# Short Communication

# Acute Pancreatitis Is Associated with Ser608Leu *iNOS* Polymorphism

(*iNOS* / acute pancreatitis / genotype frequency)

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**Abstract. Acute pancreatitis is an initially localized inflammation of the pancreatic gland. The precise mechanisms by which aetiological factors induce acute pancreatitis are not yet known, but when initiated, common inflammatory pathways seem to be involved, with cytokines being their components of major importance. The inducible nitric oxide synthase gene (*iNOS*) encodes an enzyme involved in the pathway of reactive oxygen species and induced in response to infection, cytokines. iNOS is capable of generating large quantities of nitric oxide produced during inflammation. The objective of this study was to investigate the association between acute pancreatitis risk and *iNOS* polymorphisms. The studied single-nucleotide polymorphisms (SNPs) were Ser608Leu, resulting in an amino acid substitution, and 1173C/T and 954G/C, both in the gene promoter region that is linked to increased enzyme expression, leading to higher NO production. The genotypes for the three SNPs were determined in 93 patients with acute pancreatitis and 60 controls without pancreatitis or cancer that were matched for age and gender. Data analysis was done by conditional logistic regression. It was found that the Ser608Leu polymorphism was more frequent among cases with acute pancreatitis compared to controls (OR = 2.88; 95% CI: 1.49–5.57; P = 0.002), although no individually statistically significant associations for the other SNPs studied were detected. We suggest that iNOS Ser608Leu can be used as a marker to define the risk of acute pancreatitis.**

## Introduction

Acute pancreatitis is a multifactorial disease that develops due to pancreatic ischaemia, pancreatic bile duct obstruction, and activation of pancreatic protease and inflammatory cytokines. The mechanisms responsible for the development of pancreatitis have not yet been fully elucidated. However, it has been clearly shown that among the important factors activating pancreatic stellate cells during pancreatic injury are proinflammatory cytokines known to be up-regulated early in the course of acute pancreatic inflammation. Genetic factors, especially related to cytokines, may play important roles in susceptibility to pancreatic injury, as well as in the severity and evolution of the inflammatory process (Weiss et al., 2006; Rios et al., 2011).

Nitric oxide, a highly reactive free radical, is produced from the amino acid L-arginine by nitric oxide synthase (NOS) classified into at least three isozymes: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). A small amount of nitric oxide derived from the first two isozymes (nNOS and eNOS) accounts for the protective action through the regulation of various housekeeping functions, while a large amount of nitric oxide derived from iNOS, induced by inflammatory cytokines and endotoxin, mediates the deleterious action through cytotoxic influences (Moncada and Higgs, 1993; Cheng et al., 2010). It is known that nitric oxide has protective and deleterious effects under pathophysiological conditions in various organs, such as the cardiovascular, neuronal, digestive, and immunological systems. Acute pancreatitis is associated with raised serum nitric oxide levels in its early stage. Patients with higher serum nitric oxide levels are at a significantly higher risk of sepsis and mortality (Al Mufti et al., 1998). While some studies show a similar increase in plasma and pancreas nitric oxide as well as an elevation in NOS activity, the studies are still divided on whether nitric oxide plays a beneficial

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Abbreviations: CI – confidence interval, *eNOS* – endothelial NOS gene, *iNOS* – inducible NOS gene, *nNOS* – neuronal NOS gene, NOS – nitric oxide synthase, SNP – single-nucleotide polymorphism, OR – odds ratio.

or detrimental role in acute pancreatitis (Dabrowski and Gabryelewicz, 1994; Liu et al., 1995; Weidenbach et al., 1995).

Genetic association studies involving single-nucleotide polymorphisms (SNPs) and repeat polymorphisms have become increasingly important for the study of human diseases. Several *iNOS* variants may alter gene expression and nitric oxide production (Hancock, et al., 2008). Three SNPs are the C/T substitution in exon 16, which results in an amino acid substitution (Ser608Leu, dbSNP rs2297518), and -954G/C (dbSNP rs1800482) and -1173C/T (dbSNP rs9282799), both in the promoter region of the gene that is linked to increased enzyme expression, resulting in higher nitric oxide production (Kun et al., 1998; Hobbs et al., 2002; Shen et al., 2004). In the Ser608Leu polymorphism, Leu/Leu homozygote has been reported to confer higher enzymatic activity and *iNOS* expression (Shen et al., 2004). Boutlis et al. (2003) observed higher expression of *iNOS* when either allele -954C or allele -1173T was present. For -954G/C, the substitution of G to C results in a phenotype with a 7-fold higher baseline NOS activity (Kun et al., 1998). *iNOS* polymorphisms have been studied in several types of diseases, including malaria (Kun et al., 1998; Hobbs et al., 2002), diabetes (Morris et al., 2002), rheumatoid arthritis (Gonzalez-Gay et al., 2004), osteomyelitis (Canzian et al., 2008) and asthma (Holla et al., 2006), where nitric oxide production has been implicated in the disease pathogenesis. However, the role of *iNOS* promoter region variants -954G/C and -1173C/T and of the Ser608Leu substitution in exon 16 in acute pancreatitis is still unclear. Therefore, we investigated whether *iNOS* polymorphisms affect the risk of developing acute pancreatitis in the present study.

