Invited paper

Title: **Glycemic control predicts the risk of hepatic fibrosis in biopsy-proven NAFLD: a possible mediating role for leukemia inhibitory factor?**

Short Title: **LIF-mediated HbA1c-hepatic fibrosis association**

Authors' names

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Author's contributions

Feng Gong and Ming-Hua Zheng designed the study. Gang Li and Ou-Yang Huang collected the data. Feng Gong, Hong-Lei Ma, Liang-Jie Tang, Na He, and Man, Mi analyzed the data. Kenneth I. Zheng, Ming-Hua Zheng, Christopher D. Byrne, Giovanni Targher and Rafael S Rios contributed to writing and proof reading the manuscript. Administration, technique and materials were supported by Yang-Yang Li. All authors contributed to the manuscript for important intellectual content and approved the submission.

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List of Abbreviations

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HbA1c, haemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; LIF, leukemia inhibitory factor; NAFLD, nonalcoholic fatty liver disease; TC, total cholesterol; TG, triglycerides.

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None.

Conflict of Interest Statement

Nothing to declare.

Keywords: NAFLD; leukemia inhibitory factor; hepatic fibrosis

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a growing epidemic, affecting up to 30% of world's adults and around 55% of people with type 2 diabetes mellitus (T2DM).[1] T2DM is a well-established risk factor for the development of NAFLD, and is a strong predictor of liver-related morbidity and mortality.[2] Despite the strong link between NAFLD and T2DM, little is known about how the degree of glycemic control may impact the severity of NAFLD histology. Recently, Alexopoulos et al. reported a significant and independent association between the degree of glycemic control, as reflected by haemoglobin A1c (HbA1c) levels, and the severity of liver fibrosis.[3] However, as the authors mentioned, a causal relationship between HbA1c and the histologic severity of liver fibrosis has yet to be established. The aim of the present research was to further explore the relationship between HbA1c and the histologic severity of liver fibrosis in a Chinese cohort of well-phenotyped individuals with biopsy-proven NAFLD.[4,5]

Materials and methods

Patient population

Participants were prospectively recruited from the well-characterized PERSONS cohort in Wenzhou between June 2017 and July 2020. This study cohort included patients from a previously published study.[6-8] Enrollment criteria for the study included individuals with age between 18 and 75 years; evidence of hepatic steatosis

on imaging techniques (mainly ultrasonography), and then confirmed by liver biopsy. The exclusion criteria were as follows: (1) individuals who refused liver biopsy examination; (2) those with missing data for HbA1c levels; (3) those with secondary causes of chronic liver diseases (virus, drugs, autoimmunity); and (4) those who refused to sign the informed consent. A total of 568 subjects with NAFLD were initially included in the study, but we measured serum levels of cytokines only in a subset of 310 patients. The study protocol was approved by the ethics committee of the First Affiliated Hospital of Wenzhou Medical University (2016-246, 1 December 2016).[9,10] Written informed consent was obtained from each participant.

Clinical and laboratory data

Clinical and laboratory data were collected from all participants within 24 hours from liver biopsy. In all participants, demographic data, anthropometry, clinical parameters, as well as concomitant diseases were recorded by standard methods, as reported previously.[10] Hypertension was diagnosed by blood pressure ≥140/90 mmHg and/or use of any antihypertensive drugs. Homeostasis model assessment for insulin resistance (HOMA-IR) was used for assessing insulin resistance. Type 2 diabetes mellitus (T2DM) was diagnosed with at least one of the following widely accepted criteria: self-reported history of diabetes, use of any glucose-lowering agents, fasting glucose levels ≥7.0 mmol/L or hemoglobin A1c ≥6.5% (≥48 mmol/mol). Hyperlipidemia was defined as presence of total cholesterol (TC) ≥6.2 mmol/L, low-density lipoprotein (LDL-C) ≥4.1 mmol/L, triglycerides (TG) ≥2.3 mmol/L or use of

any lipid-lowering agents. Obesity was defined as a body mass index (BMI) ≥25 kg/m^2.

Liver biopsy

Ultrasound-guided liver biopsy was performed under local anesthesia using a 16-gauge Hepafix needle. Liver biopsy specimens were analyzed by experienced pathologists, who were blinded to participants' clinical details. Grading of NAFLD histology was determined by NAFLD activity score (NAS) according to the NASH Clinical Research Network scoring system.[11] The NAS score included three histologic features, including the presence of steatosis, lobular inflammation, as well as balloon-like hepatocytes. Liver fibrosis was staged from zero to four as follows: 0 = no fibrosis, 1 = perisinusoidal or portal fibrosis; 2 = perisinusoidal and portal/periportal fibrosis; 3 = bridging fibrosis; and 4 = highly suspicious or definite cirrhosis, respectively.[12]

Serum cytokines

Serum samples were collected from patients in the fasting state on the day of liver biopsy and stored at -80°C prior to testing. Serum cytokines were measured using the Bio-plex 200 system and matched kits (Bio-Rad, Hercules, California, USA). All measurements were performed only once. The intra- and inter-assay coefficients of variations were ⩽15% and ⩽25%, respectively.[13] Specifically, we measured the following serum cytokines: leukemia inhibitory factor (LIF), interleukin-1β (IL-1β),

interleukin-1 receptor antagonist (IL-1RA), IL-6, IL-7, IL-8, IL-9, IL-13, IL-16, IL-18 and interferon-inducible protein-10 (IP-10).

