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Association between the CYBA and NOX4 genes of NADPH oxidase and its relationship with metabolic syndrome in non-alcoholic fatty liver disease in Brazilian population

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ABSTRACT

Background: Oxidative stress has been implicated in the progression of severe forms of non-alcoholic fatty liver disease (NAFLD). NADPH oxidase produces reactive oxygen species. In the present study, we investigated for the first time two single nucleotide polymorphisms (SNPs) in the regulatory region of genes encoding NADPH oxidase 4 (NOX4) and p22phox (CYBA) in NAFLD.

Methods: A total of 207 biopsy-proven NAFLD patients [simple steatosis ($n=27$); nonalcoholic steatohepatitis (NASH) ($n=180$)] were evaluated. Genomic DNA was extracted from peripheral blood cells, and polymorphisms in CYBA (unregistered) and NOX4 (rs3017887) were determined by direct sequencing of PCR.

Results: Associations of CYBA-675 T/A with high-density lipoprotein (HDL) (TT vs TA vs AA; $P<0.01$) and triglycerides (TGL) (TT vs XA; $P<0.01$) were observed only in NASH patients. For polymorphisms in the NOX4 gene, NOX4 (rs3017887) CA + AA genotypes was significant associated with alanine aminotransferase (ALT) (CA + AA vs CC; $P=0.02$). However, there was no association of SNPs in the CYBA and NOX4 genes encoding the NADPH oxidase system proteins and the presence of NASH. Regarding the clinical results, it was observed that the most advanced degrees of fibrosis occurred in patients diagnosed with type 2 diabetes mellitus (66.9% vs 37.5%, $P<0.01$) and those who were more obese (32.2 vs 29.0 kg/m^2, $P<0.01$). In addition, serum glucose and insulin levels increased significantly in the presence of NASH.

Conclusions: There were associations between the presence of the allele A in the NOX4 SNP and a higher concentration of ALT in the NAFLD population; between the presence of the AA genotype in the polymorphism of the CYBA-675 T/A CYBA gene and a higher level of TGL and lower HDL in NASH patients. The presence of metabolic syndrome was associated with advanced degrees of fibrosis in NAFLD patients.

Introduction

Non-alcoholic fatty liver disease (NAFLD) encompasses a large spectrum of disease, from simple steatosis that demonstrates excessive lipid deposition (> 5%) in hepatocytes of the liver parenchyma, to the potentially progressive form known as nonalcoholic steatohepatitis (NASH), histologically characterized by hepatocyte ballooning, inflammation, and frequently, associated with fibrosis, cirrhosis and hepatocellular carcinoma (HCC) [1–3]. NAFLD is a complex entity, with varied development and progression among individuals. The reason for these diversities is not fully known; however, environmental influences, such as eating habits, intestinal microbiota, and multiple genetic factors have been described [4–6]. It is believed that genetic factors contribute to 30%–50% of the risk in cases of high prevalence of the disease, such as obesity, cardiovascular disease and cirrhosis [7].

Several enzymatic systems, at different locations, may contribute to the formation of reactive oxygen species (ROS) in

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the liver, including endothelial cells synthesizing nitric oxide, cytochrome P450, monooxygenases, and the nitrogen oxidative nicotinamide adenine dinucleotide phosphate (NADPH) system. The accumulation of ROS in hepatocytes can cause cell death, which stimulates Kupffer cells to recruit immune cells to produce pro-fibrogenic mediators, including myofibroblasts [8]. NADPH oxidases, also known as NOX, are proteins that transfer electrons through membranes and catalyze the NADPH-dependent reduction of O_2 to O_2^-, generating ROS [9]. Thus, the NADPH system may play a role both in defense against viruses and bacteria, and in acting as a pro-inflammatory agent through the overproduction of ROS.

Among the seven members of the NOX family, the classic phagocytic NOX complex consists of the catalytic subunit gp91phox (the term "phox" derives from "phagocytic oxidase"), renamed NOX2, along with the regulatory subunit p22phox located in the membrane. The other regulatory components are p47phox, p40phox, p67phox and the small Rac GTPase, and they are usually located in the cytoplasm. The p22phox protein is encoded by the CYBA gene (cytochrome b-245α), located on chromosome 16q24, and it contains six exons. P22phox requires the presence of a NOX isoform and NOX p22phox for expression, since they act to mutually stabilize one another on the membrane. Thus, variation in the structure or function of p22phox has the potential to influence the activity of a number of NOX isoforms, and this could influence the generation of ROS in several tissues and under different conditions [10].

