Iron overload is associated with Non-alcoholic Fatty Liver Disease (NAFLD): Results from The NHANES III survey

Xin Yang1*, Fengyan Deng2

Abstract: Although several studies have showed that serum ferritin or iron overload was associated with non-alcoholic fatty liver disease, those studies may have bias due to the potential confounding and selection biases in observational data. In this study, we aimed to investigate relationship between iron overload and Non-alcoholic fatty liver disease using propensity score matching analysis to minimize the selection biases. A total of 12677 subjects were selected from National health and Nutrition Examination Survey (NHANES III) from 1988 to 1994, with 2741 non-alcoholic fatty liver disease patients determined by ultrasonography. After propensity score matching, 1317 cases and 1317 controls were selected based on greedy matching algorithm. Both multiple logistic regression and conditional logistic regression were conducted using original eligible data and propensity score matching data to assess the association. Before propensity score matching, the iron overload population was 1.53 (95% CI [1.27, 1.85]) times more likely to have non-alcoholic fatty liver disease than the non-iron overload population (adjusted for socio-economic factors and risk factors). After propensity score matching, the odds ratio of having non-alcoholic fatty liver disease in iron overload versus non-iron overload population was 1.45 (95% CI [1.43, 1.47]). Sensitivity analysis based on propensity score matched data also showed similar odds ratios in models adjusted for diabetes status or excluding diabetes patients. Propensity score matching analysis showed that iron overload is independently associated with non-alcoholic fatty liver disease, which is consistent with previous studies based on cross-sectional or cohort study.

Key words: Propensity score; NAFLD; iron overload; NHANES III.

Introduction

Non-alcoholic fatty liver disease (NAFLD), defined by excessive liver fat deposition, is an expression of metabolic syndrome in liver (1). NAFLD represents a spectrum of diseases including simple steatosis, steatohepatitis, fibrosis and cirrhosis. The prevalence of NAFLD increases dramatically in patients with diabetes and obesity and increases with age by 20% from 20 to 60 years old (2). The burden of NAFLD in US is significant and the prevalence of hepatic steatosis and NAFLD were about 21.4% and 19.0% (3). In the past 20 years, the prevalence of NAFLD among adolescents increased from 3.9% in 1988-1994 to 10.7% in 2007-2010. The prevalence also increased broadly in various groups such as race/ethnic subgroups, male and female and obesity status (4). Study shows that NAFLD is also associated with higher overall liver related mortality in the US population between 1988 and 1994 (5). Several risk factors have been reported to have association with NAFLD such as occupational stress, excessive calorie intake(6,7). Polymorphisms in Patatin-like phospholipase domain-containing protein 3 gene was shown to be associated with NAFLD(8). So far, interventions such as obeticholic acid (OCA), thiazolidinedione (TZD) and vitamin E have been shown to be effective in improving NAFLD conditions(9).

Ferritin is the primary iron-storage protein in the liver and serum ferritin reflects body iron stores and systemic inflammation (10). In recent years, serum ferritin was reported to be an independent predictor of advanced hepatic fibrosis and increased NAFLD activity score (NAS) in a study with 1635 subjects (11). Fasting serum ferritin and transferrin-iron saturation were reported to be positively associated with NAFLD in a cross-sectional study with 30 non-diabetic subjects (12). A cohort study of 2410 non-obese, healthy Korean male showed that serum ferritin was an independent risk factor of NAFLD (13). However, other studies found conflicting results in relationship between serum ferritin and histologic severity in NAFLD (14). Also, these studies are limited due to a lack of randomized control trial design and thus difficult to adjust for known and unknown confounding factors. Although it is impossible to randomly assign subjects to certain treatments or conditions, propensity score matching analysis can be applied to minimize selection bias and confounding variables in observational studies (15,16). Propensity score matching analysis has many advantages compared to conventional logistic regression and provide more reliable evidence to determine the causality than observational studies (17). To our best knowledge, no study has applied propensity score matching analysis to investigate the association between NAFLD and iron overload status, particularly using data from National health and Nutrition Examination Survey 1988-1994(NHANES III). Thus the purpose of the study is to assess the association between NAFLD and iron overload in a population-based, cross-sectional US civilian, non-institutionalized sample using propensity score matching analysis.
Materials and Methods

We extracted national data of participants from 20 to 74 years in National Health and Nutrition Examination Survey 1988-1994 (NHANES III). The NHANES III is a cross-sectional survey based on complex multistage, clustered, stratified probability-sample design, which represents a sample of US non-institutionalized population living in household. Detailed sampling method is described elsewhere (18).

