



UNIVERSITÀ
DEGLI STUDI
FIRENZE

FLORE

Repository istituzionale dell'Università degli Studi di Firenze

Platelet aggregability is modulated by eNOS locus in non-type 2 diabetic patients with acute coronary syndrome

Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

Original Citation:

Platelet aggregability is modulated by eNOS locus in non-type 2 diabetic patients with acute coronary syndrome / C.Fatini; E.Sticchi; P.Bolli; R.Marcucci; B.Giusti; R.Paniccia; A.M.Gori; G.F.Gensini; R.Abbate. - In: NMCD. NUTRITION METABOLISM AND CARDIOVASCULAR DISEASES. - ISSN 0939-4753. - STAMPA. - 21:(2011), pp. 11-17.

Availability:

This version is available at: 2158/592530 since:

Terms of use:

Open Access

La pubblicazione è resa disponibile sotto le norme e i termini della licenza di deposito, secondo quanto stabilito dalla Policy per l'accesso aperto dell'Università degli Studi di Firenze (<https://www.sba.unifi.it/upload/policy-oa-2016-1.pdf>)

Publisher copyright claim:

(Article begins on next page)



Platelet aggregability is modulated by eNOS locus in non-type 2 diabetic patients with acute coronary syndrome[☆]

C. Fatini^{a,b,*}, E. Sticchi^{a,b}, P. Bolli^c, R. Marcucci^{a,b}, B. Giusti^{a,b},
R. Paniccia^{a,b}, A.M. Gori^{a,b}, G.F. Gensini^c, R. Abbate^{a,b}

^a Department of Medical and Surgical Critical Care, Department of Heart and Vessels, Azienda Ospedaliero-Universitaria Careggi, University of Florence, Florence, Italy

^b Center for the Study at Molecular and Clinical Level of Chronic, Degenerative and Neoplastic Diseases to Develop Novel Therapies, University of Florence, Florence, Italy

^c Fondazione Don Carlo Gnocchi ONLUS, Centro S.Maria degli Ulivi- IRCCS, Florence, Italy

Received 17 March 2009; received in revised form 16 June 2009; accepted 6 July 2009

KEYWORDS

eNOS locus;
Platelet aggregability;
Diabetes;
Acute coronary
syndrome

Abstract *Background and aim:* Platelet nitric oxide (NO) synthesis is compromised in patients with acute coronary syndrome (ACS), and platelet NO availability may be critically relevant in determining the extent of thrombosis in ACS patients. It has been demonstrated that an impaired responsiveness to the antiaggregatory effects of NO may affect platelet dysfunction in diabetic patients with ACS. Since NO availability may be genetically determined, we have investigated the role of endothelial nitric oxide synthase (eNOS) gene in influencing platelet aggregability in relation to the presence ($n = 247$) or absence ($n = 883$) of type 2 diabetes in ACS patients.

Methods and results: We have genotyped 1130 consecutive high risk ACS patients on dual antiplatelet therapy, previously investigated in relation to platelet function. eNOS 4a allele frequency was significantly higher in diabetic vs. non-diabetic patients ($p = 0.02$). In non-diabetic patients the eNOS 4a allele significantly modulated platelet aggregability in response to arachidonic acid (AA), but not to collagen and adenosine diphosphate (ADP) stimulus, after Bonferroni correction for multiple testing. After adjustment for age, gender, smoking habit, hypertension and ejection fraction $\leq 40\%$, the eNOS 4a allele remained significantly and independently associated with platelet aggregability in response to AA stimulus [β (SE) = 0.17 (0.07), $p = 0.01$]. When platelet aggregation values were considered according to the presence or absence of high residual platelet reactivity (RPR) eNOS 4a, but not -786C and 894T,

[☆] Sources of support for research: This study was supported in part by the Genopolis government FIRB project, and in part by grant to the FiorGen Foundation by Ente Cassa di Risparmio Florence, Italy.

* Corresponding author. Department of Medical and Surgical Critical Care, University of Florence, Viale Morgagni 85, 50134 Florence, Italy. Tel.: +39 055 794 9417; fax: +39 055 794 9418.

E-mail address: cinziafatini@hotmail.com (C. Fatini).

allele was significantly associated with RPR by AA stimulus. The haplotype reconstruction analysis for eNOS gene showed that the $-786C/894G/4a$ and $-786C/894G/4b$ haplotypes significantly influenced platelet aggregation after AA stimulus.

Conclusions: Our study indicates that eNOS 4a allele, may be a determinant of higher platelet aggregability and residual platelet reactivity in non-diabetic ACS patients.

© 2009 Elsevier B.V. All rights reserved.

Introduction

Platelet nitric oxide (NO) synthesis is compromised in patients with acute coronary syndrome (ACS) [1], and platelet NO availability may be critically relevant in determining the extent of thrombosis in ACS patients, possibly related to an abnormal platelet activation. An *in vivo* platelet activation may be related not only to a decreased NO production, but also to increased formation of reactive oxygen species (ROS) [2], as observed in diabetes. Recently it has been demonstrated that an impaired responsiveness to the antiaggregatory effects of NO, probably related to increased NO clearance by O_2^- , may affect platelet dysfunction in diabetic patients with acute coronary syndrome (ACS) [3].

