

Natural History of Nonalcoholic Fatty Liver Disease: A Prospective Follow-Up Study With Serial Biopsies

Patrik Nasr,¹ Simone Ignatova,² Stergios Kechagias,^{1*} and Mattias Ekstedt^{1*}

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease in the world. The complete natural history of NAFLD is unknown because few high-quality follow-up studies have been conducted. Our aim was to find variables predicting disease severity through an extended follow-up with serial biopsies. In a prospective cohort study, 129 patients who enrolled between 1988 and 1993 were asked to participate in a follow-up study on two occasions; biochemical, clinical, and histologic data were documented. The mean time between biopsies was 13.7 (± 1.7) and 9.3 (± 1.0) years, respectively. At the end of the study period, 12 patients (9.3%) had developed end-stage liver disease and 34% had advanced fibrosis. Out of the 113 patients with baseline low fibrosis (<3), 16% developed advanced fibrosis. Fibrosis progression did not differ among the different stages of baseline fibrosis ($P = 0.374$). Fifty-six patients (43%) had isolated steatosis, of whom 9% developed advanced fibrosis (3 patients with biopsy-proven fibrosis stage F3-F4 and 2 patients with end-stage liver disease). Fibrosis stage, ballooning, and diabetes were more common in patients who developed end-stage liver disease; however, there were no baseline clinical, histologic, or biochemical variables that predicted clinical significant disease progression. **Conclusion:** NAFLD is a highly heterogeneous disease, and it is surprisingly hard to predict fibrosis progression. Given enough time, NAFLD seems to have a more dismal prognosis than previously reported, with 16% of patients with fibrosis stage <3 developing advanced fibrosis and 9.3% showing signs of end-stage liver disease. (*Hepatology Communications* 2018;2:199-210)

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease in the Western world, affecting up to 25% of the world's population⁽¹⁾ and expected to rise in parallel with the epidemic of obesity. NAFLD is commonly associated with components of metabolic syndrome⁽²⁾ and is an independent risk factor for cardiovascular disease.^(3,4)

The histologic features of NAFLD range from isolated steatosis to nonalcoholic steatohepatitis (NASH), the latter being characterized by hepatic steatosis, lobular inflammation, and signs of hepatocyte injury (ballooning) with or without fibrosis.⁽⁵⁾ Some patients with NAFLD develop progressive fibrosis and

subsequent cirrhosis with an increased risk of hepatocellular carcinoma.⁽⁶⁾ The prevalence of cirrhosis among patients with NAFLD is approximately 5%, and patients with NAFLD have an increased mortality compared to control populations.⁽⁷⁻⁹⁾ Today, NAFLD is the second leading etiology among adult patients awaiting liver transplant in the United States.⁽¹⁰⁾

NASH has long been associated with a worse prognosis, speculated to catalyze fibrosis progression⁽¹¹⁾ and increase mortality. However, recent longitudinal studies have shown that only fibrosis and no other histologic features predicts mortality in patients with NAFLD.^(12,13)

There are few paired biopsy studies with approximately 520 patients in total,^(7,8,14-24) and all but two studies have a median follow-up time of less than 7 years (range, 3.0-7.0 years). Albeit, some studies only

Abbreviations: ALT, alanine aminotransferase; APRI, AST to platelet ratio index; AST, aspartate aminotransferase; BARD, BMI, AST/ALT ratio, diabetes; BMI, body mass index; CI, confidence interval; FIB-4, fibrosis-4; GUCI, Göteborg University cirrhosis index; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

Received October 3, 2017; accepted November 14, 2017.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1134/full.

Supported by ALF Grants from Region Östergötland and funding from the Research Council of Southeast Sweden (FORSS).

*These authors contributed equally to this work.

include patients with NASH, the rate of fibrosis progression does not seem to be affected by the presence of steatohepatitis. Moreover, some studies also show that simple steatosis can progress to steatohepatitis and advanced fibrosis.⁽²²⁻²⁴⁾

The natural history of NAFLD is not completely understood. NAFLD is a slowly progressive disease,⁽¹¹⁾ and most follow-up studies have too short a follow-up time to assess fibrosis progression.⁽²⁴⁾ Fibrosis progression is seen in 27%-52% of patients^(7,8,19,21,22,24) over a mean of 3.0-13.8 years of follow-up, with the majority of patients with NAFLD having static or regressive fibrosis. Baseline variables predicting fibrosis progression have been hard to determine.

In this study, we present a large cohort of patients with NAFLD with repeat liver biopsy with a follow-up time of almost 3 decades. Our aim was to define the clinical, biochemical, and histologic disease progression of patients with NAFLD and to assess predictors of disease progression.

Patients and Methods

SUBJECTS

In this prospective longitudinal cohort study, we included patients referred between 1988 and 1993 to the Department of Gastroenterology and Hepatology, University Hospital, Linköping, or to the Department of Internal Medicine, Oskarshamn County Hospital, for evaluation of chronically (>6 months) elevated serum alanine aminotransferase (ALT; defined as

>41 U/L), and/or aspartate aminotransferase (AST; defined as >41 U/L), and/or serum alkaline phosphatase (defined as >106 U/L for both sexes). At inclusion, a diagnostic workup was performed, including physical examination, laboratory investigation, and liver biopsy, as well as a review of their medical chart. After excluding other chronic liver diseases and significant alcohol consumption (≥ 140 g per week), 137 patients were diagnosed with NAFLD. During follow-up, 8 patients were reclassified as alcoholic fatty liver disease, and therefore the final cohort constituted of 129 patients with NAFLD. A previous follow-up study of this cohort (first follow-up) was conducted between 2003 and 2005 (details of data collection and results have been reported elsewhere⁽⁸⁾). The current follow-up (second follow-up) was conducted between 2013 and 2015.

