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Prognostic value of liver fibrosis and steatosis biomarkers in type-2 diabetes and dyslipidaemia

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SUMMARY

Background
In cardiometabolic disorders, non-alcoholic fatty liver disease is frequent and presumably associated with increased mortality and cardiovascular risk.

Aim
To evaluate the prognostic value of non-invasive biomarkers of liver fibrosis (FibroTest) and steatosis (SteatoTest) in patients with type-2 diabetes and/or dyslipidaemia.

Methods
A total of 2312 patients with type-2 diabetes and/or dyslipidaemia were included and prospectively followed up for 5–15 years. The cardiovascular Framingham-risk score was calculated; advanced fibrosis and severe steatosis, were defined by FibroTest >0.48 and SteatoTest >0.69, respectively, as previously established.

Results
During a median follow-up of 12 years, 172 patients (7.4%) died. The leading causes of mortality were cancer (31%) and cardiovascular-related death (20%). The presence of advanced fibrosis [HR (95% CI)] [2.98 (95% CI 1.78–4.99); $P < 0.0001$] or severe steatosis [1.86 (1.34–2.58); $P = 0.0002$] was associated with an increased risk of mortality. In a multivariate Cox model adjusted for confounders: the presence of advanced fibrosis was associated with overall mortality [1.95 (1.12–3.41); $P = 0.02$]; advanced fibrosis at baseline [$n = 50/677$; 1.92 (1.04–3.55); $P = 0.04$] and progression to advanced fibrosis during follow-up [$n = 16/127$; 4.8 (1.5–14.9); $P = 0.007$] were predictors of cardiovascular events in patients with type-2 diabetes. In patients with a Framingham-risk score ≥20%, the presence of advanced fibrosis was predictive of cardiovascular events [2.24 (1.16–4.33); $P < 0.05$].

Conclusions
Liver biomarkers, such as FibroTest and SteatoTest, have prognostic values in patients with metabolic disorders. FibroTest has prognostic value for predicting overall survival in patients with type-2 diabetes and/or dyslipidaemia. In type-2 diabetes, FibroTest predicted cardiovascular events and improved the Framingham-risk score.

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INTRODUCTION

Subjects with metabolic disorders such as type-2 diabetes or dyslipidaemia, have a high risk of both cardiovascular disease and non-alcoholic fatty liver disease (NAFLD). NAFLD begins with steatosis, i.e. fat accumulation in hepatocytes, and can progress to liver fibrosis, cirrhosis and/or hepatocellular carcinoma, which are associated with life-threatening complications.[1] Evidence has accumulated to indicate that, in patients with metabolic risk factors, NAFLD by itself was associated with an increased prevalence of cardiovascular disease and overall mortality.[2]

Historically, the severity of liver diseases has been assessed by liver biopsy. However, the usefulness of liver biopsy has been challenged by limited feasibility, adverse effects, sampling error and interobserver variability. Non-invasive markers have been proposed to replace liver biopsy. FibroTest, one of the most widely used markers of liver fibrosis, has demonstrated a prognostic value for the prediction of liver-related death in chronic hepatitis B and C, and in alcoholic liver disease.[3–6] FibroTest is equally performant as liver biopsy for the prediction of overall mortality in these different diseases.[7] Another marker, SteatoTest, has been validated as a marker of hepatic steatosis.[8, 9] So far, the prognostic values of FibroTest and SteatoTest have not been evaluated in NAFLD or metabolic-related disorders. The strong links between hepatic and cardiovascular diseases in patients with metabolic disorders led us to postulate that such markers would have a global prognostic value in these populations.

The primary aim of this study was to evaluate the 10-year prognostic value of FibroTest and SteatoTest, for overall survival, survival without liver-related death, and survival without cardiovascular-related death, in patients with type 2-diabetes and/or dyslipidaemia. The secondary aim was specifically addressed in patients with type 2-diabetes, and was to determine whether combining the Framingham-risk score, which is based on classical cardiometabolic risk factors, with FibroTest, would improve the 5-year prediction of cardiovascular events, and if the progression of liver fibrosis based on this test, was associated with a higher incidence of cardiovascular events.

PATIENTS AND METHODS

Study design

A longitudinal observational study was conducted at the Institute of Cardiometabolism and Nutrition (ICAN), Pitié-Salpêtrière Hospital, Paris, France. Patients from two cohorts, initially diagnosed either with dyslipidaemia[10] (cohort 1 from the Dyslipidaemia Department, $n = 1916$) or with type-2 diabetes[11] (cohort 2 from the Diabetology Department, $n = 747$), followed from January 1999 to December 2012, were eligible to participate ($n = 2663$ in total). Dyslipidaemia was defined as plasma low-density lipoprotein (LDL)-cholesterol levels above 4.1 mmol/L (160 mg/dL) or triglyceride levels above 1.70 mmol/L (150 mg/dL). Diabetes was defined by fasting glucose ≥7.0 mmol/L (126 mg/dL) or 2-h post-prandial glucose ≥11.1 mmol/L (200 mg/dL). The exclusion criteria were: liver disease other than NAFLD, the absence of follow-up and missing data.

The study protocol was conducted in accordance with the Helsinki Declaration, and was approved by the local Ethics Committee. All patients signed an informed consent upon enrolment in the study. The cohorts belong to FIBROFRANCE, a program organised in 1997 to assess the burden of chronic liver diseases in France (Clinical trial French registry no.: DRCD-2013-1 and ClinicalTrials·org no.: NCT01927133).

Evaluation of cardiometabolic risk factors

Clinical records included the measures of body mass index (BMI), waist circumference, blood pressure (measured by electronic Dinamap Pro 300; General Electric Healthcare, Little Chalfont, UK), and the reports on alcohol consumption (quantified in g/day during the past 2 years) and smoking (quantified in pack-years). Excessive alcohol intake was defined as more than 30 g/day in men and 20 g/day in women.[12] Venous blood samples were collected after 12-h overnight fasting. Plasma levels of cholesterol and triglycerides were determined by enzymatic methods (Kone Lab, Thermoclinical Labsystems, Cergy Pontoise, France and Biomerieux, Marcy L'Etoile, France), and high-density lipoprotein (HDL)-cholesterol by an enzymatic procedure after phosphotungstic acid/magnesium chloride precipitation. Serum glucose, alanine transaminase (ALT), aspartate transaminase (AST), gamma-glutamyltransferase (GGT) and total bilirubin were measured by a Hitachi 917 Analyzer or Modular DP Analyzers (Roche Diagnostics, Mannheim, Germany). Metabolic syndrome was defined according to the International Diabetes Federation criteria.[13] The Framingham-risk score was calculated including gender, age, blood pressure, total cholesterol, HDL-cholesterol, smoking and diabetes history.[14] Patients with a Framingham-risk score <20% and ≥20% were classified into a low-to-moderate and high risk of cardiovascular disease, respectively.

