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BRIEF ARTICLE

# Nonalcoholic fatty liver disease and *HFE* gene mutations: A Polish study

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## Abstract

**AIM:** To describe a Polish population with nonalcoholic fatty liver disease (NAFLD) with regard to *HFE* gene mutations, as well as analyzing demographic and clinical data.

**METHODS:** Sixty-two consecutive patients with biopsy-proven NAFLD were included in the study. Demographic, clinical, and laboratory data were summarized in a database. C282Y and H63D mutations of the *HFE* gene were analyzed using polymerase chain reaction-restriction fragment lenght polymorphism.

**RESULTS:** The analyzed cohort consisted of 62 homogeneic Caucasian participants, 66.1% men and 33.9% women, with a median age of 48 years. The median body mass index was 29.05 kg/m$^2$. Hypercholesterolemia was observed in 74.2% of patients and hypertriglyceridemia in 32.2%; 16.1% had type 2 diabetes mellitus (DMt2). On liver biopsy, 22.6% of NAFLD patients were found to have severe fibrosis. There were no differences between frequencies of *HFE* gene mutations in subgroups of NAFLD patients with less and more severe liver fibrosis. Obesity, older age, female gender and DMt2 were associated with more advanced fibrosis in this Polish cohort, as well as higher glucose level, serum iron and transaminase aspartate aminotransferase/alanine aminotransferase ratio.

**CONCLUSION:** *HFE* mutations conferred no additional hepatic fibrosis risk in NAFLD, but higher serum iron was a risk factor for severe liver damage in NAFLD, regardless of *HFE* mutations.



**Key words:** Nonalcoholic fatty liver disease; *HFE* gene mutations; Liver fibrosis

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## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is now the most common liver disease in Western countries, affecting up to a third of individuals. It covers a spectrum of liver problems including benign simple steatosis (fatty liver) and steatohepatitis [nonalcoholic steatohepatitis (NASH)] with hepatic injury, inflammation, and fibrosis, which can lead to cirrhosis, liver failure, and hepatocellular carcinoma[1]. NAFLD is strongly associated with metabolic syndrome and is now regarded as its liver manifestation. Hyperinsulinemia is a risk factor for the development of NASH. A moderate increase of iron stores is observed in NASH, and its presence may contribute to the development of insulin resistance (IR)[2,3].

Some studies have identified high ferritin level and/or increased prevalence of hyperferritinemia in patients with metabolic syndrome[4] or its single components[5]. Serum ferritin could be a marker of IR[6]. Increased ferritin levels, together with high transferrin saturation, are typical features of *HFE* hereditary hemochromatosis (HHC). However, hyperferritinemia with normal or slightly increased transferrin saturation has also been described in non-C282Y homozygotes with NASH and has been designated "IR hepatic iron overload syndrome" because of a frequent association with hepatic steatosis and metabolic abnormalities[7].

The mechanism determining the progression from fatty liver to steatohepatitis is still unclear, and there is a conflict of evidence regarding inheritance of the *HFE* gene mutation and the influence of hepatic iron deposition as cofactors for developing fibrosis in NAFLD. Family studies and inter-ethnic variations in susceptibility suggest that genetic factors may be important in determining disease risk or the clinical course of the disease. Thus, the aim of this study was to evaluate the frequency of the *HFE* gene polymorphisms C282Y and H63D in Polish patients with NAFLD with respect to demographic, clinical and laboratory data.

## MATERIALS AND METHODS

Sixty-two consecutive patients with biopsy-proven NAFLD were included in the study between 2007 and 2009. Patients were recruited consecutively by means of liver biopsy. We analyzed only the patients with a negative history of alcohol intake; those consuming more than 20 g/d were excluded from the study. All patients tested negative for HBV antigen and anti-HCV antibodies. Those aged 40 years or less had their ceruloplasmin checked and confirmed to be normal. γ-globulins, immunoglobulins, and auto-antibodies were tested and imaging diagnostics were performed to exclude autoimmune hepatitis as well as primary biliary cirrhosis and primary sclerosing cholangitis. Advanced fibrosis in liver biopsy was defined as bridging fibrosis F3 and cirrhosis F4. Demographic and clinical data were summarized in a database.

