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Influence of rs5065 Atrial Natriuretic Peptide Gene Variant on Coronary Artery Disease

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Objectives The aim of this study was to investigate the impact of rs5065 atrial natriuretic peptide (ANP) gene variant on coronary artery disease (CAD) and its outcomes and to gain potential mechanistic insights on the association with CAD.

Background Either modified ANP plasma levels or peptide structural alterations have been involved in development of cardiovascular events.

Methods Three hundred ninety-three control subjects and 1,004 patients undergoing coronary angiography for suspected CAD (432 stable angina [SA], 572 acute coronary syndrome [ACS]) were genotyped for rs5065 ANP gene variant. Data in SA and ACS groups were replicated in an independent population of 482 stable angina patients (rSA) and of 675 ACS patients, respectively. Clinical follow-up was available for both SA and rSA patients. Plasma N-terminal-proANP, myeloperoxidase, lipoprotein-associated phospholipase A2, and oxidized low-density lipoprotein were assessed in a subgroup of rSA patients.

Results rs5065 minor allele (MA) was an independent predictor of ACS (odds ratio: 1.90; 95% confidence interval: 1.40 to 2.58, $p < 0.001$). At follow-up, rs5065 MA was independently associated with a significantly higher rate of major adverse cardiovascular events in the SA group, $p < 0.001$. Data were replicated in the rSA group at follow-up ($p = 0.008$). Cox proportional hazard analysis tested by 4 models confirmed higher major adverse cardiovascular events risk in rs5065 MA carriers in both SA and rSA cohorts. Significantly higher myeloperoxidase levels were detected in rs5065 MA carriers ($n = 597$ [345 to 832 μg/l] vs. $n = 488$ [353 to 612 μg/l], $p = 0.038$). No association of rs5065 was observed with N-terminal-proANP levels.

Conclusions The MA of rs5065 ANP gene variant associates with increased susceptibility to ACS and has unfavorable prognostic value in CAD. (J Am Coll Cardiol 2012;59:1763–70) © 2012 by the American College of Cardiology Foundation

Atrial natriuretic peptide (ANP) is a hormone synthesized in cardiac atria and released into the circulation exerting relevant natriuretic, diuretic, and vasodilatory effects (1). Elevated ANP levels are associated with increased cardiovascular risk in the general population (2–4) and with increased mortality in patients with heart failure, ischemic heart disease, and stroke (5,6). The rs5065 ANP gene variant, corresponding to a T to C substitution at position 2238 of the pre-proANP gene, leads to an alteration of ANP primary sequence and translates into an altered ANP peptide. The latter has been shown to reduce endothelial cell viability, proliferation and endothelial cell tube forma-

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Abbreviations and Acronyms

ACE-I = angiotensin-converting enzyme inhibitors

ACS = acute coronary syndrome(s)

ANP = atrial natriuretic peptide

BMI = body mass index

CAD = coronary artery disease

CVA = cerebrovascular accident

ELISA = enzyme-linked immunoadsorbent assay

Lp-PLA2 = lipoprotein-associated phospholipase A2

MA = minor allele

MACCE = major adverse cardiac and cerebrovascular event(s)

MACE = major adverse cardiovascular event(s)

MI = myocardial infarction

MPO = myeloperoxidase

NT = N-terminal

OxLDL = oxidized low-density lipoprotein

rACS = independent patient population with acute coronary syndrome

rSA = independent population of stable angina patients

SA = stable angina

WT = wild-type

tion in vitro and to modulate common mechanisms underlying the transition from stable to unstable plaque (7). The frequency of the rs5065 variant in the general population ranges from 14% to 23%, and it has been associated with increased risk of stroke and myocardial infarction (MI) in previous studies (8–11). Nevertheless, the impact of rs5065 variant on susceptibility to develop cardiovascular events has never been investigated through a prospective type of approach. In addition, it is not clear whether this ANP variant, apart from predisposing to increased incidence of acute cardiovascular events due to its actions on mechanisms of plaque vulnerability, might also affect development of atherosclerotic disease. In this regard, emerging biomarkers of vascular atherosclerosis and plaque instability (i.e., lipoprotein-associated phospholipase A2 [Lp-PLA2], oxidized low-density lipoprotein [OxLDL], and myeloperoxidase [MPO]) (12–14) might help our understanding of the real impact of rs5065 on atherosclerotic plaque progression/vulnerability.

In the present study we aimed at: 1) investigating the role of rs5065 variant on susceptibility to acute coronary syndrome (ACS) through the analysis of 2 independent ACS populations and 2 independent cohorts of stable angina (SA) patients; 2) assessing its effect on coronary artery disease (CAD)-related outcomes through a prospective follow-up approach in SA patients; and 3) exploring the impact of rs5065 on atherosclerotic plaque progression/instability within the context of ischemic heart disease through the determination of vascular biomarkers like Lp-PLA2, OxLDL, and MPO.

