The association between white blood cell subtypes and prevalence and incidence of nonalcoholic fatty liver disease

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Abstract
The association between white blood cell (WBC) and nonalcoholic fatty liver disease (NAFLD) has been studied before, but whether different WBC subtypes were related to NAFLD was not detailed. The aim of our study was to investigate the relationship between WBC subtypes and NAFLD cross-sectionally and prospectively. The detailed research design has been described previously. At baseline, there were 9930 participants who had complete information, in the end a total of 8079 participants (2588 men and 5491 women) were eventually included in this study. Hepatic ultrasound examination was performed on each participant at baseline and at the end of follow-up. Alcohol abuse and hepatitis were excluded. WBC subtypes and other serum indices were measured at baseline. We found that the total WBC, neutrophil, and lymphocyte counts were independently associated with the prevalence and incidence of NAFLD. After multiple adjustments for age, gender, body mass index (BMI), insulin, HOMA-IR, TG, TC, LDL, and HDL, increased odds ratios (ORs) for new onset NAFLD were observed from the 1st to the 4th quartiles of WBC, neutrophil, and lymphocyte (all $P < 0.001$ for trend). In conclusion, total WBC counts, neutrophils, and lymphocytes were all independent risk factors for NAFLD in the rural Chinese population. The association was independent of insulin resistance.

Keywords
lymphocytes, NAFLD, neutrophils, WBC

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Introduction
Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disease that is characterized by excessive fat deposition in the liver and is not caused by alcohol consumption. NAFLD is the most common cause of liver disease worldwide and it is estimated that the prevalence of NAFLD globally is up to 1 billion.1 The effects of NAFLD is not limited to the liver, it is linked with metabolic diseases and is an independent risk factor for cardiovascular disease (CVD) and type 2 diabetes.2,3 NAFLD has become one of the most important conditions that
influence public health. The pathogenetic mechanisms of NAFLD remain to be determined. Lipotoxicity, insulin resistance, and inflammation are all considered to be associated with the disease.\textsuperscript{4} White blood cell (WBC) count is routinely tested in the clinical practice as a marker of systemic inflammation. A rise in WBC count is related to many conditions associated with insulin resistance and chronic low-grade inflammation.\textsuperscript{5} Elevated WBC counts, even within normal range, have been reported to be associated with metabolic syndrome (MS) and NAFLD.\textsuperscript{6} To date, only a cross-sectional study has shown a notable relationship between WBC count and NAFLD,\textsuperscript{7} and two cohort studies revealed association between elevated WBC counts and the incidence of NAFLD.\textsuperscript{8,9} But in these studies, only total WBC counts were considered, whether different WBC subtypes were related to NAFLD was not reported. And the Chinese longitudinal cohort study was about an urban Han Chinese population, no research has reported the relationship between WBC subtypes and NAFLD in the rural Chinese population. Besides, the serum insulin levels and the common homeostasis model of insulin resistance index (HOMA-IR) were not measured in all the above studies, and whether the association between WBC counts and NAFLD was mediated by insulin resistance remains unexplained. So in this study, we conducted a longitudinal cohort study in the rural Chinese population to determine the relationship between WBC subtypes and NAFLD. We also tested serum insulin levels and HOMA-IR to prove if insulin resistance was involved.

**Subjects and methods**

**Subjects**

This study was one part of the survey from Risk Evaluation of cAncers in Chinese diabeTic Individuals: a IONGitudinal (REACTION) study, which was conducted among 259,657 adults, aged 40 years and above in 25 communities across mainland China, from 2011 to 2014. The sample groups were from the Chongming District of Shanghai, one of the 25 communities. Informed consents were obtained from all the participants, and approval was given by the Institutional Review Board of Xinhua Hospital affiliated to Shanghai Jiaotong University School of Medicine. We set missing anthropometry as an exclusion criteria. Other exclusion criteria were (1) those with a known history of liver diseases such as hepatitis, cirrhosis, or malignancy; (2) those with a consumption of alcohol greater than 140 g/week for men and 70 g/week for women; (3) those with more than three times the normal range of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), or $\gamma$-glutamyltransferase (GGT) and (4) acute or chronic inflammatory diseases obtained from the medical history. At baseline, there were 9930 participants who had complete information, in the end a total of 8079 participants (2588 men and 5491 women) were eventually included in this study. Hepatic ultrasound examination was performed on each participant. The subjects without NAFLD at baseline were followed for 3 years and at the end of follow-up, hepatic ultrasound examination was performed on each participant again.

