Performance of the SteatoTest, ActiTest, NashTest and FibroTest in a multiethnic cohort of patients with type 2 diabetes mellitus

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ABSTRACT

Fibromax is a diagnostic tool composed of the combination of 4 non-invasive biomarker panels for the diagnosis of steatosis (SteatoTest), necrosis and inflammation (ActiTest and NashTest-2) and fibrosis (FibroTest). The purpose of this study was to assess the performance of these biomarker panels in patients with type 2 diabetes mellitus (T2DM). All patients underwent routine labs, a 75 g oral glucose tolerance test, a liver proton magnetic resonance spectroscopy (^{1}H-MRS) to measure intrahepatic triglyceride content, and a percutaneous liver biopsy to establish the diagnosis of non-alcoholic steatohepatitis (NASH) and to grade and stage the disease in those patients with non-alcoholic fatty liver disease (NAFLD) by ^{1}H-MRS. For determination of the scores, plasma samples were blindly provided to establish the SteatoTest, ActiTest, NashTest-2 and FibroTest scores. A total of 220 patients with T2DM were included in this study. When the ability of the SteatoTest to identify patients with T2DM with NAFLD by ^{1}H-MRS was assessed, the overall performance expressed as the area under the receiver operating characteristic curve was 0.73 (95% CI 0.65 to 0.81). The performance of the ActiTest and NashTest-2 to diagnose definite NASH among patients with T2DM was 0.70 (95% CI 0.63 to 0.77) and 0.69 (95% CI 0.62 to 0.76), respectively. Regarding the FibroTest score, its performance to identify patients with moderate or advanced fibrosis was 0.67 (95% CI 0.58 to 0.76) and 0.72 (95% CI 0.61 to 0.83), respectively. Non-invasive panels for the diagnosis of steatosis, NASH and/or fibrosis, which were developed and validated in non-diabetic cohorts, underperformed when applied to a large cohort of patients with T2DM. Results from non-diabetic populations should not be extrapolated to patients with T2DM.

Significance of this study

What is already known about this subject?
- Liver biopsy remains the gold standard to diagnose non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH) and the presence of liver fibrosis.
- Clinical panels are usually used to non-invasively diagnose NASH and liver fibrosis in clinical practice.
- SteatoTest, ActiTest, NashTest-2 and FibroTest are patented scores that have successfully been used to diagnose these conditions in patients with NAFLD.

What are the new findings?
- In a cohort of patients with type 2 diabetes mellitus (T2DM), the overall performance of the SteatoTest for detection of NAFLD had an area under the receiver operating characteristic (AUROC) curve of 0.73 (95% CI 0.65 to 0.81).
- The ActiTest and NashTest-2 showed AUROCs of 0.70 (95% CI 0.63 to 0.77) and 0.69 (95% CI 0.62 to 0.76), respectively, to diagnose definite NASH.
- FibroTest had an AUROC of 0.72 (95% CI 0.61 to 0.83) for the diagnosis of advanced fibrosis in this cohort of patients.

How might these results change the focus of research or clinical practice?
- In this study, we showed that many of these panels may underperform in patients with T2DM.
- These results suggest that patients with T2DM may need different panels, specifically developed for this population.
- Results from studies in non-diabetic populations should not be directly extrapolated to patients with T2DM.

INTRODUCTION

The relationship between non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes mellitus (T2DM) is complex, with each condition negatively affecting the other one.[1] While the presence of NAFLD is associated with an increased risk of developing T2DM,[2–5] patients with T2DM and NAFLD progress faster to advanced fibrosis, cirrhosis and hepatocellular carcinoma.[6–10]

Despite being a high-risk population for liver-related complications, patients with T2DM and NAFLD have frequently been excluded

from clinical studies, or included only as a minority in a larger cohort. Therefore, much of the diagnostic and therapeutic information available for patients with T2DM and NAFLD comes from the extrapolation of information obtained from patients without diabetes. However, recent reports have shown marked differences in the diagnosis, progression and treatment of non-alcoholic steatohepatitis (NASH) in patients with T2DM.[11 12]

Fibromax is a diagnostic tool composed of the combination of four non-invasive biomarker panels for the diagnosis of steatosis (SteatoTest), necrosis and inflammation (ActiTest and NashTest-2) and fibrosis (FibroTest or FibroSURE).[13] While these biomarker panels were originally used for populations with different liver conditions (alcoholic fatty liver disease, hepatitis B and C),[14–16] they have also been more recently validated for patients with NAFLD.[13 17] However, these validation cohorts only included ~30% of patients with T2DM, and the performances of the biomarker panels were not specifically assessed in this subgroup of patients. Therefore, while these biomarker panels have been used in patients with T2DM to estimate the prevalence of advanced fibrosis,[18–20] a proper validation in a T2DM cohort is missing. The purpose of this study was to assess the performance of these biomarker panels in patients with T2DM.

