Atrial Natriuretic Peptide Gene Polymorphisms and Risk of Ischemic Stroke in Humans

Speranza Rubattu, MD; Rosita Stanzione, PhD; Emanuele Di Angelantonio, MD; Bastianina Zanda, MD; Anna Evangelista, BS; David Tarasi, MD; Bruna Gigante, MD; Angelo Pirisi, MD; Ercole Brunetti, MD; Massimo Volpe, MD

Background and Purpose—A precise definition of genetic factors responsible for common forms of stroke is still lacking. The purpose of the present study was to investigate the contributory role of the genes encoding atrial natriuretic peptide (*ANP*) and type A natriuretic peptide receptor (*NPRA*) in humans' susceptibility to develop ischemic stroke.

Methods—Allele and genotype frequencies of *ANP* and *NPRA* were characterized in an Italian case-control study with patients affected by vascular disease or risk factors. Subjects were recruited from the island of Sardinia (206 cases, 236 controls).

Results—A significant association between the *ANP*/TC2238 polymorphic site and stroke occurrence was found when a recessive model of inheritance was assumed. The risk conferred by this mutant genotype, when estimated by multivariate logistic regression analysis, was 3.8 (95% confidence interval, 1.4 to 10.9). A significantly increased risk of stroke recurrence was observed among cases carrying the *ANP*/CC2238 genotype compared with cases carrying the *ANP*/TT2238 genotype ($P=0.04$). No direct association of *NPRA* with stroke occurrence was detected. However, a significant epistatic interaction between the *ANP*/CC2238 genotype and an allelic variant of *NPRA* led to a 5.5-fold increased risk of stroke (95% confidence interval, 1.5 to 19.4).

Conclusions—Our findings support a direct contributory role of *ANP* to stroke in humans. A significant interaction between *ANP* and *NPRA* on stroke occurrence was found. **(*Stroke*. 2004;35:814-818.)**

Key Words: cerebrovascular disorders ■ gene mutation ■ genetics ■ natriuretic peptides, atrial ■ receptors, atrial natriuretic factor

Stroke represents a common cardiovascular complex trait resulting from complex gene-gene and gene-environment interactions.[1,2] Among other genes, the atrial natriuretic peptide (*ANP*) gene has been involved in the pathogenesis of stroke. In fact, structural abnormalities of *ANP* are significantly associated with stroke in both an animal model, the stroke-prone spontaneously hypertensive rat,[3] and the North American white population of male physicians recruited in the Physicians Health Study (PHS).[4] Indeed, the *ANP* gene represents a candidate in vascular diseases. In fact, the ANP peptide plays a pivotal role in the regulation of electrolyte and water balance through its known cardiovascular effects.[5] Moreover, recent studies have shown that it exerts a significant impact on cardiac and vascular remodeling through its direct modulatory role on cellular growth.[6–9] Two coding mutations of human *ANP* are known: an exon 1 mutation responsible for a Val/Met transposition within the 1-30 proANP (long-acting natriuretic peptide)[4] and a stop codon mutation responsible for the synthesis of a 30 rather than 28 aa mature ANP peptide.[10] In particular, we reported a 2-fold increased risk of stroke independent of hypertension, obesity, and diabetes in subjects carrying the exon 1 mutation described in the PHS.[4]

Abnormalities of the gene encoding type A natriuretic peptide receptor (*NPRA*) with which ANP peptide primarily interacts may, in turn, facilitate occurrence of hypertension and related phenotypes, as already documented in an animal model.[11]

We conducted a case-control study to investigate the role of *ANP* on ischemic stroke predisposition. Cases and controls were recruited from Sardinia, a large Mediterranean island with a well-known elevated degree of genetic homogeneity. In addition, we tested the role of *NPRA* and the possible interactions among the 2 genes on the final phenotype. Moreover, we compared the incidence of stroke recurrence among *ANP*/CC2238 mutant cases and *ANP*/TT2238 wild-type cases over a 5-year follow-up period. Finally, direct sequencing of *ANP* in CC2238 individuals was performed to rule out the existence of other coding mutations.

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From IRCCS Neuromed, Pozzilli (S.R., R.S., A.E., M.V.); Cardiology Division, Second Faculty of Medicine, University La Sapienza, Rome (S.R., D.T., M.V.); Clinica Neurologica, University of Sassari, Sassari (B.Z., A.P.); Department of Internal Medicine, First Faculty of Medicine, University La Sapienza, Rome (E.D.A.); Research Center, St Peter Hospital AFAR Fatebenefratelli, Rome (E.B.); and Biogem Scacl c/o IGB A. Buzzati-Traverso, Naples (B.G.), Italy.