NO is a physiologically relevant inhibitor of platelet aggregation, and a reduction in NO bioavailability may represent a contributing factor in affecting platelet phenotype in diabetes. Platelets have the capacity to synthesize and release bioactive NO, and the role of platelet-derived NO has been receiving increasing attention. NO is released simultaneously with platelet aggregation and may regulate platelet recruitment [4]. NO release from activated human platelets inhibits platelet recruitment, thus possibly limiting progression of intra-arterial thrombosis [4]. An *in vivo* study provided evidence that systemic infusion of the nitric oxide synthase (NOS) inhibitor L-N-mono-methyl-arginine citrate (L-NMMA) enhances platelet reactivity to various agonists [1], supporting the clinical relevance of platelet-derived NO. Data from experimental studies showed controversial findings concerning the existence of platelet NOS. Both constitutive and inducible NO synthases have been identified in human platelets [5], and data from an experimental study performed on eNOS deficient mice demonstrated that stimulated platelets were found to lack stimulation-dependent NO release [6]. Nevertheless, recently it has been demonstrated that human and mouse platelets do not express eNOS/iNOS [7].

NO availability may be genetically determined, and there is documented evidence linking reduction in NO production, induced by eNOS gene polymorphisms, with endothelial dysfunction. eNOS gene (7q35–q36) exhibits several polymorphisms, some of which appear to be related with the variability in NO plasma levels: a substitution of guanine to thymine at nucleotide 894 in exon 7 of the eNOS gene (894G > T polymorphism) is associated with reduced basal NO production [8]; the rare C allele of the $-786T > C$ polymorphism in the 5'-flanking region of the gene results in a significant reduction in eNOS promoter activity [9]; and, finally, a 27-base pair (bp) variable tandem repeats polymorphism in intron 4 (also called eNOS 4a/4b) has been

associated with variations in NO, nitrite and nitrate plasma levels [10]. Recently, it has been demonstrated a functional role for the eNOS 4a/4b polymorphism: subjects carrying the 4a/4a genotype have lower NO-producing activity comparing to subjects carrying the wild-type allele, even if higher eNOS mRNA levels were observed [11].

We previously demonstrated that the eNOS gene and in particular the 4a/4b polymorphism represents a predisposing condition to ACS [12], possibly related to endothelial dysfunction. Thus, it would be intriguing to investigate the role of eNOS gene in influencing the platelet aggregation, as a marker of platelet dysfunction, in a large cohort of previously investigated ACS patients on dual antiplatelet therapy [13–15] in relation to the presence or absence of type 2 diabetes.

Methods

Study population

This analysis includes 1130 consecutive patients admitted to the Department of Medical and Surgical Critical Care, Coronary Care Units of the Azienda Ospedaliero-Universitaria Careggi, University of Florence with the diagnosis of acute coronary syndromes (ACS), previously investigated [13–15]. Two hundred and forty-seven out of 1130 patients analyzed were diagnosed with type 2 diabetes. Acute myocardial infarction was diagnosed on the basis of an increase in creatine kinase MB isoenzyme to at least twice the upper normal limits (3.6 ng/ml), and/or elevated cardiac troponin I (cTnI) (>0.15 ng/ml) levels with at least one of the following: acute onset of prolonged (≥ 20 min) typical ischemic chest pain; ST-segment elevation of at least 1 mm in two or more contiguous electrocardiographic leads; ST-depression of ≥ 0.5 mm, 0.08 s after the J point in ≥ 2 contiguous leads or T waves inversion >1 mm in leads with predominant R waves. All patients underwent coronary angiography performed by the Judkins' technique and PCI. Patients were considered to have hypertension if they had been diagnosed as hypertensive according to the European Society of Hypertension/European Society of Cardiology guidelines or were taking anti-hypertensive drugs. Dyslipidemia was defined according to the Third report of the National Cholesterol Education Program, and diabetes according to the American Diabetes Association.

The exclusion criteria included history of bleeding diathesis, platelet count $\leq 100,000/mm^3$ hematocrit $\leq 30\%$ and serum creatinine ≥ 4.0 mg/dl. All patients received a loading dose of 600 mg of clopidogrel orally before the procedure and of 500 mg of ASA intravenously, followed by 75 mg of clopidogrel and 100–325 mg of ASA daily. Unfractionated heparin 70 IU/Kg was used during the

procedure as an anticoagulant. The use of glycoprotein (Gp) IIb/IIIa inhibitors was at the discretion of the operating surgeon. For patients receiving the IIb/IIIa inhibitor in the catheterization laboratory, blood samples were obtained after 6 days. Informed written consent was obtained from all patients, and the study was approved by the local Ethical Review Board.

Blood sampling and assessment of platelet aggregation on platelet-rich plasma

Venous blood samples were taken from each patient at 15–24 h after PCI intervention in tubes containing 3.2% trisodium citrate.

