Risk of Severe Liver Disease in Nonalcoholic Fatty Liver Disease with Normal Aminotransferase Levels: A Role for Insulin Resistance and Diabetes

Anna Ludovica Fracanzani,[1] Luca Valenti,[1] Elisabetta Bugianesi,[2] Marco Andreoletti,[3] Agostino Colli,[3] Ester Vanni,[2] Cristina Bertelli,[1] Erika Fatta,[1] Daniela Bignamini,[1] Giulio Marchesini,[4] and Silvia Fargion[1]

It is uncertain whether patients with nonalcoholic fatty liver disease (NAFLD) and normal alanine aminotransferase (ALT) have a milder disease and should undergo liver biopsy. We reviewed the histological data of 458 Italian patients with NAFLD in whom liver biopsy was indicated by altered liver enzymes (395 cases, 86%), or persistently elevated ferritin or long-lasting severe steatosis (63 cases). Factors associated with nonalcoholic steatohepatitis (NASH) and fibrosis ≥ 2 were identified by multivariate analysis. Patients with normal ALT were significantly older, had lower body mass index, fasting triglycerides, insulin resistance according to homeostasis model assessment (HOMA-IR), ALT, and gamma-glutamyltransferase, but a higher prevalence of hypertension. NASH was diagnosed in 59% and 74% of the patients with normal and increased ALT, respectively ($P = 0.01$). In the overall series of patients, NASH was independently predicted by ALT (odds ratio [OR], 1.11; 95% confidence interval [CI], 1.04-1.19 per 10-IU/mL increase) and diabetes (OR, 1.5; 95% CI, 1.1-2.0). The same variables were selected in patients with increased ALT, whereas in those with normal ALT, HOMA-IR and ALT were independent predictors. Severe fibrosis was independently predicted by serum ferritin (OR, 1.04; 95% CI, 1.001-1.08 per 50-ng/mL increase), ALT (OR, 1.07; 95% CI, 1.02-1.14), and diabetes (OR, 1.8; 95% CI, 1.4-2.3) in the overall series, serum ferritin and diabetes in those with increased ALT, and only HOMA-IR (OR, 1.97; 95% CI, 1.2-3.7) in patients with normal ALT. *Conclusion:* Normal ALT is not a valuable criterion to exclude patients from liver biopsy. Alterations in glucose metabolism and insulin resistance in subjects with normal ALT should also be considered in the selection of NAFLD cases for histological assessment of disease severity and progression. (HEPATOLOGY 2008;48:792-798.)

Nonalcoholic fatty liver disease (NAFLD) includes a wide spectrum of liver diseases, ranging from pure fatty liver, which is usually a benign and nonprogressive condition, to nonalcoholic steatohepatitis (NASH), which may eventually progress to liver cirrhosis, portal hypertension, and hepatocellular carcinoma.[1-7] Hepatic insulin resistance (IR), associated with obesity, type 2 diabetes, and dyslipidemia is the underlying metabolic condition favoring the occurrence of NAFLD.[8] Consequently, NAFLD is now considered the hepatic expression of the metabolic syndrome,[9,10] accounting for the risk of advanced liver disease observed in these patients,[9,10] in addition to the well-established cardiovascular risk.[11-13] Several studies have provided detailed information on the relationship between histological and clinical findings in patients with NAFLD, demonstrating that the higher the number of components of the metabolic syndrome, the higher the risk of fibrosis and advanced disease,[14] thus pinpointing a criterion for selecting candidates for invasive diagnostic procedures.

However, the most common criterion for referral to Liver Units is the presence of elevated liver enzymes, and

Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; CI, confidence interval; HOMA, homeostasis model assessment; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OR, odds ratio.

From the [1]Department of Internal Medicine, Scientific Institute of Recovery and Cure Foundation (IRCCS), Hospital Maggiore Policlinico, University of Milan, Milan, Italy; [2]Division of Gastroenterology, Torino University, Torino, Italy; [3]Department of Internal Medicine, Hospital Manzoni Lecco, Lecco, Italy; [4]Department of Medicine, University Alma Mater Studiorum, Bologna, Italy.

