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Glycemic Control Predicts Severity of Hepatocyte Ballooning and Hepatic Fibrosis in Nonalcoholic Fatty Liver Disease

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Abbreviations:

BMI, body mass index

BP, blood pressure

DUHS, Duke University Health System

DPP4, dipeptidyl peptidase 4

eGFR, estimated glomerular filtration rate HbA1c, hemoglobin A1c

GLP-1RA, glucagon-like peptide-1 receptor agonist

T2D, type 2 diabetes

NASH CRN, Nonalcoholic Steatohepatitis Clinical Research Network

NAS, NAFLD Activation Score

RCS, restricted cubic splines

SGLT2i, sodium-glucose cotransporter 2 inhibitor.

ABSTRACT

BACKGROUND AND AIMS: Whether glycemic control, as opposed to diabetes status, is associated with the severity of nonalcoholic fatty liver disease (NAFLD) is unknown. We aimed to evaluate whether degree of glycemic control in the years preceding liver biopsy predicts the histologic severity of nonalcoholic steatohepatitis (NASH).

METHODS & RESULTS: Using the Duke NAFLD Clinical Database we examined patients with biopsy-proven NAFLD/NASH (n=713) and the association of liver injury with glycemic control as measured by hemoglobin A1c (HbA1c). The study cohort was predominantly female (59%), Caucasian (84%) with median (IQR) age of 50 (42, 58) years; 49% had diabetes (n=348). Generalized linear regression models adjusted for age, sex, race, diabetes, body mass index, and hyperlipidemia were used to assess the association between mean HbA1c over the year preceding liver biopsy and severity of histologic features of NAFLD/NASH. Histologic features were graded and staged according to NASH Clinical Research Network system. Group-based trajectory analysis was used to examine patients with ≥3 HbA1c (n=298) measures over 5 years preceding clinically indicated liver biopsy. Higher mean HbA1c was associated with higher grade of steatosis and ballooned hepatocytes, but not lobular inflammation. Every 1% increase in mean HbA1c was associated with 15% higher odds of increased fibrosis stage (OR 1.15, 95% CI 1.01, 1.31).

As compared with good glycemic control, moderate control was significantly associated with increased severity of ballooned hepatocytes (OR 1.74, 95% CI 1.01, 3.01, p=0.048) and hepatic fibrosis (OR 4.59, 95% CI 2.33, 9.06, p<0.01). **CONCLUSIONS:** Glycemic control predicts severity of ballooned hepatocytes and hepatic fibrosis in NAFLD/NASH, and thus optimizing glycemic control may be a means of modifying risk of NASH-related fibrosis progression.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a growing epidemic, affecting one in four people worldwide (1, 2) and approximately 60% of patients with type 2 diabetes (T2D). (3, 4) The term NAFLD encompasses a disease spectrum with isolated steatosis on the most benign end, to nonalcoholic steatohepatitis (NASH) characterized by steatosis, inflammation and ballooned hepatocytes, with or without fibrosis.(5) NASH increases the risk of fibrosis progression to cirrhosis with risk of hepatic decompensation and hepatocellular carcinoma, making NASH the leading indication for liver transplantation in the United States. (6) Higher grades of steatosis, inflammation and ballooned hepatocytes render increased steatohepatitis severity, which, in turn, is strongly associated with progressive hepatic fibrosis (7), the primary predictor of liver-related morbidity and mortality.(8)

T2D is a well-established risk factor for the development of NAFLD, and is a strong predictor of advanced hepatic fibrosis, complications of cirrhosis, such as hepatocellular carcinoma and liver-related mortality.(9-12) Despite the clear link between NAFLD and T2D, little is understood about how glycemic control impacts histological severity and associated risk for NAFLD progression. While glucose-lowering drugs have been utilized as therapeutic approaches for NASH, it is unclear whether the therapeutic benefit is attributable to the glucose-lowering effect of such interventions. Further, liver aminotransferases in patients with diabetes are particularly insensitive (13) and are normal in greater than 70% of patients with biopsy-proven NASH (14), limiting the ability to ascertain the effect of glucose lowering on NASH using non-invasive surrogates for disease activity. Therefore, studies which directly examine the association of glycemic control, as opposed to diabetes status alone, on histologic features of liver injury in NAFLD/NASH are needed.

In order to address this evidence gap, we investigated the association between degree of glycemic control (assessed using mean HbA1c), as well as pre-biopsy trends in glycemic control in the years preceding a clinically indicated liver biopsy in a large cohort of well-phenotyped patients with biopsy proven NAFLD/NASH.

MATERIALS AND METHODS

Study Design and Data Source

We conducted a longitudinal cohort study utilizing retrospectively / prospectively collected data from NAFLD subjects in the Duke University Health System NAFLD Biorepository (Duke University, Durham, NC). The DUHS NAFLD Clinical Database, established in 2007, is a prospective, open-enrolling and well-annotated clinical database of patients who underwent clinical and histologic evaluations of the suspected diagnosis of NASH as part of standard of care. The DUHS NAFLD Clinical Database was approved by the Duke University Institutional Review Board (Protocol # 00102631) and conducted in accordance with the Declaration of Helsinki ethical guidelines. For the present study, NAFLD was defined as (1) presence of >5% hepatic steatosis on liver biopsy and (2) absence of histologic and serologic evidence for other chronic liver disease in a patient with risk factors for metabolic syndrome.

Demographic data (i.e., height, weight, BMI, age, gender, race, ethnicity, smoking status, and comorbid illnesses) and laboratory studies (i.e., lipids, glucose, hemoglobin A1c [HbA1c], liver aminotransferases, and measures of liver synthetic function) were obtained within 6 months of liver biopsy in all patients and extracted from the medical record, as otherwise available, for the preceding 5 years prior to liver biopsy for evaluation of NAFLD/NASH.

Study Population

We examined 713 patients enrolled into the DUHS NAFLD Clinical Database between January 2007 and October 2019, who met the following inclusion criteria for our study: 1) age ≥ 18 years old, 2) histologic diagnosis of NAFLD and 3) at least 1 documented HbA1c value in the year preceding a clinically indicated liver biopsy for evaluation of NAFLD/NASH. Exclusion criteria included the following: 1) current alcohol consumption of ≥ 14 servings per week (for men) and ≥ 7 servings per week (for women), 2) serologic evidence of alternative forms of chronic liver disease (e.g., chronic viral hepatitis, primary biliary cirrhosis, autoimmune hepatitis, hemochromatosis, Wilson's disease, alpha-1-antitrypsin deficiency), 3) histologic features suggesting co-existing liver diseases, 4) history of bariatric surgery, or 5) liver transplantation. No patients with decompensated or overt features of cirrhosis were included, as none of these patients would have undergone a clinically indicated liver biopsy.

