Atrial Natriuretic Peptide Genetic Variant rs5065 and Risk for Cardiovascular Disease in the General Community
A 9-Year Follow-Up Study

Valentina Cannone, Brenda K. Huntley, Timothy M. Olson, Denise M. Heublein, Christopher G. Scott, Kent R. Bailey, Margaret M. Redfield, Richard J. Rodeheffer, John C. Burnett Jr

Abstract—We analyzed the phenotype associated with the atrial natriuretic peptide (ANP) genetic variant rs5065 in a random community-based sample. We also assessed and compared the biological action of 2 concentrations (10⁻¹⁰ mol/L, 10⁻⁸ mol/L) of ANP and ANP-RR, the protein variant encoded by the minor allele of rs5065, on activation of the guanylyl cyclase (GC)-A and GC-B receptors, production of the second messenger 3′,5′-cGMP in endothelial cells, and endothelial permeability. rs5065 genotypes were determined in a cross-sectional adult cohort from Olmsted County, MN (n=1623). Genotype frequencies for rs5065 were 75%, 24%, and 1% for TT, TC, and CC, respectively. Multivariate analysis showed that the C allele was associated with increased risk of cerebrovascular accident (hazard ratio, 1.43; 95% confidence interval, 1.09–1.86; P=0.009) and higher prevalence of myocardial infarction (odds ratio, 1.82; 95% confidence interval, 1.07–3.09; P=0.026). ANP-RR 10⁻⁸ mol/L activated the GC-A receptor (83.07±8.31 versus no treatment 0.18±0.04 per 6 wells; P=0.006), whereas ANP-RR 10⁻¹⁰ mol/L did not. Neither 10⁻⁸ mol/L nor 10⁻¹⁰ mol/L ANP-RR activated GC-B receptor (P=0.10, P=0.35). ANP 10⁻⁸ mol/L and ANP-RR 10⁻⁸ mol/L stimulated 3′,5′-cGMP production in endothelial cells similarly (P=0.58). Both concentrations of ANP-RR significantly enhanced human aortic endothelial cell permeability (69 versus 29 relative fluorescence units [RFUs], P=0.012; 58 versus 39 RFUs, P=0.015) compared with ANP. The minor allele of rs5065 was associated with increased cardiovascular risk. ANP-RR activated the GC-A receptor, increased 3′,5′-cGMP in endothelial cells, and when compared with ANP, augmented endothelial cell permeability. (Hypertension. 2013;62:860-865.) • Online Data Supplement

Key Words: atrial natriuretic peptides ■ cardiovascular diseases ■ natriuretic peptides ■ stroke

In 1981 with the report of DeBold on the isolation of atrial natriuretic peptide (ANP), a substance with natriuretic properties extracted from rat atrial tissue, the concept of the heart as an endocrine organ emerged.¹ ANP is a 28-amino-acid peptide synthesized and secreted by the atrial and ventricular myocardia in response to wall stress.² The actions of ANP are mediated by the transmembrane guanylyl cyclase (GC)-A receptor, which once activated catalyzes the synthesis of the natriuretic peptide second messenger, 3′,5′-cGMP. Vasodilation, natriuresis, suppression of renin and aldosterone, inhibition of cardiomyocyte hypertrophy, cardiac fibroblast proliferation, collagen synthesis, and enhanced lipolysis represent the cardiorenal protective properties of ANP.³ Besides the above well-known cardiorenal actions, ANP participates in the regulation of systemic blood pressure and intravascular volume by enhancing vascular permeability.⁴ Importantly, the biological action of ANP on endothelial cell permeability is mediated by the GC-A receptor,⁵ which is highly expressed in endothelial cells, and cGMP⁶ Measurements with iodinated albumin have revealed that ANP stimulates endothelial macromolecule permeability in rats and mice,⁵,⁷,⁸ whereas mice with endothelium-restricted deletion of GC-A gene have shown chronic arterial hypertension and hypervolemia.⁸ In humans, the minor allele of a genetic variant of the ANP gene (NPPA) is associated with higher plasma levels of ANP, lower blood pressure values, and reduced risk for hypertension.⁹,¹⁰¹¹

The single nucleotide polymorphism of NPPA, rs5065, is located in the stop codon of exon 3. The T-to-C nucleotide substitution replaces the stop codon with an arginine amino acid, leading to the extension of ANP by 2 additional arginines at the C terminus, ANP-RR.

The rs5065 genetic variant has been previously investigated in selected populations of subjects affected by cardiovascular diseases, including coronary artery disease and stroke. Rubattu et al¹² revealed that the minor C allele of rs5065 was more prevalent in patients with ischemic stroke than in control subjects.

