The amount of liver fat predicts mortality and development of type 2 diabetes in non-alcoholic fatty liver disease

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Funding Information
ALF Grants, Region Östergötland, Medical Research Council of Southeast Sweden.

Handling Editor: Luca Valenti

Abstract

Background & Aims: Non-alcoholic fatty liver disease (NAFLD) is a risk factor for development of type 2 diabetes mellitus (T2DM). We aimed to evaluate whether conventional histological grading of steatosis and accurate quantification of fat content in liver biopsies using stereological point counting (SPC) can predict mortality and future development of T2DM in NAFLD patients.

Methods: 129 patients with biopsy proven NAFLD, enrolled between 1988 and 1992, were re-evaluated on two occasions, after 13.7 (±1.5) and 23.2 (±6.8) years. In patients accepting to undergo the procedure, repeat liver biopsies were performed on each follow-up and were evaluated with conventional histopathological methodology and SPC.

Results: Of the 106 patients without T2DM at baseline, 66 (62%) developed T2DM during a mean follow-up of 23.2 (± 6.8) years. Steatosis grade and liver fat measured with SPC independently (adjusted for age, BMI, fibrosis stage) predicted development of T2DM with an aHR of 1.60 per grade and 1.03 for each SPC percentage increase respectively. Overall mortality and development of T2DM was more common in patients with grade 3 steatosis compared to lower grades of steatosis. Liver fat measured with SPC was significant for overall mortality (aHR 1.04). In patients that underwent repeat biopsy, reduction in liver fat measured with SPC was associated with decreased risk of developing T2DM (aHR 0.91 for each SPC percentage decrease).

Conclusion: Steatosis grade and liver fat measured with SPC predict mortality and the risk of developing T2DM in NAFLD. Reduction in liver fat decreases the risk of developing T2DM.

Keywords
hepatic steatosis, quantitative steatosis, stereological point count

Abbreviations: 1H-MRS, proton magnetic resonance spectroscopy; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HTGC, hepatic triglyceride content; IGT, impaired glucose tolerance; MR, magnetic resonance; MRI, magnetic resonance imaging; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; OGTT, oral glucose tolerance test; PDFF, proton density fat fraction; SPC, stereological point counting; T2DM, type 2 diabetes mellitus.

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Liver International. 2020:00:1–10. wileyonlinelibrary.com/journal/liv
Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease, affecting approximately 25% of the global population. Insulin resistance is a crucial pathophysiological factor in NAFLD reflected in the high prevalence of type 2 diabetes mellitus (T2DM) amongst NAFLD patients. Moreover, many NAFLD patients without T2DM will develop T2DM. Therefore, the paradigm of NAFLD being a consequence of the metabolic syndrome or T2DM has been questioned. Instead, NAFLD has been suggested to precede the above-mentioned conditions.

Liver biopsy is considered the reference method for diagnosing and assessing the severity of NAFLD. A prerequisite for the histopathological diagnosis of NAFLD to be established is that >5 percent of the hepatocytes contain fat globules. The conventional histopathological methodology used to quantify liver fat content consists of a visual semiquantitative approach in which the histopathologist uses a four-graded scale (0-3). It has previously been demonstrated that semiquantitative assessment of steatosis by a histopathologist frequently overestimates hepatic fat content when measured quantitatively. An alternate approach is to assess steatosis quantitatively with stereological point counting (SPC), a method with higher reproducibility suggested to be preferable when accurate measurements of liver steatosis are required. Liver biopsy also offers evaluation of other histopathological features of NAFLD such as lobular inflammation, ballooning, and fibrosis stage.

The clinical application of quantifying hepatic steatosis has increased in recent years, mainly because of the access to magnetic resonance (MR) techniques as non-invasive alternatives for diagnosing NAFLD and monitoring changes in hepatic steatosis during clinical trials. SPC correlates excellently with hepatic triglyceride content (HTGC) measured with MR.

The primary aim of our study was to assess if the amount of hepatic fat assessed with SPC and histopathological grading is associated with future development of T2DM in NAFLD. Moreover, we evaluated if change in quantity of hepatic fat content affects the risk of developing T2DM.

