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Natriuretic Peptide Val7Met Substitution and Risk of Coronary Artery Disease in Greek Patients With Familial Hypercholesterolemia

George V.Z. Dedoussis,[1]* Sandy Maumus,[2] John Skoumas,[3] Despoina M. Choumerianou,[1] Christos Pitsavos,[3] Christodoulos Stefanadis,[3] and Sophie Visvikis-Siest[2]

[1]Laboratory of Molecular Biology, Department of Dietetics and Nutrition, Harokopio University of Athens, Athens, Greece
[2]INSERM Unité 525, Faculté de Pharmacie, Centre du Médicament, Université Henri Poincaré Nancy 1, Nancy, France
[3]Department of Cardiology, Hyperlipidemic Clinic Hippocratio Hospital, Medical School of Athens, Athens, Greece

Atrial natriuretic peptide (ANP or NPPA) is the precursor protein of the form of amyloidosis called isolated atrial amyloid (IAA), which is related to the increased incidence of cardiac pathological conditions with age. Familial hypercholesterolemia (FH) patients are characterized by high concentrations of low-density lipoprotein cholesterol (LDL-C), which frequently gives rise to premature coronary artery disease (CAD). However, not all FH patients have the same clinical phenotype. The aim of the present study was to assess the relationship between ANP polymorphisms and apolipoprotein (Apo) A1 levels and CAD risk in FH patients. Transition T2238C, which leads to ANP with two additional arginines, and G664A (Val7Met) were investigated with lipid values and clinical phenotype in 83 FH patients. ApoA1 and HDL cholesterol levels were lower in GA patients compared to GG homozygotes for the G664A polymorphism. No association was found between the G664A polymorphism and CAD in our population. Moreover, ApoA1 and high-density lipoprotein cholesterol (HDL-C) levels did not differ among the different genotypes of the T2238C polymorphism, even after adjusting for age and sex. The 664A allele of the ANP polymorphism is associated with lower levels of ApoA1 and HDL-C in FH patients, but not with CAD risk. Concerning the T2238C polymorphism, no effect was found on lipid parameters or CAD incidence. J. Clin. Lab. Anal. 20:98–104, 2006. © 2006 Wiley-Liss, Inc.

Key words: ANP; polymorphism; G664A; FH; Apo-A1; HDL-C; coronary artery disease

INTRODUCTION

Familial hypercholesterolemia (FH, MIM♯ 143890) is an autosomal-dominant disorder that results from mutations in the low-density lipoprotein receptor (*LDLR*) gene (LDLR; MIM♯143890, GenBank accession number 000527) or within the apolipoprotein B-100 (*apoB*) gene. It affects approximately one in 500 individuals worldwide (1) and is characterized by a high concentration of low-density lipoprotein cholesterol (LDL-C), which frequently gives rise to tendon xanthomas (TX) and premature coronary artery disease (CAD).

Since CAD is a multifactorial disease resulting from both genetic and environmental factors, one approach toward understanding its etiology is to study candidate genes involved in CAD pathogenesis.

Atrial natriuretic polypeptide (ANP) is the precursor protein of the form of amyloidosis called isolated atrial amyloid (IAA) (2). It was previously shown that human plasma atrial K-natriuretic peptide (K-hANP)

*Correspondence to: George Dedoussis, Asst. Prof. of Biology, Department of Nutrition and Dietetics, Harokopio University of Athens, 70 El.Venizelou, 17671 Kallithea-Athens, Greece.
E-mail: dedousi@hua.gr

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sequestering is a protective phenomenon against amyloid aggregation (3). Although the clinical importance of IAA is still poorly defined, several studies have shown that significantly raised plasma levels of ANP may be related to the increased incidence of cardiac pathological conditions, rather than resulting from aging alone.

Human high-density lipoproteins (HDLs) are a fraction of plasma lipoproteins that do not constitute stable entities in vivo, but rather are continuously modified in the bloodstream through the action of specific factors. In their mature form, HDLs consist of spherical particles with a electrophoretic mobility containing two main apolipoproteins (apo A-I and apo A-II) that account for 70% and 20% of the HDL proteins, respectively. Other minor apos are present, including apo A-IV, apos C, apo D, apo E, and apo J (4,5). A small amount of HDL consists of discoidal particles with pre-β electrophoretic mobility containing two molecules of apo A-I complexed with phospholipids but little or no apo A-II, triglyceride, or cholesterol (6). Although reverse cholesterol transport is probably their main function, it is currently thought that HDL may mediate several still poorly defined physiological functions (7,8). A recent development in this field is the discovery that HDLs serve to protect against amyloidosis by inhibiting or strongly reducing the formation of amyloid fibrils (9).