Methods

Study population. The study population consisted of 1,004 consecutive Caucasian patients referred to the Cardiovascular Center OLV Aalst (Belgium) for cardiac catheterization from January 2000 to December 2002 because of: 1) SA with at least 1 significant coronary stenosis (diameter >50% as assessed by quantitative coronary angiography) and positive functional stress test; or 2) ACS (e.g., unstable angina, non–ST-segment elevation MI or ST-segment elevation MI) (15). Control population consisted of 393 healthy Caucasian volunteers. To replicate the findings observed in SA patients, genotype analysis was also performed in an independent population of 482 consecutive stable angina (rSA) Caucasian patients with at least 1 significant coronary stenosis (diameter >50% as assessed by quantitative coronary angiography) and positive functional stress test referred for cardiac catheterization from January 2007 to December 2009. To replicate the findings observed in ACS patients, genotype analysis was also performed in an independent population of 675 Caucasian patients with ACS, originating from the Belgian cohort of the GRACE registry (Global Registry of Acute Coronary Events) (16). Caucasian ethnicity of the subjects included in the study was established on the basis of a questionnaire to exclude non-Caucasian origin up to 3 generations of ancestors. Only white European subjects and patients from 3 generations of ancestors were included in the study.

Systemic arterial hypertension was defined as systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg on at least 2 separate occasions (17) or ongoing antihypertensive treatment. Diabetes mellitus was defined as the presence of an active treatment with insulin or an oral antidiabetic agent (18). Patients were defined as hypercholesterolemic when they had total cholesterol plasma concentrations ≥220 mg/dl or they were receiving lipid-lowering drugs. Patients reporting regular smoking in the previous 6 months were considered current smokers.

Clinical endpoints were evaluated at clinic visits alternated with phone contacts for up to 6 years in the SA population and 4 years in the rSA population. The primary endpoint was major adverse cardiovascular event (MACE), defined as the composite of: 1) mortality from all causes (Death); 2) MI; and 3) revascularization by percutaneous coronary intervention or bypass surgery (Revasc). Secondary endpoints were death, MI, revascularization, cerebrovascular accidents (CVAs), and major adverse cardiac and cerebrovascular event (MACCE) (composite of death, MI, revascularization, and CVA).

The study was approved by the Ethical Committees of the participating institutions, and informed consent was obtained from all subjects.

Genetic analysis. A blood sample collection was performed from a peripheral vein for deoxyribonucleic acid analysis. Deoxyribonucleic acid was extracted from whole blood with a commercially available kit (Qiagen). The ANP gene single nucleotide polymorphism rs5065 was characterized as previously described (8,19).

Biomarker assessment. Blood sampling for biomarker assessment was performed at the time of study enrolment in rSA patients. The N-terminal (NT)-proANP levels were assessed by a commercially available enzyme-linked immunoadsorbent assay (ELISA) kit (Gruppe Biomedica, Vienna, Austria). The MPO levels were measured by Mercodia MPO ELISA kit (Mercodia, Uppsala, Sweden);

Lp-PLA2 levels were assessed by Lp-PLA2 PLAC test Diadexus ELISA kit (Diadexus, South San Francisco, California); OxLDL levels were determined by a sandwich ELISA kit with the monoclonal antibody 4E6 (Mercodia). The antibody 4E6 is especially against an epitope in the apoB-100 moiety of OxLDL that is formed from substitution of lysine residues of apoB-100 with aldehydes (20).

Statistical analysis. All data analyses were performed with SPSS software package (version 13.0, SPSS, Inc., Chicago, Illinois). Normal distribution was assessed by Kolmogorov-Smirnov test. Continuous variables are expressed as mean ± SD or median [interquartile ranges]; categorical variables are expressed as frequencies and percentages. Student *t* test or Mann-Whitney test was used to compare continuous variables, as appropriate. Comparisons between categorical variables were evaluated with 2-tailed Fisher exact test or Pearson's chi-square test, as appropriate. The NT-proANP levels within the different genotypes were compared with Kruskal-Wallis test. Predictors of biomarkers elevation were assessed by linear regression analysis for continuous variables adjusted for age, sex, body mass index (BMI), hypertension, hypercholesterolemia, diabetes, and smoking. Genotype frequencies and Hardy-Weinberg equilibrium were estimated with chi-square test.

The risk associated with ANP variant in the occurrence of ACS and risk of significant coronary stenosis at angiography were estimated by logistic regression analysis, computing the odds ratio with the respective 95% confidence interval under the assumption of a dominant model (score of 0 for WT; 1 for heterozygous and double mutant). Due to the low number of homozygotes for the rs5065 minor allele (MA), neither additive nor recessive models of inheritance were included. A multivariate logistic regression analysis model was built including the following variables as covariates: age, sex, BMI, hypertension, hypercholesterolemia, diabetes, and smoking.

