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Association between TNF-α −308G>A polymorphism and the development of acute coronary syndromes in Greek subjects: The CARDIO2000-GENE Study

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Purpose: We investigated the association of a polymorphism within the promoter of TNF-α locus at the position −308 on the likelihood of having acute coronary syndromes (ACS) in Greek adults. **Methods:** We studied demographic, lifestyle, and clinical information in 237 hospitalized patients (185 males) with a first event of an ACS and 237 matched by age and sex (controls) without any clinical evidence of coronary heart disease. Genotyping was performed by PCR-RFLP analysis. **Results:** The genotype frequencies were in patients, 87% (n = 206), 12% (n = 29), and 1% (n = 2) for G/G, G/A, and A/A, and in controls, 96% (n = 227), 4% (n = 10), and 0% (n = 0) for G/G, G/A, and A/A, respectively (P = 0.04). After adjusting for age and sex, as well as various potential confounders, we observed that G/A or A/A genotypes were associated with 1.94-fold higher odds (95% CI 1.06 to 3.68) of ACS compared to G/G homozygotes. No gene to—gender or to—clinical syndrome interactions were observed. Further subgroup analysis showed that the distribution of TNF-α −308G>A polymorphism was associated with the presence of family history of CHD in patients, but not in controls. In particular, in G/A and A/A patients 17.2% reported family history of CHD, whereas in G/G patients, 34.5% reported family history (P = 0.036). **Conclusions:** Our findings may state a hypothesis of an association between the −308G>A TNF-α polymorphism the development of ACS and the presence of family history of CHD, in Greece. ***Genet Med*** **2005:7(6):411–416.**

Key Words: *inflammation, TNF-α, acute coronary syndrome, coronary heart disease, polymorphism*

Tumor necrosis factor-α (TNF-α) belongs to the TNF ligand family and has multiple biologic activities. Although initially TNF-α evoked attention as a factor able to elicit hemorrhagic necrosis of tumors in recipient animals,[1] it is now believed that TNF-α is one of the main proinflammatory cytokines and plays a central role in initiating and regulating the cytokine cascade during an inflammation response and is involved in local and systemic events attendant an inflammation.[2]

Inflammation plays an important role in the pathogenesis of atherosclerosis and acute coronary syndromes.[3–5] The presence of TNF-α in the majority of atherosclerotic lesions and absence from normal tissues suggests its involvement in atherogenesis.[6,7] TNF-α may contribute to the inflammatory process of atherosclerosis by activation of growth factors, cytokines, and by effecting the synthesis and stimulation of adhesion molecules.[8,9] Secretion of TNF-α from mononuclear leukocytes of patients with stable and unstable angina pectoris is elevated when compared with control individuals.[10] In addition, TNF-α possibly increases the risk of thrombotic events by stimulation of procoagulant activity and suppression of antithrombotic pathways in endothelial cells.[11] Moreover, it has been suggested that TNF-α plays a key role in cardiovascular pathophysiology as it has potent metabolic effects. It affects lipid metabolism and predispose to obesity related insulin resistance.[12–14]

Monocytes/macrophages mainly produce this cytokine, although other cell types, such as T and B cells, also produce considerable amounts. The *TNF-α* gene lies in the class III region of the major histocompatibility complex (MHC) and consists of four exons, which are interrupted by three introns.[15] Several polymorphisms have been identified in the gene encoding TNF-α. One of the most extensively investigated polymorphism in the promoter region of the *TNF-α* gene is this at position −308. It involves the substitution of guanine by adenosine in the uncommon allele.[16] High TNF-α

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synthesis seems to be associated with the presence of the rare allele and appears to influence the clinical outcome of several diseases in which inflammation plays a predominant role.[17–19] However, data regarding the association between this polymorphism with the development of coronary heart disease are conflicting and not well understood. Moreover, the association of *TNF-α* gene and various chronic diseases, including cardiovascular, seems to vary from country to country. On the other hand, it is well known that the Mediterranean population has a lower risk of coronary disease in comparison with the remaining European Caucasian population, with an important contribution of environmental and lifestyle factors.[20–22] Thus the impact of the genetic predisposition on the likelihood of having acute coronary syndromes (ACS) would be of considerable interest.

In this work, we evaluated the association between the *TNF-α −308G>A* polymorphism and the probability of having ACS, in Greek adults, after taking into account various clinical, and lifestyle characteristics of the participants.

