

Observational Study

Genetic ancestry analysis in non-alcoholic fatty liver disease patients from Brazil and Portugal

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Abstract

AIM: To study the association between genetic ancestry, non-alcoholic fatty liver disease (NAFLD) metabolic characteristics in two cohorts of patients, from Brazil and Portugal.

METHODS: We included 131 subjects from Brazil [$n = 45$ with simple steatosis (S. Steatosis) and $n = 86$ with nonalcoholic steatohepatitis (NASH)] and 90 patients from Portugal ($n = 66$, S. Steatosis; $n = 24$, NASH). All patients had biopsy-proven NAFLD. In histologic evaluation NAFLD activity score was used to assess histology and more than 5 points defined NASH in this study. Patients were divided into two groups according to histology diagnosis: simple steatosis or non-alcoholic steatohepatitis. Genetic ancestry was assessed using real-time polymerase chain reaction. Seven ancestry informative markers (AT3-I/D, LPL, Sb19.3, APO, FY-Null, PV92, and CKMM) with the greatest ethnic-geographical differential frequencies ($\geq 48\%$) were used to define genetic ancestry. Data were analyzed using R PROJECTS software. Ancestry allele frequencies between groups were analyzed by GENEPOP online

and the estimation of genetic ancestry contribution was evaluated by ADMIX-95 software. The 5% alpha-error was considered as significant ($P < 0.05$).

RESULTS: In the Brazilian sample, NASH was significantly more frequent among the elderly patients with diabetes (NASH 56 ± 1.1 years old *vs* S. Steatosis 51 ± 1.5 years old, $P = 3.7 \times 10^{-9}$), dyslipidemia (NASH 63% *vs* S. Steatosis 37%, $P = 0.009$), higher fasting glucose levels (NASH 124 ± 5.2 *vs* S. Steatosis 106 ± 5.3 , $P = 0.001$) and Homeostatic Model of Assessment index > 2.5 [NASH 5.3 (70.8%) *vs* S. Steatosis 4.6 (29.2%) $P = 0.04$]. In the Portuguese study population, dyslipidemia was present in all patients with NASH ($P = 0.03$) and hypertension was present in a larger percentage of subjects in the S. Steatosis group ($P = 0.003$, respectively). The genetic ancestry contribution among Brazilian and Portuguese individuals with NASH was similar to those with S. Steatosis from each cohort (Brazilian cohort: $P = 0.75$; Portuguese cohort: $P = 0.97$). Nonetheless, the genetic ancestry contribution of the Brazilian and Portuguese population were different, and a greater European and Amerindian ancestry contribution was detected in the Portuguese population while a higher African genetic ancestry contribution was observed in Brazilian population of both NASH and S. Steatosis groups.

CONCLUSION: There was no difference between the genetic ancestry contribution among Brazilian and Portuguese individuals with NASH and S. Steatosis from each cohort.

Key words: Ancestry; Nonalcoholic fatty liver disease; Simple steatosis; Nonalcoholic steatohepatitis; Admixed population

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Core tip: Nonalcoholic fatty liver disease (NAFLD) is frequent and may lead to cirrhosis and hepatocellular carcinoma. Awareness about its risk factors and predictive markers of severity is important in the management of this infirmity. Self-reported ancestry may also influence NAFLD outcomes in homogeneous populations and African descendants appear to have milder disease than Caucasians. However, there are no available data that demonstrate the relationship between ancestry and NAFLD in admixed populations. This is the first study to evaluate the possible association between ancestry analyzed for genetic markers and biopsy-proven NAFLD in a homogeneous and a highly admixed population.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) may vary from simple steatosis across steatohepatitis (NASH) to hepatic fibrosis and, finally, cirrhosis and hepatocellular carcinoma. NAFLD is characterized by a potential and substantial seriousness and by variability inter-patient disease progression degree. However, while a meaningful percentage of the population is in risky of worsening condition, just a subgroup of patients will advance to NASH, liver fibrosis and cirrhosis^[1].

