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Clinical Research Article

Adipose Tissue Insulin Resistance Predicts the Severity of Liver Fibrosis in Patients With Type 2 Diabetes and NAFLD

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Abstract

Context: Although type 2 diabetes (T2D) is a risk factor for liver fibrosis in nonalcoholic fatty liver disease (NAFLD), the specific contribution of insulin resistance (IR) relative to other factors is unknown.

Objective: Assess the impact on liver fibrosis in NAFLD of adipose tissue (adipose tissue insulin resistance index [adipo-IR]) and liver (Homeostatic Model Assessment of Insulin Resistance [HOMA-IR]) IR in people with T2D and NAFLD.

Design: Participants were screened by elastography in the outpatient clinics for hepatic steatosis and fibrosis, including routine metabolites, cytokeratin-18 (a marker of hepatocyte apoptosis/steatohepatitis), and HOMA-IR/adipo-IR.

Setting: University ambulatory care practice.

Participants: A total of 483 participants with T2D.

Intervention: Screening for steatosis and fibrosis with elastography.

Main outcome measures: Liver steatosis (controlled attenuation parameter), fibrosis (liver stiffness measurement), and measurements of IR (adipo-IR, HOMA-IR) and fibrosis (cytokeratin-18).

Results: Clinically significant liver fibrosis (stage F ≥ 2 = liver stiffness measurement ≥8.0 kPa) was found in 11%, having more features of the metabolic syndrome, lower adiponectin, and higher aspartate aminotransferase (AST), alanine aminotransferase, liver fat, and cytokeratin-18 ($P <$ 0.05-0.01). In multivariable analysis including just clinical variables (model 1), obesity (body mass index [BMI]) had the strongest association with fibrosis (odds ratio, 2.56; CI, 1.87-3.50; $P < 0.01$). When metabolic measurements and cytokeratin-18 were included (model 2), only BMI, AST, and liver fat remained significant. When fibrosis stage was adjusted for BMI, AST, and steatosis (model 3), only Adipo-IR remained strongly associated with fibrosis (OR, 1.51; CI, 1.05-2.16; $P = 0.03$), but not BMI, hepatic IR, or steatosis.

Conclusions: These findings pinpoint to the central role of dysfunctional, insulin-resistant adipose tissue to advanced fibrosis in T2D, beyond simply BMI or steatosis. The clinical implication is that targeting adipose tissue should be the priority of treatment in NAFLD.

Key Words: nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), liver fibrosis, obesity, type 2 diabetes mellitus (T2D), vibration controlled transient elastography (VCTE), adipose tissue insulin resistance index (Adipo-IR)

Abbreviations: adipo-IR, adipose tissue insulin resistance index; ALT, alanine aminotransferase; APRI, AST to platelet radio index; AST, aspartate aminotransferase; AUC, area under the curve; BMI, body mass index; CAP, controlled attenuation parameter; FIB-4, Fibrosis-4; FFA, free fatty acid; HDL, high-density lipoprotein; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; IR, insulin resistance; LSM, liver stiffness measurement; MetS, metabolic syndrome; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis; OR, odds ratio; T2D, type 2 diabetes.

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent worldwide chronic liver disease in overweight and obese individuals (1, 2). It often progresses to nonalcoholic steatohepatitis (NASH), a more severe form of the disease in which hepatic steatosis is associated with inflammation and hepatocyte injury (also known as hepatocyte ballooning), with or without fibrosis. Overall, NAFLD is more common in patients with type 2 diabetes (T2D), with about 70% of the patients

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having steatosis (3-5), about 30% to 40% having NASH (6), and 12% to 20% having clinically significant fibrosis (4, 5, 7). Some genetic polymorphisms involved in lipid metabolism and droplet trafficking (eg, PNPLA3, TM6SF2, MBOAT7, HSD17B13) have been linked to an increased susceptibility and progression of steatohepatitis (7). Genetic and acquired risk factors such as age, fibrosis stage at diagnosis, insulin resistance, obesity, and T2D interact to influence progression of steatohepatitis to cirrhosis, but the underlying mechanisms and their relative contribution remain uncertain (8).

To prevent cirrhosis in people with T2D, the American Diabetes Association has raised awareness among clinicians about the high risk of NASH-related advanced fibrosis. In 2022, it incorporated guidance for the diagnosis and management of liver fibrosis in patients with T2D from a multidisciplinary panel of experts, including those from the American Diabetes Association (9, 10). More recently, the American Association of Clinical Endocrinology (11) recommended that primary care physicians and endocrinologists screen all high-risk individuals for liver fibrosis (ie, those with prediabetes/T2D, obesity associated with metabolic syndrome, alanine aminotransferase (ALT) > 30 U/L or steatosis on imaging) with the Fibrosis-4 (FIB-4) index, followed by imaging with transient elastography (or plasma biomarkers if imaging not available) if patients are at indeterminate- or high-risk of developing future cirrhosis. Vibration-controlled transient elastography (VCTE; FibroScan) has been the most commonly used and best validated screening test in hepatology clinics for liver fibrosis (12). A pulse-echo ultrasonic acquisition technique is used to quantify the speed of mechanically induced shear wave in liver tissue. Liver stiffness measurement (LSM) by VCTE has been validated to correlate with the severity of fibrosis (13). Hepatic steatosis can also be quantified by measuring the ultrasonic attenuation of the echo wave, termed as the controlled attenuation parameter (CAP) (11). Recently, Lomonaco et al (4) reported that in the United States prevalence of steatosis by CAP was 70% and that of moderate-to-advanced fibrosis (≥F2) by VCTE was 15% in patients with T2D. Comparable results have been reported in the United States by others (5) and worldwide with an overall prevalence of moderate-to-advanced stage fibrosis ranging from 12% to 21% in patients with T2D (7).