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The CC genotype has also been significantly associated with a higher incidence of history of myocardial infarction (MI) and multivessel coronary atherosclerosis in the analysis conducted by Gruchala et al\textsuperscript{13} in patients with coronary artery stenosis. Moreover, Zhang et al\textsuperscript{14} reported that in a Chinese population of patients with coronary artery disease the C allele is more frequent than in control subjects. Importantly, the rs5065 minor allele is also an independent predictor of acute coronary syndrome and is associated with increased risk of major adverse cardiovascular events in a population of patients with coronary artery disease.\textsuperscript{15} It should be noted that from a mechanistic perspective, previous in vitro studies investigated the effect of ANP-RR on oxidative stress, cell proliferation and migration, angiogenesis, and vascular remodeling in human umbilical vein endothelial cells, suggesting that ANP-RR may lead to enhanced susceptibility to vascular damage.\textsuperscript{16}

To date, no data have been reported about the clinical phenotype and cardiovascular risk associated with rs5065 genotypes in the general population or their relation to circulating ANP and B-type natriuretic peptide (BNP). The goal of the current study was 3-fold. First, in a large well-characterized general adult population in Olmsted County, MN, we sought to define associations of rs5065 with cardiovascular and metabolic phenotypes, including myocardial structure, function, and circulating natriuretic peptides. Further, we performed a follow-up analysis to evaluate whether this NPPA genetic variant might be associated with an increased risk of cardiovascular disease. Last, we defined whether ANP-RR activates the natriuretic peptide receptor GC-A or GC-B, if it increases cGMP production in human aortic endothelial cells, and importantly we assessed the biological action of ANP and ANP-RR on endothelial cell permeability, recognizing that endothelial cell leakiness is also involved in the development of atherosclerosis, especially in the setting of cardiovascular risk factors.\textsuperscript{17–20}

**Methods**

Methods are available in the online-only Data Supplement.

**Results**

**Frequency of rs5065 Genotypes and Natriuretic Peptides Plasma Levels**

A total of 1623 subjects were successfully genotyped. Frequencies of rs5065 were TT: 75% (n=1219), CT: 24% (n=384), and CC: 1% (n=20). Minor allele frequency was 13%, and distribution was in Hardy–Weinberg equilibrium (P=0.093).

In consideration of the low frequency of the C allele, all analyses were performed after combining TC and CC genotypes. Table S1 in the online-only Data Supplement illustrates the characteristics of the study population. The 2 groups (TT versus TC+CC genotypes) did not differ in terms of age (sex-adjusted P=0.224). The C allele tended to be slightly less frequent for women (49% versus 54%, age-adjusted P=0.088). An age- and sex-adjusted analysis showed that the minor C allele was significantly associated with higher BNP plasma levels measured using both Biosite (median 27.6 versus 23.2 pg/mL; P<0.0001) and Shionogi assays (16.9 versus 14.7 pg/mL; P=0.006; Table S2). After including body mass index (BMI), hypertension, coronary artery disease, MI, heart failure, ejection fraction <50% or 40%, atrial fibrillation, and cerebrovascular accident (CVA) as confounding factors in the regression model, it was found that there was a positive association of the C allele with BNP Biosite (parameter estimate=0.17; 95% confidence interval [CI], 0.08–0.26; P=0.0003) and BNP Shionogi (parameter estimate=0.11; 95% CI, 0.02–0.20; P=0.022). No difference was detected in ANP plasma levels.

**Cardiovascular Phenotype**

**Blood Pressure, Echocardiographic Parameters, and Renal Function**

The age- and sex analysis of echocardiographic parameters revealed that the C allele was significantly associated with a higher prevalence of ejection fraction <40% (3% versus 1%; P=0.018; Table S1). After controlling for BMI, hypertension, coronary artery disease, MI, heart failure, and atrial fibrillation, the association was attenuated (odds ratio, 2.39; 95% CI, 0.98–5.84; P=0.056). Median values of ejection fraction and prevalence of ejection fraction <50% were similar between groups. Moreover, rs5065 genotype was not associated with left atrial volume, left ventricular structure, and prevalence of moderate-to-severe diastolic dysfunction. The 2 groups did not differ in terms of systolic and diastolic blood pressure, serum creatinine values, and glomerular filtration rate. Values of C-reactive protein were similar between groups.

**Cardiovascular Diseases**

After adjusting for age and sex, history of MI was more prevalent for the carriers of the minor allele (7% versus 5%; P=0.023; Table S3). A similar result was obtained with regard to CVA because the C allele was associated with higher prevalence of stroke or transient ischemic attack in a regression model adjusted for age and sex (2% versus 1%; P=0.024). Importantly, the C allele was significantly associated with higher prevalence of MI (odds ratio, 1.82; 95% CI, 1.07–3.09; P=0.026) and CVA (odds ratio, 2.52; 95% CI, 1.03–6.13; P=0.042) even after adjusting for BMI, diabetes mellitus, hyperlipidemia, smoking, and hypertension. Survival free of CVA was decreased for the TC or CC genotypes (Figure S1). Moreover, analysis controlling for age, sex, and BMI showed that the carriers of the minor allele of rs5065 had an increased risk of developing CVA (hazard ratio, 1.43; 95% CI, 1.09–1.86; P=0.009) during a mean follow-up of 9 years (Table S4). Multivariate adjustment including age, sex, BMI, diabetes mellitus, hyperlipidemia, smoking, and hypertension confirmed higher risk of CVA (hazard ratio, 1.48; 95% CI, 1.12–1.94; P=0.005) in the carriers of C allele.

The 2 groups did not differ in terms of prevalence of hypertension, coronary artery disease, congestive heart failure, or atrial fibrillation. The follow-up analysis did not reveal any significant association between genotype and MI, heart failure, and all-cause mortality.