Several studies suggest the presence of diverse risks factors for NAFLD and differences in clinical features based upon ancestry, as well as the potential role of ancestry as an independent risk factor associated to disease gravity^[2]. In particular, Hispanic Americans and Caucasians have the larger frequency of NAFLD while African Americans have the lowest^[2]. However, few data are available about ancestry contribution in NAFLD using ancestry informative markers (AIMs). These AIMs are powerful tools for inferring the genetic composition of admixed populations. The assessment of accurate admixture estimates is important in population genetic studies, particularly in the context of highly admixed populations as those of Brazil and most American countries^[3-5]. In Brazil, a country that was colonized by Portugal, the admixtures of three main parental groups (Amerindians, Europeans and Sub-Saharan Africans) have originated the current Brazilian population. Thus, Brazilian population is admixed, tri-hybrid, with a great heterogeneity, resulting of inter-ethnic mating between individuals from three major ancestries contributors: the Amerindians, the Europeans who colonized South America in the 1500s and the Africans who arrived through the slave trade over a span of more than 300 years^[3,6]. Furthermore, the diverse regions of the country underwent different colonization processes, which to some extent shaped their genetic backgrounds, nowadays characterized by different proportions of Amerindian, European and African contribution^[7].

To investigate the possible association between genetic ancestry, NAFLD severity (simple steatosis or NASH) and metabolic characteristics in two cohorts with biopsy-proven NAFLD from Brazil and Portugal we then aimed to identify, for the first time, the ancestry influence in NAFLD and the possible role of genetic ancestry as an independent risk factor associated with non-alcoholic fatty liver disease.

MATERIALS AND METHODS

Subjects

We investigated 131 NAFLD patients whose diagnosis was confirmed by liver biopsy, ($n = 45$ had simple steatosis and $n = 86$ had NASH) from Brazil and 90 NAFLD patients, ($n = 66$, simple steatosis and $n = 24$, NASH) who had undergone bariatric surgery from Portugal. NASH was diagnosed based upon the pathologic criteria, and NAFLD activity score (NAS) was used to evaluate NASH diagnosis. All included patients

were at least 18 years old. The inclusion criterion was to have a liver biopsy-proven NAFLD. Exclusion criteria were the presence of concomitant known liver disease comprising viral hepatitis, Wilson's disease, hemochromatosis, or/and autoimmune liver diseases; reporting of methotrexate, tamoxifen or corticosteroids intake; or alcohol drinking ≥ 140 g ethanol weekly.

Ethics Committees from University of São Paulo School of Medicine and from the Hospital of Santa Maria University Lisbon have approved this study. The written informed consent was obtained from subjects, or their legal guardian, prior to study inclusion.

Laboratory evaluation

Laboratory tests were performed from peripheral blood sample, including: fasting glucose, plasma insulin, total cholesterol, high density lipoprotein-, low density lipoprotein (LDL)-, very LDL-cholesterol, triglycerides, aspartate aminotransferase and alanine aminotransferase (ALT). Insulin resistance calculi was performed using Homeostatic Model of Assessment (HOMA) [(fasting insulin mU/L) \times (fasting glucose mmol/L)/22.5]. The cut-off of HOMA ≥ 2.5 was used to define insulin resistance, a value that has been assessed at prior studies realized in Brazilian population^[8].

Histological evaluation

Hepatic biopsy fragments were fixed in formaldehyde saline (4%) and processed using hematoxylin-eosin and picosirius stains. A blinded single pathologist with liver expertise in each center performed histological analyzes.

NASH was described as increased hepatic steatosis with centrilobular ballooning and/or Mallory-Denk bodies or any level of steatosis beside pericellular or centrilobular fibrosis perisinusoidal or bridging fibrosis^[9,10]. We used the NAS to assess histology and more than 5 points defined NASH in this study.

DNA extraction and AIMS

Peripheral blood samples were collected and DNA extraction was performed from mononuclear cell by Pure Link Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, United States) following the manufacturer's guidelines.