Platelet aggregation was assessed using platelet-rich plasma (PRP) by the turbidimetric method in a four-channel aggregometer (APACT 4, Labor Biomedical Technologies GmbH, Ahrensburg, Germany) as previously reported [16]. Platelet agonists included 10 μ M ADP, 2 μ g/mL collagen, and 1mM arachidonic acid (AA).

Antiplatelet response

For antiplatelet response, the following definitions were used:

1. Residual platelet reactivity (RPR) evaluated by 10 μ M ADP-induced platelet aggregation (ADP-RPR) = 10 μ M ADP-induced platelet aggregation \geq 70% [13].
2. Residual platelet reactivity (RPR) evaluated by 1 mM AA-induced platelet aggregation (AA-RPR) = AA-induced platelet aggregation \geq 20% [17].
3. Residual platelet reactivity (RPR) evaluated by 2 μ g/mL collagen-induced platelet aggregation (collagen-RPR) = 2 μ g/mL collagen-induced platelet aggregation \geq 56% (90th percentile).

Analysis of eNOS –786T > C, 894G > T and 4a/4b polymorphisms

Genomic DNA was isolated from whole blood by using the FlexiGeneDNA kit (QIAGEN, Germany).

eNOS–786T > C (rs2070744) and 894G > T (rs1799983) polymorphisms were evaluated through electronic microchip technology (NanoChip Molecular Biology Workstation; 10 \times 10 array NanoChip cartridge; Nanogen, San Diego, CA, USA), by using the Universal Reporting System, as previously described [18]. The amplification of the sequence containing each SNP has been performed through PCR reaction in a MJ thermocycler (MJ research, Waltham, MA, USA). Primers used in the amplification process and Cy3 and Cy5-labelled universal reporter oligonucleotides are reported in Appendix 1.

The eNOS 4a/4b polymorphism was analyzed as previously described [12].

Statistical analysis

Statistical analysis was performed by using the SPSS (Statistical Package for Social Sciences, Chicago, USA)

software for Windows (Version 11.5). The χ^2 -test was used to test for deviation of genotype distribution from Hardy–Weinberg equilibrium for all polymorphisms analyzed. Genotype and allele frequencies were compared between groups by using the χ^2 -test. Categorical variables were expressed as frequencies and percentages. Unless otherwise indicated, data are given as median values and range. Log-transformed values for all circulating parameters were used in the analysis, and back transformed for data presentation. General linear model was carried out to investigate the possible relationship between eNOS polymorphisms and maximal platelet aggregation.

To test the association between platelet function and the eNOS polymorphisms we performed the univariate linear regression analysis with 2 μ g/mL collagen or 10 μ M ADP or log(AA) platelet aggregation as dependent variable and eNOS polymorphisms as independent variables. Age, gender, hypertension, smoking habit and ejection fraction \leq 40%, which were previously demonstrated to influence platelet aggregation [19], were included in the multivariate linear regression analysis as independent variables. The Bonferroni correction was used for multiple testing (the 3 candidate polymorphisms were treated as 3 independent statistical tests) by multiplying the nominal *p*-value of each test by the number of tests conducted. To test the influence of eNOS polymorphisms on the presence or absence of residual platelet reactivity (RPR), we performed the logistic regression analysis.

In genotyping the eNOS 4a/4b polymorphism we found the presence of two rarer alleles (c and d alleles), which have been previously described in ACS population, in six patients [20]. In order to avoid confounding effects on statistical analysis, we excluded these patients from the analysis concerning 4a/4b polymorphism. We performed the analysis under a dominant genetic model of inheritance. The dominant genetic model compares individuals with one or more polymorphic alleles with a baseline group with no polymorphic alleles (e.g. eNOS 4a4a + 4a4b vs. 4b4b). The recessive genetic model compares the 4a4a genotype with the combined 4a4b + 4b4b genotypes, which form the baseline group. The additive genetic model assumes that there is a linear gradient in risk between the 4a4a, 4a4b and 4b4b genotypes (4b4b genotype baseline). This is equivalent to a comparison of the 4a allele vs. the 4b allele (baseline). A *p*-value <0.05 was considered to indicate statistical significance. Haplotype reconstruction and frequency estimation were performed using the R (<http://www.r-project.org>) package haplo.stats by expectation–maximization strategy (EM algorithm) [21]. The haplo.stats package was also used to identify statistically significant associations between haplotypes and disease risks by means of generalized linear models.

Results

Demographic and clinical characteristics of patients are reported in Table 1.

All three eNOS polymorphisms analyzed were in Hardy–Weinberg equilibrium. As concerns the eNOS 4a4b polymorphism, we excluded from the analysis 4 patients carrying rarer alleles (i.e. c and d) previously identified [20], in order to avoid confounding effects.

Table 1 Demographic and clinical characteristics of ACS patients with and without diabetes.

