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Clinical studies

Low hepatic copper content and *PNPLA3* polymorphism in non-alcoholic fatty liver disease in patients without metabolic syndrome

Albert Friedrich Stättermayer[a], Stefan Traussnigg[a], Elmar Aigner[b], Christian Kienbacher[a], Ursula Huber-Schönauer[c], Petra Steindl-Munda[a], Andreas Stadlmayr[c], Friedrich Wrba[d], Michael Trauner[a], Christian Datz[c], Peter Ferenci[a,*]

[a] Department of Internal Medicine III, Division of Gastroenterology and Hepatology, Medical University of Vienna, Vienna, Austria
[b] Department of Internal Medicine I, Paracelsus Private Medical University, Salzburg, Austria
[c] Department of Internal Medicine, KH Oberndorf, Oberndorf, Austria
[d] Institute of Clinical Pathology, Medical University of Vienna, Vienna, Austria

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ABSTRACT

Introduction: The pathogenesis of non-alcoholic fatty liver disease (NAFLD) is multifactorial including metabolic, genetic (e.g. *PNPLA3* [patatin-like phospholipase domain-containing 3 gene]), viral factors and drugs. Besides, there is evidence for a role of copper deficiency. Aim of the study was to evaluate the role of hepatic copper content, *PNPLA3* in NAFLD patients with and without metabolic syndrome (MetS).
Methods: One-hundred seventy-four NAFLD patients, who underwent liver biopsy for diagnostic work-up, were studied. Diagnosis of MetS was based on the WHO Clinical Criteria. Steatosis was semiquantified as percentage of fat containing hepatocytes and was graded according to Brunt. Histological features of non-alcoholic steatohepatitis (NASH) were assessed using the Bedossa classification. Hepatic copper content (in μg/g dry weight) was measured by flame atomic absorption spectroscopy. SNP rs738409 in *PNPLA3* was investigated by RT-PCR.
Results: Mean hepatic copper content was 22.3 (19.6–25.1) μg/g. The mean percentage of histologically lipid containing hepatocytes was 42.2% (38.3–46.0) and correlated inversely with hepatic copper content ($\rho = -0.358$, $P < 0.001$). By subgroup analysis this inverse correlation remained significant only in patients without MetS (OR: 0.959 [CI95%: 0.926–0.944], $P = 0.020$). Presence of minor allele (G) of *PNPLA3* was also associated with moderate/severe steatosis (≥33%) both in patients with (OR: 2.405 [CI95%: 1.220–4.744], $P = 0.011$) and without MetS (OR: 2.481 [CI95%: 1.172–5.250], $P = 0.018$), but was only associated with NASH (OR: 2.002 [CI95%: 1.062–3.772], $P = 0.032$) and liver fibrosis (OR: 2.646 [CI95%: 1.299–5.389], $P = 0.007$) in patients without MetS.
Conclusion: Hepatic copper content and *PNPLA3* mutations are associated with disease activity in NAFLD patients without MetS. Presence of MetS appears to mask the effects of hepatic copper and *PNPLA3*.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a rapidly increasing disease worldwide with estimates on prevalence varying between 20–50 percent [1–3] depending on study population and the used definition on diagnosis. NAFLD is usually histologically categorized into the benign form of simple steatosis and its more progressive form of non-alcoholic steatohepatitis (NASH). It is now supposed to be the most frequent liver disease in industrialized countries. Especially NASH is generally thought to be associated with progression to liver cirrhosis, development of hepatocellular carcinoma (HCC)

Abbreviations: IR, insulin resistance; MetS, metabolic syndrom; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLDactivity score; NASH, non-alcoholic steatohepatitis; *PNPLA3*, patatin-like phospholipase domain-containing 3 gene; T2DM, type 2 diabetes mellitus; *TM6SF2*, transmembrane 6 superfamily member 2 gene.

* Corresponding author at: Department of Internal Medicine III, Division of Gastroenterology and Hepatology, Medical University of Vienna, Währinger Gür 18 20, A 1090 Vienna, Austria.
E-mail address: peter.ferenci@meduniwien.ac.at (P. Ferenci).

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[4] and increased liver-related mortality, whereas simple steatosis appears to have only very slow – if any – progression of histological changes [5], although there is a substantial variation among individual patients [6].

The diagnosis of NAFLD [7] is based on the evidence of hepatic steatosis by histology or imaging, the absence of significant alcohol intake and of liver disease of other etiologies (e.g. viral hepatitis, Wilson disease) or causes (toxin/drug exposure, parenteral nutrition, starvation etc.). Diagnosis of NASH requires a liver biopsy showing hepatic steatosis with necro-inflammatory activity and hepatocyte ballooning with or without fibrosis. The prevalence of NASH is lower than that of NAFLD ranging from 3 to 5 percent [8] of the overall population.

The mechanisms responsible for NAFLD progression are still poorly understood. Hepatocellular fat accumulation is of multifactorial origin and can be due to metabolic [9], viral [10], toxic [11] and genetic factors [12,13]. Among the best investigated NAFLD associated pathophysiologic factors are the metabolic syndrome (MetS) [14–17], obesity, diabetes and mutations of *PNPLA3*. In a large proportion of patients NAFLD is considered to be the hepatic manifestation of the MetS and its features are common and well documented risk factors for the progression of NAFLD although there is also a substantial number of patients without MetS. Several important genetic factors associated with the development of fatty liver were identified. The isoleucine to methionine substitution in position 148 of rs738409 in the patatin-like phospholipase domain-containing 3 gene (*PNPLA3*) is strongly associated with increased hepatic fat content [13,18] with or without NASH [19], leading to increased risk for liver fibrosis [20] and HCC [21]. The rs58542926 E167K variant in transmembrane 6 superfamily member 2 (TM6SF2) promotes steatosis and lipid abnormalities in part by altering *TM6SF2* and *MTTP* expression, and is also associated with inflammation and fibrosis [22].

