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Original Article

# Serum homocysteine levels, oxidative stress and cardiovascular risk in non-alcoholic steatohepatitis

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ABSTRACT

*Introduction:* Hyperhomocysteinemia is considered an independent risk factor for cardiovascular disease. Oxidative stress is one of the major pathogenic mechanisms in non-alcoholic fatty liver disease and atherosclerosis.
*Aim:* Our study aimed to evaluate serum homocysteine levels and oxidative stress in patients with biopsy-proven non-alcoholic steatohepatitis and possible association with cardiovascular risk measured by carotid artery intima-media thickness (c-IMT).
*Patients and methods:* 50 patients with non-alcoholic steatohepatitis and 30 healthy controls, age and gender matched, were recruited. Lipid profile, liver biochemical markers, serum homocysteine, vitamins B6 and B12, folic acid, glutathione (reduced and total), erythrocyte superoxide dismutase, whole blood glutathione peroxidase, malondialdehyde and carotid intima-media thickness were assayed.
*Results:* Patients had an altered lipid profile and liver biochemical markers; carotid intima-media thickness and serum homocysteine levels were significantly higher compared to controls, but there were no differences in folate, B12 and B6 vitamins levels. Patients had significantly lower levels of glutathione peroxidase activity, total and reduced glutathione and higher levels of malondialdehyde, but unchanged superoxide dismutase activity compared to control group. Also, serum homocysteine level showed significant positive correlation with waist circumference, body mass index, free cholesterol, triglycerides, LDL-cholesterol, amino transferases and negative correlation with reduced and total glutathione, superoxide dismutase and γ-GT.
*Conclusion:* Non-alcoholic steatohepatitis is an independent cardiovascular risk factor, associated with elevated homocysteine levels, oxidative stress and c-IMT. c-IMT could be used as an indicator of early atherosclerotic changes initiated by dyslipidemia and oxidative stress, while higher level of homocysteine might be an effect of liver damage.


## 1. Introduction

Non-alcoholic fatty liver disease (NAFLD), the most common liver disease in the world, presents a wide spectrum of liver damage ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) and cirrhosis, in the absence of excessive alcohol consumption [1].

NASH is characterized by the presence of steatosis, lobular inflammation and hepatocyte ballooning with or without fibrosis in liver biopsies, which is the gold standard for diagnosis [2]. Several studies have reported that subjects with NAFLD, especially NASH, have a higher cardiovascular risk, beyond that conferred by the presence of metabolic syndrome alone. The prevalence of cardiovascular disease (CVD) is increased in patients with NAFLD and represents the first cause of mortality in these patients [3–5]. The main pathogenic features in NASH are the oxidative stress and insulin resistance, conditions also involved in cardiovascular disease [6]. Patients with NAFLD have an increased occurrence of subclinical atherosclerosis proved by increased carotid intima-media thickness (c-IMT) [7,8].

NAFLD association with coronary heart disease strongly suggests that NAFLD plays a proatherogenic role [9]. A number of studies have implicated elevated levels of plasma homocysteine (Hcy) in premature atherosclerosis development mainly by intensification of the oxidative stress [10,11].

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Hcy, a sulfur containing, non standard amino acid, is an intermediate in methionine metabolism, being located at a critical metabolic crossroad. It can be remethylated back to methionine (through remethylation pathway requiring folate and vitamin B12) or irreversibly degraded to cysteine and then to taurine, through the transsulfuration pathway (a vitamin B6-dependent process) [12]. The liver plays a central role in the production and catabolism of Hcy, due to the fact that it metabolizes the majority of dietary methionine and harbors 85% of the body capacity for transmethylation [13]. Thus, it is plausible that in situations of liver damage, alterations of Hcy level may occur. In fact, hyperhomocysteinemia (HHcy) has been reported in chronic alcoholism and alcoholic cirrhosis [14–16].

To date, conflicting data concerning serum Hcy levels in patients with NAFLD/NASH has been published [9,17–21] and none of these studies have measured c-IMT and oxidative stress markers.

The aim of this study was to evaluate serum Hcy levels and oxidative stress status in patients with biopsy-proven NASH and to check their possible association with cardiovascular risk reflected by c-IMT.

## 2. Patients and methods

We performed a cross-sectional study which included 50 patients (45.7 ± 10.9 years, 35 males) diagnosed with biopsy-proven NASH and 30 healthy subjects (44.9 ± 7.7 years, 20 males). Inclusion criteria for NASH patients were age >18 years, biopsy-proven NASH (NAFLD activity score ≥3) [22] within previous 12 months, bright liver on ultrasound and persistent elevated amino transferases (≥1.5 times the upper normal limit) for 6 months before liver biopsy.

Exclusion criteria for NASH patients were daily alcohol consumption >20 g, presence of hepatitis B infection serological markers (hepatitis B surface antigen), hepatitis C virus infection (anti-HCV antibody), Epstein–Barr virus (EBV) and Cytomegalovirus (CMV) antibodies, other liver diseases (i.e. autoimmune hepatitis, hemochromatosis, primary biliary cirrhosis, Wilson's disease, α1-antitrypsin deficiency, drug-induced liver disease), liver carcinoma or liver cirrhosis.