A better understanding of the underlying factors for disease progression would allow to better target screening and treatment to the "rapid progressors" (9, 14). Insulin resistance (IR) plays an important role at the level of liver, muscle, and adipose tissue in the development of hepatic steatosis in patients with NAFLD (1, 15, 16). It is believed that free fatty acid (FFA) overload from dysfunctional adipose tissue in the setting of IR leads to development of hepatic "lipotoxicity" (1), culminating in the accumulation of lipotoxic lipid intermediates such as diacylglycerols and ceramides in NASH. However, there are few population-based studies that have carefully separated hepatic from adipose tissue insulin resistance to understand their hierarchy and relative contribution on top of traditional risk factors (ie, obesity) in patients with T2D. Insights into their relative relevance would allow to better identify and target treatment to the population at the highest risk of cirrhosis in the "real world."

Our aim was to assess the impact on fibrosis stage of IR at the level of the liver and adipose tissue measured by Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) and the adipose tissue insulin resistance (adipo-IR) index, respectively, along with other better studied clinical risk factors for fibrosis, like obesity. To this end, we recruited a large population of people with T2D from outpatient general medicine and endocrine outpatient clinics, gathered extensive clinical and metabolic information, screened them by VCTE to stage fibrosis, and assessed the role of hepatic and adipose IR in relation to the severity of liver fibrosis.

Study Design and Methods

Participants

A total of 634 subjects were recruited from internal medicine, family medicine, and endocrinology clinics at the University of Florida, Gainesville, Florida. All patients (adults between 21 and 79 years of age) signed an informed consent and underwent a careful review of their medical history (both from electronic medical record and personal interview), screening for alcohol intake, physical examination, and routine chemistries. Subjects were excluded if they had an established history of NAFLD, history of alcohol abuse (≥30 g/d for males and ≥20 g/d for females), type 1 diabetes mellitus, any liver disease other than NAFLD (ie, hepatitis B or C, autoimmune hepatitis, hemochromatosis, drug-induced hepatitis, other), were on medications that could induce hepatic steatosis (eg, estrogen, glucocorticoids), on pioglitazone or GLP1-RA within 6 months before enrollment, pregnancy/lactation, presence of implanted electronic medical device (ie, pacemaker, because VCTE cannot be performed). This study was approved by the university institutional review board. Results on the prevalence of NAFLD and of liver fibrosis in this cohort of patients have been previously reported by our group (4).

Study Design

Liver fibrosis was assessed by LSM and liver steatosis by CAP in all patients fasting for ≥3 hours. Advanced fibrosis was also predicted by calculating aspartate aminotransferase-to-platelet ratio index (11) and FIB-4 index (see the following section). Plasma was collected from patients who were fasting for at least 10 hours to measure metabolites like glucose, insulin, FFA, A1c, lipid profile, and plasma aminotransferases, CK-18, and adiponectin. Patients with results suggestive of clinically significant fibrosis were offered to have a liver biopsy.

Study Measures

Vibration-controlled transient elastography

VCTE was performed by a FibroScan 530 (Echosens, Paris, France) equipped with both M (medium; ultrasound center frequency: 3.5 MHz and depth: 25–65mm) and XL (extra-large; ultrasound center frequency: 2.5 MHz and depth: 35–75mm) probes. Details about this technique are described elsewhere (4, 13). Reliable values for CAP and LSM, expressed in dB/m and kilopascals (kPa), respectively, were obtained as mean from at least 10 measurements and considered reliable only if interquartile range/median <30% and success rate >60%. Steatosis was defined as a CAP ≥ 274 dB/m and clinically significant liver fibrosis as a LSM ≥ 8.0 kPa (13). Fibrosis stage was defined based on LSM cutoffs measured by VCTE that have been validated with histology from liver biopsies (13): mild fibrosis (F1: ≥ 7.0 and ≤8.1 kPa), clinically significant/moderate fibrosis (F2: ≥ 8.2

and ≤9.6 kPa), advanced fibrosis (F3: ≥ 9.7 and ≤13.5 kPa), and hepatic cirrhosis (F4: ≥ 13.6 k).

Liver biopsy

All patients that underwent a liver biopsy had at least LSM ≥ 8.0 kPa plus belonging to a high-risk group. Patients with LSM ≥ 8.0 kPa in the clinic (fasting ≥3 hours) and aspartate aminotransferase (AST) ≥ 20 U/L had a repeat VCTE to confirm the finding after an overnight fast, and laboratories such as AST/ALT and biomarkers of liver fibrosis repeated. An ultrasound-guided liver biopsy was performed in patients with moderate-to-severe risk of fibrosis. Biopsy specimens were analyzed by a single experienced pathologist (P.B.) using standard CRN (Clinical Research Network) criteria (12). The pathologist was blinded to the subjects' identity, elastography results, or any other clinical information.

Analytical methods

Fasting plasma insulin and FFA were measured by commercially available colorimetric ELISA kits from ALPCO (New Hampshire, USA) and Fujifilm-Wako diagnostics (California, USA), respectively. CK-18 was measured by colorimetric ELISA (M30 Apoptosense ELISA PEVIVA, Diapharma group Inc, Ohio, USA). Fasting plasma adiponectin levels were quantified using ELISA (Bio-techne, R&D Systems, Minnesota, USA). Fasting plasma glucose, A1c, lipid profile, and aminotransferase levels were measured using standard laboratory methods.

Calculations

We calculated indexes of IR as follows.

- HOMA-IR, which indicates primarily fasting hepatic tissue IR, was calculated as fasting plasma glucose (mg/dL) × insulin (μU/L)/405.
- Adipo-IR, which is a measure of fasting adipose tissue insulin resistance, was evaluated as fasting FFA (mmol/L) × insulin (μU/L).

We calculated diagnostic panels of fibrosis as follows.

- The AST to platelet radio index (APRI) score was calculated as [(AST/upper limit of normal)/platelet count (10^9/L)] × 100.
- The FIB-4 was calculated as (age × AST/(platelet count [10^9/L] × square root of ALT).

Both APRI and FIB-4 were chosen as diagnostic panels because they have been well-validated and are supported by the literature (9, 11, 12).