Non-invasive liver biomarkers

FibroTest, ActiTest and SteatoTest (BioPredictive Paris, France; FibroSURE LabCorp Burlington, NC, USA) are algorithms including the serum concentrations of α2-macroglobulin, apolipoprotein A1, haptoglobin, total bilirubin and GGT, adjusted for age and gender. In addition to these five components, SteatoTest includes the serum concentrations of ALT, fasting glucose, triglycerides and cholesterol, adjusted for age, gender and BMI, while ActiTest, a biomarker of hepatic necrotic/inflammatory lesions, includes FibroTest components plus ALT. Alpha-2-macroglobulin, apolipoprotein A1 and haptoglobin were measured using an automatic nephelometer BNII (Dade Behring, Marburg, Germany).

The recommended pre-analytical and analytical procedures were applied.[15] The scores of these biomarkers range from 0 to 1.00, the highest scores being attributed to the most severe lesions. We previously established that advanced fibrosis (F2F3F4 according to the METAVIR histological scoring system)[16] could be determined by a score of FibroTest >0.48[17] and severe steatosis (>32% of hepatocytes, i.e. approximately S2S3 according to the SAF histological scoring system),[18, 19] by SteatoTest >0.69.[8]

Follow-up and outcomes

Patients attended the Dyslipidaemia (cohort 1) or Diabetology Department (cohort 2), and the Hepatology Department when justified by abnormal liver tests, at least once a year. The duration of follow-up was calculated from the baseline date, defined as the date when the serum used for the first analyses of liver biomarkers was collected, to the date a non-lethal or lethal event occurred. This interval was censored at the time of last follow-up. If several events occurred, the first one was taken into account.

The mortality rate and incidence of cardiovascular events were determined during follow-up. The causes of death were collected from the French national registry (CépiDc Inserm), according to the 10th International Classification of Diseases (http://apps.who.int/classifications/icd10/browse/2010). The codes considered as diagnosis of cardiovascular-related death were: ischaemic heart diseases (I20–I25), cardiac arrest (I46), heart failure (I50), cerebrovascular diseases (I63 and I64) and cardiogenic shock (R57.0). The codes for liver-related death were: liver fibrosis and cirrhosis of liver (K74), non-alcoholic steatohepatitis (K75.8), portal hypertension (K76.6), oesophageal varices bleeding (I85.0), hepatocellular carcinoma (C22.0) and cholangiocarcinoma (C22.1). Death coded as R99 (other ill-defined and unspecified causes of mortality) was considered as unknown cause of death. The survival curve of the French population was calculated on the basis of age, gender, follow-up period and conditional probabilities of death from official published census tables.[20]

Patients with type-2 diabetes from cohort 2 were evaluated for cardiovascular events, i.e. myocardial infarction, unstable angina, coronary by-pass (either angioplasty or surgical) and stroke. Information on treatments and cardiovascular events was collected by a trained investigator (HP) based on an extensive review of the medical records, an interview with patients and their primary care physicians. In addition, the coronary angiography database of the Cardiology Department was manually screened to identify interventional procedures. The analysis of liver biomarkers was repeated in these patients, during follow-up.

Statistical analysis

Continuous variables are reported as mean ± standard deviation if normally distributed or as median (range) if not. Discrete variables are reported as absolute and relative frequency. The χ^2 or Fisher's exact tests were used for qualitative comparisons. The analysis of variance, Kruskal–Wallis, Student's t or Mann–Whitney tests were used for quantitative comparisons. The Kaplan–Meier curves were plotted and the log-rank test was calculated for univariate analysis. We used the time to event Cox proportional-hazard model for uni- and multivariate analyses after checking that the main variables verified the proportional-hazard assumption using the Schoenfeld residuals. Model 1 for prediction of mortality was adjusted for age, gender, metabolic factors, tobacco and alcohol consumption, as well as for haemoglobin A1c (HbA1c) in type-2 diabetic patients. Model 2 for prediction of mortality in type-2 diabetic patients was adjusted as Model 1 plus treatment factors. The model for prediction of cardiovascular events was also adjusted for the Framingham-risk score and HbA1c in Model 3 and also for treatment in Model 4. Significance level was determined when $P \leq 0.05$ assuming two-tailed tests. Analysis was performed using Number Cruncher Statistical Systems 2008 (NCSS, Kaysville, UT, USA) and STATA package 2012 (StataCorp LP, College Station, TX, USA)

RESULTS

Among 2663 eligible patients, 2312 patients with type-2 diabetes and/or dyslipidaemia, i.e. 1635 patients from cohort 1 and 677 patients from cohort 2, were included (Figure 1). The entire population (52% male; mean age

of 55 years; 36% with metabolic syndrome; mean BMI of 27.2 kg/m^2) comprised 1401 dyslipidaemic patients without type 2 diabetes and 911 patients with type-2 diabetes including 644 with and 267 without dyslipidaemia (Figure 1; Table 1). Patients with advanced fibrosis (F2F3F4 METAVIR), estimated by FibroTest (>0.48), were older, had higher BMI, higher fasting glucose and triglycerides levels, and more metabolic risk factors compared to those with no advanced fibrosis (Table 1). Among patients with type-2 diabetes, 37% were treated with insulin and 64% with statins or fibrates for associated dyslipidaemia. Median HbA1c in these patients was 8.1% (4.6–24.0), with levels <7.0% defining well-controlled disease, and ≥9.5% defining decompensated disease, in 21% and 25% of the cases, respectively. Patients having both dyslipidaemia and type 2 diabetes displayed the highest rates of advanced fibrosis and of severe steatosis (Table S1).

