Metformin Use Is Associated with Hepatic Fibrosis Regression in Nonalcoholic Fatty Liver Disease

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) will soon become the leading etiology of end-stage liver disease, yet there are currently no approved therapies to reduce hepatic fibrosis in NAFLD. Animal studies have shown that metformin can reduce hepatic fibrosis, yet human data is limited, and metformin's potential as a treatment for NAFLD remains controversial. A commonly cited meta-analysis of 52 patients concluded that metformin does not have histologic benefits in NAFLD, while a pilot clinical trial that found histologic benefits attributed this effect to metformin-induced weight loss. To clarify the effect of metformin on hepatic fibrosis in NAFLD, we evaluated the association between metformin use and fibrosis regression in a cohort of non-cirrhotic NAFLD patients with paired liver biopsies at least one year apart.

Using a database of clinical pathology reports, we identified patients who had undergone two clinically indicated liver biopsies at least one year apart, with baseline biopsy demonstrating non-cirrhotic NAFLD, no history of excessive alcohol consumption, and no histological or serological evidence of a competing etiology of chronic liver disease. Among these, we identified 101 patients with either continuous metformin use or no metformin use at either baseline or follow-up time points. The primary outcome was a decrease in fibrosis stage of ≥1 between baseline and follow-up biopsy. We used Cox proportional hazards regression analysis to assess the association between metformin use and fibrosis regression among subjects with non-cirrhotic NAFLD at baseline.

Fibrosis regression occurred in 16/30 (53%) metformin users and 28/71 (39%) non-users. The unadjusted hazard ratio (HR) for fibrosis regression among metformin users compared to non-users was 2.21 (95% CI 1.17-4.15). Moreover, after adjustment for established predictors of histologic fibrosis regression, including baseline fibrosis stage, age, diabetes status, body mass index, interval total body weight loss of ≥10%, as well as weight loss surgery, this association remained significant (HR 3.72, 95% CI 1.53-9.06). Among those who regressed, weight loss surgery was less common in metformin users (25%) compared to non-users (43%). The mean annual change in Brunt fibrosis stage between baseline and follow-up biopsy was -0.32 (SE 0.08) in metformin users and -0.11 (SE 0.05) in non-users ($p = 0.04$). Fibrosis progression was less frequent in metformin users, occurring in 1/30 (3%) metformin users and 18/71 (25%) non-users. However, this association was not statistically significant on Cox regression analysis.

Metformin use is associated with an increased frequency of hepatic fibrosis regression in non-cirrhotic NAFLD, even after adjustment for interval weight loss. Our findings support a potential role for metformin in fibrosis regression in non-cirrhotic NAFLD.

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GLOSSARY OF ABBREVIATIONS

ALT	Alanine aminotransferase
AMPK	Adenosine monophosphate-activated protein kinase
AST	Aspartate aminotransferase
BMI	Body mass index
CCR	C-C motif chemokine receptor
CI	Confidence interval
DPP-4	Dipeptidyl peptidase-4
EHR	Electronic health record
FFPE	Formalin-fixed paraffin-embedded
FPR	Fibrosis progression rate
GES	Gene expression signature
GLP-1	Glucagon-like peptide-1
HCC	Hepatocellular carcinoma
HIV	Human immunodeficiency virus
HR	Hazard ratio
HSC	Hepatic stellate cell
MCD	Methionine- and choline-deficient
MGH	Massachusetts general hospital
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
SD	Standard deviation
SE	Standard error
SGLT-2	Sodium-glucose cotransporter-2
T2DM	Type 2 diabetes mellitus
TBWL	Total body weight loss
TGFβ	Transforming growth factor β (TGFβ)
TZD	Thiazolidinedione
WLS	Weight loss surgery

SCHOLARLY PROJECT QUESTION AND CONTRIBUTION TO THE WORK

I initially set out to identify and validate a prognostic gene expression signature (GES) for incident cirrhosis using archival liver tissue from a previously-identified cohort of 129 adults with non-cirrhotic NAFLD. However, as I reviewed the liver biopsy pathology reports from this cohort, I determined that most patients would have to be excluded because they had other etiologies of liver disease. To identify a new cohort of NAFLD patients who had undergone serial liver biopsies and had formalin-fixed paraffin-embedded (FFPE) liver tissues available, I collaborated with a pathologist (R. Masia) to design a search query in a pathology report database. We applied this query to all reports from liver biopsies that took place at MGH between 2000 and 2017, identifying 10,881 non-allograft liver biopsies with pathology report text broadly consistent with NAFLD. By programmatically searching for duplicate medical record numbers in R, I identified a subset of 1,178 patients who had undergone two liver biopsies.

Using an electronic health record (EHR) viewer designed for research use, I reviewed the clinical pathology reports for these liver biopsies to exclude those without NAFLD. I completed exhaustive clinical data collection for both baseline and follow-up time points via manual HER review for approximately 80% of these patients. Two other study contributors (T.G. Simon, Z.P. Gitto) assisted with the chart review for the remaining 20% of patients. We collected data on demographics, habits, biometrics, lab results, medication use, bariatric surgical history, mortality, cause of death, and any evidence of another etiology of liver disease. In total, we identified 137 adult patients with baseline non-cirrhotic NAFLD with sufficient baseline and follow-up clinical data. Using the text of the pathology reports, I coded each of the 274 liver biopsies for Brunt fibrosis stage, as did a second observer (Y.D. Kim). Other collaborators contributed to study design (R.T. Chung, K. Corey, J. Luther, S.J. Patel) or sample identification (S. Rwema, S. Osganian).

In batches of 50-54 blocks, I have used a microtome to cut tissue slices from these FFPE blocks and sent these tissue samples offsite for RNA extraction. To date, I have completed processing of two batches. All available quality assessments have been highly promising (96% of samples have passed, including samples as old as 14 years). Once RNA extraction is completed for all samples, we will proceed with RNA sequencing and the GES analysis described in my original project proposal. As sample processing was delayed by bureaucratic restrictions limiting retrieval of FFPE blocks from storage, I concurrently conducted an analysis of the available clinical data. I assessed the relationship between metformin use and fibrosis regression among patients with baseline non-cirrhotic NAFLD, and the manuscript attached herein as an appendix details this project, with submission for publication pending confirmation of fibrosis stages from clinical pathology reports via blinded pathology re-reads. I was responsible for all data analysis and manuscript writing, with helpful guidance provided by K.C. Corey and R. T. Chung.

Metformin use is associated with hepatic fibrosis regression in nonalcoholic fatty liver disease

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Background: Nonalcoholic fatty liver disease (NAFLD) will soon become the leading etiology of end-stage liver disease, yet there are currently no approved therapies to reduce hepatic fibrosis in NAFLD. Animal studies have shown that metformin can reduce hepatic fibrosis, yet human data is lacking, and metformin's potential as a treatment for NAFLD remains controversial. To clarify the effect of metformin use on hepatic fibrosis in NAFLD, we evaluated the association between metformin use and fibrosis regression in a cohort of non-cirrhotic NAFLD patients with paired liver biopsies at least one year apart.

Methods: Using a database of clinical pathology reports, we identified patients who had undergone two clinically-indicated liver biopsies at least one year apart, with baseline biopsy demonstrating non-cirrhotic NAFLD, no history of excessive alcohol consumption, and no histological or serological evidence of a competing etiology of chronic liver disease. Among these, we identified 101 patients with either continuous metformin use or no metformin use at either baseline or follow-up time points. The primary outcome was a decrease of 1 or more Brunt fibrosis stages between baseline and follow-up biopsy. We used to Cox proportional hazards regression analysis to assess the association between metformin use and fibrosis regression among subjects with non-cirrhotic NAFLD at baseline.

Results: Fibrosis regression occurred in 16/30 (53%) metformin users and 28/71 (39%) of non-users. The unadjusted hazard ratio (HR) for fibrosis regression among metformin users compared to non-users was 2.21 (95% CI 1.17-4.15). This association remained significant after adjusting for established predictors of histologic fibrosis progression or regression, including baseline fibrosis stage, age, diabetes status, body mass index, interval total body weight loss of 10% or more, as well as bariatric surgery (HR 3.72, 95% CI 1.53-9.06). The mean annual change in Brunt fibrosis stage between baseline and follow-up biopsy was -0.32 (SE 0.08) for metformin users compared to -0.11 (SE 0.05) for non-users (p = 0.04).

