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PAPER

Insulin resistance and ferritin as major determinants of nonalcoholic fatty liver disease in apparently healthy obese patients

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OBJECTIVES: The aims of this study were to test the possible association between nonalcoholic fatty liver disease (NAFLD) and iron and insulin resistance, and to determine the prevalence of NAFLD in apparently healthy obese subjects.
DESIGN: Cross-sectional, clinical epidemiologic study.
SUBJECTS: A total of 210 apparently healthy obese patients, aged from 18 to 65 y, with a body mass index (BMI) of 28 kg/m^2 or more, were enrolled in a body weight reduction program in our hospital.
MEASUREMENTS: All the subjects underwent screening and preprogram examinations, including anthropometric data measurements, biochemistry testing, and ultrasonography of the liver. NAFLD was defined as fatty liver diagnosed by ultrasonograpgy plus persistent elevation of alanine aminotransferase (ALT) levels.
RESULTS: Of the 210 patients, 80% (168/210) had fatty liver. Persistent ALT elevation in two separate tests was further detected in 25.6% (43/168) of patients. Multiple logistic regression analysis showed waist circumference and insulin resistance to be independently associated with fatty liver. Serum ferritin level and insulin resistance were two major risk factors predicting NAFLD.
CONCLUSION: The prevalence of NAFLD was 20.5% (43/210) in obese patients. As both hyperinsulinemia induced by insulin resistance and iron overload represented by ferritin elevation might damage hepatocytes, we concluded that these two factors were significantly associated with NAFLD in obese patients.
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Introduction

Nonalcoholic fatty liver disease is an increasingly recognized condition that may progress to liver cirrhosis,[1,2] and hepatocellular carcinoma only occurs in patients who developed liver cirrhosis.[3] A variety of terms have been used to describe this entity, but nonalcoholic fatty liver disease (NAFLD) seems to become the preferred term, which refers to a wide spectrum of liver damage, ranging from simple steatosis to steatohepatitis, advanced fibrosis, and cirrhosis.[4] The prevalence of NAFLD is about 20% (range, 15–39%) and the prevalence of nonalcoholic steatohepatitis (NASH) is 2–3% (range, 1.2–4.8%), making NAFLD the most common form of liver disease in the United States of America.[5]

Many recent studies mentioned about the possible role of iron in the pathogenesis of NAFLD. George *et al*[6] in Australia studied 51 NASH patients and found that 31% of them were either homozygous or heterozygous for the C282Y mutation of the hemochromatosis gene. Furthermore, they found that increased hepatic iron had the greatest association with the severity of fibrosis. However, Younossi *et al*[7] in the United States analyzed the clinicopathologic data of 65 NASH patients and showed that iron might not be associated with poor clinical or pathological outcomes. One of the possible reasons for reporting different results between these two studies is that the percentage of hemochromatosis gene mutation in the two study populations might have been different. Although the frequency of heterozygosity is 10% and the frequency of homozygosity is 0.3–0.5% for the

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C282Y hemochromatosis gene mutation[8] in Caucasians, people in Taiwan have seldom been reported to have the gene mutation. As we encounter many patients with fatty liver and iron overload in our clinical practice, the objectives of this study were to test the hypothesis that iron overload and other risk factors such as insulin resistance and obesity are related to NAFLD, and to determine the prevalence of NAFLD in obese patients.

Materials and methods

In total, 210 obese patients who met the criteria for the hospital body weight reduction program were enrolled from 2000 to 2002. The inclusion criteria were: age between 18 and 65 y and body mass index (BMI) of 28 kg/m^2 or more. The exclusion criteria were: any history of major cardiac, pulmonary, hepatic, gastroenterologic, neurologic, psychiatric, renal, or endocrine disease. Uncontrolled hypertension with systolic blood pressure greater than 165 mmHg or diastolic pressure greater than 105 mmHg was also an exclusion criterion. Diabetes mellitus patients under medication, any medication taken 7 days before the screening test, hepatitis B surface antigen (HBsAg) or antihepatitis C virus antibody (anti-HCV) positivity, antinuclear antibody (ANA) titer greater than 1:320, abnormal thyroid stimulating hormone test, and habitual alcohol intake once or more frequently per week were also exclusion criteria. A total of 250 patients underwent screening, which included a questionnaire and tests for HBsAg, anti-HCV, ANA, and creatinine. Among them, four were excluded because of alcoholism. Finally, 210 patients who met the above inclusion and exclusion criteria entered the body weight reduction program.