Each subject in the study cohort was currently identified by linking his or her unique personal identification number to the National Registry of Population. All medical records from primary health care centers and hospitals were reviewed. Subjects who had died during follow-up were identified, and their causes of death were obtained by reviewing their medical records and the information obtained from the Registry of Causes of Death. All participants in the study cohort who were still living were asked to participate in this second follow-up study. Those who accepted were offered clinical and biochemical investigation and a biopsy of the liver.

All participating subjects gave written informed consent. The study design was approved by the ethics

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DOI 10.1002/hep4.1134

Potential conflict of interest: Nothing to report.

ARTICLE INFORMATION:

From the ¹Department of Gastroenterology and Hepatology, Department of Medical and Health Sciences, Linköping University, Linköping, Sweden; ²Department of Clinical Pathology and Clinical Genetics, Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Mattias Ekstedt, M.D., Ph.D.
Department of Gastroenterology and Hepatology
University Hospital, SE-581 85

Linköping, Sweden
E-mail: mattias.ekstedt@liu.se
Tel: +46 10 103 8670

committee at the University Hospital in Linköping (02-454, amendments: 2011/468-32, 2012/229-32, and 2013/72-32).

DATA COLLECTION

At second follow-up, a hepatologist (S.K., M.E., or P.N.) recorded each patient's medical history, including pharmacologic treatments. Alcohol consumption was assessed through an oral interview and the AUDIT-C questionnaire.⁽²⁵⁾ Blood pressure, waist circumference, body weight, and height were measured. Blood samples were taken from all subjects for analysis of routine biochemical parameters. At second follow-up, transient elastography was available and performed during the clinical evaluation. All patients without contraindications were asked to undergo a liver biopsy. The liver biopsy was performed within 3 months of the hepatologist's evaluation.

As outcome, patients were evaluated for signs of fibrosis progression and clinically significant disease progression. Fibrosis progression was defined as an increase of ≥ 1 in fibrosis stage between first and second/third liver biopsy. Patients with end-stage liver disease were defined as fibrosis stage 4. Clinically significant disease progression was defined as: 1) an increase in fibrosis stage from none (F0) to significant (F2); 2) development of advanced fibrosis (F3-F4); 3) development of end-stage liver disease; 4) NAFLD fibrosis score >0.676 or 5), liver stiffness >7.2 kPa as measured with transient elastography.

BIOCHEMICAL ANALYSIS

Subjects had blood drawn in the fasting state for a complete blood count, analysis of prothrombin, iron, transferrin, transferrin saturation, ferritin, AST, ALT, alkaline phosphatase, gamma glutamyl transferase, bilirubin, thyroid stimulating hormone, anti-transglutaminase antibodies, total cholesterol, low- and high-density lipoprotein, glucose, serum insulin, ceruloplasmin, and plasma protein electrophoresis, including, among others, albumin, α_1 -antitrypsin, and immunoglobulins. Patients who had not previously been diagnosed with diabetes mellitus type 2 underwent a 75-g oral glucose tolerance test. In addition, blood was drawn for detection of hepatitis B surface antigen, hepatitis B virus DNA, anti-hepatitis C virus antibodies, hepatitis C virus RNA, anti-nuclear antibodies, smooth muscle antibodies, and anti-

mitochondrial antibodies. In addition, all patients had blood obtained to identify the C282Y, H63D, and S65C mutations in the human hemochromatosis (*HFE*) gene as well as the S and Z mutations in the serine protease inhibitor A1 (*SERPINA1*) (*Pi*) gene.

Overweight was defined as body mass index (BMI) ≥ 25 kg/m² but <30 kg/m², obesity as BMI ≥ 30 kg/m², diabetes as fasting plasma glucose ≥ 126 mg/dL requiring treatment or plasma glucose >199 mg/dL 2 h after oral administration of 75 g of glucose, impaired glucose tolerance as plasma glucose >140 mg/dL but ≤ 199 mg/dL 2 h after oral administration of 75 g of glucose, hypertension as blood pressure $\geq 130/85$ or requiring treatment, and hypertriglyceridemia as fasting triglycerides ≥ 150 mg/dL. Metabolic syndrome was defined as having a BMI >30 kg/m² or waist circumference ≥ 94 cm in men or ≥ 80 cm in women plus two of the following four factors: (1) fasting triglycerides ≥ 150 mg/dL or treatment for this lipid abnormality; (2) reduced fasting high-density lipoprotein <40 mg/dL in men or <50 mg/dL in women or treatment for this lipid abnormality; (3) systolic blood pressure ≥ 130 mm Hg or diastolic blood pressure ≥ 85 mm Hg or treatment of previously diagnosed hypertension; (4) fasting plasma glucose ≥ 100 mg/dL or previously diagnosed diabetes mellitus type 2.

NAFLD fibrosis score,⁽²⁶⁾ BMI, AST/ALT ratio, diabetes (BARD) score,⁽²⁷⁾ Göteborg University cirrhosis index (GUCI) score,⁽²⁸⁾ fibrosis-4 (FIB-4) score,⁽²⁹⁾ and AST to platelet ratio index (APRI) score⁽³⁰⁾ were calculated using biochemical and clinical data.

TRANSIENT ELASTOGRAPHY

Transient elastography was performed using FibroScan (Echosens, Paris, France) by experienced hepatologists (S.K., M.E., or P.N.) as part of the clinical workup, according to the described method.⁽³¹⁾

LIVER BIOPSY

All liver biopsies were performed percutaneously with ultrasonography guidance and a 1.6-mm Biopince needle. All biopsies were read by two experienced liver pathologists who were blinded to patient details. All biopsies were graded according to NAFLD activity score and fibrosis stage according to Kleiner et al.⁽³²⁾

