

Circulating Atrial Natriuretic Peptide Genetic Association Study Identifies a Novel Gene Cluster Associated With Stroke in Whites

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Background—The goal of this study was to identify genetic determinants of plasma N-terminal proatrial natriuretic peptide (NT-proANP) in the general community by performing a large-scale genetic association study and to assess its functional significance in *in vitro* cell studies and on disease susceptibility.

Methods and Results—Genotyping was performed across 16 000 genes in 893 randomly selected individuals, with replication in 891 subjects from the community. Plasma NT-proANP₁₋₉₈ concentrations were determined using a radioimmunoassay. Thirty-three genome-wide significant single-nucleotide polymorphisms were identified in the *MTHFR-CLCN6-NPPA-NPPB* locus and were all replicated. To assess the significance, *in vitro* functional genomic studies and clinical outcomes for carriers of a single-nucleotide polymorphism rs5063 (V32M) located in *NPPA* that represented the most significant variation in this genetic locus were assessed. The rs5063 variant allozyme in transfected HEK293 cells was decreased to 55±8% of wild-type protein ($P=0.01$) as assessed by quantitative western blots. Carriers of rs5063 had lower NT-proANP levels (1427 versus 2291 pmol/L; $P<0.001$) and higher diastolic blood pressures (75 versus 73 mmHg; $P=0.009$) and were at an increased risk of stroke when compared with wild-type subjects independent of age, sex, diabetes mellitus, hypertension, atrial fibrillation, and cholesterol levels (hazard ratio, 1.6; $P=0.004$).

Conclusions—This is the first large-scale genetic association study of circulating NT-proANP levels performed with replication and functional assessment that identified genetic variants in the *MTHFR-CLCN6-NPPA-NPPB* cluster to be significantly associated with NT-proANP levels. The clinical significance of this variation is related to lower NT-proANP levels, higher blood pressures, and an increased risk of stroke in the general community. (*Circ Cardiovasc Genet.* 2015;8:141-149. DOI: 10.1161/CIRCGENETICS.114.000624.)

Key Words: atrial natriuretic factor ■ heart failure ■ hypertension ■ genetic association studies ■ stroke

Atrial natriuretic peptide (ANP) has vasodilator and natriuretic effects and plays an important role in the pathophysiology of heart failure (HF) and hypertension.¹ The *NPPA* gene encodes the 151-amino acid prepropeptide that is cleaved, resulting in proANP₂₆₋₁₅₁ which, in turn, undergoes proteolysis to form N-terminal proANP (NT-proANP₂₆₋₁₂₃) and ANP₁₂₄₋₁₅₁. NT-proANP levels correlate well with ANP levels; however, the advantage of measuring NT-proANP level is that it is technically easier, more stable, reproducible, and has a longer half-life than ANP.^{2,3} We have previously shown that the propensity to develop hypertension is because of a relative deficiency in ANP.⁴ We have also demonstrated in subjects free of HF in the Olmsted County Community Cohort that the NT-proANP level is independently predictive of death, development of HF, and myocardial infarction (MI).⁵

Clinical Perspective on p 149

Variation in ANP levels may be due in part to genetic variation. Disruption of the *NPPA* gene in mice leads to alteration of circulating ANP levels and hypertension, and heterozygous mutants develop salt-sensitive hypertension.⁶ Conversely, gains of functional genetic variants in the *NPPA* gene have been shown to be associated with higher NT-proANP levels, lower systolic and diastolic blood pressures, and a decreased risk of hypertension.⁷ There have been no large-scale genetic association studies performed for circulating NT-proANP concentrations. Identifying the genetic determinants of NT-proANP levels may help us understand the pathophysiology of hypertension and sequelae, such as stroke, MI, and HF. Therefore, we undertook a genetic association study to identify possible

Received March 27, 2014; accepted October 10, 2014.

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The Data Supplement is available at <http://circgenetics.ahajournals.org/lookup/suppl/doi:10.1161/CIRCGENETICS.114.000624/-DC1>.

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Circ Cardiovasc Genet is available at <http://circgenetics.ahajournals.org>

DOI: 10.1161/CIRCGENETICS.114.000624

genetic determinants of circulating NT-proANP and assess its significance by performing functional genomic studies in vitro and analyzing clinical outcomes in 1784 randomly selected subjects from Olmsted County, MN.

Methods

The Mayo Clinic Institutional Review Board approved this study, and written informed consent was obtained from all subjects.

Study Population

The cohort studied consisted of a random sample of residents from Olmsted County, MN, aged 45 years or older who were first characterized as part of the National Institutes of Health–funded Prevalence of Left Ventricular Dysfunction Study (PAVD) and has been previously described.⁸ There were 2027 subjects in this cohort who had adequate quality and quantity of DNA samples for genotyping. After quality control, there were 1784 subjects in whom plasma NT-proANP levels had been measured who were included in the final analysis. The subjects recruited were randomly separated into 2 cohorts. The discovery cohort that comprised of 893 randomly selected subjects from the PAVD study and the Replication Cohort consisting of the remaining 891 samples were used for the replication study.

Genotyping

Genotyping was performed using the Metabochip, a custom Illumina iSelect genotyping array. A total of 2112 samples comprising both the discovery and replication cohorts were genotyped, including duplicates and Centre Etude Polymorphism Humain DNA controls. Samples were dropped if the call rate was <98% or if there were sex errors or duplicates. A high rate of concordance (99%) was observed for intentionally duplicated samples. PLINK software was used to estimate the relatedness between samples and those with a PI HAT value of >0.2, reflecting twins, parent-offspring or full sibling, or other close relatedness were identified, and 1 sample from each of those pairs was excluded. Multidimensional scaling was performed to identify nonwhite subjects who were then dropped from the analysis (n=2). There were 196725 single-nucleotide polymorphisms (SNPs) that were genotyped across ≈16000 genes. Using a per-SNP call rate of >98%, 5.7% of the samples were dropped. There were 879 SNPs that failed the Hardy Weinberg equilibrium model and were flagged. SNPs with a minor allele frequency of <0.01 were also dropped. After quality control, 124590 SNPs and 1931 samples remained for further analysis. NT-proANP data were available for 1784 of these samples. SNPs in the autosomal chromosomes were phased using SHAPEIT version 1 and imputed using IMPUTE version 2.2.2 with the 1000 genomes PHASE 1 data as a reference. Genetic variation in the natriuretic peptide system, including the *NPR1* (45 SNPs), *NPR2* (44 SNPs), *NPR3* (85 SNPs), and *MME* (747 SNPs) genes, was covered by genotyping and imputation.

Natriuretic Peptide Assay

Plasma NT-proANP₁₋₉₈ levels were measured in our laboratory using a radioimmunoassay (Phoenix Pharmaceuticals, Belmont, CA).⁹ Interassay and intra-assay coefficients of variation for NT-proANP were measured and were 9% and 6% respectively.