*HFE* gene analysis was performed by polymerase chain reaction (PCR) amplification of total genomic DNA of two regions of the gene carrying the mutations C282Y and H63D. The PCR primers for amplifications of the C282Y locus were 5'-TCCGTCTTAGCTGAGTGGAACTACTACCCCCAGAACATCACC-3' and 5'-AGGCAGAATCGACTCACCTGGCTCTCATCAGTCACATACCC-3'. For detection of H63D, the sense primer sequence was 5'-ATGGTTAAGGCCTGTTGCTCTGTC-3' and the antisense primer sequence was 5'-CCCTTGCTGTGGTTGTGATTTTC-3'. PCR amplification was performed in a total volume of 12.5 μL containing 10 to 20 ng genomic DNA, 2.5 nmol of each deoxynucleotide triphosphate, 4 pmol of each primer, 1.5 mmol/L magnesium chloride, 1 × PCR buffer solution, and 0.3 U *Taq* polymerase (MBI Fermentas, Vilnius, Lithuania). PCR amplification consisted of initial denaturation for 3 min at 94℃, followed by 35 cycles of denaturation at 94℃ for 30 s, annealing at 56℃ for 30 s, and extension at 72℃ for 30 s. The terminal elongation was performed at 72℃ for 7 min.

PCR products were digested with *Rsa* I (MBI Fermentas, Vilnius, Lithuania) for detection of C284Y and with *Mbo* I (MBI Fermentas, Vilnius, Lithuania) for H63D. The G-to-A transition (aa282) creates a new *Rsa* I site, and a second *Rsa* I site in this fragment acts as internal control within the restriction fragment length polymorphism. The C-to-G transversion (aa63) results in loss of one of two *Mbo* I sites in the amplified product. DNA fragments generated after digestion were separated on 3% agarose gels.

### Statistical analysis

The statistical analysis was performed using SPSS v 15.0. The independent-samples *t* test, preceded by Levene test for equality of variances test, was used to compare means in continuous data. Pearson $\chi^2$ test was used to check the association with advanced fibrosis. The Pearson correlation coefficient and Spearman rho coefficient of correlation were also calculated. Multivariate logistic regression was used to determine the set of data independently correlated with advanced fibrosis.

## RESULTS

The analyzed cohort consisted of 62 racially homogeneic Caucasian patients. There was a predominance of males over females (66.1% *vs* 33.9%). The median age of patients was 48 years (ranges, women: 30-75 years; men: 25-64 years). Body mass index (BMI) in women ranged from 21.32 to 39.3 kg/m$^2$ and in men from 21.9 to 37.7 kg/m$^2$. More than half of the analyzed group was obese (BMI > 30 kg/m$^2$ in 50.4%), and 38.1% of patients were overweight (25 kg/m$^2$ < BMI < 30 kg/m$^2$).

**Table 1 Selected characteristics of patients with NAFLD**

| | Minimum | Median | Maximum | mean ± SD |
|---|---|---|---|---|
| Age (yr) | 25 | 48 | 75 | 46.56 ± 11.91 |
| Fe (10.56-228.3 μmol/L) | 4.12 | 20.9 | 68.4 | 21.4 ± 9.79 |
| Transferrin saturation (< 45%) | 7.6 | 36 | 206 | 40.59 ± 30.46 |
| Ferritin (629.2-7999.3 pmol/L) | 629.2 | 5336.6 | 129584.4 | 9714.9 ± 18409.2 |
| AST (< 38 IU/L) | 16 | 45.5 | 275 | 57.44 ± 43.91 |
| ALT (< 41 IU/L) | 15 | 73 | 281 | 86.37 ± 57.35 |
| AST/ALT ratio | 0.36 | 0.62 | 2.43 | 0.79 ± 0.47 |
| PLT (150-400 × $10^9$/L) | 93 | 218 | 376 | 224.98 ± 67.47 |
| Albumin (34-48 g/L) | 36.8 | 46.2 | 54.8 | 45.8 ± 4.0 |
| Glycemia (3.89-5.83 mmol/L) | 3.44 | 5.6 | 11.8 | 581.7 ± 1.27 |
| BMI (19-25 kg/$m^2$) | 21.2 | 29.05 | 39.3 | 29.10 ± 4.17 |
| HOMA (> 1.8) | 0.44 | 4 | 35.63 | 7.44 ± 9.25 |
| $HBA_{1c}$ (4.8%-5.9%) | 4.99 | 5.76 | 9.76 | 6.20 ± 1.26 |

NAFLD: Nonalcoholic fatty liver disease; Fe: Iron; AST: Aspartate transaminase; ALT: Alanine transaminase; PLT: Platelet count; BMI: Body mass index; HOMA: Homeostatic model assessment; $HBA_{1c}$: Glycated hemoglobin.