Survival curves for MACE were constructed with the Kaplan–Meier method. Clinical endpoints were evaluated with the Cox proportional hazard analysis. Cox proportional hazard analysis for cumulative MACE at follow-up was performed with 4 multivariate models: 1) adjusted for age, sex, BMI, hypertension, diabetes, hypercholesterolemia, smoking habit, multivessel disease; 2) adjusted for age, hypertension, diabetes, hypercholesterolemia, smoking habit, therapy with angiotensin-converting enzyme inhibitors (ACE-I), diuretic agents, aspirin; 3) adjusted for sex, hypertension, diabetes, hypercholesterolemia, smoking habit, therapy with ACE-I, diuretic agents, aspirin; and 4) adjusted for multivessel disease, hypertension, diabetes, hypercholesterolemia, smoking habit, therapy with ACE-I, diuretic agents, aspirin. Results are expressed as hazard ratios (diuretic agents) and 95% confidence intervals. For all tests, a 2-tailed $p < 0.05$ was considered significant.

Results

Clinical characteristics. Clinical characteristics of the patients are shown in Table 1. With the exception of BMI and therapy with diuretic agents, clinical features and medical therapy of patients with SA and ACS were significantly different as compared with the control population. The SA patients were characterized with higher frequency of multivessel disease as compared with ACS.

The rSA patients were younger, with higher incidence of hypertension and hypercholesterolemia, as compared with the SA group. By contrast, smoking habit, multivessel disease, and treatment with ACE-I and aspirin at discharge were lower as compared with the SA group.

The rACS patients were slightly older and more frequently smokers, whereas they presented with lower frequency of male sex, hypertension, diabetes, and hypercholesterolemia, compared with ACS patients. Medical therapy also differed at discharge, as compared with ACS patients (Table 1).

rs5065 ANP gene polymorphism analysis. Genotype and allele frequency of the patients is reported in Table 1. The observed genotype distribution for rs5065 in the overall population was in Hardy-Weinberg equilibrium ($p = 0.48$). Frequency of the rs5065 MA in control subjects was similar to that previously published in other control populations (8,10,16). As shown in Table 1, both SA and rSA patients presented an rs5065 MA frequency similar to that observed in control subjects. In contrast, both ACS and rACS patients presented higher frequency of rs5065 MA, as compared with control and SA patients.

rs5065 ANP gene polymorphism and circulating biomarkers. The NT-proANP plasma levels were assessed in 428 rSA patients. No differences were detected among the 3 genotypes: 4,138 (2,988 to 6,578) fmol/ml in rs5065 WT, 4,266 (2,833 to 6,373) fmol/ml in rs5065 heterozygotes, 4,677 (2,531 to 8,137) fmol/ml in rs5065 mutant homozygotes ($p = 0.92$).

The MPO, Lp-PLA2, and OxLDL were assessed in 212 rSA patients (Fig. 1). The MPO levels were significantly higher in rs5065 MA carriers as compared with WT (597 [345 to 832] μg/l vs. 488 [353 to 612] μg/l, $p = 0.038$). At the linear regression analysis, rs5065 MA was the only independent predictor of MPO elevation (unadjusted: beta = 0.21, t = 3.04, $p = 0.003$; adjusted: beta = 0.18, t = 2.66, $p = 0.008$). No significant difference was observed between rs5065 WT and rs5065 MA carriers with regard to Lp-PLA2 (251 [204 to 311] μg/l vs. 252 [215 to 315] μg/l, $p = 0.59$) and OxLDL (60 [43 to 79] U/l vs. 55 [45 to 75] U/l, $p = 0.78$) levels.

Correlation of rs5065 ANP gene polymorphism with angiographic and clinical outcome. Predictors of ACS are shown in Table 2. The multivariate adjusted analysis confirmed the rs5065 MA to be an independent predictor of ACS.

