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The association between chronic pancreatitis and the iNOS-2087A>G polymorphism

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Introduction. Chronic pancreatitis is morphologically characterized by ductal dysplasia, breeding grounds for the proliferation of the ductal cells, the degenerative changes in pancreatic acinar cells and fibrosis, and it is defined on the basis of the clinical, morphological and functional criteria.

Aim. The aim of our study is to examine the existence of a possible correlation between the iNOS-2087A>G polymorphism and chronic pancreatitis by means of the genetic analysis.

Material and method. We have conducted the study at the *Gastroenterology Clinic* and the *Research Center of Gastroenterology and Hepatology* of the *University of Medicine and Pharmacy, Craiova*, between March 2015 – September 2016. The study had a prospective character. Both for the 58 patients diagnosed with chronic pancreatitis and for the 132 patients in the witness group, the biological material was represented by blood, (around 2.5 – 5 milliliters of venous blood) let on EDTA and kept at 4°C up to the separation of the DNA molecule. All the patients were genotyped for the iNOS – 2087A>G polymorphism, by means of the Real Time PCR technique with TaqMan probes.

Results. Analysing the prevalence of the iNOS genotypes within the study group and witness group, we have noticed that, statistically speaking, there are no significant differences between the two groups.

Conclusion. As a conclusion, in the study lot we can sustain that the risk of developing chronic pancreatitis is not increased by the presence of the iNOS-2087A>G polymorphism.

Keywords: chronic pancreatitis, genotype, iNOS-2087A>G polymorphism, genetic, alcohol.

INTRODUCTION

Chronic pancreatitis is a chronic inflammatory affection, characterized by the inflammation, fibrosis and progressive destruction of the pancreatic parenchyma, (focal, segmentary, or diffuse), associated with anomalies of the pancreatic channels and the relative conservation of the Langerhans islets [1]. Chronic pancreatitis is defined on the basis of clinical, morphological and functional criteria. Chronic pancreatitis has a variable and irregular geographical distribution, according to the emergence of the major risk factors involved, such as alcoholism and malnourishment, with increasing incidence. The risk of pancreatitis is two or three times higher at black people than in white population [2-4]. The incidence of chronic pancreatitis is higher in the case of black people [2], representing a risk factor for the development of pancreatic adenocarcinoma. The risk is even higher in the case of hereditary etiology [5].

Alcohol continues to be the major risk factor for chronic pancreatitis [2]. In the case of those patients that have experienced a first episode of ethanolic acute pancreatitis, there is a risk of progression towards chronic pancreatitis of about 14% in the case of total abstinence from alcohol or of the occasional consumption of alcohol, a risk of 23% in the case of the reduction of daily consumption and 41% if the consumption of alcohol is maintained at the same level as before the attack of acute pancreatitis [6].

An independent risk factor for both acute and chronic pancreatitis is smoking, and its effects could be synergistic with those of alcohol [2]. The abstinence from alcohol and giving up smoking can modify the progression of pancreatitis and reduce its recurrence, whereas giving up smoking is the most efficient strategy to reduce the risk of pancreatic cancer [2].

The pancreatitis caused by alcohol consumption is more frequent among men, although the gender differences disappear with similar levels of alcohol consumption [7]. This is the reason why studies are necessary to determine whether the genetic factors increase the risk of chronic pancreatitis. In one of the studies made in the USA, no significant association between blood type and chronic pancreatitis has been noticed [8]. Gallstones cannot cause chronic pancreatitis.

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Although less frequently, hypertriglyceridemia can cause chronic pancreatitis [9]. The risks of acute and chronic pancreatitis are also increased among patients with intestinal inflammatory diseases, systemic lupus erythematosus, although there are no exact estimations available [2]. The low incidence of pancreatic cancer for patients with chronic pancreatitis (<5%) shows that chronic pancreatitis is a rare cause of pancreatic cancer [2]. Most of the studies involving chronic pancreatitis have focused on the identification of the risk factors, the elucidation of the relation between risk factors and the disease, but also on the discovery of better diagnostic and management methods, as well as methods of pancreatitis prevention. Less expensive methods are needed for genetic analysis, which become readily available and provide much needed answers to many unsolved problems on all types of disorders of chronic pancreatitis.