MATERIALS AND METHODS

The gene symbols used in this article follow the recommendations of the HUGO Gene Nomenclature Committee.[23]

Participants

First, we randomly selected from the daily listing of the Cardiology clinics the patients. Particularly, from June 2002 to December 2002, 237 patients (185 males) who had just entered to the preselected hospitals for a first event of an ACS, from various Greek regions, entered into the study. These patients did not have any evidence for coronary heart disease before this target event that caused their admission to the hospital. Any patient with history of coronary heart disease in the past (e.g., stable angina) was excluded from the study. After the 3rd day of the hospitalization medical information was retrieved through hospital or insurance records, whereas the demographic and lifestyle data were obtained through a specific confidential questionnaire including structured questions concerning living habits and sociodemographic background factors.

The inclusion criteria for the cardiac patients were as follows: (1) acute myocardial infarction diagnosed by two or more of the following features: typical electrocardiographic changes, compatible clinical symptoms, specific diagnostic enzyme elevations, or (2) first diagnosed unstable angina corresponding to class III of the Braunwald[24] classification.

Afterward, we randomly selected 237 subjects (185 males) without any clinical symptoms or suspicious of cardiovascular disease in their medical history, matched to the patients by age (± 3 years), sex, and region. The controls were patients in surgical clinics (urology, ophthalmology, or orthopedic) of the same hospital and at the same period with the patients. We used this type of controls in order to have more accurate medical information, to eliminate the potential adverse effect of several, unknown, confounders and to increase the likelihood that cases and controls share the same study base.

Informed consent was obtained from all subjects and the study was approved by the Medical Research Ethics Committee of our clinics, and was performed in accordance with the Declaration of Helsinki (1989) of the World Health Organization.

Genotyping

Genomic DNA was extracted from whole blood leukocytes with a DNA extraction kit (Nucleospin Blood kit, Düren, Germany). *TNF-α* (*−308*) genotyping was performed as previously described by Ishii et al.[25] *Nco*I restriction digest yields DNA fragments of 87-bp and 20-bp (GG), 107/87/20 bp (G/A), and 107 bp for (AA), which were visualized using a 4% agarose gel stained with ethidium bromide. Genotypes were determined and confirmed by two experienced technicians blinded to all study data.

Demographic, lifestyle, and behavioral characteristics

The study's questionnaire included demographic characteristics, like age, gender, the average annual income during the past three years (in Euros), and education level (in years of school) of the patients and controls. Moreover, lifestyle habits, like smoking, food items consumed, and physical activity status were evaluated in all participants. Particularly, the quantification of smoking status was based on the calculation of pack-years adjusted for nicotine containment equal to 0.8-mgr/cigars. Former smokers were defined as the subjects who stopped smoking for over 1 year. Physical activity was defined as any type of nonoccupational physical exercise, at least once per week during the past year and was graded in qualitative terms such as light (expended calories < 4 Kcal/min, i.e., walking slowly, stationary cycling, light stretching, etc.), moderate (expended calories 4–7 Kcal/min, i.e., walking briskly, outdoor cycling, swimming moderate effort, etc.), and vigorous (expended calories > 7 Kcal/min, i.e., walking briskly uphill, long distance running, cycling fast or racing, swimming fast crawl, etc).[26] The rest of the subjects were defined as physically inactive. Also, the duration (minutes per time) and the years of continuous physical exercise were taken into account in all analyses. The evaluation of the nutritional habits (consumption of nonrefined cereals and products, vegetables, fruits, olive oil, dairy products, fish, poultry, pulses, and nuts, potatoes, eggs, sweets, and red meat and meat products) was based on a special and validated food frequency questionnaire.[27] Alcohol consumption was measured by daily ethanol intake, in wine glasses (100 mL per 12% ethanol concentration). Evaluation of depressive symptoms was based on a special and validated questionnaire provided by the Center of Epidemiological Studies (CES-D, range 0–60, cut-off point for depression > 45 score).[28] The previous questionnaire assessed the occurrence of symptoms during the past month through a multilevel set of topics which they reflect subject's emotional characteristics.