Ancestry for genetic markers was evaluated by analysis of seven specific alleles of population. These alleles, AIM, were chosen based on the earlier studied panel of 48 AIMS determined in diverse populations. The AIM allele* 1 was defined as the existence of insertion or the absence of restriction enzyme site^[4,5].

Seven AIM with the greatest ethnic-geographical differential frequencies ($\geq 48\%$) were chosen in this study. Polymorphisms as *Alu* insertion and *insertion-deletion* (*indel*, I/D) were identified by conventional polymerase chain reaction (PCR); single nucleotide polymorphisms were performed by real-time PCR (TaqMan™ System, Applied Biosystems).

The examined AIM were: African ancestry *AT3-I/D*

(rs3138521) and *LPL* [rs285]^[11]; European ancestry *Sb19.3* (rs3138524), *APO* (rs3138522) and *FY-Null* (rs2814778); and Amerindian ancestry markers *PV92* (rs3138523) and *CKMM* (rs4884)^[4,11]. The frequency of allele* 1 in AIM was considered in the analysis.

Ancestral population characteristics

Four hundred eighty-two ($n = 482$) subjects from homogeneous populations comprised ancestral populations. These groups were used as a referral to genetic ancestry assessment. Ancestral African population included 134 Nigerians subjects; European ancestral population included 23 Germans and 83 Spanish; and Amerindian ancestral population had 242 Native Americans. The ancestral samples were friendly provided by Mark Shriver, MD. The analyzed AIM from NAFLD studied samples were verified in the ancestral population.

Statistics analysis

Data were analyzed using R PROJECTS software version 2.11.1 for Windows (May 31 2010, Statistics Department of the University of Auckland, Auckland, New Zealand, <http://www.r-project>). The continuous variables were expressed as mean \pm SD; data comparison was executed by the Mann-Whitney *U*-test. Categorical variables were presented as number of cases and percentage and analyzed by Pearson's χ^2 test. Since the sample size assessment could not be performed previously, power was calculated posteriorly. The 5% alpha-error was considered as significant ($P < 0.05$) (two-sided).

The Hardy-Weinberg equilibrium assessment was calculated by GENEPOP online version 4.0.10 (Laboratoire de Genetique et Environment, Montpellier, France)^[12]. Ancestry allele frequencies evaluation and variation between groups were analyzed utilizing GENEPOP online version 4.0.10^[12]. Estimation of genetic ancestry contribution was computed by ADMIX-95 software (Departamento de Genética de la Facultad de Medicina, Universidad de la Republica, Montevideo, Uruguay, <http://www.genetica.fmed.edu.uy>)^[13]. Stimatives of genetic ancestry contribution from each individual were determined through STRUCTURE software version 2.2 (Human Genetics Department, University of Chicago, Chicago, IL, United States).

A biomedical statistician performed the statistical review of the study.

RESULTS

We analyzed 131 Brazilian patients with NAFLD. Forty-five patients were identified with simple steatosis and 86 had NASH by histologic liver analysis (Table 1). These results were compared with patients from Portugal who had undergone bariatric surgery; 90 had histologic liver information available to analysis. Among Portuguese subjects 66 had simple steatosis, and 24 had NASH (Table 1). In this sample, NAS criteria were used as parameters to Simple Steatosis and NASH

Table 1 Baseline characteristics of Brazilian and Portuguese study population according to non-alcoholic fatty liver disease status *n* (%)