	ACS patients with diabetes	ACS patients without diabetes
	<i>n</i> = 247	<i>n</i> = 883
Age (years) ^a	64 (39–90)	68 (27–94)
Gender (M/F)	171/76	668/215
Hypertension, <i>n</i> (%)	174 (70.4)	556 (63.0)
Smoking habit, <i>n</i> (%)	76 (30.8)	377 (42.7)
Dyslipidemia, <i>n</i> (%)	150 (60.7)	477 (54.0)
BMI > 25 Kg/m ² , <i>n</i> (%)	30 (12)	124 (14)
Renal failure, <i>n</i> (%)	64 (25.9)	216 (24.5)
Ejection fraction (EF) ≤ 40%, <i>n</i> (%)	87 (35.2)	225 (25.5)
Family history of CAD, <i>n</i> (%)	20 (8.1)	75 (8.5)
Previous AMI, <i>n</i> (%)	25 (10)	79 (8.9)
Multivessel disease, <i>n</i> (%)	116 (46.9)	291 (32.9)
Bifurcation lesions, <i>n</i> (%)	96 (38.8)	309 (35)
Drugs		
β-blockers, <i>n</i> (%)	143 (58.9)	547 (62.0)
Ca-antagonists, <i>n</i> (%)	43 (17.4)	145 (16.4)
Statins, <i>n</i> (%)	209 (84.6)	745 (84.3)
ACE-inhibitors, <i>n</i> (%)	206 (83.4)	725 (82.1)
Nitrates, <i>n</i> (%)	62 (25.1)	187 (21.2)
Diuretics, <i>n</i> (%)	65 (26.3)	181 (20.5)
Proton pump inhibitors, <i>n</i> (%)	232 (93.9)	825 (93.4)

^a Median (range).

Genotype distribution and allele frequency of the eNOS polymorphisms are reported in Table 2. As concerns the 4a/4b polymorphism, a significant difference in genotype distribution and allele frequency between diabetic and

Table 2 Genotype distribution and allele frequency of eNOS –786T > C, 894G > T and 4a/4b polymorphisms in ACS patients with and without diabetes.

Genotype	Allele	ACS patients with diabetes	ACS patients without diabetes	<i>p</i> -value	
		<i>n</i> = 247	<i>n</i> = 883		
eNOS –786TT	–786TC	75 (30.4)	257 (29.1)		
	–786CC	127 (51.4)	452 (51.2)	0.8	
	–786C	45 (18.2)	174 (19.7)	0.6	
eNOS 894GG	894GT	0.44	0.45		
	894TT	111 (44.9)	361 (40.9)		
	894T	108 (43.7)	404 (45.8)	0.5	
eNOS 4b/4b ^a	4a/4b	28 (11.3)	118 (13.4)		
	4a/4a	894T	0.33	0.36	0.2
	4a	161 (65.2)	621 (70.3)		
4a/4b	4a/4a	72 (29.1)	242 (27.4)		
	4a	13 (5.3)	17 (1.9)	0.01	
	4a	0.20	0.16	0.02	

^a 4 patients heterozygous for rarer alleles (i.e. c and d allele) are not included in the analysis.

non-diabetic patients ($p = 0.01$ and $p = 0.02$, respectively) was found.

Platelet phenotype in diabetic and non-diabetic patients

Maximal platelet aggregation values [geometric mean (95% CI)] were significantly higher in diabetic in comparison to non-diabetic patients, in response to collagen: [37.8 (34.5–41.07) vs. 30.4 (28.7–32.1), $p < 0.000$], ADP: [54.7 (51.8–57.6) vs. 46.8 (45.3–48.3), $p < 0.0001$] and AA stimuli: [26.4 (23.3–29.4) vs. 19.8 (19.2–21.4), $p < 0.0001$].

eNOS –786T > C, 894G > T and 4a/4b polymorphism and platelet phenotype in diabetic and non-diabetic patients

In diabetic patients carrying eNOS rare alleles for all polymorphisms analyzed, the difference in platelet aggregation values was not statistically significant in comparison to wild-type allele carriers, after AA, collagen and ADP stimuli ($p > 0.05$). On the contrary, we found significantly higher platelet aggregation values in response to collagen and AA, but not ADP stimulus, in non-diabetic patients carrying the eNOS 4a rare allele. Moreover, a significantly higher platelet aggregation value in response to collagen, but not to AA and ADP stimuli, was observed in non-diabetic patients carrying the eNOS –786C allele. The eNOS 894G > T polymorphism did not influence platelet aggregation in response to all stimuli analyzed in non-diabetic patients.

In non-diabetic patients, the univariate linear regression analysis model with collagen, ADP and AA-induced platelet aggregation values analyzed as continuous variables, was performed according to dominant, recessive and additive model of inheritance. We observed a significant association between eNOS 4a allele and both AA and collagen-induced platelet aggregation and between eNOS –786C allele and collagen-induced platelet aggregation under a dominant, but not recessive and additive model of inheritance. After Bonferroni correction for multiple testing, only eNOS 4a allele remained significantly associated with AA-induced platelet aggregation. The eNOS 894T allele did not affect platelet aggregation under all genetic models investigated (Table 3).