Since there are quite a few patients without MetS and/or genetic risk profile, there is a need to explore further factors involved in the pathogenesis of NAFLD/NASH. In this study we investigated the role of low hepatic copper content in patients with fatty liver. For many years we investigated the association of low hepatic copper content with the degree of hepatic steatosis [23]. These studies showed that copper deficiency is a yet neglected factor leading to NAFLD. Patients with NAFLD had overall lower hepatic copper concentrations than control subjects [24] and lower hepatic copper content was more pronounced in patients with a high degree of steatosis or NASH [25]. Rats fed a copper restricted diet developed marked hepatic steatosis and insulin resistance, whereas high-normal dietary copper intake was linked to improved glucose and lipid parameters and low liver fat [24].

The aim of this study was therefore to investigate the role of hepatic copper content and its interaction with MetS and genetic factors with histologic features in NAFLD patients.

2. Methods

2.1. Patients

Overall, 174 adult Caucasian patients from three tertiary referral centers in Austria, who underwent liver biopsy for diagnostic workup of elevated liver enzymes and/or ultrasound proven hepatic steatosis were included into this study. In all patients infectious (e.g. viral hepatitis, HIV), immunological, drug-induced hepatic steatosis (e.g. amiodarone, methotrexate, steroids, valproate, etc.) or hereditary causes (hereditary hemochromatosis, Wilson disease) of liver disease were excluded. Alcohol consumption was examined by self-reporting; subjects with an average alcohol consumption of more than 30 g/day (in men) or 20 g/day (in women) were excluded from this study.

All patients gave their written informed consent prior to liver biopsy and for genetic testing.

2.2. Laboratory assessment

Blood samples were drawn in the morning shortly before liver biopsy under fasting conditions for at least 8 h by standard laboratory techniques. All patients were tested for fasting blood glucose (mg/dL), total serum cholesterol (mg/dL), HDL- and LDL-cholesterol (mg/dL), as well as serum triglycerides (mg/dL). Further, alanine aminotransferase (ALT; U/L), aspartate aminotransferase (AST; U/L) and γ-glutamyl-transpeptidase (γGT; U/L) were determined. Testing for gycosylated hemoglobin ($HbA_{1c;}$ in%) was performed in 93 (53.4%) patients. Insulin (μU/mL) was measured in 92 (52.9%) of the subjects, and the homeostasis model assessment for insulin resistance (HOMA-IR) was calculated according to the formula glucose (mg/dL) x insulin (μU/mL)/405 [26]. Insulin resistance (IR) was considered in patients with a HOMA-IR ≥2.50.

2.3. Clinical assessment

Anthropometric data like age, sex, body weight (in kg) and size (in m) at time of liver biopsy were collected. The body mass index (BMI) was calculated accordingly (kg/m^2). Overweight was considered as a BMI of 25.0–29.9 kg/m^2, obesity as a BMI of equal to or more than 30.0 kg/m^2. All patients were evaluated for type 2 diabetes mellitus (T2DM). Diagnosis of T2DM was established according to the 2012 consensus guidelines of the Austrian Diabetes Society [27] if fasting blood glucose levels were above 125 mg/dL on two different days, glycosylated hemoglobin (HbA_{1c}) was equal to or higher than 6.5% or in patients that were already under antidiabetic treatment. Diagnosis of metabolic syndrome (MetS) was based on the WHO Clinical Criteria [28] in patients with insulin resistance (HOMA-IR ≥2.50), or diagnosis of T2DM, or impaired fasting glucose [101–125 mg/dL]) and any two of the following: central obesity (BMI ≥30.0 kg/m^2), arterial hypertension (systolic blood pressure: ≥140 mmHg, or diastolic blood pressure: ≥90 mmHg, or antihypertensive treatment), or dyslipidemia (triglycerides: ≥150 mg/dL, or HDL-cholesterol: <35 mg/dL in males or <39mg/dL in females, or medical treatment for dyslipidemia).

2.4. Histological assessment

Liver biopsies were obtained in all patients by the Menghini technique. Biopsy samples were routinely processed (formalin-fixed and paraffin embedded) and stained with hematoxylin/eosin and chromatrope aniline blue for assessment of steatosis, fibrosis and inflammation.

Fibrosis was staged on a five-point scale: no fibrosis (stage 0), pericellular fibrosis (stage 1), pericellular and portal fibrosis (stage 2), bridging fibrosis (stage 3) and cirrhosis (stage 4). Steatosis was semiquantified as proposed by Brunt et al. [29] on a three-point scale by calculating the percentage of lipid containing hepatocytes at a 40 x magnification: mild (G1: 5–33%), moderate (G2: 34–66%), or severe steatosis (G3: >66%). According to the study design, patients with less than 5% of fat containing hepatocytes (G0) were not enrolled. Hepatocyte ballooning was graded as absent (0), rare (1), or prominent ballooning (2). Necro-inflammatory activity was graded as absent (0), mild (1), moderate (2) or severe (3). The NAFLD activity score (NAS) [30] was calculated as the sum of steatosis (1–3), hepatocyte ballooning (0–2) and inflammation (0–3) as a score from 1 to 8. NASH was diagnosed in those patients with presence of both, hepatocyte ballooning and inflammation with or without fibrosis, as suggested by Bedossa et al. [31] in the SAF-score.