**Data collection**

Peripheral venous blood samples were collected after fasting overnight and tested for fasting plasma glucose, lipids profile including triglycerides (TG), cholesterol (TC), high-density lipoprotein cholesterol (HDL), and low-density lipoprotein cholesterol (LDL), ALT, AST, GGT (all measured on an automatic analyzer, Hitachi 7080; Tokyo, Japan). Fasting serum insulin concentration was determined by RIA (Linco Research, St. Charles, MO). Plasma hemoglobin A1c (HbA1c) was determined by HPLC method (BIO-RAD, D10, CA). The total WBC counts and differential leukocyte counts were tested by an automatic blood cell analyzer (Beckman-Coulter LH750). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated based on the equation described by Matthews et al.\textsuperscript{10} 

**Definition of NAFLD**

Liver ultrasound examination was carried out on all participants by two experienced ultrasonographists without knowledge of the clinical and laboratory information using a high-resolution B-mode tomographic ultrasound system (EsaoteBiomedicaSpA, Italy) with a 3.5 MHz probe. Diagnostic criteria of fatty liver by ultrasonography were the existence of at least two of the three abnormal findings: diffusely enhanced echogenicity in the liver compared with the kidney, deep ultrasonic attenuation, and vascular blurring. NAFLD was defined by hepatic ultrasound
after the exclusion of alcohol abuse and other liver diseases.

Statistical analysis
Data were presented as means ± SD. One-way analysis of variance (ANOVA) was used to compare the differences between groups. The association of WBC subtypes and other parameters was tested using multiple stepwise regression analysis. Multivariate logistic regression models were used to calculate the odds ratios (ORs) for NAFLD. Potential confounders including age, gender, body mass index (BMI), blood pressure, serum lipids, insulin, and HOMA-IR were adjusted in the regression models. All statistical analysis was conducted using the SPSS Statistical Package (version 18.0; SPSS Inc., Chicago, IL). *P* values < 0.05 were considered statistically significant.

Results
At baseline, there were 9930 participants who had complete information; in the end a total of 8079 participants (2588 men and 5491 women) were eventually included in this study. Of the 8079 subjects, 3526 were with NAFLD (935 men and 2591 women), and 4553 were non-NAFLD (1556 men and 2997 women). Of the 4553 participants who were non-NAFLD initially, 1257 (303 men, 954 women) developed NAFLD after 3 years of follow-up, 2385 remained non-NAFLD (694 men, 1691 women), and 911 were lost. For the 4553 participants who were non-NAFLD at baseline, comparisons between participants who completed the 3-year follow-up (n = 3642) and participants who were lost to follow-up (n = 911) showed that these two groups were not statistically different on any of the variables examined in this study, the proportion of NAFLD was not different.