The research program was approved by the Institutional Review Board (IRB) of Tao-Yuan General Hospital (TYGH) before commencing. Each patient underwent a preprogram physical examination on the starting day of the program. After informed consent was obtained from each subject, the patients underwent fasting blood tests for aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, blood urea nitrogen, cholesterol, high-density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol, triglycerides, glucose, uric acid, ferritin, serum iron, transferrin iron binding capacity (TIBC), and complete blood count, after overnight fasting. Complete blood count was carried out on a Sysmex XE 2100 analyzer (Sysmex Corp, USA) with EDTA-treated whole blood. The serum biochemical tests above were conducted by Hitachi autoanalyzer model 7150 (Hitachi Corp, Tokyo, Japan). Insulin, HBsAg, and Anti-HCV were measured by microparticle enzyme immunoassay (MEIA) using the Abbott Axsym System instrument (Abbott Laboratories Services Corporation, Taipei, Taiwan) with serum. Ferritin, serum iron, and TIBC were measured by Roche Integra 700 system (Roche diagnostics, Taipei, Taiwan). The clinical laboratory of TYGH has joined international laboratory control program since 2000, and has kept the standard to within 2–7% of coefficients of variation. Abnormality of any of the above tests was defined as having any value greater than the normal range established by the Department of Laboratory Diagnosis of TYGH. Insulin resistance was measured by the homeostasis model assessment method (HOMA),[9] which was calculated as [insulin]/($22.5 \times e^{-\ln[\text{glucose}]}$) in percentage, where insulin concentration is in U/ml and glucose is in mmol/l. Anthropometric measurements of body height, body weight, waist circumference, and hip circumference were also made on the same day. Body weight was measured to the nearest 0.1 kg and height to the nearest 0.5 cm. BMI was calculated as weight (kg) divided by height squared (m^2). Waist circumference was measured midway between the lowest rib and the iliac crest; hip circumference at the level of the great trochanters was measured to the nearest 0.1 cm with flexible tape.[10] They were further asked to complete an interviewer-administered questionnaire, which inquired about histories of alcohol consumption, tobacco smoking, medication, and liver disease, family history of obesity and patient body weight history.

Ultrasonography (Toshiba, model SSA-340 equipped with a 3.75 MHz convex-type transducer) of the liver and spleen was also performed in each patient by a single hepatologist, who applied the criteria described previously[11–13] to make the diagnoses of fatty liver, parenchymal liver disease, liver cirrhosis, and splenomegaly. The scores of fatty liver was assessed by three hepatologists using the following five parameters without knowing the name and clinical data: liver–kidney contrast, gall bladder wall masking, blurring of wall of hepatic and portal veins, and far attenuation of the diaphragm. Each patient was examined in the morning on the beginning day of body weight reduction program under fasting status, and five images were recorded by the same operator and stored for further scoring and analysis. Each item of the five parameters was scored as 0, 1, 2, or 3, representing no, mild, moderate, or severe, respectively; the sum of the scores ranged from 0 to 15. The scoring jobs were done by comparing the previous stored ultrasonographic pictures as the standard ones demonstrating 0–3 of each item. The average of the sum of scores for each patient by the hepatologists was used for final diagnosis. The diagnosis of fatty liver was made if the sum of the scores equaled 2 or more. When mild (total score of 2–6), moderate (7–10), or severe (11–15) fatty liver was diagnosed, the corresponding histological grades of steatosis were about <30, 30–50, and 50% or more.[13] As IRB of TYGH did not agree to perform liver biopsy in apparently healthy obese patients, we use noninvasive method to diagnose fatty liver disease. 'Presumed' NAFLD was defined as fatty liver diagnosed by ultrasonography plus persistent elevation of ALT levels (>40 IU/l) in both screening and preprogram tests, performed two times 1–4 weeks apart.[14,15]

Statistical analysis was performed using Statistical Analysis System (SAS) edition 6.12 software. Student's *t*-test and the χ^2

test were used to compare the groups with and without NAFLD. Multiple logistic regression analyses were conducted to assess the variables associated with fatty liver alone and NAFLD. A *P*-value of less than 0.05 was considered statistically significant.