Clinical characteristics

Detailed information regarding their medical and psychosocial status and various lifestyle habits related to coronary risk was previously recorded.[29]

Statistical analysis

According to the power analysis the number of the studied patients and controls was adequate to evaluate two-sided standardized differences between the frequency of genotypes and groups of the study > 0.5, achieving statistical power > 85% at 5% probability level (*P* value). Continuous variables are presented as mean ± standard deviation, whereas categorical variables are presented as absolute and relative frequencies. Pearso correlation coefficient was used in order to measure associations between normally distributed continuous variables. Contingency tables with the calculation of a chi-squared was used to evaluate the associations between categorical variables. However, due to the small number of observations in some cases, Fisher exact test, with the calculation of exact *P* values, was applied to evaluate the association between the investigated polymorphism and group of study. The application of Student *t* test evaluated the associations between categorical and normally distributed continuous variables. The distribution of the TNF-α polymorphism in our population was compared with the expected frequency through the Hardy-Weinberg equilibrium. Estimations of the relative risks of developing ACS were performed by the calculation of odds ratio (OR) and the corresponding confidence intervals through multiple conditional logistic regression analysis, after controlling for various potential confounders. Deviance residuals assessed model's goodness-of-fit. Regression analysis was used to evaluate the interaction between *TNF-α* polymorphism and group of the study on blood glucose levels. All reported P-values are two-sided and STATA 6 software was used for the calculations (STATA Corp. College Station, TX).

RESULTS

Table 1 presents various characteristics of the subjects. As expected, patients were more likely to have the common cardiovascular risk factors (i.e., hypertension, hypercholesterolemia, diabetes, smoking habits, family history of CHD, and obesity), as compared to the controls. Moreover, patients had lower income and education level, and were more frequently to be physically inactive and depressed.

The distribution of the *TNF-α G>A* polymorphism in our population was compatible with the Hardy-Weinberg equilibrium ($P > 0.7$). Table 2 illustrates the distribution of *TNF-α −308G>A* polymorphism in patients and controls. A significant association was observed between the *TNF-α* polymorphism and the group of study (exact $P = 0.027$). In particular, the genotype frequencies were, in patients, 87% ($n = 206$), 12% ($n = 29$), and 1% ($n = 2$) for G/G, G/A, and A/A, as well as, in controls, 96% ($n = 227$), 4% ($n = 10$), and 0% ($n = 0$) for G/G, G/A, and A/A, respectively. Moreover, the G allele frequency was 93% in patients and 98% in controls ($P = 0.002$). The *TNF-α G>A* polymorphism was similarly distributed between males and females (*P* for gender differences = 0.86), as well as types of the clinical syndrome (i.e., unstable angina or myocardial infarction, $P = 0.88$).

Table 3 presents the results from the multivariate analysis that assessed the relationship between *TNF-α* G>A polymor-

Table 1
Demographic, lifestyle, and clinical characteristics of the participants (mean ± SD)

	Patients ($n = 237$)		Controls ($n = 237$)		
	Males ($n = 185$)	Females ($n = 52$)	Males ($n = 185$)	Females ($n = 52$)	P^a
Age	58 ± 5	67 ± 4	57 ± 5	66 ± 5	0.56
Income (× 1000 Euros)	17 ± 8	15 ± 6	19 ± 7	16 ± 8	0.04
Education (y of school)	12 ± 4	11 ± 5	13 ± 5	12 ± 4	0.03
Current or former smokers (%)	68	41	45	35	0.001
Physical inactivity (%)	61	67	55	58	0.001
Alcohol consumption (mL/day)	280 ± 45	140 ± 60	190 ± 55	80 ± 50	0.04
Depression scale (0–60)	38 ± 20	45 ± 30	29 ± 20	36 ± 40	0.002
Hypertension (%)	60	48	36	23	0.001
Hypercholesterolemia (%)	57	46	33	34	0.001
Obesity (%)	28	24	18	12	0.001
Blood glucose (mg/dl)	168 ± 24	190 ± 26	101 ± 33	98 ± 32	0.001
Diabetes (%)	45	48	13	5	<0.001
Family history of premature CHD (%)	35	32	24	21	0.02

[a]*P*, values for the differences between groups after adjusting for gender.