Several polymorphisms have already been described in the promoter region and in the coding region of the CYBA gene, and some of them are able to influence gene expression, which results in significant functional variation in the level of oxidative stress among individuals. Some polymorphisms are associated with systemic arterial hypertension, coronary artery disease, myocardial infarction, and diabetic and non-diabetic nephropathy [11, 12].

On the other hand, the NOX4 gene is located on chromosome 11 and contains 19 exons. NOX4 is expressed in hepatocytes, Kupffer cells and culture-activated (but not quiescent) hepatic stellate cells (HSC) from mice [13]. The physiological functions of NOXs in the liver are still only partially understood.

Considering the association of hepatic fibrosis in NAFLD patients and the role of oxidative stress in its evolution, it is important to search for genetic markers capable of identifying individuals with a greater or lesser risk of disease progression. We designed a cross-sectional study to evaluate for the first time the influences of these polymorphisms in Brazilian patients with NAFLD. This study aimed to evaluate the possible association of SNPs of genes encoding proteins of the NADPH oxidase system in the CYBA (unregistered) gene and the NOX4 gene (rs3017887), and the presence of NASH in NAFLD, and also to estimate the possible association between these polymorphisms and hepatic fibrosis, insulin resistance, metabolic syndrome and NASH.

Methods

Subjects and study design

This was a cross-sectional study with biopsy-proven NAFLD patients who were followed at the Hepatology Outpatient Unit in the Hospital das Clínicas of School of Medicine of University of São Paulo (HCFMUSP), São Paulo, Brazil, between January 2009 and August 2012. A total of 207 patients were selected. They all agreed to participate in the study and signed the informed consent form. The study was approved by the Ethics Committee of the Hospital das Clínicas (435.621) and was conducted following the ethical guidelines of *the Declaration of Helsinki*.

The diagnosis of NAFLD was made by a liver biopsy. The inclusion criteria were as follows: 1) NAFLD patients with age of 18–75 years; 2) hepatic biopsy compatible with NAFLD. The exclusion criteria were as follows: 1) presence of other causes of chronic liver disease, such as schistosomiasis, viral hepatitis, autoimmune hepatitis, Wilson's disease, alpha-1-antitrypsin deficiency, hemochromatosis; 2) alcohol intake (>100 g ethanol/week); 3) refusal to consent to the research.

Clinical variables

On the day of liver biopsy or near the date of the biopsy (within 3 months), demographic data such as gender, age and anthropometric data [weight, height, body mass index (BMI) and waist circumference] were collected. Metabolic syndrome was defined using the National Cholesterol Education Program/Adult Treatment Panel III (NCEP/ATPIII) criteria: fasting glucose $\geq$ 100 mg/dL; systolic blood pressure $\geq$ 130 mmHg or diastolic blood pressure $\geq$ 85 mmHg; triglycerides $\geq$ 150 mg/dL; low high-density lipoprotein cholesterol (HDL-cholesterol: men $<$ 40 mg/dL, women $<$ 50 mg/dL); abdominal obesity, given as waist circumference (men $>$ 102 cm/40 in., women $>$ 88 cm/35 in.). The definition used for type 2 diabetes was fasting glucose $\geq$ 126 mg/dL according to American Diabetes Association 2017 [14]. For the evaluation of insulin resistance, Homeostasis Model Assessment (HOMA-IR) was used [fasting glucose (mg/dL)/ $18 \times$ fasting insulin (μU/mL)/22.5]. The HOMA-IR, when greater than or equal to 2.513, was used as the marker of insulin resistance [15].

Biochemical analysis

Serum tests are routinely performed in patients who undergo liver biopsy. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), total bilirubin and fractions, glucose, insulin, markers for hepatitis B and C, hepatic autoantibodies, copper and serum ceruloplasmin, and an iron profile were all measured. In addition, a parasitological examination of feces and a search for eggs of *Schistosoma mansoni* (Kato-Katz) were requested if this had not yet been performed.