Two-Dimensional and Doppler Echocardiography

Two-dimensional and Doppler imaging echocardiography was performed as per a standardized protocol as previously described.⁸ Left ventricular (LV) dimension and mass left atrial volume were calculated from M-mode and 2-dimensional measurements, respectively, and were indexed to body surface area.¹⁰ The presence of LV hypertrophy was defined based on an LV mass index of >130 g/m² for men and of >100 g/m² for women.¹¹

In Vitro ANP Protein Expression Analysis

The human NPPA cDNA and mutant rs5063 constructs were obtained from OriGene (Rockville, MD). HEK293 cells were cultured in

DMEM (ATCC, 30-2002) with 10% fetal bovine serum. Transfections were performed with an empty vector, wild-type (WT) and variant allozyme for NPPA using Lipofectamine 2000 (Invitrogen, Grand Island, NY). β-Galactosidase DNA (Promega, Madison, WI) was co-transfected to correct for variation in transfection efficiency. The cells were harvested, and whole cell lysate was prepared after 48 hours. Experiments were performed in triplicate.

Protein expression was determined by quantitative western blot analysis. Whole cell lysates were subjected to electrophoresis on TGX gels and then transferred to polyvinylidene fluoride membranes (BioRad, Hercules, CA), which were blocked with a purified rabbit anti-NPPA antibody (Phoenix Pharmaceuticals, Burlingame, CA), followed by incubation with a secondary antibody. Immunoreactive proteins were detected with the Immun-Star WesternC kit (BioRad, Hercules, CA), and Gel Doc XR system (BioRad, Hercules, CA) was used to quantify proteins; data were normalized to β-galactosidase concentrations.

Clinical Outcome Measures

Participants were followed for a median follow-up time of 12.2 years (interquartile range, 10.6–13.0, maximum=14.5). In addition to all-cause mortality, participants were monitored with respect to HF, MI, and cerebrovascular accident. HF was defined as International Classification of Diseases-Ninth Revision (ICD-9) code 402 or 428. Stroke and transient ischemic attack were grouped together under the term cerebrovascular accident and included ICD-9 codes 430 to 438. MI was defined as ICD-9 code 410 or 412.

Statistical Analysis

Association analyses were performed using PLINK and R, separately for each cohort. The relationship between NT-proANP and genetic markers was modeled using ordinary multiple linear regression. NT-proANP was transformed on the log scale to remove skewness; SNP genotype effects were assumed to be additive, and age and sex were included in models to control for their effects on the phenotype.

Group characteristics, based on cohort and genotype subgroups, are presented with frequency and percentage and compared between groups using Pearson χ^2 test for categorical variables. Continuous variables are presented as mean and SD or median and quartiles and compared between groups using 2-sample *t* test or nonparametric Wilcoxon signed-rank sum test, depending on distribution. Because of the low minor allele frequency of rs5063, analyses presented in this report use the dominant genotype model. Time to event variables was defined based on date of event or last known follow-up for those without event. Kaplan–Meier curves were created to show the relationship between genotype and outcomes, including death, MI, stroke, HF, and atrial fibrillation. The association of genotypes and other baseline characteristics were analyzed using Cox proportional hazards regression methods. The proportional hazards assumption was tested by plotting and visual examination of scaled Schoenfeld residuals versus time, and significance was tested via correlation of residuals with rank time. No violations of the proportional hazards assumption were noted. All tests were conducted using 2-sided *P* values. Mean protein values were compared using the standard 2-sample *t* test.

Results

Study Population

There were 1784 subjects who were randomly divided into discovery (n=893) and replication (n=891) cohorts. Baseline demographics for these subjects are listed in Table I in the Data Supplement. There were no significant differences between the 2 groups in age, sex, or body mass index or comorbidities, including hypertension, diabetes mellitus, and coronary artery disease. Renal function measured by creatinine and calculated glomerular filtration rate were within normal limits and similar between the 2 groups. LV ejection fraction was preserved, LV mass, and diastolic function parameters, such as E/e' ratio,

were also similar in both groups. Plasma NT-proANP measurements, including the variability in these measurements, were similar in the discovery and replication cohorts, with a median NT-proANP level in the discovery cohort of 2215 pmol/L (interquartile range, 1372–3337) and 2174 pmol/L (interquartile range, 1436–3190) in the replication cohort.

Genotyping Results in the Discovery and Replication Cohorts

The Manhattan plot of data for both genotyped and imputed SNPs for the discovery cohort is presented in Figure 1. The QQ plot that displays the potential for P value inflation is shown in Figure I in the Data Supplement with a genomic inflation factor of 0.994, demonstrating adequate adjustment for population stratification with no apparent inflation of type 1 error. The significance threshold after correcting for multiple testing was 3.9×10^{-7} . This threshold was also used for the replication study because the entire analysis that was performed in the discovery cohort was repeated in the replication cohort. In both cohorts, as shown in the Manhattan plot in Figure 1 for the discovery cohort and in Table I in the Data Supplement for both cohorts, the genome-wide highly significant SNPs were mapped to chromosome 1. Table 1 shows the results from combined analyses of all SNPs with association, $P \leq 9.6 \times 10^{-7}$, in the discovery cohort. There were 38 SNPs on chromosome 1, which were significant in the discovery cohort, 33 of which were validated in the replication cohort with similar P values. Among these 33 SNPs, 24 SNPs were genotyped and 9 were imputed using 1000 Genomes Project data as a reference. The top 10 SNPs were all genotyped SNPs and had association P values in the range of $\leq 10^{-13}$. The SNP with the lowest P value, rs75747410, in the discovery cohort had a minor allele frequency of 5.3% and was located in an intron of *CLCN6*, a chloride ion channel. The effect of all of the significant SNPs on NT-proANP levels was in the range of 0.63- to 0.76-fold change per minor allele in the discovery cohort and 0.62- to 0.81-fold change

per minor allele in the replication cohort. The directionality of the effect of these SNPs on NT-proANP levels was identical in both the discovery and replication cohorts. The location of these SNPs and information on their linkage disequilibrium are shown graphically in the locus zoom plot in Figure 2. Most of the significant SNPs mapped to the *MTHFR-CLCN6-NPPA-NPPB* locus were in linkage disequilibrium ($r^2 \geq 0.6$) with each other, as seen in Figure II in the Data Supplement. The 33 significant SNPs in both the discovery and replication cohorts that were not within this gene cluster were present in *LOC100506273*, *LOC100506273*, and a small nuclear RNA gene, *RNU5E-1*. Among the significant variants, 15 were present in the 5'-upstream or promoter regions, 12 were in introns, and 6 were present in exons of various genes within the cluster. There were 2 nonsynonymous SNPs, rs2274976 (R594Q) in *MTHFR*, a gene involved in the homocysteine pathway, and rs5063 (V32M) in the *NPPA* gene encoding the precursor peptide of NT-proANP. The SNP rs5063 was the most significant SNP ($P = 1.14 \times 10^{-26}$) in the combined analysis (Table 1).