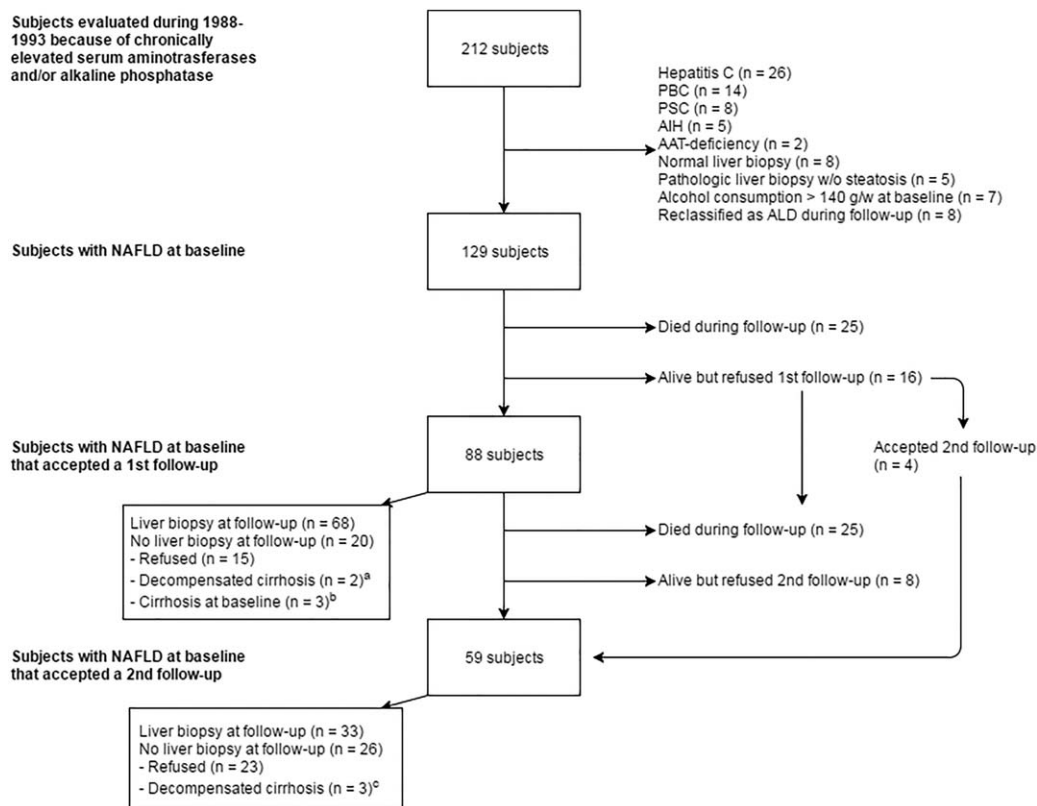


FIG. 1. Details about patients studied, showing reasons for exclusions. ^aBoth patients were diagnosed with hepatocellular carcinoma at follow-up; 1 patient died shortly after diagnostic workup at follow-up. ^bOne patient developed hepatocellular carcinoma and underwent orthoptic liver transplantation during follow-up. ^cOne patient developed hepatocellular carcinoma, 1 developed ascites, and 1 developed gastric antral vascular ectasia. Abbreviations: AAT, α 1-antitrypsin; AIH, autoimmune hepatitis; ALD, alcoholic liver disease; g/w, grams per weight; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; w/o, without.

STATISTICAL ANALYSIS

All statistical analyses were performed using SPSS version 24.0 (SPSS Inc., Chicago, IL). Data are expressed as mean \pm SD or as total numbers with percentages if applicable. To compare the means of normally distributed variables between groups, the independent two-sided Student *t* test was performed. Longitudinal changes in continuous variables were assessed by paired-sample *t* test or Wilcoxon rank-sum test. The Mann-Whitney test was used to compare continuous data between two groups; if more than two groups were analyzed, the Kruskal-Wallis one-way analysis of variance was used. The χ^2 test was used for categorical data. Survival curves were made according to the Kaplan-Meier method, and the log-rank test was applied for determination of difference in survival. $P < 0.05$ was considered significant.

Results

STUDY POPULATION AND CLINICAL CHARACTERISTICS

Originally, 129 patients with NAFLD were prospectively enrolled in a clinical study between 1988 and 1993, and all patients alive were invited for a first and second follow-up. At the end of the study, 50 patients had died. Mean follow-up time was 19.8 ± 6.0 years with 2,587 person-years of follow-up. At first follow-up, 104 patients were alive and 88 patients (85%) accepted clinical evaluation; at second follow-up, 79 patients were alive and 59 patients (75%) agreed to participate (Fig. 1). At inclusion, patients were middle aged (51.0 ± 12.9 years) with a male predominance (67%), 11 patients (8.5%) had previously diagnosed diabetes, and 14 patients (11%) had

