

# Association of Tumor Necrosis Factor $\alpha$ and Manganese Superoxide Dismutase Polymorphisms in Patients with Non-Alcoholic Steatohepatitis from Northeast Mexico

Karina Trujillo-Murillo<sup>1,6</sup>, Francisco J. Bosques-Padilla<sup>2</sup>, Irma Calderón-Lozano<sup>2</sup>, Sirelda Navar-Vizcarra<sup>2</sup>, Elvira Garza-González<sup>3</sup>, Alberto Niderhauser-García<sup>4</sup>, Juan P. Flores-Gutiérrez<sup>4</sup>, Pablo Zorrilla-Blanco<sup>5</sup>, Ricardo Salinas-Garza<sup>2</sup>, Ana M. Rivas-Estilla<sup>1</sup> and Herminia G. Martínez-Rodríguez<sup>\*,1</sup>

<sup>1</sup>Departments of Biochemistry and Molecular Medicine, <sup>2</sup>Gastroenterology, <sup>3</sup>Microbiology and <sup>4</sup>Pathology, School of Medicine and University Hospital "Dr. Jose E. Gonzalez", Autonomous University of Nuevo Leon, and the <sup>5</sup>Hidalgo Clinic, Monterrey, Nuevo Leon, Mexico. <sup>6</sup>Mesoamerican Center Studies in Public Health and Disasters (CEMESAD), Autonomous University of Chiapas, Mexico

**Abstract:** *Background and Aims:* Environmental and genetic factors play a role in the pathogenesis and natural history of non-alcoholic steatohepatitis (NASH). The aim of this study was to determine if the G-238A (allele TNFA) and G-308A (allele TNF2) tumor necrosis factor-alpha (TNF- $\alpha$ ) and Ala9Val manganese superoxide dismutase (MnSOD) polymorphisms were associated with NASH in a case-control study of subjects from Northeast Mexico. *Methods:* We analyzed DNA samples from 68 patients with NASH (50 women and 18 men; mean age  $\pm$  SD, 33.9  $\pm$  10.8 years; BMI 43.9  $\pm$  7.2 kg/m<sup>2</sup>) and 100 healthy subjects (44 women and 56 men; mean age  $\pm$  SD, 27.8  $\pm$  10.8 years; BMI 23  $\pm$  1.6 kg/m<sup>2</sup>). The diagnosis was based on liver biopsy reviewed by two pathologists blinded to clinical data. The polymorphisms were evaluated using the polymerase chain reaction-restriction fragment length polymorphism method. *Results:* The frequency of G-238A TNF- $\alpha$  and Ala9Val MnSOD polymorphisms in NASH patients was significantly higher in comparison with control subjects (OR = 3.06, 95% CI 1.29-7.33,  $p$  = 0.0047 and OR = 6.28, 95% CI 2.95-13.55,  $p$  = 0.001, respectively). In contrast, the G-308A TNF- $\alpha$  polymorphism did not show a statistical difference between either group (OR = 1.04, 95% CI 0.45-2.38,  $p$  = NS). Analyzed populations were in Hardy-Weinberg equilibrium. *Conclusions:* Our results suggest that G-238A TNF- $\alpha$  and Ala9Val MnSOD polymorphisms, which are molecules involved in inflammation and cellular oxidative stress, could be associated with NASH. These data may contribute to understanding the genetic susceptibility to NASH.

**Keywords:** Non-alcoholic steatohepatitis (NASH), morbidly obese, tumor necrosis factor alpha (TNF- $\alpha$ ), manganese superoxide dismutase (MnSOD), polymorphisms.

## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most frequent cause of abnormal hepatic steatosis in the absence of alcohol abuse world-wide. Non-alcoholic steatohepatitis (NASH) is the most progressive form of NAFLD. Little is known about the pathogenesis and natural history of NASH; however, it has been reported that environmental and genetic factors act in concert. Various risk factors have been associated with NASH, such as insulin resistance (IR) [1], obesity, type 2 diabetes mellitus, hypercholesterolemia, drugs, toxins [2,3], mitochondrial dysfunction, and oxidative stress [4]. Recently, polymorphisms in genes that play a role in lipid metabolism and steatosis, the insulin signaling pathway, reactive oxygen species (ROS) formation and degradation, cytokines and endotoxin receptors, and profibrogenic

mediators are of special interest because it has been proposed that they increase the risk of developing NASH [5, 6].