MATERIALS AND METHODS

Subjects

A total of 220 patients with T2DM were included in this study. Briefly, patients were recruited from the general population and from hepatology and endocrinology clinics at the University of Florida (Gainesville, Florida, USA) and at the University of Texas Health Science Center (San Antonio, Texas, USA).

Participants with any liver disease other than NASH (ie, hepatitis B or C, autoimmune hepatitis, hemochromatosis, Wilson's disease or drug-induced hepatitis) or significant alcohol consumption (≥30 g/day for males and ≥20 g/day for females) were excluded from the study. Other exclusion criteria included type 1 diabetes mellitus, any evidence of clinically significant renal, pulmonary or heart disease, use of prohibited medications (ie, vitamin E, pioglitazone, weight loss medications, amiodarone, glucocorticoids, methotrexate, olanzapine, protease inhibitors). Patients receiving pharmacological treatment for T2DM were only allowed in the study if they were taking only metformin, sulfonylureas and/or insulin, and on stable doses for at least 3 months prior to enrollment. Most patients (~90%) have been previously included in a report assessing the performance of a lipidomic model for the diagnosis of NASH in patients with T2DM,[12] as well as in prior reports examining metabolic characteristics of patients with NAFLD.[21 22] A written informed consent was obtained from each patient prior to their participation.

Study design

As previously described,[22] patients underwent routine labs, a 75 g oral glucose tolerance test with blood draws at −15, 0, 30, 60, 90 and 120 min to confirm the diagnosis of T2DM and estimate insulin secretion and insulin resistance, a liver proton magnetic resonance spectroscopy (^{1}H-MRS) to measure intrahepatic triglyceride content, and a percutaneous liver biopsy to establish the diagnosis of NASH and to grade and stage the disease in those patients with a diagnosis of NAFLD by ^{1}H-MRS.

For determination of the scores, a plasma sample was blindly provided to Quest Diagnostics (San Juan Capistrano, California, USA) to measure haptoglobin, α_2-macroglobulin, apolipoprotein A1 (apo A1), bilirubin, γ-glutamyl transferase (GGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST), fasting plasma glucose, triglycerides and total cholesterol. These measurements, combined with age, sex, height and weight, were used by Biopredictive to establish the SteatoTest, ActiTest, NashTest and FibroTest scores.

Oral glucose tolerance test and measurements of insulin resistance

Oral glucose tolerance tests were performed after at least 10 hours of fasting. A 75 g beverage of glucose (Fisher-Brand, Fisher Scientific, Pittsburgh, Pennsylvania, USA) was used. Serum glucose was measured bedside with the Analox GM9 (Analox Instruments, Hammersmith, UK), and plasma for each time point was stored at −80°C for measurement of insulin and free fatty acids (FFA). Insulin resistance was estimated by homeostatic model assessment of insulin resistance (HOMA-IR) and Matsuda index. Adipose tissue insulin resistance was calculated as fasting FFA×fasting plasma insulin (Adipo-IR_{index}) based on the linear relationship between the rise in fasting plasma insulin levels and inhibition of the rate of basal (ie, fasting) plasma FFA release from the adipose tissue.[23]

Measurements of intrahepatic triglyceride content

Intrahepatic triglyceride content was measured by ^{1}H-MRS in a 3 T MRI scanner. Two or three areas with a volume of 30×30×30 mm were selected for voxel placement within the right lobe of the liver. A single experienced observer analyzed the spectra using commercial software (NUTS, Acorn NMR, Livermore, California, USA). Intrahepatic triglyceride content was calculated as fat fraction (area under the curve [AUC] fat peak/[AUC fat peak+water peak]). Measurements were corrected for T1 and T2 relaxation using methods previously described.[24] A liver fat content of >5.56% was considered diagnostic of NAFLD.[25 26]

Percutaneous liver biopsy

An ultrasound-guided liver biopsy was performed in patients with a diagnosis of NAFLD by ^{1}H-MRS. Histological characteristics for the diagnosis of NASH were determined using standard criteria.[27] Briefly, a diagnosis of definite NASH was made based on the presence of: zone 3 accentuation of macrovesicular steatosis (any grade), hepatocellular ballooning (of any degree) and lobular inflammatory infiltrates (of any amount). Borderline NASH was defined when some, but not all of the features of definite steatohepatitis were present.[28]

Laboratory assays

Assays used in the calculation of the BioPredictive algorithms were run on routine automated platforms using standard reagents. Total cholesterol, triglycerides, glucose, ALT, AST, γ-glutamyl transpeptidase (GGT) and total bilirubin were run on a Beckman Coulter (Beckman Coulter, Brea, California, USA) AU series instruments. Haptoglobin,

