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# $\alpha_{2A}$ -Adrenergic Receptor Polymorphism Potentiates Platelet Reactivity in Patients With Stable Coronary Artery Disease Carrying the Cytochrome P450 2C19\*2 Genetic Variant

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**Objective**—Platelet  $\alpha_{2A}$ -adrenergic receptors (ARs) mediate platelet aggregation in response to sympathetic stimulation. The 6.3-kb variant of  $\alpha_{2A}$ -AR gene is associated with increased epinephrine-induced platelet aggregation in healthy volunteers. The cytochrome P450 2C19\*2 (CYP2C19\*2) loss-of-function allele influences P2Y12-mediated platelet inhibition and hence the rate of major adverse cardiovascular events. We assessed the influence of 6.3-kb  $\alpha_{2A}$ -AR gene variant on platelet aggregation and its interaction with CYP2C19\*2 loss-of-function allele in patients with stable angina on aspirin and clopidogrel (dual antiplatelet therapy).

**Approach and Results**—Aggregation to 5 increasing doses of epinephrine (from 0.156 to 10  $\mu\text{mol/L}$ ) was assessed in aggregation units by Multiplate Analyzer and platelet reactivity in P2Y12 reactivity units and % inhibition by VerifyNow P2Y12 assay before percutaneous revascularization. Gene polymorphisms were analyzed with TaqMan Drug Metabolism assay. Of 141 patients, aggregation was higher in 6.3-kb carriers ( $n=52$ ) when compared with wild types ( $n=89$ ) at all epinephrine doses ( $P<0.05$ ) apart from 10  $\mu\text{mol/L}$  ( $P=0.077$ ). Percentage inhibition was lower ( $P=0.048$ ) in 6.3-kb  $\alpha_{2A}$ -AR carriers. Percentage inhibition was lower ( $P=0.005$ ) and P2Y12 reactivity units was higher ( $P=0.012$ ) in CYP2C19\*2 allele carriers. Higher P2Y12 reactivity units ( $P=0.037$ ) and lower percentage inhibition ( $P=0.009$ ) were observed in carriers of both 6.3-kb  $\alpha_{2A}$ -AR variant and CYP2C19\*2 allele when compared with wild-type or with either mutation on its own.

**Conclusions**—The 6.3-kb  $\alpha_{2A}$ -AR variant is associated with increased platelet reactivity to epinephrine and has an additive effect along with CYP2C19\*2 loss-of-function allele on P2Y12-mediated platelet responses in patients with stable angina on dual antiplatelet therapy. (*Arterioscler Thromb Vasc Biol.* 2014;34:1314-1319.)

**Key Words:** adrenergic alpha-2A receptors ■ coronary artery disease ■ CYP2C19, human ■ genetic polymorphism ■ platelet aggregation

Despite dual antiplatelet therapy (DAPT) with aspirin and clopidogrel, there is a considerable interindividual variability of ADP-induced, purinergic receptor P2Y12 (P2Y12)-mediated platelet aggregation. The explanation for such wide interindividual variability remains unclear but is felt to be important because suboptimal platelet inhibition in some patients equates to an increased risk of cardiovascular events (ie, patients with insufficient platelet inhibition from the P2Y12 antagonist, clopidogrel, have been shown to have an increased risk of myocardial infarction and stent thrombosis after coronary stent implantation).<sup>1-4</sup> Although the interindividual variability has been reduced with the introduction of more potent P2Y12 inhibitors, such as prasugrel or ticagrelor,<sup>5,6</sup> variability continues to exist,<sup>7</sup> suggesting that

other signaling pathways or genetic polymorphisms may be important.<sup>8,9</sup>

Variability in platelet response has been partly attributed to the influence of environmental factors, such as smoking, body weight, and alcohol; however, genetic polymorphisms may play a crucial role as well. The cytochrome P450 2C19\*2 (CYP2C19\*2) loss-of-function allele exemplifies this with its known influence on ADP-induced platelet aggregation.<sup>10</sup> Carriers of this particular variant have been shown to be less responsive to clopidogrel, resulting in suboptimal platelet inhibition and hence an increased rate of cardiovascular events.<sup>11-16</sup> In the same way that the platelet possesses multiple receptors and signaling pathways important in platelet activation, aggregation, and arterial thrombus development, there

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Nonstandard Abbreviations and Acronyms	
$\alpha_{2A}$ -AR	$\alpha_{2A}$ adrenergic receptor
AU	aggregation unit
CYP2C19*2	cytochrome P450 2C19* 2
DAPT	dual antiplatelet therapy
PRU	P2Y12 reactivity unit

are several genetic polymorphisms that influence platelet biology.<sup>17</sup> Information as to whether these genetic polymorphisms act in a synergistic or additive manner remains poorly understood, particularly in patients taking DAPT.