Metabolic syndrome (MetS) is a continuous MetS-severity Z-score that is sex and race/ethnicity specific (17). The MetS-severity score is associated with increased risk of future T2D, cardiovascular disease, chronic kidney disease, and, in patients with elevated ALT, with advanced fibrosis (18) and is therefore a useful index for better identifying individuals at risk for multiple MetS-associated chronic diseases.

Statistical Analysis

All continuous variables are reported as mean ± SD and categorical variables are presented as percentages. Mean comparisons compared between those with and without fibrosis were done via *t* tests, accommodating for unequal variances when deemed necessary, and a P value ≤ 0.05 was considered statistically significant. Sex and race/ethnicity distributions between the groups were compared via χ^2 tests. Logistic regression was first used to estimate univariate associations between all variables and odds of fibrosis individually. Odds ratios relative to a 1-SD change in each variable were calculated to facilitate comparisons across variables. Multivariable logistic regression was then used to develop models of odds of fibrosis. Three sets of models were developed to parallel 3 categories of variables. Those clinical variables that were significantly ($P < 0.05$) associated with fibrosis were initially included in model 1, removing variables 1-by-1 until only significant variables remained. Any significant "clinical" variables from model 1 were forced into model 2, which then considered routine laboratory results and other variables. A similar stepwise procedure was performed, starting with those with significant univariate associations. Model 3 was developed beginning with these model 1 and 2 variables and using the backwards stepwise process to select any additional research laboratory variables independently associated with fibrosis. For model 3, only those patients who were not on insulin were considered. Correlation analyses between LSM or CAP and histology scores were conducted by Pearson correlation.

Results

Patient Characteristics

A total of 634 subjects were recruited and had a liver stiffness measurement by VCTE. Among them, 116 were lost to follow-up or not interested in having blood testing after LSM (signed consent in the clinic but were not fasting and did not return to participate in the other study procedures), 17 subjects were excluded because they did not meet inclusion/exclusion after initial laboratory results or secondary review, and 18 subjects were excluded for other reasons (ie, started exclusion medications after initial recruitment, moved, other; see Supplementary Fig. S1 (19) (10.6084/m9.figshare.21396960). Thus, a cohort comprising 483 patients were included. Table 1 summarizes the clinical and metabolic profile of the 483 subjects. An LSM cutoff of ≥8.0 kPa is considered to represent a stage in which the risk for developing future cirrhosis greatly increases (12). Therefore, patients were divided based on LSM ≥ 8.0 kPa (stage F ≥ 2) as having clinically significant liver fibrosis (n = 54 or 11%) or without clinically significant fibrosis (LSM < 8.0 kPa; n = 429). Patients with F ≥ 2 fibrosis stage had higher body mass index (BMI; 38.0 ± 6.3 vs 32.5 ± 5.8 kg/m^2, $P < 0.0001$) and elevated AST and ALT (30 ± 17 vs 20 ± 8 and 37 ± 30 vs 21 ± 12 U/L, respectively; both $P < 0.001$) compared with patients without fibrosis. The mean values for CAP and LSM in these groups were 351 ± 47 vs 298 ± 62 dB/m and 11.0 ± 3.8 vs 5.1 ± 1.1, respectively; both $P < 0.001$.

Patients who were on exogenous insulin were excluded from the analysis of HOMA-IR and adipo-IR (n = 135) because the presence of exogenous insulin renders these equations invalid. Therefore, 348 patients (age, 61 ± 11 years; BMI, 32.8 ± 6.2 kg/m^2; A1c, 7.0 ± 1.4%) were included for assessing insulin resistance at the level of the liver (HOMA-IR) and adipose tissue (adipo-IR). Their clinical characteristics are available in Supplementary Table S1 (19) (10.6084/m9.figshare.21396960). Among them, 67% had NAFLD and 15% had F ≥ 1 fibrosis. A total of 34 patients

Table 1. Clinical characteristics of all patients

	Overall sample (n = 483)			
	Overall n = 483	Fibrosis (kPa ≥8.0) n = 54	Without fibrosis (kPa <8.0) n = 429	*P* value (with vs without fibrosis)
Clinical variables				
Age, y	60 ± 12	57 ± 12	60 ± 11	0.06
Female, n (%)	264 (55)	30 (56)	234 (55)	0.89[a]
Race/ethnicity, n (%)				0.07[a]
Non-Hispanic White	273 (57)	39 (72)	234 (55)	
Non-Hispanic Black	141 (29)	10 (19)	131 (31)	
Hispanic or Latino	38 (8)	4 (7)	34 (8)	
Unknown/other	31 (6)	1 (2)	30 (7)	
BMI, kg/m^2	33.1 ± 6.1	38.0 ± 6.3	32.5 ± 5.8	<0.01
Systolic blood pressure, mmHg	133 ± 16	138 ± 16	133 ± 16	0.03
Diastolic blood pressure, mmHg	79 ± 9	82 ± 10	79 ± 9	0.02
Routine laboratory values + other variables				
Total cholesterol, mg/dL	164 ± 45	163 ± 48	164 ± 44	0.82
LDL-C, mg/dL	89 ± 38	82 ± 36	89 ± 39	0.18
HDL-C, mg/dL	47 ± 12	42 ± 11	47 ± 12	0.01
Triglycerides, mg/dL	153 ± 97	201 ± 120	147 ± 92	<0.01[b]
FPG, mg/dL	131 ± 39	138 ± 43	130 ± 38	0.21
FFA, mmol/L	0.30 ± 0.16	0.34 ± 0.18	0.30 ± 0.16	0.03
A1c, %	7.5 ± 1.8	7.7 ± 1.6	7.5 ± 1.8	0.48
AST, U/L	21 ± 10	30 ± 17	20 ± 8	<0.01[b]
AST >30	11%	33%	8%	<0.01[a]
AST >40	5%	19%	3%	<0.01[a]
ALT, U/L	23 ± 16	37 ± 30	21 ± 12	<0.01[b]
ALT >30	19%	37%	17%	<0.01[a]
ALT >40	9%	33%	6%	<0.01[a]
FIB-4	1.2 ± 0.5	1.3 ± 0.6	1.2 ± 0.5	0.11
APRI	0.23 ± 0.13	0.33 ± 0.21	0.22 ± 0.12	<0.01[b]
CAP, dB/m	304 ± 63	351 ± 47	298 ± 62	<0.01[b]
CAP ≥274 dB/m	67%	89%	65%	<0.01[a]
CK-18, U/L	166 ± 160	270 ± 209	153 ± 148	<0.01
Adiponectin, µg/mL	5.2 ± 3.3	4.2 ± 2.3	5.3 ± 3.3	<0.01[b]
LSM, kPa	5.8 ± 2.5	11.0 ± 3.8	5.1 ± 1.1	<0.01[b]
Mild fibrosis (F1 = kPa ≥ 7.0 and ≤8.1)	5%	6%	5%	0.95
Significant fibrosis (F2 = kPa ≥ 8.2 and ≤9.6)	5%	44%	0	<0.01[a]
Advanced fibrosis (F3 = kPa ≥9.7 and ≤13.5)	5%	41%	0	<0.01[a]
Hepatic cirrhosis (F4 = kPa ≥13.6)	1%	9%	0	<0.01[a]