Table 1 Demographic and clinical characteristics of patients

	Overall cohort (n=220)	No NAFLD (n=69)	NAFLD (n=151)	P values between NAFLD and no NAFLD
Age, years	58±9	60±9	57±9	0.013
Gender, male %	83	86	82	0.53
Ethnicity, n (%)				<0.001
Caucasian	131 (60)	44 (64)	87 (58)	
Hispanic	64 (29)	9 (13)	55 (36)	
African-American	24 (11)	16 (23)	8 (5)	
Other	1 (0)	0 (0)	1 (1)	
Weight, kg	101±16	97±16	104±16	0.004
Body mass index, kg/m^2	33.7±4.8	31.5±4.5	34.7±4.6	<0.001
Total body fat, %	36±7	34±7	36±7	0.047
Hemoglobin A1c, %	7.1±1.2	7.0±1.3	7.1±1.1	0.71
Fasting plasma glucose, mg/mL	147±44	146±48	148±42	0.82
Fasting plasma insulin, µU/mL	15±11	9±7	17±12	<0.001
HOMA-IR	5.5±4.9	3.2±3.1	6.4±5.2	<0.001
Adipo-IR$_{index}$, mmol/L×µU/mL	6.7±6.5	3.2±3.5	8.1±6.9	<0.001
Matsuda index	3.4±3.2	2.5±1.6	2.5±1.6	<0.001
Diabetes medications				
Metformin, %	74	76	73	0.70
Sulfonylurea, %	42	41	42	0.86
Insulin, %	24	31	22	0.19
Intrahepatic triglyceride content, %	10±8	3±1	15±8	<0.001
Aspartate aminotransferase, U/L	36±24	24±11	41±26	<0.001
Alanine aminotransferase, U/L	47±37	26±18	57±39	<0.001
Cytokeratin-18 fragments, U/L	290±303	136±123	360±334	<0.001
Biopsy performed, n (%)		0 (0%)	151 (100%)	
Patients with NASH, n (%)		–	96 (64%)	
Patients with NAFLD activity score≥4, n (%)		–	92 (61%)	
Patients with advanced fibrosis, n (%)		–	25 (17%)	
NAFLD activity score		–	3.9±1.6	
0/1/2/3/4/5/6/7/8			0/0/29/31/28/33/24/5/1	
Steatosis grade		–	1.7±0.8	
S0/S1/S2/S3, n		–	0/61/60/30	
Inflammation grade		–	1.4±0.6	
I0/I1/I2/I3, n		–	1/77/69/4	
Ballooning grade		–	0.7±0.7	
B0/B1/B2, n		–	55/75/21	
Fibrosis stage		–	1.2±1.1	
F0/F1/F2/F3/F4, n		–	38/63/25/19/6	

HOMA-IR, NAFLD, NASH.

alpha-2-macroglobulin and apo A1 were run on the Siemens (Siemens Healthcare Diagnostics, Tarrytown, New York, USA) BNII instrument.

FibroMax test

The FibroTest, NashTest, NashTest-2 and SteatoTest are patented as 'in vitro diagnostic multivariate index assays' for the diagnosis of METAVIR fibrosis stages and CRN-equivalent stages including cirrhosis, for CRN-equivalent activity and for CRN-equivalent steatosis grades, respectively.[29] A quantitative NashTest-2 was constructed, and internally validated in 1081 patients at risk of metabolic liver disease.[30] These tests are exclusively available online and include clinical security algorithms.[31]

The FibroTest includes serum α_2-macroglobulin, apo A1, haptoglobin, total bilirubin and GGT. The ActiTest includes the same components plus ALT. The SteatoTest adds to the prior components the body mass index (BMI), cholesterol, triglycerides and fasting glucose. The semi-quantitative NashTest included the five components of the FibroTest plus AST, cholesterol, triglycerides, glucose and BMI. The NashTest-2 included the five components of the FibroTest plus AST, cholesterol and triglycerides. All these tests were adjusted for age and gender.

Statistical analysis

Data were summarized as number (percentages) for categorical variables and as mean±SD for numeric variables.