**Metabolic Phenotype**

Carriers of the C allele showed significantly higher levels of total cholesterol when compared with the homozygotes for the
major T allele after controlling for age, sex, and BMI (204 versus 199 mg/dL; \( P = 0.037 \)). Plasma values of the other elements of the lipid panel, triglycerides, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol, as well as glucose and insulin levels, did not differ between genotypes (Table S1). The percentage of subjects treated with antihypertensive agents was similar between the groups. The analysis with regard to BMI values, prevalence of obesity, metabolic syndrome, and diabetes mellitus did not reveal any significant association with rs5065 alleles.

**In Vitro Studies**

**Activation of GC-A or GC-B Receptors by ANP or ANP-RR**

In human embryonic kidney 293 cells expressing GC-A, 10\(^{-8}\) mol/L of both ANP and ANP-RR activated cGMP production (10\(^{-8}\) mol/L ANP: 77.50±7.95 versus no treatment 0.18±0.04 per 6 wells, \( P = 0.006 \); 10\(^{-8}\) mol/L ANP-RR: 83.07±8.31 versus no treatment 0.18±0.04 per 6 wells, \( P = 0.006 \); Figure S2). Conversely, 10\(^{-10}\) mol/L ANP or ANP-RR did not significantly increase the production of cGMP in human embryonic kidney 293 cells expressing GC-A when compared with no treatment (10\(^{-10}\) mol/L ANP 0.22±0.01 versus no treatment 0.18±0.04 per 6 wells, \( P = 0.39 \); 10\(^{-10}\) mol/L ANP-RR 0.18±0.02 versus no treatment 0.18±0.04 per 6 wells, \( P = 0.95 \)). There was no significant difference in cGMP production between ANP and ANP-RR at either tested concentration.

Incubation of human embryonic kidney 293 cells expressing GC-B with 10\(^{-10}\) mol/L ANP or 10\(^{-8}\) mol/L ANP determined an increase in cGMP production (ANP 10\(^{-10}\) mol/L: 0.21±0.015 versus no treatment 0.15±0.001 per 6 wells, \( P = 0.02 \); ANP 10\(^{-8}\) mol/L: 0.17±0.005 versus no treatment 0.15±0.001 per 6 wells, \( P = 0.03 \)) although the amount of cGMP produced was minimal. Neither 10\(^{-10}\) mol/L ANP-RR nor 10\(^{-8}\) mol/L ANP-RR activated cGMP in human embryonic kidney 293 cells expressing GC-B (ANP-RR 10\(^{-10}\) mol/L: 0.35±0.18 versus no treatment 0.15±0.001 per 6 wells, \( P = 0.35 \); ANP-RR 10\(^{-4}\) mol/L: 0.20±0.03 versus no treatment 0.15±0.001 per 6 wells, \( P = 0.10 \)). There was no statistical difference between ANP and ANP-RR cGMP production in GC-B transfected cells.

**Production of cGMP in Human Aortic Endothelial Cells by ANP or ANP-RR**

At 10\(^{-4}\) mol/L, both ANP and ANP-RR activated cGMP in human aortic endothelial cells (ANP 10\(^{-4}\) mol/L: 0.18±0.05 versus no treatment 0.02±0.005 pmol/mL, \( P = 0.01 \); ANP-RR 10\(^{-4}\) mol/L: 0.22±0.04 versus no treatment 0.02±0.005 pmol/mL, \( P = 0.0009 \)) at statistically similar levels (\( P = 0.58 \); Figure S3). Neither ANP nor ANP-RR induced cGMP at 10\(^{-10}\) mol/L concentration compared with no treatment (ANP 10\(^{-10}\) mol/L: 0.03±0.002 versus no treatment 0.02±0.005 pmol/mL, \( P = 0.52 \); ANP-RR 10\(^{-10}\) mol/L: 0.03±0.003 versus no treatment 0.02±0.005 pmol/mL, \( P = 0.1 \)).

**Endothelial Cell Permeability**

In this study, we evaluated the ability of 2 concentrations of ANP and ANP-RR (10\(^{-10}\) mol/L, 10\(^{-8}\) mol/L) to increase endothelial cell permeability. Importantly, both concentrations of ANP-RR significantly increased human aortic endothelial cell permeability (68 versus 28 relative fluorescence units [RFUs], \( P = 0.012 \); 56 versus 37 RFUs, \( P = 0.015 \)) compared with ANP (Figure S4). When compared with no treatment, ANP 10\(^{-8}\) mol/L significantly enhanced endothelial permeability (37 versus 20 RFUs, \( P = 0.005 \)), whereas ANP 10\(^{-10}\) mol/L tended to increase endothelial permeability (\( P = 0.176 \)). Both concentrations of ANP-RR (10\(^{-10}\) mol/L, 10\(^{-8}\) mol/L) augmented endothelial permeability when compared with control (68 versus 20 RFUs, \( P = 0.012 \); 56 versus 20 RFUs, \( P = 0.0005 \)).