Table 2
Distribution of TNF-α genotype in patients and controls, by gender

	Patients ($n = 237$)		Controls ($n = 237$)	
Genotype (%)	Males ($n = 185$)	Females ($n = 52$)	Males ($n = 185$)	Females ($n = 52$)
G/G	85	92	95	98
G/A	14	6	5	2
A/A	1	1	0	0

Table 3
Results from the multivariate logistic regression model[a]

Variable	Odds ratio	95% Confidence interval		*P* value
G/G	1.00	—	—	
G/A or *G/A*	1.94	1.06	3.68	0.04

[a]After taking into account the effect of age and sex (by design), pack-years of smoking, presence of hypertension, hypercholesterolemia, diabetes, obesity, family history of CHD, food items and alcohol consumption, physical activity level, depression scale, years of school, and annual income.

phism and the likelihood of having ACS, after controlling for the effect of several potential confounders. For this analysis, we combined G/A and A/A genotypes because of the small numbers of patients and controls into an A/A genotype. Thus, after adjusting for age and sex (by design), as well as pack-years of smoking, presence of hypertension, hypercholesterolemia, diabetes, obesity, family history of CHD, food items and alcohol consumption, physical activity level, depression scale, years of school, and annual income we observed that G/A or A/A genotypes were associated with 1.94-fold higher likelihood of having ACS, as compared to G/G homozygotes. This association was not influenced by the type of clinical syndrome (*P* for the interaction between syndrome and genotype = 0.67).

Further subgroup analysis showed that the distribution of *TNF-α −308G>A* polymorphism was associated with the presence of family history of CHD in ACS patients, but not in controls. In particular, in G/A and A/A patients 17.2% reported family history of CHD, whereas in G/G patients, 34.5% reported family history ($P = 0.036$). No differences were observed in controls (i.e., frequency distribution of family history of CHD in G/A and A/A subjects was 25% and in G/G subjects was 23.9%, $P = 0.86$).

Moreover, in ACS patients, G/A and A/A genotypes were associated with higher blood glucose levels (patients G/A and A/A vs. patients G/G: 171 ± 24 vs. 117 ± 31 mg/dL, $P < 0.001$), whereas no differences were observed in controls regarding their blood glucose levels (controls G/A and A/A vs. controls G/G: 102 ± 32 vs. 86 ± 43 mg/dL, $P = 0.112$). In addition, the previous interaction between TNF-α *−308G>A* polymorphism and group of study on blood glucose levels was confirmed using multiple linear regression analysis (beta coefficient for the interaction term ± SE: 0.23 ± 0.03, $P = 0.02$). However, it should be noted that the insignificant trend observed in the controls group, might simply be an issue of a lower frequency of the allele rather than an absence of effect.

No associations were observed between the distribution of *TNF-α −308 G>A* polymorphism and presence of hypertension, hypercholesterolemia, and obesity, in both patients and controls (all $P > 0.4$).

DISCUSSION

The aim of the present study was to investigate the association of the *TNF-α −308G>A* polymorphism in the development of ACS in Greek adults. We revealed a strong association of the investigated polymorphism with the presence of ACS, irrespective of various potential confounders. Furthermore, our analysis showed that the distribution of *TNF-α −308G>A* polymorphism was associated with the presence of family history of CHD in ACS patients, but not in controls, and with increased blood glucose levels.

Looking on the TNF-α genotyping, the frequency for the A allele, among healthy individuals, in this survey was 0.02, which was similar to the allelic distribution observed by Costeas et al. in healthy Greek Cypriots.[30] Moreover, we observed that the genotype and allele frequencies for A carriers were higher in CHD patients compared to these in the healthy control group, which is in accordance with the study of Vendrell et al.[31] However, the results from other studies that investigated the genotypic and allelic distribution between CHD patients and healthy individuals are contradictory.[32–35] The stage of disease, the geographical origin, the age, as well as sex and the number of participants, are dynamics to read between the lines in order to explain the contrasting findings. The effect of these biological factors on TNF-α levels was extensively evaluated very recently[36] underlying the differentially result of each one in a total of 171 healthy families selected from the STANISLAS cohort.

In this study, multivariate analysis showed that the presence of G/A or A/A genotypes were associated with 2-fold higher likelihood of having ACS, as compared to G/G homozygotes, even after adjusting for age, sex, pack-years of smoking, presence of hypertension, hypercholesterolemia, diabetes, obesity, family history of CHD, food items and alcohol consumption, physical activity level, depression status, years of school, and annual income. Therefore we may state a hypothesis, for the first time in Greek adults that the presence of the minor *TNF-α*-308 allele of the polymorphism predisposes to the development of ACS. In another study,[37] in 299 hospitalized French patients with coronary artery disease a higher frequency of carriers of the A allele was observed in patients with unstable angina when compared to control patients with stable angina but not in patients with myocardial infarction.