2 | PATIENTS AND METHODS

2.1 | Subjects

In this prospective longitudinal study we included patients referred between 1988 and 1993 to the Department of Gastroenterology and Hepatology, University Hospital, Linköping or the Department of Internal Medicine, Oskarshamn County Hospital, for evaluation of chronically (>6 months) elevated serum alanine aminotransferase (ALT, defined as >41 U/L for both genders), and/or aspartate aminotransferase (AST, defined as >41 U/L for both sexes), and/or serum alkaline phosphatase (ALP, defined as >106 U/L for both sexes). A diagnostic work-up was performed including review of medical charts, physical examination, laboratory investigation and liver biopsy. After excluding other chronic liver diseases and significant alcohol consumption (>140 g per week), 129 patients were diagnosed with NAFLD and constituted the cohort of this study. All NAFLD patients were later asked to participate in two follow-up studies (after 13.7 ± 1.5 years and 23.2 ± 6.8 years) including clinical, laboratory and histopathological evaluation. Patients who had died or did not accept participation in the follow-up studies were evaluated with an extensive review of their medical charts in order to extract the diagnosis of T2DM and time and cause of mortality. End of follow-up was death or, if alive, December 31, 2017.

2.2 | Clinical evaluation

Subjects had blood drawn in the fasting state for routine biochemical evaluation and to exclude other liver diseases as reported previously. 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 hours after oral administration of 75 g of glucose, hypertension as blood pressure ≥130/85 mmHg or requiring treatment, hypertriglyceridemia as fasting triglycerides ≥150 mg/dL and manifest cardiovascular disease (CVD) as the presence of coronary heart disease, cerebrovascular disease or peripheral arterial disease.

2.3 | Liver biopsy and histopathological evaluation

Liver biopsies were performed percutaneously with ultrasonography guidance and a 1.4-1.6 mm Biopince needle. All biopsies were read by an experienced liver pathologist who was blinded to the treatment group.
patients' details. Biopsies were graded according to NAFLD activity score (NAS) in which steatosis grades 0-3 are considered to correspond to fat deposition in <5%, 5%-33%, 34%-66% and >66% of the hepatocytes respectively. Fibrosis stage was assessed according to Kleiner et al.]

2.4 | Stereological point counting (SPC)

This method has been described elsewhere. In short, a Nikon Eclipse E800 microscope with a Nikon DS-Ri1 digital camera was used to capture all tissue images for histopathology analysis. Ten images from each biopsy sample were captured and stored in a computer using NIS-Elements BR v 4.0 (Nikon Corporation). A point grid was later superimposed upon each image. The number of fat vacuoles in the hepatocytes were counted manually. The number of hits on fat vacuoles divided by the total number of hits on liver tissue represented the ratio of steatotic area within the section (expressed in %).

2.5 | Statistical analysis

All statistical analyses were performed using SPSS version 24.0 (SPSS Inc) and STATA version 15.1. Data are expressed as mean ± standard deviation (SD), median with range or as total number with percentages when applicable. When comparing means of normally distributed variables between groups, the independent two-sided Student t test was used if two groups were analysed, otherwise ANOVA was performed. Longitudinal changes in continuous variables were assessed by paired-sample t test or Wilcoxon rank-sum test. The Mann-Whitney U test was used to compare continuous data between groups; if more than two groups were analysed, the Kruskal-Wallis one-way analysis of variance was used. The chi-squared ($\chi^2$) test was used for categorical data. A Cox regression model was used. Gender, age, BMI, liver fat measured with SPC and the histological scores for steatosis (0-3), lobular inflammation (0-3), ballooning (0-2) and fibrosis stage (0-4) were all tested separately in univariate analyses for T2DM or overall mortality (Model 1). We later used a stepwise-forward approach to select potential demographic or anthropometric covariates (ie age, gender, BMI) by using P value <0.20 as significant (Model 2). Finally, we selected the significant demographic and anthropometric covariates from Model 1 together with fibrosis stage, irrespective of its significance (Model 3). A separate multivariate model was used when calculating difference in quantitative steatosis at inclusion and follow-up and the impact on development of T2DM. In this model gender, difference in BMI and presence of progressive fibrosis was used as a priori covariates. Overall mortality and proportion free of T2DM was calculated according to the Kaplan-Meier method, and the log-rank test was applied for determination of statistical significance. $P < .05$ was considered significant.

