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 MI[3] 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 ≥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.[5] Hypertension was diagnosed using Joint National Committee VI criteria.[6]

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).[7] 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.[8]

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/m^2 for men and greater than 100 g/m^2 for women.[11] Presence of left atrial enlargement was defined as left atrial volume index >33 ml/m^2 in men and >30 ml/m^2 in females.[12]

***In vitro* Studies**

Human embryonic kidney 293cells 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 250ug/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.[13] 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-methylzanthine (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.[14]

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.

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Table S1. Characteristics of Study Population according to rs5065 Genotypes

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

Table S1. Characteristics of Study Population according to rs5065 Genotypes

Characteristic	TT (n=1219)	TC or CC (n=404)	Unadjusted p value*	Adjusted p value†
Metabolic Syndrome (at least 3 of 5 criteria met)	239 (20%)	78 (19%)	0.88	0.85

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

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

* 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

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

* 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

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

***** p values obtained from univariate model. †p values obtained from age- gender- body mass index adjusted hazard ratio analysis

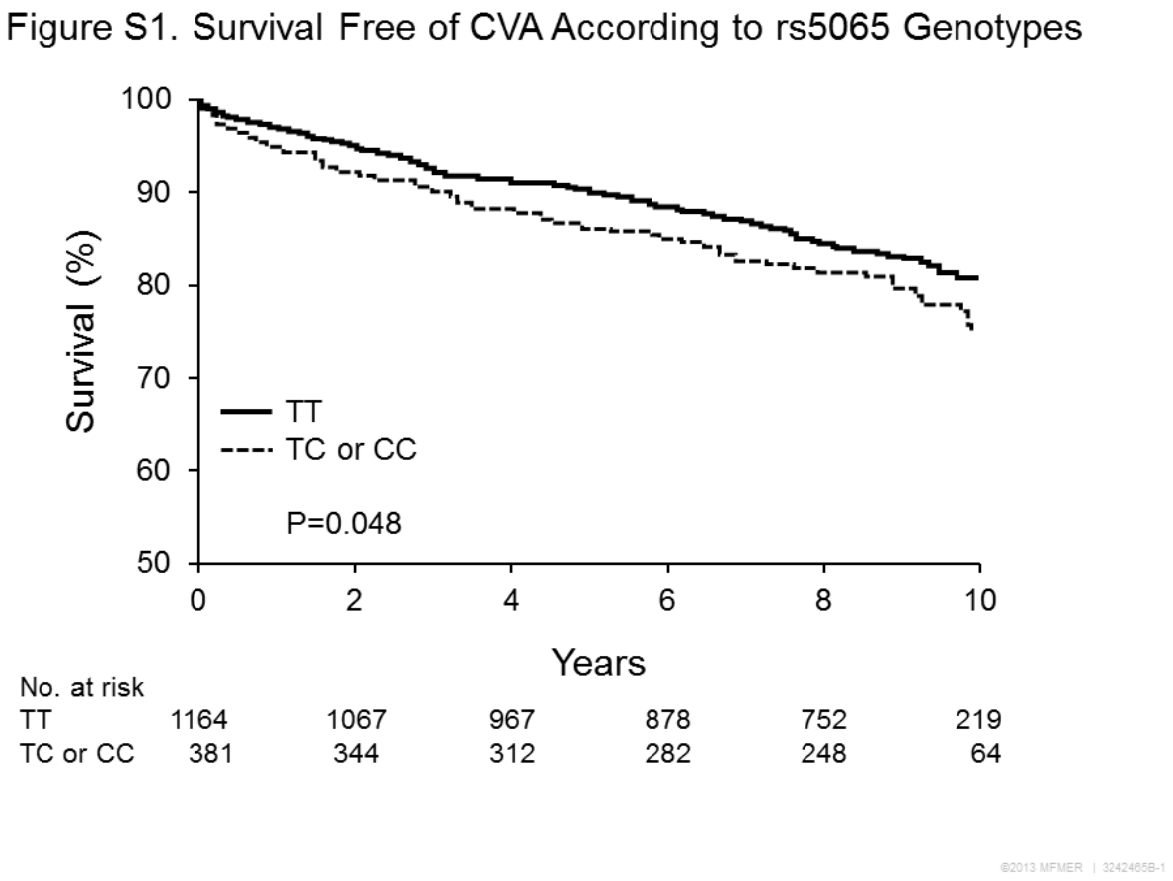

Figure S1: Survival Free of Cerebrovascular Accident According to rs5065 Genotypes

