The Gene Encoding Atrial Natriuretic Peptide and the Risk of Human Stroke

Speranza Rubattu, MD; Paul Ridker, MD, MPH; Meir J. Stampfer, MD; Massimo Volpe, MD; Charles H. Hennekens, MD, DrPH; Klaus Lindpaintner, MD

**Background**—Recent evidence from an animal model of stroke, the stroke-prone spontaneously hypertensive rat, implicated the gene encoding atrial natriuretic peptide (ANP) as a possible candidate contributing to the likelihood of experiencing a stroke. The purpose of the present study was to investigate the role of ANP in the pathogenesis of cerebrovascular accidents in humans.

**Methods and Results**—We investigated 2 previously known markers at ANP, G1837A and T2238C, for their possible association with the occurrence of stroke. This was the largest matched case-controlled sample studied thus far; the sample was drawn from a large prospective study (the Physician’s Health Study). When assuming a dominant mode of inheritance, a statistically significant positive association was observed for the G1837A allele, indicating an odds ratio of 1.64 (95% confidence interval, 1.01 to 2.65) for stroke. This observation led to the discovery of a new molecular variant in exon 1, G664A, which was responsible for a valine-to-methionine substitution in the proANP peptide. This mutation, which was in linkage disequilibrium with the G1837A marker, was associated with the occurrence of stroke (odds ratio, 2.0; 95% confidence interval, 1.17 to 3.19; *P=0.01*).

**Conclusions**—Our findings suggest that molecular variants of the ANP gene may represent an independent risk factor for cerebrovascular accidents in humans. The strong parallelism to the experimental data obtained in the stroke-prone animal model provides assurance for the relevance of our observation. (*Circulation*. 1999;100:1722-1726.)

**Key Words:** cerebrovascular disorders • genetics • natriuretic peptides

Stroke represents a major cause of death and disability. Known risk factors such as hypertension, diabetes, lipid abnormalities, and smoking account for a portion of overall disease risk; in addition, an important role of heritable factors predisposing to stroke has been recognized. Thus, a positive maternal history of stroke is associated with a doubling of stroke incidence, independent of other risk factors, whereas being an identical, as compared with a dizygotic, twin of a stroke victim raises the risk of stroke 5-fold. Similarly, a number of large epidemiologic studies documented a statistically significant familial aggregation of stroke. In addition, the existence of rare monogenic disorders in which stroke segregates in a Mendelian fashion emphasizes the potential pathogenic role of even single gene mutants to cause stroke.

Understanding the genetic factors that contribute to stroke will advance efforts to develop specific diagnostic, preventive, and therapeutic strategies to combat this disease. The late onset of stroke, the common presence of other risk factors (such as those enumerated above), and the probably modest contribution of individual gene variants to a disorder considered a complex, polygenic, and etiologically heterogeneous trait present major impediments in the recognition of disease-relevant genes in human populations. Animal models of inherited forms of stroke have thus been used as a reductionist approach to the problem. Recently, we reported the identification of 3 chromosomal regions carrying stroke-relevant genes in the stroke-prone, spontaneously hypertensive rat, a model that resembles some forms of human stroke. One of them, on rat chromosome 5, colocalized with the gene encoding atrial natriuretic peptide (ANP). On the basis of the well-recognized vascular properties of this peptide, which is also well-represented in the cerebral areas involved in cardiovascular regulation, we proposed it as a possible candidate disease gene. Indeed, further work in this animal strain disclosed point mutations affecting both regulatory and coding regions of ANP that resulted in a structurally and functionally different protein and in gene expression in the brain that differs from the stroke-resistant spontaneously hypertensive rat (S. Rubattu, MD, et al, unpublished data.)
1999). Prompted by these findings, we investigated the possible association of genetic variants of ANP with the occurrence of stroke in humans by using a prospectively collected, matched, case-controlled sample.

Methods

Study Subjects
Our study sample was drawn from the Physician’s Health Study, an ongoing prospective study originally conceived to determine the effects of aspirin and beta carotene on the incidence of myocardial infarction and colon cancer, respectively.14 It was initiated in 1982 in 22,071 male, predominantly white, US physicians, who were 40 to 84 years of age at study entry. Before randomization, 14,916 participants provided an EDTA-anticoagulated blood sample that was stored at −80°C. All participants were free from prior history of stroke or transient ischemic attacks. Yearly follow-up questionnaires allowed for updated information on newly developed diseases. Stroke was ascertained from a blinded review of medical records and autopsy results in 353 male subjects for whom blood samples were available. For 348 of the cases, a control matched by age, smoking history, and time of randomization into the study (to ensure comparable length of follow-up) was chosen among subjects eligible on the basis of health status (no reported signs of cerebrovascular disease).