Conclusions: Metformin use is associated with an increased incidence of hepatic fibrosis regression in non-cirrhotic NAFLD, even after adjusting for interval weight loss. Our findings support a potential role for metformin in inducing fibrosis regression in non-cirrhotic NAFLD.

Introduction

Nonalcoholic fatty liver disease (NAFLD) affects 24% of adults worldwide and will soon become the leading etiology of end-stage liver disease requiring liver transplantation.[1,2,3] While simple steatosis is typically benign, nonalcoholic steatohepatitis (NASH) progresses to cirrhosis in up to 25% of patients,[4] and those without NASH but with hepatic fibrosis at baseline are also at risk of progressing to cirrhosis.[5] Patients with NAFLD are at a significantly increased risk of both all-cause and liver-specific mortality, and the risk of liver-specific mortality, in particular, has been found to increase exponentially as hepatic fibrosis increases from the F0 stage, or no fibrosis, to the F4 stage, or cirrhosis.[6] Furthermore, several studies have indicated that fibrosis stage, but not steatohepatitis or any other histological characteristic, is predictive of both all-cause and disease-specific mortality in NAFLD.[5] In the United States, where rates of obesity and diabetes mellitus remain high, the overall prevalence of NAFLD is projected to reach 33.5% by 2030, and the incidence of decompensated cirrhosis and that of hepatocellular carcinoma (HCC) will increase to 105,430 and 12,240 new cases per year, respectively, by 2030.[7] Despite the significant morbidity and mortality associated with NAFLD fibrosis, at present, there are no approved anti-fibrotic therapies for NAFLD.[8] Although cenicriviroc, a dual C-C motif chemokine receptor-2/5 (CCR2/CCR5) inhibitor, has shown promise as a treatment for NASH fibrosis and is currently in a phase III clinical trial,[8,9] given the current lack of long-term follow-up data, questions remain regarding the durability of the antifibrotic response that this new drug appears to induce after a one year of treatment.[10]

The pathogenesis of NAFLD is now most often conceptualized by a "two-hit" model, in which insulin resistance leads to hepatic lipid accumulation (the first hit), which may, in turn, lead to activation of multiple pathways resulting in oxidative stress, inflammation, and fibrosis (the second hit).[11] Given the central role of insulin resistance in both the development and progression of NAFLD,[12] there has been much interest in insulin sensitizers as potential disease-modifying treatments.[13] The most commonly-prescribed antidiabetic medication, metformin, is an insulin sensitizer that acts to decrease hepatic gluconeogenesis and increase skeletal muscle glucose uptake.[13] Unlike other insulin sensitizers, the biguanide metformin is associated with weight loss,[14] and furthermore is a low-cost drug with favorable long-term safety and tolerance profiles.[15] Several animal studies have shown that metformin can reduce hepatic fibrosis. In a non-diabetic methionine- and choline-deficient (MCD) plus high-fat diet mouse model of NASH, metformin reduced hepatic steatosis, inflammation, and fibrosis without affecting glucose tolerance or insulin sensitivity.[16] Metformin was found to lessen intrahepatic fibrosis in rats with biliary cirrhosis induced by common bile duct ligation,[17] and to ameliorate carbon tetrachloride-induced TGF-β1 activation and resultant liver fibrosis in mice,[18] suggesting potential anti-inflammatory and or anti-fibrotic

effects independent of metformin's ability to reduce intrahepatic steatosis. Additionally, metformin has been observed to have anti-fibrotic effects in mouse models of both renal[19] and pulmonary fibrosis.[20,21]

Based on the results of several large population-based cohort studies, metformin use is known to reduce the risk of HCC in patients with type II diabetes mellitus (T2DM).[22,23,24] More recently, an association has been reported between metformin and longer median overall survival in those with T2DM and who had developed HCC, as well as improved overall hepatic function.[14] In patients with cirrhosis and T2DM, continuing metformin after cirrhosis is diagnosed is also associated with improved survival.[25] In a 48-week pilot clinical trial in which the majority patients were non-diabetic at baseline, investigators found that metformin treatment resulted in decreased levels of alanine aminotransferase (ALT) and improvements in hepatic inflammation and hepatocellular injury in patients with non-cirrhotic NASH.[15] However, subsequent clinical trials investigating metformin as a potential treatment for non-cirrhotic NAFLD have had less promising results, and consequently, it is widely considered to have only minimal effects on liver histology when used as a single agent and is not currently recommended as a treatment for NAFLD.[11] In a meta-analysis commonly cited by clinical practice references such as UpToDate that describe metformin as an ineffective treatment for NAFLD,[26] the authors concluded that metformin is not an effective treatment for NASH, yet this was based on an examination of three trials that included a total of 52 patients and an average study length of only 36 weeks.[13] In contrast, the time to progress one fibrosis stage in NAFLD is has been shown to be 7.1 years on average, even when there is hepatic inflammation at baseline.[27] In a more recent systematic review and meta-analysis of metformin in NAFLD, which included nine studies with a maximum study period of 12 months, the authors concluded that "the effect of metformin on liver histology remains unclear."[28]

Because the results of prior clinical trials examining the effects of metformin in non-cirrhotic NAFLD have been variable, its potential as an effective, safe, and low-cost treatment remains controversial. These trial findings are at odds with animal studies, which have reliably demonstrated antifibrotic effects of metformin the liver, kidney, and lungs.[29] We hypothesized that the disconnect between animal studies and human studies, as well as the heterogeneity in findings among clinical trials investigating metformin as a treatment for NAFLD, may be at least in part due to insufficient length of treatment with metformin, given that fibrosis takes years to develop, and that metformin may exert an anti-fibrotic effect that is more prominent than effects on other histologic parameters in human NAFLD. To clarify the effect of longer-term metformin use on fibrosis in non-cirrhotic NAFLD, we sought to evaluate the association between metformin use and fibrosis regression in a cohort of 137 non-cirrhotic NAFLD patients with paired liver biopsies at least one year apart, with a mean interval between biopsies

of 3.5 years and a maximum follow-up interval of 13.4 years. Given the scarcity of longitudinal data on histologic fibrosis stage in NAFLD patients, data from this cohort is particularly useful for assessing a potential antifibrotic effect of metformin over a longer time period than ever previously examined.

Methods

Cohort Identification

The process we used to identify a cohort of adult patients with baseline non-cirrhotic NAFLD who had undergone two clinically-indicated liver biopsies at least one year apart (i.e., histologic data available from two distinct time points) at Massachusetts General Hospital (MGH) is detailed in Figure 1. We first designed and applied a search query to an institutional database of clinical pathology reports, with the initial goal of identifying all liver biopsies conducted at MGH between 2000 and 2017 that had clinical pathology report text broadly consistent with a diagnosis of NAFLD. We consulted with a hepato-pathologist (RM) to design this search query. The year 2000 was chosen as a starting point because NASH and especially NAFLD were not regularly recognized or described by pathologists as a distinct clinical entity prior to this year.[30] Using the appropriate Boolean logic, we searched for clinical pathology reports that included the terms 'steatohepatitis' or 'cirrhosis' or 'hepatocellular carcinoma', but not the term 'allograft'. We based this search query on the terms that, based on empiric prior experience, would most often appear in a pathology report in which the diagnosis is NAFLD, or a report in which the diagnosis could be a sequala of NAFLD found on a second biopsy, such as cirrhosis or HCC. This strategy was necessitated by the search functionality of this database, which does not allow searches for particular diagnoses or ICD codes. This initial search yielded a total of 10,881 candidate liver biopsies.