None of the patients were taking medication that could cause steatosis (e.g. corticosteroids, estrogens, methotrexate, tamoxifen, tetracycline, metronidazole, calcium channel blockers, amiodarone, tuberculostatic drugs) or influence serum Hcy levels (vitamins B, C and E, folate, aspirin or oral anticoagulants, L-DOPA, nicotinic acid, theophylline).

Inclusion criteria for controls were age >18 years, normal liver ultrasound imaging, negative serology for viral hepatitis and normal liver biochemical tests.

The study protocol has been designed in accordance with the WMA Declaration of Helsinki and was approved by the University Ethics Committee. Informed consent was obtained, prior to any clinical and biological testing, from all individuals included in the study.

All subjects underwent a complete clinical, anthropometric and laboratory investigation.

### 2.1. Clinical evaluation

For all patients we used a structured questionnaire referring to personal (gender and age), anthropometric (weight, height, waist circumference, waist/hip ratio) and demographic characteristics, some cardiovascular risk factors (e.g. smoking, unhealthy diet, sedentary lifestyle, history of diabetes, dyslipidemia and high blood pressure), and daily alcohol consumption. Sedentary lifestyle or physical inactivity was defined as less than five times 30 min of moderate activity per week, or less than three times 20 min of vigorous activity per week, or equivalent. Unhealthy diet was considered as high dietary intakes of saturated fat, trans-fats and salt, low intake of fruits, vegetables and fish [23].

BMI ($kg/m^2$) was calculated as the ratio of the weight (expressed in kilograms) to the square of height (expressed in meters). The diagnosis of metabolic syndrome (MS) and its components was based on the criteria proposed by The Joint Interim Statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity in 2009. In accordance with this definition, patients were classified as having MS if at least three of the following five criteria were present (the first one being mandatory): (1) waist circumference (WC) ≥94 cm in men or ≥80 cm in women, (2) triglycerides (TG) ≥150 mg/dl or treatment with fibrates, (3) HDL-cholesterol ≤40 mg/dl in men and ≤50 mg/dl in women or cholesterol-lowering treatment, (4) fasting glucose ≥100 mg/dl or previously diagnosed type 2 diabetes mellitus, (5) blood pressure (BP) ≥130/85 mm Hg or antihypertensive treatment [24].

### 2.2. Laboratory investigations

Blood samples were collected in the morning, after a minimum 12 hour-overnight fasting, using 3 types of vacutainers: with clot-activator, heparin and EDTA, respectively. Serum obtained after centrifugation (3 min at 3000 rpm) was frozen at −80 °C until analysis. Erythrocytes obtained from 0.5 ml EDTA-blood were washed three times with a NaCl solution (9 g/L) and then stored at −80 °C until analysis. Common serum biochemical markers: total cholesterol, HDL-cholesterol, LDL-cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GT) and glucose were assayed on an automatic analyzer (Konelab 301-Thermo Electron Corp., Finland) using commercially available kits. Oxidative stress status was evaluated by measuring the activities of erythrocyte superoxide dismutase (SOD) and whole blood glutathione peroxidase (GPx), whole blood levels of total (GSSG + GSH) and reduced (GSH) glutathione and serum malondialdehyde (MDA) concentration. Serum Hcy, vitamin $B_6$, MDA and whole blood glutathione (reduced and total) were quantified, after protein precipitation and derivatization, using an isocratic high performance liquid chromatography (HPLC) with fluorescence detection, on an Agilent chromatographic system, with specific Chromsystems kits (Chromsystems Instruments & Chemicals GmbH, Germany). Serum levels of vitamin B12 and whole blood folic acid were measured on an Immulite 1000 analyzer, with specific ELISA kits (DPC-Siemens, Los Angeles, CA, USA) according to the manufacturer's instructions.

SOD and GPx activities were measured on Cobas Mira Plus analyzer (Roche, France) with SD125-RANSOD and respectively RS505-RANSEL kits (Randox Laboratories, UK).

Serum insulin antibodies were quantified with competitive ELISA, following the kit protocol (Diametra, Italy).

Insulin resistance (IR) was estimated with the homeostasis model assessment formula HOMA-IR = fasting plasma glucose (mg/dl) × fasting serum insulin (μU/ml)/405 [25]. The cut offs of ≥2 and >4 were chosen as criteria for IR and prediabetes, respectively [26].

Type 2 diabetes and dyslipidemia were assessed by using the American Diabetes Association criteria: fasting glucose ≥100 mg/dl, triglycerides ≥150 mg/dl and HDL-cholesterol <40 mg/dl in men and <50 mg/dl in women, respectively [27].

### 2.3. Ultrasound evaluation

All subjects underwent abdominal and carotid Doppler ultrasound investigation in order to assess hepatic steatosis, c-IMT and the presence of carotid plaques. All investigations were performed by an experienced operator who was blinded to clinical presentation and laboratory findings. Ultrasound imaging was performed using a high resolution ultrasonographic system (General Electric Logiq7), with convex (5 MHz) and high frequency linear probes (7.5 MHz) to scan the liver and carotid arteries, respectively.