Determination of the HpaII and ScaI ANP Genotypes
DNA was extracted from peripheral whole blood according to standard protocols.15,16 For the characterization of the ANP genotype, we used 2 previously described markers, G1837A and T2238C. G1837A is located in the second intron of the gene at position 1837 of the published sequence17; at this point, either a guanine or an adenine base is encountered, which results in the presence or absence, respectively, of a recognition site for the restriction endonuclease HpaII. T2238C is located within the third exon at position 2238, where the presence of a variant C instead of the wild-type T changes the tga stop-codon of the ANP open reading frame to a cga, resulting in a peptide that has 2 supernumerary arginine residues at its carboxy terminal; this change also inactivates a wild-type recognition site for the restriction endonuclease ScaI. For the G1837A marker, we amplified a 446 bp product from genomic DNA that contained either 4 (1837G) or 3 (1837A) restriction sites for HpaII, T2238C is located within the third exon at position 2238, where the presence of a variant C instead of the wild-type T changes the tga stop-codon of the ANP open reading frame to a cga, resulting in a peptide that has 2 supernumerary arginine residues at its carboxy terminal; this change also inactivates a wild-type recognition site for the restriction endonuclease ScaI. For the G1837A marker, we amplified a 446 bp product from genomic DNA that contained either 4 (1837G) or 3 (1837A) restriction sites for HpaII, respectively, using 2 flanking oligonucleotides (and AS-I: 5′-gaggagccagggaatgcat-3′ and AS-II: 5′-ccccaccaccgctctg-3′). For the T2238C marker, we followed a previously reported procedure.18 All polymerase chain reactions (PCR) were performed as previously described.19 Digestion with the corresponding enzyme was carried out as recommended by the manufacturer (NEB); the PCR products were loaded onto 3.5% and 2% submarine polyacrylamide gels were run at room temperature and low voltage. Subsequently, repetitive sequencing of case and control samples using fluorescent-labeled dideoxy terminators on an ABI377 apparatus (Perkin Elmer) was performed for the relevant PCR segment generated using the oligonucleotide primers S613 (5′-tggcattccagctcctaggt-3′) and AS684 (5′-gattaaggcaggaggccag-3′). PCR conditions were as mentioned above.

Statistical Analysis
Allele and genotype frequencies among cases and controls were counted and compared with values predicted by Hardy-Weinberg equilibrium using the χ² test. Odds ratios (ORs) were calculated as a measure of the association of genotype with stroke phenotype under assumptions of additive (assigning scores of 0, 1, and 2 for homozygous 1837GG, 2238TT, or 664GG; heterozygous 1837GA, 2238TC, or 664GA; and homozygous 1837AA, 2238CC, or 664AA, respectively), dominant (score of 0 for 1837GG, 2238TT, or 664GG and 1 for 1837GA and 2238TA and 2238CC, or 664GA and 664AA combined, respectively), and recessive (score of 0 for 1837GG and 1837GA or 2238TT and 2238TC combined, and 1 for 1837AA or 2238CC, respectively) effects of either allele. A recessive model of the G664A marker was not tested due to the low prevalence of the allele. Because of the potential confounding effects of aspirin and beta carotene treatments, all analyses were adjusted for these variables. For each OR, we calculated 2-tailed probability values and 95% confidence intervals (CI). We performed both matched-pair and unmatched analyses, with adjustment for possible confounding factors (body mass index, systolic and diastolic blood pressures, smoking, history of hypertension, history of diabetes, and history of hypercholesterolemia) by conditional and unconditional logistic regression, respectively.20 To examine a “low-risk” group, we excluded all individuals with a history of hypertension and/or diabetes mellitus and conducted the same analyses.

The magnitude of linkage disequilibrium between markers was evaluated using the program EH (Estimating Haplotype-Frequencies).21 P<0.05 was considered statistically significant.

Results

Association of G1837A and T2238C Variants of ANP With Stroke
The main characteristics of our study sample are summarized in Table 1. The data (adjusted in all analyses for treatment with aspirin and beta carotene) reflect the expected, recognized risk factors, with a significantly higher prevalence of hypertension and diabetes mellitus among cases compared with controls. Both systolic and diastolic blood pressure levels and body mass index were significantly higher among cases (P<0.001). In a low-risk subgroup, defined by exclusion of all individuals with a history of hypertension and/or diabetes, differences among cases and controls in systolic blood pressure were less pronounced (P<0.05), and differences in diastolic blood pressure and body mass index were no longer significant (P>0.05).