Mean ± SD unless otherwise noted.
Abbreviations: ALT, alanine aminotransferase; APRI, AST to platelet radio index; AST, aspartate aminotransferase; BMI, body mass index; CAP, controlled attenuation parameter; FIB-4, Fibrosis-4; FFA, free fatty acid; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LSM, liver stiffness measurement.
[a]*P* value from χ^2 test; other *P* values from *t* tests.
[b]*t* test *P* value allowing for unequal variances.

(10%) had clinically significant fibrosis (F ≥ 2), whereas 314 patients did not (LSM < 8.0 kPa).

Prevalence of Liver Steatosis and Fibrosis

The average liver fat measurement by CAP was 304 ± 63 dB/m. The prevalence of hepatic steatosis (CAP ≥ 274 dB/m) was 67% and with most having severe steatosis. Eight percent of patients had mild steatosis (274-289 dB/m), 7% moderate steatosis (290-301 dB/m) and 52% severe steatosis (≥302 dB/m). The average LSM for the overall cohort was 5.8 ± 2.5 kPa. The presence of clinically significant fibrosis (≥F2), defined as a kPa ≥ 8.0, was 11%. Prevalence of mild fibrosis (F1 = kPa ≥ 7.0 and ≤8.1) was 5%, moderate fibrosis (F2 = kPa ≥ 8.2 and ≤9.6) was 5%, advanced fibrosis (F3 = kPa ≥9.7 and ≤13.5) was 5%, and 1% had values suggestive of hepatic cirrhosis (F4 = kPa ≥13.6).

Table 2. Unadjusted odds of fibrosis

	Unadjusted OR					
	Overall sample				Patients not on insulin	
	SD	OR	95% CI	*P* value	OR	95% CI	*P* value
Clinical variables							
Age, y	12	0.78	(0.60, 1.02)	0.07	0.79	(0.57, 1.11)	0.18
Female	—	0.96	(0.54, 1.70)	0.89	0.92	(0.45, 1.88)	0.81
BMI, kg/m^2	6	2.56	(1.87, 3.50)	<0.01	2.14	(1.48, 3.08)	<0.01
Systolic blood pressure, mmHg	16	1.36	(1.03, 1.78)	0.03	1.63	(1.16, 2.28)	<0.01
Diastolic blood pressure, mmHg	10	1.39	(1.06, 1.83)	0.02	1.46	(1.05, 2.04)	0.02
Routine laboratory values + other variables							
Total cholesterol, mg/dL	45	0.97	(0.72, 1.29)	0.82	0.98	(0.68, 1.42)	0.92
LDL, mg/dL	38	0.80	(0.57, 1.11)	0.18	0.76	(0.50, 1.16)	0.21
HDL, mg/dL	12	0.63	(0.45, 0.87)	<0.01	0.65	(0.43, 0.98)	0.04
TG, mg/dL	97	1.50	(1.20, 1.88)	<0.01	1.55	(1.18, 2.03)	<0.01
FPG, mg/dL	39	1.20	(0.91, 1.59)	0.21	1.37	(0.96, 1.94)	0.08
FFA, mmol/L	0.16	1.30	(1.01, 1.67)	0.04	1.42	(1.06, 1.91)	0.02
Metabolic syndrome Z-score	1.1	1.77	(1.32, 2.38)	<0.01	1.89	(1.29, 2.76)	<0.01
A1c, %	1.8	1.11	(0.84, 1.46)	0.48	1.27	(0.86, 1.87)	0.24
AST, U/L	10	1.96	(1.54, 2.48)	<0.01	1.97	(1.45, 2.69)	<0.01
ALT, U/L	16	1.97	(1.53, 2.54)	<0.01	1.67	(1.24, 2.26)	<0.01
FIB-4	0.5	1.22	(0.96, 1.56)	0.11	1.42	(1.03, 1.94)	0.03
APRI	0.13	1.79	(1.40, 2.28)	<0.01	2.08	(1.48, 2.92)	<0.01
CAP, dB/m	63	3.03	(2.04, 4.49)	<0.01	2.48	(1.58, 3.88)	<0.01
CK-18, U/L	160	1.63	(1.30, 2.05)	<0.01	1.61	(1.22, 2.13)	<0.01
Adiponectin, µg/mL	3.3	0.64	(0.44, 0.93)	0.02	0.75	(0.49, 1.14)	0.17
Research laboratory values							
Insulin, µU/mL	6.6	—	—	—	2.05	(1.41, 2.99)	<0.01
HOMA-IR, mg/dL x µU/mL	2.5	—	—	—	1.85	(1.30, 2.63)	<0.01
Adipo-IR, mmol/L x µU/mL	3.1	—	—	—	1.85	(1.35, 2.54)	<0.01

Abbreviations: ALT, alanine aminotransferase; APRI, AST to platelet radio index; AST, aspartate aminotransferase; BMI, body mass index; CAP, controlled attenuation parameter; FIB-4, Fibrosis-4; FFA, free fatty acid; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LSM, liver stiffness measurement.
1-SD increase odds ratio for continuous variables.
SD calculated for overall sample, except for Research Lab variables (only those not on insulin).