G to A polymorphic sequence at position -308 (TNF1 to TNF2 allele) and -238 (TNF $\beta$  to TNFA allele) in the tumor necrosis factor alpha (TNF- $\alpha$ ) gene promoter region has been shown to influence TNF- $\alpha$  gene expression and may induce IR [7]. Moreover, these polymorphisms have been involved in the pathogenesis and progression of NASH [8, 9]. Valenti *et al.* [9] reported that Italian NAFLD patients have a high frequency of G-238A, but not of G-308A TNF- $\alpha$  polymorphisms in comparison with controls. In addition, these polymorphisms were associated with IR. Another report by Tokushige *et al.* [10] found that there were no significant differences in the genotype frequency of any of the five TNF- $\alpha$  polymorphisms (T-1031C, C-863A, C-857T, G-308A and G-238A) analyzed among Japanese NAFLD patients compared with healthy controls; however, the polymorphisms T-1031C and C-863A were associated with IR. On the other hand, a potentially functional amino acid polymorphism (Ala9Val) described in the mitochondrial targeting sequence (MTS) of manganese superoxide

\*Address correspondence to this author at the Department of Biochemistry and Molecular Medicine, School of Medicine, Autonomous University of Nuevo Leon, Monterrey, NL, Mexico; Tel: (52-81) 8329-4174; Fax: (52-81) 8333-7747; E-mail: herminiamar@gmail.com

dismutase (MnSOD) has been associated with NASH development [11]. This polymorphism leads to a valine-to-alanine amino acid change in the MTS [12,13]. This amino acid substitution affects the secondary structure of the MTS causing partial arrest of the precursor within the inner membrane and a decreased formation of the active MnSOD tetramer in the mitochondrial matrix, thus causing induced accumulation of ROS within the cells [11,14]. Based on this, the aim of this study was to determine whether the G-238A (allele TNFA), G-308A (allele TNF2) TNF- $\alpha$ , and Ala9Val MnSOD polymorphisms were associated with NASH in a case-control study of subjects from Northeast Mexico.

## MATERIAL AND METHODS

### Patients and Controls

We included 92 patients over 18 years of age with a body mass index (BMI)  $\geq 35 \text{ kg/m}^2$  who underwent gastric bypass surgery at a single medical center in Monterrey, Mexico that cares for patients from the State of Nuevo Leon and surrounding states in Northeast Mexico (Coahuila, Tamaulipas, and San Luis Potosi). Studied patients underwent hepatic wedge biopsy at the time of gastric bypass surgery and a peripheral blood sample was drawn to perform molecular and laboratory tests.

NASH was diagnosed in 68 patients (50 women and 18 men; mean age  $\pm$  SD,  $33.9 \pm 10.8$  years; BMI  $43.9 \pm 7.2 \text{ kg/m}^2$ ). The following laboratory tests were performed in all patients: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP),  $\gamma$ -glutamyltransferase (GGT), as well as total cholesterol and triglycerides. Insulin resistance (IR) was evaluated by Quantitative Insulin Sensitivity Check Index (QUICKI) =  $1/[\log(I_0) + \log(G_0)]$ , where  $I_0$  is the fasting plasma insulin level (microunits per mL), and  $G_0$  is the fasting blood glucose level (mg/dL) [16].

The control group included 100 unrelated healthy subjects who were residents of Northeast Mexico. A peripheral

blood sample was collected from each. Forty-four were women and 56 were men. Mean age was  $27.8 \pm 10.8$  years and mean BMI was  $23 \pm 1.6 \text{ kg/m}^2$ . All control subjects were confirmed to have normal liver function, no viral hepatitis infection by blood test, and no history of alcohol abuse. The research protocol was reviewed and approved by the institutional review board. At enrollment, all participants provided written informed consent.

### Biopsy Analysis

Tissues were fixed in formaldehyde, embedded in paraffin, and stained with hematoxylin-eosin, silver reticulin, Sirius red for collagen, Perls' for iron and Masson's trichrome for assessment of liver fibrosis. All specimens were considered sufficient for analysis. Two pathologists blinded to clinical data reviewed biopsy specimens for hepatic steatosis, necroinflammatory activity, and liver fibrosis/cirrhosis according to the Brunt classification [3]. Fibrosis was scored using a 5-grade scale: F0, none or normal connective tissue; F1, foci of perivenular or pericellular fibrosis in zone 3; F2, perivenular or pericellular fibrosis confined to zones 3 and 2, with or without portal/periportal fibrosis; F3, bridging or septal fibrosis; and F4, cirrhosis [15].

### DNA Extraction

Genomic DNA was obtained from peripheral blood leukocytes from patient's and control's samples using the standard phenol-chloroform method [17].