Samples of blood collected after a 12-hour fast were centrifuged within one hour after collection for separation of plasma, serum and leukocyte cells and stored at -80 °C. Biochemical analyses were performed at the HCFMUSP central laboratory. Glucose concentrations were determined by the enzymatic method of hexokinase (Cobas, Roche, Switzerland); insulin by the chemiluminescence method (Cobas, Roche); total cholesterol, HDL cholesterol and triglycerides by the enzymatic colorimetric method (Cobas, Roche); and low-density lipoprotein (LDL) cholesterol by using the Friedwald equation.

NADPH oxidase genotype analysis

Genomic DNA was extracted from peripheral blood leukocytes by the salting-out method. Two SNPs in the putative regulatory region of the genes encoding NADPH oxidase 4 catalytic subunit (NOX4) (rs3017887) and its regulatory subunit p22phox (CYBA) were selected based on previous studies [16, 17]. The samples were genotyped by real-time PCR using fluorescent-labeled probes according to the procedures recommended by the manufacturer. NOX4 rs3017887 was genotyped by assay type validation (C_15762095; Applied Biosystems, Foster City, USA), and CYBA-675 T$\rightarrow$A (unregistered) was genotyped by PCR using specific primers (sense: 5′-GCGCTGGCTCACCAC-3′ and antisense: 5′-ACTGGGAAAGCACAGAATGCA-3′) and fluorescent-labeled probes

(VIC: 5′-CCTCCCGAACCCAGG-3′ and FAM: 5′-CCTCCCGTACCCAGG-3′) (TaqMan, Applied Biosystems). Amplifications were performed on an Applied Biosystems Step One Plus Real-Time PCR System [30 s at 60 °C, 10 min at 95 °C, followed by 40 cycles of PCR (15 s at 95 °C, followed by 60 s at 60 °C) and 30 s at 60 °C]. Genotyping success rates were 98% for both SNPs, and the distribution of genotypes was consistent with the Hardy-Weinberg equilibrium for the two evaluated SNPs.

Histological analysis

Fragments of hepatic tissue previously fixed in 4% formalin solution were processed and submitted to hematoxylin-eosin (HE) staining, Masson's trichrome and Perls for pigments. Liver samples were evaluated by an experienced liver pathologist blinded to clinical information. The following histological parameters were quantified: balloonization, micro and/or macro steatosis and its zonal distribution, inflammatory infiltrate and its zonal distribution, portal and perivenular fibrosis, and focal necrosis.

The histopathological variables for activity were graded according to the NAFLD Activity Score (NAS) defined by the Pathology Committee of the NASH Clinical Research Network (CRN) [18]. The score is defined as the sum of the degrees of steatosis (0–3), lobular inflammation (0–3), and balloonization (0–2). Values range from 0 to 8. NAS values ≥ 5 correlate with the diagnosis of NASH and < 4 was diagnosed as non-NASH according to the News clinical trials. Fibrosis, which is less reversible and results from disease activity, is not included in the score. The separation of fibrosis from other histological variables facilitates understanding of staging ("degree of fibrosis") and what indicates necroinflammatory activity ("score") in NASH. For macro- and microvesicular steatosis, considering the percentage of infiltrated hepatocytes, the intensity considered was as follows: Grade 0, < 5%; Grade I, 5–33%; Grade II, 34–66%; and Grade III > 66%. In the staging of the structural alterations, Grade IV was assigned to cases with septa of fibrosis-determining nodules or when there was already established cirrhosis.