Several polymorphisms have been described in the human *ANP* gene, and have been investigated mainly as potential markers of salt-sensitive hypertension (10,11). One of these polymorphisms, the T2238C ANP gene stop codon polymorphism, has been suggested to be associated with myocardial infarction (11). The loss of the ScaI restriction site in the *ANP* precursor gene because of T2238C transition within a stop codon leads to the extension of the human atrial natriuretic peptide (i.e., the peptide of 28 amino acids is extended to 30 amino acids by two additional arginines). Another polymorphism, the G664A nucleotide substitution in exon 1 of the gene, results in the amino-acid change of Val to Met at residue 7 (12).

In this study we aimed to correlate the incidence of ANP polymorphisms among FH patients with the lipid parameters and the outcome of the disease.

MATERIALS AND METHODS

Study Population

A total of 83 patients (males and females) were investigated. Patients with clinically diagnosed FH, who were referred to us for genetic analysis, had elevated total cholesterol (TC) (<290 mg/dL) and LDL-C (<200 mg/dL) above the 95th percentile for age and sex with normal triglyceride (TG) levels (<175 mg/dL). The end point of this analysis was a first, fatal, or nonfatal coronary heart disease event (according to the World Health Organization International Coding of Disease), since most of the subjects had a history of CAD and a family history of hypercholesterolemia and CAD (in first- or second-degree relatives). Tendon xanthomas were present in some families and absent in others. Secondary causes for hypercholesterolemia, such as hypothyroidism, diabetes, and renal or hepatic disease, were absent. Smoking habit was determined from the patients' notes and each subject was defined as a smoker, ex-smoker, or nonsmoker. Two recent blood pressure measurements were taken from the patients' and controls' medical records. As is commonly done in epidemiological studies, and according to the guidelines provided by the seventh report of the Joint National Committee on the Prevention, Detection, Evaluation, and Treatment of High Blood Pressure, patients who had an average blood pressure level of $\geq 140/90$ mmHg or were receiving antihypertensive medication were classified as hypertensives. A family history of premature coronary heart disease in first-degree relatives was recorded in all participants (male relative <45 years old, female relative <55 years old). An institutional review committee approved this study, and informed consent for participation was obtained from all subjects.

Biochemical Analysis

Blood samples from the antecubital vein were collected between 8 and 10 a.m. from subjects in a sitting position after 12–14 hours of fasting and avoiding alcohol. TC, high-density lipoprotein cholesterol (HDL-C), and triglycerides were measured by means of a chromatographic enzymatic method using a Technicon automatic analyzer RA 1000 (Dade Boehringer, Mannheim, Germany), and LDL-C was calculated with the Friedewald formula. Apolipoproteins A1 and B were measured by a BNII Dade Behring automatic nephelometer.

Mutation Analysis and ANP Genotyping

Genomic DNA was extracted from peripheral leukocytes using the Gentra DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN). ANP genotyping was performed using a prototypic multilocus genotyping assay focused on cardiovascular diseases essentially as described by Cheng et al. (13). Briefly, each sample was amplified by a 33-cycle polymerase chain reaction (PCR; 25 ng of genomic DNA). One of the biotinylated primer pairs in the multiplex was designed to amplify the gene of ANP. The PCR product was then hybridized to oligonucleotide probes that were immobilized in a linear array on a backed nylon membrane strip.

The colorimetric detection was based on streptavidin-horseradish peroxidase conjugate and substrates. *LDLR* genotyping was performed by denaturing gradient gel electrophoresis (DGGE) analysis as previously described (14). Automated sequencing of DGGE patterns representing *LDLR* polymorphisms was carried out using an ABI PRISM DyeDeoxy Terminator Cycle Sequencing Kit with a ABI 3700 Genetic Analyzer (Perkin Elmer, Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

Statistical Analysis

Continuous variables are presented as mean values ± standard deviation (SD), while qualitative variables are presented as absolute and relative frequencies. Statistically significant differences among groups were evaluated by Mann-Whitney test due to the small number of cases and the skewed distribution of the lipid values. The odds ratios (OR) and 95% confidence intervals (CI) are also shown. Differences between genotypes were assessed by analysis of variance (ANOVA). Because of the multiple significance tests performed and the inflation of type I error, the Bonferroni criterion was applied in all post hoc analyses. Moreover, relationships between the investigated variables and ANP genotypes were also assessed by multiple linear regression analyses after adjusting for age and sex. The results from the regression models are presented as β-coefficients and standard error of the coefficient and their R^2.