| Characteristics | Prevalent NAFLD | Incident NAFLD | Non-NAFLD | *P* value |
|-----------------|-----------------|----------------|-----------|-----------|
| N               | 3526            | 1257           | 2385      | <0.001    |
| Age (yr)        | 57.04 ± 7.41    | 55.38 ± 7.52   | 55.66 ± 8.05 | <0.001    |
| Sex (male/female)| 935/2591       | 339/918        | 818/1567  | 0.002     |
| BMI (kg/m²)     | 26.48 ± 6.62    | 24.43 ± 2.75   | 22.79 ± 2.95 | <0.001    |
| SBP (mmHg)      | 133.66 ± 18.84  | 129.54 ± 18.73 | 126.75 ± 18.94 | <0.001    |
| DBP (mmHg)      | 81.72 ± 9.88    | 80.08 ± 10.03  | 78.20 ± 10.38 | <0.001    |
| FBG (mmol/L)    | 6.61 ± 1.89     | 6.10 ± 1.40    | 5.96 ± 1.44 | <0.001    |
| HbA1C (%)       | 6.23 ± 1.13     | 5.85 ± 0.87    | 5.78 ± 0.87 | <0.001    |
| Insulin (pmol/L)| 9.58 ± 5.15     | 6.60 (5.00, 8.53) | 5.00 (3.80, 6.66) | <0.001    |
| HOMA-IR         | 2.89 ± 1.37     | 1.73 (1.34, 2.44) | 1.34 (0.96, 1.78) | <0.001    |
| WBC (10⁹/L)     | 6.22 ± 1.52     | 5.82 ± 1.40    | 5.53 ± 1.42 | <0.001    |
| Neutrophil (10⁹/L)| 3.63 ± 1.16   | 3.43 ± 1.09    | 3.26 ± 1.12 | <0.001    |
| Lymphocyte (10⁹/L)| 2.13 ± 0.61   | 2.00 ± 0.85    | 1.86 ± 0.55 | <0.001    |
| Monocyte (10⁹/L)| 0.31 ± 0.17     | 0.30 ± 0.09    | 0.29 ± 0.09 | <0.001    |
| Eosinophil (10⁹/L)| 0.12 ± 0.10   | 0.12 ± 0.00    | 0.12 ± 0.09 | <0.001    |
| Basophil (10⁹/L)| 0.015 ± 0.01    | 0.015 ± 0.01   | 0.015 ± 0.01 | 0.004     |
| HDL (mmol/L)    | 1.15 ± 0.28     | 1.23 ± 0.29    | 1.33 ± 0.34 | <0.001    |
| LDL (mmol/L)    | 2.70 ± 0.80     | 2.63 ± 0.76    | 2.55 ± 0.73 | <0.001    |
| TC (mmol/L)     | 4.77 ± 1.06     | 4.67 ± 1.04    | 4.57 ± 0.98 | <0.001    |
| TG (mmol/L)     | 2.13 ± 1.54     | 1.35 (0.99, 1.90) | 1.05 (0.80, 1.45) | <0.001    |
| ALT (U/L)       | 20.84 ± 15.63   | 15.40 ± 10.96  | 13.52 ± 9.56 | <0.001    |
| AST (U/L)       | 21.98 ± 11.71   | 19.68 ± 8.79   | 19.60 ± 8.87 | <0.001    |
| GGT (U/L)       | 33.44 ± 22.63   | 25.65 ± 17.46  | 20.32 ± 11.61 | <0.001    |

NAFLD: nonalcoholic fatty liver disease; BMI: body mass index; FBG: HOMA-IR: homeostasis model of insulin resistance index; WBC: white blood cell; HDL: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol; TG: triglycerides; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: γ-glutamyltransferase.

Data are means ± SD or median (interquartile range).
 incident NAFLD were more likely being female, with higher BMI, increased blood pressure, higher blood glucose levels, insulin levels, HOMA-IR, and blood lipids levels. WBC levels, neutrophil counts, and lymphocyte counts were significantly higher in prevalent and incident NAFLD subjects compared with non-NAFLD ones. The prevalent group had the highest levels of all these clinical and laboratory characteristics.

**Associations between WBC, neutrophils, and lymphocytes and NAFLD at baseline**

Table 2 displays the adjusted ORs and 95% confidence intervals (CIs) for prevalent NAFLD at baseline according to WBC, neutrophil, and lymphocyte quartiles. As can be seen, increased ORs for NAFLD were observed from the 1st to the 4th quartiles of WBC, neutrophil, and lymphocyte (all $P < 0.001$ for trend). In the highest quartile of WBC, neutrophil, and lymphocyte, after adjusting for age, gender, BMI, insulin, HOMA-IR, HDL, LDL, TC, and TG, the adjusted OR for NAFLD was 2.29 (CI, 1.98–2.66), 1.82 (CI, 1.58–2.10), and 2.10 (CI, 1.79–2.47), respectively.