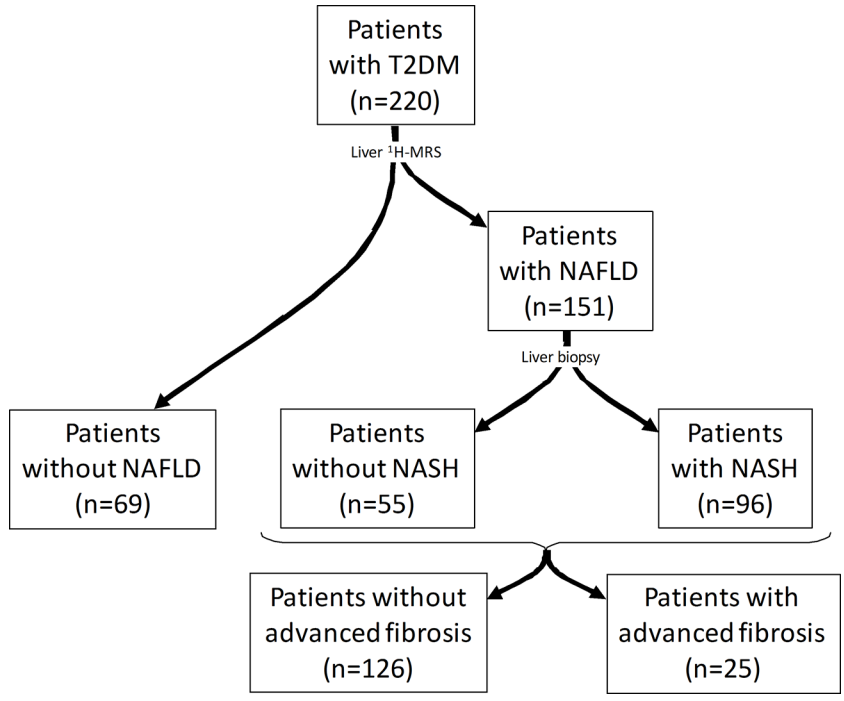

Figure 1 Flow chart of patient recruitment and the number of patients in each group. Patients without non-alcoholic fatty liver disease (NAFLD) by liver proton magnetic resonance spectroscopy were considered as steatosis grade 0, and included in the rest of the analyses as not having non-alcoholic steatohepatitis (NASH), or not having significant activity or fibrosis. T2DM, type 2 diabetes mellitus.

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for the biomarker panels were assessed considering liver ^{1}H-MRS and/or liver histology as the gold standard reference.

Due to the absence of biopsy in patients with T2DM without NAFLD by ^{1}H-MRS, these patients in whom liver diseases had been excluded, were used as controls to assess the specificities of tests. They were considered as not having NASH, not having significant activity and not having significant fibrosis.

Receiver operating characteristic curves were plotted and the area under the receiver operating characteristic curves (AUROC) calculated to represent their performance to predict binary outcomes, such as NAFLD, NASH, any fibrosis (defined as fibrosis stage ≥1), moderate fibrosis (defined as fibrosis stage ≥2) or advanced fibrosis (defined as fibrosis stage ≥3), among others. The concordance C-statistics (or C-index) was also calculated for ordinal outcomes as an accuracy measure. Comparisons between AUROCs were performed with the *roccomp* command (test of equality of ROC areas) in Stata. A two-tailed value of $p<0.05$ was considered to indicate statistical significance. Analyses were performed with Stata V.11.1 (StataCorp, College Station, Texas, USA) and graphs with Prism V.6.0 (GraphPad Software, La Jolla, California, USA).

RESULTS

Demographic and clinical characteristics of patients

In table 1, patients' demographic, clinical and biochemical characteristics were summarized. This multiethnic cohort of patients was composed of middle-aged (58±9 years), mostly obese individuals (BMI: 33.7±4.8 kg/m^2) with relatively well-controlled T2DM (hemoglobin A1c: 7.1%±1.2%, fasting plasma glucose: 147±44 mg/dL). As can be observed, this cohort of patients with T2DM included patients with relatively more severe liver disease as evidenced by high basal plasma AST (36±24 IU/L) and ALT (47±37 IU/L) levels, as well as high proportion of patients with NAFLD (69%).

When patients were divided based on the presence or absence of NAFLD by ^{1}H-MRS, we observed no clinically relevant differences in age, gender, diabetes control (fasting plasma glucose or hemoglobin A1c) or antihyperglycemic medications. Patients with NAFLD had higher BMI and higher fasting plasma insulin levels (both $p<0.001$). A larger proportion of patients of African-American origin were present in the no NAFLD group ($p<0.001$).

Histological characteristics of patients with biopsy

Details of histological characteristics are provided in table 1. As can be observed, no patient had absence of lobular inflammation, and 55 patients had no ballooning. A total of 25 (17%) patients had advanced fibrosis at enrollment, despite being completely asymptomatic (stage 3 [n=19] and stage 4 [n=6]). Figure 1 represents a flow chart summarizing patient recruitment and the number of patients in each group. Overall, 96 patients had a diagnosis of NASH, which constitutes 44% of the entire cohort or 64% of the patients with a diagnosis of NAFLD.

Role of SteatoTest for the diagnosis of NAFLD

In figure 2, we have plotted the performance of the SteatoTest for the identification of all patients with NAFLD (panel A), patients with >10% based on ^{1}H-MRS (panel B), patients with steatosis grade 2 by histology (panel C) and