	Brazil (<i>n</i> = 131)			Portugal (<i>n</i> = 90)		
	NASH	S. Steatosis	<i>P</i> -value	NASH	S. Steatosis	<i>P</i> -value
Gender	86 (65.6)	45 (34.4)		24 (26.7)	66 (73.3)	
Female	61 (70.1)	26 (29.9)	0.2	20 (28.6)	50 (71.4)	0.6
Male	25 (56.8)	19 (43.2)		4 (20.0)	16 (80.0)	
Age (yr)	56 ± 1.1	51 ± 1.5	² 0.006	47 ± 12.4	47 ± 10.2	0.3
Diabetes-2	71 (82.4)	8 (17.6)	² 3.7 × 10 ⁻⁹	14 (33.3)	28 (66.7)	0.2
Fasting glucose (mg/dL)	124.9 ± 5.2	106.0 ± 5.3	² 0.001	97.3 ± 28.2	92.6 ± 18.4	0.3
HOMA ≥ 2.5 - mean	5.3	4.6	² 0.04	4.9	4.8	0.5
HOMA ≥ 2.5 - <i>n</i>	75 (70.8)	31 (29.2)	² 0.02	15 (45.5)	18 (54.5)	0.5
Hypertension	59 (68.2)	14 (31.8)	² 8.0 × 10 ⁻⁵	12 (20.1)	34 (73.9)	² 0.003
ALT (IU/L)	38.3 ± 0.6	33 ± 5.2	0.2	31.7 ± 30.6	30.56 ± 15.9	0.7
AST (IU/L)	22 ± 22.5	32.8 ± 3.2	² 0.009	25.9 ± 24.8	25.06 ± 11.2	0.7
¹ Dyslipidemia	54 (63.0)	17 (37.0)	² 0.009	24 (100)	55 (83)	² 0.03

¹High levels of cholesterol and/or triglyceride; ²Statistically significant results, *P* < 0.05. NASH: Nonalcoholic steatohepatitis; S. Steatosis: Simple steatosis; HOMA: Homeostatic Model of Assessment; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

Table 2 Genetic ancestry contribution in Brazilian and Portuguese populations with non-alcoholic fatty liver disease

	Brazil			Portugal		
	NASH	S. Steatosis	<i>P</i> -value	NASH	S. Steatosis	<i>P</i> -value
African	41.7%	37.7%	0.75	6.4%	6.1%	0.97
Amerindian	9.5%	12.7%		37.8%	39.4%	
European	48.8%	49.6%		55.9%	54.5%	

NASH: Nonalcoholic steatohepatitis; S. Steatosis: Simple steatosis.

diagnosis: 65.6% of Brazilians (*n* = 86) had NAS score ≥ 5 defining NASH and 26.7% Portuguese people had NAS score ≥ 5, classified as NASH.

In the Brazilian sample, NASH was more frequent among the elderly with diabetes, dyslipidemia, high fasting glucose levels and HOMA index ≥ 2.5. In the Portuguese study population, dyslipidemia was present in all patients (*n* = 24) with NASH and hypertension was present in a superior number of patients in the simple steatosis group compared to NASH (Table 1). ALT levels were similar between NASH and simple steatosis patients.

The genetic ancestry contribution among Brazilian and Portuguese individuals with NASH was similar to those with simple steatosis from each cohort (Table 2). Furthermore, the genetic ancestry contribution of the Brazilian and Portuguese population were different and a greater European and Amerindian ancestry contribution was detected in the Portuguese population while a higher African genetic ancestry contribution was observed in Brazilian population of both NASH and simple steatosis subgroups (Table 2).

In Brazilian population, genetic ancestry did not influence known risk factors for NAFLD as gender, hypertension, diabetes, HOMA-index or dyslipidemia. However, in Portugal sample there was a trend for European genetic ancestry being greater among subjects with HOMA ≥ 2.5 and Amerindian ancestry contribution among those with HOMA-index < 2.5 (Table 3).

DISCUSSION

Of our knowledge, this is the first study that investigated the possible association between genetic ancestry, NAFLD and metabolic characteristics using AIM in two cohorts with biopsy-proven NAFLD from Brazil and Portugal. However, our results showed that these genetic ancestry differences do not appear to be associated with the development of NASH. Genetic ancestry distribution was quite different between Brazilian and Portuguese patients. Unexpectedly, there was a higher prevalence of an Amerindian ancestry in the Portuguese group than in the Brazilian. On the other hand, as expected there was a high prevalence of African ancestry in the Brazilian patients. However, interestingly, no differences in this distribution were found when NASH or simple steatosis was compared, either in the Brazilian or the Portuguese group.