Correspondence to Speranza Rubattu, MD, IRCCS Neuromed, Localitá Camerelle, 86077 Pozzilli, Italy. E-mail rubattu.speranza@neuromed.it

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Methods

Study Population

A hospital-based stroke register has been established in the Neurological Department of the University of Sassari. Almost 100% of these patients originated from North Sardinia (180 000 inhabitants). For this study, we considered 206 consecutive patients with ischemic stroke admitted between September 1996 and March 2003. Diagnosis of ischemic stroke was based on the presence of rapidly developing clinical symptoms and/or signs of focal and at times global loss of cerebral function, with signs and symptoms persisting for >24 hours. Clinical diagnosis was confirmed in each individual case by the presence of a well-defined cerebral lesion documented either in the early phase or at least 5 to 10 days after the acute event by CT scan and/or nuclear MR imaging. Cardioembolic forms of stroke were excluded. Controls (n=236), drawn from the same geographical area and identified among an age-matched population of patients admitted to the same hospital, were included if they had vascular risk factors or a history of myocardial infarction or peripheral vascular disease, but they were excluded if they had either current or previous cerebrovascular disease. The study protocol was approved by the local ethics committee, and all participants gave written informed consent.

Cardiovascular risk factors were evaluated for both cases and controls on the basis of the criteria shown below. Hypercholesterolemia was defined as the presence of total cholesterol blood levels >220 mg/dL. Hypertension was defined as present if subjects had been previously diagnosed according the World Health Organization/International Society of Hypertension guidelines and were routinely receiving antihypertensive therapy. Subjects were defined as current smokers or ex-smokers if they had stopped smoking >2 months before the study. Alcohol consumption was considered when subjects used to drink >30 g/d. We also recorded history of myocardial infarction (documented by medical records with clinical, laboratory, and ECG changes), presence of peripheral arteriopathy, and presence of type 1 or 2 diabetes mellitus.

Finally, incidence of stroke recurrence among *ANP*/CC2238 cases (n=14) was recorded and compared with that in *ANP*/TT2238 cases (n=14) followed up over a period of 5 years.

Analysis of the *ANP* and *NPRA* Polymorphic Markers

DNA was extracted from peripheral whole blood by use of a commercially available kit (Qiagen). Characterization of *ANP* polymorphic markers was performed by previously reported procedures. Briefly, we analyzed a −G664A promoter mutation by an *Rsa*I restriction fragment length polymorphism (RFLP) assay,[12] an intronic G1837A mutation by an *Hpa*II RFLP assay,[13] and a T2238C coding mutation by an *Sca*I RFLP assay.[10] For *NPRA*, we used a previously reported microsatellite marker localized within the promoter region.[14] All polymerase chain reactions (PCRs) were performed with a PTC-100 thermal cycler, and any unclear result was resolved by new PCR. Digestion with the corresponding enzyme was carried out for the *ANP* markers as recommended by the manufacturer (NEB). The PCR products were resolved on agarose gels and visualized by ethidium bromide staining. The *NPRA* microsatellite was amplified with the ^{32}P-labeled forward primer and unlabeled reverse primer and visualized by autoradiography. The genotypes were read by 2 blinded independent investigators.

Sequencing of the ANP Gene

The genomic DNA from 6 cases carrying the *ANP* CC2238 genotype was amplified with specific set of oligos for exon 1, 2, and 3 of *ANP* and subjected to direct sequencing. The oligo sequences were the following: exon 1S (position 514 to 533), AACCAGAGGGGAGAGACAGA; exon 1AS (position 775 to 794), GTGAGGTTTATCCCTTTCCC; exon 2S, first part of the exon (position 636 to 657), ACCAGAGCTAATCCCATGTACA; exon 2AS, first part of the exon (position 963 to 982), AGAGAGATGGAGGTGCCCTC; exon 2S, second part of the exon (position 951 to 970), TCAGCCCAGCCCAGAGAGAT; exon 2AS, second part of the exon (position 1149 to 1168), GAACTGGGGATGGAAATGGG; exon 3S (position 2195 to 2214), CCAGGTCACCAAGCCAGATA; and exon 3 AS (position 2280 to 2299), GACAGACTGCAAGAGGCTCC.

Sequencing was conducted with a dye-terminator cycle sequencing method (Perkin-Elmer) and performed on an automatic sequencing apparatus (ABI Prism 373A sequencer, Perkin Elmer).