**Discussion**

rs5065 is a genetic variant of NPPA, and its minor allele encodes for an ANP with 2 additional arginines at the C terminus, ANP-RR.\(^{21}\) For the first time, in a random sample of the general adult population in Olmsted County, MN, we defined the cardiovascular and metabolic phenotype associated with this variant of the ANP gene during a mean follow-up of 9 years. Here, we report that the minor C allele of rs5065 is associated with higher prevalence of MI, and carriers of such allele are at higher risk to develop CVA independent of several risk factors. Moreover, the minor allele of rs5065 is associated with higher BNP plasma values, and prevalence of impaired ejection fraction <40% tends to be higher in the TC+CC group. The carriers of the C allele also have higher total cholesterol plasma levels. Our in vitro studies showed that ANP-RR activates the GC-A receptor similar to ANP but does not activate GC-B. Both ANP and ANP-RR activate human aortic endothelial cells with similar increase in cGMP production. Last, comparison of native ANP and ANP-RR demonstrated that the molecular form encoded by the minor allele of rs5065 enhances in vitro endothelial cell permeability to a greater magnitude than native ANP.

Previous studies involving patients with cardiovascular diseases showed that the minor C allele of rs5065 is associated with higher frequency and risk of coronary heart disease,\(^{14,15}\) history of MI, and multivessel atherosclerosis.\(^{13}\) In a case–control study conducted on patients with ischemic stroke, the C allele of rs5065 was significantly more prevalent for cases and was associated with increased recurrence of stroke in a 5-year follow-up analysis.\(^{13}\) To date, the phenotypic characteristics of rs5065 in a general adult population are undefined. The most striking finding of the current study is that in a cross-sectional community-based cohort the minor allele of the ANP genetic variant rs5065 is significantly associated with increased risk of stroke even during a mean follow-up analysis of 9 years. Thus, in our epidemiological analysis, the clinical phenotype observed in the carriers of the minor allele coding for ANP-RR was characterized by an increased risk for atherosclerotic disease. More specifically, the univariate and multivariate analyses adjusted for traditional cardiovascular risk factors showed a significant association between the C allele of rs5065 and a higher prevalence of both CVA and MI. Moreover, we performed a follow-up analysis using data for a mean of 9 years, which is the longest follow-up analysis performed to date with regard to this genetic variant, and confirmed an increased risk to develop CVA for the carriers of the minor allele even after including age, sex, BMI, diabetes mellitus, hyperlipidemia, smoking, and hypertension.
in the multivariate model. Our study confirms the association between the C allele of rs5065 and cardiovascular disease for the first time in a random sample of the general community from Olmsted County, MN; such association was previously found only in selected populations of patients with cardiovascular disease.12–14 Our data in the general population suggest that the protein variant ANP-RR might act noxiously on the vascular wall and endothelial cells, leading to hyperpermeability and the consequent increased risk of atherosclerotic disease. Indeed, recent studies demonstrated that ANP-RR may lead to atherosclerotic plaque formation and instability in vitro as well as to increased risk of cardiovascular disease in vivo.12–15

In our study, the in-depth characterization of the population analyzed included not only cardiovascular characteristics but also the metabolic phenotype. Evaluating the metabolic phenotype associated with rs5065 genotypes was relevant because natriuretic peptides have lipolytic actions3 and protect against obesity, as shown in mouse models.22 Furthermore, we have reported that the ANP genetic variant rs5068 is associated with increased circulating ANP and BNP plasma levels is also associated with lower BMI and prevalence of metabolic syndrome in the general population.10,11 In the current study, after controlling for age, sex, and BMI, a significant association was found between the rs5065 minor allele and higher values of total plasma cholesterol. A difference in treatment cannot explain such association because subjects treated with antilipemic agents were similar across genotypes. We tend to exclude the possibility that this association might have influenced the higher prevalence of MI and increased risk for CVA observed in the carriers of the C allele because our multivariate analysis was adjusted for hyperlipidemia. Moreover, in both groups, median values of total cholesterol were close to the cutoff level of 200 mg/dL, which is considered a desirable treatment outcome by the third report of the National Cholesterol Educational Program.23 A possible relationship between the minor allele of rs5065 and plasma cholesterol levels thus remains unclear, and more studies are needed to confirm our finding.

In the current study, we also investigated natriuretic peptide plasma levels and echocardiographic parameters according to rs5065 genotypes. In our analysis, both groups revealed median plasma levels of BNP in the normal range. Despite being within the normal range, carriers of the minor allele showed higher BNP values by both the Biosoite and Shionogi assays in a multivariate-adjusted analysis. We hypothesize that higher levels of BNP might be a reflection of deleterious effects exerted by ANP-RR on the heart, although echocardiography did not reveal any atrial or ventricular dilatation or hypertrophy. It is also possible that in the carriers of the C allele the deleterious vascular effect of ANP-RR is strengthened by a reduced production of ANP, which possesses cardiovascular protective properties. Furthermore, ejection fraction <40% tended to be more prevalent in the group characterized by the presence of the C allele on the adjusted logistic regression analysis. Ellis et al24 had previously assessed natriuretic peptide concentrations and echocardiographic indices according to rs5065 genotypes in patients with coronary artery disease, and no significant association was observed. Whether rs5065 is associated with impaired systolic function and the association between the C allele and higher circulating levels of BNP still remain unclear. Further studies are certainly warranted to investigate more in this regard.