In the Brazilian sample, known risk factors as diabetes, dyslipidemia, higher fasting glucose levels and HOMA index ≥ 2.5 were more frequent among patients with NASH than simple steatosis. In the Portuguese study population, dyslipidemia was frequent among patients with NASH; and hypertension was present in a higher proportion among subjects with simple steatosis in comparison to those with NASH, possibly due to larger sample size in simple steatosis group. However, there were no differences regarding other metabolic risk factors, what may be due to the fact that the entire Portuguese cohort had morbid obesity. In the Portugal sample, the genetic ancestry did not have a significant influence in the prevalence of insulin resistance as evaluated by HOMA. Several studies have showed that individuals with European ancestry have a higher risk of progression to NASH; however, these studies used self-reported ancestry classification. To our knowledge, no previous study has evaluated the influence of genetic ancestry in NASH progression in admixed populations.

The heterogeneity of the Brazilian population presents an additional difficulty in pharmacogenetic and clinical

Table 3 Genetic ancestry influence in risk factors for non-alcoholic fatty liver diseases in Brazilian and Portuguese study populations

	Genetic ancestry contribution							
	Brazil				Portugal			
	African	Amerindian	European	P-value	African	Amerindian	European	P-value
Diabetes								
No	11.7%	39.8%	48.5%	1.00	6.1%	37.5%	56.4%	0.69
Yes	12.1%	39.6%	48.3%		7.2%	31.6%	61.2%	
Dyslipidemia								
No	11.8%	39.5%	48.6%	0.99	6.0%	37.4%	56.5%	0.90
Yes	11.7%	39.6%	48.7%		6.6%	33.6%	59.8%	
Gender								
No	11.8%	39.3%	48.9%	0.99	6.2%	37.9%	55.9%	0.97
Yes	11.7%	39.4%	48.9%		6.2%	39.5%	54.3%	
HOMA-index								
> 2.5	11.7%	39.7%	48.6%	0.94	3.4%	33.9%	62.7%	0.07
< 2.5	10.6%	38.3%	51.1%		7.8%	43.5%	48.7%	
Hypertension								
No	45.9%	20.4%	21.0%	0.16	4.7%	31.4%	63.9%	0.18
Yes	44.0%	18.7%	37.3%		7.6%	41.2%	51.1%	

genetic research. Studies have demonstrated only a weak association between skin pigmentation, self-reported ancestry and the ancestrality determined by DNA markers in admixed populations, as is the Brazilian case^[3,6,14]. Therefore, assessment of an individual's genetic ancestry might be the best way to assess the possible relation among disease factors and ethnic influence in association studies from admixed populations as the Brazilian.

Brazilian and Portuguese populations have different ancestry's contributions. More even proportions of ethnic contribution were observed in the Brazilian population, reflecting the greater racial admixing, while a higher genetic European contribution and a small African contribution was observed among the Portuguese, probably reflecting the historic context of both countries.

European and Amerindian come from the same chain migration and then some specific allele populations could be difficult to differentiate similar ancestries. So we believe that a larger number of AIMs with a great differential of frequency (> 40%) among Amerindians and Europeans or mitochondrial DNA analysis could be able to better differentiate the contribution of each of these ancestries in the Portuguese sample^[15,16].

Limitations of the present study could be the differences in the studied cohorts. While the Brazilian cohort consisted of a group of patients being evaluated for suspected NAFLD, the Portuguese cohort was a cohort of morbid obese patients, with the usual distribution of about a third having NASH and two thirds having simple steatosis. Also, we are not aware what the ancestry distribution of the normal population of either country is. Regarding the Portuguese cohort it would be tempting to speculate that this high prevalence of Amerindian ancestry could be involved in the fact that they have morbid obesity, since it has been recognized that Amerindian subjects have an increased susceptibility to develop obesity^[17,18].