Epinephrine is an important mediator of platelet aggregation, and through the stimulation of  $\alpha_{2A}$ -ARs on the platelet membrane surface<sup>18</sup> plays a crucial role in the pathogenesis of ischemic syndromes.<sup>19</sup> Previous studies suggest that  $\approx 30\%$  of healthy volunteers<sup>20,21</sup> and patients with stable coronary artery disease taking aspirin and clopidogrel<sup>22</sup> have increased platelet reactivity in response to epinephrine. Furthermore, Yee et al<sup>21</sup> previously demonstrated that healthy volunteers who have the greatest platelet reactivity to epinephrine aggregate more in response to other agonists, such as ADP. Work by Freeman et al<sup>20</sup> in healthy volunteers suggests that this increased platelet reactivity in response to epinephrine may be attributable to a genetic variant of the  $\alpha_{2A}$ -ARs (6.3-kb variant). Therefore, the primary aim was to investigate the functional effect of the 6.3-kb  $\alpha_{2A}$ -AR variant in patients with stable coronary disease taking aspirin and clopidogrel. The secondary aim was to investigate the potential interaction on platelet aggregation between the 6.3-kb  $\alpha_{2A}$ -AR variant and CYP2C19\*2 loss-of-function allele.

### Materials and Methods

Materials and Methods are available in the online-only Supplement.

### Results

#### Genotype and Clinical Characteristics of the Patients

A total of 141 patients were enrolled. The allele and genotype frequencies for both the  $\alpha_{2A}$ -AR and the CYP2C19 polymorphism are reported in Table 1. The observed frequencies for both polymorphisms were in Hardy–Weinberg equilibrium

**Table 1. Allele and Genotype Frequency of  $\alpha_{2A}$ -AR and CYP2C19 Polymorphisms**

	$\alpha_{2A}$ -AR	CYP2C19
Allele frequency, n=282		
Wild-type allele	229 (81)	234 (83)
Mutated allele	53 (19)	48 (17)
Genotype frequency, n=141		
Wild type	89 (63)	95 (67)
Heterozygote	51 (36)	44 (31)
Homozygote mutated	1 (1)	2 (2)

$\alpha_{2A}$ -AR indicates  $\alpha_{2A}$ -adrenergic receptor; and CYP2C19, cytochrome P450 2C19.

and similar to that previously reported.<sup>16,20,23</sup> The clinical characteristics are summarized in Table 2. There were no differences between the wild-type group and the patients carrying the  $\alpha_{2A}$ -AR mutation.

#### Correlation Between VerifyNow and Multiplate Analyzer

There was a significant correlation between P2Y12 reactivity unit (PRU) and ADP-induced aggregation from the Multiplate Analyzer ( $r=0.59$ ;  $P<0.0001$ ), as well as a significant negative correlation between percentage inhibition and ADP-induced aggregation (aggregation unit [AU]) from the Multiplate Analyzer ( $r=-0.63$ ;  $P<0.0001$ ) in patients taking both aspirin and clopidogrel. There was no significant correlation seen either between PRU and 2.5- $\mu\text{mol/L}$  epinephrine-induced aggregation ( $r=0.08$ ;  $P=\text{ns}$ ) or percentage inhibition and 2.5- $\mu\text{mol/L}$  epinephrine-induced aggregation ( $r=-0.13$ ;  $P=\text{ns}$ ) or at any other concentration tested on the Multiplate. However, there was a significant correlation between ADP and epinephrine-induced aggregation using the Multiplate Analyzer ( $r=0.44$ ;  $P<0.0001$ ).

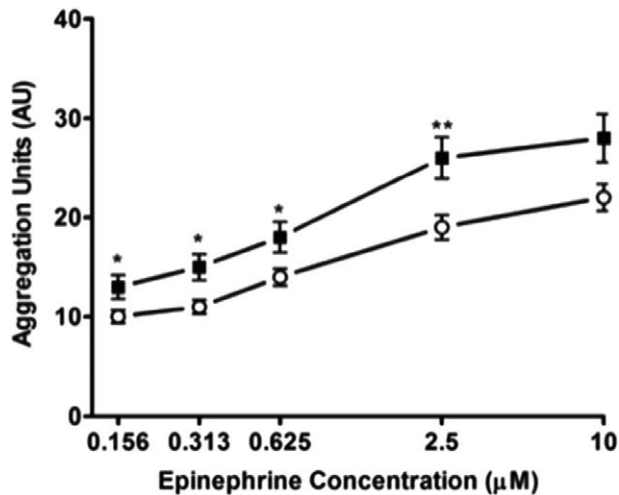
#### Platelet Aggregation Response to Epinephrine

Dose–response aggregation curve to increasing doses of epinephrine is shown in Figure 1. In wild-type patients, the aggregation response increased to increasing concentrations of epinephrine (from  $10\pm 6.3$  to  $22\pm 12.6$  AU; ANOVA,