Statistical analysis

Quantitative data are expressed as the mean ± SD except as otherwise indicated. Continuous variables were log-transformed for analysis when the normality of distribution was rejected by the Shapiro–Wilk's test. Fisher's Chi-square test, ANOVA and ANCOVA were used for comparisons between groups. Hardy–Weinberg equilibrium between the expected and observed genotype distributions was assessed using the Chi-square test. The association between metabolic syndrome and NASH was evaluated by regression models. Logistic regression analyses were used for cross-sectional analyses. The odds ratio (OR) with its 95% confidence intervals (95% CI) was calculated for the minor allele of each SNP in a codominant or dominant model. Adjustments for clinical and laboratory parameters were performed by including these variables as covariates in the regression model. The interaction between NASH and genotype (in the CYBA analysis) was evaluated by inclusion of a covariate composite (NASH/genotype) in the regression model (ANOVA or logistic regression). The effects related to NASH were then evaluated by the nesting of the genotypic variable within the NASH variable in the analysis model. This resulted in the calculation of the statistical effects for steatosis and NASH separately and adjusted for multiple comparisons due to stratification by this variable. Correction for multiple comparisons due to multiple SNP tests were performed by Bonferroni correction. A $P \leq 0.025$ was considered significant.

Table 1
Clinical characteristics of the patients according to the presence of NASH.

Characteristics	Steatosis ($n = 27$)	NASH ($n = 180$)	P value
Age (yr)	52.1 ± 12.6	56.0 ± 9.4	0.21
Male (%)	32.0	24.0	0.35
Dyslipidemia (%)	66.7	72.9	0.62
Type 2 diabetes mellitus (%)	37.5	66.9	**0.007**
SAH (%)	75.0	67.9	0.64
Metabolic syndrome (%)	62.5	82.4	0.03
BMI (kg/m^2)	29.0 ± 5.4	32.2 ± 5.2	**0.003**
Fasting glucose (mg/dL)	106.8 ± 41.1	118.6 ± 39.9	**0.02**
Insulin(μU/mL)	15.4 ± 10.7	20.9 ± 15.3	**0.02**
HOMA-IR	5.0 ± 5.0	6.2 ± 5.2	0.06
Total cholesterol (mg/dL)	193.5 ± 43.7	197.2 ± 45.0	0.73
LDL cholesterol (mg/dL)	110.8 ± 38.0	117.0 ± 38.6	0.36
HDL cholesterol (mg/dL)	48.0 ± 10.0	48.3 ± 14.9	0.81
Triglycerides (mg/dL)	168.0 ± 97.2	174.4 ± 100.5	0.59
ALT (U/L)	45.2 ± 29.3	56.7 ± 105.6	0.73
AST (U/L)	28.7 ± 12.7	39.2 ± 32.7	0.07
GGT (U/L)	68.0 ± 81.7	89.5 ± 94.8	0.59

NASH: nonalcoholic steatohepatitis; SAH: systemic arterial hypertension; BMI: body mass index; HOMA: Homeostasis Model Assessment; LDL: low-density lipoprotein; HDL: high-density lipoprotein; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamyl transpeptidase.

Results

Patients characteristics according to the presence of NASH

Demographics, clinical and biochemical characteristics according to the presence of steatosis or NASH are shown in Table 1. Among the total of 207 NAFLD patients included in the evaluation, 180 (87.0%) had NASH. NASH patients had more comorbidities of type II diabetes mellitus (T2DM, 66.9% vs 37.5%, $P < 0.01$) and obese (mean BMI 32.2 vs 29.0 kg/m^2, $P < 0.01$). Serum glucose and insulin levels increased significantly. The frequency of metabolic syndrome was higher in NASH patients (82.4% vs 62.5%, $P = 0.03$), but without statistical significance. Other clinical characteristics did not differ significantly between patients with and without NASH.

Patient characteristics according to the presence of metabolic syndrome

The demographics, clinical and laboratory characteristics of NAFLD patients according to the presence or absence of metabolic syndrome are described in Table 2. Compared with patients without metabolic syndrome, those with metabolic syndrome had a higher prevalence of NASH (90.0% vs 76.0%, $P = 0.03$), T2DM (74.0% vs 20.0%; $P < 0.01$) and hypertension (79.0% vs 26.0%, $P < 0.01$), a higher BMI (32.5 vs 28.8 kg/m^2; $P < 0.01$), a higher incidence of dyslipidemia (79.0% vs 41.0%, $P < 0.01$) and older age (57.0 vs 51.4 years, $P < 0.01$).