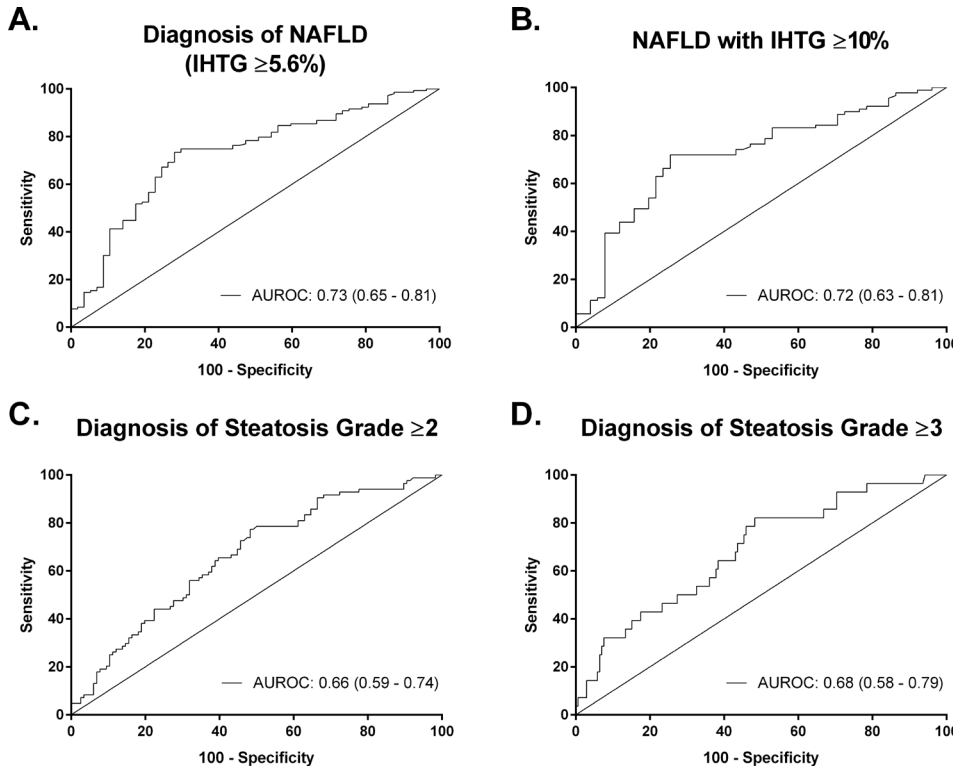

Figure 2 Performance of the SteatoTest for the diagnosis of non-alcoholic fatty liver disease (NAFLD) (panel A), for the identification of intrahepatic triglyceride (IHTG) content ≥10% (panel B), steatosis grade ≥2 (panel C), and steatosis grade ≥3 (panel D). Patients with a negative liver proton magnetic resonance spectroscopy were considered as steatosis grade 0 for panels B and C. Figure based on data from n=220. AUROC, area under the receiver operating characteristic curve.

finally those with steatosis grade 3 by histology (panel D). Patients with <5.56% of intrahepatic triglyceride content based on ^{1}H-MRS were considered as steatosis grade 0. As can be observed, when applied to a cohort of patients with T2DM, this non-invasive panel had AUROCs of 0.73 (95% CI 0.65 to 0.81), 0.72 (95% CI 0.63 to 0.81), 0.66 (95% CI 0.59 to 0.74) and 0.68 (95% CI 0.58 to 0.79) for the different steatosis-related outcomes (figure 2). In the best scenario, with an optimal cut-off point of 0.52, the sensitivity, specificity, NPV and PPV of the SteatoTest for the diagnosis of NAFLD was: 73% (65%–80%), 72% (58%–83%), 51% (40%–63%) and 87% (79%–92%), respectively. The performance of this biomarker panel did not improve when used to identify higher amounts of intrahepatic triglyceride accumulation (ie, ≥10% by ^{1}H-MRS or grade 2 or 3 by histology). Of note, the correlation of the SteatoTest with measurements of insulin resistance was statistically significant: HOMA-IR (r=0.50, p<0.001), Adiopo-IR (r=0.35, p<0.001) and Matsuda index (r=−0.42, p<0.001). This translated into a sensitivity of 77% (68%–84%), a specificity of 67% (54%–79%), PPV of 81% (72%–88%) and NPV of 61% (49%–72%) to identify insulin-resistant patients (defined as HOMA-IR >3). Of note, these associations were independent of fasting plasma glucose levels (a component of the SteatoTest; all p<0.001).

Role of ActiTest, NashTest and NashTest-2 for the identification of inflammation, ballooning and/or NASH

We then attempted to assess whether the ActiTest, NashTest, or the NashTest-2 could be used to identify those patients with definite NASH (vs those without definite NASH), or detect those patients with more severe liver disease (ie, significant inflammation [defined as grade ≥2], any hepatocyte ballooning or NAFLD activity score ≥4). In figure 3, we have plotted the ROC curves for the ActiTest to detect the above outcomes. Patients with a negative ^{1}H-MRS were included in the control groups (ie, in the 'no NASH' group, or in the ballooning <1). For the diagnosis of definite NASH, the ActiTest achieved a sensitivity of 74% (64%–82%), specificity of 62% (53%–70%), PPV of 60% (50%–69%) and NPV of 75% (66%–83%). Moreover, we did not observe any significant improvement if patients with borderline NASH were excluded from the analysis. As for the classification of inflammation and ballooning, the C-statistics were 0.72 and 0.67 for the ActiTest, respectively.