At the multivariate linear regression analysis model with AA-induced platelet aggregation as dependent variable, and age, gender, smoking habit, hypertension and ejection fraction ≤ 40% as independent variables, eNOS 4a significantly and independently influenced platelet aggregation in response to AA stimulus (Table 3).

When platelet aggregation values were considered according to the presence or absence of high residual platelet reactivity (RPR), at logistic regression analysis, eNOS 4a, but not –786C and 894T, allele was significantly and independently associated with RPR by AA, but not by collagen and ADP, under the dominant model of inheritance (Table 4).

The haplotype reconstruction analysis for eNOS gene showed that the –786C/894G/4a and –786C/894G/4b haplotypes significantly and independently influenced platelet aggregation after AA, but not ADP and collagen, stimulus (Appendix 2).

Table 3 Univariate and multivariate linear regression analysis with 1 mM AA, 2 µg/mL Collagen, and 10 µM ADP platelet aggregation values analyzed as continuous variables in non-diabetic patients, according to genetic models of inheritance.

	log (AA)		Collagen		ADP	
	β (SE)	p	β (SE)	p	β (SE)	p
<i>Univariate linear regression analysis (Dominant Model)</i>						
eNOS -786C	0.12 (0.06)	0.06	4.18 (1.89)	0.03 ^a	2.98 (1.69)	0.08
eNOS 894T	-0.05 (0.06)	0.4	0.32 (1.76)	0.9	1.52 (1.57)	0.3
eNOS 4a	0.18 (0.06)	0.006 ^a	3.85 (1.87)	0.04 ^a	2.40 (1.69)	0.2
<i>Univariate linear regression analysis (Recessive Model)</i>						
eNOS -786CC	0.03 (0.07)	0.7	2.20 (2.16)	0.3	0.22 (1.95)	0.9
eNOS 894TT	-0.03 (0.09)	0.7	0.88 (2.53)	0.7	-1.90 (2.29)	0.4
eNOS 4a4a	-0.05 (0.23)	0.8	-7.50 (6.40)	0.2	-8.92 (5.80)	0.1
<i>Univariate linear regression analysis (Additive Model)</i>						
eNOS -786CC vs. TT	0.11 (0.09)	0.2	4.74 (2.55)	0.06	2.28 (2.32)	0.3
eNOS 894TT vs. GG	-0.06 (0.10)	0.6	0.96 (2.86)	0.7	-0.75 (2.47)	0.8
eNOS 4a4a vs. 4b4b	0.005 (0.22)	0.9	-6.25 (6.40)	0.3	-8.05 (5.80)	0.2
<i>Multivariate linear regression analysis (Dominant Model)</i>						
eNOS 4a	0.17 (0.07)	0.01	—	—	—	—
Age	0.01 (0.003)	<0.0001	—	—	—	—
Gender	0.05 (0.07)	0.5	—	—	—	—
Smoking habit	0.09 (0.06)	0.2	—	—	—	—
Hypertension	0.12 (0.06)	0.05	—	—	—	—
Ejection fraction _{≤40%}	-0.06 (0.07)	0.4	—	—	—	—

^a Bonferroni correction for multiple testing by multiplying the nominal *p*-value of each test by 3 (i.e. the number of tests conducted): eNOS 4a and log (AA) *p* = 0.02; eNOS 4a and collagen *p* = 0.12; eNOS -786C and collagen *p* = 0.09.

Discussion

The novelty of our findings lies in searching for the role of eNOS gene in influencing the platelet phenotype in high coronary risk diabetic and non-diabetic patients on dual antiplatelet treatment. We have provided evidence of a contribution of eNOS gene in affecting platelet aggregation in ACS non-diabetic patients, possibly reducing NO availability, and thus conferring a “hyperaggregable phenotype” to platelets in spite of the dual antiplatelet therapy. Of interest, the present study shows that the eNOS 4a allele may modulate residual platelet reactivity to AA in non-diabetic patients, thus suggesting a decreased response to aspirin therapy in 4a allele carriers. Really, aspirin highly improves the NO production in platelets, by increasing eNOS expression, and maximal NO bioavailability almost doubles after incubation with aspirin [22]. It has been suggested that the high NO production could counteract the effect of thromboxane and inhibit platelet activation and aggregation, thus compensating for the reduction in prostacyclin concentrations by aspirin [22].

Finally, our results show a significantly higher prevalence of the eNOS 4a allele in diabetics in comparison to non-diabetic ACS patients, so confirming data from literature reporting its role in increasing susceptibility to both diabetes and its complications [23,24].

Platelet NO appears to play a role in inhibiting recruitment of platelets to the growing thrombus after platelet activation [4]. NO inhibits platelet aggregation and adhesion via activation of soluble guanylyl cyclase (sGC), which increases intracellular cGMP levels [25]. Data from an

experimental study performed on collagen-stimulated rabbit platelets [26] reported that cGMP, the NO effector, may have an inhibitory effect on collagen-induced aggregation by inhibiting AA release. Moreover, data from an *in vivo* study reported that the NO/cGMP pathway may inhibit the signaling cascade of thromboxane A₂ receptor through phosphorylation by cGMP-dependent protein kinase (cGK), and through collagen-induced AA release by suppressing Ca²⁺-activated phospholipase A₂ activity [27]. Changes in the activity of platelet eNOS are responsible for abnormal platelet activation: recent evidence indicates a complex biphasic interaction between NO and platelet activation and aggregation; platelet-derived NO may stimulate platelets [28] through an initial transient stimulatory response, able to promote platelet aggregation, and a subsequent inhibitory response that limits the size of thrombi. Additional evidence indicates that low NO concentrations promote a platelet degranulation; on the contrary, high NO concentrations inhibit the exocytosis of dense granules [29].