Study subjects were excluded according to the following criteria: (1) HBV infection (presence of serum hepatitis B surface antigen); (2) HCV infection (presence of serum hepatitis C antibody); (3) Excessive drinking (>10 drinks/week for women, >20 drinks/week for men); (4) Missing value on ultrasonography or serum iron or ferritin.

Measurements

We defined NAFLD as moderate-severe hepatic steatosis based on ultrasonography in subjects without excessive alcohol use. Several parameters were used to determine the liver steatosis status including liver to kidney contrast, parenchymal brightness, deep beam attenuation, vessel walls definition, and gallbladder wall definition. Liver steatosis conditions were defined by a standardized algorithm as normal, mild, moderate, and severe steatosis according to the parameters above. Binary liver steatosis status was defined as either absent (normal-mild) or present (moderate or severe) (18). The excessive alcohol use was defined as more than 20 drinks for men and 10 drinks for women in the prior 12 months.

Body mass index (BMI) was calculated as weight (kg)/height (m²). We then categorized participants as “underweight” (<18), “Normal” (18-25), “Overweight” (25-30) and “Obese” (>30) based on their BMI. Metabolic syndrome was defined as meeting two or more following criteria: (1) waist circumference >102 cm in men or >88 cm in women; (2) Triglyceride level ≥150 mg/dL; (3) HDL <<40mg/dL in men and ≤50mg/dL in women; (4) SBP ≥130 mm Hg or DBP ≥85 mm Hg; (5) Fasting plasma glucose level ≥110 mg/dL. Participants are defined as “sedentary” if they said “no” to all the questions regarding physical activity last month, including jogging/running, bicycling, swimming, aerobics/aerobic dancing, other dancing, calisthenics, garden work, weight lifting or other sports. We defined smoking status on the basis of self-report smoking status. People were “current smokers” if they had smoked >100 cigarettes and currently smoke someday. “Former smoker” was defined as have smoked >100 cigarettes in their life but no current smoking. People who had smoked less than 100 cigarettes during lifetime are classified as “Non-smoker”. Diabetes was defined based on self-reported physician diagnosis, use of insulin, fasting plasma glucose ≥126 mg/dL or glucose tolerance test ≥200 mg/dL. For people without diabetes, Homeostasis model assessment (HOMA) was used to evaluate their insulin resistance using the following formula: fasting serum insulin (µU/mL) × fasting plasma glucose (mmol/L)/22.5. Insulin resistance was defined as HOMA>3. We then further classified participants into three groups: “Diabetes”, “No diabetes but have insulin resistance”, and “No diabetes and no insulin resistance”.

Hypertension was defined based on self-reported physician diagnosis, systolic blood pressure ≥140 mm Hg, or diastolic blood pressure ≥90 mm Hg.

Elevated alanine aminotransferase (ALT) or aspartate aminotransferase (AST) were defined base on the upper limit of normal value range (level of alanine aminotransferase >40 U/L for men and >31 U/L for women; level of aspartate aminotransferase >37 U/L for men and >31 U/L for women).

Iron overload was defined on the basis of serum iron/ferritin level obtained from NHANES III laboratory test. The criteria for iron overload is serum iron >190 µg/dl or serum ferritin >300 µg/L for men; serum iron >175 µg/dl or serum ferritin >200 µg/L for women.

Statistical analysis

Complex survey procedures in SAS 9.3 (SAS Institute, Cary, North Carolina) were used in all analysis by incorporating sampling weights to obtain unbiased estimates. The standard errors were estimated by Taylor series (linearization). Population characteristics were compared between groups using Rao-Scott modified chi-square tests for categorical variables or t-tests for continuous variables. Since NHANES III is a cross-sectional observational data, we used the propensity score matched data to account for the potential confounding and selection biases in the selected populations. A multiple logistic regression, of which iron overload status was used as dependent variable and other variables as independent variables, was used to determine the propensity score (c statistics is 0.712). Independent variables included above variables except diabetes. The propensity score matching procedure was conducted using Greedy algorithm based matching procedure in order to reduce the bias (http://www2.sas.com/procedings/sugi26/p214-26.pdf). The 1317 iron overload cases were matched to 1317 non-iron overload case with the maximum difference of 0.0657 in propensity score between each matched pair. The characteristics in the eligible study sample (n =12677) and propensity score matched sample (n = 2634) were described in table 2. To assess the association between iron overload status and NAFLD status, we conducted logistic regression models for initial eligible sample and conditional logistic regression for propensity score matched sample (table 3).