**Table 2. Adjusted ORs and 95% CIs for prevalent NAFLD according to WBC, neutrophil, and lymphocyte quartiles.**

| WBC | ORs (95% CI) |
|-----|-------------|
| Q1 ($<4.80 \times 10^9/\text{l}$) | Q2 (4.80–5.69 $\times 10^9/\text{l}$) | Q3 (5.70–6.79 $\times 10^9/\text{l}$) | Q4 ($\geq 6.80 \times 10^9/\text{l}$) |
| NAFLD/non-NAFLD | 539/1,289 | 813/1,182 | 1,055/1,155 | 1,119/927 |
| Model 1 | 1 | 1.75 (1.53–1.99) | 2.25 (1.98–2.55) | 2.96 (2.60–3.36) | $P < 0.001$ |
| Model 2 | 1 | 1.55 (1.34–1.80) | 1.89 (1.63–2.18) | 2.29 (1.98–2.66) | $P < 0.001$ |
| Neutrophil | Q1 ($<2.70 \times 10^9/\text{l}$) | Q2 (2.70–3.29 $\times 10^9/\text{l}$) | Q3 (3.30–4.09 $\times 10^9/\text{l}$) | Q4 ($\geq 4.10 \times 10^9/\text{l}$) |
| NAFLD/non-NAFLD | 653/1,315 | 792/1,145 | 1,040/1,097 | 1,041/996 |
| Model 1 | 1 | 1.40 (1.23–1.58) | 1.93 (1.71–2.18) | 2.12 (1.87–2.40) | $P < 0.001$ |
| Model 2 | 1 | 1.33 (1.15–1.53) | 1.63 (1.41–1.87) | 1.82 (1.58–2.10) | $P < 0.001$ |
| Lymphocyte | Q1 ($<1.60 \times 10^9/\text{l}$) | Q2 (1.60–1.89 $\times 10^9/\text{l}$) | Q3 (1.90–2.29 $\times 10^9/\text{l}$) | Q4 ($\geq 2.30 \times 10^9/\text{l}$) |
| NAFLD/non-NAFLD | 570/1,252 | 694/1,060 | 970/1,217 | 1,292/1,024 |
| Model 1 | 1 | 1.45 (1.27–1.65) | 1.78 (1.57–2.02) | 2.03 (1.79–2.47) | $P < 0.001$ |
| Model 2 | 1 | 1.28 (1.07–1.53) | 1.37 (1.16–1.63) | $P < 0.001$ |

ORs: odds ratios; CIs: confidence intervals; NAFLD: nonalcoholic fatty liver disease; WBC: white blood cell; Q: quartile. Model 1 not adjusted. Model 2 adjusted for age, gender, BMI, insulin, HOMA-IR, HDL, LDL, TC, TG.