Results on a possible association between the genetic variants rs5065 and hypertension have been controversial. Although some of them showed the minor C allele of rs5065 to be associated with a lower prevalence of hypertension,24,25 in accordance with our analysis, many case–control studies did not find any significant relationship.26–30 A recent meta-analysis suggests that the carriers of the C allele might be at a moderately decreased risk of developing hypertension, but the meta-analysis also identified a significant heterogeneity in the design of the studies evaluated, leaving the controversy still unresolved.31

Atherosclerosis plays a major role in the pathophysiological mechanism leading to cardiovascular disease and is associated with increased vascular permeability.19,20 With this key information in mind, in our in vitro studies we assessed and compared the biological action of ANP and ANP-RR on endothelial cell permeability. We also evaluated whether these 2 peptides activate the natriuretic peptide receptors GC-A and GC-B similarly. Our findings show that both ANP and ANP-RR generate cGMP in human aortic endothelial cells and activate GC-A receptor with no difference between the 2 peptides. Incubation of human embryonic kidney 293 cells expressing GC-B with ANP and ANP-RR determined a similar but minimal increase in cGMP production, suggesting that biological actions of ANP and ANP-RR are predominantly mediated by GC-A but not by GC-B receptor. Interestingly, Siciarretta et al12 showed that ANP-RR probably exerts its detrimental vascular effect not through GC-A or GC-B receptors but through an inappropriate activation of the natriuretic peptide receptor C and cAMP pathway. Clarifying the receptor and mechanisms through which ANP-RR exerts its noxious action is certainly a crucial point that requires further studies.

When human aortic endothelial cells were incubated with ANP-RR, a significant increase in permeability occurred, and it was greater when compared with ANP. Several studies showed that ANP exerts a biological action on endothelial cell permeability, regulating transvascular fluid and protein transport.4,5,7,8 Intravenous infusion of ANP at high dose increases albumin shift out of the systemic circulation in rats,7 and local superfusion of ANP enhances microvascular albumin extravasation in mice.4 ANP exerts its biological action by binding to the GC-A receptor that is densely expressed in vascular endothelium.9 Indeed, acute vascular volume expansion in mice with endothelium-restricted deletion of GC-A gene results in rapid and significant increases in central venous pressure and decreases in hematocrit when compared with control mice, suggesting a lack of ANP modulation on oncostic intravascular pressure and fluid transport in mice with endothelium-restricted deletion of GC-A gene.3 In our study, 2 concentrations of ANP-RR significantly increased human aortic endothelial cell permeability when compared with equimolar concentrations of native ANP. We hypothesize that chronic exposure to ANP-RR may lead to a condition of hyperpermeability and predispose the subject to atherosclerotic disease.19,20 Such state of hyperpermeability might be a
result of the noxious effect exerted by ANP-RR on endothelium. In a recent study conducted by Scarpino et al. on human umbilical vein endothelial cells, ANP-RR reduced endothelial cell viability and proliferation, increased reactive oxygen species production, and stimulated gene expression of molecules involved in atherogenesis. The protein variant ANP-RR probably impairs endothelial function through an altered natriuretic peptide receptor C signaling.

Perspectives

The minor C allele of the NPPA genetic variant rs5065 codes for an ANP with 2 additional arginines at the C terminus, ANP-RR. Our analysis of a well-characterized random sample of the general population from Olmsted County, MN, showed that the C allele of rs5065 is associated with higher prevalence of MI and risk of CVA. Furthermore, the carriers of the minor allele presented higher BNP and total cholesterol plasma values. Ejection fraction <40% tends to be more frequent between the carriers of the minor allele. In vitro studies showed that ANP-RR activates GC-A receptor similarly to ANP and induces an increase in cGMP production in human aortic endothelial cells. Moreover, ANP-RR significantly increases human aortic endothelial cell permeability when compared with ANP. Additional studies are warranted to confirm the associations found in a general US population and to investigate in-depth the biological action of this ANP genetic and protein variant. The prognostic implication of the above associations might be of relevant importance in terms of assessment of cardiovascular disease risk, and further analyses are clearly needed to evaluate it.

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Disclosure

None.

References


**Novelty and Significance**

**What Is New?**
- No previous studies have investigated the cardiometabolic phenotype and cardiovascular risk associated with rs5065 in a general population.

With regard to vascular permeability, the biological action of atrial natriuretic peptide (ANP)-RR, which is the peptide variant encoded by the minor allele of rs5065, is still unknown.

**What Is Relevant?**
- The C allele is associated with increased risk of cerebrovascular accident (hazard ratio, 1.48; 95% confidence interval, 1.12–1.94; \(P=0.005\)), higher prevalence of myocardial infarction (odds ratio, 1.82; 95% confidence interval, 1.07–3.09; \(P=0.026\)), and higher B-type natriuretic peptide plasma levels measured using Biosite (\(P=0.0003\)) and Shionogi assays (\(P=0.022\)). ANP-RR significantly enhanced human aortic endothelial cell permeability (\(P=0.012\), \(P=0.015\)) compared with ANP.

**Summary**
In a general US population, the minor allele of rs5065 is associated with increased cardiovascular risk. In vitro studies showed that when compared with ANP, ANP-RR determines augmented endothelial cell permeability.
THE ATRIAL NATRIURETIC PEPTIDE GENETIC VARIANT RS5065
AND RISK FOR CARDIOVASCULAR DISEASE IN THE GENERAL COMMUNITY: A
NINE-YEAR FOLLOW-UP STUDY RR

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METHODS

This study was approved by the Mayo Clinic Institutional Review Board and subjects gave informed consent. Our study adhered to the principles of the Declaration of Helsinki and Title 45, U.S. Code of Federal Regulation, Part 46, Protection of Human Subjects, Revised November 13, 2001, effective December 13, 2001. All procedures followed were in accordance with institutional guidelines.