In Vitro ANP Protein Expression Levels

The nonsynonymous SNP rs5063 located in the *NPPA* gene was in high linkage disequilibrium ($r^2 > 0.8$) with 21 other genotyped SNPs that were significant in both cohorts and hence represented most of the significant genetic variations in the *MTHFR-CLCN6-NPPA-NPPB* locus. To determine the functional effect of this SNP on *NPPA* expression, WT cDNA and rs5063 variant construct were transfected into HEK293 cells, and protein levels were measured by quantitative western blots. The rs5063 allozyme was decreased to $55 \pm 8\%$ of WT protein ($P = 0.01$; Figure 3).

Genetic Association With Clinical Outcomes

To assess its functional significance on clinical outcomes and disease susceptibility, subjects homozygous for the minor allele (T/T) for rs5063 were pooled with heterozygous

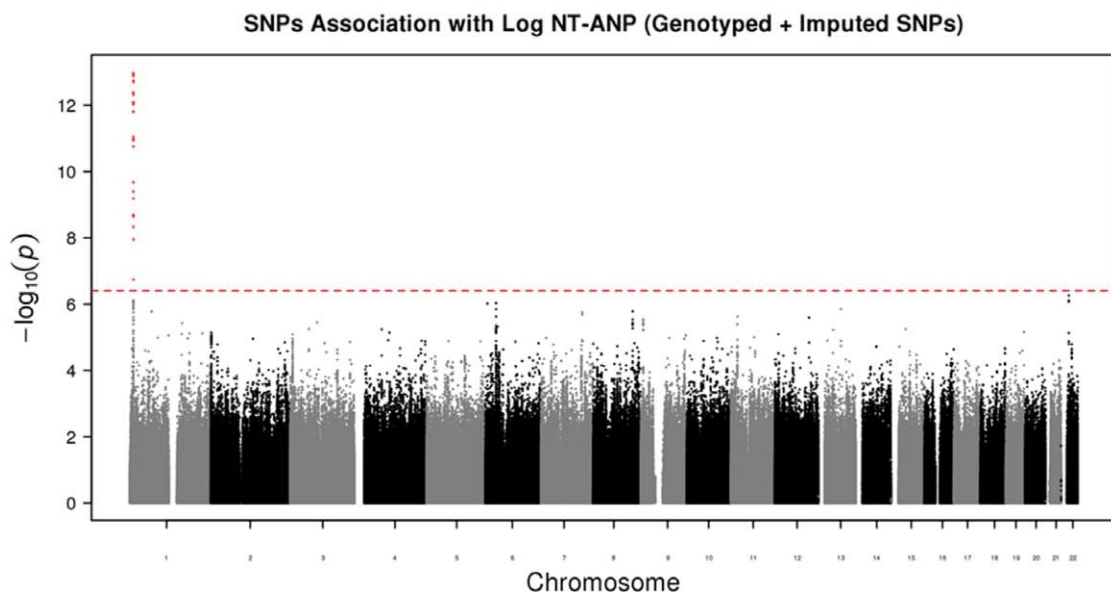


Figure 1. Manhattan plot of the results of the large-scale genetic association study of circulating atrial natriuretic peptide levels in the general community. The red line represents a P value of 3.9×10^{-7} , the level required for statistical significance. NT-ANP indicates N-terminal atrial natriuretic peptide; and SNPs, single-nucleotide polymorphisms.

Table 1. Significant Genetic Associations With N-Terminal proAtrial Natriuretic Peptide Concentrations in the Combined Cohort

