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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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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 two 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-A (GC-A) and B (GC-B) receptors, production of the second messenger 3',5'cyclic guanosine monophosphate (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% CI, 1.09 to 1.86; p= 0.009) and higher prevalence of myocardial infarction (odd ratio = 1.82; 95% CI, 1.07 to 3.09; p= 0.026). ANP-RR 10^{-8}mol/L activated the GC-A receptor (83.07 ±8.31 vs no treatment 0.18±0.04 6-per well, p=0.006), 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 cGMP production in endothelial cells similarly (p=0.58). Both concentrations of ANP-RR significantly enhanced human aortic endothelial cell permeability (69 vs 29 RFUs, p=0.012; 58 vs 39 RFUs, p= 0.015) compared to ANP. The minor allele of rs5065 was associated with increased cardiovascular risk. ANP-RR activated the GC-A receptor, increased cGMP in endothelial cells and when compared to ANP, ANP-RR augmented endothelial cell permeability.

Keywords

rs5065; atrial natriuretic peptide; natriuretic peptides; cardiovascular risk; cerebrovascular accident

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Conflict of Interest/Disclosure Statement
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INTRODUCTION

In 1981 with DeBold's report of 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-A (GC-A) receptor, which once activated catalyzes the synthesis of the natriuretic peptide second messenger, 3',5'cyclic guanosine monophosphate (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 cGMP.[6] Measurements with iodinated albumin have revealed that ANP stimulates endothelial macromolecule permeability in rats and mice[5, 7, 8] while mice with endothelium-restricted deletion of GC-A gene (EC GC-A KO) 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.[9–11]

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 two additional arginines at the carboxyl 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 al. revealed that the minor C allele of rs5065 was more prevalent in ischemic stroke patients than in control subjects.[12] 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. in patients with coronary artery stenosis.[13] Moreover, Zhang et al. reported that in a Chinese population of coronary artery disease patients the C allele is more frequent than in control subjects.[14] Importantly, the rs5065 minor allele is also an independent predictor of acute coronary syndrome and associated with increased risk of major adverse cardiovascular events in a population of coronary artery disease patients.[15] It should be noted that from a mechanistic perspective, previous *in vitro* studies investigated the impact 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.[16]

To date, no data have been reported regarding the clinical phenotype and cardiovascular risk associated with rs5065 genotypes in the general population or its relation to circulating ANP and BNP. The goal of the current study was threefold. First, in a large well characterized general adult population in Olmsted County, MN we sought to define associations of rs5065 with cardiovascular and metabolic phenotype 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. Lastly, we defined whether ANP-RR activates the natriuretic peptide receptor GC-A or guanylyl cyclase-B (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.[17–20]

METHODS

See on-line supplements.

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. Supplementary Table S1 illustrates the characteristics of the study population. The two groups (TT vs TC + CC genotypes) did not differ in terms of age (gender-adjusted p= 0.224). The C allele tended to be slightly less frequent for females (49% vs 54%, age-adjusted p= 0.088). An age- and gender- adjusted analysis showed that the minor C allele was significantly associated with higher BNP plasma levels measured with both Biosite (median 27.6 vs 23.2 pg/mL, p< 0.0001) and Shionogi assay (16.9 vs 14.7 pg/mL, p= 0.006) (supplementary table S2). After including body mass index (BMI), hypertension, coronary artery disease, MI, heart failure, ejection fraction below 50% or 40%, atrial fibrillation and cerebrovascular accident (CVA) as confounding factors in the regression model, there was a positive association of the C allele with BNP Biosite (parameter estimate = 0.17; 95% CI, 0.08 to 0.26; p= 0.0003) and BNP Shionogi (parameter estimate= 0.11; 95% CI, 0.02 to 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 gender- analysis of echocardiographic parameters revealed that the C allele was significantly associated with a higher prevalence of ejection fraction < 40% (3% vs 1%, p= 0.018) (supplementary table S1). After controlling for BMI, hypertension, coronary artery disease, MI, heart failure and atrial fibrillation the association was attenuated (odd ratio = 2.39; 95% CI, 0.98 to 5.84; p= 0.056). Median values of ejection fraction and prevalence of ejection fraction below 50% were similar between groups. Moreover, rs5065 genotype was not associated with left atrial volume, LV structure and prevalence of moderate to severe diastolic dysfunction. The two 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 gender, history of MI was more prevalent for the carriers of the minor allele (7% vs 5%, p= 0.023) (supplementary table S3). A similar result was obtained in regard to CVA, as the C allele was associated with higher prevalence of stroke or transient ischemic attack in a regression model adjusted for age and gender (2% vs 1%, p= 0.024). Importantly, the C allele was significantly associated with higher prevalence of MI (odd ratio = 1.82; 95% CI, 1.07 to 3.09; p= 0.026) and CVA (odd ratio = 2.52; 95% CI, 1.03 to 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 (supplementary figure S1). Moreover, analysis controlling for age, gender 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 to 1.86; p= 0.009) over a mean follow-up of 9 years (supplementary table S4). Multivariate adjustment including age, gender, BMI but also diabetes mellitus, hyperlipidemia, smoking and hypertension confirmed higher risk of CVA (hazard ratio 1.48; 95% CI, 1.12 to 1.94; p= 0.005) in the carriers of C allele.