**Table 2. Clinical Characteristics of Patients According to  $\alpha_{2A}$ -Adrenergic Receptor Polymorphism**

	Entire Cohort (n=141)	Wild Type (n=89)	Carriers (n=52)	P Value
Age, y	65 $\pm$ 12	64 $\pm$ 12	66 $\pm$ 11	NS
Male sex, n (%)	101 (71)	64	37	NS
Weight, kg	79 $\pm$ 14	80 $\pm$ 13	76 $\pm$ 14	NS
BMI, kg/m <sup>2</sup>	27 $\pm$ 4	28 $\pm$ 4	26 $\pm$ 4	NS
Risk factors				
Hypertension	69 (48)	44 (49)	25 (47)	NS
Diabetes mellitus	35 (25)	25 (28)	10 (19)	NS
Dyslipidemia	80 (56)	51 (57)	29 (55)	NS
Smoking history	61 (42)	42 (47)	19 (36)	NS
Family history	40 (28)	22 (25)	18 (34)	NS
Vascular disease	94 (66)	62 (70)	32 (60)	NS
Medical therapy, n (%)				
Aspirin	100 (100)	100 (100)	100 (100)	NS
Clopidogrel	100 (100)	100 (100)	100 (100)	NS
$\beta$ -Blockers	89 (64)	53 (60)	36 (68)	NS
ACE-inhibitors	57 (41)	37 (42)	20 (38)	NS
AT2-antagonists	28 (20)	20 (22)	8 (15)	NS
Statins	117 (84)	72 (81)	45 (85)	NS
$\alpha$ -Blockers	2 (1)	1 (1)	1 (2)	NS
Nitrates	41 (29)	27 (30)	14 (26)	NS
Ca-channel blockers	27 (19)	16 (18)	11 (21)	NS

ACE indicates angiotensin-converting enzyme; AT2, angiotensin 2 receptor; and BMI, body mass index.



**Figure 1.** Platelet aggregation response (aggregation unit [AU]) to increasing doses of epinephrine in function of the  $\alpha_{2A}$ -adrenergic receptor (AR) polymorphism. Wild-type patients,  $\circ$ ; carriers of the 6.3-kb  $\alpha_{2A}$ -AR variant,  $\blacksquare$ . \* $P < 0.05$  and \*\* $P < 0.01$  vs wild type.

$P < 0.0001$ ). Likewise in patients carrying the mutation, the same pattern of aggregation increase was observed (from  $13 \pm 8.6$  to  $28 \pm 17.4$  AU; ANOVA,  $P < 0.0001$ ) although this was significantly higher at all epinephrine concentrations when compared with wild-type patients (epinephrine, 0.156  $\mu\text{mol/L}$ ;  $P = 0.023$ ; epinephrine, 0.313  $\mu\text{mol/L}$ ;  $P = 0.013$ ; epinephrine, 0.625  $\mu\text{mol/L}$ ;  $P = 0.03$ ; epinephrine, 2.5  $\mu\text{mol/L}$ ;  $P = 0.0099$ ), with the exception of the highest concentration (epinephrine, 10  $\mu\text{mol/L}$ ;  $P = 0.08$ ).

At the multivariable analysis (Table 3), the presence of the  $\alpha_{2A}$ -AR mutation (carrier status) was the strongest independent predictor of epinephrine-induced aggregation. Likewise, a significant association with epinephrine-induced aggregation was also observed with angiotensin-converting enzyme-inhibitors and  $\beta$ -blockers.

### P2Y12-Mediated Aggregation With 6.3-/6.7-kb $\alpha_{2A}$ -AR and CYP2C19\*2 Polymorphisms

The presence of the 6.3-kb  $\alpha_{2A}$ -AR mutation influenced P2Y12-mediated aggregation (Figure 2A). Percentage P2Y12-mediated platelet inhibition was significantly lower in 6.3-kb  $\alpha_{2A}$ -AR carriers when compared with the wild types ( $37.6 \pm 26.7\%$  versus  $47.6 \pm 28.5\%$ ;  $P = 0.048$ ), with a trend toward higher PRU values ( $208 \pm 97$  versus  $179 \pm 107$  U;  $P = 0.127$ ).

The presence of the CYP2C19\*2 loss-of-function allele significantly influenced P2Y12-mediated aggregation (Figure 2B). Percentage P2Y12-mediated platelet inhibition

was significantly lower in the carrier group when compared with the wild-type group ( $34 \pm 25.8\%$  versus  $48.6 \pm 28.2\%$ ;  $P = 0.005$ ), with significantly higher PRU values ( $222 \pm 94$  versus  $174 \pm 105$  U;  $P = 0.012$ ).

### Additive Effect of the 6.3-kb $\alpha_{2A}$ -AR Variant and CYP2C19\*2 Allele on P2Y12-Mediated Aggregation

A total of 63/141 (44%) of the study group did not carry either the 6.3-kb  $\alpha_{2A}$ -AR variant or the CYP2C19\*2 loss-of-function allele; 32/141 (21%) patients were 6.3-kb  $\alpha_{2A}$ -AR carrier but wild type for CYP2C19 polymorphism; 26/141 (18%) patients were wild type for  $\alpha_{2A}$ -AR polymorphism but were carrier for the CYP2C19\*2 loss-of-function allele; finally, 20/141 (14%) patients were carrying both the 6.3-kb  $\alpha_{2A}$ -AR variant and the CYP2C19\*2 loss-of-function allele. There was no association between the 2 mutations ( $P = 0.258$ ).

When we explored the effect of each mutation alone and then in combination, we found that carriers of both the 6.3-kb  $\alpha_{2A}$ -AR variant and the CYP2C19\*2 loss-of-function allele have an additive effect on P2Y12-mediated platelet aggregation (Figure 3). In the presence of both mutations, a significantly higher level of PRU ( $P = 0.037$ ) and significantly lower percentage P2Y12-mediated platelet inhibition ( $P = 0.009$ ) were observed when compared with wild-type patients or with either mutation on its own.