The NashTest, which provides a classification of 'no NASH', 'possible NASH' or 'NASH', classified the majority of patients (n=156, 78%) in the intermediate group (NashTest results were available for only 200 patients). From these 156 patients, only 42 (27%) were actually classified as borderline NASH in the liver biopsy. From those patients classified as 'no NASH' or 'NASH' by the test, only 41% were correctly labeled. Overall, only 30% of patients were correctly classified by the test. Results from the NashTest-2, a quantitative test constructed with a simplified version of CRN NASH definition[30] are summarized in figure 4. The AUROC for the diagnostic of NASH was 0.69 (95% CI 0.62 to 0.76). Using the NashTest-2 to identify patients with definite NASH, we obtained a sensitivity of

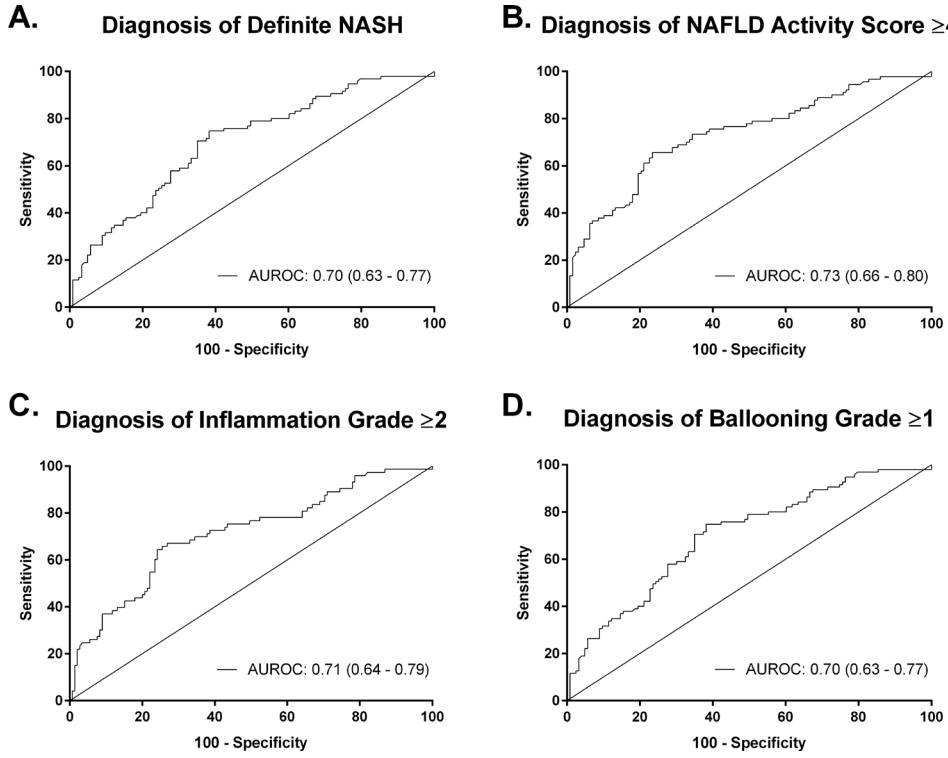

Figure 3 Performance of the ActiTest for the diagnosis of non-alcoholic steatohepatitis (NASH) (prevalence=44%; panel A), identification of non-alcoholic fatty liver disease (NAFLD) activity score ≥4 (prevalence=41%; panel B), inflammation grade ≥2 (prevalence=33%; panel C) and ballooning grade ≥1 (prevalence=44%; panel D). Patients with a negative liver proton magnetic resonance spectroscopy were considered as not having NASH, or as not having any of the outcomes analyzed in the figure. Figure based on data from n=220. AUROC, area under the receiver operating characteristic.

71% (61%–80%), specificity of 60% (50%–69%), PPV of 59%, (49%–68%) and NPV of 72% (61%–81%).

Role of FibroTest for the diagnosis of fibrosis in patients with NASH

The AUROC of the FibroTest panel for the diagnosis of any fibrosis (stage ≥1) was 0.61 (95% CI 0.54 to 0.69), for moderate fibrosis (stage ≥2) was 0.67 (95% CI 0.58 to 0.76) and for advanced fibrosis (stage ≥3) was 0.72 (95% CI 0.61 to 0.83) (figure 5), when applied as a stand-alone test in patients with T2DM and NAFLD. The overall C-statistics for fibrosis stage (stages 0–4) was 0.63. A summary with all results is shown in table 2.

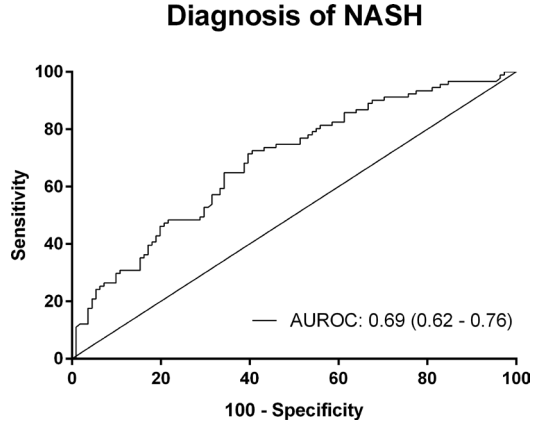

Figure 4 Performance of the NashTest-2 for the diagnosis of non-alcoholic steatohepatitis (NASH) (prevalence=44%). Patients with a negative liver proton magnetic resonance spectroscopy were considered as not having NASH. Figure based on data from n=202. AUROC, area under the receiver operating characteristic.