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Address reprint requests to: Silvia Fargion, Prof., Dipartimento Medicina Interna, Fondazione IRCCS Ospedale Maggiore Policlinico, Via F Sforza 35, 20122, Milano, Italy. E-mail: silvia.fargion@unimi.it; fax: 39 02 50320296.

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only subjects with increased alanine aminotransferase (ALT) were enrolled in the large majority of NAFLD studies. In several series it was demonstrated that the higher the ALT levels, the higher the risk of NASH,[15-16] but some data indicate that patients with normal ALT may also have histological features at risk for disease progression.[17-18] The lack of a systematic association between out-of-normal ALT and severe NAFLD is crucial for diagnostic purposes, since it raises the issue of whether NAFLD patients with normal ALT should also undergo liver biopsy and be investigated for extrahepatic manifestations of the metabolic syndrome.

The aim of this study was to compare the clinical presentation and liver histological findings in patients with NAFLD with and without increased ALT, as well as the prevalence of metabolic alterations related to the metabolic syndrome, to determine whether normal ALT is a valuable criterion to exclude patients from liver biopsy.

Patients and Methods

Patients. We merged the databases of consecutive patients with liver biopsy-confirmed NAFLD observed in four liver units. All consecutive patients who underwent liver biopsy between January 2003 and June 2006 were included in the study unless tissue sample size was <1.7 cm. The final cohort consisted of 458 cases; 395 (86%) had abnormalities in liver function tests justifying liver biopsy, and the remaining 63 patients had permanently normal ALT. The indications for liver biopsy in the latter subgroup are shown in Table 1. Persistent increases in serum ferritin and a long-lasting history of severe steatosis were the main reasons for biopsy in this subgroup. Other causes of liver diseases (viral, autoimmune, cholestatic, drug-induced, hereditary hemochromatosis, or Wilson's disease) were systematically excluded, as were patients with clinical or imaging evidence of decompensated cirrhosis. Finally, only subjects with daily alcohol intake <20 g (confirmed by at least one family member) were considered. Clinical and laboratory data were collected at the time of liver biopsy.

All patients had given informed, written consent to data handling according to a protocol approved by the Senior Staff Committee of our Institutions, a board comparable to an Institutional Review Board.

Table 1. Indications for Liver Biopsy in Patients with Normal ALT (63 Cases)

Indication	Number of Cases
Persistently increased serum ferritin with/without ultrasonographic evidence of steatosis	29/6
Persistent evidence of severe steatosis at ultrasonography	25
Persistently increased γ-glutamyl-transferase levels	3

Methods. Lifestyle habits, anthropometric and clinical data, and a complete drug history were available for all subjects. Waist circumference was measured in a standing position at the level of the umbilicus, and arterial blood pressure was defined as the mean of the second and third reading of three consecutive blood pressure measurements. Routine tests included a complete blood count, ALT and gamma-glutamyltransferase, fasting glucose, total and high-density lipoprotein cholesterol, triglycerides, and uric acid. All tests were determined by standard laboratory procedures, insulin by a commercially purchased radioimmunoassay (RIA; Biochem Immunosystems, Bologna, Italy). The upper normal limit of ALT levels was set at 40 U/L, but a sensitivity analysis was also carried out using the lower range of ALT normality, which was more recently suggested by Prati et al.[19] The diagnosis of the metabolic syndrome was carried out according to Adult Treatment Panel III criteria,[20] and based on the presence of three or more of the following criteria: (1) fasting glucose $\geq$110 mg/dL; (2) central obesity (waist circumference $>$102 cm (men) and $>$88 cm (women); (3) arterial pressure $\geq$130/85 mmHg; (4) triglyceride levels $\geq$150 mg/dL or current use of fibrates; or (5) high-density lipoprotein-cholesterol $<$40 mg/dL (men) and $<$50 mg/dL (women).

IR was evaluated according to homeostatic model assessment (HOMA),[21] as fasting serum insulin (in μIU/mL) multiplied by fasting serum glucose (in mmol/L), divided by 22.5. The oral glucose tolerance test was performed with 75 g of glucose according to World Health Organization criteria. The presence of diabetes mellitus (fasting glucose $\geq$126 mg/dL, 120-minute glucose $\geq$200 during oral glucose tolerance test, or treatment with antidiabetic drugs), obesity (body mass index [BMI] $>$30 kg/m^2) and overweight (BMI, 25-29.9 kg/m^2) status were also recorded.