A series of genetic factors, cytokines (IL-1; IL-1 antagonist of receivers; IL-6; IL-10; TNF-α; IL-1β), factors related to the angiogenesis (VEGFR-2; CXCR-2 ; PAR-1; EGF, TGF-β), genes belonging to the receivers of (CD 14) model reconnaissance, but also iNOS can play an important part in the seriousness and the evolution of the inflammatory process [10].

The gene for inducible nitric oxide synthase (iNOS) is to be found on the chromosome 17q11.2 [11, 12]. It codifies an enzyme involved within the reactive oxygen species and it induces cytokines as a response to infections. iNOS can generate big quantities of nitric oxide during inflammatory processes. The enzymatic activity expression and the production of nitric oxide could be influenced and modified by several polymorphisms of the iNOS gene [13]. The increased production of nitric oxide has been associated with an increased enzymatic expression determined by single nucleotide polymorphisms (SNP) localized in the promoter region of the iNOS gene, (iNOS - 954G> C or -2087A> G) [14, 15]. Variations in the DNA sequence and the mononucleotide polymorphisms can suggest a high sensitivity and a specific response to the disease [16-18].

According to some recent studies, molecular markers can serve as prognostic factors and they can be used to identify aggressive phenotypes and to choose the best individual therapies [16, 18, 19]. The iNOS-2087A>G polymorphism has been significantly associated with the risk of developing pancreatitis, showing a higher frequency of the TT genotype in the case of patients diagnosed with pancreatitis, compared to the healthy patients [10]. The iNOS-2087A>G (rs2297518) can also be used to evaluate the risk of pancreatitis [10].

All studies in recent years have shown that the assessment of cytokines, factors associated with angiogenesis, various polymorphisms can provide a key tool for genetic and molecular level, which can be used to identify various subsets of the population at high risk for pancreatic disorders, fix prognosis and choose the optimal course of treatment.

Objectives

Our research theme is currently relevant, as it explores and improves the recent discoveries concerned with chronic pancreatitis, focusing especially on the evaluation of the roles that certain genetic versions have in the initiation and progression of this disease.

The aim of our study is to investigate the existence of a possible correlation between the iNOS-2087A>G mononucleotide polymorphism and chronic pancreatitis by means of the genetic analysis.

MATERIAL AND METHODS

We have conducted the study at the *Gastroenterology Clinic* and the *Research Center of Gastroenterology and Hepatology of the University of Medicine and Pharmacy*, *Craiova*, between March 2015 – September 2016. We have included in the study 58 patients diagnosed with chronic pancreatitis, on the basis of the clinical, paraclinical and imagistic criteria. All the cases have been confirmed and hospitalized Gastroenterology and the Medical II Clinics, of the County Emergency Clinical Hospital of Craiova. The study has a control group including 132 patients that have not been diagnosed with chronic pancreatitis, hospitalized in the same hospital.

Th research activities in the field of Genetics have been carried out in the *Molecular Genetics Laboratory of the Research Center of Gastroenterology and Hepatology Craiova*,which has all the equipment necessary to identify genetic polymorphisms investigated.

The biological material necessary to study the investigated polymorphism has been represented by blood on the (EDTA) dispersion stabilizer, obtained from the patients in the study and witness groups. All the patients have signed an informed consent before sampling of the biological material necessary for the study. The Commission of Ethics of the University of Medicine and Pharmacy, Craiova has authorized the design of the study.

We have collected data from each of the patients in the two study groups, data concerned with the age, gender, demographical data, blood

type, the mass body index, ethanol consumption, pathological personal and heredocollateral history and medication.

We have established criteria of inclusion and exclusion for both groups. The criteria of inclusion for the studied group have been represented by patients diagnosed with chronic pancreatitis and > 18 years old, whereas for the control group, the criteria of inclusion have been represented by patients without chronic pancreatitis, identical socio-economic environment and similar ages. We have established one criterion of exclusion for the study group including patients < 18 years old, whereas for the witness group the only criterion of exclusion has been the diagnostic of chronic pancreatitis. The diagnosis of chronic pancreatitis was established by imagistic assessment: CT scan and EUS in patients with chronic alcohol con-sumption. The EUS criteria for chronic pancreatitis, according to Rosemont classification, are listed in Table 1. According to this consensus there are 3 types of results: consistent, suggestive and indeterminate chronic pancreatitis [20]. We included in the study only patients with consistent and suggestive chronic pancreatitis.