## Discussion

Our study demonstrates that not only genetic polymorphisms of the  $\alpha_{2A}$ -AR are relatively common but also the presence of this polymorphism modulates platelet reactivity in response to epinephrine in patients with stable angina, despite loading with 600 mg of clopidogrel and 500 mg of aspirin. In addition, the 6.3-kb  $\alpha_{2A}$ -AR variant exerts an additive effect with CYP2C19\*2 loss-of-function allele further increasing residual platelet reactivity.

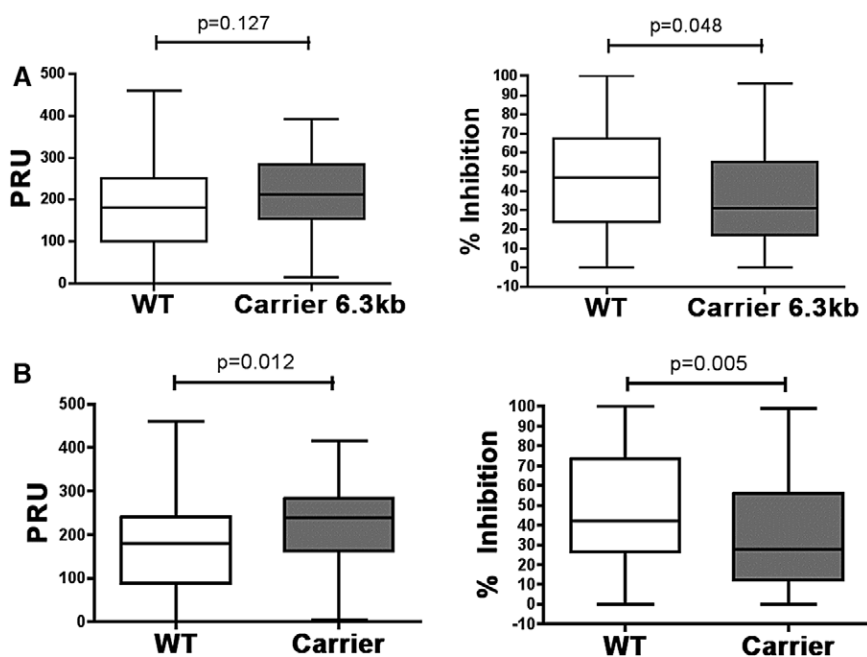
### Prevalence and Role of $\alpha_{2A}$ -AR Polymorphism

Epinephrine is a potent mediator of platelet aggregation through the stimulation of  $\alpha_{2A}$ -ARs on the platelet membrane surface.<sup>18</sup> Of note, this receptor presents a relatively common 6.7-/6.3-kb polymorphism able to modulate platelet function that is detectable in approximately one third of healthy volunteers and patients with acute coronary syndromes (ACS).<sup>20,23</sup> In the current study, we found again that approximately one third of our patients with stable angina was either heterozygous or homozygous for the  $\alpha_{2A}$ -AR polymorphism.

**Table 3. Multivariable Linear Regression Analysis to Assess the Influence of the  $\alpha_{2A}$ -AR Polymorphism on Epi-Induced Aggregation**

Epi, 2.5 $\mu\text{mol/L}$	Coef.	SE	<i>t</i>	95% CI	<i>P</i> Value
$\alpha_{2A}$ -AR carriers	8.249	2.316	3.562	3.663 to 12.834	0.001
$\beta$ -Blockers	-5.762	2.394	-2.407	-10.500 to -1.023	0.018
ACE-inhibitors	6.346	2.343	2.709	1.708 to 10.984	0.008

$\alpha_{2A}$ -AR indicates adrenergic receptor; ACE, angiotensin-converting enzyme; CI, confidence interval; and Epi, epinephrine.



**Figure 2.** P2Y12-mediated aggregation with  $\alpha_{2A}$ -adrenergic receptor (AR) or cytochrome P450 2C19 (CYP2C19) polymorphisms. **A**, Platelet reactivity unit (PRU) and percentage inhibition in wild-type (WT) patients and carrier of the 6.3-kb  $\alpha_{2A}$ -AR variant. **B**, PRU and percentage inhibition in WT patients and carrier of the CYP2C19\*2 loss-of-function allele.

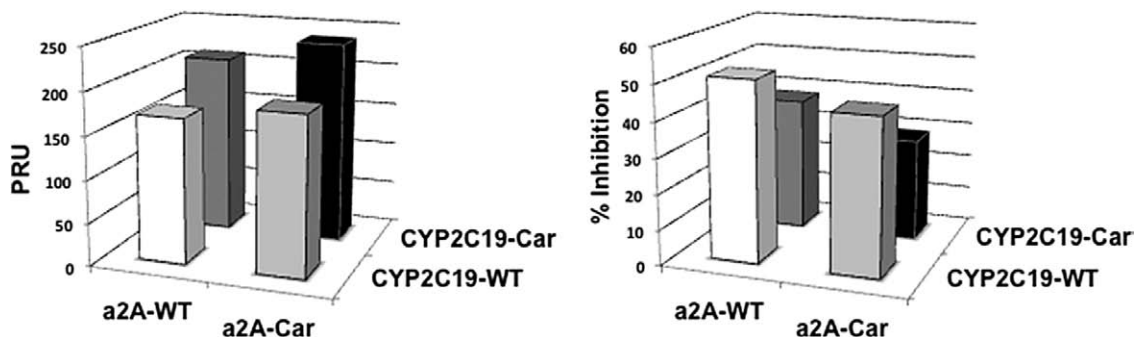
**Influence of DAPT on Epinephrine-Induced Aggregation**