In summary, the genetic ancestry contribution among

Brazilian and Portuguese individuals with NASH was similar to those with simple steatosis from each cohort. On the other hand, a greater European and Amerindian ancestry contribution was detected in the Portuguese population while a higher African genetic ancestry contribution was observed in Brazilian population of both NASH and simple steatosis groups.

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COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) is a frequent liver disease and may vary from simple steatosis through steatohepatitis, fibrosis and, finally, cirrhosis and hepatocellular carcinoma. Several studies suggest the presence of diverse risks factors for NAFLD and differences in clinical features based upon ancestry, as well as the potential role of ancestry as an independent risk factor for disease severity. Hispanic Americans and Caucasians have the highest prevalence of NAFLD whereas African Americans have the lowest; however, few data about ancestry contribution in NAFLD patients are available. Brazil is a country that was colonized by Portugal and its population genetic background ancestry is an admixture of three main parental groups (Amerindians, Europeans and Sub-Saharan Africans). Portugal has a more homogeneous population of European ancestry. In order to analyze the influence that ancestry of admixed populations has over a specific disease it is necessary to assess genetic ancestry markers. The ancestry informative markers (AIMs) is powerful tools for inferring the genetic composition of admixed populations. In this study the authors investigated the possible association between genetic ancestry, NAFLD severity [simple steatosis and nonalcoholic steatohepatitis (NASH)] and metabolic characteristics in two cohorts with biopsy-proven NAFLD from Brazil and Portugal.

Research frontiers

To their knowledge this is the first study that have utilized genetic ancestry markers to compare the ancestry influence of an admixed population with a more homogeneous one such as the Portuguese population.

Innovations and breakthroughs

The authors results showed that these genetic ancestry differences do not appear to be related with NASH; no differences in genetic ancestry distribution were found when NASH or simple steatosis were compared, either in the

Brazilian or the Portuguese study samples. The genetic ancestry distribution among Brazilian and Portuguese patients were different and they observed a greater prevalence of Amerindian ancestry among Portuguese compared to the Brazilians. On the other hand there was a marked prevalence of African ancestry in the Brazilian patients. However, interestingly, no differences in this distribution were found when NASH or simple steatosis was compared, either in the Brazilian or the Portuguese samples. Previous studies have showed that individuals with European ancestry have a higher risk of progression to NASH; however, these studies used self-reported ancestry classification, which may not be accurate when analyzing admixed populations. Known risk factors as diabetes, dyslipidemia, higher fasting glucose levels and Homeostatic Model of Assessment index ≥ 2.5 were more frequent among patients with NASH than simple steatosis in the Brazilian sample. In the Portuguese analyzed sample, dyslipidemia was present in a higher proportion in the NASH group compared to simple steatosis. They did not find any association among genetic ancestry and those risk factors.

Applications

This study suggests that ancestry analyzed by genetic markers possibly do not add as a risk factor in the evaluation of NAFLD disease. However, other known risk factors were present in this study. The high Amerindian ancestry among Portuguese is an issue to be studied in future analyses.

Terminology

NAFLD comprises two major presentations, simple steatosis and NASH. The former is a mild clinical condition characterized by fatty deposits in the hepatocyte cytoplasm while the latter is defined by the presence of any degree of steatosis along with centrilobular ballooning and/or Mallory-Denk bodies or any degree of steatosis along with centrilobular pericellular/perisinusoidal fibrosis or bridging fibrosis. The NAFLD activity score is a tool to assess NASH diagnosis and NAFLD severity. AIMs are genetic polymorphisms, which have a frequency differential of at least 30% in order to differentiate two distinct populations. They have chosen AIMs with high frequency differential in this analysis ($\geq 48\%$).

Peer-review

In the original article of Cavalcante *et al.*, the authors investigated the possible association between genetic ancestry, NAFLD severity and metabolic characteristics in two cohorts with biopsy-proven NAFLD from Brazil and Portugal. They found that ancestry markers are not different between subjects with steatohepatitis and ones suffering from hepatic steatosis in the investigated populations. They concluded that genetic ancestry is not associated with a higher risk of NASH in their study.

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