### Determination of TNF- $\alpha$ and MnSOD Polymorphisms

Polymorphisms were detected in each DNA sample by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. For the polymorphism of TNF- $\alpha$  promoter at position -238 we used the following primers: sense primer 5'-AGACCCCCCTCGGAACC-3' and antisense primer 5'-ATCTGGAGGAAGCGGTAGTG-3' [18]; while for the -308 TNF- $\alpha$  polymorphism we used the

**Table 1. Demographic and Clinical Characteristics of NASH Patients and Controls**

Characteristics	NASH patients n = 68	Controls n = 100
Age (years; mean $\pm$ SD)	$33.9 \pm 10.8$	$28 \pm 11$
BMI ( $\text{kg}/\text{m}^2$ )	$43.9 \pm 7.2$	$23 \pm 2$
<b>Gender</b>		
Female	50 (74%)	44 (44%)
Male	18 (26%)	56 (56%)
<b>Laboratory data</b>		
*Aspartate aminotransferase (U/L)	$7 (58.7 \pm 10.9)$	-----
*Alanine aminotransferase (U/L)	$8 (98.5 \pm 34.8)$	-----
* $\gamma$ -glutamyltransferase (U/L)	$5 (148.6 \pm 108.9)$	-----
*Cholesterol (mg/dL)	$22 (236.5 \pm 32)$	-----
*Triglycerides (mg/dL)	$19 (203.3 \pm 41)$	-----
*Alkaline phosphatase (U/L)	$7 (179.8 \pm 36.1)$	-----

\* Patients with abnormal values above (at least one time) upper normal limit. Results are expressed as means  $\pm$  SD in parenthesis. All control subjects had normal values.

sense primer 5'-CAATAGGTTTGAGGGCCAT-3' (modified from Day *et al.* 1998) and the antisense primer describe above. These primers that contain a single base-pair mismatch adjacent to the polymorphism site to introduce a restriction site for the enzymes *Msp I* and *Nco I* into the wild-type nucleotide sequences after amplification, were used to detect the polymorphisms -238 and -308, respectively [7,19]. The amplified 151 bp and 223 bp products were digested with *Msp I* and *Nco I* restriction enzymes (New England BioLabs, Beverly, MA, USA) and analyzed using 2% agarose gel.

For the MnSOD MTS polymorphism (Ala9Val) we used the following primers [12]; sense primer 5'-CAGCCCAG CCTGCGTAGACGG-3' and antisense primer 5'-CTTGGCC AACGCCTCCTGGTACTT-3'. A PCR product of 267 bp was obtained and then digested with *Bsa WI* restriction enzyme (New England BioLabs, Beverly, MA, USA) and analyzed using 2% agarose gel.

### Statistical Analysis

Results are expressed as means and standard deviations and were considered significant when  $p < 0.05$  (2-tailed). Hardy-Weinberg equilibrium was determined for these polymorphisms within the control group using  $X^2$  test. Multivariate analysis was performed to assess the association

between polymorphisms and several variables of phenotype expression of NASH. Data analysis was performed using Epi-Info version 3.3.2 and SPSS version 13.0 software.

## RESULTS

### Demographic and Clinical Characteristics

The demographic and clinical data of the 68 patients with NASH and 100 healthy subjects are shown in Table 1. All patients referred alcohol abstinence for a minimum of 4 years and none had ascites clinically or by ultrasonography, or obvious peripheral edema.

### TNF- $\alpha$ and MnSOD Polymorphisms

The frequency of the -238 and -308 TNF- $\alpha$  polymorphisms (with the prevalence of TNFA and TNF2 alleles) in patients with NASH and controls are shown in Tables 2 and 3. No significant difference in the prevalence of -308 TNF- $\alpha$  polymorphism was observed among patients and controls; however, the prevalence of patients who carried the -238 TNF- $\alpha$  polymorphism was 29% and 12% in controls ( $p = 0.0047$ ) (Table 2). The allele frequencies of the TNFA and TNF2 alleles were 0.06 and 0.10, respectively, and were in Hardy-Weinberg equilibrium ( $X^2 = 0.407$ ;  $p = 0.5233$  and  $X^2 = 1.235$ ;  $p = 0.2665$ , respectively).