The diagnosis of diabetes was defined as an HbA1c ≥ 6.5% (≥ 48 mmol/mol), the presence of a diagnosis of diabetes as detailed in the medical history by a provider, at least 2 fasting glucose values ≥126 mg/dL (7.0 mmol/L) in excess of 6 months apart, or the use of glucose-lowering agents. The study scheme is detailed in Figure 1.

HbA1c measures

We used manual electronic health record chart review to extract additional HbA1c data from 5 years pre- to 90 days post-liver biopsy. All patients meeting inclusion and exclusion criteria for the study were examined in the mean HbA1c analysis, which utilized HbA1c data over 1 year pre- to 90 days post-biopsy. Only patients with three or more HbA1c levels from 5 years pre- to 90-days post-liver biopsy were examined by group-based trajectory analysis. We allowed HbA1c data up to 90 days post-biopsy as levels drawn in this time period (within 3 months) may still reasonably reflect glycemic control at time of biopsy.

Liver Histology

All liver biopsy specimens were stained with hematoxylin-eosin and Masson's trichrome stains and reviewed and scored by a hepatopathologist according to the published Nonalcoholic Steatohepatitis Clinical Research Network (NASH CRN) grading and staging system (15). The hepatopathologist was blinded to clinical phenotype of the patient and associated laboratory data. For the analyses, fibrosis stages 1a, 1b, and 1c were combined and treated as stage 1. To address our research interest in evaluating the association of portal inflammation with glycemic control, the grades of portal inflammation were defined as grade 0 (absent) and grade 1 (present).

Primary Outcomes

The primary outcome was severity of hepatic fibrosis stage as defined by the NASH CRN (stage 0-4).(15). The severity of individual histologic features of steatohepatitis (grade of steatosis, lobular inflammation, and ballooned hepatocytes) and the composite assessment of severity of NASH as defined by NAFLD Activity Score (NAS) were analyzed as secondary outcomes. Presence of NASH was defined as NAS ≥4.

Statistical analysis

Demographic, clinical, and hepatic histological data were summarized as a count (percent) or median (interquartile range). Wilcoxon rank sum tests and Kruskal-Wallis tests were used to compare continuous variables between patients with and without diabetes, or among different HbA1c trajectory groups, respectively. Cochran-Mantel-Haenszel tests were employed to compare categorical variables. A two-sided test with *p* value of <0.05 was considered statistically significant for all analyses. Statistical analyses were conducted using SAS statistical software (version 9.4, SAS Institute Inc., Cary, NC).

Mean HbA1c analysis

The association between mean HbA1c in the year preceding biopsy and hepatic histological features was investigated with ordinal logistic regression after testing for proportional odds assumption, multicolinearity and linear association. The relationship between mean HbA1c and outcome measures was in some cases linear and in other cases non-linear. For linear associations between mean HbA1c and severity of histological outcomes, odds ratios and 95% confidence intervals were estimated using regular ordinal logistic regression analyses.

In order to ensure that the non-linear relationship between HbA1c and certain histological outcomes was adequately captured, we utilized restricted cubic spline (RCS) method for continuous variable transformation using R package rms followed by original logistic regression analysis.(16) RCS with 3 knots (corresponding to the 10th, 50th, and 90th percentiles of mean HbA1c on the basis of the distribution of our data) was employed to estimate the dose-response relationship using an HbA1c reference of 5.0% (31 mmol/mol).(17) We chose a reference of 5.0% as this represents a low-normal HbA1c value against which to examine the impact of higher HbA1c values commonly seen in clinical practice. Odds ratios and 95% confidence intervals before and after the turning point (HbA1c of 7.0% [53 mmol/mol]), according to dose-response plots (Figure 2), were estimated using an ordinal logistic regression model after transforming mean HbA1c values using the method described by Singer and Willett.(18) All analyses were adjusted for age, sex, race, body mass index (BMI), diabetes status and hyperlipidemia status. The number of glucose-lowering drugs used by each patient did not impact the model and therefore was excluded as a covariate.

Group-based trajectory analysis

To examine the association between hepatic histological features and trajectory pattern of HbA1c 5 years preceding (and up to 90 days post-) biopsy, a group-based trajectory model (19) was employed using SAS Proc Traj macro.(20) Bayes information criterion (BIC) was used to assess the optimal number of trajectory groups, where lower BIC values point to a better model. Other criteria for ascertaining the best fitting model included non-overlapping confidence intervals, reasonable sample sizes in each identified trajectory group (each group should include at least 5% of the subjects), and distinct average posterior probabilities across groups. Finally, a multivariable ordinal logistic regression model was applied to investigate the association between HbA1c trajectory groups and all histological features, adjusted for age, sex, race, BMI, diabetes status and hyperlipidemia status.

In both mean HbA1c and group-based trajectory analyses, glucagon-like peptide-1 receptor agonist (GLP-1RA) use, thiazolidinedione use and ethnicity were examined as covariates in the model (see Supplemental Tables 2 and 3), however they did not substantially impact results, so were excluded as covariates in the main analysis to avoid over-fitting. Sodium-glucose cotransporter 2 inhibitor (SGLT2i) use was not examined as a covariate given its low prevalence in our cohort (0.4%).

RESULTS

A total of 713 patients met inclusion and exclusion criteria for the study. Of the 713 patients, 49% (n=348) had diabetes and 51% (n=365) did not have diabetes. Table 1 describes patient characteristics of the cohort, with stratification by diabetes status. The median age of our cohort was 50 years, and most (59%, n=417) were female. Patients with diabetes were older than those without (53 vs 47 years, p<0,0001) and were more likely to be female (67% vs 50%, p<0.0001). The majority of patients were Caucasian (84%) and non-Hispanic (72%), though there were proportionally more African American patients in the diabetes group (13% vs 6.3%, p=0.007). Cirrhosis (stage 4 fibrosis) was noted in 3.8%. Of patients with T2D, 66% were on metformin, and nearly 1 in 4 were on insulin therapy at time of liver biopsy. Median HbA1c was 6.9% (52 mmol/mol) in the diabetes group, and 6.0% (42 mmol/mol) in the whole cohort (see Supplemental Figure 1 for distribution of HbA1c values). Compared to individuals without diabetes, those with diabetes had higher median BMI (35 vs 32 kg/m^{2,} p<0.0001) and triglycerides (166 vs 144 mg/dL, p=0.0027). The overwhelming majority, 98% of those with diabetes, were diagnosed with T2D while only 2% were diagnosed with type 1 or indeterminate /mixed-type diabetes (n=8).