Analyses in subpopulations

In order to assess the impact of demographic and clinical characteristics on the performance of these biomarker panels, we repeated the analyses in different subgroups, based on age, gender, ethnicity, presence of obesity and diabetes control.

None of these covariates significantly affected the performance of the SteatoTest for the diagnosis of NAFLD. However, there was a non-significant trend towards a worse performance among African-Americans versus Caucasians versus Hispanics: 0.66 (95% CI 0.41 to 0.90) vs 0.76 (95% CI 0.67 to 0.85) vs 0.81 (95% CI 0.68 to 0.95), p=0.54, respectively as well as among those with a better glycemic control (HbA1c <7% vs ≥7%). As for the ActiTest, none of the covariates had a major influence on its association with the presence of NASH. However, once again, we observed a non-significant variation among the ethnic groups, with a better performance among African-American patients (0.85 [95% CI 0.68 to 1.00] vs 0.71 [95% CI 0.62 to 0.80] vs 0.66 [95% CI 0.52 to 0.79], p=0.20), respectively. No significant differences were observed among the different subgroups in the performance of the FibroTest.

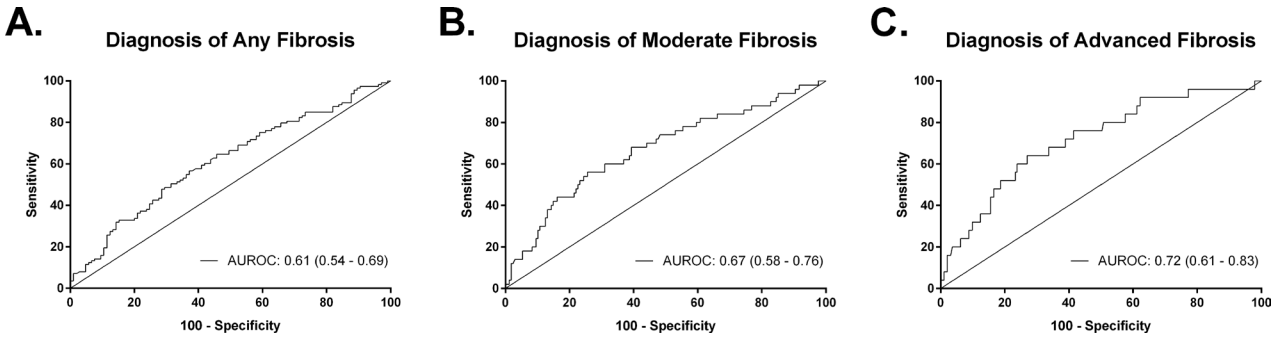

Figure 5 Performance of the FibroTest for the identification of any fibrosis (stage ≥1; prevalence=70%; panel A), moderate fibrosis (stage ≥2; prevalence=31%; panel B) and advanced fibrosis (stage ≥3; prevalence=16%; panel C). Patients with a negative liver proton magnetic resonance spectroscopy were considered as not having fibrosis for the purposes of these analyses. Figure based on data from n=220. AUROC, area under the receiver operating characteristic.

DISCUSSION

While the percutaneous liver biopsy remains the gold standard for the diagnosis of NASH and to determine the fibrosis stage, it is frequently avoided in clinical practice for a number of reasons (ie, cost, potential risks, lack of well-accepted and FDA-approved treatments, etc). As a consequence of this, many non-invasive panels have been developed and validated in order to avoid the need of a liver biopsy.[17 32] However, in most cases these panels were either developed in patients without diabetes, or included only a minority of patients with T2DM. Moreover, presence of diabetes or hyperglycemia is sometimes one of the factors included in these panels to identify patients at higher risk. Whether results from these studies can be directly extrapolated to a large cohort of patients with T2DM is unknown.

In the current work, we assessed well-validated panels for frequent chronic liver diseases, such as FibroTest, ActiTest and SteatoTest, as well as the less validated NashTest and NashTest-2, in a multiethnic cohort of patients with T2DM receiving metformin, sulfonylureas and/or insulin therapy. The performances (ie, AUROCs) seemed in the lower range compared with those observed in patients with chronic hepatitis C, the most validated population for FibroTest and ActiTest, or to patients with NAFLD without T2DM for SteatoTest and NashTest. The same trend was observed with non-proprietary clinical models (ie, AST to platelet ratio index (APRI), fibrosis-4 index [FIB-4]), with overall worse performances in patients with NAFLD and T2DM compared with patients without diabetes.[33]

Several reasons could explain the relatively low performance of these tests in patients with T2DM. First, as mentioned above, the mechanisms promoting NASH in patients with T2DM may be different, and therefore, these patients may have a different metabolic and biochemical fingerprint compared with patients with NAFLD without T2DM. Second, while the presence of diabetes or hyperglycemia may contribute to identify patients at higher risk in a mixed population, we cannot rely on this parameter when facing only patients with T2DM. Third, glycemic control can vary over relatively short periods of time based on compliance with the diet, affecting different parameters frequently used to develop these panels (eg, ALT, triglycerides, fasting plasma glucose, etc). Finally, use of hypoglycemic agents (as well as lipid-lowering and blood pressure medications) can also affect many of the parameters used to develop these panels.