Recent work shows that<sup>22,24</sup> aspirin and clopidogrel can significantly modulate not only ADP but also epinephrine-induced platelet aggregation. The average aggregation response to epinephrine is significantly reduced in patients with coronary artery disease taking both aspirin and clopidogrel when compared with control healthy volunteers naïve to therapy. Notably in patients with coronary artery disease, the response elicited by epinephrine is markedly heterogeneous, and in treated patients a significant interaction was observed in platelet aggregation response to epinephrine and ADP (ie, those patients with increased epinephrine-induced platelet response presented also an increased response to ADP). This interaction is limited to these 2 pathways and not observed with other pathways, such as arachidonic acid, thrombin receptor-activating peptide, and collagen. These data fit with our current observation in that this heterogeneity can be partly attributed to the presence of  $\alpha_{2A}$ -AR polymorphism and to its interaction with CYP2C19\*2 allele.

The presence of the 6.3-kb  $\alpha_{2A}$ -AR variant was associated with increased responsiveness to epinephrine-induced aggregation in the initial healthy volunteer cohort.<sup>20</sup> In keeping

with this study, we found in our patients with stable angina that the presence of the  $\alpha_{2A}$ -AR polymorphism was associated with increased epinephrine-induced aggregation, despite DAPT. At the multivariable analysis, carrying the 6.3-kb  $\alpha_{2A}$ -AR variant was the strongest predictor of the enhanced epinephrine-induced platelet aggregation.

In a previous study, we were unable to detect any major effect of the 6.3-kb  $\alpha_{2A}$ -AR variant on platelet response in patients with non-ST-segment-elevation myocardial infarction ACS treated with DAPT.<sup>23</sup> This apparent discrepancy with the current findings can have different explanations. First, in the non-ST-segment-elevation myocardial infarction ACS study, platelet responsiveness was not evaluated in response to epinephrine but only in the context of ADP-induced aggregation as measured by light transmission aggregation and using the vasodilator-stimulated phosphoprotein phosphorylation assay. Second, lack of interaction between  $\alpha_{2A}$ -AR polymorphism and ADP-induced aggregation could be because of the fact that patients with ACS have higher levels of circulating catecholamines, in general, and epinephrine, in particular. This prolonged or repeated exposure to epinephrine leads to downregulation of ARs.<sup>25-27</sup> Therefore, patients with ACS may



**Figure 3.** Platelet reactivity unit (PRU) and percentage P2Y12-mediated platelet inhibition with combined 6.3-/6.7-kb  $\alpha_{2A}$ -adrenergic receptor and CYP2C19\*2 polymorphisms. Post-test ANOVA confirmed that the presence of both mutations leads to a significant increase in PRU value (left; \*P=0.037) and a significant decrease in percentage inhibition (right; \*\*P=0.009).

be less responsive to the effects of catecholamines and other mediators, such as ADP, making it difficult to identify differences. Finally, previous evidence shows that baseline platelet reactivity in patients with ACS is already elevated, so detecting subtle differences in platelet reactivity in response to various agonists may be more difficult.<sup>28,29</sup>

### Clinical Implications

Our study further adds on the available literature showing for the first time that the  $\alpha_{2A}$ -AR polymorphism influences P2Y<sub>12</sub>-mediated platelet aggregation. More importantly, a clear functional interaction was observed with CYP2C19\*2 loss-of-function polymorphism. The CYP2C19\*2 allele has been shown to be an important genetic mediator of P2Y<sub>12</sub>-mediated platelet responses in patients taking clopidogrel, which in turn translates into a higher rate of stent thrombosis and cardiovascular death.<sup>11–16</sup> This variant seems to have little effect, if any, in patients with ACS taking more potent P2Y<sub>12</sub> inhibitors, such as prasugrel or ticagrelor.<sup>5,6</sup> Yet, recent reports have shown some residual interindividual variability, despite prasugrel that could be because of other signaling pathways or genetic polymorphisms.<sup>8,9</sup> In this study, we corroborate with previous work that the presence of the CYP2C19\*2 loss-of-function allele influences on treatment P2Y<sub>12</sub>-mediated platelet responses, and provide novel insights that  $\alpha_{2A}$ -AR polymorphism mediates P2Y<sub>12</sub>-mediated platelet responses in stable coronary artery disease patients taking clopidogrel. Furthermore, in those individuals who carry both mutations, it is notable that there is an additive effect on the P2Y<sub>12</sub>-mediated platelet responses. This raises the question as to whether this genetic combination could have a clinical effect on major adverse cardiovascular events, and whether this enhanced platelet reactivity would benefit from more potent P2Y<sub>12</sub> inhibitors also in patients with stable coronary disease.