By programmatically searching for medical record numbers that appeared at least two times among these candidates, we identified 1,178 candidate patients with data available from two or more liver biopsies and age of 18 or older at the time of baseline biopsy. If more than two liver biopsies were available, we considered those with the greatest time interval between them. We excluded those with an interval between baseline and follow-up liver biopsy of fewer than 12 months. Subsequently, we performed electronic health record (EHR) review to exclude patients with histological or serological evidence of a non-NAFLD etiology of liver disease, including but not limited to baseline cirrhosis or HCC, hepatitis B or C-related liver disease, autoimmune liver diseases, human immunodeficiency virus (HIV), alcohol-related liver diseases. Those with minimal baseline hepatic steatosis (<5% of hepatocytes) were also excluded, as they would not qualify for a diagnosis of NAFLD. Those with a chart history of significant alcohol consumption (more than four drinks in a single day or seven drinks pin a week for women, or more

than four drinks in a day or 14 drinks per week in men) were also excluded. Patients were also excluded if they did not have pertinent lab data, including complete blood counts and liver function tests, within three months of each liver biopsy. This yielded 137 adult patients with paired liver biopsy data, baseline non-cirrhotic NAFLD, sufficient clinical covariate data, and no evidence of another etiology of liver disease.

Metformin Use

The use of metformin was ascertained from a comprehensive medication history obtained by review of physician notes and medication list entries in the EHR dated within one month of baseline and follow-up liver biopsies. Continuous metformin use was defined as the use of metformin at the time of baseline biopsy and throughout the interval up to and including the time of follow-up biopsy. Thirteen patients who either initiated or discontinued metformin during the interval between baseline and follow-up liver biopsy (i.e., those with non-continuous metformin use) were thus excluded from subsequent analyses. Metformin dose at the time of baseline biopsy was also recorded. Information on the use of other potentially disease- or fibrosis risk-modifying medications, including vitamin E, aspirin, statins, and glitazones,[31,32] as well as use of any other anti-diabetic medications, including insulin, sulfonylureas, meglitinides, glucagon-like peptide-1 (GLP-1) analogs and agonists, dipeptidyl peptidase-4 (DPP-4) inhibitors, and sodium-glucose cotransporter-2 (SGLT-2) inhibitors, was also collected.

Collection of Histopathological Data

Histopathological data were obtained from the clinical pathology reports of patients' clinically-indicated liver biopsies performed as a part of routine clinical care at MGH. Clinical pathology reports from both baseline and follow-up liver biopsy were reviewed. The primary outcome of fibrosis regression was defined as a decrease in the Brunt fibrosis stage of one or more full stages between baseline and follow-up liver biopsy (i.e., a decrease from stage 2 to stage 1A would be considered fibrosis regression, but not a decrease from stage 1C to 1A). Similarly, fibrosis progression was defined as an increase in the Brunt fibrosis stage of one or more full stages between baseline and follow-up biopsy. A biopsy was considered positive for NASH if the clinical pathology report indicated that both lobular inflammation and hepatocyte ballooning were present.[33] As the outcome of interest was fibrosis regression among patients with baseline non-cirrhotic NAFLD, 23 patients with a diagnosis of NAFLD, but with no fibrosis at baseline per the clinical pathology report, were excluded from subsequent analyses. In total, 101 adult patients with non-cirrhotic NAFLD at baseline, paired liver biopsies at least one year apart, and either continuous or no metformin use were ultimately included in our analyses. The majority of clinical pathology reports

explicitly noted a Brunt fibrosis stage, but for those that did not, we assigned a Brunt fibrosis stage based on matching the pattern of fibrosis described in detail in the clinical pathology report to the staging criteria published by Brunt et al.[34,35] For example, a report in which the biopsy was noted to have perisinusoidal and portal or periportal fibrosis, but no bridging fibrosis, the biopsy would be assigned a Brunt fibrosis stage of 2. Two independent observers (ACE and YDK) assessed these descriptions in all clinical pathology reports to assign a stage and to ensure that the stated stage matched the histological description, as well as to ensure that no histological evidence of another etiology of liver disease was mentioned in the report. Of the 202 paired liver biopsy reports reviewed, there was a disagreement between observers in only 9/202 (4.5%) of cases, and these ties were broken by review of the clinical pathology report text by a clinical hepatologist specializing in NAFLD (KEC).

Statistical Analysis

Baseline demographic, clinical, and histological characteristics of these 101 non-cirrhotic NAFLD patients were assessed. In order to compare the distribution of these baseline variables patients with continuous metformin use (continuous metformin users) and those with no metformin use (non-users), univariate analyses were carried out using the Welch t-test for continuous data and the Fischer exact test for categorical data. Univariate associations between metformin use and histological outcomes (e.g., fibrosis regression) were determined by the hazard ratio (HR) and 95% confidence interval (CI) calculated using Cox proportional hazards regression. To ensure any observed associations were not attributable to other confounding variables, we also performed a multivariate Cox proportional hazards regression analysis, including as covariates previously established predictors or known influencers of fibrosis progression in NAFLD: baseline fibrosis stage, age, body mass index (BMI), diabetes mellitus status, interval total body weight loss (TBWL) ≥ 10%, and weight loss surgery (WLS).[36, 37] Patients who had a chart history of WLS at or after the time of baseline biopsy, but before the time of follow-up biopsy, were considered positive for WLS for the purposes of this analysis.

We also performed a multivariate analysis that included aspirin and statin use as covariates to ensure that observed associations were independent of the use of these frequently co-prescribed medications, which have been found in population studies to be associated with decreased incidence and slowed progression of hepatic fibrosis.[38,39] Mean fibrosis progression rates (i.e., change in fibrosis stage per year) were calculated for each group, with the statistical significance of the difference between groups assessed via the Welch t-test. A two-tailed p-value <0.05 was considered statistically significant. R version 3.5.1 (Berkley, CA) was used for all statistical analysis.

Results

Baseline Characteristics

The demographic, clinical, and histologic data of 101 patients with baseline non-cirrhotic NAFLD fibrosis, grouped by metformin use, is shown in Table 1. Thirty patients used metformin continuously over an average interval of 2.8 years between baseline and follow-up biopsy. Seventy-one patients had no metformin use and averaged an interval of 3.7 years between biopsies. Continuous metformin users were more likely to be older, have diabetes mellitus, and use aspirin, statins, and sulfonylureas compared with non-users. Baseline BMI, interval weight loss, and the incidence of weight loss were similar between the two groups. Moreover, thiazolidinedione (TZD) and vitamin E use were similarly low in both groups, with only 1/71 non-users (1.4%) and 1/30 metformin users (3.3%) also using a TZD, and only 8/71 non-users (11.3) and 4/30 metformin users (13.3%) also using vitamin E. The baseline Brunt fibrosis stage was significantly higher (p = 0.032) in continuous metformin users (mean Brunt stage of 1.9) than in non-users (mean Brunt stage of 1.5). A similar proportion of patients in each group had steatohepatitis at baseline (93.3% in metformin users vs. 84.5% in non-users, p = 0.334). Continuous metformin users had lower total bilirubin levels at baseline, but there was no significant difference in baseline alanine aminotransferase (ALT) or aspartate aminotransferase (AST) levels between the two groups.

Incidence of Fibrosis Regression

Fibrosis regression occurred in 16 of the 30 (53%) continuous metformin users compared to 28 of the 71 (39%) non-users. The distribution of fibrosis progression, regression, or stability in each group is displayed in Figure 2. As reported in Table 2, the unadjusted hazard ratio (HR) for fibrosis regression among continuous metformin users compared to non-users was 2.21 (95% confidence interval 1.17-4.15, p = 0.014). The association between metformin use and fibrosis regression remained significant after adjusting for established positive or negative predictors of histologic fibrosis regression, including baseline fibrosis stage, age, diabetes status, BMI, and interval total body weight loss (TBWL) of 10% or more, with an HR of 3.72 (95% CI 1.53-9.06, p = 0.004). Among those who regressed, weight loss surgery was less common in metformin users (25%) compared to non-users (43%). When only a two-point or more decrease in Brunt fibrosis stage was considered (regression of two or more fibrosis stages), there was also a significant association of this outcome with metformin use on Cox proportional hazards regression analysis (unadjusted HR 3.57, CI 1.13-11.32, p = 0.030). ALT levels at follow-up biopsy were similar between groups, with a mean of 56.2 U/L in metformin users and 53.7 U/L in non-users (p = 0.823).