Results

The average age of the 210 obese patients was 35.6 ± 10.0 y (range, 18–61 y). More than three-fourths were women. The mean BMI was 31.5 ± 3.5 kg/m^2 for women and 32.3 ± 3.4 kg/m^2 for men, but there was no statistical difference between them. The mean waist circumferences were 91.1 ± 8.5 cm for women and 104.9 ± 8.4 cm for men; the difference between the results for women and men was statistically significant (*t*-test, $P<0.05$). Among the blood tests, the variables below were statistically higher in men: uric acid, triglyceride, ALT, and ferritin levels. On the other hand, HDL-cholesterol was higher in women than in men.

NAFLD was found in 43 (20.5%) out of the 210 subjects. Among these 43 patients, 20 ($20/47 = 42.6\%$) were men and 23 were women ($23/163 = 14.1\%$). NAFLD was significantly associated with following characteristics: male gender, taller height, heavier weight, larger BMI, and larger waist circumference, hip circumference, waist to hip ratio (WHR), higher uric acid, insulin resistance, hemoglobin, ferritin, serum iron, transferrin saturation, AST, and ALT levels, and lower HDL-cholesterol level (Table 1).

Fatty liver was found in 168 (80%) out of the 210 obese patients, including 58 with mild, 79 with moderate, and 31 with severe fatty liver. Nonetheless, only 43 (25.6%) out of the 168 fatty liver patients had persistent ALT elevation and were, thus, diagnosed with NAFLD. There was an increasing trend of ALT abnormality as the severity of fatty liver increased (Table 2). On the other hand, ALT elevation (>40 IU/l) was found in 45 (21.4%) out of the 210 obese patients; 43 (95.5%) of these 45 patients had fatty liver diagnosed by ultrasonography. None of our subjects had liver cirrhosis.

Owing to the positively skewed data for AST and ALT, logarithmic transformation was used to transform the data into nearly normal distributions. Through linear regression modeling, ferritin level, insulin resistance, BMI, and male gender were associated with log ALT after adjusting for age. Moreover, ferritin and insulin resistance were also significantly associated with log AST after controlling gender, age, and BMI.

Logistic regression analyses were conducted to determine the most powerful model to predict the diagnoses of fatty liver alone and NAFLD. The selected models are shown in Tables 3 and 4. We found that insulin resistance and waist circumference were two independent factors associated with the development of fatty liver, after controlling ferritin, BMI, sex, and age (Table 3). Insulin resistance and ferritin level were two independent factors significantly associated with NAFLD, after controlling for BMI, waist circumference, gender, and age (Table 4).

Table 1 Comparison of characteristics of obese patients with NAFLD and obese patients without NAFLD

Variable	*NAFLD*	*Without NAFLD*
No. of subjects	43	167
% Male**	46.5 (20/43)	16.2 (27/167)
Age (y)	32.9 ± 10.7[a]	36.3 ± 9.8
Body height (cm)**	165.9 ± 10.4	159.8 ± 7.9
Body weight (kg)**	94.3 ± 15.1	79.4 ± 11.7
BMI (kg/m^2)**	34.2 ± 4.0	31.0 ± 3.0
Waist circumference (cm)**	103.3 ± 9.6	91.9 ± 9.0
Hip circumference (cm)**	114.7 ± 7.6	109.3 ± 7.3
Waist–hip ratio**	0.90 ± 0.06	0.84 ± 0.07
Uric acid (μmol/l)**	505.6 ± 124.9	398.5 ± 89.2
Cholesterol (mmol/l)	5.3 ± 1.1	5.1 ± 0.9
Triglyceride (mmol/l)**	2.0 ± 0.9	1.4 ± 0.8
HDL-cholesterol (mmol/l)**	1.1 ± 0.2	1.3 ± 0.3
LDL-cholesterol (mmol/l)	3.3 ± 0.9	3.1 ± 0.8
Fasting sugar (mmol/l)	5.9 ± 1.4	5.6 ± 0.9
Insulin (pmol/l)**	195.9 ± 166.5	114.8 ± 60.3
Insulin resistance (%)**	7.2 ± 6.3	4.0 ± 2.4
Hemoglobin (g/l)*	138 ± 16	131 ± 13
Serum ferritin (μg/l)**	113.8 ± 99.2	38.4 ± 36.7
Serum iron (μmol/l)*,[b]	20.6 ± 8.2	16.8 ± 7.0
TIBC (μmol/l)[b]	67.5 ± 11.5	67.2 ± 9.8
Transferrin saturation (%)*,[b]	31.6 ± 12.8	25.8 ± 11.6
AST (IU/l)**	49.4 ± 28.7	19.3 ± 4.7
ALT (IU/l)**	86.7 ± 48.9	22.7 ± 10.9

Interval variables were tested using Student's *t*-test; nominal variables were tested using the χ^2 test. ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein. [a]Mean $\pm$ s.d., as appropriate throughout the table. [b]The levels of serum iron, TIBC, and transferring saturation were only available in 139 subjects, which included 29 in NAFLD group and 110 in non-NAFLD group.*$P<0.05$, **$P<0.01$.