Table 1 Clinical Characteristics and Genotypes of the Study Groups

	CTRL (n = 393)	SA (n = 432)	p Value (vs. CTRL)	rSA (n = 482)	p Value (vs. CTRL)	p Value (vs. SA)	ACS (n = 572)	p Value (vs. CTRL)	p Value (vs. SA)	rACS (n = 675)	p Value vs. CTRL	p Value vs. ACS
Age, yrs	69 ± 13	76 ± 10	<0.001	65 ± 11	<0.001	<0.001	64 ± 12	<0.001	<0.001	66 ± 12	0.001	0.037
Male	247 (63)	306 (71)	0.017	313 (65)	0.525	0.065	493 (86)	<0.001	<0.001	507 (75)	<0.001	<0.001
BMI, kg/m^2	26 ± 4	27 ± 4	0.109	27 ± 5	0.01	0.122	27 ± 4	0.121	0.815	27 ± 4	0.147	0.894
Hypertension	164 (42)	222 (51)	0.004	281 (58)	<0.001	0.037	362 (63)	<0.001	<0.001	372 (55)	<0.001	0.004
Diabetes	60 (15)	90 (21)	0.037	119 (25)	<0.001	0.179	181 (32)	<0.001	<0.001	127 (19)	0.156	<0.001
Hypercholesterolemia	118 (30)	249 (58)	<0.001	324 (67)	0.003	0.003	348 (61)	<0.001	0.434	289 (43)	<0.001	<0.001
Smoking habit	115 (29)	197 (46)	<0.001	186 (39)	0.003	0.03	277 (48)	<0.001	0.482	397 (59)	<0.001	0.001
Multivessel disease	—	288 (67)	—	179 (37)	—	<0.001	296 (52)	—	<0.001	360 (50)	—	0.538
Genotype			0.522		0.901	0.599		0.002	0.001		0.002	0.703
rs5065 WT	284 (72)	324 (75)		358 (74)			359 (63)			424 (63)		
rs5065 heterozygote	103 (26)	99 (23)		109 (23)			198 (35)			238 (35)		
rs5065 mutant homozygote	6 (2)	9 (2)		15 (3)			15 (2)			13 (2)		
Allele			0.571		0.946	0.637		0.003	0.001		0.005	0.854
WT	671 (85)	747 (86)		825 (86)			916 (80)			1,086 (80)		
MA	115 (15)	117 (14)		139 (14)			228 (20)			264 (20)		
Treatment at discharge												
Beta-blockers	91 (23)	203 (47)	<0.001	253 (52)	<0.001	0.098	326 (57)	<0.001	0.002	475 (70)	<0.001	<0.001
ACE-I	143 (36)	186 (43)	0.054	163 (34)	0.434	0.004	332 (58)	<0.001	<0.001	405 (60)	<0.001	<0.488
Diuretic agents	117 (30)	112 (26)	0.243	120 (25)	0.109	0.761	194 (34)	0.183	0.007	—		
Calcium antagonists	41 (10)	69 (16)	0.0238	73 (15)	0.043	0.784	132 (23)	<0.001	0.005	—		
Statins	99 (25)	259 (60)	<0.001	287 (60)	<0.001	0.946	463 (81)	<0.001	<0.001	391 (58)	<0.001	<0.001
Aspirin	108 (27)	384 (89)	<0.001	338 (70)	<0.001	<0.001	543 (95)	<0.001	<0.001	589 (87)	<0.001	<0.001

Values are mean ± SD or n (%). Fisher or chi-square test performed as appropriate.

ACE-I = angiotensin-converting enzyme inhibitors; ACS = acute coronary syndrome(s); BMI = body mass index; CTRL = control; MA = minor allele; rACS = independent patient population with acute coronary syndrome; rSA = independent population of stable angina patients; SA = stable angina; WT = wild-type.

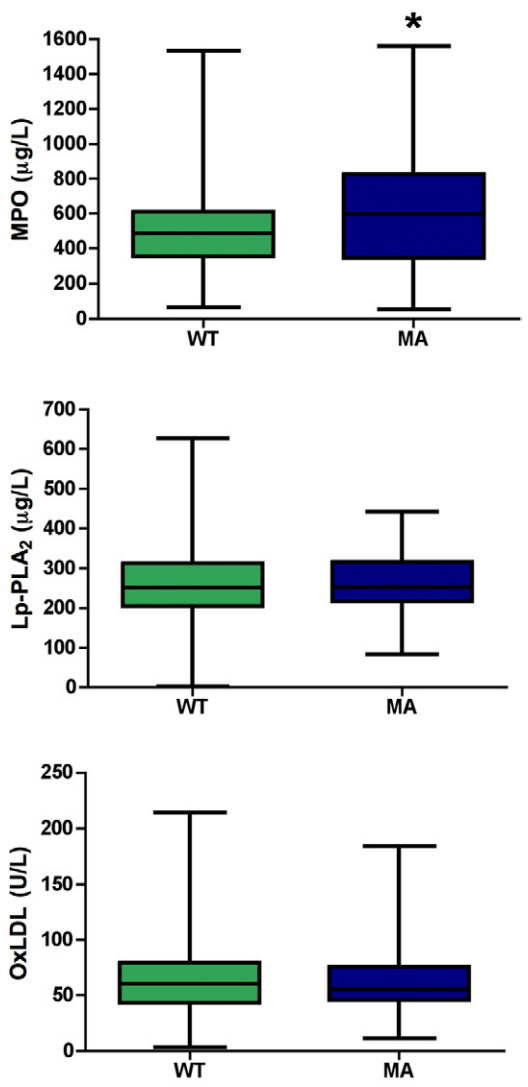

Figure 1 **Biomarkers in rs5065 Homozygotes and in MA Carriers**

Myeloperoxidase (MPO), lipoprotein-associated phospholipase A2 (Lp-PLA2), and oxidized low-density lipoprotein (OxLDL) plasma levels in rs5065 WT (WT) **(green)** versus minor allele (MA) carriers **(blue)**. The OxLDL was determined by 4E6 Mercodia antibody (Mercodia, Uppsala, Sweden).

The rs5065 variant neither showed an effect on the risk of significant coronary artery stenosis at the angiography ($p = 0.15$) nor was associated with the number of diseased vessels ($p = 0.32$).