Mortality

During a median follow-up of 12.2 (0.1–14.5) years, 172 (7.4%) patients died. Nonliver cancer (31.2%) was the leading cause of death followed by cardiovascular-related death (20.2%). Liver-related death accounted for 4.1% of all deaths (Table 2). In patients with type-2 diabetes, with or without dyslipidaemia (n = 911 in total), cardiovascular disease was the leading cause of death (27.4%) (Table S2).

Patients who died compared to those who survived, were significantly older; they had higher systolic blood pressure, serum fasting glucose, HbA1c, triglyceride levels and GGT, more frequent arterial hypertension, type-2 diabetes and metabolic syndrome. Framingham-risk score, Fibrotest and SteatoTest were all higher in these patients (Table S3).

The overall mortality rate was 7.0 (95% CI 6.0–8.1) per 1000 person-year. Patients with advanced fibrosis or severe steatosis, as determined by FibroTest/SteatoTest, had an overall mortality rate of 18.9 (11.6–30.8) per 1000 person-year and 11.0 (8.4–14.6) per 1000 person-year respectively. Therefore, the presence of advanced fibrosis or severe steatosis was associated with increased risk of all-cause mortality, by threefold [relative Risk (RR) = 2.9 (95% CI 1.7–4.8); $P < 0.0001$] and twofold [RR=1.8 (1.3–2.5); $P = 0.0003$] respectively (Table S4). The presence of type-2 diabetes was also associated with an increased mortality risk [RR=1.7 (1.4–2.0); $P < 0.0001$].

Patients with advanced fibrosis had a significantly lower 10-year overall survival than those without [85% (95% CI 75–91) vs. 94% (93–95); $P < 0.0001$] (Figure 2a; Table S5). Patients with severe steatosis also had a 10-year overall survival lower than those without [89% (85–92) vs. 95% (94–96); $P = 0.0002$] (Figure 2b; Table S5). The presence of advanced fibrosis [Hazard Ratio (HR) = 2.98 (95% CI 1.78–4.99); $P < 0.0001$] or severe steatosis [HR = 1.86 (95% CI 1.34–2.58); $P = 0.0002$] was associated with an increased risk of mortality. Advanced liver fibrosis, estimated by FibroTest,

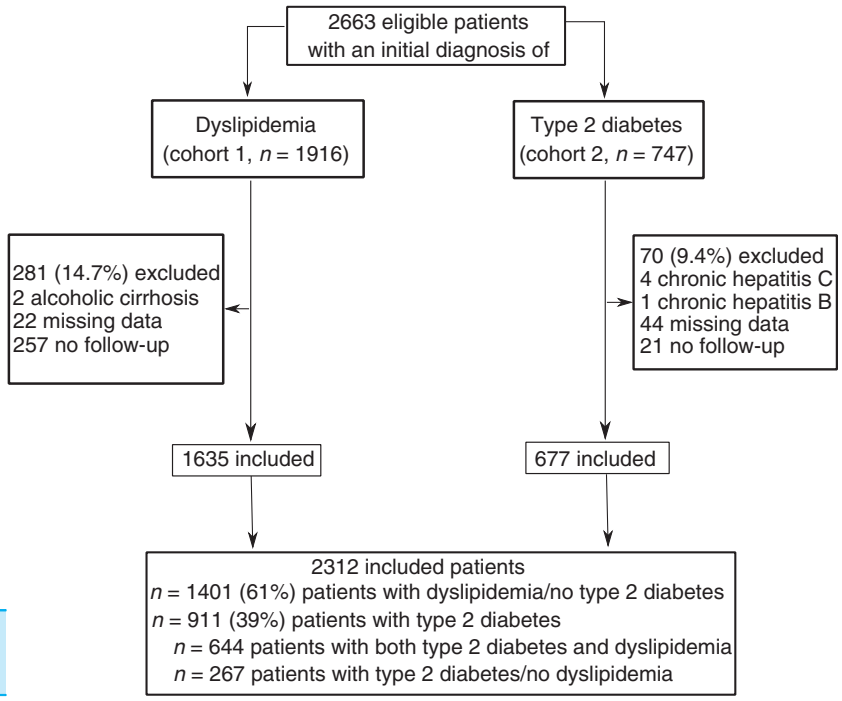

Figure 1 | Study flow chart of patient's recruitment.

Table 1 | Baseline characteristics of patients according to liver fibrosis stage estimated by FibroTest

	No advanced fibrosis (FibroTest ≤0.48) (n = 2217)	Advanced fibrosis (FibroTest >0.48) (n = 95)	P value
Age (years)*	55 ± 12	62 ± 11	<0.0001
Male gender†	1115 (50)	78 (82)	<0.0001
Follow-up, years‡	12.2 (0.1–14.5)	7.6 (1.3–13.6)	<0.0001
Liver biomarkers			
SteatoTest score*	0.47 ± 0.23	0.61 ± 0.22	<0.0001
Severe steatosis (ST>0.69)‡	434 (20)	36 (39)	<0.0001
Liver tests (U/L)			
ALT*	31 ± 20	46 ± 33	<0.0001
AST*	28 ± 15	36 ± 16	<0.0001
GGT*	39 ± 46	98 ± 125	<0.0001
Cardiometabolic risk factors			
BMI (kg/m^2)*	27.1 ± 5.3	28.5 ± 4.8	0.01
Current smoking†	935 (42)	45 (47)	0.32
Alcohol consumption (g/day)‡	0 (0–230)	0 (0–84)	0.67
Systolic blood pressure (mmHg)*	130 ± 16	133 ± 16	0.04
Diastolic blood pressure (mmHg)*	77 ± 10	76 ± 12	0.44
Arterial hypertension†	1218 (55)	69 (75)	0.0002
Total cholesterol (mmol/L)*	6.0 ± 1.5	5.6 ± 1.7	0.01
LDL-cholesterol (mmol/L)*	3.7 ± 1.3	3.2 ± 1.6	0.002
HDL-cholesterol (mmol/L)*	1.5 ± 0.4	1.2 ± 0.4	<0.0001
Triglycerides (mmol/L)*	1.6 ± 1.5	2.5 ± 2.1	<0.0001
Fasting blood glucose (mmol/L)*	6.4 ± 2.8	7.8 ± 3.8	<0.0001
Fasting insulin (microU/mL)*	9.4 ± 8.3	13.7 ± 5.2	0.002
HOMA*	2.3 ± 2.2	3.6 ± 1.8	0.002
HbA1c (%)‡	8.1 (4.6–24.0)	8.4 (6.3-13.3)	0.15
Diabetes†	846 (38)	65 (68)	<0.0001
Dyslipidaemia treatment † (statins or fibrates)	1228 (56)	56 (61)	0.33
Insulin treatment†	234 (36)	25 (50)	0.06
Metabolic syndrome†,§	683 (36)	38 (50)	0.009
Framingham-risk score (%)*	17 ± 13	31 ± 15	<0.0001