| TABLE 1 | Clinical, biochemical and histopathological data of the cohort at baseline |
|----------|---------------------------------------------------------------|
| At baseline (n = 106) | |
| Age (y) | 49.3 ± 12.9 |
| Gender (male) | 73 (69%) |
| BMI (kg/m$^2$) | 28.2 ± 3.8 |
| Overweight | 59 (56%) |
| Obese | 29 (27%) |
| Hypertension | 76 (72%) |
| Manifest cardiovascular disease | 7 (7.0%) |
| Hypertriglyceridaemia | 56 (53%) |
| ALT (U/L) | 80 ± 45 |
| AST (U/L) | 46 ± 23 |
| AST/ALT ratio | 0.6 ± 0.2 |
| ALP (U/L) | 58 ± 30 |
| Bilirubin (mg/dL) | 0.64 ± 0.31 |
| Albumin (g/dL) | 4.1 ± 0.3 |
| Platelet count (x 10$^9$/L) | 227 ± 61 |
| Prothrombin (INR) | 1.0 ± 0.1 |
| Ferritin (µg/L) | 246 ± 346 |
| Triglycerides (mg/dL) | 183 ± 124 |
| Cholesterol (mg/dL) | 231 ± 51 |
| SPC (%) | 13.0 ± 10.2 |
| Steatosis grade | |
| 0 | 3 (1-3) |
| 1 | 0 (0%) |
| 2 | 24 (23%) |
| 3 | 23 (22%) |
| 4 | 59 (56%) |
| Lobular inflammation | |
| 0 | 0 (0-1) |
| 1 | 97 (91.5%) |
| 2 | 9 (8.5%) |
| 3 | 0 (0%) |
| Ballooning | |
| 0 | 0 (0-2) |
| 1 | 97 (91.5%) |
| 2 | 8 (7.5%) |
| 3 | 1 (0.9%) |
| Fibrosis stage | |
| 0 | 0.5 (0-4) |
| 1 | 53 (50%) |
| 2 | 28 (26%) |
| 3 | 14 (13%) |
| 4 | 9 (8.5%) |
| 2 | 2 (1.9%) |

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; INR, international normalized ratio; SPC, stereological point counting.
2.6 | Ethical consideration

All participating subjects gave written informed consent. The study design was approved by the Regional Ethical Review Board in Linköping, Sweden (02-454, amendments: 2011/468-32, 2012/229-32 and 2013/72-32).

3 | RESULTS

3.1 | Study population and clinical characteristics

At inclusion, 14 patients (10.9%) had T2DM and were excluded from further evaluation in this study. Moreover, nine patients (7.0%) were excluded because of missing quantitative liver fat measurement with SPC at baseline. Thus, at inclusion 106 patients had complete clinical, biochemical and histopathological data for final analysis. Mean age at inclusion was 49.3 years (±12.9) and most patients were male (n = 73, 69%). Patients were in general overweight with a mean BMI of 28.2 kg/m$^2$ (±3.8). Clinical and biochemical data of the study cohort at inclusion are presented in Table 1. At first follow-up, 104 patients were alive, of whom 88 (85%) accepted re-evaluation. At second follow-up, 79 patients were alive of whom 59 (75%) accepted re-evaluation (Figure 1). At first and second follow-up, 59 and 32 patients underwent a second and third biopsy respectively.

Mean follow-up time until development of T2DM, death or end of study was 17.5 years (±6.0; range 0-28) or 1859 person-years of follow-up. During this period 66 patients developed T2DM, corresponding to an incidence rate of 40 cases per 1000 person-years. The unadjusted cumulative incidence of T2DM after 5, 10 and 20 years was 11.3%, 31.1% and 50.0% respectively. Mean follow-up time until death or end of study was 23.2 years (±6.8; range 2-30) or 2457 person-years of follow-up. During follow-up, there were 40 deaths in total; 24 among the 66 patients that developed T2DM (36.4%) and 16 among patients without T2DM (40.0%). Of the 40 patients that died, 16 patients died from cardiovascular disease (40%), 11 from extrahepatic malignancies (27.5%), 5 from infectious diseases (12.5%), 4 from end-stage liver disease, including hepatocellular carcinoma (10%), 2 from kidney disease (5%), 1 from respiratory disease (2.5%) and 1 from neurological disease (2.5%). Development of T2DM was not associated with an increased risk of death \(P = .836\).