Use of Other Medications, Diabetes Status, and Dosage Effects

When aspirin and statin use are added as covariates the multivariate model, the association also remains significant, with an HR of 4.76 (95% CI: 1.74-13.06, p = 0.002). The use of sulfonylureas was not significantly associated with hepatic fibrosis regression (unadjusted HR = 1.75, 95% CI 0.84-3.66, p = 0.14).

Furthermore, hepatic fibrosis regression was not associated with the use of either aspirin (HR = 1.35, 95% CI 0.74-2.48, p = 0.331) or the use of statins (unadjusted HR = 1.34, 95% CI 0.74-2.45, p = 0.335). As shown in Table S3, when analysis was limited to patients with a diagnosis of diabetes at baseline (n = 44), there was still a greater incidence of fibrosis regression in metformin users (14/27, or 52%) compared to non-users (7/17, or 41%), but this association was not statistically significant on Cox proportional hazards regression analysis (unadjusted HR 2.35, p = 0.07; adjusted HR 3.49, p = 0.06). Among patients without diabetes at baseline (n = 57), there was also a greater incidence of fibrosis regression in metformin users (2/3, or 67%) than in non-users (21/54, or 39%), but this was not significant on proportional hazards regression analysis (unadjusted HR 2.33, p = 0.259; adjusted HR 3.29, p = 0.138). As shown in Figure 3, among metformin users, there was a relatively greater incidence of fibrosis regression in those taking a dose of 2,000 or more mg/day of metformin (8/13, or 62% regressed) compared to those taking <2,000 mg/day (8/17, or 47% regressed).

Incidence of Fibrosis Progression

As reported in Table S2, there was a lower incidence of fibrosis progression in metformin users (1/30, or 3%) compared to non-users (18/71, or 25%), but this association was not statistically significant on Cox proportional hazards regression analysis (unadjusted HR = 0.27, 95% CI 0.04-2.02, p = 0.201; adjusted HR 0.16, 95% CI 0.01-1.94, p = 0.150). The mean fibrosis progression rate (FPR) was -0.319 stages/year (95% CI -0.489 to -0.148) in metformin users, compared to -0.111 stages/year (95% CI -0.217 to -0.004), and the difference in FPRs between the two groups was borderline statistically significant (p = 0.041 on Welch t-test), as reported in Table 3. The changes in fibrosis stages from baseline to follow-up biopsy in metformin users and non-users are also detailed in Table 3.

Discussion

As diet and lifestyle modifications are difficult for patients to maintain, and existing NAFLD therapies such as vitamin E and TZDs do not reduce hepatic fibrosis, anti-fibrotic therapies for NAFLD are urgently needed. One of the most commonly prescribed medications, metformin is an anti-diabetic drug that has gained increasing attention in recent years for its potential non-glycemic benefits.[40] Like other insulin-sensitizers, metformin has long been of interest as a potential NAFLD therapy, given the likely central role that insulin insensitivity plays in the pathogenesis and progression of NAFLD.[12] Metformin is also a low-cost drug with a favorable safety profile that has also been associated with a reduced risk of HCC in patients with T2DM,[22,23,24] decreased all-cause mortality in those with cirrhosis continuing

metformin after diagnosis,[25] and increased median overall survival in those with T2DM and HCC.[14] However, meta-analyses of available clinical trials have concluded that metformin does not offer histologic benefits in NAFLD[13] and clinical practice guidelines do not recommend metformin as treatment for NAFLD on this basis.[26] Yet in the limited number of small trials considered, patients were often treated with metformin for fewer than six months, and none were treated with metformin for more than one year.[28] This is at odds with the fact that the time to progress a single fibrosis stage in NASH is at least seven years,[27] a much longer natural history of fibrosis progression (and likely regression as well) than suggested by these treatment times.

Using a cohort of non-cirrhotic NAFLD patients with paired, clinically indicated liver biopsies at least one year apart (with a mean interval of 3.5 years), we found an association between metformin use and hepatic fibrosis regression. This association remained statistically significant after adjusting for age, baseline fibrosis, BMI, diabetes, interval weight loss, and weight loss surgery (WLS). The likelihood of fibrosis regression in a given time interval among continuous metformin users was more than double that of non-users in our unadjusted analysis. In our multivariate analysis adjusting for previously established fibrosis predictors and risk-modifiers, this likelihood of fibrosis regression was almost four times higher in continuous metformin users compared to non-users.

Given that our primary outcome of interest was fibrosis regression, our cohort was selected to only include patients with NAFLD fibrosis, but not cirrhosis, at baseline. Metformin users had a slightly higher mean baseline Brunt fibrosis stage (1.9) compared to that of non-users (1.5), and this difference was statistically significant. Baseline fibrosis stage is a known predictor of progression to cirrhosis in NAFLD.[41] There also is data to suggest that the rate of fibrosis progression is faster in those with higher baseline fibrosis. Singh et al., for example, found that those with NAFLD without baseline inflammation progressed at a faster rate if they had fibrosis at baseline (0.15 stages/year vs..07 stages/year).[27] This higher baseline fibrosis stage, which may be attributable to the predictably higher prevalence of diabetes among metformin users (90.0% vs. 23.9% in non-users), makes the observed increased incidence of fibrosis regression among metformin users all the more striking.

Overall, our cohort had a greater incidence of fibrosis regression than other previously studied paired biopsy cohorts, which is likely attributable to a higher frequency of weight loss and weight loss surgery (WLS) in this cohort. Metformin users and non-users lost a similar amount of weight in the interval between baseline and follow-up biopsy, around 8 kg in each group, and had a similarly high incidence of WLS, with close to one-third of patients in each group undergoing WLS. This disproportionately high frequency of WLS is due to the fact that baseline biopsies for patients in this cohort were routinely

performed in patients undergoing WLS at our center at the time of the operation. In a 48-week pilot clinical trial that reported an association between metformin and improvements in hepatic inflammation and hepatocellular injury in patients with non-cirrhotic NASH, these improvements in liver histology were attributed entirely to the weight loss induced by metformin use.[15] However, in our cohort, weight loss was similar in each group, and the association between fibrosis regression and metformin use remained significant even when adjusted for TBWL >10% and WLS. Moreover, among those who experienced fibrosis regression, WLS was less common in continuous metformin users than in non-users.

There is evidence to suggest that statins may have antifibrotic effects independent of anti-inflammatory effects in other causes of hepatic fibrosis such as chronic hepatitis C.[39,31] Aspirin use has also been associated with lower levels of hepatic fibrosis.[38] Hyperlipidemia and other cardiac risk factors are more common among those with diabetes, and predictably, in our cohort, both statin and aspirin use were higher in metformin users compared to non-users. However, neither aspirin use nor statin use was associated with fibrosis regression in our cohort, and the association between metformin use and fibrosis regression remained after adjustment for aspirin and statin use. TZDs, a medication class thought to have potential disease-modifying effects in NAFLD, was used at a similarly low rate in each group. The dose-dependent increase in fibrosis regression, as well as the association between metformin use and two-point decreases in fibrosis stage, further supports the observed association with fibrosis regression.

When our analysis was restricted to only individuals with diabetes, however, there was a greater incidence of fibrosis regression in metformin users, but this association was not statistically significant on either unadjusted or adjusted proportional hazards regression analysis. Similarly, in the non-diabetic patients, there was a greater proportion of fibrosis regression events among those on metformin, but this difference was also not statistically significant. We suspect that this lack of statistical significance is related to limited power to detect an association due to the decreases in sample size to only 44 patients with T2DM and 57 patients without T2DM. Only three non-diabetic patients were continuous metformin-users, for example, greatly limiting the statistical power of this sub-analysis. Similarly, although only one metformin user experienced fibrosis progression (and increase in Brunt fibrosis stage by one or more full stages) compared to one-fourth of non-users, this association was not statistically significant on proportional hazards regression analysis. Again, we hypothesize that this may be due to a limited number of observed fibrosis progression events. As paired biopsy studies have shown that it takes an average of 8.3 years to progress one fibrosis stage in NAFLD,[27] it is possible that the follow-up interval was not long enough to capture progression events, and that regression events may take less time to occur.

