

Relationship Between Immune Parameters and Non-alcoholic Fatty Liver Disease in Obese Children

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Received: May 27, 2016; Initial Review: November 03, 2016; Accepted: June 30, 2017.

Objective: To investigate the relationship between immune parameters and non-alcoholic fatty liver disease (NAFLD) in obese children.

Design: Cross-sectional study.

Setting: Hospital-based study in Zhejiang Province, China between July to September 2015.

Participants: A total of 117 obese children and 209 healthy non-obese children were studied as the obese and control groups. Depending on the severity of NAFLD, the obese group was divided into subgroups 1 (without NAFLD), 2 (with simple fatty liver) and 3 (with steatohepatitis).

Outcome Measures: Glucose metabolism, lipid metabolism and immune parameters.

Results: In the obese group, body mass index (BMI), waist-and hip-circumferences, fasting insulin, Homeostasis model of

assessment for insulin resistance (HOMA-IR), triglyceride, total cholesterol, low density lipoprotein cholesterol (LDL-C), apolipoprotein (Apo)B/ApoA1, alanine aminotransferase, uric acid, white blood cells, neutrophils percentage, platelet and interleukin (IL)-6 were significantly higher than those in the controls ($P<0.05$), while lower high density lipoprotein cholesterol and lymphocyte percentage were noted ($P<0.05$). IL-10 in the subgroup 3 was higher than those in the control group, subgroup 1 and 2 ($P<0.05$). Logistic regression analysis showed that BMI, LDL-C, HOMA-IR and IL-10 were independent factors of NAFLD ($P<0.05$).

Conclusion: These results support a low-grade chronic inflammation in obese children. Moreover, obesity, dyslipidaemia and IR are risk factors while IL-10 may be a protective factor for NAFLD.

Keywords: Cytokine, Interleukin-6, Interleukin-10, Metabolic syndrome, Overweight

Published online: July 11, 2017. PII:S097475591600073

Non-alcoholic fatty liver disease (NAFLD) is a severe complication of obesity. It is a kind of hepatic steatosis, inflammation and fibrosis, which is characterized by fatty infiltration in over 5% hepatocytes without a history of alcohol intake, viral infections, autoimmune hepatitis or drug-induced liver disease. According to pathologic changes and liver function, NAFLD is divided into simple fatty liver (SFL), non-alcoholic steatohepatitis (NASH) and cirrhosis [1]. With the increase of childhood obesity, the incidence of NAFLD has increased recently. Studies on NAFLD have suggested that free fatty acid, triacylglycerol and insulin resistance (IR) were involved in pathogenesis of NAFLD [2,3]. However, the pathogenesis of NAFLD is not fully understood till now.

Recently, multitude evidence has revealed that obesity was a low-grade chronic inflammation process. Interleukin (IL)-6, tumor necrosis factor-alpha (TNF- α) may contribute to inflammation [4-6]. TNF- α was reported to

be closely related to IR. In addition, some cytokines may play a protective role in the progression of NAFLD, such as IL-4 and IL-10 [7]. However, studies on the cytokines and NAFLD in children were rare. We studied the relationship between immune parameters and NAFLD to explore the risk and protective factors for NAFLD.

METHODS

A total of 117 obese children (81 males), were studied as the obese group aged between 5.2 to 14.8 years. They were hospitalized in the Endocrinology Department of Children's Hospital, Zhejiang University School of Medicine during July to September 2015 to definite etiology and investigate the complications of obesity, such as disorder of glucose metabolism or lipid metabolism disorders. Their body mass index (BMI) ranged from 22.2 to 42.2 kg/m². Children with viral hepatitis, drug-induced hepatitis, autoimmune liver disease, infection, severe heart and kidney disease, or alcohol consumption were excluded. Another 209 non-obese healthy children aged

6-14 year from Children Health Care Department in our hospital were enrolled as the control group. Their BMI ranged from 11.6 to 20.3 kg/m². The age difference between the two groups was significantly different ($P=0.03$) (**Table I**).

Depending on the severity of NAFLD, the obese group was divided into three subgroups: *Subgroup 1* (Obesity without NAFLD, $N=23$), *Subgroup 2* (Obesity with SFL, $N=43$) and *Subgroup 3* (Obesity with NASH, $N=51$). Subgroup 1 (mean age, 9.8 (2.5)y, 14 males) had BMI from 22.4 to 32.0 kg/m² (mean BMI, 26.1 (2.5) kg/m²). Subgroup 2 (mean age, 10.8 (2.2)y, 30 males) had BMI from 22.2 to 34.9 kg/m² (mean BMI, 28.2 (3.1) kg/m²). Subgroup 3 (mean age, 11.3 (1.9) y, 37 males) had BMI from 22.5 to 42.2 kg/m² a (mean BMI, 29.7 (4.2) kg/m²). The difference of age between the groups was statistically significant ($P=0.003$).