Statistical analysis was performed using the SPSS 10.0 program (SPSS Inc.). All reported *P*-values are based on two-sided tests and compared with a significance level of 5%.

RESULTS

The clinical characteristics of the study group are reported in Table 1. Genotype and allele frequencies are given in Table 2. The allele frequencies for G664A and T2238C were 0.04 for the A allele and 0.16 for the C allele, respectively. The distributions of ANP polymorphisms in our population were compatible with the Hardy-Weinberg equilibrium (G664A, T238C; $P = 0.92$, $P = 1$, respectively). Participants with the GA genotype of the G664A ANP polymorphism were found to have lower levels of TC, LDL-C, HDL-C, and ApoA1 compared to the more frequent GG genotype (Table 3). Regarding the remaining biochemical indices, no differences were detected between the two groups. No

TABLE 1. Anthropometrical and Clinical Parameters of the FH Patients*

Patients (n = 83)	Males (46)/females (37)
Age (years)	40.5 ± 16.2/41.9 ± 12.7
BMI (kg/m^2)	25.4 ± 4/24.1 ± 3.1
Waist/hip	0.88 ± 0.09/0.74 ± 0.08
Hypertension (mmHg)	6/5
Coronary artery disease	10/4
Type II diabetes	1/1
Tendon xanthomata	10/2
Former smokers	10/4
Current smokers	12/7
LDLR mutation	19/25

*Data are means ± SD values for continuous data and absolute/relative frequencies for qualitative data.
SD, standard deviation; BMI, body-mass index.

TABLE 2. ANP Genotypes and Allele Frequencies

Genotype Frequency n (%)		Allele Frequency n (%)			
T2238C	G664A	T238	238C	G664	664A
TT	GG				
57 (70.3)	76 (91.4)	136 (84)	26 (16)	159(96)	7 (4)
TC	GA				
22 (27.2)	7 (8.6)				
CC	AA				
2 (2.5)	0 (0)				

TABLE 3. Lipid Profiles of FH Patients Depending on the ANP Genotyping *P* Values are Estimated by Mann-Whitney Test

	Genotype					
	G664A			T2238C		
Parameters (mg/dL)	GG (n = 70)	GA (n = 6)	*P**	TT (n = 52)	TC/CC (n = 24)	*P**
TC	340.6 ± 79.3	277.8 ± 40.8	0.03	343.7 ± 86.5	320.7 ± 57.6	0.36
LDL-C	266.7 ± 71	210.4 ± 45	0.03	269.2 ± 75.2	249.7 ± 60.1	0.42
HDL-C	48.1 ± 15.4	37.2 ± 5.9	0.05	45.7 ± 13.2	50 ± 18.7	0.48
Triglycerides	142 ± 178	143.3 ± 76.1	0.63	127.5 ± 58.8	176.1 ± 294.7	0.82
ApoA1	141.4 ± 23.6	121.7 ± 16.1	0.02	135.2 ± 20.7	147.6 ± 27.5	0.15
ApoB	193 ± 40.2	177.2 ± 37	0.33	191.5 ± 42.6	192.2 ± 35.6	0.96
Lp(a)	49 ± 49.8	22.5 ± 20	0.11	47.2 ± 52.2	46.3 ± 41.2	0.90

TABLE 4. Relative Risk of CAD According to ANP Genotyping*

Genotype	Prevalence (%)	OR	95% CI	*P*
G664A				
GG	11/66 (16.6)	5.15	0.684–38.78	0.11
GA	3/5 (60)			
T2238C				
TT	11/47 (23.4)	0.395	0.98–1.58	0.19
CC/CT	3/23 (13)			

*Adjustment for age and sex was performed.