3.2 | Predicting the risk of developing T2DM in NAFLD patients

During a mean follow-up of 17.5 years (±6.0; range 0-28), 66 patients developed T2DM. The 66 patients who developed T2DM were compared to the 40 patients who did not develop T2DM during follow-up. Those that developed T2DM had higher baseline BMI \((28.8 ± 3.8 \text{ vs } 27.2 ± 3.5 \text{ kg/m}^2, P = .032)\) and higher SPC \((14.4 ± 10.2 \text{ vs } 10.6 ± 10.0\%, P = .043)\). There was a significant difference in follow-up time between steatosis grade 1 \((19.3 ± 1.9\text{ years}), 2 (18.6 ± 1.7\text{ years})\) and 3 \((13.1 ± 1.0\text{ years}) (P = .002)\). There were no other biochemical, clinical or histopathological differences between the two groups (Table 2).

Statistically significant univariate hazard ratios for development of T2DM were found for age, BMI, steatosis grade 3, quantitative liver fat measured with SPC and fibrosis stages 2 and 4. Steatosis grade 3 and quantitative liver fat measured with SPC were independently (adjusted for age, BMI, fibrosis stage) associated with development of T2DM (Table 3). Univariate and multivariate hazard ratios for stepwise increase in steatosis grade, quantitative liver fat measured with SPC, lobular inflammation, ballooning and fibrosis stage were calculated (Table 3). For each SPC percentage increase at baseline the risk for developing T2DM during follow-up increased with an adjusted hazard ratio (aHR) of 1.03 (95% confidence interval [CI] 1.00-1.05 \(P = .02\)). Furthermore, an SPC threshold of 5.2% was associated with a significantly increased risk of developing T2DM (cHR 1.88, 95% CI 1.07-3.31, \(P = .02\)), with a sensitivity and specificity of 76% and 45%, respectively. Moreover, a stepwise increase in steatosis grade showed an increased aHR of 1.60 (95% CI 1.13-2.28, \(P < .01\)).

Fifty-nine patients underwent repeat liver biopsy at first follow-up with a mean interval between biopsies of 13.9 years (±1.2).
TABLE 2  Comparison of baseline data in patients with and without T2DM at follow-up

|                        | With T2DM (n = 66) | Without T2DM (n = 40) | P value |
|------------------------|--------------------|-----------------------|---------|
| Age (y)                | 48.8 ± 11.6       | 50.0 ± 14.8           | .643    |
| Gender (male)          | 44 (67%)          | 29 (73%)              | .666    |
| BMI (kg/m²)            | 28.8 ± 3.8        | 27.2 ± 3.5            | .032    |
| Overweight             | 35 (53%)          | 24 (60%)              | .548    |
| Obese                  | 22 (33%)          | 7 (18%)               | .115    |
| Hypertension           | 51 (77%)          | 25 (63%)              | .683    |
| Manifest CVD           | 4 (6.1%)          | 3 (7.5%)              | 1.000   |
| Hypertriglyceridaemia  | 37 (56%)          | 19 (48%)              | .223    |
| ALT (U/L)              | 83 ± 50           | 75 ± 35               | .323    |
| AST (U/L)              | 46 ± 26           | 45 ± 19               | .829    |
| AST/ALT ratio          | 0.6 ± 0.1         | 0.6 ± 0.2             | .051    |
| ALP (U/L)              | 54 ± 24           | 65 ± 36               | .071    |
| Bilirubin (mg/dL)      | 0.66 ± 0.35       | 0.61 ± 0.22           | .387    |
| Albumin (g/dL)         | 4.1 ± 0.3         | 4.1 ± 0.4             | .903    |
| Platelet count (×10⁹/L) | 222 ± 61        | 236 ± 60              | .299    |
| Prothrombin (INR)      | 1.0 ± 0.1         | 1.0 ± 0.1             | .981    |
| Ferritin (µg/L)        | 257 ± 393         | 228 ± 253             | .671    |
| Triglycerides (mg/dL)  | 191 ± 140         | 169 ± 92              | .380    |
| Cholesterol (mg/dL)    | 231 ± 54          | 232 ± 46              | .898    |
| SPC (%)                | 14.4 ± 10.2       | 10.6 ± 10.0           | .043    |
| Steatosis grade        |                    |                       |         |
| 0                      | 3 (1-3)           | 2 (1-3)               | .104    |
| 1                      | 0 (0%)            | 0 (0%)                |         |
| 2                      | 12 (18.2%)        | 12 (30.0%)            |         |
| 3                      | 12 (18.2%)        | 11 (27.5%)            |         |
| Lobular inflammation   | 0 (0-1)           | 0 (0-1)               | .251    |
| Ballooning             | 0 (0-2)           | 0 (0-2)               | .312    |
| Fibrosis stage         | 1 (0-4)           | 0 (0-4)               | .204    |

Note: 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 chi-squared (χ²) test. Numbers in bold are significant. Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CVD, cardiovascular disease; INR, international normalized ratio; SPC, stereological point counting.