Study population

We analyzed a subset of clinically well-characterized community-based sample of the general population 45 years or older living in Olmsted County, MN in 1997-2000. This population was first characterized as part of the National Institutes of Health-funded Prevalence of Left Ventricular Dysfunction Study and Cardiac Peptides in Cardiorenal Regulation (RO1 HL55502 and HL36634). The design and selection criteria of the above study as well as the characteristics of the Olmsted County population have been previously described.1, 2 This population was characterized clinically, biochemically, and by echocardiography. Each subject’s medical record was reviewed by trained nurse chart abstractors using established criteria for MI3 and congestive heart failure.4 In addition, clinical diagnoses of stroke, transient ischemic attack and diabetes mellitus type 2 were recorded. Coronary artery disease was defined as a clinical diagnosis in the medical records with confirmation by exercise treadmill test, angiogram, or echocardiogram. Each participant underwent a focused physical examination that included measurement of blood pressure, height, and weight. The mean follow-up for the participants was 8.9 years, standard deviation= 1.7 years, median= 9.1, maximum = 11 years. From collected
DNA samples on 2,027 subjects, a total of 1,623 subjects were successfully genotyped and included in this study.

Body mass index (BMI) was measured as kilograms per (meter)$^2$. Obesity was defined as BMI $\geq 30$ kg/m$^2$. Waist circumference was measured in centimeters at the top of the umbilicus. In accordance with the National Cholesterol Education Program Adult Treatment Panel III criteria, metabolic syndrome was defined by the presence of 3 or more of the following criteria: (1) central obesity defined as a waist circumference greater than 102 cm in men and greater than 88 cm in women, (2) triglyceride level higher than 150 mg/dL (to convert to mmol/L, multiply by 0.0113), (3) high-density lipoprotein cholesterol level less than 40 mg/dL (to convert to mmol/L, multiply by 0.0259) in men and less than 50 mg/dL in women, (4) blood pressure of 130/85 mm Hg or higher, and (5) fasting glucose level of 110 mg/dL (to convert to mmol/L, multiply by 0.0555) or higher. Hypertension was diagnosed using Joint National Committee VI criteria.

**Genotyping**

Genotyping of rs5065 was carried out on 1623 subjects using TaqMan (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions, using 10-20ng DNA. Primers and probes were Assay-by Design (Applied Biosystems). Following PCR amplification, end reactions were read on the ABI Prism 7900ht using Sequence Detection Software (Applied Biosystems). The quality value percentage is a quality metric that indicates the reliability of called genotypes generated by the SDS software. The quality value was calculated by using ABI’s proprietary calling algorithm determining how well that sample fits into the cluster.
Genotypes less than 95% are located further from their clusters and have a lower reliability. An electronic data file was generated that contains genotypes and the quality value.

**Natriuretic peptide assays**

Plasma ANP levels were measured in 1529 subjects respectively using radioimmunoassay (Phoenix Pharmaceuticals, Belmont, Ca). Plasma B-type natriuretic peptide (BNP) levels were determined by fluorescence immunoassay (Biosite Diagnostic) in a subgroup of 1523 subjects and by an immunoradiometric assay (Shionogy Co Ltd) in 1622 subjects.

**Doppler echocardiography**

All echocardiograms were performed with the same echocardiographic instrument (HP-2500, Palo Alto, California) and were interpreted by a single echocardiologist blinded to clinical data. Two-dimensional and color Doppler imaging were performed to screen for valvular stenosis and regurgitation. In each subject, ejection fraction was measured and diastolic function was classified as mild, moderate, and severe as previously described.1 Left ventricular (LV) dimension and mass and left atrial volume were calculated from M-mode and 2-D measurements, respectively, and were indexed to body surface area.8-10 Left ventricular mass was calculated according to the Devereux formula. Presence of LV hypertrophy was defined on the basis of LV mass index greater than 130 g/m² for men and greater than 100 g/m² for women.11 Presence of left atrial enlargement was defined as left atrial volume index >33 ml/m² in men and >30 ml/m² in females.12
**In vitro Studies**

Human embryonic kidney 293 cells were stably transfected with either GC-A (HEK-GC-A) or GC-B (HEK-GC-B) using Lipofectamine (Invitrogen, Grand Island, NY). Transfected cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100U/ml penicillin, 100U/ml streptomycin, and 250µg/ml G418 (all reagents from Invitrogen, Grand Island, NY).

**Cell cGMP Assay**

HEK-GC-A, HEK-GC-B or human aortic endothelial cell were plated in 6-well plates and treated as previously described. Briefly, cells were incubated in Hank's balanced salt solution (Invitrogen, Carlsbad, CA) containing 20mmol/L N-[2-hydroxyethyl]piperazine-N'[2-ethanesulfonic acid], 0.1% bovine serum albumin, and 0.5 mmol/L 3-isobutyl-1-methylxanthine (Sigma, St. Louis, MO). Treated cells received 10^{-10} mol/L ANP, 10^{-8} mol/L ANP, 10^{-10} mol/L ANP-RR or 10^{-8} mol/L ANP-RR for 10 minutes. Cells were lysed in 300ul 6% TCA and sonicated for 10 min. The samples were ether extracted four times in 4 volumes of ether, dried, and reconstituted in 300µl cGMP assay buffer. The samples were assayed using a competitive RIA cGMP kit (Perkin-Elmer, Boston, MA) as previously described.