TABLE 1. Characteristics of the Study Population

	Ischemic Stroke Cases (n=206)	Controls (n=236)
Age (mean±SD), y	72.1±11.8	71.9±12.8
Range	50–97	50–93
Male sex, n (%)	120 (58.3)	132 (55.9)
Smoking, n (%)	81 (42.2)	90 (39)
Alcohol (>30 g/d), n (%)	23 (11.2)	32 (13.6)
Diabetes, n (%)	53 (25.7)	66 (28.1)
Hypercholesterolemia,* n (%)	54 (26.2)	33 (14)
Hypertension,† n (%)	129 (62.9)	110 (46.8)
History of MI, n (%)	25 (12.1)	32 (13.6)
Peripheral arteriopathy, n (%)	9 (4.4)	13 (5.5)

MI indicates myocardial infarction.
*P=0.01; †P<0.001.

Statistical Analysis

Allele and genotype frequencies were computed for each locus, and their distributions in cases and controls were analyzed by χ^2 test with 2 and 1 *df*, respectively. Concordance to the frequency predicted by the Hardy-Weinberg equilibrium was assessed by χ^2 test with 1 *df*. The magnitude of linkage disequilibrium between *ANP* markers was estimated by use of the GDA program.[15]

The risk associated with each *ANP* genotype in the occurrence of ischemic stroke was estimated by logistic regression analysis computing the odds ratio (OR) with the respective 95% confidence interval (CI) under the assumption of an additive (assigning scores of 0, 1, and 2 for homozygous TT2238, GG1837, −GG664; heterozygous TC2238, GA1837, −GA664; and homozygous CC2238, AA1837, −AA664, respectively), a dominant (score of 0 for TT2238, GG1837, or −GG664; 1 for TC2238 and CC2238, GA1837 and AA1837, or −GA664 and −AA664 combined, respectively), and a recessive (score of 0 for TT2238 and TC2238 combined; 1 for CC2238) effect of each allele. A recessive model of the G1837A and −G664A markers was not tested because of the low prevalence of the alleles. For each OR, we calculated 2-tailed probability values and 95% CIs.

Multivariate logistic regression analysis was used to control for possible confounding factors. The multivariate model included variables (hypertension and hypercholesterolemia) that were significant (P<0.02) in the univariate analysis or any potential confounder that changed the unadjusted OR for *ANP* genotype by >5% after adjustment (smoking and peripheral arteriopathy).[16]

Finally, the presence of possible interactions between *ANP* and *NPRA* was assessed by adding an interaction term to the logistic model and adjusting for possible confounders (age, hypercholesterolemia, hypertension, and peripheral arteriopathy). All calculations were considered significant at P<0.05.

Results

ANP Gene Polymorphism Analysis

Table 1 shows the main characteristics of the study population. No statistically significant differences were observed for age and sex distributions between cases and controls. A significantly higher prevalence of hypertension and of hyper-

TABLE 2. Genotype and Allele Frequencies for the 3 *ANP* Markers in Cases and Controls

	Ischemic Stroke Cases, n (%)	Controls, n (%)
Genotype		
T2238C		
TT	141 (68.5)	171 (72.5)
TC	47 (22.8)	60 (25.4)
CC	18 (8.7)	5 (2.1)
Allele		
T	329 (79.9)	402 (85.2)
C	83 (20.1)	70 (14.8)
G1837A		
Genotype		
GG	198 (96.6)	227 (96.6)
GA	6 (2.9)	8 (3.4)
AA	1 (0.5)	...
Allele		
G	402 (98.0)	462 (98.3)
A	8 (2.0)	8 (1.7)
−G664A		
Genotype		
GG	200 (97.1)	227 (96.2)
GA	6 (2.9)	9 (3.8)
AA	...	...
Allele		
G	406 (98.5)	463 (98.1)
A	6 (1.5)	9 (1.9)

cholesterolemia was detected among cases compared with controls. The stroke subtype distribution of the population considered for the present analysis was as follows: large-vessel disease caused by relevant atherothrombotic lesions of the carotid and/or main cerebral arteries, 64%; small-artery disease in the presence of lacunar infarction and of a characteristic disease of the penetrating arteries, 36%.