Among these, 31 (52.5%) exhibited reduction and 28 (47.5%) exhibited increase in quantitative liver fat measured with SPC. Patients that had reduced the amount of quantitative liver fat, between baseline and first follow-up, had significantly lower risk of developing T2DM in a univariate hazard ratio (HR 0.91, 95% CI 0.85-0.97, P = .002). When adjusted for gender, difference in BMI and presence of progressive fibrosis the aHR was 0.84 (95% CI 0.72-0.99, P = .039). Moreover, patients that had increased amount of quantitative liver fat measured with SPC, between baseline and first follow-up, did not have a significantly increased risk of developing T2DM (aHR 1.03, 95% CI 0.93-1.13, P = .62 and aHR 1.09, 95% CI 0.96-1.23, P = .19). Complete clinical, biochemical and histological data at inclusion and first follow-up are shown in Table S1. Twenty-nine patients (27%) underwent three consecutive biopsies. No statistical calculations were made based on these biopsies because of the low number and subsequently insecure statistical calculations.

3.3  Steatosis as a predictor of mortality and development of T2DM

Of the 106 patients included at baseline, 24 patients had steatosis grade 1 (SPC 1.1 ± 1.3%), 23 patients had steatosis grade 2 (SPC 8.1 ± 4.8%) and 59 patients had steatosis grade 3 (SPC 19.7 ± 8.1%). The Pearson's and Spearman's correlation between steatosis grade and SPC was 0.77 and 0.83, respectively (P < .0001). During a mean follow-up of 23.2 years (±6.8; range 2-30), there were 40 deaths in total. There was no significant difference in follow-up time between steatosis grade 1 (24.3 ± 6.2 years), 2 (24.8 ± 5.7 years) and 3 (22.1 ± 7.4 years) (P = .194). Furthermore, there was no significant difference in baseline steatosis grade or SPC regarding the different causes of mortality (Table S2). Hypertriglyceridaemia and BMI were significantly associated with baseline steatosis grade (P = .01 and P = .016 respectively). Mortality was 21%, 22% and 41% in patients with steatosis grade 1, 2, and 3, respectively. Kaplan-Meier survival estimate showed decreased survival rate and increased risk for development of T2DM in patients with grade 3 steatosis compared to steatosis grade 1 and 2 individually (Figures 2 and 3). Patients with higher grade of steatosis had significantly higher BMI, higher frequency of hypertriglyceridaemia and higher SPC value. Although there was no difference in fibrosis stage between the groups (P = .112), patients with steatosis grade 3 had more often fibrosis stage ≥1 compared to mild/moderate (ie ≤2) steatosis grade (61% vs 36%, P = .002) (Table S3). No other clinically significant differences were observed.

Univariate and multivariate hazard ratios were calculated for overall mortality. Statistically significant univariate hazard ratios for overall mortality was observed for gender, age, steatosis grade, lobular inflammation, ballooning and fibrosis stage. However, after adjusting for gender and age, only liver fat measured with SPC was significant for overall mortality (aHR 1.04, 95% CI 1.00-1.07, P = .03). Furthermore, after adjusting for fibrosis stage, liver fat measured with SPC remained significant (aHR 1.04, 95% CI 1.00-1.07, P = .04) (Table 4). Moreover, an SPC threshold of 7.6% was associated with a significantly increased risk of all-cause mortality (CHR 1.72, 95% CI 1.00-2.95, P < .05), with a sensitivity and specificity of 71% and 60%, respectively.

4  DISCUSSION

This is the first study investigating the predictive value of quantitative liver fat on the risk of developing T2DM in biopsy proven
NAFLD. The main finding was that a stepwise increased amount of liver fat, expressed as percentage of steatotic area in liver biopsy, predicted an increased risk of developing T2DM (aHR 1.03 per 1% increase, 95% CI 1.00-1.05, \( P = .02 \)). In addition, a reduction in liver fat percentage between baseline and first follow-up was associated with a decreased risk of developing T2DM (aHR 0.91 per 1% reduction, 95% CI 0.85-0.98). Moreover, semiquantitative assessment of liver fat content by a histopathologist using steatosis grade was independently (adjusted for age, BMI, fibrosis stage) associated with development of T2DM (aHR 1.60 per 1 grade increase, 95% CI 1.13-2.28).