Univariate and Multivariable Logistic Regression Results

To estimate the associations between all variables and odds of fibrosis individually, a series of logistic regression models were fit, calculating odds ratios (ORs) relative to 1-SD changes in those variables along with corresponding 95% CIs (Table 2). Clinical variables such as BMI, high-density lipoprotein (HDL), triglyceride, AST, ALT, and CAP were significant ($P < 0.01$) and research laboratory variables like FFA, CK-18, and adiponectin were also significant ($P < 0.05$). For other research laboratory variables such as insulin, HOMA-IR, and adipo-IR only a restricted sample size (patients who were not on insulin) was considered for the analysis. They remained significant ($P < 0.01$) when calculating ORs (and 95% CIs) (Table 2).

Using these unadjusted ORs from Table 2, multivariable logistic regression models of odds of fibrosis, which was of primary interest, were developed (Tables 3 and 4). Model 1 focused only on clinical variables (Table 3) in all 483 patients, with obesity (BMI) being the only clinical variable that remained as a strong predictor of fibrosis (area under the curve [AUC]: 0.73; $P < 0.01$). Model 2 added routine laboratory values and other variables to the clinical variables, with BMI, AST, and CAP being significant predictors with AUC = 0.81 and $P < 0.01$ (Table 3). We then refit variables from model 1 and model 2: BMI, AST, and CAP (without a separate selection process) on the restricted sample (n = 348) of those individuals not on insulin, and developed model 3 using the backwards stepwise process to select any additional research laboratory variables (insulin, HOMA-IR, and adipo-IR) that could be independently associated with fibrosis. Adipose tissue insulin resistance remained as the only significant predictor ($P = 0.03$) with an AUC = 0.75 (Table 4).

When beginning with the full set of identified variables for each model, collinearity was examined via variance inflation factors. The only variance inflation factor > 5 occurred when examining AST and ALT simultaneously. As a result, we only considered AST in building models 2 and 3 (Tables 3 and 4). However, we performed a sensitivity analysis by replicating the entire modeling process with ALT instead of AST. The same variables remained as

Table 3. Multivariable logistic regression results in all patients (n = 483): odds of fibrosis (LSM ≥8.0 kPa)

	SD[a]	Model 1: clinical variables[b]			Model 2: clinical + routine NAFLD variables[c]		
		OR	95% CI	*P* value	OR	95% CI	*P* value
Clinical variables							
BMI, kg/m^2	6	2.56	(1.87, 3.50)	<0.01	2.16	(1.50, 3.13)	<0.01
Routine laboratory values other variables							
AST, U/L	10	—	—	—	1.84	(1.40, 2.41)	<0.01
CAP, dB/m	63	—	—	—	1.82	(1.19, 2.80)	<0.01
Cross-validation AUC		0.73			0.81		

Abbreviations: AST, aspartate aminotransferase; AUC, area under the curve; BMI, body mass index; CAP, controlled attenuation parameter; HDL, high-density lipoprotein; LSM, liver stiffness measurement; MetS, metabolic syndrome; NAFLD, nonalcoholic fatty liver disease; TG, triglyceride.
[a]SD calculated for overall sample; odds ratios presented as a 1-SD increase
[b]All clinical variables included in the initial model and removed 1 by 1 until only significant ($P < 0.05$) predictors remained.
[c]BMI was included, along with all routine labs associated with fibrosis in the general sample (Table 2; HDL, TG, AST, CAP, MetS Z-score, CK-18, and adiponectin). Routine variables were removed 1 by 1 until only significant ($P < 0.05$) predictors remained.

Table 4. Multivariable logistic regression results in patients not on insulin (n = 343): odds of fibrosis (LSM ≥8.0 kPa)

	SD[a]	Model 1: clinical variables[b]			Model 2: clinical + routine NAFLD variables[c]			Model 3: clinical + routine NAFLD + research variables[d]		
		OR	95% CI	*P* value	OR	95% CI	*P* value	OR	95% CI	*P* value
Clinical variables										
BMI, kg/m^2	6	2.13	(1.48, 3.08)	<0.01	1.86	(1.23, 2.82)	<0.01	1.34	(0.79, 2.26)	0.28
Routine laboratory values + other variables										
AST, U/L	10				1.86	(1.32, 2.62)	<0.01	1.68	(1.11, 2.55)	0.01
CAP, dB/m	63				1.65	(1.02, 2.67)	0.04	1.51	(0.85, 2.67)	0.16
Research laboratory values										
Adipo-IR, mmol/L x µU/mL	3.1							1.51	(1.05, 2.16)	0.03
Cross-validation AUC		0.68			0.78			0.75		

Abbreviations: AST, aspartate aminotransferase; AUC, area under the curve; BMI, body mass index; CAP, controlled attenuation parameter; LSM, liver stiffness measurement; NAFLD, nonalcoholic fatty liver disease; OR, odds ratio.
[a]SD calculated for overall sample; odds ratios presented as a 1-SD increase.
[b]Final model from general sample (Table 1) replicated (no model selection).
[c]Final model from general sample (Table 1) replicated (no model selection).
[d]BMI, AST, and CAP were included in the model for patients not on insulin. Additional research laboratory values associated with fibrosis in this sample (Table 2: insulin, HOMA-IR, adipo-IR) were then included; these variables were removed 1 by 1 until only significant ($P < 0.05$) predictors remained.

predictors of fibrosis with almost equal level of significance and area under curve (Supplementary Tables S2A and 2B (10.6084/m9.figshare.21396960)) (19).