Patients were first analyzed as a single group, then as two separate groups according to persistently normal ALT (63 cases) or increased ALT levels (395 cases).

Liver Histology. Liver biopsies were processed routinely, and scored by a single pathologist in each center. To control for biopsy size, the length of the biopsy was measured with a hand ruler, and the number of portal areas on a cross-section was counted. The minimum biopsy size was 1.7 cm and the number of portal areas was 10. The diagnosis of NASH was based on the Brunt criteria,[22] recently modified by Kleiner et al.[23] The stage of fibrosis was scored based on a five-point scale, as follows: stage 0, absence of fibrosis; stage 1, perisinusoidal or portal fibrosis; stage 2, perisinusoidal and portal/periportal

Table 2. Characteristics of Patients with NAFLD, Divided According to ALT Levels (Mean ± SD) or Number of Cases (%)

Variables	All Cases (n = 458)	Normal ALT (n = 63)	Increased ALT (n = 395)	*P* Values*
M/F	370/85	44/16	326/69	
Age (years)	44 ± 11	49 ± 11	43 ± 11	0.001
BMI (kg/m^2)	27.3 ± 3.9	26.0 ± 4.0	27.4 ± 3.7	0.04
Total cholesterol (mg/dL)	206 ± 43	204 ± 36	207 ± 44	NS
HDL (mg/dL)	47 ± 13	50 ± 14	47 ± 13	NS
Triglycerides (mg/dL)	146 ± 85	124 ± 70	150 ± 87	0.03
Fasting glucose (mg/dL)	97 ± 27	98 ± 23	97 ± 28	NS
Fasting insulinemia (μU/mL)	18.3 ± 14.3	11.8 ± 6.2	19.1 ± 14.8	0.001
HOMA-IR	4.5 ± 4.4	2.9 ± 1.4	4.6 ± 3.9	0.006
ALT (U/L)	76 ± 45	28 ± 7	84 ± 44	0.0001
GGT (U/L)	97 ± 108	64 ± 61	101 ± 114	0.02
Serum ferritin (ng/mL)	316 ± 280	309 ± 273	317 ± 287	NS
Transferrin saturation (%)	34 ± 11	33 ± 13	35 ± 12	NS
Impaired glucose tolerance	61 (13)	10 (16)	53 (13)	NS
Diabetes	47 (10)	7 (11)	38 (9)	NS
Hypertension	98 (21)	22 (36)	78(21)	0.03
Metabolic syndrome	86 (19)	12 (19)	83 (21)	NS

SD, standard deviation; M, male; F, female; NS, not significant.
**P* indicates differences between patients without and with increased ALT.

fibrosis; stage 3, septal or bridging fibrosis; and stage 4, cirrhosis. The severity of steatosis was graded 1 to 3 according to the percentage of cells with fatty droplets (1, 10%-33%; 2, 33%-66%; and 3, >66%). The presence of siderosis was evaluated by Perl's staining.

Statistical Analysis. Results are expressed as means ± standard deviations for continuous variables and as frequencies for categorical variables. Mean values were compared by *t* test for unequal variances. Frequencies were compared by chi-square test. *P* values ≤ 0.05 were considered statistically significant. Continuous variables were correlated by Spearman test. Two separate logistic regression analyses were performed to assess the variables independently associated with (1) presence of NASH and (2) presence of advanced fibrosis (≥2). Variables significant at univariate analyses were entered in a multivariate analysis, whenever possible. All statistical analyses were performed with the JMP statistical discovery software system (SAS Institute, Cary, NC).

Results

Patients with normal ALT were significantly older, had lower BMI, triglycerides, ALT, and gamma-glutamyltransferase, and milder IR compared to patients with increased ALT, and a higher prevalence of hypertension (Table 2). The prevalence and the number of the components of the metabolic syndrome were similar in patients with normal ALT (19%) or increased ALT (21%).