Mean HbA1c in the year preceding biopsy was significantly (linearly) associated with severity of liver fibrosis at time of biopsy, even after adjusting for age, sex, race, BMI, diabetes and hyperlipidemia status. Every 1% increase in mean HbA1c was associated with 15% higher odds of increased hepatic fibrosis, analyzed by original logistic regression model (OR 1.15, 95% CI 1.01, 1.31, *p* =0.039). The dose-response plot for hepatic fibrosis, derived from RCS methods, also showed a linear trend (Supplemental Figure 2), though this did not reach statistical significance (p = 0.097). When assessing individual covariates from the model, presence of diabetes was associated with the highest odds of increased fibrosis severity (OR 1.71, 95% CI 1.20, 2.44, *p* =0.003). Higher mean HbA1c preceding biopsy was also associated with higher grades of steatosis, ballooned hepatocytes and portal inflammation, but not with lobular inflammation (Table 2).

Dose-response plots derived from RCS methods demonstrated that the associations were non-linear and approximated inverted L-shaped curves for the relationships between mean HbA1c and steatosis, hepatocellular ballooning, and NAS (Figure 2). A V-shaped dose-response curve was observed for the association between HbA1c and portal inflammation (Figure 2). For mean HbA1c < 7.0% (53 mmol/mol), positive linear associations were detected for outcomes of steatosis, hepatocyte ballooning, and NAS; the significance of these linear associations were lost beyond a HbA1c of 7% (53 mmol/mol), Table 2 reports odds ratios and 95% confidence intervals for these histological outcomes after adjusting for age, sex, race, BMI, diabetes and hyperlipidemia status.

Group-based trajectory analysis was conducted for those patients with ≥ 3 measures of HbA1c values (n=298); how this group compares to those with < 3 HbA1c measures (i.e., those excluded from group-based trajectory analysis) can be seen in Supplemental Table 1. Three HbA1c control groups were identified by group-based trajectory analysis: good (group 1), moderate (group 2), and poor (group 3) glycemic control (Figure 3). As seen in Table 3, a higher proportion of patients with poor glycemic control self-identified as 'Other' race (31.8%) compared to the moderate (12.5%) and good (8.5%) glycemic control groups. Median HbA1c values were 6.0% (42 mmol/mol), 7.6% (60 mmol/mol) and 10.0% (86 mmol/mol) in the good, moderate and poor glycemic control groups, respectively. GLP-1RA use increased as glycemic control worsened (2.7% vs 10.2%, vs 13.6%, in groups 1, 2, and 3, respectively), as did insulin use (4.8% vs 43.2%, vs 77.3%, in groups 1, 2, and 3, respectively). Likewise, median BMI increased as glycemic control worsened (34.4 vs 35.3 vs 36.8 kg/m2, in groups 1, 2, and 3, respectively). Triglycerides were similar in moderate and poor control groups (median of 163 mg/dL and 166 mg/dL, respectively), and were lower in the good glycemic control group (median 147.5 mg/dL), although not significant between groups.

Compared to the good glycemic control group, patients with moderate control had substantially higher odds of advanced versus mild fibrosis (OR 4.59, 95% CI 2.33, 9.06), as well as higher odds of more severe hepatocyte ballooning (OR 1.74, 95% CI 1.01, 3.01) and higher NAS score (OR 2.49, 95% CI 1.25, 4.95). (Table 4) There was no significant difference between groups with good and moderate control as it related to severity of steatosis, portal or lobular inflammation (data not shown). Comparisons with the poor control group were limited by small sample size (n=22).

DISCUSSION

To the best of our knowledge, this is the first study to examine, in a large cohort of well-phenotyped patients with biopsy-proven disease, the association between cumulative glucose exposure and glycemic trajectories on the histologic severity of NAFLD/NASH. We found glycemic control preceding biopsy to be linearly associated with severity of fibrosis. HbA1c was also associated with severity of steatosis, hepatocyte ballooning, portal inflammation and likelihood of NASH, but this relationship was non-linear and varied depending on degree of glycemic control.

We found every 1% increase in mean HbA1c preceding biopsy to be associated with 15% higher odds of increase in fibrosis stage. Mean HbA1c was also associated with severity of ballooned hepatocytes (for HbA1c <7.0% [53 mmol/mol]; OR 1.62, 95% CI 1.15, 2.28). A similar pattern was seen in group-based trajectory analysis, where patients with moderate glycemic control had higher odds of increased stage of hepatic fibrosis and grade of ballooned hepatocytes than those with good control. The ability to discern an association between poor versus good/moderate glycemic control and the odds of more severe hepatic fibrosis or ballooned hepatocytes in group-based trajectory analysis was limited by small sample size, though this is a question of great importance and should be explored in future studies. Notably, evidence suggests that the severity of ballooned hepatocytes is a strong predictor of hepatic fibrosis.(7, 21) Therefore, the strong association of glycemic control with the severity of both ballooned hepatocytes and stage of hepatic fibrosis supports existing knowledge of T2D being a strong predictor of progressive NAFLD/NASH.

A number of studies have reported on the link between the presence of T2D and increased fibrosis severity, though data are mixed, and the influence of HbA1c on fibrosis is unclear.(22-26) A recent epidemiological study by Tanaka *et al* found glycemic control to be associated with advanced fibrosis (as defined by the lab-based fibrosis-4 index) up to an HbA1c of 7.9% (63 mmol/mol), but not beyond.(27) A similar finding was also reported in another study that utilized NAFLD fibrosis score to define advanced fibrosis, except that HbA1c was only predictive of advanced fibrosis in patients with HbA1c under 6.5% (48 mmol/mol).(26)

We observed a linear association between HbA1c and fibrosis stage over a broad range of HbA1c, from approximately 5 to 11% (31 to 97 mmol/mol). However, the association between HbA1c and likelihood of NASH, as well as severity of other histological features of NASH (i.e. steatosis, ballooning) was weaker beyond an HbA1c level of 7% (53 mmol/mol), similar to the HbA1c cut-offs noted in the literature. The reason for this discrepancy, particularly between ballooned hepatocytes and fibrosis, is unclear. One possible explanation is the phenomenon of “burnt-out NASH”, whereby patients lose characteristic features of NASH with worsening fibrosis severity.(28, 29) Since HbA1c preceding biopsy is linked with fibrosis severity, patients with poor glycemic control and severe fibrosis may have lost histologic features of steatohepatitis, resulting in a non-linear relationship between these histological features and HbA1c.