In support of this, recent studies have shown that patients with T2DM may respond differently to pharmacological therapy (ie, pioglitazone) compared with patients with prediabetes.[11] Also, lipidomics approaches designed to predict presence of NAFLD and/or NASH failed to maintain their performance in a cohort of patients with T2DM.[12] In light of our results, biomarker panels specifically developed in patients with T2DM may need to be considered. Bazick

Table 2 Summary of all biomarkers panels

Panel	Components	Outcome	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
SteatoTest	α_2-Macroglobulin, apo A1, haptoglobin, total bilirubin, GGT, BMI, cholesterol, triglycerides and fasting glucose	NAFLD (intrahepatic triglyceride content≥5.56%)	73% (65%–80%)	72% (58%–83%)	87% (79%–92%)	51% (40%–63%)
ActiTest	α_2-Macroglobulin, apo A1, haptoglobin, total bilirubin, GGT, ALT	NASH	74% (64%–82%)	62% (53%–70%)	60% (50%–69%)	75% (66%–83%)
NashTest-2	α_2-Macroglobulin, apo A1, haptoglobin, total bilirubin, GGT, AST, cholesterol, and triglycerides	NASH	71% (61%–80%)	60% (50%–69%)	59% (49%–68%)	72%(61%–81%)
FibroTest	α_2-Macroglobulin, apo A1, haptoglobin, total bilirubin, GGT	Advanced fibrosis (stage≥3)	64% (42%–82%)	73% (66%–79%)	23% (14%–35%)	94% (89%–97%)

apo A1, apolipoprotein A1; ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; GGT, γ-glutamyl transferase; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NPV, negative predictive value; PPV, positive predictive value.

et al[34] attempted this in a large cohort of patients with T2DM using numerous demographic and metabolic biomarkers, without much success, as still 44% and 87% of the patients fell in the grey zone (ie, undetermined group) for the diagnosis of NASH and advanced fibrosis, respectively.

Preventing the short-term and long-term consequences of NASH in patients with T2DM requires an early diagnosis and safe/effective treatments. New imaging techniques and biomarker panels, as well as new effective treatments are likely to play a major role.[8 35 36] However, before these advances can be recommended for routine use, validation in multiethnic cohorts should also be considered. In this study, we observed a strong trend towards a distinctive performance of the biomarker panels among African-American patients, especially for the SteatoTest and ActiTest. This is in line with several prior studies that have suggested that African-Americans may behave differently compared to other ethnicities in regard to liver fat accumulation.[22 37]

This study has some limitations that should be taken into consideration. The relative number of patients in the control and advanced fibrosis groups was limited, with wide AUROCs CIs. The same limitations were present for the small number of cases in different stages of steatosis and inflammatory activity. Indirect comparisons between different patients are limited by the impact of the prevalence of liver disease, as well as the spectrum effect on AUROCs. In patients with chronic hepatitis C, the FibroTest's AUROC varied from 0.60 to 0.90 depending on the prevalence of F0 and F4 in the population of interest. However, as the study includes a cohort of unselected patients with T2DM, the distribution of the severity of liver disease in our cohort is likely to match the distribution of the overall population of T2DM. Proof of this is that the prevalence of NAFLD in our cohort: 69%, is similar to the one reported by other groups.[38 39] Furthermore, as it would be unethical to obtain liver biopsies in all the spectrum of NAFLD to assess the sensitivity of new tests in patients with and without NAFLD, there is always some selection bias in this kind of studies. In order to minimize this, all patients with T2DM were included in the analysis regardless of their NAFLD status.

Of note, FibroTest and ActiTest were constructed, and mostly validated, in chronic hepatitis C and B, with larger spectrum of fibrosis from F0 to F4, and with more severe activity (necrosis and inflammation) compared to patients with T2DM. This could explain the relatively lower AUROCs observed in NAFLD in our manuscript versus chronic hepatitis C and B, suggesting that results in one population cannot be directly extrapolated to other populations.

In summary, in the current study we have shown that well-validated biomarker panels for the diagnosis of NAFLD, NASH, advanced fibrosis (SteatoTest, ActiTest, NashTest and FibroTest) may underperform in patients with T2DM. No particular demographic or clinical parameter explained this difference in the performance compared with prior reports. These results suggest that patients with T2DM may require predictive models that have been specifically developed for them. However, further comparisons with well-matched patients without diabetes are still required to really understand the different behavior of these different cohorts. Until then, extrapolation of results from patients without diabetes may result in significant misclassification.

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Competing interests MJM and MPC are employees and stock holders of Quest Diagnostics. JMC is full employee of BioPredictive. TP is the inventor of the tests assessed in the study and founder of BioPredictive. The patents belong to the public organization Asssitance Publique Hôpitaux de Paris.

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