Analysis of polymorphisms in CYBA and NOX4 genes according to the presence of metabolic syndrome or NASH

The genotypic frequencies of the SNPs in the CYBA and NOX4 genes do not correlated with the frequencies of metabolic syndrome (Table 3). Also, there was no statistically significant difference in the frequency of the CYBA gene polymorphism-675 T/A → (TT vs TA + AA) and the NOX4 SNP genotypes rs3017887 (CC vs CA + AA) between the steatosis population and the NASH population (Table 4).

The relationship between polymorphisms in CYBA and NOX4 genes and HDL, triglycerides and ALT

An analysis of the biochemical characteristics of patients with steatosis and NASH according to the genotype was also performed

Table 2
Clinical characteristics of the patients according to the presence of metabolic syndrome.

Characteristics	Metabolic syndrome ($n=152$)	No-metabolic syndrome ($n=39$)	*P* value
Age (yr)	57.0 ± 9.2	51.4 ± 10.9	**0.003**
Male (%)	77.0	64.0	0.10
Dyslipidemia (%)	79.0	41.0	**0.0001**
Type 2 diabetes mellitus (%)	74.0	20.0	**0.0001**
SAH (%)	79.0	26.0	**0.0001**
NASH (%)	90.0	76.0	0.03
BMI (kg/m^2)	32.5 ± 5.2	28.8 ± 5.1	**0.0001**
Fasting glucose (mg/dL)	123.3 ± 43.4	98.8 ± 17.3	**0.0004**
Insulin (μU/mL)	21.4 ± 16.2	14.8 ± 8.2	**0.02**
HOMA-IR	6.6 ± 5.6	3.9 ± 2.5	**0.004**
Total cholesterol (mg/dL)	197.6 ± 46.8	193.7 ± 38.3	0.83
LDL cholesterol (mg/dL)	116.7 ± 40.4	115.6 ± 35.9	0.89
HDL cholesterol (mg/dL)	47.5 ± 12.8	49.4 ± 17.4	0.68
Triglycerides (mg/dL)	181.5 ± 107.8	154 ± 66.8	0.11
ALT (U/L)	44.4 ± 47.0	47.8 ± 33.0	0.51
AST (U/L)	36.8 ± 31.8	34.6 ± 20.5	0.70
GGT (U/L)	83.9 ± 86.3	77.5 ± 90.0	0.11

SAH: systemic arterial hypertension; NASH: nonalcoholic steatohepatitis; BMI: body mass index; HOMA: Homeostasis Model Assessment; LDL: low-density lipoprotein; HDL: high-density lipoprotein; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamyl transpeptidase.

Table 3
Genotype frequencies of CYBA and NOX4 polymorphisms according to presence or absence of metabolic syndrome.

Genotype frequencies	No-metabolic syndrome	Metabolic syndrome	OR (95% CI)	*P* value
[a]CYBA			2.04 (0.64–7.85)	0.26
TT	0.865	0.835		
TA	0.108	0.159		
AA	0.027	0.006		
MAF	0.081	0.085		
[b]NOX4			0.54 (0.20–1.48)	0.23
CC	0.639	0.699		
CA	0.333	0.273		
AA	0.028	0.028		
MAF	0.194	0.164		

[a] Odds ratio (OR) for the minor allele in a dominant model obtained in logistic regression analyses adjusted for gender, age and T2DM.
[b] OR for the minor allele in a dominant model obtained in logistic regression analyses adjusted for gender, age, T2DM and dyslipidemia. A *P* value of < 0.025 was considered significant. MAF: minor allele frequency.

Table 4
Genotype frequencies of CYBA and NOX4 polymorphisms according to presence or absence of NASH.

Genotype frequencies	Steatosis	NASH	OR (95% CI)	*P* value
[a]CYBA			3.42 (0.84–25.97)	0.14
TT	0.923	0.837		
TA	0.077	0.151		
AA	0.000	0.012		
MAF	0.038	0.087		
[b]NOX4			0.43 (0.17–1.03)	0.06
CC	0.593	0.707		
CA	0.333	0.269		
AA	0.074	0.024		
MAF	0.240	0.158		

[a] Odds ratio (OR) for the minor allele in a dominant model obtained in logistic regression analyses adjusted for gender, age, T2DM and hypertension.
[b] OR for the minor allele in a codominant model obtained in logistic regression analyses adjusted for gender, age, T2DM, hypertension, dyslipidaemia and BMI. A *P* value of < 0.025 was considered significant. NASH: nonalcoholic steatohepatitis; MAF: minor allele frequency.