Another potential explanation for lack of linear association between steatosis and ballooning above an HbA1c of 7.0% is the greater utilization of glucose-lowering drugs with increasing HbA1c. Use of diabetes medications did not impact our results when included in the multivariable model, although change in medications over time was not captured in our study and may have still influenced this relationship between HbA1c and histologic features. This is particularly relevant for steatosis, since multiple glucose-lowering agents have been associated with reduction in hepatic steatosis.(30) Since steatosis and hepatocyte ballooning severity were linearly associated with glycemic control up to an HbA1c 7.0% (but not above), our results may also suggest that patients with prediabetes and mild or well-controlled diabetes (i.e. HbA1c <7.0%) are uniquely impacted by interventions to improve glycemic control and insulin resistance to avoid progression to severe NAFLD. To this end, several pharmacotherapies which lower blood glucose levels have demonstrated efficacy in achieving the endpoint of NASH resolution. (31-34)

Notably, an inverse association was observed between glycemic control and portal inflammation, whereby greater HbA1c below 7.0% (53 mmol/mol) was associated with lower odds of portal inflammation, and greater HbA1c above 7.0% (53 mmol/mol) was associated with higher odds of portal inflammation (Figure 2). The reason for this finding is unclear. Evidence suggests that portal inflammation, unlike lobular, is not statistically linked to NASH, hence its exclusion from the NAFLD histologic scoring system.(15) Nonetheless, moderate or severe portal inflammation has been linked to several features of advanced NAFLD,(35) and improvement in fibrosis has likewise been associated with improvement in portal, but not lobular, inflammation.(36) Therefore, our finding that poor glycemic control (above an HbA1c of 7.0% [53 mmol/mol]) was associated with greater odds of portal inflammation may be of clinical importance and warrants further investigation.

Very few studies have attempted to examine the influence of HbA1c on histological components of NASH, other than fibrosis, so we are limited in drawing comparisons to the literature. One longitudinal study by Hamaguchi *et al* examined 39 patients with sequential liver biopsies and observed change in HbA1c to be associated with progression of liver fibrosis, but not liver inflammation.(22) These results parallel our findings, though they did not examine severity of steatosis or ballooned hepatocytes. These results should be interpreted with caution due to small sample size and lack of adjustment for change in weight over time (22) as a potential confounder to changes in histologic features of NASH. Our study was of sufficient sample size (n=713) and the analysis adjusted for BMI. However, our analysis was limited by smaller sample size for group-based trajectory analysis comparisons with the poor glycemic control group (n=22). Larger population-based studies with prospectively collected and uniformly spaced HbA1c data paired with liver histology data would enhance our understanding of how glycemic control impacts the natural history of NAFLD disease progression and further validate the results and interpretation of our analysis.

Our study utilized liver histology as the gold standard by which to define the severity of NASH and stage hepatic fibrosis. Prior studies assessing the association of glycemic control and features of NAFLD utilized non-invasive approaches, such as lab-based scoring systems and/or imaging modalities to define disease activity. A recent study by Wang *et al.*(37) found glycemic measures to be associated with development and resolution of NAFLD by ultrasonography. Due to the lack of liver histology data, no conclusions could be rendered regarding the effect of glycemic control on severity of necroinflammation or fibrosis.(5)

For the first time in 2019, American Diabetes Association guidelines recommended review of laboratory and imaging data to proactively identify and risk-stratify NAFLD in patients with T2D.(38) With growing appreciation of the overlap between T2D and NAFLD, it is becoming increasingly important to understand how glycemic control, as measured by HbA1c, impacts risk of NASH and fibrosis. Our study has demonstrated a 15% higher odds of increased fibrosis stage with every 1% increase in HbA1c level. Further, odds of advanced (vs mild) fibrosis were 4.5 times higher in those patients with moderate vs good glycemic control. While these findings suggest HbA1c as a potential modifiable risk factor for NASH progression, we do not establish a causal relationship between glycemic control and NAFLD/NASH in this study. We likewise do not challenge the prevailing hypothesis that diabetes and NAFLD are both consequences of prolonged adipose tissue and hepatic insulin resistance, and that subclinical NAFLD likely precedes diabetes in most cases. As such, clinicians should continue to weigh the risks and benefits of lower HbA1c targets in their patients to ensure they are not causing harm (e.g., due to hypoglycemia). Longitudinal studies are needed to better examine this complex interplay between glucose exposure, insulin resistance and NAFLD, and to guide clinical care.

Our study has a few limitations. First, we adjusted for key variables at time of biopsy, such as age, BMI and hyperlipidemia, but did not collect these data longitudinally so were unable to adjust for changes over time. Since such covariates were our main method of capturing and adjusting for insulin resistance in multivariable analysis, our assessment of this important parameter could only be done cross-sectionally near the time of biopsy. With regards to BMI, we did exclude patients who underwent bariatric surgery to avoid confounding due to substantial weight loss during the study period. Although it was a small subset of the population, we may have missed unintentional weight loss in the poor glycemic control group as this can occur with persistent, severe hyperglycemia. Second, we may not have accounted for all liver effects of glucose-lowering drugs. For instance, preliminary data suggest potential benefit of GLP-1RA and SGLT2i for NAFLD beyond their effect on glucose and weight.(30, 39) However, use of SGLT2i was very low in this study (0.4%), and inclusion of GLP-1RA (and thiazolidinedione) use as covariates in the multivariable model did not materially impact our results (Supplemental Figure 3, Supplemental Tables 2 and 3). Since autoantibodies were not routinely checked in this cohort, it is possible we misclassified latent autoimmune diabetes of the adult as T2D, and though monogenic diabetes is rare, a small number of patients may have in theory been included in the analysis. Overall, NAFLD rarely occurs in the absence of insulin resistance, so any patients with hyperglycemia and NAFLD are likely to have T2D physiology, even if they have concurrent type 1 diabetes (T1D, n=8 in this study), or other rare forms. As such, interpretation of our results were unlikely affected by misclassification of T2D, or inclusion of T1D. Furthermore, the focus of this study was on the impact of cumulative glucose exposure on NAFLD, separate from other metabolic parameters (e.g., BMI, insulin resistance) that define clinical diabetes phenotypes.

