Association of the coronary artery disease risk gene *GUCY1A3* with ischemic events after coronary intervention

Short title: *GUCY1A3* and ischemic events after PCI

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Abstract

Aim: A common genetic variant at the *GUCY1A3* coronary artery disease locus has been shown to influence platelet aggregation. The risk of ischemic events including stent thrombosis varies with the efficacy of aspirin to inhibit platelet reactivity. This study sought to investigate whether homozygous *GUCY1A3* (rs7692387) risk allele carriers display higher on-aspirin platelet reactivity and risk of ischemic events early after coronary intervention.

Methods and Results: The association of *GUCY1A3* genotype and on-aspirin platelet reactivity was analyzed in the genetics substudy of the ISAR-ASPI registry (n=1,678) using impedance aggregometry. The clinical outcome cardiovascular death or stent thrombosis within 30 days after stenting was investigated in a meta-analysis of substudies of the ISAR-ASPI registry, the PLATO trial (n=3,326) and the Utrecht Coronary Biobank (n=1,003) comprising a total 5,917 patients. Homozygous *GUCY1A3* risk allele carriers (GG) displayed increased on-aspirin platelet reactivity compared to non-risk allele (AA/AG) carriers (150 [interquartile range: 91-209] vs. 134 [85-194] AU·min, p<0.01). More homozygous risk allele carriers, compared to non-risk allele carriers, were assigned to the high-risk group for ischemic events (>203 AU·min; 29.5 vs. 24.2%, p=0.02). Homozygous risk allele carriers were also at higher risk for cardiovascular death or stent thrombosis (Hazard ratio 1.70 [95 % confidence interval: 1.08-2.68], p=0.02). Bleeding risk was not altered.

Conclusions: We conclude that homozygous *GUCY1A3* risk allele carriers are at increased risk of cardiovascular death or stent thrombosis within 30 days after coronary stenting, likely due to higher on-aspirin platelet reactivity. Whether *GUCY1A3* genotype helps to tailor antiplatelet treatment remains to be investigated.

Keywords: on-aspirin platelet reactivity; genetic variation; stent thrombosis; genome-wide association studies; platelet aggregation

1. Introduction

Percutaneous coronary intervention (PCI) with implantation of stents is the treatment of choice in acute coronary syndromes (ACS). A combination of aspirin and an adenosine diphosphate (ADP) receptor antagonist, e.g., clopidogrel, ticagrelor, or prasugrel, is used to reduce the incidence of ischemic events, especially stent thrombosis. Recent studies, however, have shown rates of definite stent thrombosis up to 1.5% in the first year after ACS [1]. It has been shown that the responsiveness to antiplatelet therapies displays large inter-individual variability. Clopidogrel, for instance, is bio-activated via the cytochrome P450 2C19 isoform, an enzyme encoded by the *CYP2C19* gene. Polymorphisms in *CYP2C19* have been shown to cause a poor metabolizer status and, therefore, reduced activation of the prodrug clopidogrel [2]. Secondary to coronary stenting, such variants led to reduced inhibition of platelet aggregation and a higher rate of cardiovascular events [3,4]. Likewise, it has been shown that high on-aspirin platelet reactivity to arachidonic acid, is associated with ischemic events including stent thrombosis [5]. Here, it is rather debated whether doses or dosing intervals affect this outcome whereas genetic determinants of response to aspirin are not known [6].

Genome-wide association studies (GWAS) led to the identification of several chromosomal loci associated with coronary artery disease (CAD) [7-9]. One of these variants on chromosome 4q32.1 tags the *GUCY1A3* (according to a new nomenclature also known as *GUCY1A1*) gene [10] which encodes the α_1-subunit of the soluble guanylyl cyclase (sGC). In platelets, upon stimulation with nitric oxide (NO), sGC produces the second messenger cyclic guanosine monophosphate (cGMP) ultimately leading to inhibition of platelet aggregation (for a review see [11]). In mice, deletion of *Gucy1a3* accelerates formation of occluding thrombi in arteries after photoexcitation. The same mechanism is also assumed to be causal for the phenotype of premature CAD and myocardial infarction (MI) in subjects with loss-of-function mutations in *GUCY1A3* [12,13]. Recently, it has also been demonstrated that the common, non-coding lead variant at the *GUCY1A3* locus identified by GWAS [10] is associated with reduced α_1-sGC protein levels in platelets of homozygous risk allele carriers leading to weaker inhibition of platelet aggregation after stimulation with a NO donor [14].

Here, we sought to investigate whether homozygous carriers of the *GUCY1A3* risk allele display altered on-aspirin platelet reactivity and worse clinical outcome after coronary stenting.

2. Methods

2.1 Measurement of arachidonic acid-induced platelet aggregation

Arachidonic-acid induced platelet aggregation measurements in the ISAR-ASPI registry were described previously [5]. Briefly, whole blood was collected in 4.5 ml plastic tubes containing the anticoagulant lepirudin (Dynabyte, Munich, Germany). Blood samples were obtained from the arterial sheath of patients after the administration of 500 mg of aspirin intravenously which had been administered a few minutes before index PCI. Quantitative determination of platelet function triggered by arachidonic acid or adenosine diphosphate was assessed using impedance aggregometry on the Multiplate analyzer (Roche Diagnostics, Basel, Switzerland).

2.2 Study populations

2.2.1 ISAR-ASPI registry

The ISAR-ASPI registry investigated the interaction between on-aspirin platelet reactivity and ischemic events secondary to PCI in CAD patients (95% of individuals were taking clopidogrel as ADP receptor antagonist after PCI) and has been described previously [5]. *GUCY1A3* lead SNP (rs7692387) genotypes after genotyping using a rs7692387 TaqMan ® Genotyping Assay (C__29125113_10; Life Technologies, Carlsbad, CA, USA) on a ViiA7 qPCR instrument (Life Technologies, Carlsbad, CA, USA) were available from 1,678 individuals.