In SA patients, median clinical follow-up was 37 months (13 to 70 months), and it was obtained in 376 patients (87%). A total of 145 (39%) patients experienced MACE during the follow-up period (Table 3). At the univariate analysis, MA carriers showed a significantly higher rate of MI, the combined endpoint of death/MI, revascularization, MACCE, and MACE as compared with WT (Table 3). No difference was observed in the rate of death and CVA. Survival analysis revealed a significantly lower MACE-free survival in MA carriers (Fig. 2A). At Cox proportional hazard analysis, irrespective of the multivariate model, rs5065 MA carriers were independently associated with significantly higher MACE rate at follow-up (Table 4). Medical treatment at follow-up did not significantly differ between rs5065 WT and MA carriers (data not shown), with the exception of diuretic agents. In particular, 123 (24%) WT patients and 156 (30%) rs5065 MA carriers were taking diuretic agents ($p = 0.02$).

In rSA patients, median clinical follow-up was 31 months (23 to 41 months), and it was obtained in 462 patients (96%). A total of 86 (19%) patients experienced MACE during the follow-up period (Table 3). At the univariate analysis, MA carriers showed a significantly higher rate of MI, the combined endpoint of death/MI, CVA, MACCE, and MACE as compared with WT (Table 3). No difference was observed in the rate of death and Revasc. Survival analysis revealed a significantly lower MACE-free survival in MA carriers (Fig. 2B). At Cox proportional hazard analysis, irrespective of the multivariate model, rs5065 MA carriers were independently associated with significantly higher MACE rate at follow-up (Table 4). Medical treatment at follow-up did not significantly differ between WT and MA carriers (data not shown).

Discussion

Our findings demonstrate that patients carrying the rs5065 MA are at higher risk of ACS and show lower MACE-free survival rate.

The ANP variant resulting from a T to C substitution at position 2238 of the pre-proANP gene is present in 14% to 23% of individuals from the general population (8,21). It leads to a mutant peptide able to induce endothelial cell damage by increasing reactive oxygen species production and reducing antioxidative stress response (7). This mutant peptide was also shown to up-regulate genes encoding enzymes involved in extracellular matrix degradation that might favor the transition from stable to unstable plaque (7).

The rs5065 ANP variant has been previously associated with increased risk of stroke and MI (8–11). In the present study, we demonstrate increased frequency of rs5065 MA in ACS patients, with a nearly 2-fold independent risk of

Table 2 **Predictors of ACS at Multivariate Analysis**

	OR	95% CI	p Value
rs5065 MA	1.90	1.40–2.58	<0.001
Hypertension	1.85	1.37–2.49	<0.001
Diabetes	1.68	1.21–2.33	0.002
Age	0.91	0.89–0.92	<0.001
Smoking	0.69	0.50–0.96	0.028
BMI	0.96	0.93–0.99	0.015
Hypercholesterolemia	0.75	0.56–1.01	0.062
Male	0.99	0.70–1.41	0.98

Patients from SA and ACS populations.
CI = confidence interval; OR = odds ratio; other abbreviations as in Table 1.

Table 3 Association of rs5065 MA With MI and MACE at Follow-Up

	SA Population				rSA Population			
	WT	MA Carriers	HR (95% CI)	p Value	WT	MA Carriers	HR (95% CI)	p Value
Death	13 (4)	3 (6)	1.73 (0.49–6.09)	0.392	18 (5)	5 (4)	0.78 (0.28–2.16)	0.635
MI	26 (8)	14 (27)	4.02 (2.10–7.71)	<0.001	0 (0)	10 (8)	65.09 (3.78–1121)	<0.001
Death/MI	39 (12)	17 (34)	3.26 (1.84–5.77)	<0.001	18 (5)	15 (12)	2.57 (1.25–5.28)	0.008
Revasc	101 (31)	25 (47)	1.81 (1.16–2.79)	0.008	36 (10)	19 (16)	1.59 (0.88–2.91)	0.122
CVA	6 (2)	1 (2)	1.15 (0.14–9.58)	0.895	1 (0)	6 (5)	16.87 (2.03–140.27)	0.009
MACCE	117 (36)	31 (60)	1.94 (1.30–2.88)	0.001	55 (16)	37 (31)	2.33 (1.43–3.77)	<0.001
MACE	114 (35)	31 (60)	2.85 (1.56–5.24)	<0.001	54 (16)	32 (27)	1.94 (1.18–3.19)	0.008

Values are n (%). Univariate analysis.

HR = hazard ratio; MACCE = major adverse cardiac and cerebrovascular event (defined as combined death, myocardial infarction [MI], revascularization by percutaneous coronary intervention or bypass surgery [Revasc], and cerebrovascular accidents [CVAs]); MACE = major adverse cardiovascular event (defined as combined death, MI, and Revasc); other abbreviations as in Tables 1 and 2.