Angiotensin-converting enzyme-inhibitors were associated with an increased response to epinephrine on platelet aggregation. This can be explained through the inhibitory modulation exerted by angiotensin-converting enzyme-inhibitors on peripheral adrenergic transmission.<sup>30</sup> We might speculate, on this background of reduced adrenergic stimulation, a hyper-responsiveness of platelet  $\alpha_2$ -ARs to epinephrine. On the contrary,  $\beta$ -blockers were associated with a reduced epinephrine-induced platelet aggregation. This finding confirms the previously described transregulation of the platelet  $\alpha_2$ -adrenergic signal transduction pathway by chronic  $\beta$ -blockade therapy.<sup>31</sup> In this work, in fact, Schwencke et al<sup>31</sup> reported a reduction of the catecholamine-induced platelet activation and aggregation in healthy volunteers under chronic  $\beta$ -blockade.

### Limitations

We recognize the limited sample size of our study, and our findings need replication in larger cohort of patients. Although we found an additive effect from the  $\alpha_{2A}$ -AR polymorphism and CYP2C19\*2 loss-of-function allele on platelet response, it is likely to be the case that other polymorphisms may have had an additional additive or possibly antagonistic effect on platelet responses; however, these were not measured. As is the case

for many studies measuring platelet function, in this study, we have not measured baseline platelet function in treatment-naïve patients. Nevertheless, high on treatment platelet reactivity has been shown to be clinically relevant.<sup>1–4</sup> Our study lacks clinical follow-up to ascertain the clinical relevance of this additive effect seen using ex vivo platelet function testing. Finally, we cannot exclude that the apparent discrepancy between the current results and our previous study<sup>23</sup> could be partly attributed to the different platelet assays used.

### Conclusions

The presence of  $\alpha_{2A}$ -AR polymorphism is associated with increased platelet reactivity in response to epinephrine and seems to have an additive effect along with the CYP2C19\*2 loss-of-function allele on P2Y<sub>12</sub>-mediated platelet responses in patients with chronic stable angina taking DAPT. Additional studies are needed to assess whether carriers of both polymorphisms are exposed to an excessive risk of thrombotic events, especially during adrenergic stress.

### Disclosures

None.

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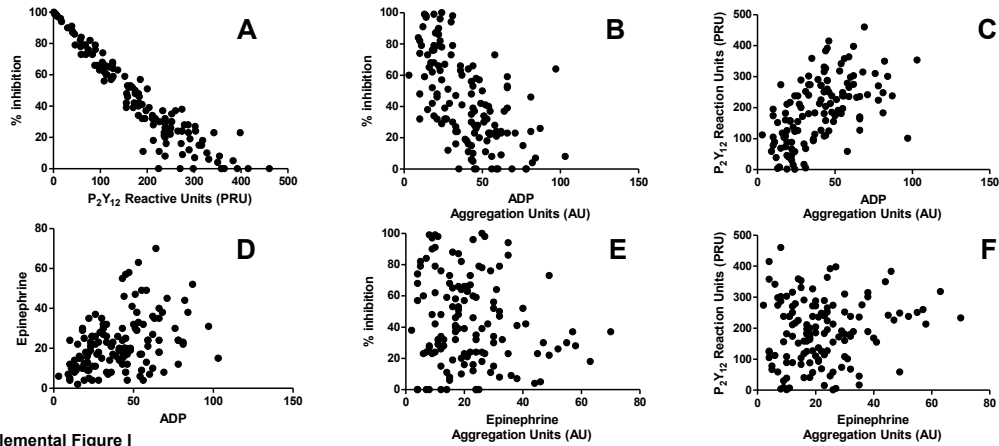
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### Significance

Despite currently available antiplatelet therapies, a proportion of patients with coronary artery disease continue to experience major adverse cardiovascular events. This is mediated by many environmental and genetic influences. Epinephrine plays a vital role in the pathogenesis of ischemic syndromes. This study shows for the first time the significant prevalence of the  $\alpha_{2A}$ -adrenergic receptor polymorphism and its crucial influence on platelet responsiveness to epinephrine in patients with coronary artery disease. Furthermore, it has an additive effect in patients with stable coronary artery disease, who also possess the CYP2C19\*2 loss-of-function allele. These patients may represent a population who are at an even higher risk of major adverse cardiovascular events.

## SUPPLEMENTAL MATERIAL



### Supplemental Figure 1

**Panel A** shows that % inhibition correlates well with P2Y<sub>12</sub> Reactive Units (PRU), ( $r = -0.94$ ,  $p < 0.0001$ ) using the Verify Now Assay.

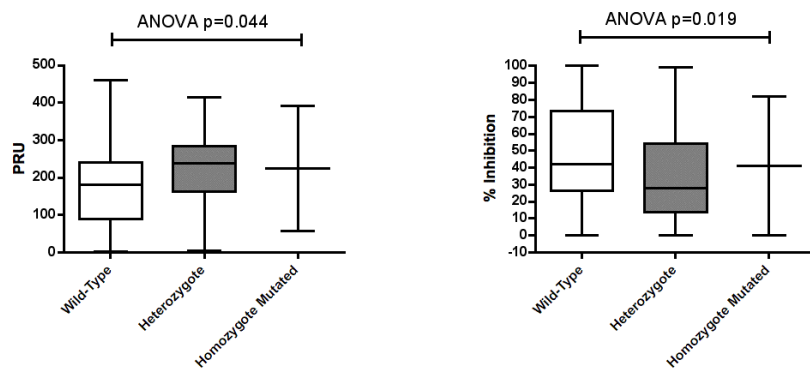
**Panel B** shows that % inhibition as measured by the Verify Now Assay significantly correlates with ADP induced platelet aggregation as measured by the Multiplate Analyser ( $r = 0.63$ ,  $P < 0.0001$ ).