In conclusion, this is the first study to *a priori* examine the effect of long-term glycemic control on histological outcomes of NAFLD/NASH. We found glycemic control preceding biopsy to be consistently associated with severity of hepatic fibrosis and hepatocyte ballooning. This study provides key insights into the relationship between glycemic control and NASH, and further research in this area will have important implications in diabetes practice, both when counseling patients at high risk of NASH, and when individualizing glycemic targets.

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Author contributions

ASA designed the study, interpreted results, and drafted the manuscript. MJC, ADC and MFA designed the study, interpreted results, and edited the manuscript. YW assisted in the design of the study, performed analyses and edited the manuscript. CAM, CDG, RH, DLP, KAS, RS, DDP, AMD, and MFA contributed to data contained in this analysis and edited the manuscript. All authors reviewed and approved final manuscript for journal submission. There were no authors who contributed to the study but did not meet requirements for authorship.

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Figure Titles and Legends

Figure 1: Flowchart of study population

Participants with alcohol consumption of ≥ 14 servings per week (for men) and ≥ 7 servings per week (for women) and those with known, pre-existing alternative chronic liver disease were excluded from the Duke NAFLD Clinical Database. Abbreviations: NAFLD=nonalcoholic fatty liver disease; HbA1c=hemoglobin A1c.

Figure 2: Dose-response relationship of mean HbA1c and the risk of severe hepatic histological outcome

Odds ratios and 95% confidence intervals (solid lines) plots derived from restricted cubic spline regression with three knots located at the 5th, 50th and 90th percentiles were shown (reference HbA1c = 5.0%, 5th percentile). Age, sex, race, BMI, T2D, and hyperlipidemia were adjusted for in the model.

Figure 3: Trajectory plots of HbA1c from 5 years preceding to 90 days post-liver biopsy

Group 1: Stable, good glycemic control (red); **Group 2**: Moderate glycemic control (green); **Group 3**: Persistently poor glycemic control (blue). For each trajectory group, the solid line represents the predicted trajectory and the dashed lines represent the 95% confidence intervals.

Table 1: **Patient characteristics of whole cohort at time of liver biopsy, and stratified by diabetes status**

Characteristics	**Whole cohort** (n=713)	**No Diabetes** (n=365)	**Diabetes** (n=348)	***P* Value**
Age (years)	50 (42, 58)	47 (39, 56)	53 (45, 59)	<0.0001*
Female sex (n,%)	417 (58.5%)	184 (50.4%)	233 (67.0%)	<0.0001†
Race (n,%)				
Caucasian	598 (83.9%)	319 (87.4%)	279 (80.2%)	0.0070†
African American	69 (9.7%)	23 (6.3%)	46 (13.2%)	
Other	46 (6.5%)	23 (6.3%)	23 (6.6%)	
Ethnicity (n,%)				
Hispanic	14 (2.0%)	8 (2.2%)	6 (1.7%)	0.0027†
Non-Hispanic	511 (71.7%)	281 (77.0%)	230 (66.1%)	
Unknown	188 (26.4%)	76 (20.8%)	112 (32.2%)	
Glucose-lowering drug use (n,%)				
Metformin	232 (32.5%)	0 (0.0%)	232 (66.7%)	<0.0001†
Sulfonylureas	68 (9.5%)	0 (0.0%)	68 (19.5%)	<0.0001†
Thiazolidinediones	21 (3.0%)	0 (0.0%)	21 (6.0%)	<0.0001†
DPP4 inhibitors	25 (3.5%)	0 (0.0%)	25 (7.2%)	<0.0001†
GLP-1 RA	22 (3.1%)	0 (0.0%)	22 (6.3%)	<0.0001†
Insulin	85 (11.9%)	0 (0.0%)	85 (24.4%)	<0.0001†
SGLT2i	3 (0.4%)	0 (0.0%)	3 (0.9%)	0.1158 ‡
Other medications (n,%)				
Statins	195 (27.4%)	61 (16.7%)	134 (38.5%)	<0.0001†
Vitamin E	43 (6.0%)	16 (4.4%)	27 (7.8%)	0.0586†
BMI (kg/m^2)	33.6 (30.3, 38.4)	32.3 (29.6, 36.3)	35.2 (31.6, 40.0)	<0.0001*
Systolic BP (mmHg)	132 (122, 141)	131 (122, 140)	133 (122, 142)	0.3838*
Diastolic BP (mmHg)	78 (71, 85)	79 (73, 86)	76 (70, 83)	0.0003*
Laboratory data				
HbA1c (%)	6.0 (5.5, 6.9)	5.6 (5.3, 5.9)	6.9 (6.4, 8.1)	<0.0001*
HbA1c (mmol/mol)	42 (37, 52)	38 (34, 41)	52 (46, 65)	<0.0001*
LDL (mg/dL)	110 (83, 139)	117 (89, 144)	103 (77, 134)	<0.0001*
HDL (mg/dL)	39 (32, 46)	40 (34, 47)	38 (31, 44)	0.0047*
Triglycerides (mg/dL)	155 (109, 225)	144 (104, 217)	166 (121, 236)	0.0027*
eGFR (mL/min/1.73m^2)	94.6 (80.8, 106.3)	92.8 (80.7, 106.5)	96.6 (81.0, 105.7)	0.5153*
Steatosis, grade (n,%)				
0	15 (2.1%)	9 (2.5%)	6 (1.7%)	
1	271 (38.0%)	128 (35.1%)	143 (41.1%)	0.2155†
2	256 (35.9%)	135 (37.0%)	121 (34.8%)	
3	171 (24.0%)	93 (25.5%)	78 (22.4%)	
Hepatocyte ballooning, grade (n,%)				
0	157 (22.1%)	107 (29.4%)	50 (14.4%)	<0.00001†
1	319 (44.9%)	169 (46.4%)	150 (43.2%)	
2	235 (33.1%)	88 (24.2%)	147 (42.4%)	
Lobular inflammation, grade (n,%)				
0	27 (3.9%)	16 (4.5%)	11 (3.3%)	0.5917†
1	455 (65.6%)	235 (66.0%)	220 (65.1%)	
2	190 (27.4%)	92 (25.8%)	98 (29.0%)	
3	22 (3.2%)	13 (3.7%)	9 (2.7%)	