NO availability has been related to several polymorphisms in the eNOS gene [8–11]. A functional role for polymorphisms at eNOS locus has been demonstrated: experimental studies evidenced that eNOS 4a/4b polymorphism in the intron 4 of eNOS gene, which represents a source of 27nt small RNA, is able to inhibit eNOS expression [30]. eNOS polymorphic variants other than 4a/4b polymorphism have been described to influence eNOS activity by different mechanisms. In particular, eNOS -786T > C SNP is reported to affect eNOS gene transcription efficiency, thus resulting in a 50% reduction in gene

Table 4 Univariate and multivariate logistic regression analysis for the presence or absence of high residual platelet reactivity (RPR), according to eNOS polymorphisms, in non-diabetic patients

	RPR by AA		RPR by Collagen		RPR by ADP	
	OR (95% CI)	<i>p</i>	OR (95% CI)	<i>p</i>	OR (95% CI)	<i>p</i>
Univariate logistic regression analysis						
<i>Dominant Model</i>						
eNOS -786C	1.39 (0.97–2.00)	0.08	1.02 (0.68–1.54)	0.9	1.00 (0.65–1.54)	0.9
eNOS 894T	0.92 (0.66–1.27)	0.6	0.81 (0.56–1.18)	0.3	1.10 (0.74–1.64)	0.6
eNOS 4a	1.51 (1.07–2.12)	0.02	1.21 (0.82–1.81)	0.3	1.34 (0.88–2.03)	0.6
<i>Recessive Model</i>						
eNOS -786C	0.88 (0.58–1.33)	0.5	1.25 (0.79–1.95)	0.3	0.94 (0.57–1.55)	0.8
eNOS 894T	0.92 (0.57–1.49)	0.7	1.24 (0.73–2.08)	0.4	0.71 (0.38–1.35)	0.3
eNOS 4a	0.49 (0.11–2.18)	0.3	0.35 (0.04–2.69)	0.3	–	–
<i>Additive Model</i>						
eNOS -786C	1.14 (0.70–1.86)	0.6	1.21 (0.71–2.04)	0.5	0.95 (0.53–1.69)	0.9
eNOS 894T	0.88 (0.53–1.47)	0.6	1.06 (0.61–1.86)	0.8	0.79 (0.39–1.55)	0.5
eNOS 4a	0.56 (0.12–2.52)	0.5	0.38 (0.05–2.91)	0.3	–	–
Multivariate logistic regression analysis for eNOS 4a allele (<i>Dominant Model</i>)						
eNOS 4a	1.51 (1.02–2.22)	0.04	–	–	–	–
Age	1.03 (1.01–1.04)	0.003	–	–	–	–
Gender	1.11 (0.73–1.69)	0.6	–	–	–	–
Smoking habit	1.19 (0.81–1.73)	0.4	–	–	–	–
Hypertension	1.36 (0.92–2.01)	0.1	–	–	–	–
Ejection fraction $\leq 40\%$	0.71 (0.46–1.08)	0.1	–	–	–	–

expression [9]. On the contrary, the eNOS 894G > T polymorphism has been recently demonstrated not to affect cellular NO synthesis *in vivo* [31].

In the present study, we confirmed results from our previous study [12], showing a similar prevalence of the 4a rare allele in ACS patients.

Findings from this study showed that eNOS locus modulated platelet aggregability in non-diabetic, but not in diabetic ACS patients on dual antiplatelet treatment, so suggesting that eNOS 4a allele could represent a predisposing factor for atherothrombotic disease in diabetes via endothelial dysfunction NO-mediated, rather than through a modulation of the platelet phenotype, which could play a major role in ACS non-diabetic patients. Actually, in diabetes the effect of a genetic predisposition in modulating platelet NO availability is of lesser impact than an altered glycemic control, which influences platelet function via several deeply investigated mechanisms [32]. Moreover, the lack of association between eNOS 4a allele and platelet aggregability in diabetic patients may be also due to the smaller sample size of the diabetic group.

This study suffers from some limitations: the effect of antiplatelet therapy, which nevertheless might have contributed to an underestimation of any true effect; the lack of a direct measure of platelets eNOS activity in addition to platelet reactivity, which would more clearly establish the relationship between eNOS alleles and eNOS and platelet function; finally, the lack of measurements of NOX levels, which might allow to evaluate the NO production and might contribute to the overall interpretation of data. Another relevant limitation is the small sample size of the diabetic group, which might have influenced the results concerning the lack of association between eNOS 4a allele and platelet aggregation in response to AA stimulus.