This study was approved by the Ethics Committee of the Children's Hospital of Zhejiang University School of Medicine. Informed consent was obtained from the parents of these children.

Obesity was diagnosed according to the criteria that BMI was above 95th percentile compared with the same age and sex children (Height and weight standardized growth charts for Chinese children and adolescents aged

0 to 18 years) [8]. NAFLD was diagnosed according to the revised definition and treatment guidelines for NAFLD by the Chinese Hepatology Association in 2010 [9]. NAFLD was diagnosed as a diffusely echogenic change on liver B-mode ultrasonography (fatty infiltration in liver), with or without elevated liver enzyme (alanine aminotransferase and aspartate aminotransferase). SFL was defined as a diffusely echogenic change with normal liver enzyme while NASH was defined as a diffusely echogenic change with elevated liver enzyme.

Anthropometric indicators were measured by professionals with unified calibrated measurement tools. Body height was measured to the nearest 0.1 cm, after subjects took off their shoes, standing on the measuring instrument. Body weight was measured to the nearest 0.1 kg on a scale. Waist circumference was measured to the nearest 0.1 cm by measuring at the midpoint between the lower rib margin and the iliac crest using a conventional tape in centimeters. Hip circumference was measured to the nearest 0.1 cm by measuring maximum hip circumference. BMI was calculated as weight (kg) divided by height (m) squared.

Blood samples were taken after overnight fasting and placed into tubes at room temperature. Then serum samples were isolated and stored at -70°C until use.

TABLE I BASELINE PARAMETERS IN THE CONTROL AND OBESE GROUPS

	Control group (n=209)	Obese group (n=117)	P value
Female/male	149/60	81/36	0.706
Age (y)	10.3 (1.8)	10.8 (2.2)	0.028
Body mass index (kg/m ²)	15.6 (1.9)	28.5 (3.8)	<0.001
Waist circumference (cm)	55.7 (5.6)	91.1 (10.7)	<0.001
Hip circumference (cm)	65.4 (6.2)	95.8 (9.3)	<0.001
*Fasting glucose (mmol/L)	5.3 (5.1-5.5)	5.3 (5.0-5.7)	0.632
*Fasting insulin (pmol/L)	6.9 (4.6-9.6)	17.8 (12.7-26.3)	<0.001
*HOMA-IR	1.6 (1.1-2.2)	4.1 (3.1-6.5)	<0.001
*Triglyceride (mmol/L)	0.8 (0.6-1.1)	1.3 (1.0-1.9)	<0.001
Cholesterol (mmol/L)	4.4 (0.8)	4.7 (0.8)	0.009
*Lipoprotein A (mmol/L)	89.0 (44.0-201.0)	79.0 (47.5-158.0)	0.369
HDL-C (mmol/L)	1.6 (0.3)	1.3 (0.3)	<0.001
LDL-C (mmol/L)	2.7 (0.5)	2.9 (0.5)	<0.001
ApoB/ApoA1	0.3 (0.1)	0.5 (0.2)	<0.001
*Alanine aminotransferase (U/L)	15 (13-20)	34 (23-69)	<0.001
*Aspartate aminotransferase (U/L)	27 (24-30)	28 (22-44)	0.120
Uric acid (μmol/L)	271.2 (65.4)	405.9 (94.2)	<0.001

HOMA-IR, homeostasis model of assessment for insulin resistance index; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; Apo, apolipoprotein; *Median (IQR).

Fasting blood glucose, insulin, triglyceride, total cholesterol, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lipoprotein A, uric acid, apolipoprotein (Apo) A1 and ApoB were measured in our clinical laboratory center. IR was individually estimated by the homeostasis model of assessment for IR (HOMA-IR) by considering as (fasting insulin×fasting glucose)/22.5.

Serum Th1/Th2 cytokines were measured by flow cytometry. IL-2, IL-4, IL-6, IL-10, TNF- α and interferon (IFN)- α were quantitatively determined by the CBA kit-BD™ CBA Human Th1/Th2 Cytokine Kit II (BD Biosciences, San Jose, CA). Peripheral blood cell count, including white blood cells, percentage of neutrophils, percentage of lymphocytes and platelet, were measured in our clinical laboratory center.