The allele and genotype frequencies of all *ANP* markers in the overall population are shown in Table 2. They were in Hardy-Weinberg equilibrium for the entire study population. The *ANP*/2238C allele was significantly more represented among cases than controls ($P=0.037$). The relative risk conferred by this mutant allele, when a recessive model of inheritance (CC2238 versus TT2238 and TC2238) was assumed, was 4.4 (95% CI, 1.6 to 12.1). Multistep logistic regression analysis identified hypertension (OR, 1.9; 95% CI, 1.3 to 2.8) and hypercholesterolemia (OR, 2.2; 95% CI, 1.3 to 3.5) as independent predictors of stroke. Adjustment for these variables and for the confounders revealed an independent OR for the *ANP*/CC2238 genotype of 3.8 (95% CI, 1.4 to 10.9; Table 3). No effect was observed when either a dominant or an additive mode of transmission was assumed. There was no difference between sexes. No association of the *ANP*/CC2238 genotype with hypertension or history of myocardial infarction was found. No association of the other 2 *ANP* markers with stroke was detected.

TABLE 3. Risk of Ischemic Stroke and *ANP* Polymorphisms in Cases and Controls

	Unadjusted		Adjusted	
	OR (95% CI)	*P*	OR (95% CI)	*P*
T2238C				
Recessive	4.4 (1.6–12.1)	0.004	3.8 (1.4–10.9)	0.006*
Additive	1.4 (0.9–1.9)	0.054	1.3 (0.9–1.9)	0.101
Dominant	1.2 (0.8–1.8)	0.356	1.2 (0.8–1.8)	0.475
G1837A				
Recessive	...		...	...
Additive	1.1 (0.4–2.9)	0.794	1.7 (0.6–4.8)	0.340
Dominant	1.0 (0.3–2.8)	0.995	1.6 (0.5–5.1)	0.462
−G664A				
Recessive	...		...	...
Additive	0.7 (0.3–2.1)	0.603	0.9 (0.3–2.7)	0.856
Dominant	0.7 (0.3–2.1)	0.603	0.9 (0.3–2.7)	0.856

Unadjusted indicates univariate logistic regression analysis; Adjusted, multivariate logistic regression analysis after adjustment for confounders.

*Confounders considered for this analysis were hypertension, hypercholesterolemia, smoking, and peripheral arteriopathy.

The T2238C locus was in linkage disequilibrium with the *Hpa*II marker [χ^2 (1 *df*), 7.5; $P<0.01$] and with the promoter polymorphism [χ^2 (1 *df*, 6.0; $P=0.01$].

Further direct screening for the G664A exon 1 mutation (that is in linkage disequilibrium with the 1837 position[4]) was not considered necessary in the present study. In fact, we verified that the genotype configuration at the 1837 and 664 loci was completely concordant by comparing a group of samples (data not shown).

Furthermore, incidence of stroke recurrence among *ANP*/CC2238 cases (n=14) was 36% compared with 0% observed among *ANP*/TT2238 cases (n=14) followed up over a period of 5 years ($P=0.04$) despite a lower incidence of hypertension (42.8% versus 71.4%, respectively) and hypercholesterolemia (0% versus 14%).

Finally, analysis of allele and genotype distributions for *NPRA* did not reveal any differences between cases and controls (Table 4). However, when the interactions between *ANP* and *NPRA* genes were tested, a significantly increased risk of stroke was found in individuals carrying the *ANP*/CC2238 genotype and allele 11 of *NPRA*, either heterozygous or homozygous (n=15 cases versus 3 controls). After adjustment for confounders, the OR was 5.5 (95% CI, 1.5 to 19.4).

Sequencing of *ANP* Gene

Direct analysis of the coding sequence in *ANP*/CC2238 cases confirmed the T/C stop codon mutation and did not reveal any additional coding mutation.

Discussion

The present study demonstrates that, among a population of patients with vascular disease, individuals with the homozygous genotype for the *ANP* stop codon mutation have an increased risk of ischemic stroke (OR, 3.8; 95% CI, 1.4 to 10.9). Thus, these findings confirm previous evidence in favor of a direct contributory role of *ANP* as a stroke gene.

TABLE 4. Genotype and Allele Frequencies of *NPRA* Microsatellite Marker in Cases and Controls

		Ischemic Stroke Cases, n (%)	Controls, n (%)
Genotype	10/10	42 (20.4)	48 (20.3)
	10/11	101 (49.0)	113 (47.8)
	11/11	45 (21.8)	45 (19.1)
	10/12	4 (1.9)	15 (6.4)
	11/12	12 (5.8)	11 (4.7)
	12/12	1 (0.5)	1 (0.4)
	9/11	...	3 (1.3)
	8/11	1 (0.5)	...
	10	189 (45.9)	224 (47.5)
	11	204 (49.5)	217 (46.0)
	12	18 (4.4)	28 (5.9)
	9	...	3 (0.6)
	8	1 (0.2)	...