Among co-morbidities, hypercholesterolemia (total cholesterol > 5.18 mmol/L) was present in 74.2% of patients; hypertriglyceridemia (> 2.03 mmol/L) in 32.2%; type 2 diabetes mellitus (DMt2) in 16.1%; and arterial hypertension in 21.0%. Selected demographic and clinical data are presented in Table 1.

Among the 62 participants, 14 patients (22.6%) had severe (F3-F4) fibrosis and 48 (77.4%) had no or mild fibrosis. Table 2 summarizes the clinical data related to NASH with no/mild (F0-F2) and advanced (F3-F4) fibrosis.

High serum aspartate aminotransferase/alanine aminotransferase ratio, and higher serum iron were strongly associated with advanced fibrosis (F3-F4). Lower levels of albumin were also indicators of more severe liver disease. There were no statistically significant interactions between gender and common co-morbidities and NAFLD, although a trend was observed in a subgroup of patients with F3-F4 fibrosis and female gender as well as DMt2. The Spearman's correlation coefficients showed higher age, higher BMI and presence of DMt2 as being associated with more advanced fibrosis. Multivariate logistic regression was provided and evidenced DMt2, higher age and female gender as good predictors of fibrosis F3-F4.

Table 3 shows the frequencies of the *HFE* gene mutations C282Y and H63D. There were no statistically significant differences between frequencies of mutated alleles in the subgroup of patients with NAFLD. The number of mutated alleles was too low to identify a Pearson correlation between lipids, iron, ferritin, and transferrin saturation or clinical course (i.e. severe fibrosis) and *HFE* gene mutations. However, a trend towards statistically significant correlations between at least one mutated allele of the H63D *HFE* gene and total cholesterol ($P = 0.034$) as well as LDL-cholesterol ($P = 0.020$) was observed in the group of NASH patients with F3-F4 fibrosis with the Pearson correlation coefficient.

**Table 2 Comparison of selected clinical data between patients with fatty liver and no/mild and advanced fibrosis**

| | Fibrosis 0-2 ($n$ = 48) | Fibrosis 3-4 ($n$ = 14) | $P$ value |
|---|---|---|---|
| Age (yr) | 45 ± 12 | 51 ± 11 | 0.096 |
| BMI (19-25 kg/$m^2$) | 28.68 ± 3.85 | 30.64 ± 5.05 | 0.135 |
| AST (< 38 IU/L) | 51.27 ± 38.75 | 80.69 ± 55.27 | 0.091 |
| ALT (< 41 IU/L) | 89.10 ± 53.64 | 75.85 ± 71.39 | 0.462 |
| AST/ALT ratio | 0.65 ± 0.31 | 1.33 ± 0.55 | 0.001 |
| PLT (150-400 × $10^9$/L) | 231.08 ± 66.51 | 201.54 ± 68.57 | 0.161 |
| Glucose (3.89-5.83 mmol/L) | 5.36 ± 0.74 | 7.08 ± 20.5 | 0.020 |
| HOMA (> 1.8) | 6.0 ± 8.4 | 13.1 ± 10.7 | 0.144 |
| Fe (10.56-228.28 μmol/L) | 19.9 ± 7.32 | 26.9 ± 15.0 | 0.028 |
| Ferritin (629.2-7999.3 pmol/L) | 742.2 ± 753.9 | 1934.4 ± 3914.3 | 0.362 |
| Albumin (34-48 g/L) | 4.64 ± 0.38 | 4.31 ± 0.37 | 0.017 |
| $HBA_{1c}$ (4.8%-5.9%) | 5.92 ± 0.96 | 7.48 ± 1.78 | 0.121 |
| Transferrin saturation (< 45%) | 38.68 ± 30.88 | 49.06 ± 28.64 | 0.361 |

**Table 3 Frequencies of C282Y and H63D *HFE* gene mutations in patients with NAFLD**

| *HFE* gene mutation | Fibrosis 0-2 ($n$ = 48) | Fibrosis 3-4 ($n$ = 14) |
|---|---|---|
| C282Y/C282Y | 1 | 0 |
| C282Y/W | 1 | 0 |
| H63D/H63D | 3 | 0 |
| H63D/W | 11 | 3 |