SNP	Chromosome	Position (bp)	Gene	Gene Type	Variant Location	Genotyped or Imputed	r ² Imputation Quality	Minor Allele	Common Allele	MAF	Fold Change	P Value
rs5063	1	11 907 648	NPPA/NPPA-AS1	Protein coding/ miscRNA	Missense/ rna_exon	Genotyped	NA	T	C	0.052	0.629	1.14×10 ⁻²⁶
rs141308438	1	11 906 469	NPPA/NPPA-AS1	Protein coding/ miscRNA	Intron/intron	Imputed	0.9983	T	C	0.052	0.630	2.03×10 ⁻²⁶
rs3737965	1	11 866 451	CLCN6	Protein coding	Intron	Genotyped	NA	A	G	0.052	0.632	3.67×10 ⁻²⁶
rs75747410	1	11 881 727	CLCN6	Protein coding	Intron	Genotyped	NA	A	G	0.053	0.633	4.21×10 ⁻²⁶
rs77072136	1	11 888 061	CLCN6	Protein coding	Intron	Genotyped	NA	T	C	0.053	0.633	4.21×10 ⁻²⁶
rs41275484	1	11 889 225	CLCN6	Protein coding	Intron	Genotyped	NA	A	G	0.053	0.633	4.21×10 ⁻²⁶
rs79811212	1	11 892 094	CLCN6	Protein coding	Intron	Genotyped	NA	C	T	0.053	0.633	4.21×10 ⁻²⁶
rs41275500	1	11 897 082	CLCN6	Protein coding	Synonymous	Genotyped	NA	T	C	0.053	0.633	4.21×10 ⁻²⁶
rs7552330	1	11 904 092	NPPA-AS1	miscRNA	rna_exon	Genotyped	NA	A	G	0.053	0.633	4.21×10 ⁻²⁶
rs3753586	1	11 864 262	MTHFR	Protein coding	Intron	Imputed	0.9994	C	T	0.052	0.633	8.85×10 ⁻²⁶
rs3753585	1	11 864 378	MTHFR	Protein coding	Intron	Imputed	0.9994	A	G	0.052	0.633	8.86×10 ⁻²⁶
rs17037397	1	11 862 163	MTHFR	Protein coding	Intron	Genotyped	NA	A	C	0.052	0.633	9.84×10 ⁻²⁶
rs2272803	1	11 898 789	CLCN6	Protein coding	Intron	Genotyped	NA	T	G	0.052	0.636	1.68×10 ⁻²⁵
rs2076003	1	11 884 147	CLCN6	Protein coding	Intron	Genotyped	NA	C	T	0.052	0.636	1.69×10 ⁻²⁵
rs2075539	1	11 897 758	CLCN6	Protein coding	Intron	Genotyped	NA	A	G	0.053	0.637	1.91×10 ⁻²⁵
rs3737967	1	11 847 449	MTHFR/ LOC100506310	Protein coding/ protein coding	3'UTR/ missense	Imputed	0.9984	A	G	0.051	0.639	3.48×10 ⁻²⁴
rs2274976	1	11 850 927	MTHFR	Protein coding	Missense	Genotyped	NA	T	C	0.051	0.643	1.26×10 ⁻²³
rs77012921	1	11 929 761	LOC390997	Pseudo	5'upstream	Imputed	1	C	T	0.043	0.626	2.22×10 ⁻²³
rs75030554	1	11 931 445	LOC390997	Pseudo	5'upstream	Genotyped	NA	T	C	0.043	0.626	2.22×10 ⁻²³
rs116525098	1	11 931 772	LOC390997	Pseudo	5'upstream	Imputed	0.9972	T	C	0.043	0.626	3.01×10 ⁻²³
rs79712000	1	11 924 030	NPPB	Protein coding	5'upstream	Genotyped	NA	C	T	0.043	0.629	3.07×10 ⁻²³
rs77003042	1	11 926 747	NPPB	Protein coding	5'upstream	Imputed	1	G	T	0.043	0.629	3.07×10 ⁻²³
rs74714707	1	11 926 758	NPPB	Protein coding	5'upstream	Genotyped	NA	T	C	0.043	0.629	3.07×10 ⁻²³
rs75540316	1	11 926 898	NPPB	Protein coding	5'upstream	Genotyped	NA	T	G	0.043	0.629	3.07×10 ⁻²³
rs79653311	1	11 927 878	NPPB	Protein coding	5'upstream	Genotyped	NA	C	T	0.043	0.629	3.07×10 ⁻²³
rs61757273	1	11 909 736	NPPA	Protein coding	5'upstream	Genotyped	NA	T	G	0.045	0.640	3.69×10 ⁻²²
rs114941496	1	11 826 085	LOC100506273	Unknown	rna_exon	Genotyped	NA	A	G	0.048	0.649	8.77×10 ⁻²²
rs79837245	1	11 932 847	LOC390997	Pseudo	5'upstream	Genotyped	NA	A	T	0.047	0.648	2.35×10 ⁻²¹
rs113823536	1	11 935 715	LOC390997	Pseudo	5'upstream	Imputed	1	G	T	0.056	0.703	3.86×10 ⁻¹⁷
rs79593079	1	11 936 644	LOC390997	Pseudo	5'upstream	Imputed	0.9953	T	C	0.056	0.703	4.64×10 ⁻¹⁷
rs60600557	1	11 935 166	LOC390997	Pseudo	5'upstream	Genotyped	NA	A	G	0.056	0.705	6.24×10 ⁻¹⁷
rs78097221	1	11 935 443	LOC390997	Pseudo	5'upstream	Imputed	1	A	C	0.056	0.706	1.16×10 ⁻¹⁶
rs58120150	1	11 925 244	NPPB	Protein coding	5'upstream	Genotyped	NA	C	T	0.057	0.711	2.57×10 ⁻¹⁶
rs80177860	1	11 934 890	LOC390997	Pseudo	5'upstream	Imputed	0.9996	C	G	0.060	0.726	3.75×10 ⁻¹⁵
rs7545290	1	11 934 928	LOC390997	Pseudo	5'upstream	Genotyped	NA	C	T	0.061	0.728	4.08×10 ⁻¹⁵
rs11803049	1	11 928 895	LOC390997	Pseudo	5'upstream	Genotyped	NA	A	G	0.070	0.744	4.62×10 ⁻¹⁵
rs112521149	1	11 889 815	CLCN6	Protein coding	Intron	Imputed	0.8924	A	G	0.108	0.783	1.60×10 ⁻¹³
rs148216773	1	11 969 569	RNU5E-1	snRNA	3'downstream	Imputed	0.9433	G	C	0.061	0.766	3.17×10 ⁻¹⁰

MAF indicates minor allele frequency; NA, not applicable; and SNP, single-nucleotide polymorphism.

subjects (T/C) and compared with individuals homozygous for the major allele (C/C). The genotype frequencies for rs5063 in these groups are as follows: C/C 90%, C/T or T/T 10%. The clinical characteristics of these subjects by genotype are presented in Table 2. Median NT-proANP levels were significantly lower, and mean diastolic blood pressures were significantly

higher in the carriers of the rs5063 minor allele (1427 pmol/L; 75±10.87 mmHg) when compared with subjects homozygous for the WT allele (2291 pmol/L; 73.4±10.18 mmHg; age and sex adjusted, $P<0.001$ and $P=0.009$, respectively). From a cardiac structural and functional perspective, there was a weak trend for a greater proportion of subjects with LV hypertrophy

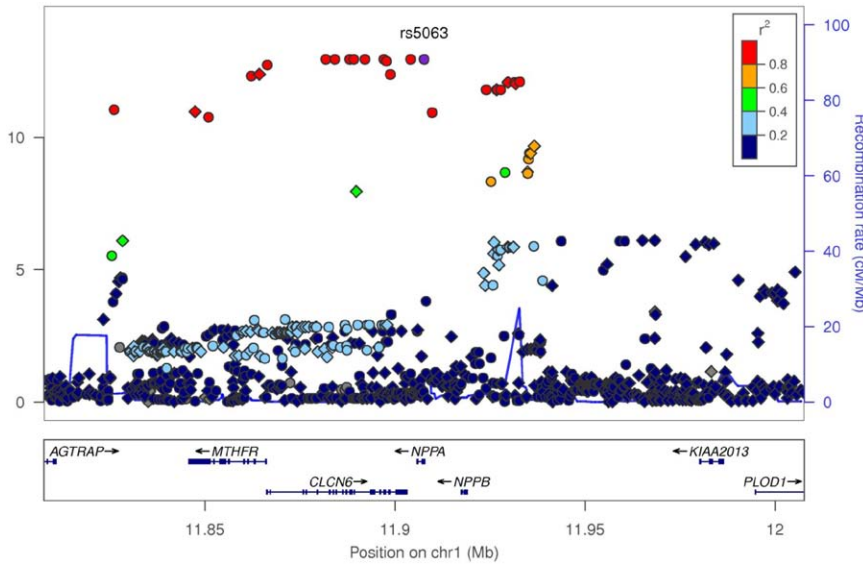


Figure 2. Genetic associations with atrial natriuretic peptide concentrations across the chromosome 1 (chr 1) region of interest. Manhattan plot of $-\log_{10}(P)$ values from conditional logistic regression for observed (blue circle) and imputed (blue diamond) single-nucleotide polymorphism (SNP) genotypes according to their physical location. The recombination rate in centimorgan per megabase (blue line) and the linkage disequilibrium (r^2) of each SNP with the rs5063 SNP are shown.

among the carriers (15%) than WT (11%) subjects ($P=0.09$), with the former group also demonstrating longer deceleration times (235 ± 43 versus 229 ± 39 ms; $P=0.03$), suggestive of early diastolic relaxation abnormalities.