TABLE 1. CLINICAL AND BIOCHEMICAL DATA OF THE COHORT AT INCLUSION, FIRST FOLLOW-UP, AND SECOND FOLLOW-UP

	Inclusion (n = 129)	1 st Follow-Up (n = 88)	2 nd Follow-Up (n = 59)
Age (years)	51.0 ± 12.9	61.0 ± 11.0	66.3 ± 10.3
Sex (male)	87 (67%)	62 (70%)	47 (81%)
BMI (kg/m ²)	28.3 ± 3.8	29.1 ± 4.7	29.1 ± 4.0
Overweight	72 (56%)	49 (56%)	25 (42%)
Obese	37 (29%)	29 (33%)	24 (41%)
Previously diagnosed diabetes	11 (8.5%)	6 (6.8%)	3 (5.1%)
Diabetes/IGT diagnosed at first follow-up	NA	69 (78%)	42 (71%)
Diabetes/IGT diagnosed at second follow-up	NA	NA	47 (80%)
Hypertensive	93 (72%)	83 (94%)	55 (93%)
Manifest cardiovascular disease	14 (11%)	16 (18%)	17 (29%)
Hypertriglyceridemia	74 (57%)	35 (40%)	39 (66%)
Metabolic syndrome	NA	70 (80%)	51 (86%)
ALT (U/L)	76 ± 43	60 ± 35	50 ± 34
AST (U/L)	45 ± 23	35 ± 15	42 ± 23
AST/ALT ratio	0.6 ± 0.2	0.7 ± 0.3	0.9 ± 0.4
ALP (U/L)	61 ± 33	65 ± 37	72 ± 31
Bilirubin (mg/dL)	0.64 ± 0.30	0.78 ± 0.33	0.8 ± 0.8
Albumin (g/dL)	4.1 ± 0.3	4.2 ± 0.4	4.0 ± 0.4
Platelet count (× 10 ⁹ /L)	235 ± 67	194 ± 94	222 ± 57
Prothrombin (INR)	1.0 ± 0.1	1.0 ± 0.2	1.1 ± 0.4
Ferritin (μg/L)	232 ± 317	192 ± 159	257 ± 230
Glucose (mg/dL)	NA	125 ± 38	135 ± 45
IR _{HOMA}	NA	3.8 ± 3.5	9.6 ± 13
Triglycerides (mg/dL)	190 ± 134	157 ± 89	170 ± 109
Cholesterol (mg/dL)	236 ± 59	202 ± 43	178 ± 53
HDL (mg/dL)	NA	51 ± 19	48 ± 16
LDL (mg/dL)	NA	123 ± 37	100 ± 44
Negative for HBsAg/ anti-HCV	NA	88/88	59/59
Negative for HBV DNA/HCV RNA	NA/NA	88/88	59/59
Positive for transglutaminase antibodies	NA	0 (0%)	1 (1.7%)
Mutation in HFE gene:			
C282Y homozygosity/C282Y heterozygosity	NA	2/6	1/3
H63D homozygosity/H63D heterozygosity	NA	3/16	1/10
S65C homozygosity/S65C heterozygosity	NA	0/2	0/2
C282Y/H63D compound heterozygosity	NA	0	0
Mutation in SERPINA1 gene (ZZ/ SZ/ MZ/ MS)	NA	0/0/10/3	0/0/8/0
Ceruloplasmin < 0.20 g/L	0 (0%)	0 (0%)	1 (1.7%)

Abbreviations: ALP, alkaline phosphatase; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HDL, high-density lipoprotein; IGT, impaired glucose tolerance; INR, international normalized ratio; IR_{HOMA}, insulin resistance according to homeostasis model assessment; LDL, low-density lipoprotein; NA, not available.

manifest cardiovascular disease. The clinical and biochemical data of the study cohort at inclusion and first and second follow-up are presented in Table 1.

CAUSE OF DEATH

Fifty patients died during follow-up: 24 patients died from cardiovascular disease (48%), 11 from extrahepatic malignancies (22%), 5 from end-stage liver disease, including hepatocellular carcinoma (10%), 5 from infectious disease (10%), 3 from kidney disease (6%), 1 from respiratory disease (2%), and 1 from neurologic disease (2%).

REPEAT LIVER BIOPSY

At inclusion, all patients underwent liver biopsy. At first follow-up, 88 patients accepted follow-up (77%) and 68 patients underwent a second liver biopsy; at second follow-up, 59 patients accepted second follow-up (76%) and 33 patients underwent liver biopsy. The mean time between first and second, second and third, and first and third liver biopsies was 13.7 ± 1.5 years, 9.3 ± 1.0 years, and 22.8 ± 1.3 years, respectively. In total, 29 patients had three consecutive liver biopsies.

Patients who accepted follow-up but showed signs of end-stage liver disease (esophageal variceal bleeds, ascites, spontaneous bacterial peritonitis, hepatic

encephalopathy, hepatorenal syndrome, or hepatocellular carcinoma) were excluded from biopsy but included as fibrosis stage 4. Therefore, fibrosis stage as well as biochemical and clinical data were available in 32 patients at inclusion and first and second follow-up.

DEVELOPMENT AND PREDICTION OF END-STAGE LIVER DISEASE AND ADVANCED FIBROSIS

At the end of follow-up, 12 patients (9.3%) had developed symptoms of end-stage liver disease. The presenting symptom was ascites ($n = 5$), hepatocellular carcinoma ($n = 4$), variceal hemorrhage ($n = 2$), and gastric antral vascular ectasia ($n = 1$). Baseline fibrosis stage in the 12 patients that developed symptoms of end-stage liver disease was: stage 0 ($n = 2$), stage 1 ($n = 2$), stage 2 ($n = 4$), stage 3 ($n = 3$), and stage 4 ($n = 1$).

At baseline, patients with NAFLD who progressed to end-stage liver disease had a higher prevalence of diabetes (33% versus 8.5%, $P = 0.026$), higher levels of AST (57 ± 30 versus 42 ± 22 U/L, $P = 0.046$), ALT (103 ± 66 versus 73 ± 40 U/L, $P = 0.022$), and ferritin (436 ± 834 versus 211 ± 201 mikrog/L, $P = 0.018$). A higher grade of ballooning (0 [0-2] versus 0 [0-1], $P = 0.015$) and fibrosis stage (2 [0-4] versus 1 [0-4], $P = 0.004$) were also seen in patients who developed end-stage liver disease. Moreover, patients with end-stage liver disease had a higher NAFLD fibrosis score (-1.055 ± 1.721 versus -2.077 ± 1.265 , $P = 0.020$) at baseline. Clinical, histologic, and biochemical data are presented in [Supporting Table S1](#).

At baseline, 16 patients (12%) had biopsy-proven advanced fibrosis stage 3 ($n = 12$) and stage 4 ($n = 4$). At first follow-up, 9 patients with previous low-stage fibrosis (<3) had developed advanced fibrosis in their second liver biopsy and 3 patients developed symptoms of end-stage liver disease. At second follow-up, 3 patients with previous low-stage fibrosis had developed advanced fibrosis in their third liver biopsy and 3 additional patients developed symptoms of end-stage liver disease.