ACS. Most importantly, the direct contributory role to cardiovascular events of rs5065 MA is supported by the observation of lower event free survival with higher MI and higher combined death/MI, revascularization, MACCE, and MACE rates in SA patients carrying rs5065 MA. Yet, no association of rs5065 MA with significant coronary artery stenosis or with the number of diseased vessels was observed. This apparent discrepancy might be related to the reported in vitro effects of this ANP variant favoring plaque instability (7).

Of note, elevated MPO levels were measured in MA carriers, as compared with WT subjects, in rSA patients, consistent with a major effect on plaque vulnerability rather than on progression of vascular atherosclerosis. Myeloperoxidase seems to play a causal role in plaque instability (22) by exerting its deleterious effects through: 1) the initiation of lipid oxidation in the subendothelial space of the vessel wall, where it generates several reactive oxygen intermediates able to induce oxidative cellular damage; and 2) the activation of matrix metalloproteinases (23) and deactivation of matrix metalloproteinase inhibitors (24), thereby promoting the weakening of the fibrous cap, potentially leading to destabilized atherosclerotic plaque. Myeloperoxidase has been associated with higher risk of developing first acute coronary event as well as subsequent cardiovascular events in patients previously admitted for ACS (25,26). Although we cannot clarify the causal interaction between mutant ANP and MPO levels, we suggest that MPO might act synergistically and exacerbate the effects of mutant ANP in CAD patients.

By contrast, we did not observe any association between rs5065 MA and levels of either OxLDL or Lp-PLA2. Lack of association with these 2 biomarkers could be partly explained by their involvement mostly in the progression, severity, and extent of atherosclerotic disease rather than on plaque vulnerability (27–30). However, with regard to our findings on OxLDL, it should also be mentioned that the 4E6 antibody from Mercodia used in our assay does not have a high specificity for OxLDL, being especially prepared against an epitope in the apoB-100 moiety of OxLDL (31,32), and consequently, it might have limited, at least in part, the results of our analysis.

Data in SA patients were replicated in an independent control patient population (rSA). It should be acknowledged that rSA patients showed lower MACEs as compared with SA patients (19% vs. 39%, $p < 0.001$), most likely due to different duration of follow-up, lower incidence

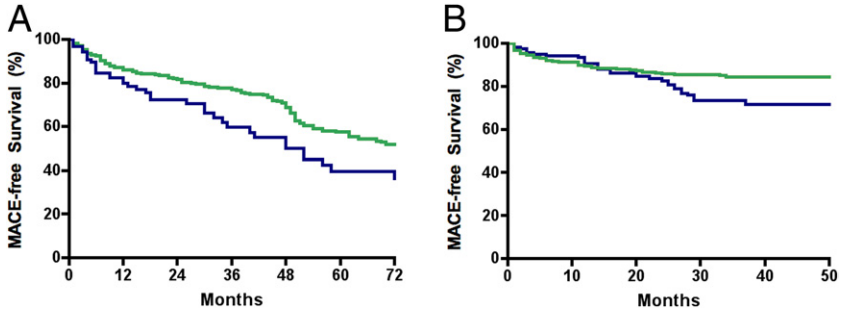

Figure 2 Survival Curves in Stable Angina and Independent Stable Angina Patient Populations

(A) Major adverse cardiovascular event (MACE)-free survival (mortality from all causes, myocardial infarction, revascularization by percutaneous coronary intervention or bypass surgery) in stable angina population (log-rank: 6.13, p = 0.013). **(B)** MACE-free survival (mortality from all causes, myocardial infarction, revascularization by percutaneous coronary intervention or bypass surgery) in an independent population of stable angina patients (log-rank: 5.71, p = 0.017). Patients WT at rs5065 are indicated with **green line**, patients carrying rs5065 MA are indicated with **blue line**.

Table 4 Cox Proportional Hazard Analysis for the Association of rs5065 MA Carriers With MACE in SA and rSA Populations

	Model 1		Model 2		Model 3		Model 4	
	HR (CI)	p Value	HR (CI)	p Value	HR (CI)	p Value	HR (CI)	p Value
SA	1.99 (1.32–2.99)	0.001	2.02 (1.35–3.02)	0.001	2.03 (1.36–3.03)	0.001	2.01 (1.34–2.99)	0.001
rSA	1.82 (1.16–2.87)	0.009	1.73 (1.07–2.79)	0.024	1.72 (1.07–2.78)	0.026	1.72 (1.06–2.80)	0.028

Model 1: adjusted for age, sex, BMI, hypertension, diabetes, hypercholesterolemia, smoking habit, multivessel disease; Model 2: adjusted for age, hypertension, diabetes, hypercholesterolemia, smoking habit, therapy with ACE-I, diuretic agents, aspirin; Model 3: adjusted for sex, hypertension, diabetes, hypercholesterolemia, smoking habit, therapy with ACE-I, diuretic agents, aspirin; Model 4: adjusted for multivessel disease, hypertension, diabetes, hypercholesterolemia, smoking habit, therapy with ACE-I, diuretic agents, aspirin. Abbreviations as in Tables 1, 2, and 3.

of multivessel disease, and adoption of drug-eluting stents, unavailable at the time SA patients were recruited. Nevertheless, rSA patients carrying rs5065 MA showed significantly higher MI, CVA, MACCE, and MACE rates as well as lower MACE-free survival at follow-up, as compared with WT patients.