**Table 2. Allele and Genotype Distribution of the -238 TNF- $\alpha$  Polymorphism in NASH Patients and Controls**

Genotype	n	Genotype frequency (%)	Allele	n	Allele frequency (%)	p-Value	OR
<b>NASH</b>							
TNFG/TNFG	48	71	TNFG	116	85	0.0047	3.06
TNFG/TNFA	20	29	TNFA	20	15		
TNFA/TNFA	0	0					
Total	68	100		136			
<b>CONTROL</b>							
TNFG/TNFG	88	88	TNFG	188	94		
TNFG/TNFA	12	12	TNFA	12	6		
TNFA/TNFA	0	0					
Total	100	100		200			

**Table 3. Allele and Genotype Distribution of the -308 TNF- $\alpha$  Polymorphism in NASH Patients and Controls**

Genotype	n	Genotype frequency (%)	Allele	n	Allele frequency (%)	p-Value	OR
<b>NASH</b>							
TNF1/TNF1	54	79	TNF1	122	90	0.925	1.04
TNF1/TNF2	14	21	TNF2	14	10		
TNF2/TNF2	0	0					
Total	68	100		136			
<b>CONTROL</b>							
TNF1/TNF1	80	80	TNF1	180	90		
TNF1/TNF2	20	20	TNF2	20	10		
TNF2/TNF2	0	0					
Total	100	100		200			

**Table 4.** Allele and Genotype Distribution of the Ala9Val MnSOD Polymorphism in NASH Patients and Controls

Genotype	n	Genotype frequency (%)	Allele	n	Allele frequency (%)	p-Value	OR
<b>NASH</b>							
Ala/Ala	15	22	Alanine	65	48	< 0.01	6.28
Ala/Val	35	51	Valine	71	52		
Val/Val	18	27					
Total	68	100		136			
<b>CONTROL</b>							
Ala/Ala	64	64	Alanine	161	80.5		
Ala/Val	33	33	Valine	39	19.5		
Val/Val	3	3					
Total	100	100		200			

On the other hand, the frequency of MnSOD MTS Ala9Val polymorphism was significantly higher in patients with NASH than in control subjects (78% vs. 36%;  $p < 0.01$ ) (Table 4). The allele frequency of the Valine allele was 0.195 and was in Hardy-Weinberg equilibrium ( $X^2 = 0.261$ ;  $p = 0.6092$ ).

The frequency of G-238A TNF- $\alpha$  and Ala9Val MnSOD polymorphisms in NASH patients was significantly higher in comparison with control subjects (OR = 3.06, 95% CI 1.29-7.33,  $p = 0.0047$  and OR = 6.28, 95% CI 2.95-13.55,  $p = 0.001$ , respectively).

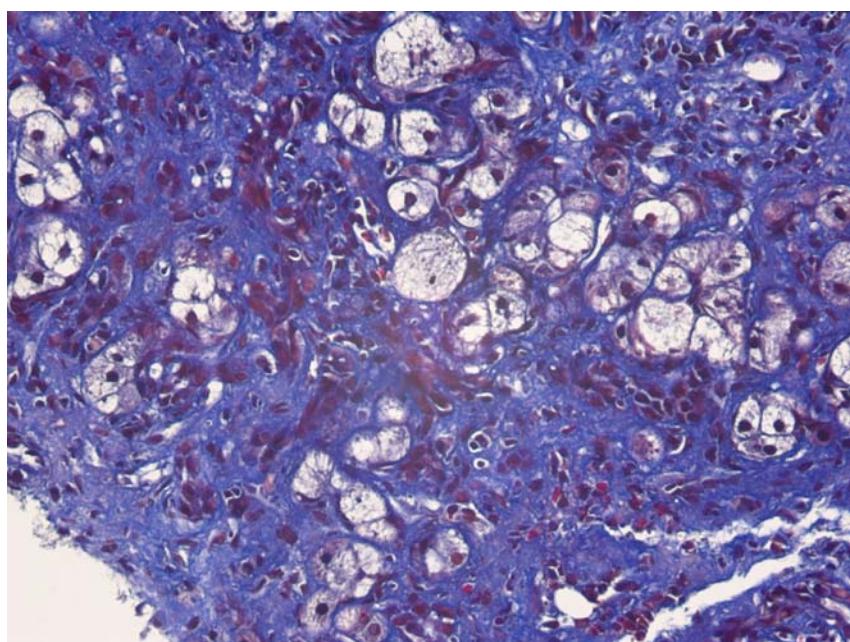
#### Relation between Polymorphisms Studied and Grade of Liver Fibrosis

Finally, we analyzed the relationship between polymorphisms studied and grade of liver fibrosis, inflammation, and

fatty deposits in NASH patients. Although fibrosis was found in all NASH patients studied (Fig. 1) we did not find any significant association between the three analyzed polymorphisms in comparison with the histological features observed (Table 5).