In total, 34 patients (26%) were diagnosed with advanced fibrosis at inclusion or developed advanced fibrosis during the study period; 28 patients had biopsy-proven fibrosis stage 3 or 4, and 6 patients showed symptoms of end-stage liver disease with previous low-stage fibrosis. In the 113 patients with initial low fibrosis stage, 18 patients (16%) developed

advanced fibrosis or end-stage liver disease. When comparing baseline data of the 13 patients who developed advanced fibrosis with the 95 patients who did not, there was no significant difference in biochemical data or clinical characteristics, although there were differences in NAFLD fibrosis, FIB-4, APRI, and GUCI scores (Table 2).

DISEASE PROGRESSION

Clinically Significant Disease Progression

During the first follow-up, 22 out of 129 patients (17%) had clinically significant disease progression (as defined in Patients and Methods; Data Collection), and during the second follow-up, an additional 13 patients (10%) had clinically significant disease progression. Two patients out of the 22 patients that initially progressed between inclusion and first follow-up also progressed between first and second follow-up. Between baseline and second follow-up, 3 (2.3%) patients had an increase of just one stage in fibrosis at each follow-up but a two-stage increase between baseline and second follow-up in total. Moreover, 2 patients who did not have a biopsy at the first follow-up accepted biopsy at the second follow-up and had clinically significant disease progression (1 from F0-F2 and 1 with F0-variceal bleeding). In total, 38 patients (29%) developed clinically significantly disease progression at some point during follow-up.

Excluding the 16 patients with advanced fibrosis at inclusion, 34 patients (30%) had clinically significant disease progression at the end of the study period. There were no statistically significant differences in baseline clinical, biochemical, or histologic characteristics between patients with clinically significant disease progression and patients with clinically stable disease ([Supporting Table S2](#)). Even when excluding the 16 patients with baseline advanced fibrosis, no biochemical, clinical, or histologic differences were seen between patients with disease progression and patients with clinically stable disease (data not presented).

Fibrosis Progression

Between the first and second biopsy, 30 (42.3%) patients progressed in fibrosis stage (median stage 1.5, range 1-3), 30 (42.3%) patients were stable, and 11 patients regressed (8.5%). Of those who regressed, no

TABLE 2. COMPARISON OF BASELINE DATA OF PATIENTS WITH AND WITHOUT ADVANCED FIBROSIS AT THE END OF THE STUDY*

	Developed Advanced Fibrosis (n = 18)	Did Not Develop Advanced Fibrosis (n = 95)	P Value
Age (years)	51 ± 11	50 ± 13	0.677
Sex (male)	12 (67%)	66 (69%)	0.788
BMI (kg/m ²)	29 ± 2.5	28 ± 3.8	0.185
Overweight	10 (56%)	57 (60%)	0.796
Obese	7 (39%)	23 (24%)	0.245
Diabetes	4 (22%)	8 (8.4%)	0.098
Hypertensive	14 (78%)	68 (72%)	1.000
Manifest cardiovascular disease	2 (11%)	9 (9.5%)	0.687
Hypertriglyceridemia	12 (67%)	46 (48%)	0.185
ALT (U/L)	82 ± 53	69 ± 29	0.148
AST (U/L)	44 ± 17	40 ± 15	0.276
AST/ALT ratio	0.6 ± 0.2	0.6 ± 0.2	0.837
ALP (U/L)	59 ± 28	60 ± 34	0.883
Bilirubin (mg/dL)	0.7 ± 0.6	0.6 ± 0.2	0.152
Albumin (g/dL)	4.1 ± 0.3	4.1 ± 0.4	0.915
Platelet count (× 10 ⁹ /L)	193 ± 49	232 ± 63	0.964
Prothrombin (INR)	1.0 ± 0.1	1.0 ± 0.1	0.201
Ferritin (µg/L)	200 ± 130	187 ± 178	0.772
Triglycerides (mg/dL)	214 ± 214	181 ± 121	0.355
Cholesterol (mg/dL)	254 ± 90	232 ± 53	0.155
NAFLD activity score	3 (1-4)	2 (1-4)	0.089
Steatosis	3 (1-3)	2 (1-3)	0.096
Ballooning	0 (0-1)	0 (0-1)	0.903
Inflammation	0 (0-1)	0 (0-1)	0.347
Fibrosis	1 (0-2)	0 (0-2)	0.015
F0	6 (33%)	54 (57%)	
F1	4 (22%)	27 (28%)	
F2	8 (44%)	14 (15%)	
F3	0 (0%)	0 (0%)	
F4	0 (0%)	0 (0%)	
NAFLD fibrosis score	-1.478 ± 1.487	-2.222 ± 1.260	0.039
BARD	1 (0-4)	0 (0-4)	0.056
FIB-4	1.7 ± 1.7	1.2 ± 0.8	0.067
APRI	0.6 ± 0.4	0.4 ± 0.3	0.023
GUCI	0.6 ± 0.5	0.4 ± 0.3	0.028

*Patients who at inclusion presented with advanced fibrosis (F3 or F4, n = 16) were excluded from this table. Continuous data were assessed with independent Student *t* test if not normally distributed, or for a nonparametric method, the Mann-Whitney *U* test was used. Dichotomous variables were assessed with the χ^2 test. Numbers in bold are significant. Abbreviations: ALP, alkaline phosphatase; INR, international normalized ratio.

patient regressed more than one fibrosis stage. Between the second and third biopsy, 12 (38.7%) patients progressed (stage 1.5, range 1-3), 14 (45.2%) patients were stable, and 5 (16.1%) patients regressed. Of those who regressed, no patient regressed more than one fibrosis stage.

Between the first and third biopsy, a comparison of fibrosis stage at inclusion with final biopsy (including counting clinical signs of end-stage liver disease as fibrosis stage 4) showed that 40 (52.6%) patients progressed (stage 2, range 1-4), 26 (34.2%) patients were stable, and 10 (13.2%) patients showed signs of regression. Of those who regressed, no patient regressed more than one fibrosis stage.