Data in ACS patients were also replicated in an independent patient population (rACS). It will be interesting in the future to assess whether rs5065 ANP variant might influence cardiovascular prognosis after acute coronary events.

A recent pharmacogenetic study has shown beneficial effects of treatment with diuretic agents in rs5065 MA carriers in terms of improved blood pressure control and reduced cardiovascular risk in hypertensive subjects (21). Thus, it is possible that a pharmacogenetic interaction might have slightly confounded our findings at least in SA patients, because rs5965 MA carriers took diuretic agents more frequently, as compared with WT patients. Therefore, the outcome of MA carriers might have been slightly improved, although it remained significantly worse as compared with that of WT patients.

We also tested the hypothesis that rs5065 ANP variant might produce its effects through an alteration of the circulating peptide levels. In fact, a substantial amount of evidence has shown that elevated ANP levels are associated with increased cardiovascular risk and with worse prognosis in patients affected by cardiovascular diseases (1). In particular, elevated NT-proANP levels were found to parallel the progression of coronary atherosclerosis (33). High ANP levels were reported in stroke (6,34), and they have been associated with increased post-stroke and post-MI mortality (6) as well as with mortality in heart failure patients (5). However, we were unable to identify significant differences among the 3 rs5065 genotypes in terms of circulating peptide concentrations. Although we cannot exclude a role of the ongoing medical therapy, the latter finding mainly suggests that the structural peptide alteration dependent from the T to C transition within exon 3 of pre-proANP gene is the predominant cause of the observed increased predisposition to ACS, consistent with our in vitro observations (7).

Conclusions

The rs5065 ANP variant leading to altered and dysfunctional ANP peptide is associated with higher risk of ACS and translates into worse long-term clinical follow-up in SA patients. Our prospective study provides the first demonstration of the important predictive role played by rs5065 ANP gene variant in CAD. We measured higher MPO levels, a biomarker associated with plaque vulnerability, in rs5065 MA carriers, corroborating previous in vitro evidence of a dysfunctional peptide.

More studies are needed to define whether systematic screening for ANP gene variants in CAD patients will help to better define the individual cardiovascular risk prediction. In particular, more aggressive and specifically tailored pharmacological treatment might be required to improve clinical outcome in patients carrying rs5065 MA.

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REFERENCES

1. Rubattu S, Sciarretta S, Valenti V, Stanzione R, Volpe M. Natriuretic peptides: an update on bioactivity, potential therapeutic use and implication in cardiovascular diseases. Am J Hypertens 2008;21:733–41.
2. Wang TJ, Larson MG, Levy D, et al. Plasma natriuretic peptide levels and the risk of cardiovascular events and death. N Engl J Med 2004;350:655–63.
3. Rubattu S, Barbato A, Marchitti S, et al. Determinants of N-terminal pro atrial natriuretic peptide plasma levels in a survey of adult male population from Southern Italy. J Hypertens 2010;28:1638–45.
4. Barbato S, Sciarretta S, Marchitti S. et al. Aminoterminal natriuretic peptides and cardiovascular risk in an Italian male adult cohort. Int J Cardiol 2011;152:245–6.
5. Volpe M, Francia P, Tocci G, et al. Prediction of long term survival in chronic heart failure by multiple biomarker assessment. A 15-year prospective follow-up study. Clin Cardiol 2010;33:700–7.
6. Makikallio AM, Makikallio TH, Korpelainen JT, et al. Natriuretic peptides and mortality after stroke. Stroke 2005;36:1016–20.
7. Scarpino S, Marchitti S, Stanzione R, et al. ROS-mediated differential effects of the human atrial natriuretic peptide T2238C genetic variant on endothelial cells in vitro. J Hypertens 2009;27:1804–13.
8. Rubattu S, Stanzione R, Di Angelantonio E, et al. Atrial natriuretic peptide gene polymorphisms and risk of ischemic stroke in humans. Stroke 2004;35:814–8.
9. Gruchala M, Ciecwierz D, Wasag B, et al. Association of the ScaI atrial natriuretic peptide gene polymorphism with nonfatal myocardial infarction and extent of coronary artery disease. Am Heart J 2003;145:125–31.
10. Lynch AI, Claas SA, Arnett DK. A review of the role of atrial natriuretic peptide gene polymorphisms in hypertension and its sequelae. Curr Hypertens Rep 2009;11:35–42.
11. Zhang I, Cheng L, He M, Hu B, Wu T. ANP T2238C, C-664G gene polymorphism and coronary heart disease in Chinese population. J Huazhong Univ Sci Technolog Med Sci 2006;26:528–30.
12. Ferrante G, Nakano M, Prati F, et al. High levels of systemic myeloperoxidase are associated with coronary plaque erosion in pa-