Histological findings are shown in Table 3. No difference in the prevalence of severe fibrosis (≥2) was observed in patients without (22%) or with (34%) increased ALT, but patients with ALT within the normal range had a significantly milder inflammation ($P = 0.0002$) and milder steatosis ($P = 0.0001$). NASH was diagnosed in 332 patients (72% of total), that is, in 37 cases (59%) with normal ALT and in 295 (75%) of those with increased ALT ($P = 0.01$). Of the 37 cases with NASH and normal ALT, 10 (27%) patients had ALT values lower than 30 U/L in men and 19 U/L in women.[19] Variables significantly associated with NASH are shown in Table 4. Parameters reflecting glucose abnormalities and IR were significantly associated with NASH in the overall series and in patients both with and without increased ALT, whereas serum ferritin was associated with NASH in the overall series and in patients with increased ALT. At logistic regression analysis, the variables independently as-

Table 3. Liver Histology in Patients with Normal or Increased ALT

Histology	All Patients (n = 458) (%)	Normal ALT (n = 63) (%)	Increased ALT (n = 395) (%)	*P* Values*
Necroinflammatory grade (0/1/2/3)	20/47/27/6	40/36/22/2	17/48/28/7	0.0002
Fibrosis stage (0/1/2/3/4)	42/25/19/9/5	51/27/13/1/8	41/25/20/10/4	NS
Steatosis grade (1/2/3)	58/26/16	83/10/7	54/28/18	0.0001

**P* indicates differences between patients without and with increased ALT. NS, not significant.

sociated with NASH in the overall series were ALT ($P = 0.002$; odds ratio [OR], 1.11; 95% confidence interval [CI], 1.04-1.19 per 10-IU/mL increase) and diabetes ($P = 0.005$; OR, 1.5; 95% CI, 1.1-2). In patients with increased liver enzymes, ALT ($P = 0.05$; OR, 1.07; 95% CI, 1.003-1.16 per 10-IU/mL increase) and diabetes ($P = 0.008$; OR, 2.4; 95% CI, 1.2-4.7) were also independent predictors of NASH, whereas in patients with normal ALT the two variables independently associated with NASH were ALT ($P = 0.02$; OR, 3.1; 95% CI, 1.2-8.9 per 10-IU/mL increase) and HOMA-IR ($P = 0.008$; OR, 1.9; 95% CI, 1.2-3.5 unit increase).

At univariate analysis (Table 5), ferritin, gender, age, BMI, fasting glucose, diabetes, and ALT were significantly associated with fibrosis ≥2 in the overall series and in patients with elevated ALT, whereas only age, fasting insulin, diabetes, and HOMA-IR were significantly associated with fibrosis in patients with normal ALT.

At multivariate logistic regression analysis, serum ferritin ($P = 0.01$; OR, 1.04; 95% CI, 1.001-1.08 per 50-ng/mL increase), ALT ($P = 0.02$; OR, 1.07; 95% CI, 1.02-1.14 per 10-IU/mL increase), and diabetes ($P = 0.0001$; OR, 1.8; 95% CI, 1.4-2.3) remained independently associated with fibrosis ≥2 in the whole series, while in those with increased ALT, only serum ferritin ($P = 0.04$; OR, 1.04; 95% CI, 1.001-1.08 per 50-ng/mL increase) and diabetes ($P = 0.0001$; OR, 3.2; 95% CI, 1.8-5.5) were independent predictors; in patients with normal ALT, HOMA-IR ($P = 0.02$; OR, 1.97; 95% CI, 1.2-3.7) was the only variable significantly associated with fibrosis ≥2.

Discussion

The present study confirms that NAFLD patients with normal ALT are at risk of progressive and severe hepatic disease, as well as of extrahepatic manifestations; this evidence could make liver biopsy mandatory in the majority of cases, unless sensitive and specific noninvasive tests currently unavailable prove their efficacy. This conclusion, however, raises a question on the feasibility of liver biopsy assessment in an extremely large at risk population, and on the cost/effectiveness of this policy.

At present, NAFLD patients with normal ALT are very rarely investigated or indicated for liver biopsy, and the utility of performing a biopsy in this situation is still debated. A very recent study of a decision-tree model supports the use of early biopsy in the majority of cases, based on evidence from the literature and a sensitivity analysis of different scenarios, but this is not current practice in most countries.[24] This is also the reason why we could analyze only 63 cases with normal ALT, out of a large series of patients with histologically-proven NAFLD. Indications to perform liver biopsy in patients with normal ALT in participating centers were similar, including persistently increased ferritin and a long history of steatosis.