Statistical analysis: Statistical analyses were conducted by using SPSS software (SPSS Inc. Chicago, USA). The Pearson Chi-square test was used to analyze the difference of categorical data between different groups. Quantitative data with normal distribution were analyzed by the independent t test or One-way ANOVA followed by LSD test when appropriate. Fasting insulin, HOMA-IR, triglyceride, lipoprotein A, ApoB/ApoA1, IL-2, IL-4, IL-6 and TNF- α were analyzed after logarithmic transformation. Skewed data were compared with Mann-Whitney U test. Logistic regression analysis was performed to analyze the independent factors of NAFLD (assignment: control group = 1, subgroup 1 =2, subgroup 2=3, subgroup 3=4). Factors with $P<0.3$ in the comparison between obese and control groups were enrolled in the logistic regression analysis. The differences were considered significant at $P<0.05$.

RESULTS

In this study, 125 obese children were screened and 8 children were excluded because of hepatitis B or Prader-Willi syndrome. Also, 221 non-obese children were screened and 12 children were excluded because of hepatitis B, atrial septal defect or hypothyroidism. A total of 117 obese children and 209 non-obese children were enrolled in the obese and control groups in the following analysis. We diagnosed NAFLD in 94 (80.3%) obese children.

Compared to the control group, higher BMI, waist circumference, hip circumference, fasting insulin, HOMA-IR, triglyceride, cholesterol, LDL-C, ApoB/ApoA1, ALT and uric acid, and lower HDL-C were found in the obese group ($P<0.05$, respectively). However, fasting glucose, lipoprotein A and AST were not significantly different between the obese and control

groups (**Table I**). Among the 117 obese children, NAFLD was found in 94 (80.3%), including 43 (36.7%) in subgroup 2 and 51 (43.6%) in subgroup 3. Comparing the control and obese subgroups, BMI, waist circumference, hip circumference, fasting insulin, HOMA-IR, triglyceride, total cholesterol, LDL-C, HDL-C, ApoB/ApoA1, uric acid and ALT were significantly different ($P<0.05$, respectively), although fasting blood glucose and lipoprotein A were not different among these groups ($P>0.05$, respectively). BMI, waist circumference, hip circumference, fasting insulin, HOMA-IR, triglyceride, LDL-C, ALT and uric acid have increased tendency while HDL-C have decreased tendency among the control group and subgroups of obese group.

Higher white blood cells, percentage of neutrophils, platelet and IL-6, and lower percentage of lymphocytes in the obese group were noted ($P<0.05$). The difference of IL-10 was marginal between the control and obese groups ($P=0.07$). IL-2, IL-4, TNF- α and IFN- α were not significantly different between the obese and control groups (**Table II**). Moreover, white blood cells and platelet in the 3 subgroups were higher than those in the control group ($P<0.05$).

Logistic regression analysis showed that BMI, LDL-C, HOMA-IR and IL-10 were independent determinants for NAFLD ($P<0.001$, $P=0.024$, $P=0.009$ and $P=0.024$) (**Web Table I**).

DISCUSSION

We found NAFLD in 94 (80.3%) of the 117 obese children. Logistic analysis revealed that BMI was the most significant independent determinant of NAFLD in

TABLE II IMMUNE PARAMETERS BETWEEN THE CONTROL GROUP AND OBESE GROUP

	Control group N=209	Obese group N=117
*White blood cell ($\times 10^9/L$)	7.5 (1.6)	9.0 (2.1)
*Neutrophils (%)	54.2 (9.7)	58.5 (7.5)
*Lymphocyte (%)	37.5 (9.0)	33.2 (6.8)
*Platelet ($\times 10^9/L$)	287.8 (63.3)	316.5 (67.4)
#IL-2 (pg/mL)	3.5 (3.1-4.0)	3.4 (3.0-4.0)
#IL-4 (pg/mL)	3.0 (2.6-3.6)	2.9 (2.5-3.3)
*#IL-6 (pg/mL)	2.7 (2.2-3.5)	3.8 (2.9-5.3)
#IL-10 (pg/mL)	2.8 (2.4-3.2)	2.9 (2.4-3.5)
#TNF- α (pg/mL)	2.8 (2.4-3.2)	2.8 (2.4-3.2)
#IFN- α (pg/mL)	6.4 (5.1-8.0)	6.1 (4.6-8.6)

IL: interleukin; TNF: tumor necrosis factor; IFN: interferon; * $P<0.001$; All values in mean (SD) except # in Median (IQR).

these children, which confirmed that obesity was a main risk factor for NAFLD in children [10,11].

There were several limitations in our study. Firstly, the study was not a prospective study, but a cross-sectional analysis. Secondly, NAFLD, SFL or NASH was diagnosed only by ultrasonic and serum liver enzymes without pathological biopsy of the liver. Thirdly, the sample size in our study was small. Also, we did not investigate the accurate role of IL-10 on the NAFLD.

In our study, we noted higher white blood cells and neutrophils percentages in obese children, which were consistent with studies from adults and mice [12-14]. Moreover, higher levels of IL-6 were noted in obese children, which suggested that IL-6 might play a role in obesity. Although the white blood cells, neutrophils, platelet and IL-6 were not independent factor by the logistic analysis, these data supported systemic low-grade inflammation in obese children and white blood cells may be a cause of increased production of cytokines [15].