#### DISCUSSION

It has been recognized that some patients with NAFLD may follow a relatively benign course, whereas some others progress to NASH and its inherent complications [20,21]. Our study demonstrates that among otherwise asymptomatic, morbidly obese individuals undergoing gastric bypass surgery, the presence of moderate to severe hepatic steatosis is quite common as shown by the diagnosis of NASH identified in 74% (68/92) of cases initially included in the



**Fig. (1).** A representative picture (20X) showing fibrosis in hepatic parenchyma from F2 patient liver biopsy. Tissue was fixed in formaldehyde, embedded in paraffin, and stained with Masson's trichrome for assessment of liver fibrosis.

**Table 5. Relationship between Liver Histological Features and TNF- $\alpha$  and MnSOD Polymorphisms in NASH**

Liver Histology	Polymorphisms		
	-238 (TNFA carrier)	-308 (TNF2 carrier)	Ala9Val (Val carrier)
<b>NASH</b>			
Mild	1.57 (0.55-4.48, $p$ = NS)	1.66 (0.50-5.46, $p$ = NS)	0.35 (0.10-1.18, $p$ = NS)
Moderate	0.60 (0.19-1.83, $p$ = NS)	0.36 (0.09-1.46, $p$ = NS)	1.31 (0.39-4.38, $p$ = NS)
Severe	1.03 (0.23-4.47, $p$ = NS)	1.83 (0.40-8.23, $p$ = NS)	0.28 (0.35-1.4, $p$ = NS)
<b>FIBROSIS</b>			
F1	1.82 (0.63-5.25, $p$ = NS)	0.80 (0.23-2.73, $p$ = NS)	0.48 (0.15-1.55, $p$ = NS)
F2	0.61 (0.21-1.76, $p$ = NS)	0.75 (0.22-2.45, $p$ = NS)	1.55 (0.48-4.99, $p$ = NS)
F3	0.77 (0.14-4.22, $p$ = NS)	2.67 (0.55-12.89, $p$ = NS)	2.13 (0.24-18.8, $p$ = NS)

Results are expressed as odds ratio, 95% confidence interval,  $p$ -Value.

$p$  = NS, not significant

study (Table 1). Furthermore, we found that 12% of the population undergoing gastric bypass surgery had advanced fibrosis (grade 3) (Fig. 1). These results are similar to those previously reported in a retrospective analysis using post-mortem data among morbidly obese patients where advanced fibrosis was found in 13.8% [22].

Although the pathogenesis of NASH in obese patients remains uncertain, it has been proposed that hepatic steatosis results from hyperinsulinemia and an inappropriately high level of free fatty acids [2,23]. Various risk factors have been associated with NASH, however, recently the role of genetic factors in the pathogenesis of this disease is receiving increasing attention [9-11]. Based on this, the aim of this study was to determine whether the G-238A (allele TNFA) and G-308A (allele TNF2) TNF- $\alpha$  and Ala9Val MnSOD polymorphisms were associated with NASH in a case-control study of subjects from Northeast Mexico. We found that the frequency of the -238 TNF- $\alpha$  polymorphism was significantly higher in patients with NASH than in controls (29% vs. 12%;  $p$  = 0.0047) (Table 2), while the prevalence of -308 was similar in patients and controls (21% vs. 20%;  $p$  = 0.925) (Table 3). Our results are in agreement with results found by Valenti *et al.* [9] who reported a high frequency of the -238 but not of the -308 TNF- $\alpha$  polymorphism in Italian NAFLD patients. Tokushige *et al.* [10] reported that there were no significant differences in the frequency of -1031, -863, -857, -308 and -238 TNF- $\alpha$  polymorphisms among Japanese patients with NAFLD and controls. These discrepancies may have been due to ethnic differences or other factors. In this study, we found that the genotype distribution of the Ala9Val polymorphism was significantly higher in patients with NASH than in controls subjects (78% vs. 36%;  $p$  < 0.01) (Table 4). Similar results were found by Namikawa *et al.* [11] who reported that NASH patients also had a higher incidence of Ala9Val MnSOD polymorphism ( $p$  = 0.016). Recently, polymorphisms in other genes have been associated with the pathogenesis of NASH, such as microsomal triglyceride transfer protein (MTP) [11], adiponectin [24], methylenetetrahydrofolate reductase (MTHFR) [25], apolipoprotein E, interleukin 1 $\beta$ , and  $\beta$ 3 adrenergic receptor [26].

In conclusion, our results suggest that G-238A TNF- $\alpha$  and Ala9Val MnSOD polymorphisms could be associated with NASH, although we did not find a direct relationship

between the three analyzed polymorphisms and histological features. Further experiments, as gene expression of studied genes, should be performed in order to probe this hypothesis. These data may contribute to the understanding of the genetic susceptibility to NASH.

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