Association of Liver Fibrosis With Hepatic and Adipose Tissue Insulin Resistance

After excluding the patients on exogenous insulin, indexes of insulin resistance were calculated in 348 patients (Supplementary Table S1 (10.6084/m9.figshare.21396960)) (19). Patients with fibrosis had worse hyperinsulinemia (17 ± 7 vs 12 ± 6 µU/mL; $P < 0.001$) and higher FFA concentration (0.38 ± 0.11 vs 0.30 ± 0.16 mmol/L; $P = 0.02$). Both HOMA-IR and adipo-IR were significantly higher in patients with fibrosis when compared with patients without fibrosis (5.6 ± 2.5 vs 3.6 ± 2.4 mg/dL × µU/mL; $P < 0.001$ and 6.5 ± 4.5 vs 3.5 ± 2.9 mmol/L × µU/mL; $P = 0.006$, respectively) (Supplementary Table S1 (10.6084/m9.figshare.21396960)) (19). This was evident when patients were grouped based on the severity of liver fibrosis as measured by LSM (Fig. 1), with worse insulin resistance across the spectrum of hepatic fibrosis (linear trend $P < 0.0001$). Both HOMA-IR and adipo-IR were significantly higher in all groups with patients whose LSM ≥ 6.0 kPa when compared with the control group without any potential of liver fibrosis with LSM < 5.0 kPa (LSM between 5.0 and 6.9 may be considered a gray zone where still a handful of patients may have early F1 fibrosis; although the majority will not have fibrosis) (Fig. 1).

To exclude the contribution of obesity and hepatic steatosis, we then adjusted the data for BMI, AST, and CAP. When doing so, HOMA-IR and CK-18 lost significance as clinical parameters related to fibrosis severity. Only adipo-IR emerged as a relevant factor in patients with clinically significant fibrosis (LSM ≥ 8 kPa) compared with healthy control patients with LSM < 5.0 kPa after adjusting to BMI, AST, and CAP ($P < 0.05$) (Fig. 1).

Patients With Liver Biopsy

A total of 27 patients agreed to a liver biopsy. Clinical characteristics of these patients were similar to those with LSM ≥

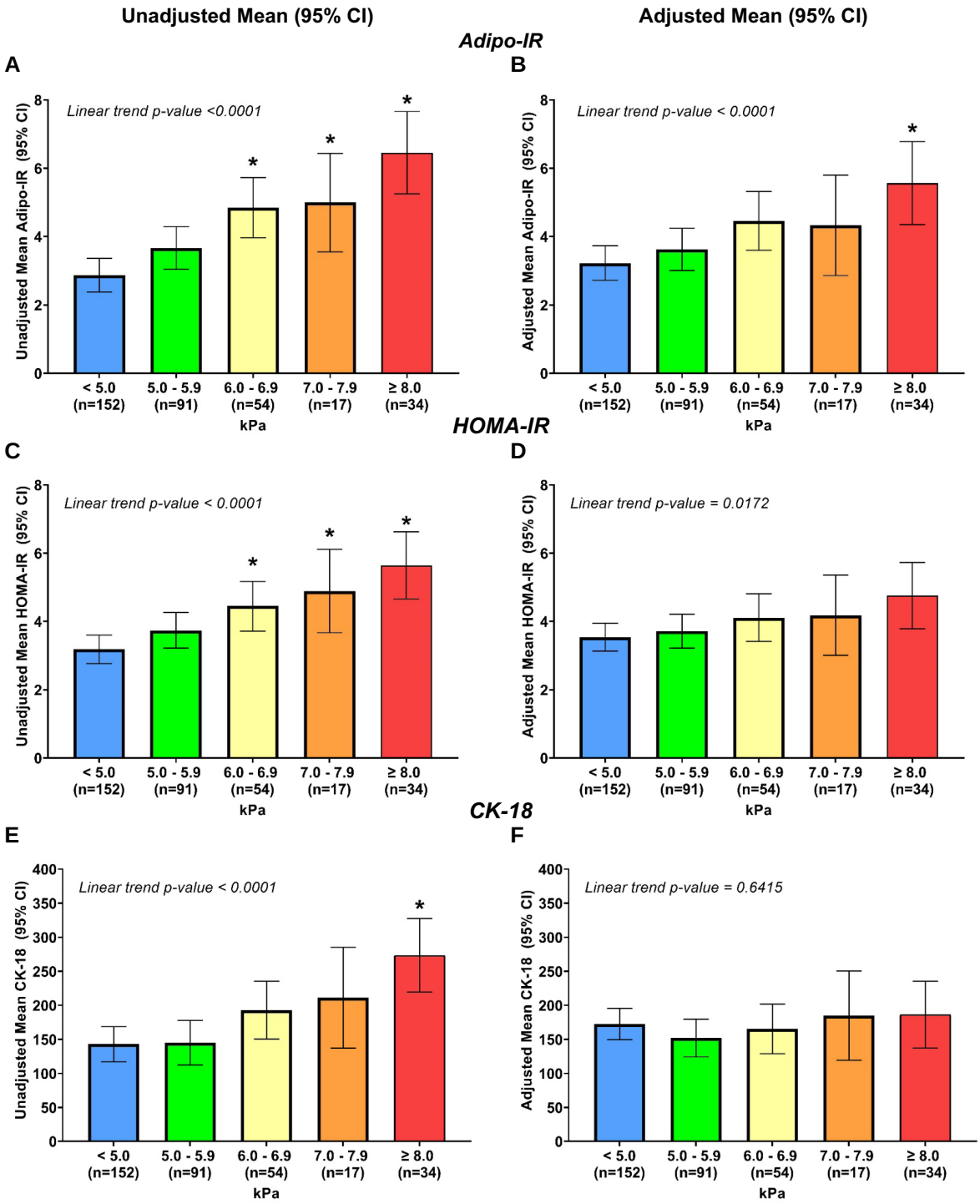

Figure 1. Adipose tissue insulin resistance (adipo-IR) (A, B), hepatic insulin resistance (HOMA-IR) (C, D), and CK-18 (E, F), across the spectrum of severity of hepatic fibrosis, unadjusted and adjusted for body mass index, plasma AST concentration, and liver fat measured by elastography (CAP), respectively. *Bonferroni adjusted $P < 0.05$ compared to kPa < 5.0 group.