The small sample size of NAFLD subjects with normal ALT represents a limitation of the present study. This hampered our attempt (results not shown) to construct a scoring system for predicting NASH and fibrosis using the variables significant at multivariate analysis to identify subjects with severe disease without the use of liver biopsy. The final risk score had a low specificity and sensitivity, insufficient for clinical purposes, also due to the small number of cases.

The issue of a noninvasive assessment of disease severity remains crucial in NAFLD, given the high number of subjects in the general population with steatosis and normal ALT. Angulo et al.[16] recently proposed a scoring system (NASH score) able to identify NAFLD patients with

Table 4. Variables Significantly Associated with NASH at Univariate Analysis

Variables	*P* Values		
	All Patients (n = 458)	Normal ALT (n = 63)	Increased ALT (n = 395)
Age (years)	NS	0.04	NS
BMI (kg/m^2)	0.04	NS	0.05
ALT (U/L)	0.007	0.02	NS
Serum ferritin (ng/mL)	0.02	NS	0.005
Fasting glucose (mg/dL)	0.001	NS	0.005
Fasting insulin (μU/mL)	0.004	0.01	NS
Diabetes or glucose intolerance (yes/no)	0.004	NS	0.003
HOMA-IR (%)	0.001	0.005	0.02

NS, not significant.

Table 5. Variables Significantly Associated with Fibrosis (≥2) in the Overall Series and in Patients Divided According to ALT Levels (Univariate Analysis)

Variables	*P* value		
	All Patients (n = 458)	Normal ALT (n = 63)	Increased ALT (n = 395)
Gender	0.01	NS	NS
Age (years)	0.001	0.03	0.002
BMI (kg/m^2)	0.02	NS	0.04
ALT (U/L)	0.01	NS	0.004
Serum ferritin (ng/mL)	0.001	NS	0.009
Fasting glucose (mg/dL)	0.002	NS	0.006
Fasting insulin (μU/mL)	NS	0.04	NS
Diabetes or glucose intolerance	0.04	0.03	0.001
HOMA-IR (%)	0.04	0.03	NS

NS, not significant.

advanced fibrosis and potentially rendering liver biopsy unnecessary in 75% of cases. In the subset of our patients with normal ALT with no missing variables in the Angulo equation (n = 63), the two cutoffs of NASH score (<-1.455 and >0.676) proposed by Angulo et al.[16] had a negative predictive value of 89.6% and a positive predictive value of 100% to identify patients without and with advanced fibrosis, respectively. Thus, with the limitation due to a different setting (the Angulo score was derived from subjects with persistently elevated ALT), our data support the hypothesis that this score could also be used in the general population, including subjects with normal ALT.

We were able to demonstrate that fibrosis, the key element that predicts the progression of fatty liver, was present with a similar prevalence in patients with normal and increased ALT. Similarly, NASH was present in more than 50% of our NAFLD patients with normal ALT and persistently increased serum ferritin and/or long-lasting steatosis. A possible limitation to this conclusion comes from the well-known sample variability in liver biopsy, which also applies to NAFLD cases.[24] However, NASH is diagnosed in the presence of fibrosis (any severity), associated with a variable degree of necroinflammation. Whereas fibrosis severity may be misdiagnosed according to sample size and chance, NASH is more likely to be underdiagnosed by sample variability. In addition, the quantification of fibrosis in patients with NAFLD has the lowest intraobserver and interobserver variability as compared to other histological features[25] and the level of experience of the pathologist is reported to impact on agreement more than the characteristics of the biopsy specimens.[26] Interestingly ALT levels were independently associated with NASH also in patients with normal ALT, indicating that even a minor elevation in ALT level, albeit within the normal limits, reflects the extent of NASH-related liver damage.