In conclusion, our study indicates that eNOS gene, and in particular 4a allele, may be a determinant of higher platelet aggregability and residual platelet reactivity in non-diabetic ACS patients. This finding may allow to recognize a mechanism, other than diabetes, able to induce platelet hyperreactivity in high risk patients and to suggest future pharmacogenetic strategies, such as platelet NO donors and NO-donating aspirin, able to offer a better control of platelet function and of endothelial dysfunction.

Finally, ACE-inhibitors therapy has to be considered in relation to NO availability. Treatment with ACE-inhibitors may be of interest to restore a physiological activity of endothelial cells of both diabetic and non-diabetic patients.

It has been demonstrated in endothelium-denuded diabetic aortas [33] that ACE-inhibitors are able to block non-endothelial NOX activity. Moreover, in diabetes the production of reactive oxygen species (ROS), further increased by the eNOS uncoupling “phenomenon”, contributes to the lack of antioxidant role of eNOS. Accordingly, ACE-inhibitors therapy may improve endothelial function via the novel mechanism of recoupling eNOS.

Appendix A Supplemental material

Supplementary information for this manuscript can be downloaded at doi: [10.1016/j.numecd.2009.07.001](https://doi.org/10.1016/j.numecd.2009.07.001).

References

- [1] Freedman JE, Ting B, Hankin B, Loscalzo J, Keaney Jr JF, Vita JA. Impaired platelet production of nitric oxide predicts