8.0 kPa but declined having a liver biopsy (n = 30) (Supplementary Table S3 (10.6084/m9.figshare.21396960)) (19), suggesting that histological results of people who were biopsied would be similar to the entire cohort of patients at risk of clinically significant fibrosis (LSM ≥ 8.0 kPa). Both groups were well matched for major variables (age, 56 ± 2 vs 58 ± 2 years; sex (male), 50 vs 40%; A1c, 7.5 ± 0.3 vs 7.8 ± 0.3%). Liver steatosis and fibrosis were not different between these groups (CAP, 358 ± 9 vs 346 ± 9 dB/m, $P = 0.37$; LSM, kPa:10.9 ± 0.6 vs 11.1 ± 0.8, $P = 0.81$).

The NAFLD activity score (NAS) was calculated as a sum of the numerical scores applied to steatosis, hepatocellular ballooning, and lobular inflammation. Eleven of 27 patients (41%) had "definite NASH" (NAS ≥ 5). Liver fibrosis measured by LSM and steatosis measured by CAP showed a positive correlation with NAS score (LSM, $r = 0.35$; $P = 0.07$;

CAP, $r = 0.46$; $P = 0.02$). Necro-inflammation score, a combined score of inflammation and ballooning, also positively correlated with liver fibrosis ($r = 0.44$; $P = 0.02$) and steatosis ($r = 0.52$; $P < 0.01$).

Patients With Elevated AST and ALT

Five percent of patients had AST > 40 U/L, with 42% patients having clinically significant fibrosis (F ≥ 2 or LSM ≥ 8 kPa) with a mean LSM, 8.0 ± 1 kPa and CAP, 335 ± 10 dB/m (92% of these having steatosis). Patients with ALT > 40 U/L were 9% in this cohort, of which 43% patients had clinically significant fibrosis (LSM, 7.7 ± 1 kPa) and 93% had steatosis measured by CAP (CAP, 342 ± 8 dB/m). Considering an aminotransferase lower cutoff of >30 U/L as abnormal (11), the number of subjects with elevated aminotransferases increased for AST from 5% to 11% (35% with ≥ F2) and for ALT from 9% to 19% (22% with ≥ F2). Plasma aminotransferases positively correlated with liver fibrosis measured by LSM in patients with AST/ALT ≥40 U/L (AST, $r = 0.67$; $P < 0.01$; ALT, $r = 0.40$; $P < 0.01$).

Discussion

The determinants of liver fibrosis in patients with NAFLD remain poorly understood, particularly in people with T2D, who are known to have the highest prevalence and worse long-term prognosis (7, 15). To gain insights into these factors, especially the role of IR as a potential target for intervention, we examined in a large number of patients with diabetes the clinical and metabolic factors associated with disease severity. Several aspects make this analysis clinically relevant: (1) Studying unselected patients in the "real world," while attending their regular primary care outpatient visits and unaware of having any liver disease (ie, not recruited late in the natural history from hepatology clinics); (2) an in-depth phenotyping by history confirmed by electronic medical record review, physical examination, clinical parameters, and measurement of specific factors with potential to modulate fibrosis progression (eg, FFA, insulin, adiponectin, CK-18); (3) a stepwise risk factor analysis, moving from clinical characteristics to routine laboratories and to metabolic determinants of NASH and fibrosis to assess their relative predictive value; and (4) comparing the relative contribution of insulin resistance at the level of the liver (ie, HOMA-IR) vs adipose tissue (ie, adipo-IR), as well as with clinical parameters, something not examined in any prior epidemiological study. Taken together, this approach allowed this work to establish that adipose tissue insulin resistance may play(s) a major role in the severity of liver fibrosis in patients with T2D and NAFLD, greater than previously outlined. This finding strongly aligns with recent guidelines (9, 11) that recommend treating people with obesity and/or T2D with agents that target adipose tissue, either by reducing fat mass with weight loss (ie, lifestyle, GLP-1Ras, and/or bariatric surgery when indicated), or with pioglitazone that reverses adipose tissue dysfunction in NASH.

The role of adipose tissue dysfunction ("lipotoxicity") in the development of NASH has been extensively studied in recent years (1, 7, 16, 20). However, its precise role in the development and progression of liver fibrosis has been difficult to establish. Although there are a number of pathways demonstrated in vitro and in animal models (16), this is more difficult to prove in humans. Some studies from our group (21-24), and from others (16, 20), have shown an association between obesity and severity of steatohepatitis or fibrosis. This study goes a step further by directly linking adipose tissue IR with liver fibrosis in a large group of unselected and carefully characterized patients with T2D. The adipo-IR index is a strong predictor of the severity of steatohepatitis (21-24) and has been well validated by comparing to direct measures of adipose tissue insulin sensitivity using the gold-standard euglycemic insulin clamp (25). Its change is also correlated with the response to pioglitazone therapy in patients with NAFLD (26) or biopsy-proven NASH (21). The current work confirms prior work on the role of obesity in promoting liver fibrosis (27) and advances the field by examining this in a larger cohort of unselected patients with T2D and pinpointing that it is adipose tissue dysfunction, carefully quantitated as adipo-IR, what plays a key role in fibrosis progression. This knowledge may assist in the clinic to predict those who are at the highest risk of having worse liver fibrosis and/or more rapid progression and target them for more aggressive intervention.