Portal inflammation, grade (n,%)				0.0032†
0	420 (60.3%)	233 (65.6%)	187 (54.7%)	
1	277 (39.7%)	122 (34.4%)	155 (45.3%)	
Fibrosis stage (n,%)				<0.0001†
0	115 (16.1%)	79 (21.6%)	36 (10.3%)	
1	232 (32.5%)	135 (37.0%)	97 (27.9%)	
2	184 (25.8%)	87 (23.8%)	97 (27.9%)	
3	155 (21.7%)	56 (15.3%)	99 (28.5%)	
4	27 (3.8%)	8 (2.2%)	19 (5.5%)	
NAS score (n,%)				0.0043†
<4 (non-NASH)	210 (30.4%)	125 (35.2%)	85 (25.2%)	
≥4 (definite NASH)	482 (69.6%)	230 (64.8%)	252 (74.8%)	

Data presented as median (IQR) unless stated otherwise. Of patients with diabetes, 98% (n=341) had T2D and 2% (n=7) had type 1 diabetes. Abbreviations: BMI=body mass index; DPP4=dipeptidyl peptidase 4; GLP-1 RA=glucagon-like peptide-1 receptor agonist; SGLT2i=sodium-glucose cotransporter 2 inhibitor; BP=blood pressure; HbA1c=hemoglobin A1c; LDL=low density lipoprotein; HDL=high density lipoprotein; eGFR=estimated glomerular filtration rate; NASH=non-alcoholic steatohepatitis. The presence of NASH was defined as a NAFLD Activation Score of >4.(14)

*Wilcoxon rank sum test.

†Cochran-Mantel-Haenszel (CMH) test.

‡ Fisher’s exact test.

Table 2*:* Association of mean HbA1c in the year preceding biopsy with severity of hepatic histological features (OR per 1% change in HbA1c)

Histological Outcomes (Linear relationship)	**OR (95% CI)**	***P -value***
Fibrosis severity (stage 0-4)	1.15 (1.01, 1.31)	0.0390
Lobular inflammation (score 0-3)	1.12 (0.96, 1.30)	0.1440

Histologic outcomes (Non-Linear relationship)	**HbA1c < 7.0% (53 mmol/mol)**		**HbA1c > 7.0% (53 mmol/mol)**	
	OR (95% CI)	***P-value***	**OR (95% CI)**	***P-value***
Hepatocellular Ballooning (score 0-2)	1.62 (1.15, 2.28)	0.0060	1.06 (0.87, 1.29)	0.5843
Steatosis (score 0-3)	1.62 (1.15, 2.27)	0.0054	0.89 (0.73, 1.08)	0.2326
Portal inflammation (score 0-1)	0.67 (0.46, 0.99)	0.0448	1.31 (1.04, 1.64)	0.0196
Definite NASH vs. no NASH (NAS ≥4 vs <4)*	1.86 (1.25, 2.78)	0.0023	0.96 (0.75, 1.23)	0.7526

Odds ratios represent odds of more severe histology for every 1% increase in HbA1c. Ordinal logistic regression model was used and was adjusted for age, sex, race, BMI, T2D, and hyperlipidemia. Restricted cubic spline regression was employed to test the linear association between mean HbA1c and histological features. For outcomes with non-linear relationship to HbA1c, ORs (95% CI) before and after an HbA1c of 7.0% were estimated using ordinal logistic regression model after transforming mean HbA1c using the method described by Singer and Willett. (17) The choice of an HbA1c cut-off of 7.0% was data-driven, based on dose-response plots (Figure 2).

*Presence of definite NASH was defined as a NAFLD Activation Score of ≥4.(14)

Table 3: Patient characteristics at time of liver biopsy by HbA1c trajectory group

Characteristics	**Group 1** Good glycemic control (n=188)	**Group 2** Moderate glycemic control (n=88)	**Group 3** Poor glycemic control (n=22)	***P* Value**
Age (years)	52 (43, 60)	54 (47, 60)	51 (46, 57)	0.4424*
Female sex (n,%)	116 (61.7%)	58 (65.9%)	16 (72.7%)	0.5268†
Race (n,%)				
Caucasian	152 (80.9%)	72 (81.8%)	14 (63.6%)	0.0718†
African American	20 (10.6%)	5 (5.7%)	1 (4.6%)	
Other	16 (8.5%)	11 (12.5%)	7 (31.8%)	
Ethnicity (n,%)				
Hispanic	2 (1.1%)	3 (3.4%)	0 (0.0%)	0.0667†
Non-Hispanic	137 (72.9%)	52 (59.1%)	12 (54.6%)	
Unknown	49 (26.1%)	33 (37.5%)	10 (45.5%)	
Diabetes (n,%)	112 (59.6%)	88 (100.0%)	22 (100.0%)	<0.0001†
HbA1c (%)	6.0 (5.6, 6.5)	7.6 (7.2, 8.1)	10.0 (9.4, 11.0)	<0.0001*
HbA1c (mmol/mol)	42 (38, 48)	60 (55, 65)	86 (79, 97)	<0.0001*
Glucose-lowering drug use (n,%)				
Metformin	73 (38.8%)	64 (72.7%)	15 (68.2%)	<0.0001†
Sulfonylureas	15 (8.0%)	30 (34.1%)	3 (13.6%)	<0.0001†
Thiazolidinediones	4 (2.1%)	7 (8.0%)	1 (4.6%)	0.0719†
DPP4 inhibitors	4 (2.1%)	13 (14.8%)	1 (4.6%)	0.0002†
GLP-1 RA	5 (2.7%)	9 (10.2%)	3 (13.6%)	0.0104†
Insulin	9 (4.8%)	38 (43.2%)	17 (77.3%)	<0.0001†
SGLT2i	0 (0.0%)	1 (1.14%)	1 (4.5%)	0.0390 †
Other medications				
Statins	69 (36.7%)	46 (52.3%)	10 (45.5%)	0.0481†
Vitamin E	18 (9.6%)	2 (2.3%)	0 (0.0%)	0.0335†
BMI (kg/m^2)	34.4 (31.0, 39.5)	35.3 (32.5, 40.5)	36.8 (33.2, 41.6)	0.1668*
Systolic BP (mmHg)	132 (123, 141)	132 (121, 142)	135 (127, 145)	0.4037*
Diastolic BP (mmHg)	77 (69, 85)	75 (70, 80)	79 (73, 84)	0.2632*
Laboratory data				
LDL (mg/dL)	106 (82, 135)	97 (73, 119)	87 (69, 156)	0.1986*
HDL (mg/dL)	38 (33, 45)	38 (30, 43)	37 (30, 43)	0.3036*
Triglycerides (mg/dL)	147.5(115.0, 219.0)	163.0 (129,0, 218.0)	166.0 (107.0, 233.0)	0.4351*
eGFR (mL/min/1.73m^2)	93.8 (77.3, 103.7)	95.4 (82.1, 104.4)	101.6 (77.0,116.0)	0.2570*
Steatosis grade (n,%)				
0	1 (0.5%)	2 (2.3%)	1 (4.6%)	
1	79 (42.0%)	37 (42.1%)	9 (40.9%)	0.4033†
2	57 (30.3%)	33 (37.5%)	8 (36.4%)	
3	51 (27.1%)	16 (18.2%)	4 (18.2%)	
Hepatocyte ballooning, grade (n,%)				
0	41 (21.9%)	9 (10.2%)	3 (13.6%)	0.0027†
1	89 (47.6%)	36 (40.9%)	8 (36.4%)	
2	57 (30.5%)	43 (48.9%)	11 (50.0%)	
Lobular inflammation,				
grade (n,%)	3 (1.6%)	6 (7.1%)	1 (4.6%)	
0	136 (73.5%)	50 (59.5%)	13 (59.1%)	0.7241†
1	43 (23.2%)	25 (29.8%)	8 (36.4%)	
2	3 (1.6%)	3 (3.6%)	0 (0.0%)	
3				