**Permeability Assay**

Cell permeability assays were performed using the Chemicon *In Vitro* Vascular Permeability Kit (Bedford, MA). Briefly, endothelial cells were seeded on semi-permeable polyethylene membranes coated with collagen and allowed to grow for 72 hours to form a monolayer. The inserts were transferred to fresh plate wells and treated with ANP or ANP-RR...
for 4 hours. The inserts were transferred to a permeability detection plate and FITC-dextran was added for 5 minutes. Permeability was assessed by fluorometry at 485nm and 530nm.

**Statistical Methods**

Patient characteristics were summarized as counts and percentages for categorical variables, or medians and interquartile ranges for continuous variables. To test for an association with the minor allele of the rs5065 genotype, each variable of interest was modeled as the dependent variable via linear or logistic regression, as appropriate, with rs5065 C allele as the explanatory variable. Results of linear regression models are reported as parameter estimates with 95% CI, results of logistic regression analyses are reported as odds ratios with 95% CI. All modeling was performed unadjusted and adjusted for potential confounding variables such as age and gender. Because some continuous variables, including C-reactive protein, serum glucose, insulin and triglycerides levels had distributions that were skewed, these variables were log-transformed to approximate normality. Distributions of ANP and BNP biomarkers were highly skewed and thus a probit transformation was applied to the ranked values of each in order to create distributions that were approximately normal.

The associations of rs5065 genotype and events occurring during follow-up were evaluated using Cox proportional hazards regression models to account for differential length of follow-up. Analyses of these events were performed both univariately with rs5065 genotype and also adjusted for potential confounding factors. Patients not observed to have an event were censored at time of last visit. A series of two sample t-tests were performed to evaluate differences in levels of cell permeability due to treatment with ANP or ANP-RR. All analyses were carried out using the SAS statistical software package (Version 9.2, SAS Institute Inc.,
Cary, NC). All tests are two-sided and p-values <0.05 were considered to be statistically significant.

References


Table S1. Characteristics of Study Population according to rs5065 Genotypes

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>TT (n=1219)</th>
<th>TC or CC (n=404)</th>
<th>Unadjusted p value*</th>
<th>Adjusted p value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>653 (54%)</td>
<td>196 (49%)</td>
<td>0.08</td>
<td>0.09</td>
</tr>
<tr>
<td>Age, yrs</td>
<td>62 (53.4, 70.5)</td>
<td>61.1 (53.2, 70.1)</td>
<td>0.20</td>
<td>0.22</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>132 (116, 146)</td>
<td>129 (116, 146)</td>
<td>0.31</td>
<td>0.54</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>73.0 (67, 80)</td>
<td>73 (67, 80)</td>
<td>0.80</td>
<td>0.43</td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>1.00 (0.90, 1.20)</td>
<td>1.00 (0.90, 1.20)</td>
<td>0.44</td>
<td>0.71</td>
</tr>
<tr>
<td>Ejection Fraction, %</td>
<td>65 (60, 68)</td>
<td>63 (60, 68)</td>
<td>0.24</td>
<td>0.50</td>
</tr>
<tr>
<td>Ejection Fraction&lt;40%</td>
<td>17 (1%)</td>
<td>13 (3%)</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>27.7 (25, 31.4)</td>
<td>27.7 (24.7, 31.2)</td>
<td>0.55</td>
<td>0.40</td>
</tr>
<tr>
<td>Obesity (BMI &gt;30 kg/m²)</td>
<td>407 (33%)</td>
<td>128 (32%)</td>
<td>0.53</td>
<td>0.44</td>
</tr>
<tr>
<td>Serum Glucose, mg/dl‡</td>
<td>94 (89, 101)</td>
<td>93 (88, 100)</td>
<td>0.60</td>
<td>0.58</td>
</tr>
<tr>
<td>Total Cholesterol, mg/dl</td>
<td>199.5 (178, 222)</td>
<td>204 (182.5, 225)</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td>HDL Cholesterol, mg/dl</td>
<td>43 (35, 54)</td>
<td>43 (36, 55)</td>
<td>0.87</td>
<td>0.27</td>
</tr>
<tr>
<td>LDL Cholesterol, mg/dl</td>
<td>124.4 (104.2, 146.6)</td>
<td>128 (107.9, 148.3)</td>
<td>0.10</td>
<td>0.11</td>
</tr>
<tr>
<td>Triglycerides, mg/dl §</td>
<td>130 (95, 183)</td>
<td>126 (91.5, 176)</td>
<td>0.78</td>
<td>0.79</td>
</tr>
<tr>
<td>Antilipemic therapy</td>
<td>210 (19%)</td>
<td>69 (18%)</td>
<td>0.93</td>
<td>0.99</td>
</tr>
<tr>
<td>Diabetes Mellitus type 2</td>
<td>93 (8%)</td>
<td>32 (8%)</td>
<td>0.85</td>
<td>0.80</td>
</tr>
<tr>
<td>Characteristic</td>
<td>TT (n=1219)</td>
<td>TC or CC (n=404)</td>
<td>Unadjusted p value*</td>
<td>Adjusted p value†</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>-------------</td>
<td>------------------</td>
<td>---------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Metabolic Syndrome (at least 3 of 5 criteria met)</td>
<td>239 (20%)</td>
<td>78 (19%)</td>
<td>0.88</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Continuous data are summarized with medians and 25th and 75th percentile values, and categorical data are summarized with counts and percentages. High-density lipoprotein, HDL; low-density lipoprotein, LDL. * p value obtained from univariate regression model. † p value obtained from regression model adjusting for age and gender. ‡p value based on logarithmic transformed variable.
Table S2. Natriuretic Peptide Plasma Values according to rs5065 Genotypes