TABLE 5. Results from Regression Analyses that Evaluated the Effect of the G664A Polymorphism on Total Cholesterol, LDL-C, HDL-C, Triglycerides ApoA1, ApoB, and Lp(a)*

Model	Parameter	β ± SE	R^2	*P*
1	TC	58.98 ± 34.81	–	NS
2	LDL-C	48.78 ± 31.32	–	NS
3	HDL-C	11.69 ± 6.61	0.082	0.081
4	Triglycerides	29.35 ± 77.2	–	NS
5	ApoA1	25.21 ± 10.10	0.138	0.015
6	ApoB	19.64 ± 18.11	–	NS
7	Lp(a)	32.90 ± 21.82	–	NS

*All models have been adjusted for age and sex. R^2 is given for significant and borderline significant *P*-values.
β, partial regression coefficient; SE, standard error, NS, not significant.

differences were observed among the different genotype groups of the T2238C polymorphism.

Table 4 presents the results from the multivariate regression analysis that assessed the relationship between the polymorphisms and the likelihood of having CAD, after adjusting for age and sex. No association was found between the G664A polymorphism and the risk for developing CAD. No effect was observed regarding the T2238C polymorphism. In addition, none of the polymorphisms were found to be associated with a higher incidence of hypertension (data not shown).

Table 5 presents the results from the multivariate regression analyses, which investigated the influence of the G664A polymorphism on lipid concentrations. Whereas the association between HDL concentration and the G664A polymorphism was of borderline significance ($P = 0.081$), the polymorphism was highly associated with ApoA1 concentration. In effect, FH patients bearing the G allele had a higher ApoA1 concentration after adjusting for age and sex ($P = 0.015$), and the polymorphism accounted for 0.138 of ApoA1 variability.

DISCUSSION

The ANP gene has important cardiovascular effects (15). Raised circulating levels of ANP have been found in stroke (16). The importance of the *ANP* gene in coronary heart disease was recently emphasized in a study that assessed the relationship between N-terminal pro-BNP (NT-pro-BNP) levels and long-term mortality from all causes in a large cohort of patients with stable coronary heart disease (17). The median NT-pro-BNP level was significantly lower among patients who survived than among those who died, rendering it a marker of long-term mortality in patients with stable coronary disease.

No association was found in this study between the ANP G664A polymorphism and CAD. This is in agreement with previous results from Kato et al. (18,19), who investigated this variant in Japanese subjects and found no association between the 664A allele and hypertension (18) or stroke (19). Similarly, Hassan et al. (20) found no association between the A allele and stroke in a well characterized population of white stroke patients. It is possible that, like Hassan and coworkers, we were unable to detect a modest effect with a noncommon polymorphism. However, a twofold increased risk of stroke was found independently of hypertension, obesity, and diabetes in white subjects carrying the Val7Met mutation who were drawn from the Physician's Health Study, in a study by Rubattu et al. (21). The same authors also demonstrated associations involving the C allele of the C2238C polymorphism and stroke in Sardinian subjects (22).

We found that FH patients heterozygous for the Val7Met polymorphism in the *ANP* gene presented lower levels of ApoA1 and HDL. Moreover, we found by multiple regression analysis that this polymorphism influenced APOA1 concentrations in FH patients, with individuals homozygous for the frequent allele presenting higher ApoA1 concentrations than patients carrying the rare allele. The link of *ANP* with ApoA1 and HDL levels was recently studied (9). In that study, it was hypothesized that the differential binding to ANP of small and medium HDLs could be the result of the exposure of a larger number of hydrophobic domains in small HDLs due to the partial delipidation that occurs during the remodeling process. Unforeseen were the lower levels of LDL-C, which could be attributed to the type of *LDLR* mutation, since as we have previously shown the type of *LDLR* defect affects the LDL-C levels (23). Additionally, other lipid-lowering genes have been reported to affect LDL-C levels in FH patients (24). G664A in exon 1 resulted in the substitution of Val for Met at amino acid residue 7 of the preprohormone of ANP, which is located in a hydrophobic leader segment and is removed from the mature ANP (12,25). Although the relevance of the amino acid substitution located in the prosegment of ANP to mature peptide function

remains to be elucidated, a possible effect could be on the post-translational level of the mature protein. The allele frequency of the above polymorphism was close to that found in a recent study of healthy Japanese students (11). In that study, the authors reported a 0.09 allele frequency, which is higher than the 0.04 frequency observed in our study group. The different rare allele frequencies that have been reported in the literature are summarized in Table 6.