There was a significant difference in the occurrence of cerebrovascular accident between the 2 groups. At 10 years, 26% of subjects who were either homozygous or heterozygous for the rs5063 minor allele had a cerebrovascular accident, compared with 17% of WT subjects (age and sex adjusted, $P=0.009$; Figure 4). As depicted in Table 3, in addition to age and sex, after adjusting for other clinical covariates, such as diabetes mellitus, hypertension, atrial arrhythmias, and serum cholesterol, the carrier status of the rs5063 genotype remained strongly associated with stroke, with a hazard ratio (HR) of 1.6 ($P=0.004$) comparable with hypertension (HR, 1.8) and atrial arrhythmias (HR, 1.6). The variables included in Table 3 are known risk factors of stroke.^{12,13} Interestingly, the effect of the genotype on stroke was independent of NT-proANP plasma concentrations, as seen in Table II in the Data Supplement. In addition to clinical covariates, after adjusting for NT-proANP levels in Table II in the Data Supplement, the rs5063 genotype remains significantly associated with stroke (HR, 1.6; $P=0.006$). Carriers of rs5063 had a higher combined composite end point of stroke, MI, HF, and death when compared with WT subjects (age and sex adjusted, $P=0.025$). However, this end point was primarily driven by an increased incidence of stroke.

Discussion

This is the first large-scale genetic association study of circulating NT-proANP levels performed, which identified and replicated genetic markers in the *MTHFR-CLCN6-NPPA-NPPB* genetic locus to be associated with variation in NT-proANP levels. The genetic regulation of ANP has important implications in the pathophysiology and treatment of cardiovascular disease. We performed this genetic association study using DNA from 893 randomly selected subjects and replicated these results in an additional 891 subjects from Olmsted County, MN. We have demonstrated the functional significance of this genetic variation by relating it to reduced

NT-proANP levels, higher blood pressures, and an increased risk of stroke in the general community. This is also the first study to demonstrate that the association of this genotype with stroke was independent of other important clinical predictors of stroke, such as age, sex, hypertension, diabetes mellitus, atrial arrhythmias, and serum cholesterol levels. The HR of the association of this allele with stroke, 1.6, was comparable with that of hypertension and atrial arrhythmias. The strength of this study is that the association of this genetic variant with stroke was evaluated in a large community-based cohort, and hence, these results may be applicable to a general and not just a disease-specific population of white ancestry. An additional strength was measurement of NT-proANP levels, and detailed phenotypic description could provide a pathophysiological explanation of the association of the rs5063 genotype with the clinical outcome of stroke. A limitation of this study is that

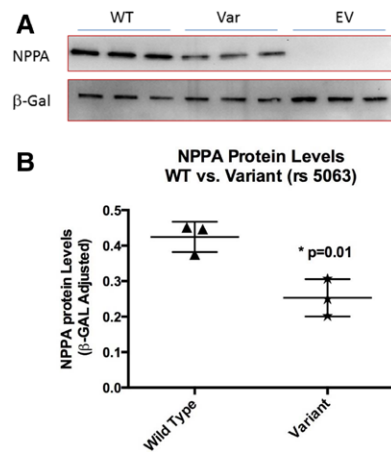


Figure 3. Atrial natriuretic peptide (*NPPA*) functional genomics. **A**, Western blot analysis showing *NPPA* wild-type (WT) protein expression when compared with rs5063 variant (Var), *NPPA* protein expression for constructs with nonsynonymous single-nucleotide polymorphisms expressed in HEK293 cells. EV denotes transfection with an empty vector. **B**, A dot plot with *NPPA* immunoreactive protein levels of rs5063 allele when compared with WT protein. Each dot represents independent transfection experiments. All values are corrected for transfection efficiency.

Table 2. Clinical Characteristics, Including Cardiovascular Outcomes of the Study Population Based on the NPPA rs5063 Genotype

Variable	rs5063		P Values	
	C/C, n=1736	C/T or T/T, n=195	Unadjusted	Age/Sex Adjusted
Age at examination	62.36±10.30	61.93±10.51	0.58	...
Sex, n (%)	906 (52%)	107 (55%)	0.48	...
BMI of patient	28.38±5.26	28.55±5.26	0.68	0.66
Diabetes mellitus, n (%)	118 (7%)	13 (7%)	0.95	0.96
CAD, n (%)	179 (10%)	26 (13%)	0.19	0.08
Verified hypertension, n (%)	480 (28%)	44 (23%)	0.13	0.14
Former/current smoker, n (%)	852 (49%)	102 (52%)	0.39	0.31
Afib/flutter, n (%)	66 (4%)	6 (3%)	0.61	0.73
Total cholesterol	203.64±35.52	202.31±36.25	0.62	0.51
HDL cholesterol	46.15±14.47	44.69±13.33	0.18	0.07
Systolic blood pressure	132.31±20.95	134.11±23.28	0.26	0.15
Diastolic blood pressure	73.40±10.18	75.14±10.87	0.024	0.009
Heart rate	66.19±11.42	66.08±10.50	0.89	0.84
Creatinine, median (Q1, Q3)	0.8 (0.7, 1.0)	0.8 (0.7, 0.9)	0.13	0.31
Calculated GFR (MDRD)	81.20±17.78	82.63±16.69	0.29	0.36
NT-proANP, median (Q1, Q3)	2291.0 (1475.0, 3388.0)	1427.0 (884.0, 2316.0)	<0.001	<0.001
Diastolic dysfunction, n (%)	445 (28%)	51 (29%)	0.93	0.98
Ejection fraction	63.18±6.48	63.17±6.16	0.99	0.82
Deceleration time	229.22±38.75	234.47±42.63	0.08	0.03
PA systolic pressure	22.49±4.81	22.73±5.16	0.59	0.61
LA volume	46.87±20.26	46.25±16.29	0.69	0.95
E/A ratio	1.10±0.39	1.10±0.37	0.95	0.85
E/e'	8.54±3.02	8.86±2.92	0.21	0.21
LV hypertrophy, n (%)	146 (11%)	23 (15%)	0.11	0.09
Average LV mass index	95.99±21.34	97.28±21.75	0.48	0.23
LVESD	2.94±0.49	2.98±0.44	0.41	0.33
LVEDD	4.89±0.49	4.96±0.47	0.08	0.04
Death, K–M estimates (cum. no of events)			0.97	0.98
1 y	1.00 (6)	1.00 (0)
5 y	0.96 (75)	0.96 (8)
10 y	0.89 (185)	0.88 (21)
CHF, K–M estimates (cum. no of events)			0.19	0.18
1 y	0.99 (21)	1.00 (0)
5 y	0.95 (79)	0.96 (6)
10 y	0.90 (152)	0.86 (21)
Stroke, K–M estimates (cum. no of events)			0.012	0.009
1 y	0.97 (46)	0.95 (9)
5 y	0.91 (139)	0.83 (30)
10 y	0.83 (256)	0.74 (43)
Afib, K–M estimates (cum. # events)			0.82	0.78
1 y	0.98 (29)	0.98 (3)
5 y	0.94 (100)	0.93 (12)
10 y	0.87 (188)	0.86 (22)
MI, K–M estimates (cum. # events)			0.86	0.81
1 y	0.98 (32)	0.99 (2)
5 y	0.95 (80)	0.95 (8)
10 y	0.90 (144)	0.90 (16)
Combined stroke, MI, CHF, Death, K–M estimates (cum. # events)			0.032	0.025
1 y	0.94 (93)	0.94 (11)
5 y	0.83 (280)	0.77 (41)
10 y	0.69 (492)	0.60 (67)

Outcomes are summarized by 1, 5, and 10 y survival estimates from Kaplan–Meier analyses with cum. numbers of events observed within each time period. Unadjusted and adjusted *P* values for outcomes from Cox regression analyses. Afib indicates atrial fibrillation; BMI, body mass index; CAD, coronary artery disease; CHF, congestive heart failure; cum, cumulative; GFR, glomerular filtration rate; HDL, high-density lipoprotein; K–M, Kaplan–Meier; LA, left atrial; LV, left ventricular; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; MDRD, Modification of Diet in Renal Disease study; MI, myocardial infarction; NT-proANP, N-terminal proatrial natriuretic peptide; and PA, pulmonary artery.