Diabetes and insulin resistance were factors most closely associated with severe liver disease in patients with normal ALT. Similarly, diabetes was the only independent factor associated with advanced fibrosis in the retrospective study by Mofrad et al.,[17] in 51 patients with normal ALT who underwent liver biopsy either for unexplained hepatomegaly or as potential living donors for transplantation. They first proved that patients with normal ALT may also have NASH and severe fibrosis. Our study expands their observation to patients who are commonly referred to dedicated outpatient services for steatosis and/or metabolic alterations, being representative of a much larger group of NAFLD patients. In the general population, the data by Mofrad et al.[17] were only partially confirmed in a recent study limited to potential liver donors, in which the prevalence of NASH in patients with or without increased ALT was only 3.4% and 2.1%, respectively.[18] Similar results were also reported by Machado et al.,[27] in a review of studies focused on the prevalence of NAFLD/NASH in obese patients undergoing bariatric surgery, but Gholam et al.[28] reported a higher prevalence of normal ALT (46%) in obese patients with NASH.

In our study, the prevalence and the number of the components of the metabolic syndrome were also similar in patients with normal or increased ALT, confirming the data previously reported by Mofrad et al.[17] The overall prevalence of the metabolic syndrome, defined according to Adult Treatment Panel III criteria, however, was lower than the numbers observed in most NAFLD series. This reflects specific characteristics of the Italian NAFLD population, different from the U.S. population in the prevalence of obesity (the mean BMI of our cases was 27.3 kg/m^2, and only 21% of our patients were in the range of obesity), and an a priori selection based on the inclusion of a high number of subjects referred to Liver Units for hyperferritinemia, which could make this patient population not truly representative of normal NAFLD patients. Interestingly, a large number of patients with normal ALT had liver siderosis and a significant correlation was demonstrated between ferritin levels and siderosis, suggesting that hyperferritinemia reflects a mild iron overload. This was also confirmed by the low prevalence of raised inflammatory indices (8%) ruling out ferritin as a marker of inflammation. In addition, experimental and clinical data point out that a mild iron overload contributes to insulin resistance.[29,30]

Fibrosis was relatively mild in our series of Italian patients with NAFLD, confirming previous data reported in a recent multicenter study indicating that advanced fibrosis is present in only 12.5% of Italian patients compared to 19.6% of Northern European, 29.8% of U.S., and 30.9 % of Australian patients.[16] Moreover, due to the low prevalence of stage 3-4 fibrosis, we had to choose stage 2 as the cutoff point for the analysis of factors associated with more severe fibrosis.

Hypertension, one of the key features of the metabolic syndrome, was surprisingly more prevalent in patients with NAFLD and normal ALT than in those with increased liver enzymes. This underlines the fact that the metabolic alterations related to steatosis and to adipose tissue–related endocrine dysfunction occur independently of overt liver damage and that even pure fatty liver is frequently associated with extrahepatic manifestations of IR syndrome. The results obtained in this cohort, which very likely reflect the characteristics of unselected NAFLD patients, indicate that Italian pa-

tients have, on average, milder liver disease, but are nonetheless exposed to all potential complications of the metabolic syndrome.

All patients within the spectrum of NAFLD should thus be considered potentially affected not only by a hepatic but also by a multisystemic disease. This suggestion could be even stronger in the presence of higher insulin resistance, which is a sensitive predictor of both progressive liver disease and severe extrahepatic disease. These data are supported by the analysis of NAFLD-related mortality at a population-based level. Data from two registry-based cohorts indicated that patients with NAFLD had a 2.6-fold increased mortality compared to the general population,[31,32] and these data were confirmed by Adams et al.,[6] who analyzed the natural history of patients with NAFLD. In patients with NAFLD there was definitely an excess mortality risk over the age-matched and sex-matched general population, and the risk increased with longer follow-up.[6] This risk includes both cardiovascular and liver-related events, as shown in cohort studies with long follow-up.[7,13,30-33,34]

In conclusion, our data indicate that more than half of NAFLD patients with persistently normal ALT have a potentially progressive liver disease. In the absence of biopsy or of an adequate score able to identify subjects at risk, these patients could miss careful follow-up and might be scarcely motivated toward lifestyle modifications that are potentially able to cure their liver disease and the extrahepatic manifestations of the metabolic syndrome. Clinicians should be aware of the importance of a complete clinical evaluation for early diagnosis and treatment of liver disease, as well as the different manifestations of the metabolic syndrome.

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