic decision flow. There is no evidence to support immunosuppressive therapy for NASH.

Indeed, an empiric steroid-based therapy in ANA+ve NAFLD patients lacking histological stigmata of AIH would be inappropriate and potentially harmful, as systemic steroid treatment could favour the onset of diabetes, the strongest clinical predictor of overall mortality in patients with NAFLD. A limitation of this study is the lack of ANA patterns as well as of a central scoring for all liver biopsies: some cases could have had histological evidence of AIH if reviewed by a different pathologist, although the lack of this becoming evident during follow up is reassuring.

In summary, ANA positivity in NAFLD patients is a relatively common finding and may be associated with NASH and fibrosis. When AIH has been histologically ruled out, the long-term clinical outcomes are not affected by the presence of ANA at baseline.

FIGURE LEGENDS

Figure 1. Survival Curve comparing ANA positive vs ANA negative NASH patients after a mean follow up of 106 ±50 months. No significant differences were observed between the two groups (log rank 0,899 P=0,343)

REFERENCES

1. Younossi Z, Anstee QM, Marietti M, et al. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. *Nat Rev Gastroenterol Hepatol.* 2017.
2. Younes R, Bugianesi E. NASH in Lean Individuals. *Semin Liver Dis.* 2019;39(1):86-95.
3. European Association for the Study of the L, European Association for the Study of D, European Association for the Study of O. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. *J Hepatol.* 2016;64(6):1388-1402.
4. European Association for the Study of the L. EASL Clinical Practice Guidelines: Autoimmune hepatitis. *J Hepatol.* 2015;63(4):971-1004.
5. Czaja AJ, Carpenter HA, Santrach PJ, Moore SB. Genetic predispositions for immunological features in chronic liver diseases other than autoimmune hepatitis. *J Hepatol.* 1996;24(1):52-59.
6. Cotler SJ, Kanji K, Keshavarzian A, Jensen DM, Jakate S. Prevalence and significance of autoantibodies in patients with non-alcoholic steatohepatitis. *J Clin Gastroenterol.* 2004;38(9):801-804.
7. Niwa H, Sasaki M, Haratake J, et al. Clinicopathological significance of antinuclear antibodies in non-alcoholic steatohepatitis. *Hepatol Res.* 2007;37(11):923-931.
8. Ravi S, Shoreibah M, Raff E, et al. Autoimmune Markers Do Not Impact Clinical Presentation or Natural History of Steatohepatitis-Related Liver Disease. *Dig Dis Sci.* 2015;60(12):3788-3793.
9. Vuppalanchi R, Gould RJ, Wilson LA, et al. Clinical significance of serum autoantibodies in patients with NAFLD: results from the nonalcoholic steatohepatitis clinical research network. *Hepatol Int.* 2012;6(1):379-385.
10. De Luca-Johnson J, Wangensteen KJ, Hanson J, Krawitt E, Wilcox R. Natural History of Patients Presenting with Autoimmune Hepatitis and Coincident Nonalcoholic Fatty Liver Disease. *Dig Dis Sci.* 2016;61(9):2710-2720.
11. Hennes EM, Zeniya M, Czaja AJ, et al. Simplified criteria for the diagnosis of autoimmune hepatitis. *Hepatology.* 2008;48(1):169-176.
12. European Association for the Study of the Liver. Electronic address eee, European Association for the Study of the L. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. *J Hepatol.* 2018;69(1):182-236.
13. Kleiner DE, Brunt EM, Van Natta M, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology.* 2005;41(6):1313-1321.