Table 1
Diagnostic EUS criteria for chronic pancreatitis [20]

Major A criteria	Major B criteria	Minor criteria
Hyperechoic foci with shadowing, main pancreatic duct (MPD) calculi	Lobularity, honeycombing type	Cysts, Dilated MPD (≥3.5 mm), irregular MPD contour, Dilated side branches (≥1 mm), hyperechoic duct wall, hyperechoic non-shadowing foci, non-honeycombing lobularity

Both for the patients diagnosed with chronic pancreatitis and for those in the witness group, the biological material has been the blood (about 2.5 – 5 milliliters of venous blood) let on the EDTA and kept at 4°C up to the separation of the DNA molecule. All the patients have been genotyped for the iNOS – 2087A>G (rs2297518).

The protocol concerned with the identification of the genetic polymorphisms has included the following phases: the separation of the DNA genome from the blood, the spectrophotometric evaluation, the identification of the allelic versions by means of the Real Time PCR technique with TaqMan probes, the interpretation of the obtained results. For the separation of the genomic DNA from the blood probes obtained from the patients in the study group, as well as from the subjects in the control group, we have used the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI), observing the producer's protocol. The Real Time PCR technique with TaqMan tubes is a modern method, compared to the conventional method. The augmented sample is or the amplicon is visualized concomitantly with the development of the process of amplification.

For the identification of the mononucleotide polymorphisms, we have used the Real Time ViiA7 system, (Applied Biosystem). Results interpretation has been achieved by means of the system's soft – ViiA™ 7 Software v1.0 and of the option called Allelic Discrimination. The reactions have taken place in plaques of 384 godets, the total volume per reaction has been of 5 µl: 2.5 µl DNA have been transferred in 2.5 µl reaction mixture, made up of: Universal Master Mix (Applied Biosystems, Foster City, CA and TaqMan® SNP Genotyping Assays 40x (Applied Biosystems, Foster City, CA). The cycling conditions for the Real Time PCR ViiA7 system are described in Table 3.

Statistical analysis

The information obtained have been stored up and then processed statistically, with a view to analyzing the relations between the clinical and paraclinical data of the patients. The secondary data processing has involved the calculation of the average, of the standard deviation, of the coefficient of variation, but also the use of the complex statistical tests (Chi square test, Student test, ANOVA test). The comparison between the frequencies of the genotypes belonging to the study and control groups has been completed by means of the Hardy-Weinberg statistical equilibrium. The genotypes have been evaluated by the indicating variables with the homozygote version as reference. A value $p < 0.05$ has been considered statistically significant.

RESULTS

All the 190 blood probes have been genotyped. The genotyping has been elaborated in the case of 58 patients diagnosed with chronic pancreatitis. We have also carried out 132 controls. The age of 56% of the patients diagnosed with chronic pancreatitis ranges between 50 and 59. Most of the patients with chronic pancreatitis are

smokers, in comparison with those in the witness group, (52.75% compared to 36.5%, $p < 0.01$). As to gender distribution, we have noticed a significant difference, chronic pancreatitis being present in a percentage of 68% in the case of men. The patients diagnosed with chronic pancreatitis have registered the highest percentage of alcohol consumers – 60%. Hypertriglyceridemia has been present in the case of 44% of the patients with chronic pancreatitis. We have not identified in our study any statistical significant association between the sanguine group and chronic pancreatitis.

According to the results obtained on the basis of the signal emitted by the TaqMan specific fluorescent probe, we have identified the two alleles for the **iNOS2 -2087A>G**: polymorphism: the **G allele** – FAMsignal and the **A allele** – VIC signal. Thus, we have established the three genotypes: GG homozygote, AG heterozygote and AA homozygote.