In patients with F0, 23 (59%) patients progressed histologically; the corresponding numbers for F1, F2, and F3 were 8 (40%), 7 (58.3%), and 2 (50%) patients, respectively; there was no significant difference between groups ($P = 0.374$). In total, 40 (52.6%) patients with repeat biopsies showed signs of fibrosis progression during the study period.

FOLLOW-UP OF PATIENTS WITH THREE CONSECUTIVE BIOPSIES

The data of the 32 patients with three consecutive biopsies are presented in Table 3. None of the patients with three consecutive biopsies had advanced fibrosis

TABLE 3. BIOCHEMICAL, CLINICAL, AND HISTOLOGIC CHARACTERISTICS OF PATIENTS THAT UNDERWENT THREE CONSECUTIVE BIOPSIES DURING THE FOLLOW-UP PERIOD

	Baseline (n = 32)	1 st Follow-Up (n = 32)	2 nd Follow-Up (n = 32)	P Value Baseline vs. 1 st Follow-Up	P Value 1 st Follow-Up vs. 2 nd Follow-Up	P Value Baseline vs. 2 nd Follow-Up
Age (years)	43.3 ± 10.6	57.0 ± 10.5	66.1 ± 10.3			
Sex (male)	28 (88%)	28 (88%)	28 (88%)			
BMI (kg/m ²)	27.9 ± 4.3	29.5 ± 5.2	29.0 ± 4.4	<0.0001	0.513	0.107
Overweight	19 (59%)	20 (63%)	15 (47%)	1.000	0.625	0.727
Obese	6 (19%)	11 (34%)	12 (38%)	0.063	1.000	0.031
Diabetes/IGT	2 (6%)	24 (75%)	24 (75%)	<0.0001	1.000	<0.0001
Hypertensive	22 (69%)	30 (94%)	29 (91%)	1.000	1.000	1.000
Manifest cardiovascular disease	0 (0%)	2 (6%)	7 (22%)	0.500	0.063	0.016
Hypertriglyceridemia	15 (47%)	17 (53%)	12 (38%)	1.000	0.344	0.289
Metabolic syndrome	NA	26 (81%)	26 (81%)	NA	0.688	NA
ALT (U/L)	74 ± 40	63 ± 33	47 ± 31	0.186	0.023	<0.0001
AST (U/L)	38 ± 13	34 ± 19	42 ± 27	0.318	0.087	0.360
AST/ALT ratio	0.5 ± 0.1	0.6 ± 0.2	0.9 ± 0.3	0.298	<0.0001	<0.0001
ALP (U/L)	53 ± 24	57 ± 17	73 ± 34	0.278	0.027	0.016
Bilirubin (mg/dL)	0.7 ± 0.5	0.7 ± 0.4	0.9 ± 1.1	0.166	0.496	0.344
Albumin (g/dL)	4.2 ± 0.3	4.3 ± 0.4	4.0 ± 0.5	0.721	0.011	0.016
Platelet count (× 10 ⁹ /L)	220 ± 60	223 ± 55	229 ± 66	0.623	0.835	0.995
Prothrombin (INR)	0.96 ± 0.10	0.98 ± 0.07	1.07 ± 0.29	0.172	0.050	0.018
Ferritin (μg/L)	202 ± 155	220 ± 167	278 ± 263	0.626	0.242	0.209
Glucose (mg/dL)	NA	119 ± 37	129 ± 40	NA	0.188	NA
IR _{HOMA}	NA	3.1 ± 1.9	8.7 ± 13.4	NA	0.031	NA
Triglycerides (mg/dL)	185 ± 178	169 ± 95	173 ± 114	0.567	0.926	0.564
Cholesterol (mg/dL)	240 ± 73	204 ± 42	188 ± 54	0.015	0.056	0.001
HDL (mg/dL)	NA	54 ± 25	50 ± 16	NA	0.203	NA
LDL (mg/dL)	NA	127 ± 37	112 ± 45	NA	0.081	NA
NAFLD activity score	3 (1-4)	2 (0-4)	2 (0-5)	0.226	0.946	0.513
Steatosis	1.5 (0-3)	3 (1-3)	2 (0-3)	0.027	0.047	0.001
Ballooning	0 (0-1)	0 (0-2)	0 (0-2)	0.014	0.038	0.013
Inflammation	0 (0)	0 (0-1)	0 (0-2)	0.317	0.011	0.007
Fibrosis	0 (0-2)	0.5 (0-4)	1.5 (0-4)	0.007	0.041	0.013
F0 (%)	20 (63%)	16 (50%)	13 (41%)			
F1 (%)	10 (31%)	9 (28%)	3 (9%)			
F2 (%)	2 (6%)	3 (9%)	9 (28%)			
F3 (%)	0	3 (9%)	4 (13%)			
F4 (%)	0	1 (3%)	3 (9%)			
NAFLD fibrosis score	-2.536 ± 1.203	-1.074 ± 1.375	-0.212 ± 1.355	<0.0001	<0.0001	<0.0001
FIB-4	1.0 ± 0.5	1.2 ± 0.6	2.0 ± 1.3	0.072	<0.0001	<0.0001
APRI	0.43 ± 0.23	0.39 ± 0.29	0.44 ± 0.32	0.560	0.197	0.614
GUCI	0.41 ± 0.23	0.38 ± 0.28	0.57 ± 0.74	0.631	0.101	0.164
BARD	0 (0-2)	1 (0-3)	2 (0-4)	0.001	0.003	<0.0001
Fibroscan (kPa)	NA	NA	8.0 ± 4.1	NA	NA	NA

Continuous data were assessed with dependent *t* test if not normally distributed, or for a nonparametric method, the Wilcoxon rank-sum test was used. Dichotomous variables were assessed with the χ^2 test. Numbers in bold are significant.

Abbreviations: ALP, alkaline phosphatase; HDL, high-density lipoprotein; IGT, impaired glucose tolerance; IR_{HOMA}, insulin resistance according to homeostasis model assessment; LDL, low-density lipoprotein; NA, not available.

at baseline. At first follow-up, 4 patients (12%) had developed advanced fibrosis, and at second follow-up, 7 patients (22%) had developed advanced fibrosis.