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

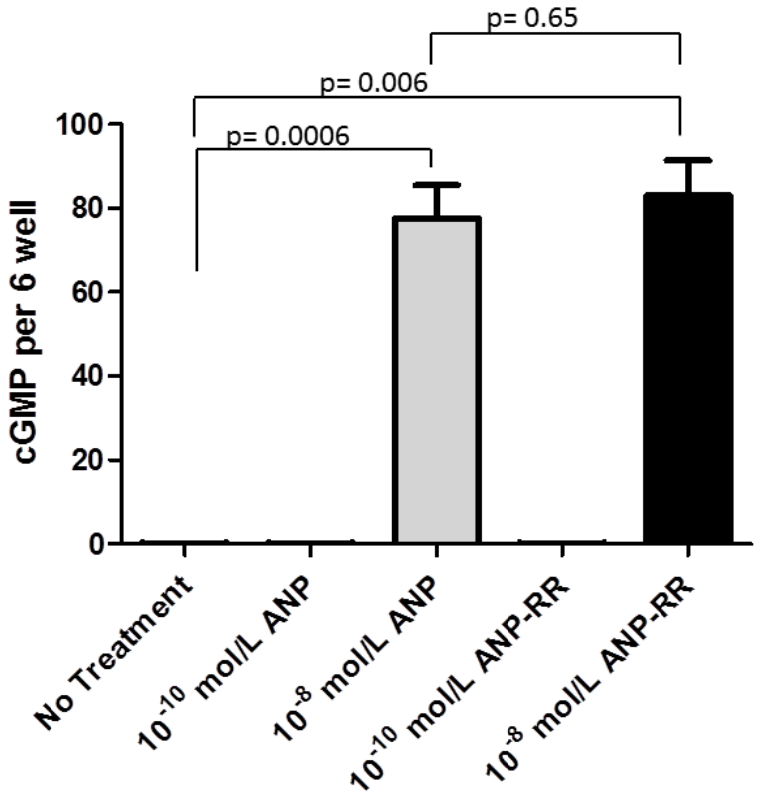

Figure S2: Activation of GC-A Receptor by ANP or ANP-RR

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

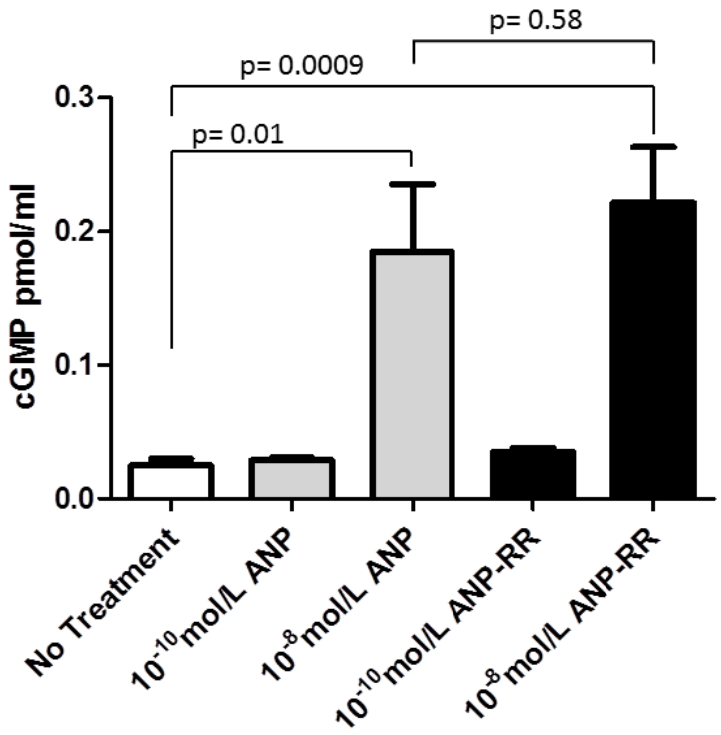

Figure S3: cGMP Production in Human Aortic Endothelial Cells by ANP or ANP-RR

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

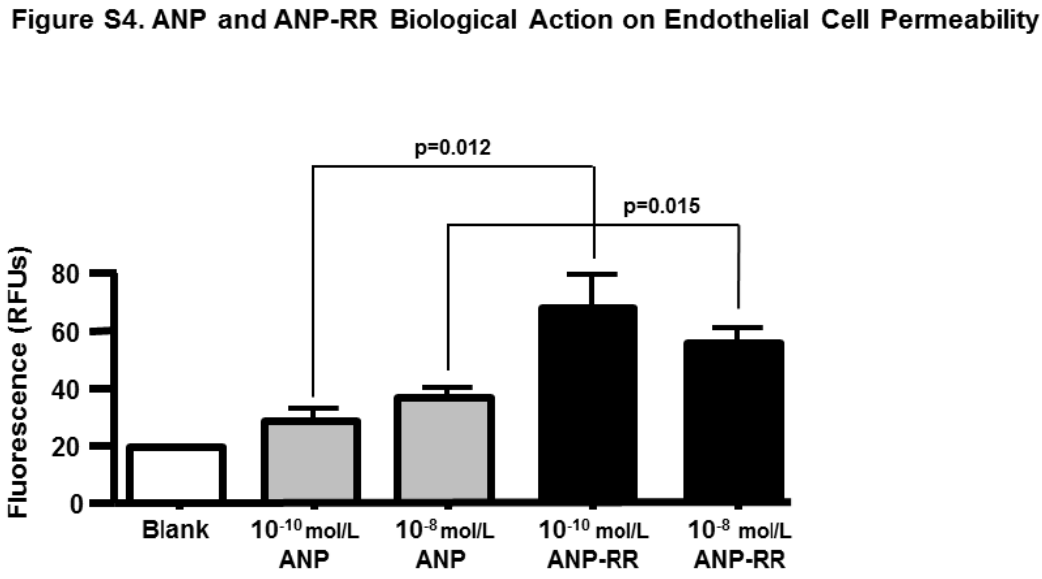

Figure S4: ANP and ANP-RR Biological Action on Endothelial Cell Permeability

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.