While analyzing the prevalence of the iNOS genotypes in the study and control groups, we have noticed that there were no significant differences between the two groups (Figure 1), or differences concerned with thehypertriglyceridemia between the patients with different iNOS genotypes (Figure 2).

As we can notice in Figure 1 that describes the distribution of the patients diagnosed with chronic pancreatitis according to the various genotypes, the GG genotype is the best represented in this group, which is confirmed both in the speciality literature and the international databases (NCBI). As the result of the Chi square test is $p = 0.749 > 0.05$, there is no significant association between the presence of this polymorphism and the patients' increased risk of developing chronic pancreatitis.

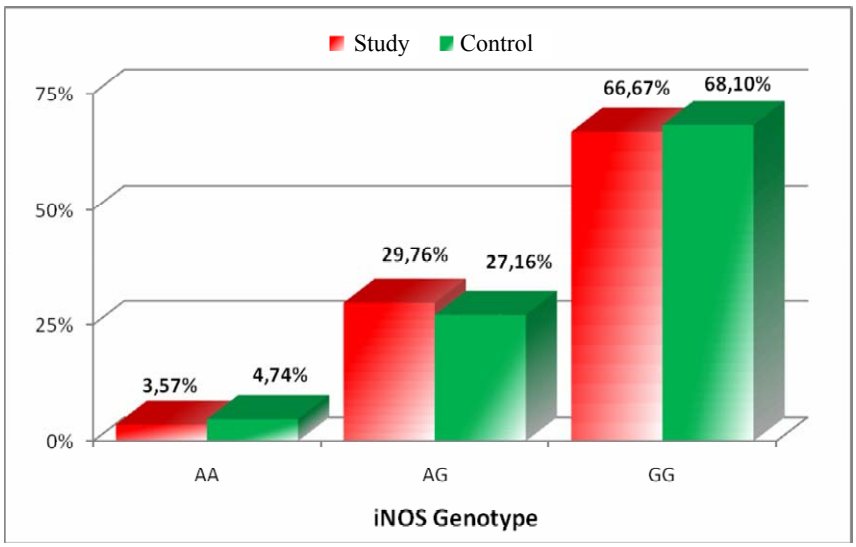

Figure 1. Prevalence of the iNOS genotypes.

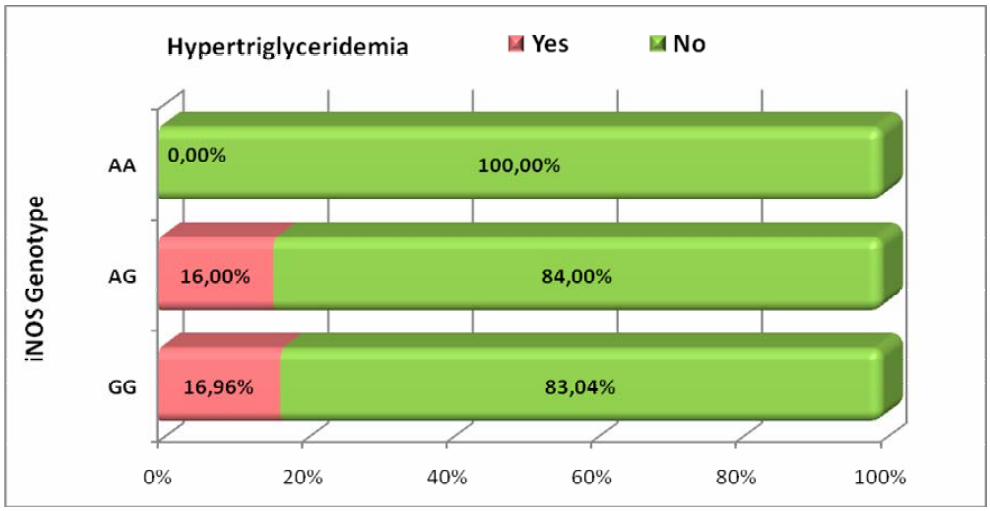

Figure 2. Hypertriglyceridemia and iNOS genotypes.