Patients with steatosis grade 3, had an increased overall mortality and increased risk of developing T2DM compared to steatosis grade 1 and 2. Albeit there was a significant difference in fibrosis stage between low grade steatosis (grades 1 and 2) and high grade steatosis (grade 3), this was mainly attributed to more patients having lower stages of fibrosis (stage 1 and 2) in those with grade 3 steatosis (Table S3). However, multivariate analysis showed that only liver fat measured with SPC predicted overall mortality after adjusting for gender and age. The increased risk was present after adjusting for fibrosis stage (aHR 1.04 per 1% increase, 95% CI 1.00-1.07, \( P = .04 \)).

There is a high association between NAFLD and T2DM, which was shown in a meta-analysis by Morrison et al, where the pooled relative risk of T2DM in NAFLD patients compared to those without, was more than two-fold (RR 2.17, 95% CI 1.77-2.65). Furthermore, in our study, we showed an incidence rate of T2DM of 40 cases per 1000 person-years, which is more than 13 times higher than that of the general nationwide population in Sweden during 2013 (ie 3.04 cases per 1000 person-years). However, the incidence rate of our study should be interpreted with caution, because of the lack of values for glucose and HbA1c at baseline. In the absence of these markers, it is hard to draw any causal conclusions because of the strong collinearity between T2DM and NAFLD. Albeit, the incidence rate seen in our study is higher than that seen in the Swedish population, it is lower than that seen in patients with impaired glucose tolerance or increased (IGT) levels of HbA1c (86 and 93.8 cases per 1000 person-years respectively).

Similar to our findings, Park et al reported an aHR of 1.30-1.64 for development of T2DM over a 5-year period in NAFLD patients diagnosed with ultrasonography. Likewise, in a study by Zelber-Sagi et al, where 141 NAFLD patients diagnosed with ultrasonography were followed for 7 years, the risk of incident prediabetes was independently increased (aOR 2.93, 95% CI 1.02-8.41). These data are in accordance with the study by Dongiovanni et al, where hepatic steatosis was associated with higher IR HOMA-2 indicating insulin resistance.

A main finding in our study was that a reduction in liver fat percentage was associated with a decreased risk of developing T2DM.

| Variable                  | Model 1a | Model 2b | Model 3c |
|---------------------------|----------|----------|----------|
|                           | cHR      | 95% CI   | P value  |
| Gender (male)             | 1.40     | 0.83-2.35| .20      |
| Age (y)                   | 1.02     | 1.00-1.04| .02      |
| BMI (kg/m²)               | 1.09     | 1.02-1.16| <.01     |
| Steatosis grade (0-3)     | 1.58     | 1.14-2.20| <.01     |
| 1 (ref)                   | 1.08     | 0.48-2.40| .85      |
| 2                         | 2.26     | 1.18-4.34| <.02     |
| SPC (%)                   | 1.03     | 1.00-1.05| <.02     |
| Lobular inflam. (0-3)     | 0.78     | 0.28-2.13| .62      |
| Ballooning (0-2)          | 2.07     | 1.04-4.14| .04      |
| Fibrosis stage (0-4)      | 1.28     | 1.03-1.58| <.01     |
| 0 (ref)                   | 1.11     | 0.59-2.08| .75      |
| 2                         | 2.11     | 1.05-4.24| <.04     |
| 3                         | 1.46     | 0.61-3.51| .40      |
| 4                         | 12.0     | 3.43-43.3| <.01     |

Note: Numbers in bold are significant.
Abbreviations: BMI, body mass index; SPC, stereotological point counting.
Unadjusted estimates.
Adjusted for age and BMI.
Adjusted for age, BMI, and fibrosis stage.
No patients had lobular inflammation grade >1.
1 patient had ballooning grade 2.
Similarly, in a recent study by Yamazaki and colleagues, hepatic steatosis resolution during a 11.3-year follow-up showed a reduction in incident T2DM (aOR 0.27, 95% CI 0.12-0.61).\textsuperscript{27} Albeit this study was performed in a large cohort, it is retrospective and used ultrasonography for the diagnosis of NAFLD—a method with difficulties in detecting low levels of hepatic steatosis and with highly variable accuracy.\textsuperscript{28}