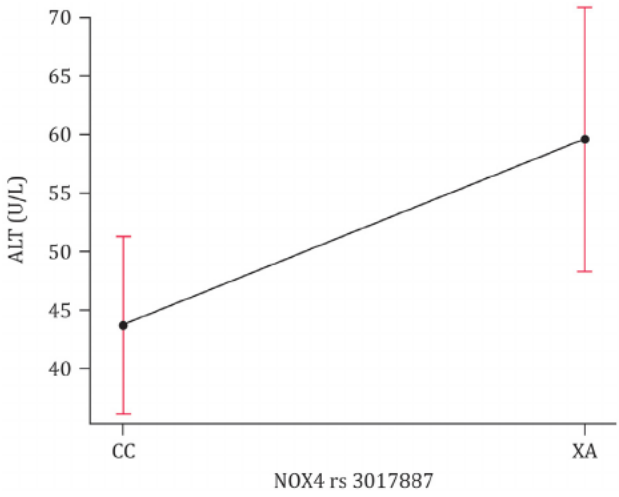

Fig. 1. Serum ALT of NAFLD patients according to SNP no NOX4 (rs 3017887) ($P=0.02$). Results were presented as mean ± SEM. Adjusted ANOVA for age, gender, T2DM, HDL and GGT. ALT: alanine aminotransferase; NAFLD: non-alcoholic fatty liver disease; T2DM: type II diabetes mellitus; HDL: high-density lipoprotein; GGT: gamma-glutamyl transpeptidase.

and demonstrated an association of SNPs-675 T/A genotypes in the CYBA gene with HDL (TT vs TA vs AA; $P<0.01$) and triglycerides (TT vs XA; $P<0.01$). There was a statistically significant difference in genotype frequency when the population was stratified into steatosis and NASH. The analysis of the biochemical characteristics of patients with steatosis and NASH according to their genotype also demonstrated an association of NOX4 (rs3017887) CA + AA genotypes with ALT level ($P=0.02$) (Fig. 1). There was no statistically significant difference in genotype frequency when the population was stratified into steatosis and NASH after adjustment for the variables of gender, age, T2DM, HDL and GGT.

Discussion

In the present study, we did not sound the difference between simple steatosis and NASH in the genotypic frequencies of NADPH NOX4 (CC vs CA + AA) and CYBA (TT vs TA + AA) polymorphisms. This can be explained because, unlike monogenic diseases in which a relatively rare mutation in a single gene confers a high risk for the development of the disease, NAFLD most likely results from common allelic variants in a large number of genes that interact with each other, each of which alone determines a modest risk, in addition to undergoing diverse influences, such as metabolic, hemodynamic and environmental. For these reasons, the identification of the factors of susceptibility to NAFLD constitutes a challenge similar to that of other polygenic diseases [19].

On the other hand, a positive finding of the present study was the nominal association of the NOX4 CA + AA genotype with ALT levels, which is a marker of inflammatory activity. It is important to note that there is a statistically significant difference in the frequency of genotypes when the population was stratified by steatosis and NASH. The association of the allele A of rs3017887 with the disease must be caused by increased necrotic activity in the context of higher oxidative stress [19]. Elevated ALT is considered a consequence of damage to the hepatocytes caused by NAFLD. Although oxidative stress is more severe in cirrhotic patients, it is not only a late-stage phenomenon, since it is supposed to occur early when transaminase levels are still elevated. These effects, in turn, may explain the fact that functional polymorphisms that modulate inflammation and/or oxidative stress may be markers that are inherited along with other allelic variants that predispose to oxidative stress and inflammation.

A cohort study of 903 overweight Korean women by Song et al. [20] found that ALT concentrations have a strong association with visceral fat accumulation, with abdominal visceral adipose tissue being the main predictor of ALT elevation in the context of NAFLD among non-diabetics. Sookoian and Pirola [21] reported that hepatic transaminases should not be considered mere biomarkers of liver damage, but the central points in the pathophysiology of NAFLD in particular or the components of metabolic syndrome in general. With respect to heredity, Makkonen et al. [22], in a study with twins, confirmed that higher levels of ALT were a hereditary trait, with genetic characteristics that explain up to 60% of the variability.