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>TT</th>
<th>TC or CC</th>
<th>Unadjusted p value*</th>
<th>Adjusted p value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNP Biosite, pg/ml‡</td>
<td>23.2 (9.2, 53.4)</td>
<td>27.6 (11.5, 66)</td>
<td>0.007</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BNP Shionogi, pg/ml‡</td>
<td>14.7 (5.7, 30.9)</td>
<td>16.9 (6.2, 40.1)</td>
<td>0.07</td>
<td>0.006</td>
</tr>
<tr>
<td>ANP, pg/ml‡</td>
<td>12 (7.5, 16.6)</td>
<td>11.2 (7.4, 16.8)</td>
<td>0.60</td>
<td>0.97</td>
</tr>
</tbody>
</table>

*p value obtained from univariate regression model. † p value obtained from regression model adjusting for age and gender. ‡ p values reflect probit transformation applied to rank-ordered B-type natriuretic peptide (BNP) Biosite, BNP Shionogy, atrial natriuretic peptide (ANP) values.
Table S3. Prevalence of Cardiovascular Diseases according to rs5065 Genotypes

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>TT (n=1219)</th>
<th>TC or CC (n=404)</th>
<th>Unadjusted p value*</th>
<th>Adjusted p value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebrovascular Accident</td>
<td>12 (1%)</td>
<td>10 (2%)</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>Myocardial Infarction</td>
<td>55 (5%)</td>
<td>29 (7%)</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>Hypertension</td>
<td>371 (30%)</td>
<td>109 (27%)</td>
<td>0.19</td>
<td>0.32</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>149 (12%)</td>
<td>55 (14%)</td>
<td>0.47</td>
<td>0.36</td>
</tr>
<tr>
<td>Congestive Heart Failure</td>
<td>25 (2%)</td>
<td>11 (3%)</td>
<td>0.43</td>
<td>0.31</td>
</tr>
<tr>
<td>Atrial Fibrillation</td>
<td>55 (5%)</td>
<td>25 (6%)</td>
<td>0.18</td>
<td>0.10</td>
</tr>
</tbody>
</table>

*p value obtained from univariate regression model. † p value obtained from regression model adjusting for age and gender.
Table S4. Risk of Cardiovascular Disease in Carriers of the C Allele

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>TT</th>
<th>TC or CC</th>
<th>HR (95% CI) [pvalue]*</th>
<th>HR (95% CI) [pvalue] †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebrovascular Accident</td>
<td>188 (16%)</td>
<td>78 (20%)</td>
<td>1.30 (1.00, 1.70) [0.05]</td>
<td>1.43 (1.09, 1.86) [0.009]</td>
</tr>
<tr>
<td>Myocardial Infarction</td>
<td>108 (9%)</td>
<td>39 (10%)</td>
<td>1.12 (0.78, 1.61) [0.55]</td>
<td>1.13 (0.78, 1.63) [0.51]</td>
</tr>
<tr>
<td>Heart Failure</td>
<td>133 (11%)</td>
<td>51 (13%)</td>
<td>1.18 (0.85, 1.62) [0.33]</td>
<td>1.28 (0.93, 1.78) [0.13]</td>
</tr>
<tr>
<td>All-cause Death</td>
<td>137 (11%)</td>
<td>50 (12%)</td>
<td>1.10 (0.80, 1.52) [0.57]</td>
<td>1.20 (0.86, 1.65) [0.28]</td>
</tr>
</tbody>
</table>

* p values obtained from univariate model. †p values obtained from age- gender- body mass index adjusted hazard ratio analysis
Figure S1: Survival Free of Cerebrovascular Accident According to rs5065 Genotypes

Kaplan Meyer curve for unadjusted survival free of cerebrovascular accident (CVA) according to rs5065 genotypes over 10 years.
Figure S2: Activation of GC-A Receptor by ANP or ANP-RR

The ability of two concentrations of atrial natriuretic peptide (ANP) and ANP-RR (10^{-10} mol/L, 10^{-8} mol/L) to stimulate guanylyl cyclase-A (GC-A) receptor. P values obtained from t test.
Figure S3: cGMP Production in Human Aortic Endothelial Cells by ANP or ANP-RR

The ability of two concentrations of atrial natriuretic peptide (ANP) and ANP-RR (10^{-10} mol/L, 10^{-8} mol/L) to stimulate 3',5' cyclic guanosine monophosphate (cGMP) in human aortic endothelial cells. P values obtained from t test
Figure S4: ANP and ANP-RR Biological Action on Endothelial Cell Permeability

The ability of two concentrations of atrial natriuretic peptide (ANP) and ANP-RR ($10^{-10}$ mol/L, $10^{-8}$ mol/L) to stimulate human aortic endothelial cell permeability. P values obtained from t test.