The development of liver fibrosis requires the transition of quiescent hepatic stellate cells (HSCs) to active myofibroblasts.[31] These activated myofibroblasts have increased expression of extracellular matrix proteins necessary for the development of fibrotic tissue, such as collagen, as well as the profibrogenic cytokine transforming growth factor β (TGFβ). There is a significant body of evidence from animal model studies to suggest that the anti-fibrotic effects of metformin operate via this same pathway in liver fibrosis, as well as in renal and pulmonary fibrosis.[16,18,19,21] The mechanism of metformin's reduction of hepatic gluconeogenesis involves activation of AMP-activated protein kinase (AMPK), which has been shown *in vitro* to suppress proliferation and activation of HSCs via inhibition of Akt, induction of antioxidant enzymes, and cell cycle arrest in the G0/G1 phase.[16] Notably, in a non-diabetic mouse model of NAFLD, metformin both reversed and prevented MCD-diet-induced hepatic steatosis, inflammation, and fibrosis.[16] Metformin has been shown to reduce carbon-tetrachloride-induced liver TGF-β1 signaling and liver fibrosis in mice,[18] and to reduce hepatic fibrosis in rats with biliary cirrhosis,[17] suggesting that the anti-fibrotic effects of metformin may not be solely dependent on improvements in insulin sensitivity or reductions in hepatic steatosis. The fact that hepatic fibrosis progresses at a faster rate in animal models may explain the discord between strong evidence of anti-fibrotic effects in animal models and the relative paucity of evidence of anti-fibrotic effects of metformin in human trials lasting less than one year.

Limitations to this study include the fact that we were unable to fully evaluate the effect of metformin on hepatic steatosis, lobular inflammation, or hepatocyte injury, as the staging of these histological features was not reliably available from clinical pathology reports. Furthermore, a significant weakness of this study is that fibrosis stages were acquired from pathology reports written as a part of routine clinical care, rather than blinded reads by an expert hepatopathologist. However, there is no reason to believe that a history of metformin use, even if known to the assigned clinical pathologist, would bias the reading of fibrosis stage, or that inter-observer variability would preferentially effect metformin users vs. non-users. Furthermore, there was a statistically significant association between a two-point decrease in Brunt fibrosis stage (regression by two or more stages) and metformin use, as well as a relatively greater incidence of fibrosis regression among those taking metformin doses of 2,000 or more mg/day. This dose-dependent effect and the association with two-stage fibrosis regression support the observed association between metformin use and fibrosis regression.

Moreover, the interval between baseline and follow-up biopsy was shorter in metformin users than in non-users. Though our method of analysis (Cox proportional hazards regression) was designed to account for this unbalanced follow-up time, which is likely inherent in clinically-indicated liver biopsies, yet ideally, follow-up time would be similar between groups. This difference in follow-up between groups

may be explained by more careful or frequent monitoring of NAFLD for those with T2DM. Overall, a prospective, randomized study with balanced follow-up times and blinded reads of liver biopsies by an hepato-pathologist to reduce inter-observer variability and allow for measurement of hepatic steatosis, inflammation, and hepatocellular injury, would be needed to confirm, validate, and better characterize the relationship between metformin use and fibrosis regression suggested by our observational data.

In conclusion, in our cohort, metformin use is associated with an increased frequency of hepatic fibrosis regression in non-cirrhotic NAFLD, even after adjustment for interval weight loss and other established fibrosis predictors and risk-modifiers. Metformin has been shown to have anti-fibrotic effects in non-diabetic NASH mouse models,[16] as well as survival benefits and dose-dependent chemo-preventive effects in humans with cirrhosis and T2DM.[22] Data from large population studies evidence the benefits of continuing metformin in diabetic patients newly diagnosed with cirrhosis.[24,42] Our findings support a potential role for long-term metformin use in fibrosis regression in non-cirrhotic NAFLD. Although our study has significant shortcomings largely attributable to the nature of retrospective clinical data, our study, along with animal data, suggests that future clinical trials of metformin as a potential anti-fibrotic drug in NAFLD should have longer treatment time than other previously conducted trials, which have had mixed results. Furthermore, there is a paucity of data evaluating metformin at a treatment for NAFLD in non-diabetic or pre-diabetic patients, which is of particular interest given the frequency with which metformin is already prescribed to diabetic patients. Our data, though limited both sample size, does suggest that metformin's potential anti-fibrotic effect in non-diabetic patients is particularly worth investigating further. Overall, metformin is an affordable and safe medication with a range of non-glycemic benefits, and further investigations into the potential efficacy of its long-term use as an anti-fibrotic agent in non-cirrhotic NAFLD are warranted.

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References

1. Younossi Z, Anstee QM, Marietti M, et al. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. *Nat Publ Gr*. 2017;14. doi:10.1038/nrgastro.2017.109
2. Pais R, Barritt AS, Calmus Y, et al. *NAFLD and Liver Transplantation: Current Burden and Expected Challenges*.; 2016. doi:10.1016/j.jhep.2016.07.033
3. Wong RJ, Aguilar M, Cheung R, et al. Nonalcoholic steatohepatitis is the second leading etiology of liver disease among adults awaiting liver transplantation in the United States. *Gastroenterology*. 2015;148(3):547-555. doi:10.1053/j.gastro.2014.11.039
4. Bertot LC, Adams LA. The natural course of non-alcoholic fatty liver disease. *Int J Mol Sci*. 2016;17(5). doi:10.3390/ijms17050774
5. Hagström H, Nasr P, Ekstedt M, et al. Fibrosis stage but not NASH predicts mortality and time to development of severe liver disease in biopsy-proven NAFLD. *J Hepatol*. 2017;67(6):1265-1273. doi:10.1016/j.jhep.2017.07.027
6. Dulai PS, Singh S, Patel J, et al. Increased risk of mortality by fibrosis stage in nonalcoholic fatty liver disease: Systematic review and meta-analysis. *Hepatology*. 2017;65(5):1557-1565. doi:10.1002/hep.29085
7. Estes C, Razavi H, Loomba R, Younossi Z, Sanyal AJ. Modeling the epidemic of nonalcoholic fatty liver disease demonstrates an exponential increase in burden of disease. *Hepatology*. 2018;67(1):123-133. doi:10.1002/hep.29466
8. Sumida Y, Yoneda M. Current and future pharmacological therapies for NAFLD/NASH. *J Gastroenterol*. 2018;53(3):362-376. doi:10.1007/s00535-017-1415-1
9. Friedman SL, Ratziu V, Harrison SA, et al. A randomized, placebo-controlled trial of cenicriviroc for treatment of nonalcoholic steatohepatitis with fibrosis. *Hepatology*. 2018;67(5):1754-1767. doi:10.1002/hep.29477
10. Tacke F. Cenicriviroc for the treatment of non-alcoholic steatohepatitis and liver fibrosis. *Expert Opin Investig Drugs*. 2018;27(3):301-311. doi:10.1080/13543784.2018.1442436
11. Chalasani N, Vuppalanchi R, Rinella M, et al. Randomised clinical trial: a leucine-metformin-sildenafil combination (NS-0200) vs placebo in patients with non-alcoholic fatty liver disease. 2018. doi:10.1111/apt.14674
12. Rouabhia S, Milic N, Abenavoli L. Metformin in the treatment of non-alcoholic fatty liver disease: safety, efficacy and mechanism. *Expert Rev Gastroenterol Hepatol*. 2014;8(4):343-349. doi:10.1586/17474124.2014.894880
13. Rakoski MO, Singal AG, Rogers MAM, Conjeevaram H. Meta-analysis: Insulin sensitizers for the treatment of non-alcoholic steatohepatitis. *Aliment Pharmacol Ther*. 2010;32(10):1211-1221. doi:10.1111/j.1365-2036.2010.04467.x
14. Schulte L, Scheiner B, Voigtländer T, et al. Treatment with metformin is associated with a prolonged survival in patients with hepatocellular carcinoma. *Liver Int*. 2019:0-2. doi:10.1111/liv.14048
15. Loomba R, Lutchman G, Kleiner DE, et al. Clinical trial: pilot study of metformin for the treatment of non-alcoholic steatohepatitis. *Aliment Pharmacol Ther*. 2009;29(2):172-182. doi:10.1111/j.1365-2036.2008.03869.x