The genotype frequencies for both markers are reported in Tables 2 and 3. They agreed with those predicted by the Hardy-Weinberg equilibrium. The 1837A allele was overrepresented among cases compared with matched controls. The relative risk conferred by this mutant allele, adjusted for the effect of aspirin and beta carotene, was 1.64 in the matched-pair analysis (95% CI, 1.01 to 2.65; P=0.046), assuming a dominant mode of inheritance (1837GG versus 1837GA and 1837AA). Similar point estimates of relative risk were obtained when an additive mode of inheritance was assumed and when the low-risk subgroup (n=206) or cases with ischemic stroke only (n=281) and their matched controls were examined separately (data not shown). The unmatched analysis confirmed these results. Multistep logistic regression analyses identified systolic blood pressure (OR=1.02; 95% CI, 1.01 to 1.04; P=0.004), history of hypertension (OR=2.2; 95% CI, 1.45 to 3.39; P=0.0002), and history of diabetes (OR=2.94; 95% CI, 1.35 to 6.38; P=0.0065) as
additional independent predictors of stroke. Adjustment for all these variables revealed an independent OR for the 1837A allele of 1.49 in the dominant model (95% CI, 0.96 to 2.31; P = 0.07) and of 1.54 in the additive model (95% CI, 1.02 to 2.32; P = 0.038). Finally, testing a recessive model did not show any significant result in either group.

Analogous analyses carried out for the ANP T2238C marker failed to show any significant association between genotype and case-control status (Table 3). As expected, the G1837A marker showed a weak but significant degree of linkage disequilibrium with the T2238C marker (X² 1df = 6.8; P = 0.01).

### Coding Sequence Polymorphism and Stroke

Because the G1837A marker is devoid of any demonstrated functional relevance, we performed single-strand conforma-

<table>
<thead>
<tr>
<th>TABLE 2. Genotype Frequencies for the G1837A Polymorphism</th>
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<tbody>
<tr>
<td>Genotype</td>
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<tr>
<td>---------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>G1837A</td>
</tr>
<tr>
<td>GG</td>
</tr>
<tr>
<td>G4</td>
</tr>
<tr>
<td>AA</td>
</tr>
<tr>
<td>All</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD or No. of subjects.

*P<0.01; †P<0.001.

### Coding Sequence Polymorphism and Stroke

Because the G1837A marker is devoid of any demonstrated functional relevance, we performed single-strand conforma-

<table>
<thead>
<tr>
<th>TABLE 3. Genotype Frequencies for the T2238C Polymorphism</th>
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</thead>
<tbody>
<tr>
<td>Genotype</td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>T2238C</td>
</tr>
<tr>
<td>TT</td>
</tr>
<tr>
<td>TC</td>
</tr>
<tr>
<td>CC</td>
</tr>
<tr>
<td>All</td>
</tr>
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</table>

Values are expressed as mean ± SD or No. of subjects.

*P<0.01; †P<0.001.
when a dominant mode of inheritance was assumed. Analyses assuming different modes of inheritance and subgroups were performed as before for the \textit{G1837A} marker; they again yielded materially similar results (Table 5). Adjustment for the other independent predictors for stroke by multivariate analysis revealed an OR for the \textit{G664A} carrier status of 2.09 in the dominant model (95% CI, 1.18 to 3.72; \( P = 0.01 \)) and of 2.03 in the additive model (95% CI, 1.20 to 3.44; \( P = 0.008 \)).

The \textit{G1837A} and \textit{G664A} polymorphisms displayed a highly significant degree of linkage disequilibrium \( (X^2_{\text{ld}} = 299.6; P < 10^{-5}) \), as expected. However, the \textit{G664A} polymorphism showed a weaker degree of linkage disequilibrium with \textit{T2238C} \( (X^2_{\text{ld}} = 6.9; P = 0.01) \).

### Table 4. Genotype Frequencies for the \textit{G664A} Polymorphism

<table>
<thead>
<tr>
<th>Genotype</th>
<th>All Subjects</th>
<th>Low-Risk Subgroup</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>Controls</td>
</tr>
<tr>
<td>GG</td>
<td>306</td>
<td>0.88</td>
</tr>
<tr>
<td>GA</td>
<td>220</td>
<td>0.57</td>
</tr>
<tr>
<td>AA</td>
<td>12</td>
<td>0.03</td>
</tr>
<tr>
<td>All</td>
<td>348</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\( n \) indicates number of subjects; and \( f \), frequency.