tients with acute coronary syndromes: a clinicopathological study. Circulation 2010;122:2505–13.
13. Serruys PW, García-García HM, Buszman P, et al. Effects of the direct lipoprotein-associated phospholipase A2 inhibitor darapladib on human coronary atherosclerotic plaque. Circulation 2008;118:1172–82.
14. Schönbeck U, Gerdes N, Varo N, et al. Oxidized low-density lipoprotein augments and 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors limit CD40 and CD40L expression in human vascular cells. Circulation 2002;106:2888–93.
15. Thygesen K, Alpert JS, White HD, et al. Universal definition of myocardial infarction. Circulation 2007;116:2634–53.
16. Buysschaert I, Carruthers KF, Dunbar DR, et al. A variant at chromosome 9p21 is associated with recurrent myocardial infarction and cardiac death after acute coronary syndrome: the GRACE Genetics Study. Eur Heart J 2010;31:1132–41.
17. World Health Organization-International Society of Hypertension guidelines for the management of hypertension. Guidelines Subcommittee. J Hypertens 1999;17:151–83.
18. Diabetes mellitus. Report of a WHO Study Group. World Health Organ Tech Rep Ser 1985;727:1–113.
19. Rubattu S, Bigatti G, Evangelista A, et al. Association of atrial natriuretic peptide and type A natriuretic peptide receptor gene polymorphisms with left ventricular mass in human essential hypertension. J Am Coll Cardiol 2006;48:499–505.
20. Holvoet P, Van Cleemput J, Collen D, Vanhaecke J. Oxidized low density lipoprotein is a prognostic marker of transplant-associated coronary artery disease. Arterioscler Thromb Vasc Biol 2000;20:698–702.
21. Lynch AI, Boerwinkle E, Davis BR, et al. Pharmacogenetic association of the NPPA T2238C genetic variant with cardiovascular disease outcomes in patients with hypertension. JAMA 2008;299:296–307.
22. Hazen SL. Myeloperoxidase and plaque vulnerability. Arterioscler Thromb Vasc Biol 2004;24:1143–6.
23. Fu X, Kassim SY, Parks WC, Heinecke JW. Hypochlorous acid oxygenates the cysteine switch domain of pro-matrilysin (MMP-7). A mechanism for matrix metalloproteinase activation and atherosclerotic plaque rupture by myeloperoxidase. J Biol Chem 2001;276:41279–87.
24. Shabani F, McNeil J, Tippett L. The oxidative inactivation of tissue inhibitor of metalloproteinase-1 (TIMP-1) by hypochlorous acid (HOCl) is suppressed by anti-rheumatic drugs. Free Radic Res 1998; 28:115–23.
25. Brennan ML, Penn MS, Van Lente F, et al. Prognostic value of myeloperoxidase in patients with chest pain. N Engl J Med 2003;349: 1595–604.
26. Baldus S, Heeschen C, Meinertz T, et al. Myeloperoxidase serum levels predict risk in patients with acute coronary syndromes. Circulation 2003;108:1440–5.
27. Navab M, Ananthramaiah GM, Reddy ST, et al. The oxidation hypothesis of atherogenesis: the role of oxidized phospholipids and HDL. J Lipid Res 2004;45:993–1007.
28. Macphee CH, Moores KE, Boyd HF, et al. Lipoprotein-associated phospholipase A2, platelet-activating factor acetylhydrolase, generates two bioactive products during the oxidation of low density lipoprotein: use of a novel inhibitor. Biochem J 1999;338:479–87.
29. Tsimikas S, Brilakis ES, Miller ER, et al. Oxidized phospholipids, Lp(a) lipoprotein, and coronary artery disease. N Engl J Med 2005; 353:46–57.
30. Ehara S, Ueda M, Naruko T, et al. Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes. Circulation 2001;103:1955–60.
31. Tsouli SG, Kiortsis DN, Lourida ES et al. Autoantibody titers against OxLDL are correlated with Achilles tendon thickness in patients with familial hypercholesterolemia. J Lipid Res 2006;47:2208–14.
32. Wu T, Willett WC, Rifai N, Shai I, Manson JE, Rimm EB. Is plasma oxidized low-density lipoprotein, measured with the widely used antibody 4E6, an independent predictor of coronary heart disease among U.S. men and women? J Am Coll Cardiol 2006;48:973–9.
33. Barbato E, Rubattu S, Bartunek J, et al. Role of cardiac natriuretic peptides in human coronary atherosclerosis. Atherosclerosis 2009;206: 258–64.
34. Estrada V, Tellez MJ, Moya J, Fernandez-Durango R, Egido J, Fernandez Cruz AF. High plasma levels of endothelin-1 and atrial natriuretic peptide in patients with acute ischemic stroke. Am J Hypertens 1994;7:1085–9.

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