A recent study of subjects from Sardinia found that the relative risk for stroke conferred by the ANP 2238C allele when a recessive model of inheritance was assumed (CC2238 vs. TT2238 & TC2238) was 4.4 (95%CI, 1.6–12.1) (22). Additionally, the T2238C polymorphism was recently shown to be associated with a positive history of nonfatal myocardial infarction and multiple-vessel coronary atherosclerosis in a large number of Polish patients with stable CAD (19). A higher allele frequency of the C allele (0.4) compared to ours (0.16) was reported in that study. However, a similar frequency (0.19) of the rare allele was observed in a small group of healthy Italian subjects (27). The allele frequencies of the C allele in the study that investigated stroke in Sardinians were 20.1% in ischemic stroke cases and 14.8% in controls. These discrepancies could be attributed to the fact that the cohorts in each study were recruited under different criteria, and to a possible ethnic difference in the allele frequency of ANP variants (28,29).

It was previously shown (30) that ApoA1 and the ApoA1/ApoB ratio are better indicators than HDL-C for assessing the severity of coronary damage. It is well known that several plasma factors, including apoproteins, lipolytic enzymes, transfer proteins, and lipoproteins acting as lipid acceptors (31), are responsible for remodeling and regulate the levels and composition of HDL subclasses. It was recently suggested (32) that ANP binds to an ApoA1 dimeric form, which probably belongs to small HDLs. HDLs are generally considered to protect against amyloidosis by inhibiting or strongly reducing the formation of amyloid fibrils. If the 664A ANP variant has greater affinity for binding HDL particles via the ApoA1 dimer, this could explain the lower levels of ApoA1 and HDL found in FH patients bearing this ANP allele.

Study Limitations

There are some limitations to this study. First, we did not measure ANP protein levels, because plasma was not available. Nevertheless, according to the limited studies in the literature (11,27), association studies of ANP plasma level variations with ANP gene polymorphisms yielded contradictory results. The absence of

TABLE 6. A Review of the Different Articles Which Have Investigated the Association Between ANP G664A Genetic Polymorphism

Article reference	Rubattu et al. (22)	Kato et al. (11)	Hassan et al. (20)	Kato et al. (19)
Study population	Sample drawn from the Physician's Health Study	Japanese subjects 1st panel: 179 healthy students	Sample drawn from a well-characterized white stroke population	Japanese subjects 1st panel: 270 brain infarction patients and 350 controls
	Males, predominantly White American physicians, aged 40–84 years at the inclusion.	2nd panel: 102 health normotensive hospital staff	436 patients with ischemic cerebrovascular disease	2nd panel: 178 patients and 163 controls
	348 stroke cases 348 controls	3rd panel: 255 hypertensive and 225 normotensive subjects	295 community controls	
Results: polymorphism: rare allele frequency:	G664A: 11.6% in cases, 6.5% in controls	G664A: Determined in the 1st panel: 9%	G664A: 4.8% in cases, 4.9% in controls	G664A: 1st panel: 8.0% in cases, 8.9% in controls 2nd panel: 6.8% to 8.7% in cases, 11.7% in controls
	Association of the A allele with the occurrence of stroke (OR = 2.0, 95%CI 1.17–3.19, P = 0.01).	2nd panel no association between ANP Val7Met polymorphism and ANP concentrations. 3rd panel: no association between Val7Met polymorphism and hypertension.	No association between the presence of the GA/AA genotype and stroke in this model	No significant association between 664A variant and stroke in the studied population, in either of panels.

a healthy group of controls precludes us from assuming a possible impact of ANP polymorphisms on lipid level variations. A prospective study performed in a large sample of the general population will be needed to accurately define the relative and absolute risks of CAD associated with ANP gene variants. We are currently genotyping ANP polymorphisms in the ATTICA study (33–35), and we hope to confirm our preliminary data from the FH patients. Finally, only a limited number of FH patients were genotyped for the two polymorphisms because of the high cost of genotyping.

In conclusion, our results suggest that the G664A polymorphism, after being tested in larger cohorts, could constitute (along with other inflammation markers) a useful predictive marker for CAD. A genome-wide scan for early-onset CAD (36), which revealed susceptibility genes for early-onset CAD, emphasized the importance of identifying genotype markers for the early prediction of atherosclerotic disease.

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