Because hepatic IR may be another important determinant of the severity of liver disease in NASH, we examined its role relative to adipose tissue insulin resistance. In univariate, unadjusted fibrosis risk factor analysis, a number of clinical (ie, BMI, hypertension, metabolic syndrome) and routine laboratory parameters (low plasma HDL-cholesterol and elevated triglycerides, or increased plasma aminotransferases) correlated with hepatic fibrosis (Table 2). However, although HOMA-IR correlated in univariate analysis with fibrosis severity (Fig. 1, panel C), once clinical parameters were incorporated such as obesity (BMI), AST, and hepatic steatosis (11), it no longer correlated with significant liver fibrosis (Fig. 1D). Diagnostic panels such as FIB-4 or APRI were also associated with fibrosis, as expected, because of AST being included in their equations and being a known direct indicator of hepatocyte injury and liver fibrosis (12). Not unexpectedly, hepatic steatosis (ie, CAP) correlated with liver fibrosis, being a direct biomarker of IR and an unfavorable metabolic milieu (4, 5). However, in stepwise multivariate analysis, a clearer picture emerged (Tables 3 and 4). Among only clinical variables (model 1), just BMI remained as relevant in predicting the severity of liver fibrosis. When routine chemistries, liver fat (11) and more specific measures blood biomarkers of liver fibrosis were included (HDL, TG, AST, CAP, MetS Z-score, CK-18, and adiponectin), only direct measures such as AST and CAP remained significant. When assessing indirect measurements linked to the pathophysiology of the disease, such as hepatic insulin resistance (HOMA-IR) or even hepatocyte injury and apoptosis (CK-18), only adipose tissue insulin resistance/lipotoxicity (adipo-IR) remained significant among patients with clinically significant fibrosis (≥F2; Fig. 1B). As shown in Fig. 1, in multivariate analysis, neither HOMA-IR (ie, hepatic insulin resistance; Fig. 1D) or CK-18 (Fig. 1F) was relevant anymore to predict the severity of liver fibrosis. This highlights the unique role of "lipotoxicity" and dysfunctional adipose tissue and suggests that hepatic IR is more a consequence rather than a primary defect in patients with NASH and fibrosis. In other words, that hepatic insulin resistance appears primarily driven by adipose tissue insulin resistance and the elevated FFA flow to the liver with accumulation of toxic lipid metabolites, such as diacylglycerols or ceramides (16). The clinical implication is that there is a need to target adipose tissue to modify the natural history of the disease and reverse steatohepatitis and profibrogenic signals that drive fibrosis.

The study had some limitations: (1) not all patients with apparent fibrosis on imaging (ie, LSM) received a liver biopsy. However, the characteristics of patients with liver fibrosis by LSM (VCTE) that underwent a liver biopsy were similar to those compared with those that did not undergo the procedure (Supplementary Table S3 (10.6084/m9.figshare.21396960)) (19). Mean LSM was 10.9 kPa vs 11.1 kPa, respectively. (2) No adipose tissue was obtained to directly assess molecular mechanisms of adipose tissue dysfunction. This work will call for additional in-depth studies that may allow targeted therapies in NASH but help further understand the success of the thiazolidinedione pioglitazone, and more recently of the pan-peroxisome proliferator–activated receptor lanifibranor (28); (3) limitations related to a cross-sectional study; (4) adipo-IR is a reliable estimate of adipose tissue insulin resistance, whereas HOMA-IR appears to be a weaker estimate of hepatic IR. We are now examining this aspect in a larger longitudinal study involving people with and without T2D; and (5) more precise measures of IR would have been ideal, but impossible to perform on a large scale in epidemiological studies. Measuring visceral adiposity will also be important in future studies because it affects both lipid and glucose metabolism, primarily increases gluconeogenic flux, and worsens insulin resistance (26), as well as cardiometabolic risk and progression of NAFLD (15). Weight loss and GLP-1RAs reduce subcutaneous and visceral fat in people with steatosis, but their relative contribution to clinical benefit is unclear (15, 29-31). However, the relevance of visceral fat has been highlighted by a recent study that concluded that following pioglitazone treatment reduction in visceral fat was strongly associated to steatohepatitis improvement in people with T2D and NASH, even as subcutaneous fat slightly increased (24). Future studies will need to address these aspects of the disease.

In summary, this study shows that among a population of people with T2D in the "real world" and at a high risk for cirrhosis, adipose tissue IR plays a central role in the development of advanced fibrosis. An index to quantify it (adipo-IR) can more specifically pinpoint this risk than just obesity by BMI *per se*. Focusing clinical efforts to reverse "sick (insulin-resistant) fat" with either lifestyle, weight loss (ie, GLP-1RA) or pioglitazone, is closely aligned with current guidelines that call for early diagnosis and intervention, and at a time when cirrhosis can still be prevented.

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Author Contributions

S.K. was responsible for laboratory measurements, data analysis, and interpretation, and writing/editing of the manuscript. R.D. assisted with laboratory measurements and data analysis. E.G.L., R.L., S.S., D.B., L.R.S., J.B., JPR, J.l., F.B., G.S., J.P., P.T., F.O., S.S., L.R.S.-S., and D.B. were responsible for patient recruitment and follow-up, data acquisition and interpretation, and critical revisions of the manuscript. X.C., M.G., and F.B. contributed to data analysis and critical revisions of the manuscript. P.B. undertook pathology reading of liver biopsies. K.C. contributed to the study design, analysis of the results and interpretation, writing, and critical revisions of the manuscript. K.C. is the guarantor of this work, and as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of Interest

K.C. has received research support as principal investigator from the University of Florida from the National Institutes of Health, Cirius, Echosens, Inventiva, Novartis, Novo Nordisk, Poxel, and Zydus. K.C. is also a consultant for Allergan, Altimmune, Arrowhead, AstraZeneca, BMS, Boehringer Ingelheim, Coherus, Eli Lilly, Fractyl, Hanmi, Genentech, Gilead, Intercept, Janssen, Pfizer, Prosciento, Madrigal, and Novo Nordisk. All other authors have no potential conflicts of interest relevant to this article.

Data Availability

Restrictions apply to the availability of some or all data generated or analyzed during this study to preserve patient confidentiality or because they were used under license. The corresponding author will on request detail the restrictions and any conditions under which access to some data may be provided.

Prior Publication

Portions of this study were presented in an abstract at the American Diabetes Association's Virtual 80th Scientific Sessions June 12-16, 2020 (1463-*P*).

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