16. Kita Y, Takamura T, Misu H, et al. Metformin prevents and reverses inflammation in a non-diabetic mouse model of nonalcoholic steatohepatitis. *PLoS One*. 2012;7(9):e43056. doi:10.1371/journal.pone.0043056

17. Ko MT, Huang HC, Lee WS, et al. Metformin reduces intrahepatic fibrosis and intrapulmonary shunts in biliary cirrhotic rats. *J Chinese Med Assoc*. 2017;80(8):467-475. doi:10.1007/s11837-017-2672-4

18. Fan K, Wu K, Lin L, et al. Metformin mitigates carbon tetrachloride-induced TGF-β1/Smad3 signaling and liver fibrosis in mice. *Biomed Pharmacother*. 2017;90(2017):421-426. doi:10.1016/j.biopha.2017.03.079

19. Feng Y, Wang S, Zhang Y, Xiao H. Metformin attenuates renal fibrosis in both AMPKα2-dependent and independent manners. *Clin Exp Pharmacol Physiol*. 2017;44(6):648-655. doi:10.1111/1440-1681.12748

20. Rangarajan S, Bone NB, Zmijewska AA, et al. Metformin reverses established lung fibrosis in a bleomycin model. *Nat Med*. 2018;24(August):1-7. doi:10.1038/s41591-018-0087-6

21. Sato N, Takasaka N, Yoshida M, et al. Metformin attenuates lung fibrosis development via NOX4 suppression. *Respir Res*. 2016;17(1):1-12. doi:10.1186/s12931-016-0420-x

22. Chen H-P, Shieh J-J, Chang C-C, et al. Metformin decreases hepatocellular carcinoma risk in a dose-dependent manner: population-based and in vitro studies. *Gut*. 2013;62(4):606-615. doi:10.1136/gutjnl-2011-301708

23. Donadon V, Balbi M, Mas MD, Casarin P, Zanette G. Metformin and reduced risk of hepatocellular carcinoma in diabetic patients with chronic liver disease. *Liver Int*. 2010;30(5):750-758. doi:10.1111/j.1478-3231.2010.02223.x

24. Lai S-W, Chen P-C, Liao K-F, Muo C-H, Lin C-C, Sung F-C. Risk of hepatocellular carcinoma in diabetic patients and risk reduction associated with anti-diabetic therapy: a population-based cohort study. *Am J Gastroenterol*. 2012;107(1):46-52. doi:10.1038/ajg.2011.384

25. Zhang X, Harmsen WS, Mettler TA, et al. Continuation of metformin use after a diagnosis of cirrhosis significantly improves survival of patients with diabetes. *Hepatology*. 2014;60(6):2008-2016. doi:10.1002/hep.27199

26. Sanjiv Chopra ML. Management of nonalcoholic fatty liver disease in adults - UpToDate. UpToDate. https://www.uptodate.com/contents/management-of-nonalcoholic-fatty-liver-disease-in-adults. Published 2018. Accessed January 30, 2019.

27. Singh S, Allen AM, Wang Z, Prokop LJ, Murad MH, Loomba R. Fibrosis Progression in Nonalcoholic Fatty Liver vs Nonalcoholic Steatohepatitis: A Systematic Review and Meta-analysis of Paired-Biopsy Studies. *Clin Gastroenterol Hepatol*. 2015;13(4):643-654. doi:10.1016/j.cgh.2014.04.014

28. Li Y, Liu L, Wang B, Wang J, Chen D. Metformin in non-alcoholic fatty liver disease: A systematic review and meta-analysis. *Biomed Reports*. 2013. doi:10.3892/br.2012.18

29. Traussnigg S, Kienbacher C, Halilbasic E, et al. Challenges and Management of Liver Cirrhosis: Practical Issues in the Therapy of Patients with Cirrhosis due to NAFLD and NASH. *Dig Dis*. 2015;33(4):598-607. doi:10.1159/000375353

30. Farrell GC, Larter CZ. Nonalcoholic fatty liver disease: From steatosis to cirrhosis. *Hepatology*. 2006;43(S1):S99-S112. doi:10.1002/hep.20973

31. Simon TG, King LY, Zheng H, Chung RT. Statin use is associated with a reduced risk of fibrosis progression in chronic hepatitis C. *J Hepatol*. 2015;62(1):18-23. doi:10.1016/j.jhep.2014.08.013

32. Singh S, Allen AM, Wang Z, Prokop LJ, Murad MH, Loomba R. Fibrosis Progression in Nonalcoholic Fatty Liver versus Nonalcoholic Steatohepatitis: A Systematic Review and Meta- analysis of Paired-Biopsy Studies. *Clin Gastroenterol Hepatol*. 2015;13(4):643-654. doi:10.1016/j.cgh.2014.04.014.Fibrosis

33. Brunt EM, Kleiner DE, Wilson LA, Belt P, Brent A, Neuschwander-Tetri. The NAS and the Histophatologic Diagnosis of NAFLD: Distinct Clinicopathologic Meanings. *Hepatology*. 2011;53(3):810-820. doi:10.1002/hep.24127.The

34. Brunt EM, Janney CG, Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol*. 1999;94(9):2467-2474. doi:10.1111/j.1572-0241.1999.01377.x

35. Kleiner DE, Brunt EM, Van Natta M, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*. 2005;41(6):1313-1321. doi:10.1002/hep.20701

36. Glass LM, Dickson RC, Anderson JC, et al. Total Body Weight Loss of ≥10 % Is Associated with Improved Hepatic Fibrosis in Patients with Nonalcoholic Steatohepatitis. *Dig Dis Sci*. 2015;60(4):1024-1030. doi:10.1007/s10620-014-3380-3

37. Schuppan D, Surabattula R, Wang XY. Determinants of fibrosis progression and regression in NASH. *J Hepatol*. 2018;68(2):238-250. doi:10.1016/j.jhep.2017.11.012

38. Jiang ZG, Feldbrügge L, Tapper EB, et al. Aspirin use is associated with lower indices of liver fibrosis among adults in the United States. *Aliment Pharmacol Ther*. 2016;43(6):734-743. doi:10.1111/apt.13515

39. Kamal S, Khan MA, Seth A, et al. Beneficial Effects of Statins on the Rates of Hepatic Fibrosis, Hepatic Decompensation, and Mortality in Chronic Liver Disease: A Systematic Review and Meta-Analysis. *Am J Gastroenterol*. 2017;112(10):1495-1505. doi:10.1038/ajg.2017.170

40. Fujita Y, Inagaki N. Metformin: New Preparations and Nonglycemic Benefits. *Curr Diab Rep*. 2017;17(1):5. doi:10.1007/s11892-017-0829-8

41. Ekstedt M, Hagstr€ H, Nasr P, et al. Fibrosis Stage Is the Strongest Predictor for Disease-Specific Mortality in NAFLD After Up to 33 Years of Follow-Up. 2014. doi:10.1002/hep.27368

42. Donadon V, Balbi M, Mas MD, Casarin P, Zanette G. Metformin and reduced risk of hepatocellular carcinoma in diabetic patients with chronic liver disease. *Liver Int*. 2010;30(5):750-758. doi:10.1111/j.1478-3231.2010.02223.x