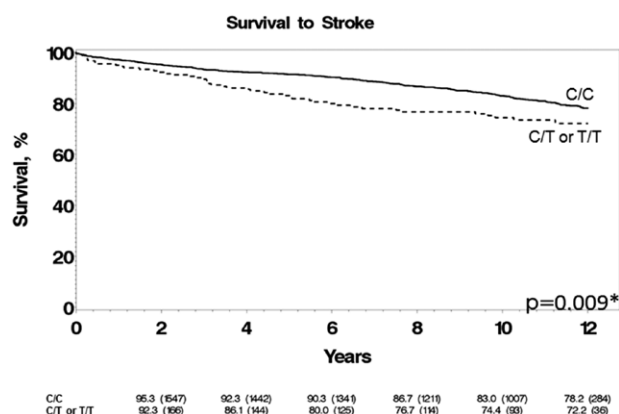


Figure 4. Kaplan–Meier curve demonstrating stroke free survival according to carrier status of the *NPPA* rs5063 genotype. Numbers below the figure represent the survival estimate (number at risk) and *P* value from Cox regression analysis, including age and sex as covariates.

subjects from Olmsted County, MN, where the study is based at are primarily whites, and therefore, results of this cannot be generalized to subjects of other ancestries.

Genetic variants in the 5′-upstream region of *NPPA* or promoter variants (eg, C-664G and A-2843G) have been associated with decreased circulating ANP levels.^{14,15} Both the 5′-upstream and 3′-downstream regions, −3.2 kbp and +3.7 kbp, relative to the transcription start site of *NPPA*, have multiple transcription factor binding sites that could serve as activator or repressor sequences and could regulate *NPPA* gene expression.¹⁶ We have identified a genetic variant, 1334 base pairs (bp) upstream from the 5′ region of *NPPA*, rs61757273 that was significantly associated with NT-proANP levels ($P=1.15\times 10^{-14}$ in the discovery cohort and $P=7.64\times 10^{-12}$ in the replication cohort). A biologically plausible nonsynonymous SNP rs5063 that substitutes valine with methionine at residue 32 in *NPPA* was also significantly associated with NT-proANP levels in our study.

A study by Newton-Cheh et al⁷ genotyped for only 13 SNPs in the *NPPA-NPPB* locus in 29 717 subjects and resulted in the identification of rs5068 ($P=8\times 10^{-70}$) in *NPPA* and rs198358 ($P=8\times 10^{-30}$) in the *NPPA-antisense RNA 1 (NPPA-ASI)* gene as being associated with higher NT-proANP levels, lower systolic blood pressure, and a reduced risk of hypertension.

Table 3. Multivariable Logistic Regression Model for Stroke

Variable	<i>P</i> Value	Hazard Ratio	95% LCL	95% UCL
Age at examination	<0.0001	1.08	1.07	1.09
Sex, female	0.07	0.81	0.65	1.02
Diabetes mellitus	0.35	1.18	0.83	1.68
Verified hypertension	<0.0001	1.80	1.44	2.25
Afib/flutter	0.02	1.58	1.07	2.34
Cholesterol	0.76	1.00	1.00	1.00
BMI	0.69	1.00	0.98	1.03
Former/current smoker	0.01	1.34	1.07	1.67
rs5063 C/T or T/T	0.003	1.61	1.18	2.21

Afib indicates atrial fibrillation; BMI, body mass index; LCL, lower control limit; and UCL, upper control limit.

In our study, when using a large-scale genotyping array covering 16 000 genes, neither of these SNPs (rs5068, $P=0.002$ and rs198358, $P=0.01$) met our study's significance threshold (3.9×10^{-7}). Newton-Cheh et al⁷ also identified those SNPs to be significant genetic determinants of brain natriuretic peptide (BNP) levels; however, a more recent genome-wide association study (GWAS) of circulating NT-proBNP levels did not validate either rs5068 or rs198358 to be significantly associated with BNP concentrations.¹⁷ Our large-scale genetic association study with NT-proANP levels and the GWAS of NT-proBNP levels highlights the importance of an agnostic approach in determining genetic variants and the potential bias of candidate gene studies.

Although the *NPPA-ASI* rs198358 SNP was not validated in our study in our cohort, rs7552330, an SNP, in that same gene was significantly associated with NT-proANP levels ($P=1.11\times 10^{-13}$ in the discovery cohort and $P=1.25\times 10^{-14}$ in the replication cohort). The transcripts for *NPPA-ASI* and *NPPA* can form RNA duplexes and result in post-transcriptional downregulation of *NPPA* expression and, therefore, could affect NT-proANP levels.¹⁸ Therefore, the *NPPA-ASI* gene could play a role in the regulation of NT-proANP levels.

The importance of the gene encoding chloride channel 6 (*CLCN6*) in the regulation of natriuretic peptides was demonstrated in a GWAS performed in 3071 subjects to identify genetic determinants of circulating BNP levels.¹⁷ An intronic SNP in *CLCN6*, rs1023252 (meta-analysis $P=3.5\times 10^{-14}$), was identified and replicated as a GWA significant determinant of BNP levels. In our study, 27% of the SNPs that met significance in both the discovery and replication cohorts were all in the intronic region of *CLCN6*. It is being increasingly recognized that introns can serve as binding sites for transcription factors, and intronic transcripts can be a type of long noncoding RNA that can regulate gene expression.¹⁹ Therefore, it is conceivable that *CLCN6* could play an important role in regulating the expression of not only *NPPB* but also *NPPA*. Finally, 6 SNPs in our study that were in the 5′ flanking region of *NPPB* were significantly associated with NT-proANP levels. It is possible that these regions share transcription factor binding motifs that regulate the expression of both natriuretic peptides.