NA, not available; ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; GGT, gamma-glutamyltransferase; HbA1c, Haemoglobin A1c; HDL, high-density lipoprotein; HOMA, homoeostatic model assessment; LDL, low-density lipoprotein. Missing data (n): dyslipidaemia treatment (19); insulin treatment (215), Metabolic syndrome (311). Data expressed as: *means ± s.d.; †absolute (%) or ‡median (range). §Insulin treatment assessed only in type-2 diabetic patients (n = 911). Fasting insulin and HOMA (n = 1954).

remained an independent factors related to overall mortality in multivariate model [FibroTest > 0.48 HR = 1.95 (1.12–3.41); P = 0.02] adjusted for confounding factors (Model 1 shown in Table 3). Fasting insulin and HOMA-index were available for only 358 patients of the cohort 1 (Table 1). In this population, HOMA-index was not associated with overall survival in uni- or multivariate analyses and did not change the prognostic significance of this population which had 28 deaths including four related to cardiovascular events (Table S6).

In patients with type-2 diabetes, advanced fibrosis was also independently associated with overall mortality after adjustment for the same variables plus HbA1c [HR = 2.54 (1.18–5.44); P = 0.017]. FibroTest was also predictive of overall mortality in patients with steatosis (>5% hepatocytes) and without excessive alcohol consumption [HR = 1.97 (1.03–3.77); P = 0.04] as well as in obese patients [HR = 2.99 (1.15–7.80); P = 0.03] independently of metabolic factors. Analyses excluding loss of follow-up and the 17 patients (0.75%) with follow-up lower than 1 year yielded results similar to those reported here. Decompensated diabetes (HbA1c ≥ 9.5%) was associated with increased mortality [HR = 1.95 (1.13–3.36); P = 0.02]. Liver fibrosis or steatosis was also associated with an increased mortality in comparison with age- and gender-paired healthy controls from the French population (Table S7).

Table 2 | 10-year specific causes of death and Kaplan–Meier mortality rate of patients with type-2 diabetes and/or dyslipidaemia

	10-year mortality	
	n (%)	(95% CI)
Nonliver cancer	54 (31.2)	2.4 (1.7–3.1)
Lung cancer	14 (26)	
Colon-rectal cancer	6 (11)	
Urinary cancer	6 (11)	
Breast cancer	5 (9)	
Pancreatic cancer	5 (9)	
Haematological cancer	4 (7)	
Cerebral cancer	4 (7)	
Upper gastrointestinal cancer	3 (6)	
Genital cancer	3 (6)	
Head and neck cancer	2 (4)	
Neuroendocrine cancer	1 (2)	
Carcinoma of unknown primary site	1 (2)	
Cardiovascular-related death	35 (20.2)	1.3 (1.1–2.2)
Ischaemic heart disease	14 (40)	
Cardiac arrest	10 (28)	
Heart failure	8 (23)	
Cardiogenic shock	2 (6)	
Cerebrovascular disease	1 (3)	
Liver-related death	7 (4.1)	0.3 (0.08–0.6)
Hepatocellular carcinoma	4 (57)	
Cholangiocarcinoma	2 (29)	
Cirrhosis	1 (14)	
Others or unknown	76 (44.5)	2.5 (1.8–3.2)

Other causes of death were [10th International Classification of Diseases (*n*)] respiratory disease [J18.9 (6), W79.9 (4)]; natural death [R09.2 (3)]; sepsis [A41.9 (3)]; pancreatitis [K85(1), K86.1(1)]; adrenocotical insufficiency [E27.4 (1)]; non-variceal gastrointestinal bleeding [K92.2 (1)]; suicide [X70 (1)]; accident [X59 (1)] and unknown [R99 (54)].

Specific liver-related and cardiovascular-related mortality

The 10-year survival without liver-related death was decreased in the presence of advanced fibrosis [93% (85–97) vs. 99% (99–100); $P < 0.0001$] (Table S5). The 10-year survival without cardiovascular-related death was decreased in the presence of severe steatosis [96% (94–98) vs. 99% (98–99); $P = 0.0012$] (Table S5). The presence of advanced fibrosis strongly increased the risk of liver-related death in univariate analysis [HR = 159.60 (19.15–1330.48); $P < 0.0001$]. Multivariate analysis for prediction of liver-related death was not performed due to the low number of events ($n = 7$). Severe steatosis increased the risk of cardiovascular-related death [HR = 2.93 (1.48–5.81); $P = 0.002$]. In a multivariate analysis, severe steatosis, estimated by SteatoTest, was not associated with cardiovascular-related death [HR = 2.27 (0.75–6.89); $P = 0.15$] after adjustment for metabolic factors (Table 3).

Cardiovascular risk and liver fibrosis progression

Fatal and non-fatal cardiovascular events were recorded in patients with type-2 diabetes from cohort 2. During a median follow-up of 7 years, 91 (13.4%) patients in this cohort developed 133 cardiovascular events (Table S8). The incidence of cardiovascular events was 22.0 (95% CI 17.9–27.0) per 1000 person-year. It was higher in patients with advanced fibrosis at baseline than in those without [45.2 (25.7–79.3) vs. 20.4 (16.4–25.4) per 1000 person-year] (Table S4). In patients with type-2 diabetes, the presence of advanced fibrosis [RR = 2.2 (1.2–4.1); $P = 0.008$] and the presence of dyslipidaemia, [RR = 2.2 (1.4–3.6); $P = 0.0007$] increased the risk of cardiovascular events.