Results

Patient characteristics in NAFLD and non-NAFLD groups

A comparison of demographic and clinical characteristics between NAFLD and non-NAFLD groups is presented table 1 based on the initial eligible sample. On average, NAFLD population was 6 years older than non-NAFLD population. About 21.6% of subjects were (2741 out of 12576) defined as NAFLD based on the definition described above. Female were less likely to have NAFLD since 47.2% of NAFLD population and 54.4% non-NAFLD population were female. Among various race groups, Mexican American was more likely to have NAFLD. Approximately 7.71% of NAFLD patients and 4.85% of Non-NAFLD were Mexican American, while non-Hispanic black (NHB) has lower chance to have NAFLD since the proportion of NHB in NAFLD group was significantly lower than that in non-NAFLD group. Most NAFLD patients were overweight or obese (81.73%...
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In NAFLD versus 48.23% in non-NAFLD). Conditions such as hypertension, metabolic syndrome and elevated ALT/AST were associated with NAFLD since NAFLD group had a significant higher percentage of population with those conditions than non-NAFLD group (all p values < 0.0001). NAFLD patients tended to have less physical activity, lower poverty-income ratio (PIR) and higher level of serum cholesterol.

Population characteristics in iron overload and normal groups before and after propensity score matching analysis

Table 2 presents the characteristics in iron overload and normal groups before and after propensity score matching analysis. Before matching, iron overload population was less likely to be female (47.38% in iron overload and 53.99% in normal groups, p value = 0.0042), 4.5 years older than normal population (p value < 0.0001) and more likely to be non-Hispanic black (15.05% in iron overload group vs 10.07% in normal group, p value < 0.0001). Iron overload population was more likely to have obesity (29.08% in iron overload group vs 21.82% in normal group, p value < 0.0001), former smoker (28.64% in iron overload group vs 24.60% in normal group, p value =0.0078) and people with higher cholesterol level (5.60mmol/L in iron overload group vs 5.34mmol/L in normal group, p value < 0.0001). Conditions such as hypertension, metabolic syndrome and elevated ALT/AST were also positively associated with iron overload while sedentary life style was negatively associated with iron overload. Factors such as poverty income ratio and anemia were not significantly associated with iron overload status.

After propensity score matching, characteristics between iron overload and normal groups were no more significantly different, indicating the sample were randomized for all other variables. For example, the average age before matching in iron overload and normal groups were 45.8 and 41.4 respectively (p value < 0.0001). After matching, the average age was 47.9 and 48.2 respectively (p value = 0.721).

Sensitivity analysis for association between iron overload status and NAFLD

Table 3 presents the odds ratio (ORs) in various logistic regression models using eligible samples before or after propensity score matching analysis. In the crude model (model 1) with iron overload status as the only predictor, iron overload was significantly associated with NAFLD.
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<th>Table 2. Characteristics by iron overload status in initial eligible sample and propensity-matched sample.</th>
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<td><strong>Initial eligible sample</strong></td>
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<td><strong>Female, Gender</strong></td>
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<td><strong>Sedentary life style</strong></td>
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<td>Age (years)</td>
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<td>Poverty Income ratio</td>
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† Defined by serum ferritin and serum iron level.
‡ P values were reported by chi-square test or t-test.
BMI, body mass index; NAFLD, non-alcoholic fatty liver disease; SE, standard error; Elevated ALT/AST, Elevated alanine aminotransferase or aspartate aminotransferase.

(ORs = 1.98, 95% CI = [1.69, 2.32]). After adjusting for socio-economic factors and all other confounding variables (model 2 and model 3), the positive association between iron overload and NAFLD remained (ORs =2.07, 95% CI = [1.72, 2.48] for model 2 and ORs =1.53, 95% CI = [1.27, 1.85] for model 3). After performing logistic regression after propensity score matching, the association was still significant but the point estimate of odds ratio was lower than models using initial eligible sample (ORs =1.45, 95% CI = [1.43, 1.47]). Similarly, sensitivity analysis showed that iron overload was positively associated with NAFLD in the models adjusted for diabetes status and excluding diabetes patients (ORs =1.36, 95% CI = [1.34, 1.38] and ORs =1.40, 95% CI = [1.37, 1.42]).