## DISCUSSION

NAFLD has become the most common liver disorder of our times. In about 15% of all NAFLD cases, steatosis may evolve into steatohepatitis, a mix of inflammation, hepatocellular injury and fibrosis, often resulting in cirrhosis and even hepatocellular carcinoma, but this process is not well understood[8]. The pathogenesis of NAFLD is often posited as the "double-hit" hypothesis, in which hepatocellular lipid accumulation is the first hit, followed by a second hit in which pro-inflammatory mediators and reactive oxygen species induce inflammation, hepatocellular injury, and fibrosis. Altered abundance and composition of liver tissue lipids may impair sinusoidal perfusion and modulate the microvascular inflammatory response[8]. On the other hand, in chronic liver disease, iron deposits are found in hepatocytes and in Kupffer/sinusoidal cells, leading to oxidative stress, cell toxicity, and genotoxicity[2]. Despite a number of reports supporting a hypothesis of an association among ferritin, iron overload, IR, and NAFLD, the mechanisms underlying this intricate and intriguing relationship remain unclear, but a likelihood is that IR, fatty acid accumula-

tion, and oxidation are the major determinants of serum ferritin levels[9].

The goal of this study was to describe a racially homogeneic cohort of Polish patients with NAFLD with respect to *HFE* gene mutations as well as their demographic, clinical, and laboratory data. The results of our study showed similar clinical and laboratory markers to those previously described as being associated with advanced fibrosis[1]. The Polish NAFLD population differs with regard to two points: our patients are less obese than the patients participating in Harrison *et al*[10] and Angulo *et al*[11] and only 16.1% of Polish patients in the current work had diabetes mellitus. In these two US studies - the largest cohort studies in NAFLD - the participants had higher BMI and DMt2 affected 35%[10] and 30%[11] of individuals, respectively.

The frequency of *HFE* gene mutations in the Polish NAFLD population is similar to the frequencies in a healthy Polish cohort[12] and other Central European countries[12] as well as in 119 Polish patients with alcoholic liver disease[13]. The first report about the connection between *HFE* gene mutations and NAFLD showed a positive correlation between levels of serum ferritin, iron, and transferrin saturation and the presence of the mutated allele, as well as between C282Y mutation and more severe fibrosis in a North American population[14]. The results of George *et al*[15] supported these findings, but subsequent larger series have not confirmed such an association[3,16-18]. The results of Chitturi *et al*[18] even brought into question the link between hepatic iron and liver fibrogenesis, and both Adams *et al*[16] and Bugianesi *et al*[3] in their series reported a lack of an association between hepatic steatosis and hepatic fibrosis in patients with HHC and iron overload. On the other hand, among patients homozygous for the C282Y *HFE* gene mutation, the degree of hepatic iron loading remains the dominant factor associated with liver fibrosis[16]. Serum ferritin is often raised in NAFLD patients[3,9] and has been associated with advanced fibrosis[18]. In our Polish cohort we also found the highest serum ferritin levels in the subgroup of NASH patients with F3-F4 fibrosis, although without statistical significance.

In the current study, higher levels of serum iron, as well as DMt2, were associated with severe liver fibrosis. There is a growing body of work showing that iron and glucose metabolism are interdependent and that an increased iron store may contribute to IR. Serum iron may contribute to IR *via* increased adipocyte lipolysis and impairment of glucose transport[19]. There is evidence from *in vitro* studies that iron can reduce binding of insulin to its receptor and reduce insulin receptor gene expression[20]. Valenti *et al*[21] showed that higher iron stores were associated with more advanced liver fibrosis.

The conflicting evidence regarding *HFE* gene mutation and NAFLD could be the result of the ethnic structure of a cohort study. The results from our study with a racially homogeneic Caucasian population showed no increased frequency of mutated alleles of the *HFE* gene in Polish NAFLD patients as well as no association between mutations and iron, ferritin, and transferrin saturation. In addition, no connection was found between *HFE* gene mutation and the clinical course of NAFLD, supporting the results of Bugianesi *et al*[3] and Yoneda *et al*[22]. An increased ferritin level is a marker of severe histological liver damage in some studies[3,9] and also in ours such a trend was observed. Together with the lack of an increased transferrin saturation and increased serum iron independently of *HFE* gene mutations, the results point strongly towards IR hepatic iron overload as a cause of progression in NAFLD. Data obtained in studies of NAFLD provide the possible mechanism of iron interfering with lipid metabolism[23] and thereby promoting hyperglycemia. There is a suggestion that the effect of iron on steatosis development may extend to other liver diseases[24].