Table 2 Prevalence of ALT elevation in different degrees of fatty liver, as observed using ultrasonography

Diagnosis of ultrasonography	*Normal*	*Mild*	*Moderate*	*Severe*	*Total*
ALT normal	40	55	59	11	165
ALT elevated	2	3	20	20	45
(%)	(4.8%)**	(5.2%)**	(25.3%)**	(64.5%)**	(21.4%)
Total	42	58	79	31	210
(%)	(20.0%)	(27.6%)	(37.6%)	(14.8%)	(100%)

**Cochran–Armitage trend test *P*-value < 0.01.

Discussion

As the gold standard for diagnosing fatty liver is histopathologic findings,[16] there is a concern about the accuracy of ultrasonographic diagnoses. We followed the procedures and criteria of Yang *et al*,[13] which showed an accuracy of 96.8% from direct comparison between histopathologic and ultrasonographic findings of 344 patients. Saverymuttu *et al*,[17] in a similar study, also demonstrated a sensitivity of 94% and

Table 3 Logistic regression analysis of the development of fatty liver alone in apparently healthy obese patients

Variable	*Range*	*Odds ratio*	*95% confidence interval*
Insulin resistance (%)**	0.15–37.83	1.16	1.06–1.27
Ferritin (ng/ml)	0.5–514.0	1.00	0.99–1.01
BMI (kg/m^2)	26.9–46.4	0.97	0.86–1.10
Waist circumference (cm)**	74.6–124.6	1.10	1.04–1.16
Gender	Male or female	0.69	0.27–1.75
Age (y)	18–64	1.01	0.98–1.04

*$P<0.05$, **$P<0.01$.

Table 4 Logistic regression analysis of nonalcoholic fatty liver disease ($n=43$) among fatty liver cases ($n=168$)

Variable	*Range*	*Odds ratio*	*95% confidence interval*
Insulin resistance (%)*	0.15–37.83	1.17	1.03–1.35
Ferritin (ng/ml)**	0.5–514.0	1.02	1.01–1.02
BMI (kg/m^2)	26.9–46.4	1.10	0.89–1.34
Waist circumference (cm)	74.6–124.6	1.04	0.94–1.14
Gender	Male or female	1.25	0.27–5.57
Age (y)	18–64	0.97	0.92–1.02

*$P<0.05$, **$P<0.01$.

specificity of 84%. Since the prevalence of fatty liver diagnosed by ultrasonography in our study was 80% in apparently healthy obese subjects, a figure similar to that reported for hospitalized patients and verified by biopsy,[18,19] we believe that our diagnoses were accurate and reliable. As the TYGH IRB would not approve of conducting liver biopsies in apparently healthy obese patients, noninvasive ultrasonographic diagnosis was the most feasible and ethical diagnostic choice.

As alcoholism is a potential major confounding factor in our study, it must be addressed appropriately. In the beginning when the obese patients were recruited to enter the body weight reduction program, they were requested to complete a questionnaire containing items related to the inclusion and exclusion criteria. But they were not told prior to filling the questionnaire that alcoholics would be excluded from the program. So we presume that most of them filled up the questionnaire with honesty and four male subjects of the original 250 volunteered to participate in the program were found and excluded. Furthermore, the prevalence of alcoholism (abuse plus dependent) was less than 10% in Taiwan and most of them were males.[20] In our study population, only 47 of the 210 patients were males. So the drinking problem is not likely to be a confounder in our study.

Although the detailed pathogenesis of hepatic steatosis remains unclear, recent studies show that insulin resistance reduces the ability of insulin to suppress lipolysis[21] and the release of very low-density lipoprotein (VLDL) from the liver,[22] and these phenomena further increase the level of free fatty acid and triglyceride in serum and hepatocytes. We found that waist circumference, in addition to insulin resistance, was independently associated with fatty liver (Table 3). A large autopsy study showed that the severity of fatty liver was associated with the degree of obesity.[23] Nonetheless, as BMI is not a direct reflection of the distribution of adipose tissue, waist circumference,[24] which represents the amount of visceral fat, was more associated with the risk of metabolic syndrome and fatty liver.[25] Thus, in the model of Table 3, waist circumference was a stronger indicator of fatty liver than BMI.