**Table 3. Adjusted ORs and 95% CIs for new-onset NAFLD according to WBC, neutrophil, and lymphocyte quartiles.**

| WBC | ORs (95% CI) |
|-----|-------------|
| Q1 ($<4.70 \times 10^9/\text{l}$) | Q2 (4.70–5.49 $\times 10^9/\text{l}$) | Q3 (5.50–6.49 $\times 10^9/\text{l}$) | Q4 ($\geq 6.50 \times 10^9/\text{l}$) |
| NAFLD/non-NAFLD | 258/663 | 288/599 | 335/591 | 376/532 |
| Model 1 | 1 | 1.24 (1.02–1.51) | 1.46 (1.23–1.76) | 1.80 (1.49–2.18) | $P < 0.001$ |
| Model 2 | 1 | 1.21 (0.98–1.49) | 1.42 (1.16–1.74) | 1.77 (1.44–2.17) | $P < 0.001$ |
| Neutrophil | Q1 ($<2.60 \times 10^9/\text{l}$) | Q2 (2.60–3.19 $\times 10^9/\text{l}$) | Q3 (3.20–3.89 $\times 10^9/\text{l}$) | Q4 ($\geq 3.90 \times 10^9/\text{l}$) |
| NAFLD/non-NAFLD | 202/672 | 327/595 | 331/544 | 397/574 |
| Model 1 | 1 | 1.37 (1.13–1.67) | 1.48 (1.22–1.80) | 1.70 (1.41–2.06) | $P < 0.001$ |
| Model 2 | 1 | 1.37 (1.12–1.69) | 1.47 (1.20–1.81) | 1.68 (1.37–2.06) | $P < 0.001$ |
| Lymphocyte | Q1 ($<1.50 \times 10^9/\text{l}$) | Q2 (1.50–1.79 $\times 10^9/\text{l}$) | Q3 (1.80–2.19 $\times 10^9/\text{l}$) | Q4 ($\geq 2.20 \times 10^9/\text{l}$) |
| NAFLD/non-NAFLD | 211/585 | 246/470 | 391/692 | 409/638 |
| Model 1 | 1 | 1.30 (1.06–1.61) | 1.49 (1.22–1.81) | 1.68 (1.38–2.05) | $P < 0.001$ |
| Model 2 | 1 | 1.31 (1.05–1.64) | 1.50 (1.21–1.85) | 1.65 (1.34–2.04) | $P < 0.001$ |

ORs: odds ratios; CIs: confidence intervals; NAFLD: nonalcoholic fatty liver disease; WBC: white blood cell; Q: Quartile; Model 1 not adjusted. Model 2 adjusted for age, gender, BMI, insulin, HOMA-IR, HDL, LDL, TC, TG.
lymphocyte quartiles. Increased ORs for new onset NAFLD were observed from the 1st to the 4th quartiles of WBC, neutrophil, and lymphocyte (all \( P < 0.001 \) for trend). In the highest quartile, the adjusted OR for NAFLD was 1.77 (CI, 1.44–2.17) for WBC, 1.68 (CI, 1.37–2.06) for neutrophils, and 1.65 (CI, 1.34–2.04) for lymphocytes after adjusting for age, gender, BMI, insulin, HOMA-IR, HDL, LDL, TC, and TG.

Discussion

NAFLD is a rising problem worldwide and is associated with harmful outcomes. Although the pathogenesis of NAFLD is still not very clear, insulin resistance, oxidative stress, and inflammation may be involved in the development and progression of NAFLD.\(^4\) WBC count is a marker of chronic low-grade inflammation.\(^5\) Elevated WBC counts, even within normal range, have been reported to be associated with NAFLD.\(^6\) WBC is also directly related to insulin resistance. But after adjusting for insulin and HOMA-IR, WBC was still an independent risk factor for NAFLD in this study. It seemed that inflammation is the possible link between WBC and NAFLD.

Neutrophils are the most abundant WBC subtypes in the human circulatory system, their contribution to chronic inflammation has only recently been noticed. Neutrophils are the immune cells that act in response first when inflammation happens in a body, and neutrophils will further promote the chronic inflammatory status by assisting macrophages recruiting and interacting with antigen presenting cells.\(^11\) Lymphocytes are spread in obese adipose tissues and regulate the production of inflammatory mediators from macrophages.\(^12\) Lymphocytes are essentially important for obesity-associated inflammation. In this study, we found neutrophils and lymphocyte are all independently related to the prevalence and incidence of NAFLD.

In sum, we found that total WBC counts, neutrophils, and lymphocytes were all independent risk factors of NAFLD in the rural Chinese population. Insulin resistance was involved in the development of NAFLD, but the association between WBC counts and NAFLD was not mediated by insulin resistance. It seemed that inflammation is the more possible link between WBC and NAFLD.

A limitation of this study is the lack of cytological and/or histopathological information because we could not carry out liver biopsy in this studied population, ultrasonic examination was used to detect NAFLD. Another limitation is that the response rate of women was high, leading to more women participants in this study. The third limitation is that there could be other environmental confounders which may influence our conclusions, and such factors, if discovered, need to be considered in future studies.

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Declaration of conflicting interests

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