### Table 5. ORs of the ANP Coding Mutation for Stroke

<table>
<thead>
<tr>
<th>Model</th>
<th>All Subjects</th>
<th>Low-Risk Subgroup</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR 95% CI</td>
<td>( P )</td>
</tr>
<tr>
<td></td>
<td>OR 95% CI</td>
<td>( P )</td>
</tr>
<tr>
<td>Additive</td>
<td>1.9 1.16–3.12</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>1.9 1.03–3.42</td>
<td>0.04</td>
</tr>
<tr>
<td>Dominant</td>
<td>2.0 1.17–3.39</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>2.0 1.03–3.94</td>
<td>0.04</td>
</tr>
</tbody>
</table>

The additive model is \textit{GG} vs \textit{GA} vs \textit{AA}, and the dominant model, \textit{GG} vs \textit{GA} and \textit{AA}.

### Discussion

The current study indicates that the carrier status for the \textit{G664A} allele of the gene encoding ANP is associated with an increased risk of stroke. Our findings were derived from a large, prospective, case-controlled study, and they provide evidence for a genetic susceptibility factor that contributes to the pathogenesis of polygenic, multifactorial, common forms of stroke independent of other, conventional risk factors. These results are of particular interest because they parallel findings in an experimental model of stroke in which the ANP gene was likewise implicated in stroke susceptibility.\(^{12}\)

ANP exerts powerful natriuretic, diuretic, and vasodilatory effects, and it is well represented in the cerebral areas involved in cardiovascular regulation. Thus, it is reasonable to consider it a logical candidate gene for vascular, including cerebrovascular, disease, particularly in light of previous evidence of significant elevations of circulating ANP levels in stroke patients.\(^{22}\) The biology of ANP has been studied extensively in cardiac atria and ventricles, where upregulation of ANP expression is considered a hallmark of ventricular hypertrophy. Much less is known about its expression and regulation in other tissues, such as the brain (where it is primarily expressed in the hypothalamus, anterior pituitary and, notably, the AV3V region that plays an important role in cardiovascular regulation) or vascular smooth muscle cells. Although extracardiac expression of ANP contributes only a rather small fraction to overall ANP synthesis, local concentrations in the brain and the cerebral vasculature can reach physiologically relevant levels.\(^{23}\)

The \textit{G1837A} polymorphism, for which we initially found a statistically significant association with the incidence of stroke, is located within an intron; it was, therefore, judged unlikely to represent a biologically/functionally relevant mutation. In contrast, the subsequently detected \textit{G664A} variation, which shows a somewhat higher OR of being associated with the phenotype, is consistent with a functionally relevant structural change in the molecule. The possible functional importance of the residue at position 7 of the prosegment of ANP may be gleaned from the fact that this amino acid is conserved in different species\(^{24}\) and that it belongs to the N-terminal region of the proANP peptide that, as cardiodilatin, has well-characterized biological activity of its own.\(^{25,26}\)

In contrast, the \textit{T2238C} mutation, which also changes the coding sequence of ANP (resulting in the addition of 2 extra arginine residues to the translation product) was not significantly associated with stroke. In rats, mice, cattle, and rabbits, the published sequence contains an arginine-arginine dibasic residue at the carboxy terminal that may undergo cleavage during the same processing step that results in the cleavage of
the prohormone to the actual, biologically active 28 amino acid peptide at a monobasic arginine-serine residue site. Therefore, it is possible that the 2 additional arginine residues at the carboxy terminal are no longer present in the mature peptide.

Because of the immediate vicinity of the genes encoding brain natriuretic peptide (BNP) and ANP on the chromosome, our findings could potentially also imply a role for BNP. We found no differences in structure or expression of BNP among stroke-prone and stroke-resistant rat strains (unpublished observations). A polymorphic marker for BNP that we typed in the study sample failed to yield sufficient information to provide additional insights (data not shown).

The full understanding of our present findings, particularly the possible functional consequences of the G664A variant and its potential application to prognosis, prevention, or therapy for stroke, await further studies. However our observations, if confirmed by replication, do provide a new molecular marker for the risk of stroke in humans. The size and prospective character of the present study and the strong parallelism to experimental data support the relevance of our observations.

Acknowledgments
Supported by a Research Career Development Award (K04- HL01318-01), grant R01-HL564101 from the National Heart, Lung, and Blood Institute (to K.L.), a Special Program Grant from the Italian Ministry of Health (IRCCS; to M.V.), a grant from the Italian Telethon (project D.45), and by grants (CA40360 and CA42182) from the National Institutes of Health.

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Circulation. 1999;100:1722-1726
doi: 10.1161/01.CIR.100.16.1722

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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