In cohort 2, patients with type-2 diabetes with advanced fibrosis compared to those without, had a lower 5-year survival without cardiovascular events [75% (95% CI 61–85) vs. 89% (86–91); $P = 0.01$]. The presence of advanced fibrosis at baseline, increased the risk of cardiovascular events, in univariate [HR = 2.18 (1.18–3.99); $P = 0.01$] and multivariate analyses [HR = 1.92 (1.04–3.55); $P = 0.04$], after adjustment for the Framingham-risk score and HbA1c (Model 3 shown in Table 3). After adjusting this model for anti-diabetic, dyslipidaemia and anti-platelet treatment (Model 4 shown in Table 3), the presence of advanced fibrosis showed a HR = 1.82 (0.96–3.43); $P = 0.06$. A high Framingham-risk score (≥20%) was associated with a lower survival without cardiovascular events, in comparison with a low-to-moderate Framingham-risk score [84% (80–87) vs. 93% (89–95); $P = 0.0002$] (Table 4). In patients with a high Framingham-risk score, those with advanced fibrosis had a lower survival without cardiovascular events than the others [67% (50–80) vs. 86% (82–89); $P = 0.007$] (Figure 3a). The combination of the Framingham-risk score with FibroTest improved the prediction of cardiovascular events [HR = 2.24 (1.16–4.33); $P < 0.05$] after adjustment for HbA1c and treatment (anti-diabetic, anti-platelet and dyslipidaemia drugs).

Among the patients with type-2 diabetes, 136 (22%) underwent a reevaluation of their liver biomarkers after a median of 7 (6–8) years. Most patients (93%) had no advanced fibrosis at baseline and 28 (21%) developed fibrosis progression of at least 0.20 units of FibroTest, equivalent of one METAVIR stage of

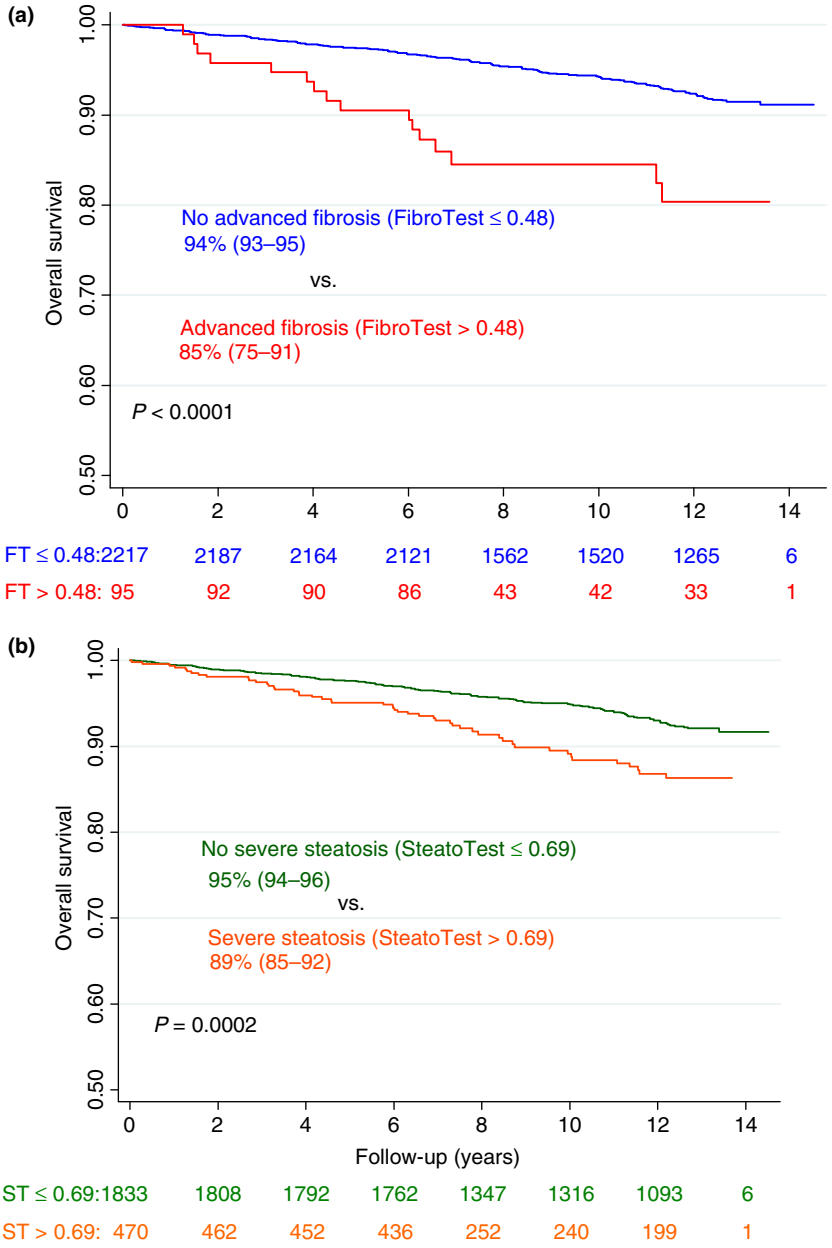

Figure 2 | Kaplan–Meier curves of 10-year overall survival according to: (a) liver fibrosis, estimated by FibroTest (FT) and (b) liver steatosis, estimated by SteatoTest (ST) (all log-rank test).

fibrosis. Among 127 patients without advanced fibrosis at baseline, 16 (13%) had progressed to advanced fibrosis upon reevaluation. Neither HbA1c nor insulin treatment were associated with liver fibrosis progression. In addition, 16 (13%) patients developed cardiovascular events (23 coronary diseases and one stroke). Survival without cardiovascular events was 69% (41–86) in patients who progressed to advanced fibrosis vs. 91% (84–95) ($P = 0.008$) in those who did not (Figure 3b). Progression to advanced fibrosis increased the risk of cardiovascular events both in uni-[HR = 3.8 (95% CI 1.3–11.0); $P = 0.01$) and multivariate analysis, after adjustment on the Framingham-risk score and alcohol consumption [HR = 4.8 (95% CI 1.5–14.9); $P = 0.007$].