Our study provides novel findings regarding *HFE* gene mutations and cholesterol levels. We found that in a subgroup of NASH patients with severe fibrosis, there were statistically significant correlations between the presence of at least one mutated allele of H63D and total cholesterol as well as LDL-cholesterol levels. Although H63D *HFE* gene mutations are quite common in the general population from the northern part of Europe, these results contrast with those of Pankow *et al*'s[25] US population-based study, but support our findings from a previous alcoholic liver disease study[13]. These novel results may be of value in explaining progressive liver fibrosis in at least some proportion of patients with NAFLD. Presence of *HFE* H63D mutation with elevated total and LDL cholesterol, together with increased iron levels, may lead to excessive free fatty acid oxidation with free oxygen radical formation, increased inflammation, and fibrogenesis. However, this novel aspect of NAFLD pathogenesis should be evaluated in a larger cohort of patients, because a previous study with Caucasians showed higher prevalence of the C282Y mutation in NAFLD[26]. In other studies, the frequencies of C282Y *HFE* gene mutation were similar in NAFLD patients and in the general population[27-29] and none of them found increased prevalence of H63D mutation in NAFLD.

In conclusion, this Polish population of patients with NAFLD was similar to populations described in the largest cohort studies from the United States, according to demographic and clinical data, although our patients were less obese and with no incidence of morbid obesity among them. The risk factors for advanced fibrosis in this Polish NAFLD cohort are also approximately similar to those previously reported. In addition, the frequency of *HFE* gene mutations was similar to that described for populations in Central-Eastern Europe, and neither the C282Y nor the H63D *HFE* gene mutations were associated with NAFLD pathogenesis in this cohort. Our study results also suggest a novel possible pathogenesis in some populations of NAFLD patients; however, these results should be validated in larger and racially heterogeneic populations of NAFLD patients.

## COMMENTS

### Background

Nonalcoholic fatty liver disease (NAFLD) is now the commonest liver disease, affecting up to a third of individuals. It includes a wide spectrum of liver diseases, ranging from pure steatosis to nonalcoholic steatohepatitis (NASH), and can eventually lead to liver cirrhosis, liver failure and hepatocelullar carcinoma. Identifying the biochemical markers and gene mutations leading to more severe liver damage in patients with NAFLD is crucial for evaluating their prognosis.

### Research frontiers

There is conflicting evidence regarding inheritance of *HFE* gene mutations [hereditary hemochromatosis (HHC)] as a cofactor for development of more severe fibrosis in NAFLD patients. Although serum ferritin is an independent predictor of hepatic fibrosis, the mechanisms underlying the intricate and intriguing relationship with NAFLD are still unclear. The authors analyzed different biochemical and clinical parameters of sixty two patients with biopsy-proven NAFLD. *HFE* gene analysis was performed by polymerase chain reaction amplification of total genomic DNA of two regions of the *HFE* gene carrying the mutations C282Y and H63D.

### Innovations and breakthroughs

Higher serum iron level is a predictor of more advanced fibrosis, in addition to the factors of obesity, older age, female gender and type 2 diabetes mellitus. This research provides novel findings regarding *HFE* gene mutations and cholesterol level. It was found that in a subgroup of NASH patients with severe fibrosis there were correlations between at least one mutated allele of H63D and total cholesterol as well as LDL-cholesterol levels.

### Applications

These findings may be of value in the explanation of progressive liver fibrosis in at least some proportion of patients with NAFLD. Higher total and LDL cholesterol levels, together with increased iron levels, may lead to excessive free fatty acid oxidation with free oxygen radical formation, increased inflammation and fibrogenesis. However, this novel aspect of NAFLD pathogenesis should be evaluated in larger cohorts of patients.

### Terminology

*HFE* gene mutation/HHC: An autosomal recessive genetic iron storage disorder, that usually results from defects in this gene. The mutation or polymorphism most commonly associated with hemochromatosis is C282Y. About 1/200 of people of Northern European origin have two copies of this variant; males are particularly at high risk of developing hemochromatosis. Organs commonly affected by hemochromatosis are the liver, heart and endocrine glands.

### Peer review

The authors described a Polish population with NAFLD and tried to analyze the correlation of different biochemical and clinical parameters with the disease. This can be considered a very well-designed study.

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