**Discussion**

In our study, we investigated the association between iron overload status and NAFLD using propensity score matched sample from NHANES III. In the propensity score matching analysis, we simulated the randomized treatment assignment to adjust for major socio-economic and risk factors in the procedure, which minimized the selection bias of observational data. To our knowledge, this is the first study using propensity score matched analysis based on NHANES III to assess the relationship between NAFLD and iron overload status. In our study, iron overload was significantly positively associated with NAFLD, although the point estimates of ORs in models using propensity score matching sample were slightly lower than those before matching. The 95% confidence intervals for models in those propensity score matched sample were narrower than those in models before matching. Our study showed that iron overload defined by higher than 1 up limit of normal (ULN) of serum ferritin or serum iron could be a useful diagnostic marker to for evaluating patients with NAFLD.

In the study by Kowdley et al. serum ferritin (SF) was an independent predictor for NAFLD, which was assessed by histological features of NAFLD (11). Although
NAFLD was determined by ultrasonography in our study, our results were consistent with the conclusion made by Kowdley et al. In their study, as the SF level increased from > 1 ULN to > 2.5 ULN, there was an increasing trend in independent association between SF level and advanced Fibrosis. In our study, we used 1 ULN of SF and serum iron as criteria to determine iron overload status, and we also showed that subjects whose SF level or serum iron were greater than 1 ULN is more likely to have NAFLD. In another cohort study where NAFLD was also measured by ultrasonography, ferritin serum was also shown to be an independent risk factor of NAFLD, even in the non-obese, healthy subjects (13). A recent study also showed that serum ferritin was positively associated with NAFLD and negatively associated with β-cell function, which suggested pathophysiological link between iron metabolism, liver fat and diabetes (12). However, serum ferritin levels may not predict the stages of NAFLD, such as simple steatosis, NASH and cirrhosis (19). A study showed that iron depletion by phlebotomy might improve histological liver damage in patients with NAFLD (20), supporting the effect of iron overload on NAFLD.

Although the molecular mechanism that connects NAFLD and iron overload is still unclear, there are several explanations for iron overload-related NAFLD. Several studies showed that increased serum ferritin level might reduce insulin sensitivity, which mediate the pathogenicity of NAFLD (21,22). Study showed that insulin resistance in skeletal muscle can promote the free fatty acids by de novo hepatic lipogenesis (23). Excess free fatty acids flow to liver can accumulate lipid in the liver and cause NAFLD (24). Interestingly, iron depletion by venesection improve the normalization of insulin resistance in non-iron overload NAFLD patients (25) and iron overload NAFLD patients (26). Our results also support that excess serum iron/ferritin level is an indicator of NAFLD.

One of the strengths of this study is the large sample size that represents the US population due to the sampling design. We also used propensity score matching analysis in order to reduce the selection bias and simulate randomized sample. There are several limitations in the present study. First, the use of alcohol is based on the self-reported that may not accurately reflect the alcohol consumption. Second, due to the nature of observational study, it is impossible to eliminate the selection bias though the propensity score matching could significantly reduce bias. For example, the genetics variables of subjects were not assessed in the study and they may play an important role in causing NAFLD. The bias will not be eliminated unless more confounding variables were included in the conditional logistic regression models to generate propensity score due to the lack of longitudinal data. It is also impossible to establish the causality and temporality between NAFLD and iron overload due to the design. Third, our study used ultrasonography to determine the NAFLD. Although ultrasonography has many advantages such as low cost and easy operation in large sample screen, it is relatively insensitive to mild liver steatosis and the determination is operator-dependent. Additionally, ultrasonography cannot determine the advanced stages of NAFLD and therefore is rather a qualitative method (27). All these limitations mentioned above may generate potential bias to the association between NAFLD and iron overload status.

In conclusion, our study showed that iron overload, measured by serum iron and serum ferritin, is an independent predictor for NAFLD when using both NHANES III data before and after propensity score matching analysis. This result is consistent with other studies and supports previous conclusions on the relationship between serum ferritin and NAFLD with relatively low biased large data. Future studies are needed to elucidate the biological mechanism and causality about relationship between NAFLD and iron overload.

### References

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