- presence of acute coronary syndromes. *Circulation* 1998;98:1481–6.
- [2] Vinik AI, Erbas T, Park TS, Nolan R, Pittenger GL. Platelet dysfunction in type 2 diabetes. *Diabetes Care* 2001;24:1476–85.
- [3] Worthley MI, Holmes AS, Willoughby SR, Kucia AM, Heresztyn T, Stewart S, et al. The deleterious effects of hyperglycemia on platelet function in diabetic patients with acute coronary syndromes mediated by superoxide production, resolution with intensive insulin administration. *J Am Coll Cardiol* 2007;49:304–10.
- [4] Freedman JE, Loscalzo J, Barnard MR, Alpert C, Keaney JF, Michelson AD. Nitric oxide released from activated platelets inhibits platelet recruitment. *J Clin Invest* 1995;100:350–6.
- [5] Mehta JL, Chen LY, Kone BC, Mehta P, Turner P. Identification of constitutive and inducible forms of nitric oxide synthase in human platelets. *J Lab Clin Med* 1995;125:370–7.
- [6] Freedman JE, Sauter R, Battinelli EM, Ault K, Knowles C, Huang PL, et al. Deficient platelet-derived nitric oxide and enhanced hemostasis in mice lacking the NOSIII gene. *Circ Res* 1999;84:1416–21.
- [7] Gambaryan S, Kobsar A, Hartmann S, Birschmann I, Kuhlencordt PJ, Müller-Esterl W, et al. NO-synthase-/NO-independent regulation of human and murine platelet soluble guanylyl cyclase activity. *J Thromb Haemost* 2008;6:1376–84.
- [8] Veldman BA, Spiering W, Doevendans PA, Vervoort G, Kroon AA, de Leeuw PW, et al. The Glu298Asp polymorphisms of the NOS3 gene as a determinant of the baseline production of nitric oxide. *J Hypertens* 2002;20:2023–7.
- [9] Nakayama M, Yasue H, Yoshimura M, Shimasaki Y, Kugiyama K, Ogawa H, et al. T-786C mutation in the 5'-flanking region of the endothelial nitric oxide synthase gene is associated with coronary spasm. *Circulation* 1999;99:2864–70.
- [10] Tsukada T, Yokoyama K, Arai T, Takemoto F, Hara S, Yamada A, et al. Evidence of association of the eNOS gene polymorphism with plasma NO metabolite levels in humans. *Biochem Biophys Res Commun* 1998;245:190–3.
- [11] Dosenko VE, Zagoriy VY, Haytovich NV, Gordok OA, Moibenko AA. Allelic polymorphism of endothelial NO-synthase gene and its functional manifestations. *Acta Biochim Pol* 2006;53:299–302.
- [12] Fatini C, Sofi F, Sticchi E, Gensini F, Gori AM, Fedi S, et al. Influence of endothelial nitric oxide synthase gene polymorphisms (G894T, 4a4b, T-786C) and hyperhomocysteinemia on the predisposition to acute coronary syndromes. *Am Heart J* 2004;147:516–21.
- [13] Buonomici P, Marcucci R, Migliorini A, Gensini GF, Santini A, Paniccia R, et al. Impact of platelet reactivity after clopidogrel administration on drug-eluting stent thrombosis. *J Am Coll Cardiol* 2007;49:2312–7.
- [14] Giusti B, Gori AM, Marcucci R, Saracini C, Sestini I, Paniccia R, et al. Cytochrome P450 2C19 loss-of-function polymorphism, but not CYP3A4 IVS10 + 12G/A and P2Y12 T744C polymorphisms, is associated with response variability to dual antiplatelet treatment in high-risk vascular patients. *Pharmacogenet Genomics* 2007;17:1057–64.
- [15] Marcucci R, Gori AM, Paniccia R, Giusti B, Valente S, Giglioli C, et al. Cardiovascular death and nonfatal myocardial infarction in acute coronary syndrome patients receiving coronary stenting are predicted by residual platelet reactivity to ADP detected by a point-of-care assay: a 12-month follow-up. *Circulation* 2009;119:237–42.
- [16] Paniccia R, Antonucci E, Gori AM, Marcucci R, Poli S, Romano E, et al. Comparison of different methods to evaluate the effect of aspirin on platelet function in high-risk patients with ischemic heart disease receiving dual antiplatelet treatment. *Am J Clin Pathol* 2007;128:143–9.
- [17] Gum PA, Kottke-Marchant K, Welsh PA, White J, Topol EJ. A prospective, blinded determination of the natural history of aspirin resistance among stable patients with cardiovascular disease. *J Am Coll Cardiol* 2003;41:961–5.
- [18] Sticchi E, Lenti M, Giusti B, Sestini I, Abbate R, Fatini C. LPA +93C > T and +121G > A polymorphisms detection by electronic microchip technology. *Mol Genet Metab* 2007;91:79–84.
- [19] Marcucci R, Gori AM, Paniccia R, Giglioli C, Buonomici P, Antonucci D, et al. Residual platelet reactivity is associated with clinical and laboratory characteristics in patients with ischemic heart disease undergoing PCI on dual antiplatelet therapy. *Atherosclerosis* 2007;195:e217–23.
- [20] Bolli P, Sticchi E, Abbate R, Fatini C. A novel allele of eNOS gene in the Italian population: the actual essence of intron 4 polymorphism. *Nitric Oxide* 2007;16:392–4.
- [21] Becker T, Cichon S, Jönson E, Knapp M. Multiple testing in the context of haplotype analysis revisited: application to case-control data. *Ann Hum Genet* 2005;69:747–56.
- [22] Madajka M, Korda M, White J, Malinski T. Effect of aspirin on constitutive nitric oxide synthase and the bioavailability of NO. *Thromb Res* 2003;110:317–21.
- [23] Ezzidi I, Mtiraoui N, Mohamed MB, Mahjoub T, Kacem M, Almawi WY. Association of endothelial nitric oxide synthase Glu298Asp, 4b/a, and –786T > C gene variants with diabetic nephropathy. *J Diabetes Complications* 2008;22:331–8.
- [24] de Syllos RW, Sandrim VC, Lisboa HR, Tres GS, Tanus-Santos JE. Endothelial nitric oxide synthase genotype and haplotypes are not associated with diabetic retinopathy in diabetes type 2 patients. *Nitric Oxide* 2006;15:417–22.
- [25] Radomski MW, Palmer RM, Moncada S. An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proc Natl Acad Sci U S A* 1990;87:5193–7.
- [26] Matsuoka I, Nakahata N, Nakanishi H. Inhibitory effect of 8-bromo cyclic GMP on an extracellular Ca²⁺-dependent arachidonic acid liberation in collagen-stimulated rabbit platelets. *Biochem Pharmacol* 1989;38:1841–7.
- [27] Wang GR, Zhu Y, Halushka PV, Lincoln TM, Mendelsohn ME. Mechanism of platelet inhibition by nitric oxide: in vivo phosphorylation of thromboxane receptor by cyclic GMP-dependent protein kinase. *Proc Natl Acad Sci U S A* 1998;95:4888–93.
- [28] Li Z, Xi X, Gu M, Feil R, Ye RD, Eigenthaler M, et al. A stimulatory role for cGMP-dependent protein kinase in platelet activation. *Cell* 2003;112:77–86.
- [29] Morrell CN, Matsushita K, Chiles K, Scharpf RB, Yamakuchi M, Mason RJ, et al. Regulation of platelet granule exocytosis by S-nitrosylation. *Proc Natl Acad Sci U S A* 2005;102:3782–7.
- [30] Zhang MX, Zhang C, Shen YH, Wang J, Li XN, Chen L, et al. Effect of 27nt small RNA on endothelial nitric-oxide synthase expression. *Mol Biol Cell* 2008;19:3997–4005.
- [31] Vignini A, Nanetti L, Moroni C, Testa R, Sirolla C, Marra M, et al. Platelet nitric oxide production and IR: Relation with obesity and hypertriglyceridemia. *Nutr Metab Cardiovasc Dis* 2008;18:553–8.
- [32] Ferretti G, Rabini RA, Bacchetti T, Vignini A, Salvolini E, Ravaglia F, et al. Glycated low density lipoproteins modify platelet properties: a compositional and functional study. *J Clin Endocrinol Metab* 2002;87:2180–4.
- [33] Oak JH, Cai H. Attenuation of angiotensin II signaling recouples eNOS and inhibits nonendothelial NOX activity in diabetic mice. *Diabetes* 2007;56:118–26.