Several studies in adults or animal model have found that imbalance between pro- and anti-inflammatory cytokines was involved in the second hit of NAFLD [16]. In this study, we noted that IL-10 and IL-6 levels increased with the severity of NAFLD in obese children. Several studies have reported that IL-10 played a role as a protective factor for liver injury induced by alcoholic, hepatitis C and liver steatosis [17]. Also, animal model studies suggested protective effect of IL-10 against liver steatosis, fibrosis and other metabolic disorders [18,19]. Our results seem to be contrary against the above studies, but consistent with a human study, which found that the IL-10 mRNA was higher in obese patients with NAFLD, and especially in obese patients with NASH [20]. Also,

higher IL-10 levels were noted in mice after long-term high fat diet fed, and IL-10^{-/-} mice displayed greater liver inflammatory response after high fat diet feeding [21]. These implied that IL-10 is a protective factor, which may increase to compensate inflammation and NAFLD in obesity.

The effect of IL-6 on the NAFLD reported from different studies was seen to be contradictory. It was reported to play a protective effect in early stage of hepatic steatosis by inhibiting oxidative stress and preventing mitochondrial dysfunction [16]. However, it was reported to mediate inflammatory of hepatocyte, induce apoptosis of hepatocytes and IR in the subsequent progress of fatty liver [22,23]. Animal model found that hepatocytes exposed to exogenous IL-6 accelerated self-repairing in short-term, but aggravated injury in long-term [24].

In summary, our data support low-grade chronic inflammation in obese children. Moreover, obesity, dyslipidemia and IR are risk factors of NAFLD in children. IL-10 may be a protective factor for liver, with increased compensatory levels in children with NAFLD.

Acknowledgments: Guan-Ping Dong, Wei Wu, and Li Zhang for patient-care and organization.

Contributors : CCZ: concept and design; JQS, WXS: data collection and analysis; JQS: drafting the manuscript; XZW, KH: help in patient organization and data collection. Jun-Qi Shi and Wen-Xia Shen contributed equally to the article.

Funding : This work is supported, in part, by National Natural Science Foundation of China (81170787 & 81670786), Zhejiang Health and Family Planning Commission (2016KYA128) and Zhejiang Provincial Program for the Cultivation of High-Level Innovative Health Talents (2015).

Competing interest: None stated.

TABLE III IMMUNE PARAMETERS IN THE THREE SUBGROUPS OF OBESE GROUP

	Subgroup 1 (n=23)	Subgroup 2 (n=43)	Subgroup 3 (n=51)	P value
White blood cell ($\times 10^9/L$)	9.4 (2.3)*	9.0 (2.2)*	8.8 (2.0)*	<0.001
Neutrophils (%)	56.7 (6.3)	59.3 (7.8)*	58.8 (7.8)*	<0.001
Lymphocyte (%)	34.6 (5.3)	32.7 (7.2)*	33.0 (7.1)*	<0.001
Platelet ($\times 10^9/L$)	334.0 (74.2)*	318.2 (64.6)*	307.3 (66.1)*	0.001
IL-2 (pg/mL)	3.4 (3.1-4.0)	3.3(3.0-3.9)	3.6 (3.0-4.2)	0.525
IL-4 (pg/mL)	2.9 (0.6)	2.8 (0.6)*	3.2 (1.2) [§]	0.026
IL-6 (pg/mL)	3.4 (2.5-4.3)	3.8 (3.1-5.1)*	4.0 (3.1-6.0)*, [#]	<0.001
IL-10 (pg/mL)	2.6 (2.3-3.0)	2.9 (2.4-3.3)	3.2 (2.6-3.7)*, ^{#,\$}	0.011
TNF- α (pg/mL)	2.8 (2.6-3.1)	2.6 (2.3-3.0)	2.8 (2.4-3.3)	0.079
IFN- α (pg/mL)	7.2 (5.2-9.5)	5.5 (4.2-7.0)	6.2 (4.8-9.2)	0.068

Subgroup 1: Without NAFLD; Subgroup 2: With simple fatty liver; Subgroup 3: With steatohepatitis; NAFLD: Non-alcoholic fatty liver disease; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon. *compared to control group ($P<0.05$); # compared to subgroup 1 ($P<0.05$); § compared to subgroup 2 ($P<0.05$).

WHAT IS ALREADY KNOWN?

- The roles of cytokines in NAFLD in obese children is not well-elucidated.

WHAT THIS STUDY ADDS?

- Obesity, dyslipidemia and insulin-resistance are risk factors of non-alcoholic fatty liver disease in children.

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