**Panel C** shows that PRU significantly correlates with ADP induced aggregation as measured by the Multiplate ( $r = 0.59$ ,  $p < 0.0001$ ).

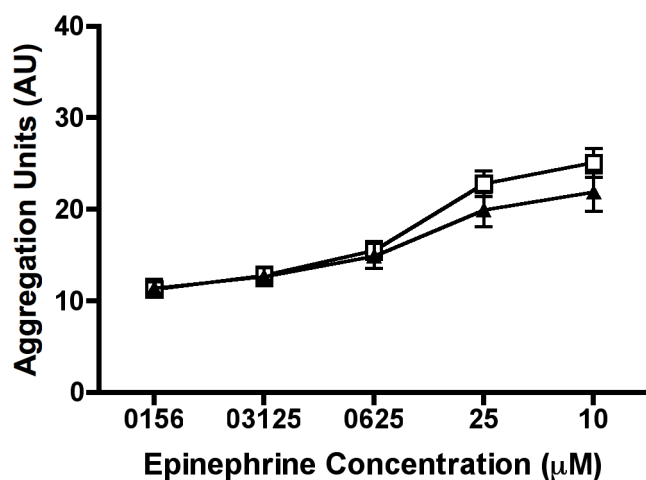
**Panel D** shows that Epinephrine induced aggregation significantly correlates with ADP induced aggregation in the Multiplate ( $r = 0.44$ ,  $p < 0.0001$ ).

**Panels E & F** there is no correlation between either % inhibition or PRU in the Verify Now and Epinephrine induced aggregation in the Multiplate ( $r = -0.13$ ,  $p = ns$  and  $r = 0.08$ ,  $p = ns$  respectively).





**Supplemental Figure II**  
**P2Y12-mediated aggregation with CYP2C19 polymorphisms.** PRU and % P2Y12-mediated platelet inhibition in patients Wild Type (WT), Heterozygote and Homozygote (n=2) for CYP2C19\*2 loss of function allele. The left panel shows that PRU levels are significantly higher in patients with the mutation. The right panel shows that the level of platelet inhibition is significantly reduced in patients with the mutation.



**Supplemental Figure III**

**Impact of CYP2C19\*2 polymorphism on epinephrine-induced platelet reactivity.** A significant dose-response increase to epinephrine-induced aggregation is observed both in CYP2C19 wild type (white boxes, from  $11 \pm 0.8$  AU to  $25 \pm 1.5$  AU, ANOVA  $p < 0.0001$ ) and in CYP2C19\*2 carriers (black triangles, from  $11 \pm 0.9$  AU to  $22 \pm 2.1$  AU, ANOVA  $p < 0.0001$ ). Though no significant difference between the 2 groups of patients at any of the epinephrine dose is observed.

## **MATERIALS AND METHODS**

### **Patient population and study design**

From October 2009 to December 2010, consecutive stable angina patients with stable coronary artery disease undergoing elective percutaneous coronary intervention (PCI) were prospectively recruited at the Cardiovascular Center Aalst OLV Clinic, Aalst, Belgium. All patients were loaded with 600 mg of clopidogrel and 500 mg of aspirin at least 12 hours prior to the procedure or were taking dual antiplatelet therapy consisting of aspirin and clopidogrel for at least 8 days. Exclusion criteria were upstream use of glycoprotein IIb/IIIa inhibitors, platelet count  $<70 \times 10^9/L$ , high bleeding risk (active internal bleeding, history of hemorrhagic stroke, intracranial neoplasm, arteriovenous malformation or aneurysm, ischemic stroke in the previous 3 months), coronary artery bypass surgery in the previous 3 months, and severe renal failure (serum creatinine  $>2$  mg/dl). This study complied with the Declaration of Helsinki and was approved by the local ethics Committee, with all patients giving written informed consent.

### **Blood sampling and platelet function analysis**

Before giving heparin, whole blood was drawn from a 6 French sheath directly before PCI and prior to the administration of any anticoagulant/antithrombotic treatment in the catheterization laboratory. Due to the lack of agreement on the ideal and most clinically relevant platelet function test or the threshold of response for each test it was felt that using two assays of platelet function would be important as opposed to using a single assay of platelet function. Previous work shows a significant correlation between the Verify Now<sup>®</sup> Assay and the Multiplate<sup>®</sup> Analyzer and so these were chosen for this study<sup>1</sup>.

After discarding the first 3 ml of blood, the next 7.5 ml was drawn and dispensed into a Lithium-Heparin tube. A further 2 ml was drawn and dispensed into 3.2% sodium citrate blood bottle. Epinephrine-induced aggregation was measured across a broad range of concentrations (0.156, 0.313, 0.625, 2.5 and 10  $\mu$ M) using the Multiplate<sup>®</sup> Platelet Function Analyzer with the results expressed in Aggregation Units (AU) (3). The Multiplate<sup>®</sup> analyzer was chosen for the following reasons: a) It allowed us to investigate epinephrine-induced platelet aggregation in whole blood; b) It was more expedient than Light Transmission Aggregometry (LTA) and used considerably less blood per patient; c) It allowed us to obtain a dose-response aggregation curve over a wide range of epinephrine concentrations; d) By using dose response curves, we could then investigate the effect of submaximal concentrations of epinephrine, in line with previous work showing a distinct hyper-reactive platelet response to epinephrine in healthy volunteers and in patients taking both aspirin and clopidogrel<sup>2,3</sup>.