*Table 1. Distribution of selected characteristics among cases with acute pancreatitis and controls*

| | Cases % (N = 93) | Control % (N = 60) | P value |
|---|---|---|---|
| **Gender** | | | |
| **Male** | 53.76 | 49.39 | 0.479 |
| **Female** | 46.23 | 50.61 | |
| **Age** | | | |
| **≤60** | 33.33 | 27.88 | 0.441 |
| **>60** | 66.67 | 72.12 | |
| **Family history** | | | |
| **No** | 82.80 | 89.17 | 0.526 |
| **Yes** | 13.98 | 10.83 | |
| **Unknown** | 3.22 | 0 | |
| **BMI*** | | | |
| **≤25** | 45.16 | 26.96 | 0.012 |
| **>25** | 54.84 | 73.04 | |
| **Smoking status**** | | | |
| **Non-smoking** | 36.26 | 48.02 | 0.115 |
| **Smoking** | 63.74 | 51.98 | |

*BMI – body mass index (kg/m$^2$)
**For smoking status, a person who had smoked at least once a day for > 1 year in his or her lifetime was regarded as a smoker.

## Material and Methods

Following the approval of the Ethics Committee of Istanbul University, blood samples were collected from 93 patients with acute pancreatitis seen at the Emergency Service, the Hospital of Istanbul University, Turkey. The criteria for diagnosis of acute pancreatitis were: a clinical history consistent with the disease, appropriate radiological evidence, and serum amylase level higher than three times the upper limit of normal. The progress of individuals with regard to the development of complications was monitored during their disease episode. The disease severity was classified as mild or severe according to the criteria by the Atlanta Consensus Conference (Bradley, 1993). Cases consisted of 58 patients with mild acute pancreatitis and 35 patients with severe acute pancreatitis. As a control group, 60 healthy ethnically matched subjects were obtained during the same period to examine association between *iNOS* genotypes and susceptibility to acute pancreatitis. Healthy individuals had no evidence of pancreatitis or pancreatic cancer. We recorded the smoking status in addition to age, BMI, sex and family history (Table 1).

Genomic DNA was extracted from blood samples (Daly et al., 1996). Genotyping of -954G/C, -1173C/T and Ser608Leu variants was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods (Table 2). Restriction enzymes were obtained from New England Biolabs (Ipswich, UK). All other molecular biology chemicals were obtained from Fermentas (Vilnius, Lithuania) and Sigma-Aldrich (St. Louis, MO). To assess the reliability of genotyping, analysis was repeated in 10 % of the samples and DNA sequencing was performed in representative samples for all genotypes and SNPs.

*Table 2. Primers, restriction enzymes, and results for iNOS variants*

| iNOS Polymorphism | Primers | T° Anneal | Restriction Enzyme | Product Size (bp)* |
|---|---|---|---|---|
| **rs1800482** (954C/G) | 5′-CATATGTATGGGAATACTGTATTTCAG-3′<br>5′-TCTGAACTAGTCACTTGAGG-3′ | 61 °C | BccI | 570 (=429, 141) |
| **rs9282799** (1173C/T) | 5′-CAAAGATCCTTGAGCTCTGA-3′<br>5′-CAACTACATTAGGGAGAAGTTGAG-3′ | 61 °C | BccI | 198 (=141, 57) |
| **rs2297518** (Ser608Leu) | 5′-TGTAAACCAACTTCCGTGGTG-3′<br>5′-GTCTCTGCGGGTCTGAGAAG-3′ | 61 °C | Tsp509I | 289 (=139, 117, 33) |

*bp – base pair

Hardy-Weinberg equilibrium was tested to compare the observed and expected genotype frequencies among cases and controls by using the $\chi^2$ test. The genotype and allele frequencies were determined by direct counting. Odds ratios (ORs) and 95 % confidence intervals (CIs) were estimated by conditional logistic regression analyses based on the comparison of genotypes between patients with acute pancreatitis and healthy controls, adjusting for the potential confounders, age, sex, and smoking. A two-sided P value < 0.05 was considered to be statistically significant. Statistical analysis was implemented in the Statistical Package for Social Sciences program (version13.0).

## Results and Discussion

No significant differences were found in the study between the cases and controls for age and sex distribution, and this suggested that the matching based on these two variables was adequate. No association between *iNOS* genotypes and family history of pancreatitis was detected. On the other hand, we observed that BMI might have slightly increased acute pancreatitis risk (OR = 1.46; 95% CI: 0.25–1.83; P = 0.012), similarly to the results obtained by Lindkvist et al. (2008). However, there was no specific interaction between any polymorphisms studied and BMI (data not shown). Smoking was associated neither with any of the studied polymorphisms (P > 0.1) nor with the risk of acute pancreatitis (P = 0.115) in the studied population (Table 1).