The prevalence of NAFLD was 20.5% (43/210) among our study subjects, which indicates that it is relatively prevalent among obese patients and deserves more attention. Nonetheless, the mechanism leading to NAFLD needs to be clarified. McGarry *et al*[26] reported that insulin inhibits β-oxidation of free fatty acids in mitochondria, which might lead to increased cellular levels of toxic free fatty acids, causing injury to hepatocytes. Wanless *et al*[27] showed that NASH histology could be induced by peritoneal infusion of insulin. Thus, persistent elevation of ALT could be induced by hyperinsulinemia resulting from insulin resistance.

Iron overload is associated with liver injury, although the mechanism also remains to be elucidated.[28] Ferritin is a high molecular weight iron storage protein occurring mainly in the cells of the liver and reticuloendothelial system.[29] Although serum ferritin levels could increase to a degree disproportionate to that of iron stores in some forms of inflammation, liver disease, and increased red-cell turnover, it closely reflects the size of the total iron burden[30] and ferritin iron.[31] As hemochromatosis is a well-known disease resulting from heavy iron overload, it might not be surprising for us to find ferritin-associated liver injury among patients with fatty liver. However, none of our subjects showed any sign consistent with hemochromatosis and the average level of ferritin was $113.8\pm99.2\,\mu g/l$. Initially, in the study, we simply wanted to monitor any possible anemia caused by the weight reduction program. Thus, we checked serum ferritin only. After the first 70 patients, we began to notice that ferritin was associated with an elevation of ALT and added the items of TIBC and serum iron for blood tests. And we successfully obtained the data of serum iron and TIBC of 139 cases (one missing) among the 210 study population. The ferritin was significantly correlated with TIBC saturation ($P<0.01$, $r=0.25$). Among the 210 patients, only five female patients had ferritin level exceeding $200\,\mu g/l$ and none of male patients were greater than $300\,\mu g/l$. Among the five female patients, we had the data of transferrin saturation in four, but no one had a saturation ratio greater than 47%. Thus, although we did not conduct gene analysis, the possibility of hereditary hemochromatotsis was very low. In fact, our finding seemed to be more consistent with the proposed new syndrome of liver iron overload with normal transferrin saturation by Moriand,[32] although the affecting level of ferritin was lower in our study.

The 'two-hit' theory of steatohepatitis was proposed under the observation that not all steatosis progresses to steatohepatitis.[33,34] The first hit means the development of fatty

liver, and the second one means the progression from fatty liver to steatohepatitis. The frequently reported first hits include obesity, diabetes mellitus, and hyperlipidemia, while reactive oxygen species induced by cytochrome *P*-450 2E1 and/or lipid peroxidation could be the major causes of a second hit.[35,36] As our study had already excluded other common causes of ALT elevation, such as viral hepatitis B or C, autoimmune hepatitis, drug or chemical induced hepatitis, alcoholic hepatitis, liver cirrhosis, and hepatocellular carcinoma, we believe that insulin resistance and ferritin are two independent risk factors predicting NAFLD (Table 4). In the future, for prevention of NAFLD, minimizing foods high in iron, such as red meat, should be considered[37] in apparently healthy obese patients with fatty liver. Additionally, alcohol, vitamin C, and nutrition supplements containing iron should probably be limited due to their effect of enhancing iron absorption.[38]

We reported a potential therapeutic effect of body weight reduction in NAFLD in obese patients with ALT elevation. In the 45 patients who completed 48 weeks' of the body weight reduction program, the ALT normalization rate was positively correlated with the body weight reduction percentage. The fatty liver score, estimated by ultrasonography, for assessing the severity of fatty liver was significantly decreased after body weight reduction.[39] One study also found that agents enhancing insulin sensitivity can treat NAFLD with 70% ALT normalization and modest histologic improvement.[40] Nonetheless, due to the small sample size, a larger, randomized study should be carried out in the future.

In conclusion, we found that NAFLD is a relatively common liver disorder among obese patients. NAFLD was significantly associated with insulin resistance and ferritin. Further studies focusing on the pathogenesis, causal–effect relationship with ferritin, and effective treatment of NAFLD is warranted.

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