Tables and Figures

Figure 1. Cohort search strategy

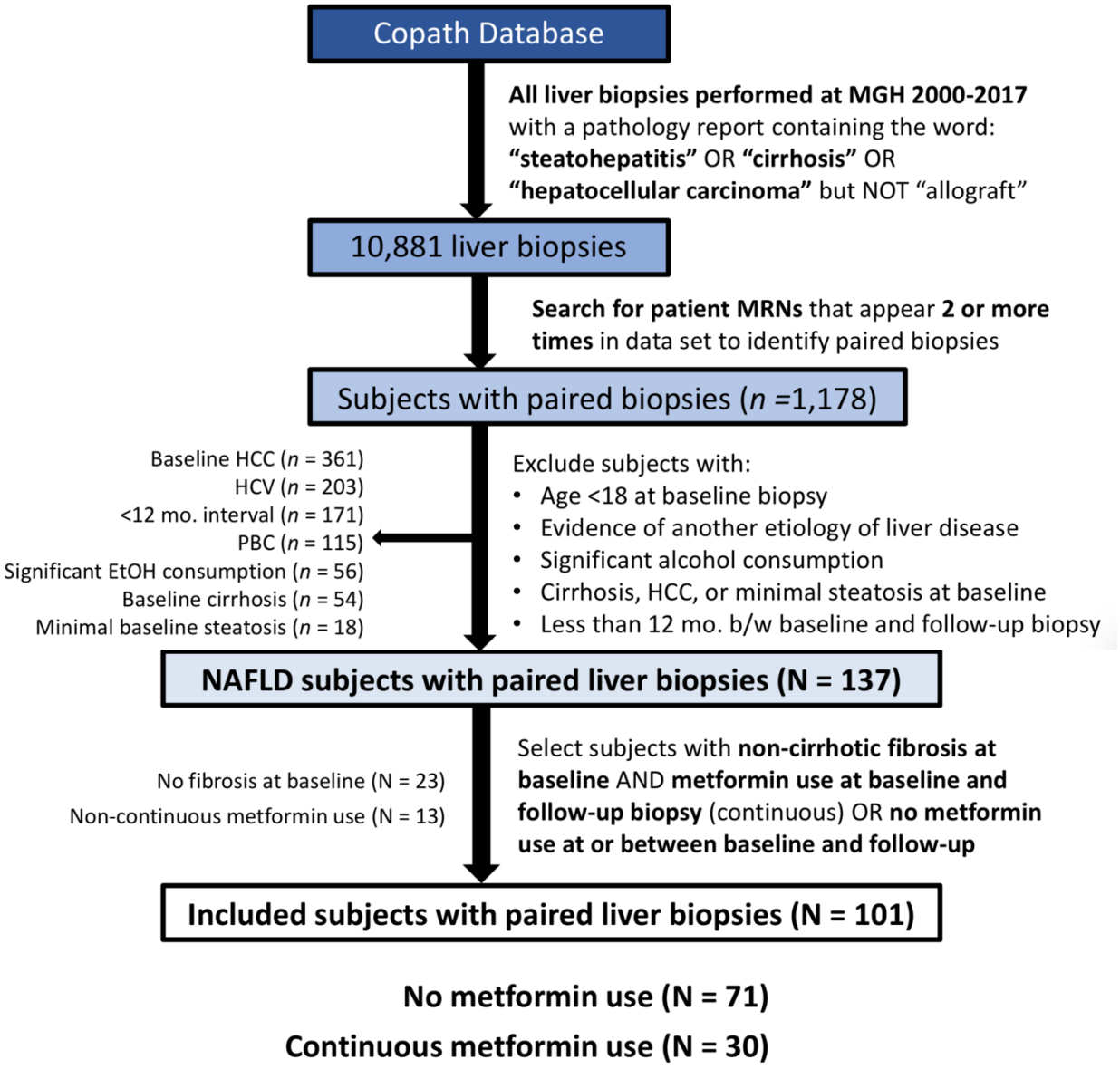


Table 1. Baseline clinical, laboratory, and histological characteristics of patients with non-cirrhotic NAFLD fibrosis (N = 101), according to continuous metformin use

Variable*	No metformin use (N = 71)	Metformin use (N = 30)	*P* value
Age, years	**48.8 (11.4)**	**53.3 (9.5)**	**0.045**
Female, %	52.1	53.3	0.999
Race and ethnicity			
Non-Hispanic White, %	76.1	70.0	0.497
Hispanic, %	21.1	23.3	
Black, %	0.0	3.3	
Other, %	2.8	3.3	
Biopsy interval, years	**3.7 (2.7)**	**2.8 (1.8)**	**0.037**
BMI, kg/m^2	37.4 (9.3)	38.6 (8.7)	0.543
Weight loss, kg	8.4 (16.9)	7.6 (12.7)	0.791
Weight loss surgery, %	29.6	30.0	0.999
Diabetes, %	**23.9**	**90.0**	**<0.001**
Hypertension, %	64.8	73.3	0.490
Past smoking history, %	53.5	53.3	0.999
Alcohol history, g/day	2.0 (3.9)	2.0 (3.2)	0.711
ALT, U/L	71.8 (50.7)	71.0 (44.8)	0.937
AST, U/L	54.1 (34.5)	55.0 (33.0)	0.885
Total bilirubin, mg/dl	**0.72 (0.94)**	**0.45 (0.18)**	**0.024**
Albumin, g/dl	4.3 (0.4)	4.4 (0.3)	0.283
Alkaline phosphatase, U/L	86.6 (26.9)	84.1 (23.1)	0.628
Platelets, x1000/mm^3	253.7 (78.4)	253.9 (74.0)	0.991
Brunt fibrosis stage	**1.5 (0.7)**	**1.9 (0.8)**	**0.032**
Brunt fibrosis stage			0.072
F1, %	59.2	36.7	
F2, %	26.8	33.3	
F3, %	14.1	30.0	
Steatohepatitis, %	84.5	93.3	0.334
Vitamin E use, %	11.3	13.3	0.746
Aspirin use, %	**31.0**	**73.3**	**<0.001**
Statin use, %	**29.6**	**70.0**	**<0.001**
Insulin use, %	11.3	16.7	0.520
Thiazolidinedione use, %	1.4	3.3	0.508
Sulfonylurea	**1.4**	**40.0**	**<0.001**
Other anti-diabetic agent use[1], %	2.8	10.0	0.154

*Variables expressed as mean (SD) unless otherwise indicated

[1]Other anti-diabetic agents include: meglitinides, GLP-1 analogs and agonists, DPP-4 inhibitors, and SGLT-2 inhibitors

Table 2. Cox proportional hazards regression analysis of the relationship between continuous metformin use, and the time to one-point decrease in Brunt fibrosis stage, among patients with baseline non-cirrhotic NAFLD fibrosis (N = 101)

		Unadjusted		Adjusted*	
Variable (N)	Fibrosis regression, % (N)	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Metformin use (30)	53 (16)	**2.21 (1.17-4.15)**	**0.014**	**3.72 (1.53-9.06)**	**0.004**
No metformin use (71)	39 (28)	1.00		1.00	

*Hazard ratio adjusted for baseline fibrosis stage, age, BMI, diabetes, TBWL ≥ 10%, and bariatric surgery

Table 3. Change in Brunt fibrosis stage between baseline and follow-up biopsy by continuous metformin use, among patients with baseline non-cirrhotic fibrosis (N = 101)

Variable (N)		Fibrosis stage at follow-up biopsy					Mean FPR* (95% CI)	Mean time to regress 1 stage, years
Metformin use (30)								
Fibrosis stage at baseline biopsy		F0	F1	F2	F3	F4	**-0.319 (-0.489 to -0.148)**	3.14
	F1 (11)	3	7	1	0	0		
	F2 (10)	3	4	3	0	0		
	F3 (9)	1	2	3	3	0		
	Sum	7	13	7	3	0		
No metformin use (71)								
Fibrosis stage at baseline biopsy		F0	F1	F2	F3	F4	**-0.111 (-0.218 to -0.004)**	8.98
	F1 (42)	13	16	7	6	0		
	F2 (19)	4	7	6	1	1		
	F3 (10)	1	1	2	3	3		
	Sum	18	24	15	10	4		