DISCUSSION

This study demonstrates the prognostic value of biomarkers assessing liver fibrosis and steatosis, i.e. FibroTest and SteatoTest, respectively, in patients with type-2 diabetes and/or dyslipidaemia. Both disorders are commonly associated with NAFLD. Our hypothesis was that liver fibrosis would be a major risk factor for liver-related mortality, and steatosis, a surrogate marker of atherosclerosis. Accordingly, we not only found a significant prognostic value of FibroTest/SteatoTest for overall

Table 3 | Prognostic value of presence of advanced fibrosis or severe steatosis, estimated by liver biomarkers for the prediction of overall and cardiovascular mortality

	Overall mortality	Cardiovascular mortality
Univariate analysis HR (95% CI)		
Advanced fibrosis (F ≥ 2) (FibroTest >0.48)	2.98 (1.78–4.99)*	1.64 (0.39–6.83)
Severe steatosis (≥32%) (SteatoTest >0.69)	1.86 (1.34–2.58)†	2.93 (1.48–5.81)‡
Multivariate analysis HR (95% CI)		
Entire population (n = 2312)		
Model 1 adjusted for age, gender, BMI, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, systolic and diastolic blood pressure, smoking and alcohol consumption and presence of type-2 diabetes		
Advanced fibrosis (F ≥ 2) (FibroTest >0.48)	1.95 (1.12–3.41)§	1.24 (0.27–5.77)
Severe steatosis (≥32%) (SteatoTest >0.69)	1.43 (0.91–2.25)	2.27 (0.75–6.89)
Type 2 diabetes (n = 911)		
Model 1 adjusted for age, gender, BMI, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, systolic and diastolic blood pressure, smoking and alcohol consumption and HbA1c		
Advanced fibrosis (F ≥ 2) (FibroTest >0.48)	2.54 (1.18–5.44)§	1.24 (0.06–8.44)
Severe steatosis (≥32%) (SteatoTest >0.69)	1.91 (0.85–4.31)	1.57 (0.21–11.52)
Model 2 adjusted for the same variables of model 1 plus treatment (statins or fibrates, anti-diabetics and anti-platelets)		
Advanced fibrosis (F≥2) (FibroTest >0.48)	2.89 (1.30–6.39)‡	1.26 (0.06–8.31)
Severe steatosis (≥32%) (SteatoTest >0.69)	2.19 (0.95–5.04)	1.46 (0.21–10.27)

Age (>55 vs. ≤55 years); gender (male vs. female); BMI (>30 vs. ≤30 kg/m²), smoking (yes vs. no); excessive alcohol consumption (yes vs. no); HbA1c (>9.5 vs. ≤9.5%); statins or fibrate (yes vs. no); anti-platelet (yes vs. no) and anti-diabetic (insulin vs. oral) were included in the multivariate models as dichotomy variables. Total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides levels, diastolic and systolic blood pressure were included as quartiles (4th quartile vs. 1st quartile). Missing data (n): models for entire population (505) and models for diabetic patients (370).

*P < 0.0001; †P < 0.001; ‡P < 0.01; §P < 0.05.

mortality but also of FibroTest for cardiovascular outcomes.

Liver-related mortality

It was previously demonstrated that the presence of liver fibrosis evaluated by FibroTest was strongly associated with increased overall mortality and liver-related death in various chronic liver diseases.[3–7] Here, the demonstration was extended to a population at high risk of NAFLD. In our study, patients who died from liver-related death had higher FibroTest score. However, the number of liver-related deaths (n = 7) was too small to perform a multivariate model and discern any trends. Recently, the prognostic value of other fibrosis markers, including NAFLD fibrosis score, AST/platelet ratio index and FIB-4, was reported in patients with ultrasound-defined NAFLD.[21, 22] Relying on these biomarkers, advanced fibrosis was also independently associated with overall mortality and liver-related death in patients with biopsy-proven NAFLD.[23] However, fibrosis may have been overestimated by these markers, which integrate transaminases in their formula, as opposed to FibroTest.[24, 25]

In patients with NAFLD, type-2 diabetes was an independent risk factor for liver fibrosis.[26] More recently, the association between metabolic features and liver fibrosis progression was also reported in patients with chronic viral hepatitis.[27] Currently, weight loss and physical exercise are the main options for treatment to prevent evolution towards the severe form of the liver disease that might be associated with severe outcomes.[28]

Cardiovascular outcomes

Steatosis is associated with coronary artery disease[29] and NAFLD patients have increased surrogate markers of atherosclerosis, such as carotid intima medial thickness or coronary artery calcification.[30, 31] Yet, so far, it was unknown if the association between steatosis and cardiovascular-related death was still significant after adjustment on a fibrosis estimate.

Our study showed that the presence of advanced fibrosis, estimated by FibroTest, was predictive of the 5-year survival without cardiovascular events in patients with type-2 diabetes after adjustment for confounding factors, such as diabetes severity, treatment and the Framingham-risk score. However, this liver biomarker has lost its prognostic value for cardiovascular events when adjusted for all confounding factors, such as those on the mortality multivariate model 1. This might be explained by the fact that the same risk factors cause liver fibrosis and cardiovascular events in this

Table 4 | Prognostic values of risk factors, including presence of advanced fibrosis and severe steatosis estimated by liver biomarkers and cardiovascular risk estimated by Framingham-risk score for the 5-year survival without cardiovascular events in patients with type-2 diabetes (cohort 2)

	CV events, *n* (%)	5-year survival (95% CI)	*P* value	Univariate Cox HR (95% CI)	Multivariate Cox Model 3 HR (95% CI)	Multivariate Cox Model 4 HR (95% CI)
Liver biomarkers						
Fibrosis						
No advanced fibrosis (FibroTest ≤0.48)	80 (13)	89 (86–91)		Reference	Reference	Reference
Advanced fibrosis (FibroTest >0.48)	12 (25)	75 (61–85)	0.01	2.18 (1.18–3.99)*	1.92 (1.04–3.55)†	1.82 (0.96–3.43)
Steatosis						
No severe steatosis (SteatoTest ≤0.69)	59 (13)	89 (86–92)		Reference	Reference	Reference
Severe steatosis (SteatoTest >0.69)	33 (16)	85 (79–89)	0.32	1.28 (0.83–1.95)	1.24 (0.80–1.90)	1.20 (0.77–1.89)
HbA1c (%)						
<9.5	67 (13)	88 (85–91)		Reference	Reference	Reference
≥9.5	23 (14)	87 (82–92)	0.95	1.02 (0.63–1.63)	1.04 (0.65–1.66)	0.91 (0.55–1.50)
Diabetes treatment						
Oral	44 (11)	90 (87–93)		Reference	Reference	Reference
Insulin	45 (18)	84 (80–89)	0.01	1.67 (1.10–2.53)†	1.66 (1.08–2.57)†	1.21 (0.79–1.88)
Statins or Fibrate						
None	22 (8)	94 (91–97)		Reference	Reference	Reference
Yes	67 (18)	83 (79–87)	0.0001	2.59 (1.60–4.20) ‡	2.32 (1.43–3.77)§	1.32 (0.78–2.25)
Anti-platelet treatment						
No	29 (7)	94 (92–97)		Reference	Reference	Reference
Anti-platelet treatment	60 (27)	74 (68–80)	<0.0001	4.74 (3.04–7.38)‡	4.32 (2.73–6.83)‡	3.75 (2.27–6.19)‡
Framingham score						
Low-moderate (<20%)	26 (8)	93 (89–95)		Reference	Reference	Reference
High (FRS ≥20%)	66 (18)	84 (80–87)	0.0002	2.34 (1.49–3.69)§	2.42 (1.52–3.83)§	1.62 (1.01–2.60)†
Combination of scores						
High Framingham and low FibroTest	54 (16)	86 (82–89)		Reference	Reference	Reference
High Framingham and high FibroTest	12 (32)	67 (49–80)	0.007	2.29 (1.22–4.29)*	2.31 (1.23–4.34)*	2.24 (1.16–4.33)†