Portal inflammation, grade (n,%)				0.2992†
0	110 (59.8%)	44 (50.6%)	11 (50.0%)	
1	74 (40.2%)	43 (49.4%)	11 (50.0%)	
Fibrosis stage (n,%)				0.0003†
0	34 (18.1%)	8 (9.1%)	1 (4.6%)	
1	64 (34.0%)	19 (21.6%)	7 (31.8%)	
2	53 (28.2%)	20 (22.7%)	8 (36.4%)	
3	34 (18.1%)	31 (35.2%)	5 (22.7%)	
4	3 (1.6%)	10 (11.4%)	1 (4.6%)	
NAS score (n,%)				0.0146†
<4 (non-NASH)	64 (34.8%)	15 (17.9%)	5 (22.7%)	
≥4 (definite NASH)	120 (65.2%)	69 (82.1%)	17 (77.3%)	

Data presented as median (IQR) unless stated otherwise. The presence of NASH was defined as a NAFLD Activation Score of >4.(14) Abbreviations: BMI=body mass index; DPP4=dipeptidyl peptidase 4; GLP-1 RA=glucagon-like peptide-1 receptor agonists; SGLT2i=sodium-glucose cotransporter 2 inhibitor; BP=blood pressure; HbA1c=hemoglobin A1c; LDL=low density lipoprotein; HDL=high density lipoprotein; eGFR=estimated glomerular filtration rate; NASH=non-alcoholic steatohepatitis.

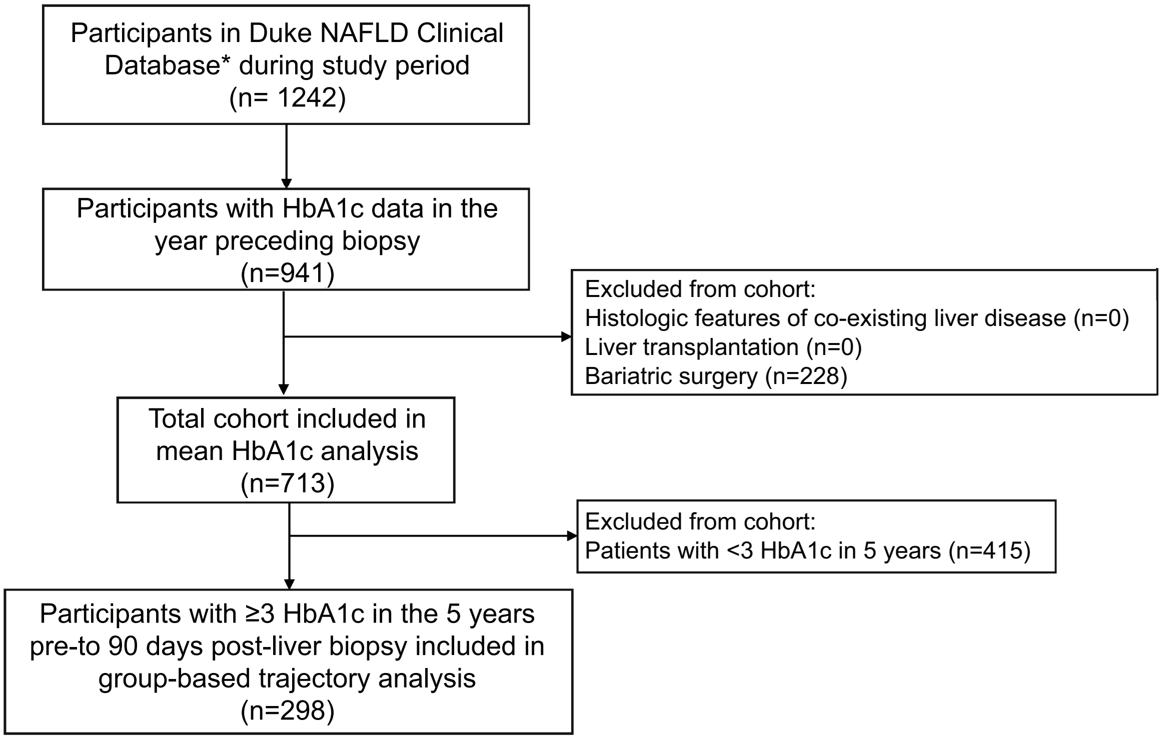
*Kruskal-Wallis test.;

†Cochran-Mantel-Haenszel (CMH) test.

Table 4: **Risk of more severe hepatic histological features on biopsy by glycemic control groups defined by HbA1c trajectory**

	OR (95% CI)	***P* value**
Fibrosis Severity (advanced vs mild; stage 3-4 vs 0-2)		
Group 2 vs Group 1 (Moderate vs Good control)	4.59 (2.33, 9.06)	*<0.0001
Group 3 vs Group 1 (Poor vs Good control)	2.52 (0.81, 7.84)	0.1117
Group 3 vs Group 2 (Poor vs Moderate control)	0.55 (0.18, 1.65)	0.2841
Hepatocellular Ballooning (grade 0-2)		
Group 2 vs Group 1 (Moderate vs Good control)	1.74 (1.01, 3.01)	*0.0479
Group 3 vs Group 1 (Poor vs Good control)	1.79 (0.72, 4.43)	0.2089
Group 3 vs Group 2 (Poor vs Moderate control)	1.03 (0.41, 2.60)	0.9525
Definite NASH versus no NASH **(NAS ≥4 vs <4)**		
Group 2 vs Group 1 (Moderate vs Good control)	2.49 (1.25, 4.95)	*0.0094
Group 3 vs Group 1 (Poor vs Good control)	1.77 (0.60, 5.23)	0.3037
Group 3 vs Group 2 (Poor vs Moderate control)	0.71 (0.22, 2.56)	0.5620

Odds ratios represent a logistic regression analysis with adjustment for age, sex, race, BMI, T2D and hyperlipidemia.