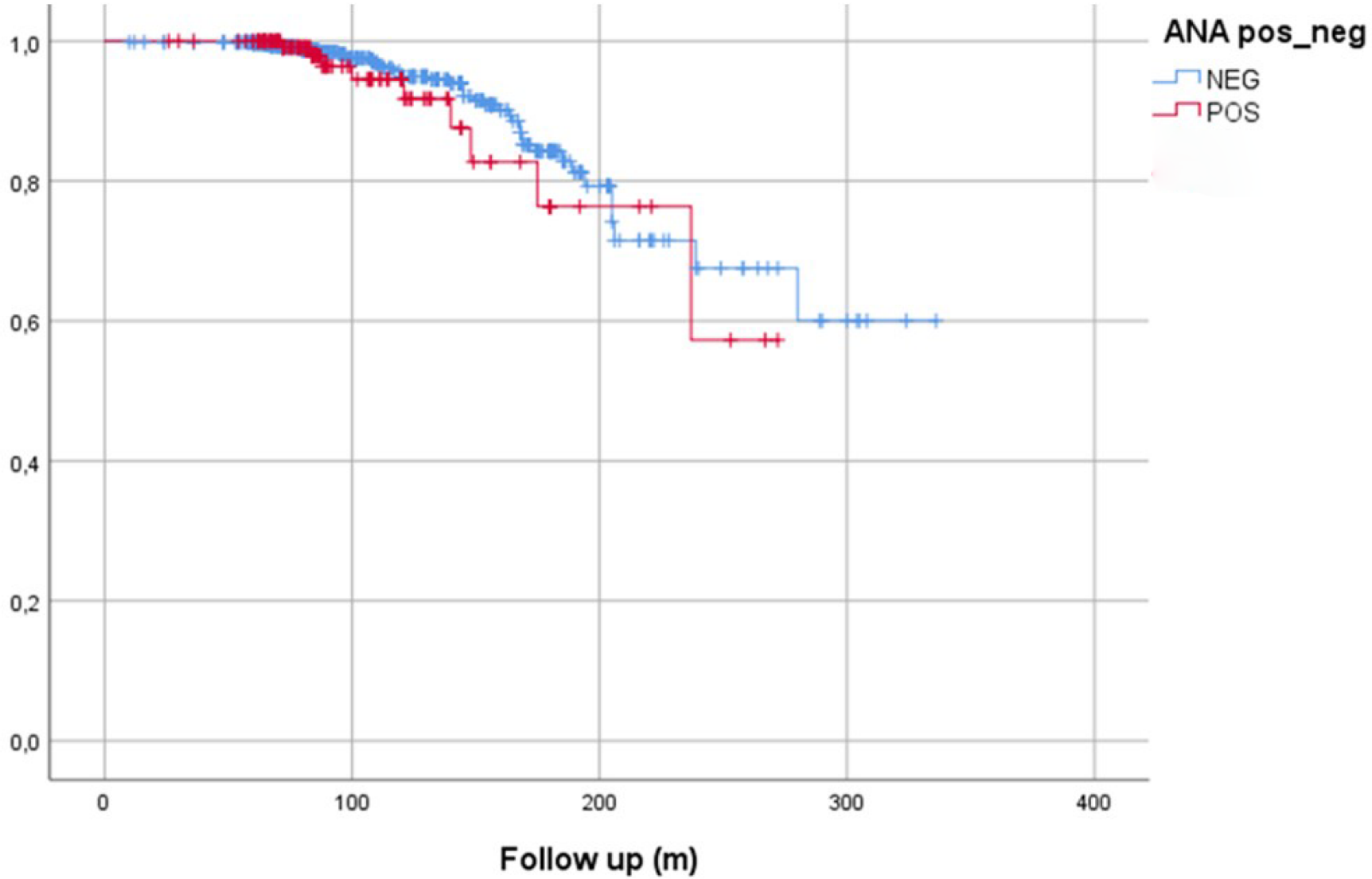
Table 1: Demographic and Biochemical Features of the total cohort and grouped according to antibody-status.

Parameter	Total Cohort	ANA positive	ANA negative	P value
N (%)	923	156 (16.9%)	767 (83.1%)	
Gender (Female) N(%)	293 (31.7%)	78 (27%)	78 (12%)	< 0.001
Age (years)	47 ± 13	50 ± 12	46 ± 13	0.002
BMI (kg/m^2)	30.2 ± 5.8	30.3 ± 5.8	30.1 ± 5.8	0.821
aSMA+ve*	24/510	5/65	19/445	0.22
Waist circumference (cm)	101 ± 12.6	100.2 ± 12.3	101.6 ± 12.7	0.265
ALT (IU/L)	75.5 ± 48.4	77.2 ± 58.3	75.2 ± 46.2	0.634
AST (IU/L)	47.2 ± 31.8	50.8 ± 42.7	46.4 ± 29	0.250
Hb (g/dl)	14.6 ± 1.5	14.2 ± 1.6	14.7 ± 1.4	0.001
Platelets (10^9/L)	227 ± 62	235 ± 75	226 ± 59	0.157
Albumin (g/dl)	45.5 ± 4	45.3 ± 3.5	45.5 ± 4	0.152
Total Bilirubin (mg/dL)	0.78 ± 0.46	0.74 ± 0.53	0.79 ± 0.44	0.730
ALP (IU/L)	104 ± 61.7	99 ± 62	105 ± 58	0.252
GGT (IU/L)	107 ± 140	106 ± 144	107 ± 125	0.901
Ferritin (ng/ml)	236 ± 253	224 ± 261	239 ± 213	0.525
Glucose (mg/dl)	104 ± 40	103 ± 34	104 ± 40	0.449
T2DM prevalence	266 (29%)	41 (26.3%)	225 (29.5%)	0.416
Hypertension	381 (41.3%)	70 (44.9%)	311 (41%)	0.369
Dyslipidaemia	438 (47.5%)	58 (54.7%)	380 (56.4%)	0.748
Follow up (months)	106 ± 50	99 ± 44	107 ± 51	0.034

*aSMA titres available for 510 patients

Table 2: Histological Features of the total cohort and according to antibody status

Histological Parameter	Total Cohort	ANA pos	ANA neg	P value
Advanced steatosis (grade 2 or 3)	568 (61.5%)	94 (60.3%)	474 (61.8%)	0.183
Ballooning (grade 1 or 2)	657 (71.4%)	124 (79.5%)	533 (69.8%)	0.014
Lobular Inflammation (grade 1 or 2)	800 (86.7%)	140 (89.7%)	660 (86.4%)	0.257
Fibrosis stage				
F0	202 (21.9%)	24 (15.4%)	178 (23.2%)	0.03
F1	254 (22.5%)	46 (29.5%)	208 (27.1%)	0.546
F2	251 (27.2%)	46 (29.5%)	205 (26.7%)	0.480
F3	138 (15.0%)	28 (17.9%)	110 (14.3%)	0.249
F1-F2-F3	643 (69.7%)	120 (76.9%)	523 (68.2%)	0.03
F4	78 (8.5%)	12 (7.7%)	66 (8.6%)	0.709
NASH (histological hallmarks: contemporary presence of steatosis, ballooning and lobular inflammation)	660 (71,5%)	124 (79.5%)	536 (70.2%)	0.019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