Additionally, in CHD patients, G/A and A/A genotypes were associated with higher blood glucose levels. A pathophysiological explanation might be that higher glucose levels could be attributed to insulin resistance in these patients. The mechanism by which TNF-α induces insulin resistance could be described as follows: after binding to the TNF-α receptor, TNF-α

phosphorylates the serine residues of insulin receptor substrate-1, and subsequently the activity of insulin receptor kinase is inhibited.[38] This causes an inhibition of the cascade of the insulin-signaling pathway distal to the insulin receptor and decreases GLUT4 translocation and final glucose uptake. Higher fasting glucose levels were found in older Japanese men bearing the A allele.[21] Given the possible relationship of the insulin resistance syndrome in CHD[38] the presence of the A allele could be the link between type 2 diabetes and CHD. Similarly, Nicaud et al.[39] among males offspring with a paternal history of premature myocardial infarction to age-matched controls recruited from 14 European university populations observed among cases, that those carrying the A allele exhibited a higher area under the curve for insulin, a higher increment between baseline concentration and peak of insulin and a greater decrease between peak and insulin at 120 minutes than those with the GG genotype. No such effect was observed in control subjects being in accordance with our results.

Also, we observed that the distribution of the *TNF-α −308G>A* polymorphism was associated with the presence of family history in CHD patients, but not in controls. Family history merits further investigation as a public health tool to identify persons with increased CHD risk that might benefit from enhanced prevention strategies.[40] The association between minor RFLP alleles and polymorphic gene variants, which enhance liability to CHD, has been previously documented.[41] According to our knowledge this is the first report implicating a TNF-α polymorphism with family history to CHD, in Greek adults. The significance of this association also has been reported in patients with myocardial infarction in Northern Ireland and France.[42] In that survey a possible relationship between obesity and the *TNF-α −308G>A* polymorphism was also reported; however, no association between the polymorphism distribution and obesity was observed in the present study, being in accordance with another study in an Hungarian case control study,[43] as well as no association between the polymorphism distribution and hypertension, hypercholesterolemia, in both patients and controls. Several studies have investigated the TNF-α polymorphism in diseases in which deregulation of TNF-α production might have played a role and several of them found no association between *TNF-α −308*A and its severity. Multiple genotyping of various inflammatory molecules would increase the possibility of identifying target groups with imbalance of inflammatory process thus raising the susceptibility to cardiovascular disease.

Limitations of the study

Because this is a retrospective case-control study, the findings can be used to state hypotheses but not claim causality. Confounding by ancestry is a concern in case-control genetic studies. Although, we have stratified our sample in all Greek regions, and we have matched patients and controls by region, confounding effect of ancestry cannot entirely be excluded in our study. Moreover, two main sources of bias may exist in this type of study, including selection and recall. As we have described in the methodology section, in order to eliminate selection bias, we tried to set objective criteria, both for patients and controls. However, insignificant misclassification may exist, because a small percentage of asymptomatic coronary patients may be wrongly assigned to controls, even though a cardiologist evaluated them. The patients, who died at entry or the day after, were not included into the study. This bias could influence our results, but because the physicians estimated the proportion of deaths of the study to be between 2% and 4% during the first two days, we believe that the inability to include the fatal events did not alter, significantly, our findings. Although we recovered detailed information, recall bias may still exist, especially in the measurement of smoking, dietary habits, duration, and intensity of physical activity and the onset of other investigated cardiovascular risk factors. Furthermore, regarding the potential effect of uncontrolled/unknown confounders, we tried to reduce it using the same study base, both for patients and controls. Lastly, due to the fact that many of our subjects were on drug therapy (statins or anti-inflammatory treatment), we decided not to measure any of the inflammatory markers like the TNF-α in the plasma.

In summary, our findings suggesting that the *−308 TNF-α* gene polymorphism could constitute a useful predictive marker for ACS, but that larger studies are necessary to confirm this association. A genome wide scan for early-onset coronary artery disease, which revealed susceptibility genes for early-onset CAD[44] also emphasizes the importance of the identification of genotypic markers for the early prediction of atherosclerotic disease.

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