CV, cardiovascular; FRS, Framingham-risk score; CI, confidence interval; HR, hazard ratio. Missing data (*n*): HbA1c (10), anti-diabetic treatment (10), statins or fibrates treatment (11), anti-platelet treatment (12).

Multivariate Cox Model 3 adjusted for the Framingham-risk score and diabetes control (HbA1c); Cox Model 4 adjusted for, Framingham-risk score, HbA1c and treatment (anti-diabetic, anti-platelet and dyslipidaemia drugs).

*$P < 0.0001$; †$P < 0.001$; ‡$P < 0.01$; §$P < 0.05$.

population. Framingham-risk score is well validated for the prediction cardiovascular events, relying on 2000 publications since 1950 (http://www.framinghamheartstudy.org/biblio/index.html). It has also been validated in NAFLD patients for the prediction of cardiovascular outcomes.[32] We confirmed the prognostic value of the Framingham-risk score for the prediction of survival without cardiovascular events, in patients with type-2 diabetes. FibroTest improved the Framingham-risk score, particularly in patients at high risk of cardiovascular complications (score ≥20%). Furthermore, liver fibrosis progression, estimated by the FibroTest, was associated with a higher incidence of cardiovascular events.

Apolipoprotein A1, the protein transporting HDL-cholesterol, is a component of FibroTest and could have been a confounding prognostic factor for cardiovascular-related complications and death. Therefore, we verified the independent performance of FibroTest and of each component of the FibroTest/SteatoTest after adjustment for apolipoproteinA1 in two multivariate models (Table S9).

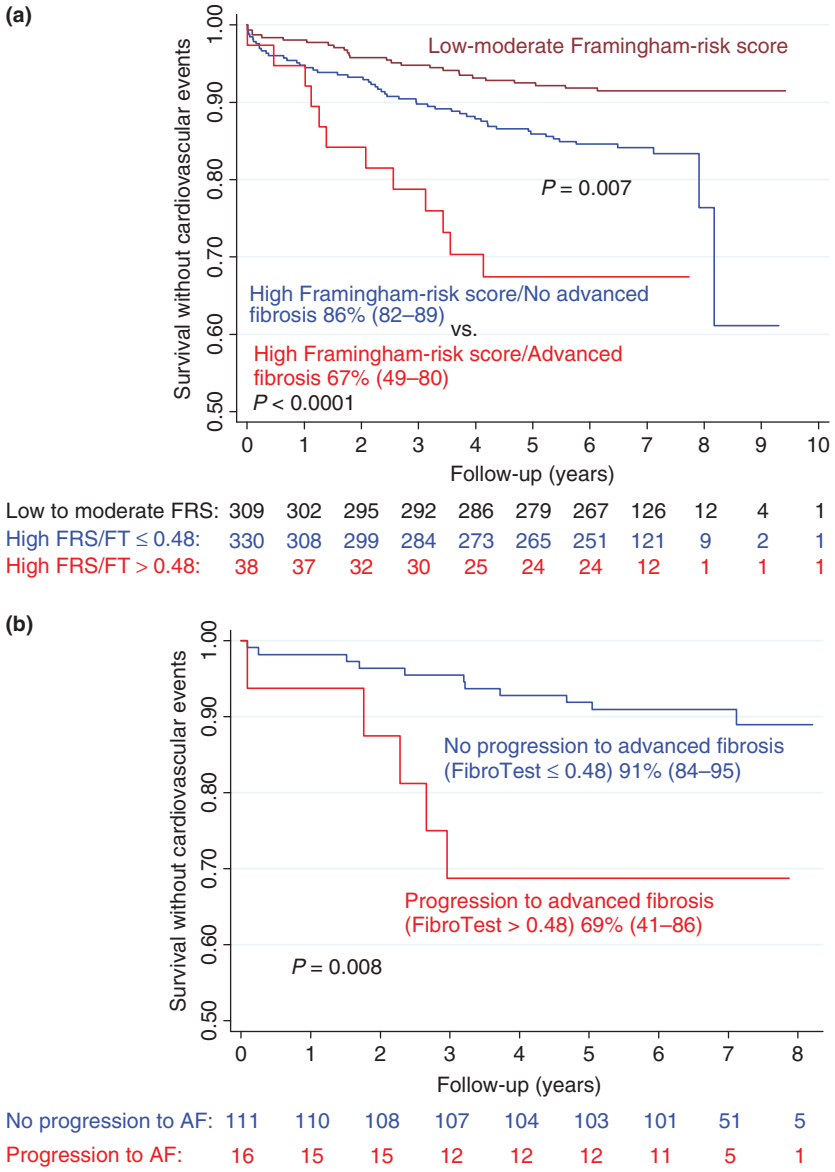

Figure 3 | Kaplan–Meier curves of the 5-year survival without cardiovascular events in patients with type-2 diabetes according to (a) the association between the Framingham-risk score (FRS) (score ≥20% defined high risk of cardiovascular disease) and liver fibrosis stage estimated by FibroTest (FT) and (b) the progression from minimal to advanced fibrosis (AF) estimated by FibroTest (FT) (all log-rank test).