Statistical analysis

Continuous variables were expressed as means±SD or medians (Q1-Q3) and categorical variables were expressed as frequencies (%). Continuous variables were compared by the unpaired Student's *t* test (for variables normally distributed) or the Kruskal-Wallis test (for variables not normally distributed), whilst categorical variables were compared using the chi-square test. The association between HbA1c and features of liver histology was investigated with ordinal logistic regression analysis. Generalized additive models (GAM) and smooth curve fittings were used to identify non-linear relationship between HbA1c and increased fibrosis stage (stage F ≥3).[14,15] Causal mediation analysis was also performed to explore the indirect effect of the relationship between HbA1c and liver fibrosis in NAFLD mediated by serum cytokines.[16] Analytical software used consisted of SPSS version 22.0 (SPSS), and R3.3.1 (R Advancement Core Group, http://www.r-project.org).

Results

Patients characteristics

Of the 590 individuals with suspected NAFLD, who were initially enrolled in the study, we excluded 22 persons for the following reasons: 10 subjects for alcoholic fatty liver, 6 subjects for autoimmune hepatitis, 3 subjects for drug-induced liver

injury and 3 subjects for missing data. As a consequence, 568 individuals with biopsy-proven NAFLD were included in the final analysis. Our cohort was predominantly male (72.9%), with a mean age of 41 years; mean HbA1c of 6.3% and 26.4% had pre-existing T2DM. **Table 1** shows a summary of patients' anthropometric, clinical, laboratory and histological characteristics.

Relationship between HbA1c and liver fibrosis stage

We found that every 1% increase in HbA1c was associated with 16% higher odds of increased liver fibrosis (odds ratio 1.16, 95%CI 1.04-1.30), after adjustment for age, sex, body mass index, serum aminotransferases, lipids, insulin resistance, presence of T2DM and diabetes treatment by ordinal logistic regression.

Moreover, we found that there was a non-linear relationship between HbA1c and increased liver fibrosis (stage F ≥3) by GAM and smooth curve fittings after adjustment for the aforementioned covariates. In particular, a non-linear relationship was detected between HbA1c and increased liver fibrosis, whose HbA1c inflection point was 9.2% (77 mmol/mol). Interestingly, the ORs on the left and right sides of the inflection point were 2.1 (95% CI 1.4-3.3) and 0.1 (95% CI 0-4.8), respectively (**Figure 1A**).

Mediating effect of cytokines

We measured serum cytokine levels only in a subset of 310 individuals with biopsy-proven NAFLD (**Table 2**). We also performed a mediation analysis to disentangle the possible mediating role of leukemia inhibitory factor (LIF) in the HbA1c-the severity of liver fibrosis relationship. Interestingly, 7% of the association (OR 1.07, 95% CI 1.01-1.12) between HbA1c and liver fibrosis was mediated by serum, while the remaining 9% (OR 1.09, 95% CI 1.03-1.84) was mediated by a direct effect of HbA1c on the risk of increased liver fibrosis (**Figure 1B**).

Discussion

In this cross-sectional study, we have scrutinized the association between the degree of glycemic control, as reflected by HbA1c levels, and the severity of hepatic fibrosis and found a non-linear association between HbA1c and severity of liver fibrosis (stage F ≥3) in a Chinese cohort of well-phenotyped individuals with biopsy-proven NAFLD. Our results show that serum LIF levels are a single mediating factor that contributes to the association between HbA1c and the severity of hepatic fibrosis. These findings corroborate and extend the results of a previous study by Alexopoulos et al. through mediating effect analysis and threshold effect analysis.[3] In particular, our study confirms glycemic control to be linearly associated with the severity of fibrosis. The OR value of our study was 1.16 (95%CI 1.04-1.30), and that of the Alexopoulos study was 1.15 (95%CI 1.01-1.31). However, further exploring the complex relationship between HbA1c and liver fibrosis by means of a mediation analysis of serum cytokines, we also found a non-linear association between HbA1c

and increased liver fibrosis. Specifically, we found that increasing serum LIF levels may play an intermediary role in the relationship between HbA1c and the severity of hepatic fibrosis. Liver fibrosis is a structural change defined by an accumulation of extracellular matrix proteins, such as collagen often triggered by chronic inflammation.[17] LIF is an IL-6 family member. Levels of LIF and IL-6 are both increased locally in adipose tissue and systemically in pre-clinical models and patients with obesity.[18] These cytokines are also associated closely with chronic inflammation, and LIF signals through its canonical receptor LIFR (LIFR gene) and co-receptor gp130 to activate the JAK/STAT inflammatory pathway.[19] Therefore, we reason that increased levels of LIF may affect liver fibrosis mainly through this inflammatory pathway.

Our study has some important limitations that should be mentioned. Firstly, our data are derived from a single-center study that includes Chinese individuals with baseline characteristics that may differ from other cohorts of different ethnicities. Secondly, the cross-sectional design of our study precludes us from any causal inference. Moreover, we cannot exclude the possibility of residual confounding or confounding by unmeasured or unknown factors. Thirdly, liver biopsy is not commonly used to diagnose all patients with NAFLD, and the sample size of our study was relatively small. Therefore, there might be a selection bias affecting our study results towards NAFLD patients with younger age, and those without T2DM. That said, further research is needed to decipher the existing but complex link between HbA1c and the risk of

NAFLD-related fibrosis progression.[20,21]

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Table legends

Table 1. Clinical, biochemical and liver histology characteristics of participants with biopsy-proven NAFLD.

Table 2. Subgroup of 310 individuals with biopsy-proven NAFLD with available serum cytokine measurements.

Figure legend

Figure 1. (A) Non-linear association between HbA1c and risk of having advanced fibrosis (defined as stage F ≥3 on liver histology); (B) Mediation analysis model of the association between the degree of glycemic control (as reflected by HbA1c levels) and the risk of having hepatic fibrosis (i.e., 1-increment between F0 and F4 stage), mediated by serum leukemia inhibitory factor (LIF) concentrations.