The two 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 to the homozygous for the major T allele after controlling for age, gender and BMI (204 vs 199 mg/dL, p= 0.037). Plasma values of the other elements of the lipid panel, triglycerides, LDL and HDL cholesterol, as well as glucose and insulin levels did not differ between genotypes (supplementary table S1). Percentage of subjects treated with antilipemic agents was similar between groups. The analysis in 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 HEK-GC-A, 10^{-8} mol/L of both ANP and ANP-RR activated cGMP production (10^{-8}mol/L ANP: 77.50±7.95 vs no treatment 0.18±0.04 per 6-well, p=0.0006; 10^{-8}mol/L ANP-RR: 83.07 ±8.31 vs no treatment 0.18±0.04 6-per well, p=0.006) (supplementary figure S2). Conversely, 10^{-10} mol/L ANP or ANP-RR did not significantly increase production of cGMP in HEK-GC-A when compared to no treatment (10^{-10}mol/L ANP 0.22±0.01 vs no treatment 0.18±0.04 per 6-well, p=0.39; 10^{-10}mol/L ANP-RR 0.18±0.02 vs no treatment 0.18±0.04 6-per well, p= 0.95). There was no significant difference in cGMP production between ANP and ANP-RR at either tested concentration.

Incubation of HEK-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 vs no treatment 0.15±0.001 per 6-well, p=0.02; ANP 10^{-8}mol/L: 0.17 ±0.005 vs no treatment 0.15±0.001 6-per well, 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 HEK-GC-B (ANP-RR 10^{-10}mol/L: 0.35±0.18 vs no treatment 0.15±0.001 per 6-well, p=0.35; ANP-RR 10^{-8}mol/L: 0.20 ±0.03 vs no treatment 0.15±0.001 per 6-well, 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^{-8} mol/L, both ANP and ANP-RR activated cGMP in human aortic endothelial cells (ANP 10^{-8} mol/L: 0.18±0.05 vs no treatment 0.02±0.005 pmol/ml, p=0.01; ANP-RR 10^{-8}mol/L: 0.22 ±0.04 vs no treatment 0.02±0.005 pmol/ml, p=0.0009) at statistically similar levels (p=0.58) (supplementary figure S3). Neither ANP nor ANP-RR induced cGMP at 10^{-10} mol/L concentration compared to no treatment (ANP 10^{-10} mol/L: 0.03± 0.002 vs no treatment 0.02±0.005 pmol/ml, p= 0.52; ANP-RR 10^{-10}mol/L: 0.03 ±0.003 vs no treatment 0.02±0.005 pmol/ml, p=0.1)

Endothelial Cell Permeability—In this study, we evaluated the ability of two 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 vs 28 RFUs, p=0.012; 56 vs 37 RFUs, p= 0.015) compared to ANP (supplementary figure S4). When compared to no treatment, ANP 10^{-8} mol/L significantly enhanced endothelial permeability (37 vs 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 to control (68 vs 20 RFUs, p=0.012; 56 vs 20 RFUs, p= 0.0005).