In a recent retrospective study by Björkström et al\textsuperscript{29} 396 patients with biopsy-proven NAFLD were followed for a mean time of 18.2 years. During follow-up 132 patients developed T2DM (incidence rate, 18 cases per 1000 person-years). The authors reported a 34% increased hazard per increase in steatosis grade in patients with fibrosis stage 0-2 (aHR 1.34, 95% CI 1.03-1.74), which is in agreement with our results (aHR 1.60, 95% CI 1.13-2.28). However, by measuring quantitative liver fat with SPC we extend these findings by reporting an aHR for each percentage increase in liver fat. Compared with the study of Björkström et al we report a two-time higher incidence rate of T2DM. This could be attributed to the prospective approach of our study and the more rigorous assessment of T2DM where we at first and second follow-up performed OGTT on all patients who did not receive antidiabetic treatment. This resulted in a de novo diagnosis of T2DM during first and second follow-up in 17 and 14 patients respectively.\textsuperscript{7,21} Nevertheless, our higher incidence rate could also be attributed to some patients having prediabetes at baseline. However, as shown in the Whitehall II Study by Tabák et al, changes in glucose concentration and insulin sensitivity was observed 3 to 6 years before the diagnosis of T2DM.\textsuperscript{30} In our study, only 10 of 66 patients (15%) had been diagnosed with T2DM after 6 years of follow-up. Moreover, in studies adjusting for baseline T2DM surrogate markers (including fasting plasma glucose, hepatic steatosis, independently associates with future development of prediabetes or manifest T2DM.\textsuperscript{8,25,29}

It has been proposed that quantitative assessment of liver fat by MRI-PDFF should be used as an endpoint in NAFLD/NASH trials\textsuperscript{31}
because of the emerging evidence of hepatic steatosis as a driver in NAFLD pathogenesis. Patel et al.\(^{33}\) used paired MRI-PDFF to demonstrate that a reduction in 29% in liver fat was associated with a histological response in NASH. Moreover, Ajmera et al.\(^{34}\) showed that patients without fibrosis but with higher percentage of fat measured by MRI-PDFF had a higher risk of fibrosis progression, a finding corroborated by Dongiovanni et al, who showed a causal relationship between hepatic fat and fibrosis independent of necroinflammation and ballooning.\(^{26}\)

However, no study has investigated the association of quantitative liver fat measured by MRI-PDFF and its effect on the development of T2DM. Although SPC and MR techniques are measuring fundamentally different properties of tissue, the correlation of hepatic fat content measured with SPC and \(^1\)H-MRS is excellent.\(^{20}\) Thus, most likely non-invasive measurement of HTGC with MR techniques in NAFLD patients can be used to assess the risk of development of T2DM in prospective cohort studies or pharmaceutical trials.

The results of our study also indicate the need for future prospective studies using MR techniques, assessing if increased amounts of HTGC entails an augmented risk of developing T2DM in NAFLD. In such case, there is a need to evaluate if pharmacological treatment of high-risk NAFLD patients could decrease the risk of developing T2DM in analogy with treatment to prevent T2DM in IGT.\(^{35-39}\)

The strength of our study is the unique cohort, which is prospectively enrolled and rigorously followed for over 20 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 for liver biopsy. Although, inclusion took place in two tertiary centres, the low frequency of NASH as well as the low mean BMI in the cohort strengthens the validity of our outcome. Also, owing to our medical chart archives and electronic medical chart systems, no patient was lost to follow-up.

This study has some limitations. Mainly, as with all studies using liver biopsy, sampling error is a possibility.\(^{40}\) Moreover, at baseline (1988-1993) we did not have data on waist or hip circumference, surrogate markers of insulin resistance (including fasting plasma glucose levels or HbA1c) nor data on physical activity and family history of diabetes, which is an important limitation. In addition, although many patients accepted re-evaluation, the high frequency of death because of the extended follow-up resulted in stagnating number of patients to follow-up as time progressed, reducing the number of patients undergoing repeat biopsies. Also, the relatively low number of patients (n = 106) makes it difficult to draw conclusions, especially when performing multivariate regression models. Finally, cardiovascular disease was the main cause of mortality in this study (40%), which it commonly is in