In a univariate analysis, the presence of severe steatosis, assessed by SteatoTest (>0.69), was associated with overall and cardiovascular mortality. The prognostic value of SteatoTest was not observed after adjustment for confounding factors. However, it might be explained by the association between biomarker parameters and metabolic factors, since SteatoTest includes BMI, fasting glucose, total cholesterol and triglycerides as its components. Liver fibrosis and atherosclerosis may be linked by common systemic factors or a causal relationship between non-alcoholic steatohepatitis, which is the necrotic/inflammatory pro-fibrogenic form of NAFLD, and a vascular proinflammatory/prothrombotic state leading to atherosclerosis.[33] We verified that a marker of necrosis/inflammation (ActiTest) had no independent prognostic value when fibrosis and steatosis were taken into account (Tables S4 and S5).

Discrepancy between studies on mortality associated with NAFLD

Conflicting results regarding the association between NAFLD and mortality have been reported, likely because neither specific marker of fibrosis, nor quantitative assessment of steatosis has been used. In three studies, NAFLD was not associated with a higher risk of mortality, except in a subpopulation over 45 years of age, more prone to fibrosis than younger subjects.[21, 34, 35] In five other studies, NAFLD was associated with mortality including in a cohort of type-2 diabetic patients.[36–40] In these latter studies, the diagnosis of steatosis was based on imaging methods or liver enzymes. Ultrasonography and ALT are less accurate than SteatoTest and FibroTest for the diagnosis of steatosis and fibrosis, respectively, which might explain conflicting results.[8, 17, 41, 42]

Limitations and strengths

The mortality status was retrieved from a national registry for all patients. Despite the high percentage of unknown cause of death (31%), the presence of unknown death cause in 54/172 patients did not affect the analysis of overall mortality. Except for few significant differences, major risk factors were similar in patients with unknown and known causes of death. Patients evaluated and non-evaluated for fibrosis progression were also not different with respect to the main prognostic factors (Tables S10 and S11). Patients were recruited in metabolic units (Dyslipidaemia and Diabetology Departments) that might lead to a pre-selection bias explaining the low number of liver-related deaths. In addition, including patients in a tertiary centre, with prophylactic treatments probably reduced the mortality risk. Whatever these spectrum effects and the limitations of the assignment of causes of death, the independent prognostic value of FibroTest observed in these cohorts seems robust, as the prognostic performance of such biomarker will be even greater with more liver events.

Data from pre-diabetes or insulin resistance status, such as HOMA-index at baseline were available for only 358 patients (22%) of the cohort 1. In these patients, HOMA-index was not associated with overall survival even after taking into account the main prognostic factors (type-2 diabetes, Framingham score, steatosis score) with still a significant prognostic of FibroTest (Table S6). These results must be verified in a larger population of patients without diabetes and more cardiovascular events.

Details on specific treatments (insulin, anti-platelet and statins or fibrates) were fully available only for patients from the cohort 2. Therefore, we did not include these parameters in the multivariate analysis for overall mortality. In addition, the present study could not evaluate the impact of treatment change during follow-up on the prognostic value of liver biomarkers.

The strengths of our study include a large size of the study population, long duration of follow-up, mortality data collection from the national registry and the confrontation of liver biomarkers with the Framingham-risk score. The mortality data enabled evaluation of the biomarker performance against death, an indisputable gold standard. Despite the need for independent validation, information provided by FibroTest could improve the Framingham-risk score for predicting cardiovascular outcome.

The study population was not confined to NAFLD defining criteria but encompassed a large spectrum of metabolic disorders. The leading cause of death was non-liver cancer in dyslipidaemia, and cardiovascular-related death in type-2 diabetes (Table S2). Consistent with previous observations, we found an impact of HbA1c on overall mortality,[43] a high prevalence of steatosis in patients with type-2 diabetes (47%)[44] and a close association between the number of metabolic factors and advanced fibrosis.[45]

Liver biomarkers, such as FibroTest and SteatoTest, are of particular interest in patients with metabolic disorders. FibroTest has prognostic value for predicting overall survival and allows a better evaluation not only of the hepatic risk, but also of the cardiovascular risk. This biomarker is useful to identify the patients with abnormal liver function tests, in whom the absence of advanced fibrosis assessed by FibroTest virtually excludes the risk of severe liver complications at 10 years. FibroTest can improve the Framingham-risk score in type-2 diabetes and pave the way for the development of a 'cardio-liver' prognostic index in patients with metabolic disorders.

AUTHORSHIP

Guarantor of the article: None.

Author contributions: Thierry Poynard: study concept and design; analysis and interpretation of data; statistical analysis; drafting; study supervision. Hugo Perazzo: acquisition of data; analysis and interpretation of data; statistical analysis; drafting. Chantal Housset: study concept and design; analysis and interpretation of data; study supervision; critical revision of the manuscript. Mona Munteanu: acquisition of data, analysis and interpretation of data; statistical analysis and critical revision of the manuscript. Yen Ngo, Pascal Lebray, Noemi Seurat, Fanny Rutka, Marion Couteau, Sophie Jacqueminet, Philippe Giral and Denis Monneret: acquisition of data. Françoise Imbert-Bismut, Vlad Ratziu and Agnes Hartemann-Huertier: critical revision of the manuscript. All authors approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Baseline characteristics of the patients according to their metabolic status.

Table S2. 10-year specific causes of death in patients with type 2 diabetes.

Table S3. Baseline characteristics of patients who died vs. those who alive during follow-up.

Table S4. Overall mortality and cardiovascular morbidity/mortality rates, as per 1000 person-year, according to presence of advanced fibrosis, severe steatosis or necro-inflammatory activity estimated by liver biomarkers.

Table S5. Overall and specific 10-year survivals according to presence of advanced fibrosis, severe steatosis or necro-inflammatory activity estimated by liver biomarkers.

Table S6. 10-year mortality analysis among 358 patients of the cohort 1 including the HOMA assessment.

Table S7. Overall survival compared to age and gender-paired controls from the French population according to fibrosis or steatosis estimated by liver biomarkers.

Table S8. Cardiovascular events in patients with type-2 diabetes (cohort 2).

Table S9. Multivariate Cox model replacing FibroTest/SteatoTest by its components for the evaluation of overall survival and survival without cardiovascular outcomes.

Table S10. Baseline characteristics of the patients who died during follow-up according to whether the cause of death was known or unknown.

Table S11. Baseline characteristics of the patients according to whether their fibrosis stage and steatosis grade were reevaluated or not by liver biomarkers.

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