The present analysis in Sardinian subjects ruled out a direct contributory role of *NPRA* on ischemic stroke predisposition. However, a significant interaction of *ANP* stop codon mutation with an allelic variant of *NPRA* led to a further increase in the risk of stroke.

The ANP peptide plays a pivotal role in the regulation of electrolytes and water balance, thus contributing to blood pressure homeostasis.[17] In fact, the *ANP* gene has long been on the list of candidate genes for cardiovascular and related diseases.[18] In this regard, evidence has been reported for systemic arterial hypertension, particularly in genetically manipulated animals,[19,20] for kidney disease in diabetes,[21] for myocardial infarction,[22] and for cerebrovascular accidents in both rats[3] and humans,[4] although with some controversies.[23,24]

In the present study, we characterized the same markers used in the PHS: G1837A, an intronic variation that reflects the effect of the exon 1 Val/Met substitution, and T2238C, which leads to the addition of 2 arginine residues to the translation product. In addition, we analyzed a polymorphic marker located within the promoter region of *ANP*.[13] The prevalence of the G1837A mutation is known to be very low among European populations.[25] Our Sardinian sample confirmed this evidence. Thus, it was difficult to assess the association of the G1837A variant with stroke.

The promoter polymorphism showed also a low frequency in Sardinians compared with a Japanese group.[13] Again, it was difficult to assess any association with stroke.

In contrast, the T2238C mutation showed a significant impact on stroke occurrence when a recessive model of inheritance was assumed, leading to an ≈4-fold increased risk of stroke. Its effect was independent from other stroke-predisposing conditions. Furthermore, we observed that Sardinian carriers of the mutant peptide had an increased risk of stroke recurrence. To exclude the possibility that other coding mutations, possibly in linkage disequilibrium with the 2238 point mutation, could be responsible for the observed effects, we explored the whole coding sequence of *ANP* in 2238 double-mutant cases. No other mutation was found.

The T2238C mutation adds 2 amino acids to the normal sequence of the human ANP peptide. The functional significance of the 30 aa peptide is at present unknown. However, a biological role of this molecular variant has been already suggested. In particular, because the 2238C allele has been reported to be significantly associated with a protective effect toward development and progression of renal damage in diabetes,[21] a selective role in the regulation of glomerular filtration rate and in the genesis of hyperfiltration has been postulated. Recently, this mutation has been found to be associated with higher circulating levels of ANP in salt-sensitive essential hypertensives[26] and with nonfatal myocardial infarction and extent of coronary artery disease.[22] Our findings suggest that the mutant ANP peptide could be involved in endothelial damage and dysfunction underlying individual susceptibility to develop stroke. In particular, the recognized modulatory role of ANP on cellular proliferation and growth[6–9] and its vasorelaxant effect could be modified in the presence of the stop codon mutation. Further studies are required to dissect out the potential pathophysiological relevance of ANP mutant peptide and its possible link with the disease. In this regard, an additional observation of the present study was the finding of a synergistic effect between the ANP mutant peptide and a molecular variant of its receptor, leading to a >5-fold increased risk of stroke. The *NPRA* marker used (located 300 bp upstream of the start codon) may well reflect different degrees of promoter gene activity between individuals. Thus, depending on the different promoter gene activity, the pathogenetic effect of the ANP mutant peptide may be amplified.

The observation that different mutations belonging to *ANP* exert a similar contributory role toward stroke occurrence in different ethnic groups (relevance of an exon 1 variant in the PHS and of an exon 3 variant in Sardinians) can be explained at least in part by the remarkable differences in allelic frequencies of the polymorphic sites in the 2 human samples and by differences in case selection (inclusion of both ischemic and hemorrhagic strokes in the PHS and of ischemic strokes only in the present study).

A potential limitation of the present study may be the recruitment of an age-matched control cohort from the same inpatient clinic. The incidence of cardiovascular diseases and related risk factors might had been higher than expected in a cohort of healthy subjects. This selection of controls restricted the domain of our study to patients with vascular disease, ie, patients with cerebrovascular disease and those with other forms of atherosclerotic disease or risk factors for it.

In conclusion, our data support the role of *ANP* as a direct contributor to stroke. The pathogenetic effect of the ANP mutant peptide may be exacerbated by the concomitant presence of molecular variants of its receptor. Mutant forms of *ANP* should be viewed as risk factors for cerebrovascular accidents, and identification of *ANP* mutation–dependent disease mechanisms may help to improve prevention and treatment of stroke.

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