### TABLE 4 Univariate and multivariate hazard ratios for overall mortality in NAFLD patients

| Variable                  | Model 1\(^a\) | Model 2\(^b\) | Model 3\(^c\) |
|---------------------------|---------------|---------------|---------------|
|                           | cHR 95% CI    | aHR 95% CI    | aHR 95% CI    |
| Gender (male)             | 0.49 0.26-0.91 | 1.05 0.97-1.14 | 1.05 0.96-1.14 |
| Age (y)                   | 1.13 1.09-1.17 | 1.50 0.92-2.44 | 1.41 0.87-2.29 |
| BMI (kg/m\(^2\))          | 1.04 0.96-1.13 | 1.05 0.92-2.44 | 1.05 0.92-2.44 |
| Steatosis grade (0-3)     | 1.78 1.11-2.80 | 1.50 0.92-2.44 | 1.50 0.92-2.44 |
| 1                         | (ref)         | (ref)         | (ref)         |
| 2                         | 1.20 0.37-3.93 | 0.59 0.17-2.10 | 0.65 0.18-2.31 |
| 3                         | 2.48 1.24-4.99 | 1.96 0.97-3.96 | 1.82 0.89-3.72 |
| SPC (%)                   | 1.03 1.00-1.06 | 1.04 1.00-1.07 | 1.04 1.00-1.07 |
| Lobular inflam. (0-3)\(^d\)| 2.71 1.34-6.48 | 1.37 0.56-3.33 | 1.27 0.52-3.13 |
| Ballooning (0-2)\(^e\)    | 1.57 0.76-3.24 | 0.73 0.35-1.50 | 0.52 0.24-1.15 |
| Fibrosis stage (0-4)      | 1.73 1.34-2.23 | 1.26 0.95-1.68 | 1.26 0.95-1.68 |
| 0                         | (ref)         | (ref)         | (ref)         |
| 1                         | 2.61 1.17-5.82 | 1.77 0.79-3.98 | 1.77 0.79-3.98 |
| 2                         | 2.44 1.08-5.49 | 1.24 0.51-3.02 | 1.24 0.51-3.02 |
| 3                         | 2.20 0.92-5.30 | 1.43 0.53-3.23 | 1.43 0.53-3.23 |
| 4                         | 4.36 1.02-18.53| 2.57 0.54-12.14| 2.57 0.54-12.14|

Note: Numbers in bold are significant.

Abbreviations: BMI, body mass index; SPC, stereological point counting.

\(^a\)Unadjusted estimates.

\(^b\)Adjusted for gender and age.

\(^c\)Adjusted for gender, age and fibrosis stage.

\(^d\)No patients had lobular inflammation grade > 1.

\(^e\)1 patient had ballooning grade 2.
observational NAFLD studies, therefore it is seen as a competing risk. Originally, Kaplan-Meier survival analysis and Cox proportional hazard regression model were developed to describe all-cause mortality, rather than incident disease (i.e. T2DM). Therefore, the use of Kaplan-Meier or Cox regression in prediction of incident disease may lead to biased results because of death as a competing risk factors.

In conclusion, we have shown that quantitative and semi-quantitative measurement of hepatic fat in NAFLD patients can be used to assess the risk of overall mortality and future development of T2DM. We have also shown that reduction in liver fat decreases the risk of developing T2DM. Our findings also suggest that quantitative measurement and monitoring of liver fat by biopsy or imaging may be of clinical importance for surveillance of NAFLD patients assessing their risk of T2DM.

CONFLICT OF INTEREST
The authors do not have any disclosures to report.

AUTHOR CONTRIBUTIONS
Conceptualization: PN, ME, SK. Data curation: PN. Formal analysis: PN, MF. Funding acquisition: ME, SK. Investigation: PN, ME, SK. Methodology: PN, ME, SK. Project administration: PN, ME, SK. Resources: PN, MF. Supervision: ME, SK. Validation: PN, MF. Visualization: PN. Writing – original draft: PN. Writing – review & editing: PN, ME, SK. Study conception and design: PN, MF. ME, SK. Methodology: PN, ME, SK. Project administration: PN, ME, SK. Funding acquisition: ME, SK. Investigation: PN, ME, SK. Conceptualization: PN, ME, SK. Data curation: PN. Formal analysis: PN. Writing – original draft: PN. Writing – review & editing: PN, ME, SK. Guarantor of the article: Stergios Kechagias.

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Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Nasr P, Fredrikson M, Ekstedt M, Kechagias S. The amount of liver fat predicts mortality and development of type 2 diabetes in non-alcoholic fatty liver disease. Liver Int. 2020;00:1-10. https://doi.org/10.1111/liv.14414