The participation of NOX4 in NASH has already been demonstrated in experimental models of NAFLD, through its prophylactic action, in HSCs [23, 24]. NOX4, a non-phagocytic NOX homolog, is expressed in the liver in hepatocytes, HSCs and endothelial cells. Jiang et al. [25] demonstrated that rats treated with an antifibrogenic drug (GKT137831) led to the suppression of the NOX1 and NOX4 gene expressions and suppression of hepatic fibrogenesis. In our study, the NOX4 polymorphism showed no association with hepatic fibrosis. In the hepatocytes, however, the role of NOX4 is not well defined and a study in NASH patients demonstrated that NOX4 levels were higher compared to those without NAFLD [26]. However, the contribution of the NOX4 gene to the genetic predisposition of NASH has never been explored before.

We did not find an association between SNP-675 T/A in the CYBA gene and NAFLD. Its implication in the pathophysiology of NAFLD and the functional repercussions in hepatocytes have been poorly explored; however, another study by our group also demonstrated a lack of association of the 645 T/A CYBA polymorphism with hepatic fibrosis in HCV patients [27]. This polymorphism was selected because it was known to be functional, as demonstrated by Moreno et al. in a study with hypertensive patients [17]. In addition, it was found that the presence of at least one A allele in the SNP-675 T/A polymorphism in the CYBA gene confers protection against diabetic nephropathy in patients with T1DM [28].

Several other polymorphisms linked to oxidative stress have already been studied, among them manganese superoxide dismutase (MnSOD), a mitochondrial protein induced by ROS production; cytochrome P4502E1, an endoplasmic monooxygenase; and iron overload, with iron being a pro-oxidant agent that acts to generate hydroxyl radicals [29]. Because it is a polygenic disease, it is difficult to correlate genetic factors with the evolution of the disease, although it is known that they are important when associated with environmental factors. However, recently, in GWAS studies, two genes were associated with NAFLD, namely PNPLA3 and TM6SF2, which are the first genes reproducibly found to be markers of the presence of steatosis, more advanced fibrosis and an elevated risk for HCC [30]. Our group demonstrated an association between the PNPLA3 gene and the degree of fibrosis in a population of patients with NAFLD (unpublished data). Although most studies have confirmed this association, the results are still controversial [31]. Considering that GWAS are expensive, require thousands of individuals and strong statistical and population genetics knowledge, the use of candidate gene studies in NAFLD is more straightforward and easier to implement [32].

The TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, USA) technique we used is still real-time PCR, the TaqMan methodology guarantees greater precision and efficiency than the technique of GWAS, with results obtained in a considerably short period of time. It is less laborious to eliminate steps requiring manipulation of amplified samples. It amplifies gene segments that are much smaller than those required by restriction fragment length polymorphism (RFLP), which makes it possible to perform genotyping on old and partially degraded samples. The protocols require less DNA, and the methodology allows the parallelization of reactions, through the multiplex tests, in real-time PCR equipment with more than two filters (unpublished data).

Due to the complexity and limitation of genetic polymorphism research in NAFLD, most studies have found a lack of statistical power, inability to achieve replication in several cohorts and the presence of heterogeneities in the disease stages. This causes research on polymorphisms in NAFLD to be filled with unknowns and challenges. And it is important to remember that SNPs are influenced by molecular factors such as the presence of other non-identified functional polymorphisms that accentuate or neutralize the variant effect, which would justify the different findings in terms of mechanism of action and function in gene/protein expression. The other interferences are environmental factors and the ethnic composition of the population.

Contributors

CGML and OCP proposed the study. RF, SJT and OCP performed the research and wrote the first draft. RF, CAM, LRVC, MDFC and CFJ collected and analyzed the data. All authors contributed to the design and interpretation of the study and to further drafts. OCP is the guarantor.

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Ethical approval

The study was approved by the Ethics Committee of the Hospital das Clínicas (435.621) and was conducted following the ethical guidelines of *the Declaration of Helsinki*.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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