Of the 11 patients (34%) that progressed between baseline and first follow-up, 5 patients (45%) also progressed during second follow-up and 1 patient went from fibrosis stage 4 to end-stage liver disease. Of the remaining 21 patients (66%) that were stable or

showed a regression in fibrosis stage between baseline and first follow-up, 8 (38%) showed signs of progression at second follow-up. The individual data of the 32 patients with three serial biopsies are shown in [Supporting Table S3](#).

There were no significant differences at baseline between those who progressed and those who were stable or regressed between baseline and first follow-up.

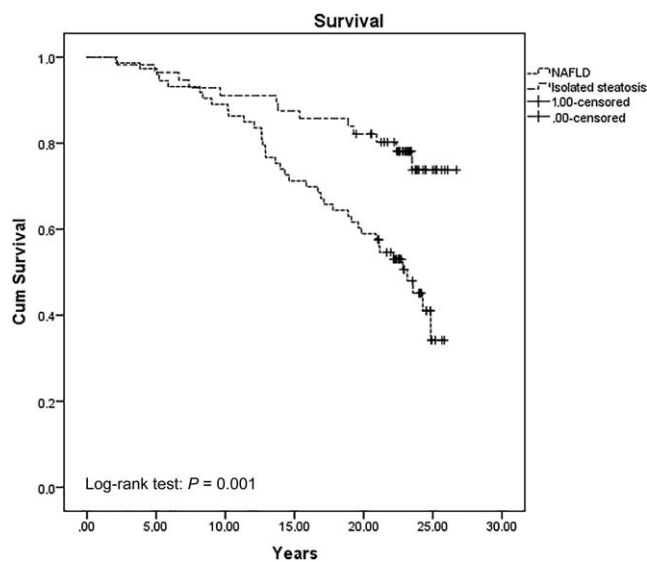


FIG. 2. Cumulative survival probability comparing patients with isolated steatosis to NAFLD over time. Abbreviation: Cum, cumulative.

Likewise, there were no significant differences at first follow-up between those who progressed and those who were stable or regressed between first and second follow-up (data not presented).

Nine patients (28%) had fibrosis stage 0 at baseline and first and second follow-up, representing a nonprogressive NAFLD. When comparing baseline variables of the nonprogressive patients with NAFLD with patients that developed any stage of fibrosis during baseline or follow-up, there was a significantly lower frequency of patients with hypertriglyceridemia (11% versus 61%, $P = 0.023$). Patients with nonprogressive NAFLD also showed lower stages of the NAFLD activity score (2 [1-3] versus 3 [1-4], $P = 0.047$), fibrosis stage (0 [0] versus 1 [0-2], $P = 0.022$), and BARD (0 [0-1] versus 1 [0-2], $P = 0.043$). There was also a trend toward lower stages of hepatic steatosis (2 [1-3] versus 3 [1-3], $P = 0.053$), APRI score (0.3 ± 0.1 versus 0.5 ± 0.2 , $P = 0.075$), and GUCI score (0.3 ± 0.1 versus 0.5 ± 0.2 , $P = 0.061$).

FOLLOW-UP OF PATIENTS WITH NAFLD AND ISOLATED STEATOSIS

Of the 129 patients in the initial cohort, 56 patients (43%) had isolated steatosis, defined as having only steatosis and no other features of NASH (lobular

inflammation and/or ballooning) or any stage of fibrosis. Generally, the 56 patients were younger and had lower AST, ALT, ferritin, and NAFLD fibrosis score (Supporting Table S4). The cumulative survival between the two groups showed better survival among patients with isolated steatosis at baseline compared with patients with NAFLD (Fig. 2).

In total, 5 patients (9%) with isolated steatosis at baseline developed biopsy-proven advanced fibrosis ($n = 3$) or clinical signs of end-stage liver disease ($n = 2$). At baseline, these patients had a higher steatosis grade (3 [2-3] versus 2 [1-3], $P = 0.032$) and also presented with a higher prevalence of type 2 diabetes (2% versus 20%, $P = 0.04$) and hypertriglyceridemia (44% versus 100%, $P = 0.021$) as well as higher levels of bilirubin mg/dL (1.1 ± 1.1 versus 0.6 ± 0.2 , $P = 0.014$) and triglycerides mg/dL (331 ± 363 versus 175 ± 125 , $P = 0.037$). There were no other baseline clinical, biochemical, or histologic differences between the groups (data not presented).

Mean follow-up from baseline in the 3 patients with biopsy-proven advanced fibrosis was 16.5 ± 4.2 years. Of the 2 patients that developed clinical signs of end-stage liver disease, 1 developed variceal bleeds 13.7 years after inclusion and died 6 months after the first sign of decompensation in an infectious disease; the second patient developed hepatocellular carcinoma 18.9 years after inclusion and died 1 month later.

Discussion

In this prospective cohort study of well-defined patients with NAFLD, we have shown that 26% of patients had advanced fibrosis and 12 patients (9.3%) had developed end-stage liver disease after an extended period of follow up. Even in patients without advanced fibrosis (fibrosis stage <3) at inclusion, a significant proportion (16%) developed advanced fibrosis.