The observed genotype frequencies of the three *iNOS* variants in 93 patients with acute pancreatitis and 60 healthy controls conformed to the Hardy-Weinberg equilibrium. We then analysed the differences between cases and controls in the distribution of genotypes. For -954G/C and -1173C/T polymorphisms, genotypes observed both in the cases and controls were only GG and CC, respectively. As for the Ser608Leu genotypes, the SNP was statistically significantly associated with the risk of developing acute pancreatitis. The association was seen with Ser608Leu (P = 0.002); specifically, the patients carrying the TT genotype in comparison with patients carrying the C allele had a significantly lower risk of disease (OR = 2.88; 95% CI: 1.49–5.57) (Table 3). When we examined the association between *iNOS* variants and disease severity, there was no significant difference in the distribution of the three *iNOS* gene polymorphisms studied between patients with mild or severe disease (data not shown).

Some studies that investigated the role of the Ser608Leu polymorphism in the risk of developing diseases related to an inflammatory process have shown conflicting results. Shen et al. (2004) found a significant association between this polymorphism and the risk of gastric cancer only in patients with a history of smoking habit or alcohol intake. On the other hand, Canzian et al. (2008) observed an association between the Ser608Leu polymorphism and atrophic gastritis, while Holla et al. (2006) found this polymorphism to be associated with susceptibility to asthma. Johannesen et al. (2001) showed that the Ser608Leu polymorphism was one of the most frequent SNPs among 10 polymorphisms of the human *iNOS* gene identified in a Danish population. They observed an association of this SNP with increased risk for a subset of type 1 diabetes in HLA DR3/4-positive individuals. The Ser608Leu polymorphism was studied in achalasia in a Spanish population (Mearin et al., 2006), in reflux oesophagitis, Barrett's oesophagus, and oesophageal adenocarcinoma in Caucasian subjects (Ferguson et al., 2008), and in prostatic cancer in non-Hispanic Caucasians and African-Americans (Lee et al., 2009). These authors reported that the 608Leu allele showed no significant association with the diseases. In our study, we observed significantly higher frequencies of Leu/Leu (TT) in patients with acute pancreatitis compared with the healthy controls (OR = 2.88; 95% CI: 1.49–5.57). Jorge et al. (2010) reported that -954G/C, along with alcohol intake and tobacco smoking, was associated with gastric cancer. However, the Ser608Leu polymorphism was not associated with gastric carcinogenesis and -1173C/T polymorphism was absent in the studied Brazil population. -954G/C and -1173C/T polymorphisms have been associated with inflammatory processes such as malaria in some populations (Kun et al., 1998; Hobbs et al., 2002), and osteomyelitis in Spanish subjects (Asensi et al., 2007). In our study, the

*Table 3. Genotype frequencies of iNOS among cases and controls and association with the acute pancreatitis risk. ORs with 95% CI and P values were calculated for the wild/wild genotype versus wild/mutant and mutant/mutant genotypes.*

| iNOS polymorphisms | Genotype | Cases (%) (N = 93) | Controls (%) (N = 60) | OR (95% CI) | P value |
|---|---|---|---|---|---|
| **rs1800482** | CC | 100.0 | 100.0 | CC vs. any G | NS |
| (954C/G) | CG | – | – | – | |
| | GG | – | – | | |
| **rs9282799** | CC | 100.0 | 100.0 | CC vs. any T | NS |
| (1173C/T) | CT | – | – | – | |
| | TT | – | – | | |
| **rs2297518** | CC | 18.9 | – | TT vs. any C | 0.0022 |
| (Ser608Leu) | CT | 26.3 | 21.0 | 2.88 (1.49-5.57) | |
| | TT | 54.8 | 79.0 | | |

* ORs were adjusted for age, sex and smoking status by conditional logistic regression analysis.
** P > 0.05 was indicated as NS (not significant).

genotyping of -954G/C and -1173C/T in patients with acute pancreatitis and healthy controls with PCR-RFLP did not yield any polymorphic variant. Our findings are in agreement with the studies of Caucasian populations. These SNPs seem to be ethnic-specific for the African population according to previous studies (Johannesen et al., 2000). Besides, there is no study about these SNPs in Turkish population. For this reason, our findings indicate that *iNOS* -954G/C and -1173C/T are rare in Turkish population similarly as in Caucasians and do not significantly contribute to the onset of acute pancreatitis risk in the investigated population.

In conclusion, iNOS Ser608Leu (rs2297518) can be used as a marker to define the risk of acute pancreatitis. Also, the results emphasize the importance of such studies in different ethnic populations. We believe that the findings may be beneficial to the development of efficacious preventive strategies and therapies for inflammation-associated cancers.

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