Platelet reactivity was then assayed using the VerifyNow<sup>®</sup> P2Y12 assay, a validated optical turbidimetric point-of-care assay specifically assessing the effects of P2Y12 receptor inhibitors with the results reported as P2Y12 reaction units (PRU) or % P2Y12-mediated platelet inhibition<sup>4</sup>. The VerifyNow<sup>®</sup> assay was also chosen for the following reasons: a) It is the most widely used and available point of care assay in the clinical arena; b) It applies a similar principle to the VASP platelet function assay thus enabling specific evaluation of the P2Y12 pathway as it uses a combination of ADP

and PGE1 as agonists in order to increase the specificity of the test <sup>5</sup>; c) It has been used previously to assess the influence of the CYP2C19\*2 loss of function allele on both ADP induced platelet aggregation and % inhibition in patients taking dual antiplatelet therapy, and it showed a good correlation with the results obtained with VASP assay <sup>6</sup>.

### **Genotyping**

The 6.7kb/6.3kb  $\alpha_{2A}$ -AR [rs553668] gene polymorphism was analyzed using sTaqMan single nucleotide polymorphism (SNP) Genotyping Assay manufactured by Applied Biosystems (C\_996424\_20). Amplification reactions were run on a ABI Prism 7000 Sequence Detection System in 25  $\mu$ l volumes using a protocol of 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, then 60°C for 1 minute. Genotyping were read after running a post-read using automated software (SDS v1.2). Patients homozygote 6.7kb/6.7kb were defined as wild-type. The 6.3kb was defined as mutated allele. Patients heterozygote 6.7kb/6.3kb and homozygote 6.3kb/6.3kb were grouped and defined carrier.

A TaqMan Drug Metabolism Genotyping Assay from Applied Biosystems (now life technologies) was used (c\_25986767-70). The ID of the SNP is: rs4244285 [681G>A]. Amplification reactions were run on an ABI Prism 7500 Sequence Detection System in 25  $\mu$ l volume with the following thermal cycler conditions: First 2 minutes at 50°C, then 10 min at 95°C followed by 50 cycles of 15 sec at 92°C and 90 sec at 60°C. Genotyping were read after running a post-read using automated software (7500 software v 2.0.6). Patients homozygote CYP2C19\*1 were defined as wild-type. The CYP2C19\*2 was defined as mutated allele. Patients heterozygote CYP2C19\*1/CYP2C19\*2 and homozygote CYP2C19\*2 were grouped and defined as carriers.

### **Statistical Analysis**

Based on previous evidences (17,18), we assumed approximately 35% of our patients to be carrier of the 6.3 kb  $\alpha_{2A}$ -AR mutation. In these patients, epinephrine-induced aggregation (at the dose of 2.5  $\mu$ M) was expected to be two-fold higher compared with wild type patients (17). Therefore, a total of 122 patients (76 Wild Types and 46 Carriers) were needed to achieve a 95% power at a 2-sided alpha of 0.05 to detect the expected difference.

Continuous variables are expressed as mean  $\pm$  SD, while categorical variables are reported as frequencies and percentages. Comparisons between continuous variables were performed using the Student's t test. Comparisons between categorical variables were evaluated using the Fisher's exact test or the Pearson's chi-square test, where appropriate. One-way ANOVA for repeated measures was used to analyze the aggregation response to increasing doses of epinephrine within the group of patients Wild Type or Carrier of the 6.3 kb  $\alpha_{2A}$ -AR mutation. Post-hoc analysis was performed with the Newman-Keuls test. One-way ANOVA with post-hoc Newman-Keuls test was used to analyze PRU and % inhibition differences according to  $\alpha_{2A}$ -AR and CYP2C19 polymorphisms. Genotype frequencies were tested for Hardy-Weinberg equilibrium using a Chi-square test. Due to the low number of homozygotes for the 6.3 kb variant of  $\alpha_{2A}$ -AR and for the CYP2C19\*2 allele, neither additive nor recessive models of inheritance were

included. Comparisons were therefore performed between patients Wild Type (Homozygote for 6.7 kb variant of  $\alpha_{2A}$ -AR or for the CYP2C19\*1 allele) and patients Carriers (grouping both patients heterozygote and homozygote for the mutated allele).

Linear regression analysis was performed to assess the association between  $\alpha_{2A}$ -AR carriers and epinephrine-induced aggregation (at 2.5  $\mu$ M dose). To adjust for potential confounders, we included in the multivariable model all factors in table 2 that changed the point estimate of the  $\alpha_{2A}$ -AR carriers' status with at least 10%. Statistical analysis was performed using GraphPAD Prism 5 and SPSS version 15.0 software (SPSS Inc., Chicago, Illinois) and p value <0.05 (two tailed) was considered significant.

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