DISCUSSION

The roles of the various iNOS polymorphisms have been studied in multiple affections. These are involved in the risk of developing different diseases, but with contradictory results. For example, Shen *et al*. (2004) have discovered a significant association between this polymorphism and the risk of gastric cancer only in the case of smokers and alcohol consumers [14], whereas Canzian *et al*. (2008) have noticed an association between polymorphism and gastric atrophy [21].

Holla *et al.* have found in 2006 that this polymorphism can be associated with asthma susceptibility [22], whereas Johannesen *et al*. (2001) have shown that polymorphism has been one of the most frequently encountered polymorphisms from among the 10 polymorphisms of the iNOS human gene, identified in the case of a Danish population. They have noticed an increased risk of hyperglycemia type 1 in a subgroup of positive HLA DR3/4 individuals [23].

Polymorphisms of the iNOS gene have been studied in achalasia in the case of a Spanish population [24], in the esophageal reflux, Barrett esophagus, the esophageal adenocarcinoma, at a Caucasian population [25], as well as in the prostate cancer at the non-Spanish Caucasians and Afro Americans [26]. As a conclusion of all these studies, there are no significant associations with these diseases.

According to the conclusion of the study published by Ozhan *et al*. in 2012, the iNOS2 – 2087A>G polymorphism can be used as a marker to define the risk of acute pancreatitis [10].

According to the data obtained at the end of our study, we have noticed that there are no significant differences between the two groups, as far as the prevalence of the iNOS genotypes is concerned, the result of the Chi square test being $p > 0.05$.

We have not observed any statistical association between the presence or the absence of the iNOS2 – 2087A>G polymorphism and the risk of developing chronic pancreatitis. These single nucleotide polymorphisms seem to be ethnically specific, taking into account the results of the previous studies [27]. As to the Romanian population, there is no study with respect to the iNOS2 – 2087A>G polymorphism.

Conclusions

Taking into account the results of our study, we can assert that the risk of developing chronic pancreatitis is not increased by the presence of the iNOS-2087A>G polymorphism in the case of the examined patients. Although the group of patients diagnosed with chronic pancreatitis has not been numerous, it has obeyed theHardy-Weinberg statistical equilibrium. This is the reason why we need thorough studies on larger groups of patients, coming from different regions, with a view to clarifying the role of the iNOS polymorphisms in the pancreatic inflammation. The impact of the study can be even bigger, if apart from the blood probes, there will also be evaluated from the genetic point of view, a complete set of biological probes, including the biopsies obtained echoendoscopically by fine-needle aspiration.

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Conflicts of interest. There are no conflicts of interest.

All authors have equal contributions.

Introducere. *Pancreatita cronică este caracterizată morfologic prin displazie ductală, focare de proliferare a celulelor ductale, degenerarea celulelor acinare şi fibroză şi este definită pe baza criteriilor clinice, morfologice şi funcționale.*

Scopul. *Scopul studiului nostru este acela de a cerceta existența unei posibile corelații între polimorfismul mononucleotidic iNOS-2087A>G şi pancreatita cronică cu ajutorul analizei genetice.*

Material şi metodă. *Studiul s-a desfăşurat în cadrul Clinicii de Gastroenterologie şi a Centrului de Cercetare în Gastroenterologie şi Hepatologie din cadrul UMF Craiova în perioada martie 2015 – septembrie 2016 având un character prospectiv. Atât pentru cei 58 de pacienți diagnosticați cu pancreatită cronică, dar şi pentru cei 132 de pacienți din lotul martor, materialul biologic a fost reprezentat de sânge (aproximativ 2,5–5 ml de sânge venos) recoltat pe EDTA*

şi menţinut la 4°C până în momentul izolării ADN-ului. Toţi pacienţii au fost genotipaţi pentru polimorfismul iNOS – 2087A>G cu ajutorul tehnicii Real Time PCR cu sondeTaqMan.

***Rezultate**. Analizând prevalenţa genotipurile iNOS în lotul de studiu şi lotul martor, am constatat că nu există diferenţe semnificative statistic între cele două loturi.*

***Concluzii**. În concluzie, putem afirma că riscul de a dezvolta pancreatită cronică nu este crescut de prezenţa polimorfismului iNOS-2087A>G la pacienţii analizaţi.*

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