Identifying significant genetic variants by genetic association studies alone does not support their causal role, and hence, functional characterization is also an important component of such association studies. To assess functional significance of these variants, in vitro cell-based studies and clinical outcomes and disease susceptibility of carriers of rs5063 that represented the most significant variation in this genetic locus were analyzed. ANP protein expression levels were reduced by 45% in rs5063 transfected HEK293 cells when compared with WT, indicative of a direct functional effect of the SNP on ANP protein levels. The most common mechanism responsible for decreased levels of protein in association with nonsynonymous SNPs is accelerated degradation involving the ubiquitin–proteasome system and autophagy as we have previously demonstrated.^{20,21}

Variation in circulating NT-proANP levels has important clinical implications, and therefore, identifying genetic modifiers of NT-proANP levels has clinical relevance. Circulating NT-proANP is significantly reduced in patients with metabolic

syndrome and in hypertensive patients and is associated with increased LV mass.²² We have also previously shown that the propensity to develop hypertension is because of a relative deficiency in natriuretic peptides, including NT-proANP.² Higher NT-proANP levels were predictive of a higher cardiovascular risk score and of cardiovascular disease independent of risk assessment profiles, such as the Progetto Cuore or Framingham risk scores.²³ We previously confirmed these findings in subjects free of HF in the Olmsted County Community Cohort by showing that NT-proANP is predictive of subsequent death, development of HF, and MI, even after adjustment for age, sex, body mass index, and other cerebrovascular risk factors.³ The NT-proANP plasma concentration is not only an independent predictor of prognosis in patients with HF with preserved ejection fraction but also a marker for asymptomatic LV dysfunction.²⁴ After MI, baseline NT-proANP is associated with adverse cardiac remodeling and is predictive of subsequent cerebrovascular events, independent of cardiac remodeling despite normal plasma NT-proBNP levels.^{25,26}

Our study is the first to show a correlation between genetic variation, in vitro NT-proANP protein and in vivo circulating plasma levels, and occurrence of subsequent adverse cardiovascular events in the general community. Our study demonstrates that genetic variation in the *MTHFR-CLCN6-NPPA-NPPB* genetic locus is associated with lower NT-proANP levels, higher diastolic blood pressure, and stroke, suggesting that these variants may be loss-of-function alleles. In our cohort, there also seems to be a trend in these subjects to have atherosclerotic disease with lower high-density lipoprotein levels and a trend toward a higher prevalence of coronary artery disease. We have previously shown that genetic variation in *NPPA* can have an effect on the cardiometabolic profile of carriers and that natriuretic peptides not only play an important role in fluid and blood pressure homeostasis but could also affect the atherosclerotic process.^{27,28} ANPs, therefore, likely play a protective role in the prevention of hypertension and its sequelae, such as stroke. Identifying high-risk patients by genotype may allow earlier intervention to prevent the occurrence of such adverse outcomes in the general community. Furthermore, in our study, the rs5063 genotype was independent of NT-proANP levels in predicting adverse outcomes. This finding is not surprising because although BNP has been a useful marker for predicting adverse cardiovascular events in community-based cohorts, the clinical use of ANP levels as a biomarker has been controversial.²⁹ Although the finding of stroke risk can be explained by lower ANP levels, such as higher blood pressures, unfavorable cardiometabolic profile, and adverse cardiac remodeling, one could speculate that a cross-sectional single value obtained by measurement of a biomarker (NT-proANP) at a given time in the general community may not reflect a true deficiency state of natriuretic peptides because of the genotype and its effect over time. Furthermore, the relationship of ANP levels and hypertension is complex and may be manifested as a lack of compensatory increase as hypertension stage progresses rather than an actual decrease in circulating levels.⁴ Finally, the genetic variation that occurs in this locus may result in the alteration of not only ANP but also other natriuretic peptides, such as BNP or other neurohormonal triggers, which could lead to adverse

cardiovascular outcomes and would not be reflected by measurement of a single biomarker.

We have identified genetic determinants of circulating NT-proANP levels in the *MTHFR-CLCN6-NPPA-NPPB* locus by performing the first large-scale genetic association study. This genetic variation was associated with lower ANP protein expression and circulating plasma levels, higher diastolic blood pressures, and stroke. Our study confirms the important role of the natriuretic peptide system in the pathophysiology of hypertension and its sequelae, such as stroke, and lays the foundation for the study of the potential therapeutic benefit of manipulating the natriuretic peptide system in treating these conditions.

Acknowledgments

We thank Luanne Wussow for her assistance with the preparation of this article.

Sources of Funding

This work was supported, in part, from Clinical and Translational Science Awards grant number UL1 TR000135 from the National Center for Advancing Translational Sciences, Heart Failure Clinical Research Network grant U01 HL 084907 and grant U10 HL11026, National Institutes of Health (NIH) grant U19 GM61388 (The Pharmacogenomics Research Network), and NIH grants R01 HL36634 and P01 HL76611.

Disclosures

None.

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CLINICAL PERSPECTIVE

The identification of genetic determinants of plasma N-terminal pro atrial natriuretic peptide (ANP) levels has implications in the understanding of the pathophysiology of hypertension and its sequelae, such as stroke and heart failure. We performed the first large-scale (across ≈ 16000 genes) genotyping study in 1784 randomly selected subjects from the general community in Olmsted County, MN. We identified genetic variants in chromosome 1 in the *MTHFR-CLCN6-NPPA-NPPB* cluster to be significantly associated with N-terminal proANP levels. The amino acid-changing single-nucleotide polymorphism, rs5063, located in *NPPA* was the most significant single-nucleotide polymorphism ($P=1.14 \times 10^{-26}$) in a combined analysis, and because of it being in high linkage disequilibrium ($r^2 > 0.8$) with other genotyped single-nucleotide polymorphisms, represented most of the significant genetic variations in this locus. To determine the functional significance of rs5063 on ANP protein expression, we performed in vitro cell studies that demonstrated that the rs5063 allozyme was reduced at $55 \pm 8\%$ of wild-type protein ($P=0.01$) as assessed by quantitative western blots that could account for lower plasma N-terminal proANP levels observed in carriers of the minor allele. Clinically, rs5063 carriers had higher diastolic blood pressures and were at an increased risk of stroke when compared with wild-type subjects independent of age, sex, diabetes mellitus, hypertension, atrial fibrillation, and cholesterol levels (hazard ratio, 1.6; $P=0.004$) over a median follow-up time of 12.2 years. Inherited deficiency of circulating ANP is associated with hypertension and stroke risk in the general community and may have implications for therapy of these diseases, especially with drugs that target the natriuretic peptide system.