Our findings showed more patients developed end-stage liver disease compared to what has been reported (1.2%-5.4%).^(7,8,13,33) Angulo et al.⁽¹³⁾ showed 4.2% of all patients developed end-stage liver disease. Theirs was a retrospective study with 619 patients and with a high loss to follow-up (15.7%). In our previous report of this cohort, 5.4% had developed end-stage liver disease after a follow-up of 13.7 years,⁽⁸⁾ which is in line with Angulo et al. who reported almost equal frequency after 12.6 years of follow-up. In addition, Adams et al.⁽⁷⁾ showed an end-stage liver disease frequency of 3% after 7.8 years. In the long-term

follow-up by Dam-Larsen et al.,⁽³³⁾ only 1.2% developed end-stage liver disease after 20.4 years of follow-up. Although this number is lower than previous studies, in the study by Dam-Larsen et al. only a minority of the patients with NAFLD were followed up and the mean age at inclusion was 39.5 years, reflecting the slow progressive nature of NAFLD and the age dependence of fibrosis progression. In our extended follow-up study, patients were prospectively enrolled and followed over a long period with enough time for liver-related events to occur. Also, the meticulous follow-up by either clinical assessment or by extensive chart review reduced the risk of misdiagnosing patients with end-stage liver disease.

In 25% of patients, three consecutive liver biopsies approximately 10 years apart were performed. Albeit, these patients originally were younger, healthier, and had a low fibrosis stage (<3), 22% developed advanced fibrosis during follow-up. No baseline biochemical, clinical, or histologic characteristic could predict progression to advanced fibrosis or significant disease progression over time. However, patients who developed end-stage liver disease had more frequent type 2 diabetes and higher levels of ferritin, AST, and ALT levels as well as higher incidence of ballooning on liver biopsy. Nevertheless, these patients also had higher levels of fibrosis stage on baseline liver biopsy, with 4 patients (33%) having advanced fibrosis, which could skewer the results.

It is generally believed that isolated steatosis is a benign condition with low clinical relevance. Patients with NAFLD with isolated steatosis showed a better overall prognosis compared to other patients with NAFLD, although 3 (5.4%) patients with isolated steatosis developed advanced fibrosis and 2 (3.6%) patients developed end-stage liver disease. Interestingly, these 5 patients showed a higher steatosis grade and a higher prevalence of diabetes mellitus type 2. However, these subanalyses should be interpreted with caution because of the inherently small number of patients. In comparison with previous studies with paired biopsies, the study by Wong et al.⁽²²⁾ showed fibrosis progression in 28% of patients with nonalcoholic fatty liver (defined as NAFLD activity score <3) over a 3-year period. The studies by Pais et al.⁽²³⁾ and McPherson et al.⁽²⁴⁾ showed fibrosis progression in 24% and 37% of patients with nonalcoholic fatty liver (defined as isolated steatosis or isolated steatosis and mild inflammation) over a follow-up time of 3.7 and 8 years, respectively. The latter study also showed a trend for more aggressive disease progression in

patients with steatosis and mild inflammation compared to patients with isolated steatosis ($P = 0.07$).⁽²⁴⁾ In our study with a mean follow-up time of 19.8 years, fibrosis progression ≥ 1 stage was seen in 61% (22 out of 36) of patients with isolated steatosis at inclusion, of whom 5 patients developed advanced fibrosis or end-stage liver disease. Although baseline characteristics for predicting histologic disease progression are hard to identify in patients with isolated steatosis with or without inflammation, patients who have fibrosis progression show a trend toward more frequent type 2 diabetes at baseline. In the studies by Pais et al.⁽²³⁾ and McPherson et al.,⁽²⁴⁾ patients who progressed also seemed to show a trend toward gaining weight or developing type 2 diabetes *a priori* to the follow-up biopsy, indicating an intricate relationship between the metabolic syndrome and histologic disease progression in patients with nonalcoholic fatty liver.

One of the main aims of this study was to determine if there were any clinical or biochemical data that could predict disease progression among patients with NAFLD. Although patients that developed end-stage liver disease showed baseline signs of higher levels of AST and ALT and necroinflammatory changes, patients with NAFLD with significant disease progression or fibrosis progression could not be distinguished from benign NAFLD.

The interval for follow-up should mainly be based on the stage of fibrosis. This is supported by a recent study of 646 patients with biopsy-proven NAFLD and a mean follow-up of 20 years.⁽³⁴⁾ In that study, the mean time for development of end-stage liver disease in 90% of patients per stage of fibrosis was 33.4 years for F0 (95% confidence interval [CI], 24.2, 42.6), 34.1 years for F1 (95% CI, 25.1, 43.2), 22.7 years for F2 (95% CI, 13.7, 31.7), 11.8 years for F3 (95% CI, 4.3, 19.4), and 5.6 years for F4 (95% CI, 0.9, 10.3). Because liver biopsy carries some procedural risks and the number of patients with NAFLD constantly increases, the need for noninvasive staging of hepatic fibrosis is imperative in a clinical setting.

The strength of our study is the unique cohort, which was prospectively enrolled and rigorously followed for 25 years. All patients included at baseline were part of a prospective study in which patients with mild to moderate elevation of liver function tests were consecutively investigated with liver biopsy, reducing selection bias. The low frequency of NASH and comorbid conditions (e.g., type 2 diabetes) as well as the low mean BMI in the cohort strengthens the

validity of our outcomes concerning mortality and liver-related events.

This study has some limitations. First, as with all studies using liver biopsy as the gold standard, sampling error as well as interassessment and intra-assessment variability is a fact.⁽³⁵⁾ Second, although many patients accepted a first and second follow-up, the high frequency of death because of the time between follow-ups resulted in a stagnating number of patients to follow-up as time progressed, reducing the number of clinical and biochemical parameters and decreasing the outcome of patients who developed severe liver disease. Moreover, 23 patients (47%) died of cardiovascular diseases, which is a competing risk for development of severe liver disease.

In conclusion, we have shown that NAFLD has a more dismal prognosis than previously reported, with 16% of patients with NAFLD with fibrosis stage <3 developing advanced fibrosis and 9.3% developing end-stage liver disease. We have also shown that it is surprisingly difficult to predict fibrosis progression in individual patients with NAFLD. There seems to be an association between a worsening of metabolic risk factors, i.e., type 2 diabetes, obesity, and fibrosis progression. These findings emphasize the need for robust noninvasive biomarkers suitable to monitor a large number of patients.

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Author names in bold designate shared co-first authorship.

Supporting Information

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