DISCUSSION

Rs5065 is a genetic variant of *NPPA* and its minor allele encodes for an ANP with two 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 over 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 independently of several risk factors. Moreover, the minor allele of rs5065 is associated with higher BNP plasma values and prevalence of impaired ejection fraction less than 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. Lastly, 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 multi-vessel atherosclerosis.[13] In a case-control study conducted on patients affected by ischemic stroke, the C allele of rs5065 was significantly more prevalent for cases and associated with increased recurrence of stroke in a 5-year follow up analysis.[12] 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 over a mean follow-up analysis of 9 years. Thus, in our epidemiologic 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 the multivariate analysis 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 over a mean 9-years, which is the longest follow up analysis performed so far in regard to this genetic variant, and confirmed an increased risk to develop CVA for the carriers of the minor allele even after including age, gender, BMI, diabetes mellitus, hyperlipidemia, smoking and hypertension in the multivariate model. Our study confirms for the first time in a random sample of the general community from Olmsted County, MN, the association between the C allele of rs5065 and cardiovascular disease, such association was previously found only in selected populations of cardiovascular disease patients.[12–14] Our data in the general population suggests 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*[16] 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 as natriuretic peptides have lipolytic actions [3] and protect against obesity, as shown in mouse models.[22] Further, we have reported that the ANP genetic variant rs5068, which 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, gender 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, since 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, as our multivariate analysis was adjusted for hyperlipidemia. Moreover, in both groups median values of total cholesterol were close to the cut-off 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 Biosite 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 less than 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 al. had previously assessed natriuretic peptide concentrations and echocardiographic indices according to rs5065 genotypes in coronary artery disease patients and no significant association was observed.[24] 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 these regards

Results regarding a possible association between the genetic variants rs5065 and hypertension have been controversial. Whereas 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 two peptides activate the natriuretic peptide receptors GC-A and/or 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 two peptides. Incubation of HEK-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 and not GC-B receptor. Interestingly, Sciarretta et al 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 cyclic adenosine monophosphate pathway.[32] 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 to 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.[5] ANP exerts its biological action by binding to the GC-A receptor that is densely expressed in vascular endothelium.[5] Indeed, acute vascular volume expansion in EC GC-A KO mice results in rapid and significant increases in central venous pressure and decreases in hematocrit when compared to control mice, suggesting a lack of ANP modulation on oncotic intravascular pressure and fluid transport in EC GC-A KO mice.[5] In our study two concentrations of ANP-RR significantly increased human aortic endothelial cell permeability when compared to 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,[16] 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.[32]

Perspectives

The minor C allele of the *NPPA* genetic variant rs5065 codes for an ANP with two 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*, 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 to ANP. Additional studies are warranted to confirm the associations found in a general USA population and to investigate more 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 cardiovascular disease risk assessment and further analyses are clearly needed to evaluate it.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ANP	atrial natriuretic peptide
BMI	body mass index
BNP	B-type natriuretic peptide
cGMP	3’,5’cyclic guanosine monophosphate
CVA	cerebrovascular accident
EC GC-A KO	endothelium-restricted deletion of the GC-A gene
GC-A	guanylyl cyclase-A
GC-B	guanylyl cyclase-B
HEK-GC-A	human embryonic kidney 293 cells expressing GC-A
HEK-GC-B	human embryonic kidney 293 cells expressing GC-B
LV	left ventricular
MI	myocardial infarction
NPPA	atrial natriuretic peptide gene

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Novelty and Significance

What is new?

No previous studies have investigated the cardiometabolic phenotype and cardiovascular risk associated with rs5065 in a general population. In regard to vascular permeability, the biological action of 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% CI, 1.12 to 1.94; p= 0.005), higher prevalence of myocardial infarction (odd ratio = 1.82; 95% CI, 1.07 to 3.09; p= 0.026) and higher B-type natriuretic peptide plasma levels measured with Biosite (p= 0.0003) and Shionogi assay (p= 0.022). ANP-RR significantly enhanced human aortic endothelial cell permeability (p=0.012; p= 0.015) compared to ANP.

Summary

In a general USA population the minor allele of rs5065 is associated with increased cardiovascular risk. In vitro, when compared to ANP, ANP-RR determines augmented endothelial cell permeability.