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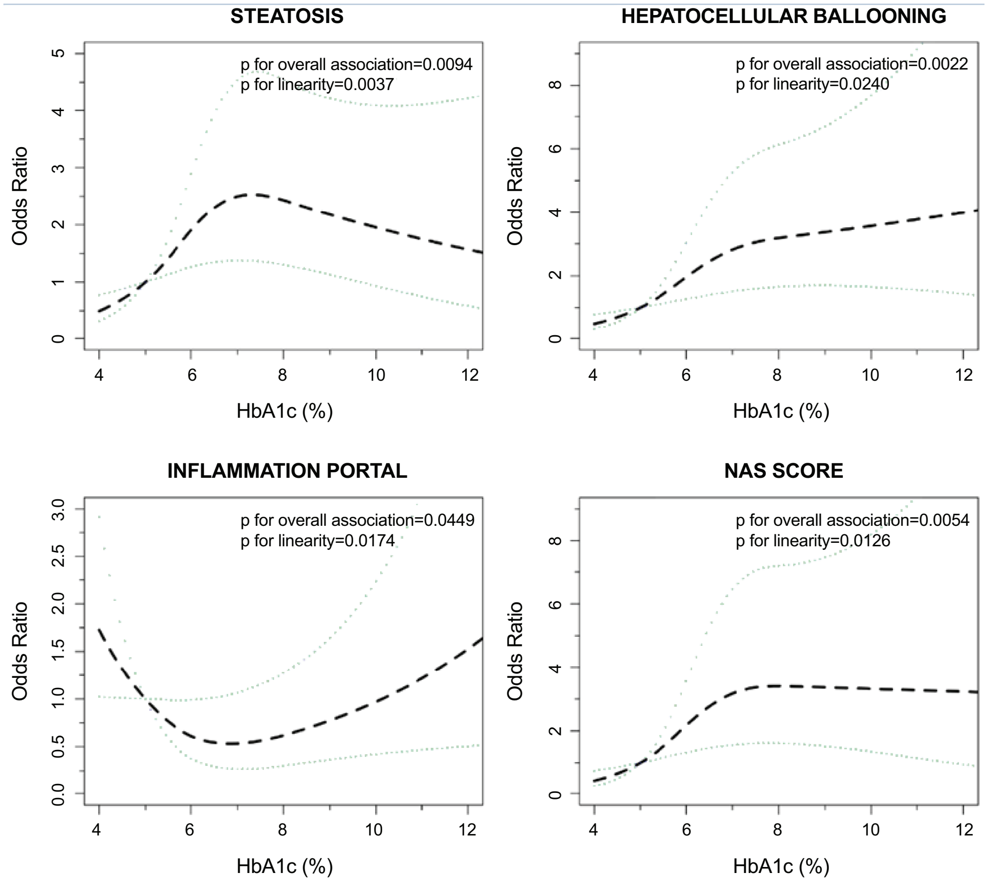

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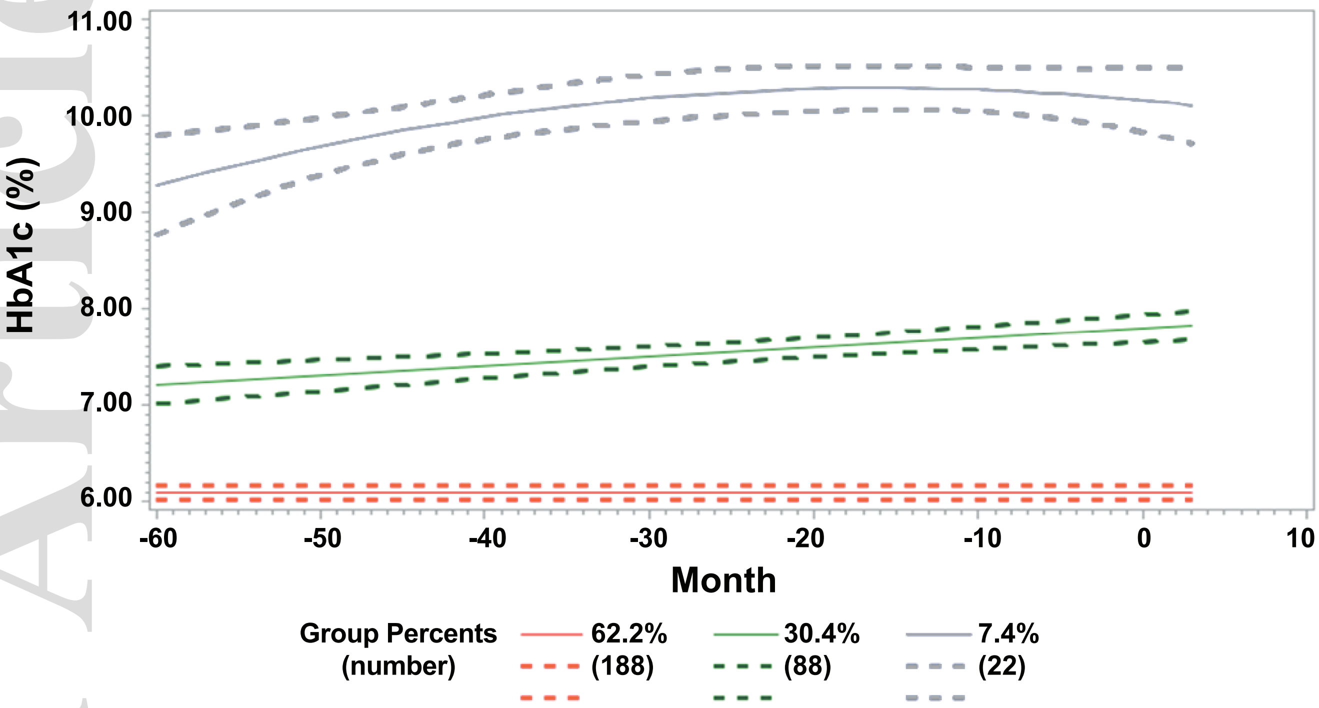
Figure 3: Trajectory plots of HbA1c from 5 years preceding to 90 days post-liver biopsy



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