*FPR = Fibrosis progression rate in stages per year, where a negative number indicates overall fibrosis regression
Green numbers indicate fibrosis regression, blue numbers indicate stability, and red numbers indicated progression

Figure 2. Change in Brunt fibrosis stage between biopsies by continuous metformin use

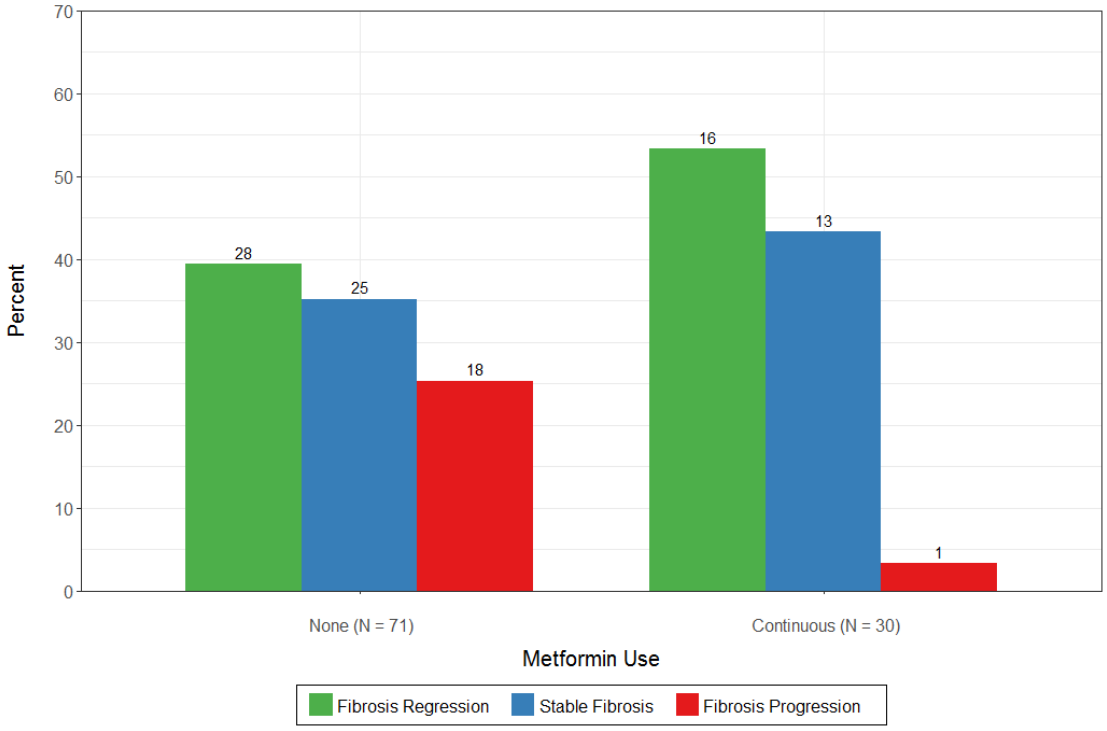
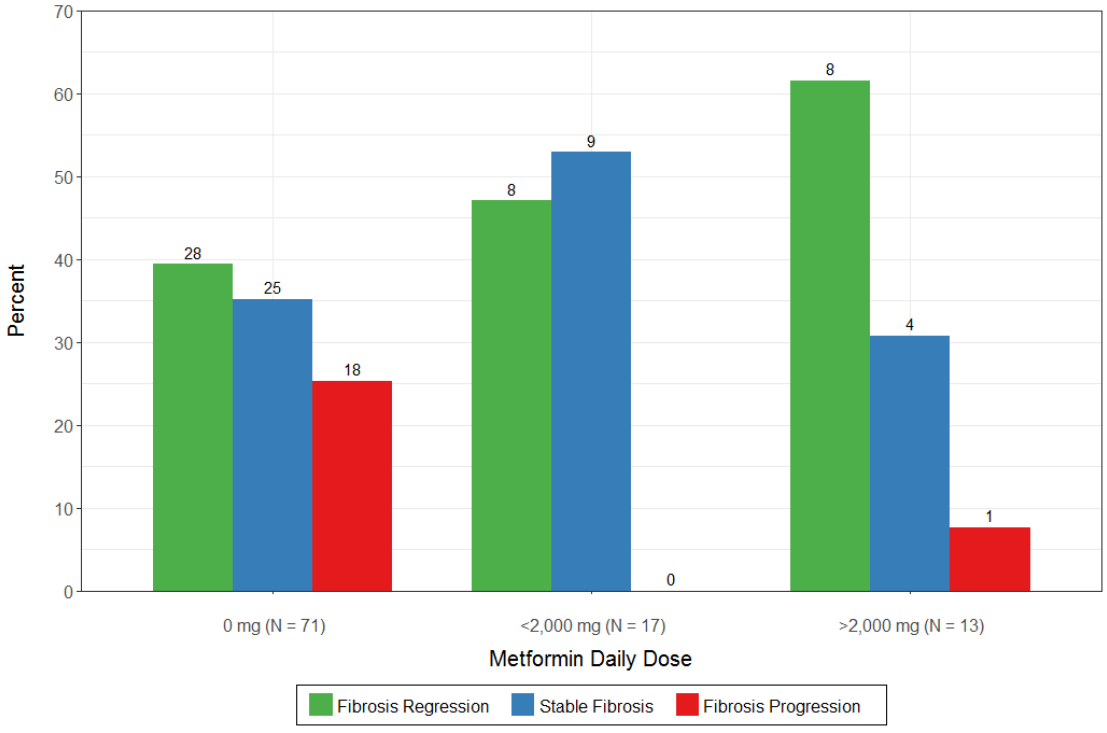


Figure 3. Change in Brunt fibrosis stage between biopsies by daily metformin dose



Supplement

Table S1. Proportional hazards regression analysis of the relationship between metformin use, and the time to two-point decrease in Brunt fibrosis stage, among patients with non-cirrhotic NAFLD (N = 101)

		Unadjusted		Adjusted*	
Variable (N)	Regression, % (N)	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Metformin use (30)	20 (6)	**3.57 (1.13-11.32)**	**0.030**	4.04 (0.62-26.38)	0.15
No metformin use (71)	8 (6)	1.00		1.00	

*Hazard ratio adjusted for baseline fibrosis stage, age, BMI, diabetes, TBWL ≥ 10%, and bariatric surgery

Table S2. Proportional hazards regression analysis of the relationship between metformin use, and the time to one-point increase in Brunt fibrosis stage, among patients with non-cirrhotic NAFLD (N = 101)

		Unadjusted		Adjusted*	
Variable (N)	Progression, % (N)	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Metformin use (30)	3 (1)	0.27 (0.04-2.02)	0.201	0.16 (0.01-1.94)	0.150
No metformin use (71)	25 (18)	1.00		1.00	

*Hazard ratio adjusted for baseline fibrosis stage, age, BMI, diabetes, TBWL ≥ 10%, and bariatric surgery.

Table S3. Proportional hazards regression analysis of the relationship between metformin use, and the time to one-point decrease in Brunt fibrosis stage, among patients with baseline NAFLD and T2DM (N = 44)

		Unadjusted		Adjusted*	
Variable (N)	Regression, % (N)	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Metformin use (27)	52 (14)	2.35 (0.92-6.00)	0.07	3.49 (0.96-13.00)	0.06
No metformin use (17)	41 (7)	1.00		1.00	

*Hazard ratio adjusted for baseline fibrosis stage, age, BMI, TBWL ≥ 10%, and bariatric surgery

Table S4. Proportional hazards regression analysis of the relationship between metformin use, and the time to one-point decrease in Brunt fibrosis stage, among patients with baseline NAFLD and no T2DM (N = 57)

		Unadjusted		Adjusted*	
Variable (N)	Regression, % (N)	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Metformin use (3)	67 (2)	2.33 (0.54-10.10)	0.259	3.29 (0.68-15.81)	0.138
No metformin use (54)	39 (21)	1.00		1.00	

*Hazard ratio adjusted for baseline fibrosis stage, age, BMI, TBWL ≥ 10%, and bariatric surgery