Circulating Atrial Natriuretic Peptide Genetic Association Study Identifies a Novel Gene Cluster Associated With Stroke in Whites

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Circ Cardiovasc Genet. 2015;8:141-149; originally published online December 1, 2014;
doi: 10.1161/CIRCGENETICS.114.000624

Circulation: Cardiovascular Genetics is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1942-325X. Online ISSN: 1942-3268

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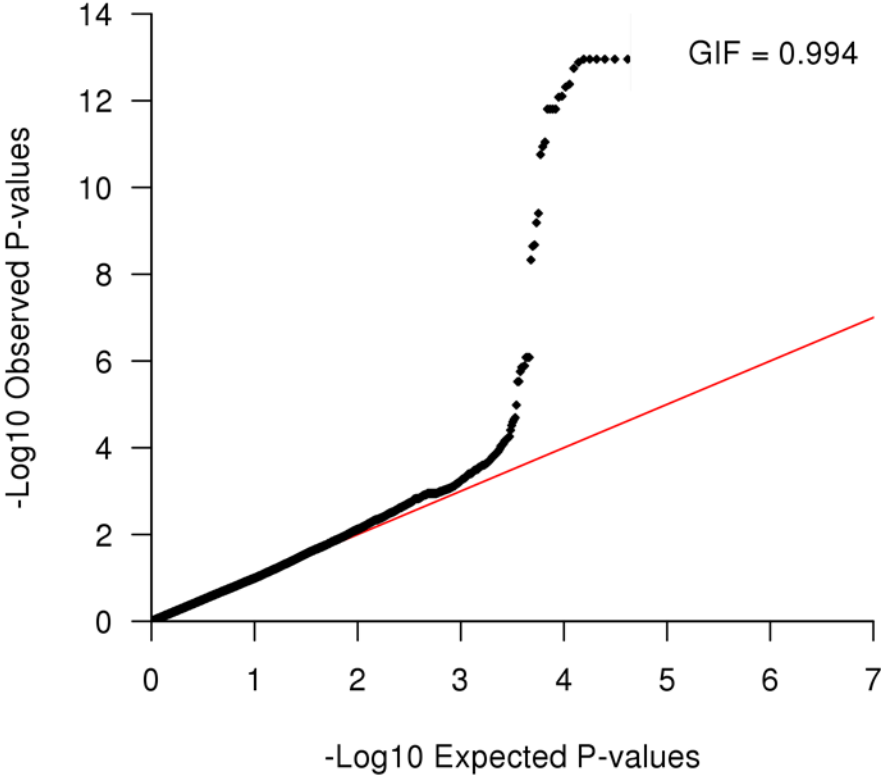
Supplemental Table 1. Significant Genetic Associations with NT-proANP Concentrations in the Discovery Cohort and the Replication Cohort

SNP	minor allele	common allele	Discovery cohort			Replication cohort		
			MAF	fold change	p-value	MAF	fold change	p-value
rs2076003	C	T	0.055	0.634	1.10E-13	0.049	0.642	5.76E-13
rs41275484	A	G	0.055	0.634	1.10E-13	0.050	0.636	1.40E-13
rs41275500	T	C	0.055	0.634	1.10E-13	0.050	0.636	1.40E-13
rs5063	T	C	0.055	0.634	1.10E-13	0.049	0.628	3.53E-14
rs7552330	A	G	0.055	0.634	1.10E-13	0.050	0.636	1.39E-13
rs75747410	A	G	0.055	0.634	1.10E-13	0.050	0.636	1.39E-13
rs77072136	T	C	0.055	0.634	1.10E-13	0.050	0.636	1.39E-13
rs79811212	C	T	0.055	0.634	1.10E-13	0.050	0.636	1.39E-13
rs2075539	A	G	0.056	0.636	1.31E-13	0.049	0.642	5.76E-13
rs3737965	A	G	0.055	0.636	1.80E-13	0.049	0.631	7.42E-14
rs141308438	T	C	0.055	0.636	2.00E-13	0.049	0.628	3.33E-14
rs3753585	A	G	0.055	0.639	4.11E-13	0.049	0.631	7.42E-14
rs3753586	C	T	0.055	0.639	4.11E-13	0.049	0.631	7.42E-14
rs2272803	T	G	0.055	0.640	4.17E-13	0.050	0.636	1.40E-13
rs17037397	A	C	0.054	0.639	4.84E-13	0.049	0.631	7.42E-14
rs79837245	A	T	0.054	0.644	7.19E-13	0.039	0.656	9.24E-10
rs75030554	T	C	0.048	0.631	8.30E-13	0.038	0.624	8.69E-12
rs77012921	C	T	0.049	0.631	8.30E-13	0.038	0.624	8.69E-12
rs116525098	T	C	0.048	0.632	9.39E-13	0.038	0.624	1.03E-11
rs74714707	T	C	0.049	0.636	1.56E-12	0.038	0.624	6.17E-12
rs75540316	T	G	0.049	0.636	1.56E-12	0.038	0.624	6.17E-12
rs77003042	G	T	0.049	0.636	1.56E-12	0.038	0.624	6.17E-12
rs79653311	C	T	0.049	0.636	1.56E-12	0.038	0.624	6.17E-12
rs79712000	C	T	0.049	0.636	1.56E-12	0.038	0.624	6.17E-12
rs114941496	A	G	0.051	0.647	8.97E-12	0.045	0.654	3.78E-11
rs3737967	A	G	0.053	0.652	1.05E-11	0.048	0.630	9.88E-14
rs61757273	T	G	0.050	0.650	1.15E-11	0.040	0.631	8.62E-12
rs2274976	T	C	0.053	0.655	1.75E-11	0.049	0.636	2.40E-13
rs79593079	T	C	0.062	0.692	2.12E-10	0.050	0.721	7.13E-08
rs113823536	G	T	0.062	0.695	3.95E-10	0.051	0.716	3.47E-08
rs78097221	A	C	0.062	0.695	3.95E-10	0.050	0.723	1.01E-07
rs60600557	A	G	0.062	0.699	6.50E-10	0.051	0.716	3.47E-08
rs80177860	C	G	0.064	0.709	2.04E-09	0.056	0.750	5.79E-07
rs11803049	A	G	0.072	0.723	2.11E-09	0.068	0.768	5.68E-07
rs7545290	C	T	0.065	0.711	2.27E-09	0.056	0.750	5.83E-07
rs58120150	C	T	0.059	0.707	4.67E-09	0.054	0.721	2.18E-08
rs112521149	A	G	0.106	0.756	1.11E-08	0.109	0.812	3.66E-06
rs148216773	G	C	0.066	0.737	1.82E-07	0.055	0.805	4.37E-04

Supplementary Table 2. Multivariable Logistic Regression Model for Stroke and Adjusted for rs5063 Genotype and NT-proANP concentrations.

Variable	p-value	Hazard Ratio	95% LCL	95% UCL
Age at Exam	<.0001	1.07	1.06	1.09
Female Gender	0.004	0.72	0.57	0.90
Verified Hypertension	<.0001	1.86	1.48	2.32
Afib/Flutter	0.02	1.63	1.08	2.45
Log (NT-ANP)	0.17	1.15	0.94	1.40
rs5063- C/T or T/T	0.006	1.61	1.14	2.27

Supplementary Figure 1



Supplementary Figure 2