2.2.2 PLATO Trial genetics substudy

The PLATelet inhibition and patient Outcomes (PLATO) trial (www.ClinicalTrials.gov, NCT00391872) assessed the benefit from treatment with ticagrelor in comparison to clopidogrel in aspirin-treated ACS [15]. Details on genotyping, quality control, and imputation in participants of the PLATO trial have been described previously [16]. The *GUCY1A3* lead risk variant (rs7692387) was imputed with good quality (impute2 info score 0.996). In this analysis, patients who underwent PCI and had been randomized to clopidogrel treatment were included. *GUCY1A3* lead SNP (rs7692387) genotypes were available from 3,236 individuals.

2.2.3 Utrecht Coronary Biobank (UCORBIO)

UCORBIO is a prospective study enrolling individuals undergoing coronary angiography for any indication. Patients were followed up for the occurrence of major cardiovascular events, as has been described previously [17,18]. In this analysis, patients who underwent PCI were included. *GUCY1A3* lead SNP (rs7692387) genotypes were available from 1,003 individuals. Genotyping for rs7692387 was performed using a customized KASP genotyping assay (LGC Group, Teddington, UK). The number of individuals taking clopidogrel or other ADP receptor antagonists at discharge were not documented. The study was approved by the Ethics Committee of the University Medical Center Utrecht and was conducted according to the Declaration of Helsinki. UCORBIO is registered with clinicaltrials.gov (ID: NCT02304744).

2.3 Study oversight

This study was performed in accordance with the Declaration of Helsinki. The institutional review board of the Technical University of Munich approved the meta-analysis study protocol (100/16s).

2.4 Clinical outcomes and sample size estimation

Clinical endpoints were defined as described previously [5,15,19]. The clinical endpoint of this study was *cardiovascular death or stent thrombosis (definite or probable) within 30 days*. The study was designed to detect statistically significant effects with power and type I error rate of 80% and 5%, respectively. Assuming a hazard ratio (HR) of 1.60, we estimated a sample size of 4,925 patients to analyze the primary endpoint. *Non-coronary artery bypass graft (CABG) major or minor bleeding within 30 days* was further analyzed to assess genotype-dependent effects on bleeding risk.

2.5 Statistical analysis

Continuous data were analyzed using t-test/ANOVA or Kruskal-Wallis Test, as appropriate. Categorical data were analyzed using Chi-squared test. Outcomes were analyzed using the

Cox proportional hazards model. Meta-analysis was performed using RevMan 5 (Review Manager (RevMan) [Computer program]. Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014) using a random-effects model.

3. Results

3.1 Baseline characteristics and genotyping

Descriptions of the study populations investigating the primary endpoints are displayed in **Table 1**. Baseline characteristics and number of events are depicted in **Supplemental Tables 1 and 2**, respectively. Genotype frequencies were in Hardy-Weinberg Equilibrium in the ISAR-ASPI (p=0.86), PLATO (p=0.95) and UCORBIO (p=0.14) studies (**Supplemental Table 3**).

3.2 *GUCY1A3* genotype and high on-aspirin platelet reactivity

To investigate whether *GUCY1A3* genotype is associated with on-aspirin platelet reactivity, we analyzed 1,678 individuals derived from the ISAR-ASPI registry. *GUCY1A3* genotype was associated with on-aspirin platelet reactivity (**Supplemental Figure 1**). Homozygous *GUCY1A3* risk allele carriers (median 150 [IQR 91-209] AU·min, n=1,145) displayed significantly higher on-aspirin platelet reactivity as compared to non-risk allele carriers (median 134 [IQR 85-194] AU·min, n=533; p=0.009; **Figure 1A**). We further analyzed the proportion of individuals displaying arachidonic acid-induced platelet aggregation values of >203 AU·min (high on-aspirin platelet reactivity). This endpoint was derived in a previous study exploring the clinical implications of high on-aspirin platelet reactivity [5]. More homozygous *GUCY1A3* risk allele carriers presented with HAPR (n=338/1,145, 29.5%) compared to non-risk allele carriers (n=129/533, 24.2%; p=0.02; **Figure 1B**). Thus, homozygous risk allele carriers were at increased risk for high on-aspirin platelet reactivity (Odds Ratio (OR) 1.31 [95% confidence interval (CI) 1.04-1.66]). ADP-induced platelet aggregation was not affected by *GUCY1A3* genotype (**Supplemental Figure 2**).

3.3 *GUCY1A3* genotype and ischemic events within 30 days after PCI

To investigate whether *GUCY1A3* genotype is associated with ischemic events secondary to coronary stenting, we performed a meta-analysis of the three study cohorts: individuals from the ISAR-ASPI registry, the clopidogrel arm of the PLATO trial (n=3,326), and the UCORBIO study (n=1,003) biobank comprising in total 5,917 patients. Homozygous risk allele carriers

were at increased risk for the endpoint *cardiovascular death or stent thrombosis within 30 days* compared to non-risk allele carriers (Hazard Ratio (HR) 1.70 [95 % CI 1.08-2.68], p=0.02; **Figure 2**). After adjustment for covariates, carriage of two risk alleles remained associated with increased risk (HR_{adj} 1.62 [95% CI 1.02-2.56], p=0.04; **Supplemental Table 4**). Trends in the same direction were observed for further outcomes (**Supplemental Figure 3**). In particular, homozygous risk allele carriers were also at increased risk for *death from any cause or stent thrombosis within 30 days* compared to non-risk allele carriers (HR 1.59 [95 % CI 1.04-2.45], p=0.03). Genes encoding for other proteins that might affect platelet function in patients taking aspirin and clopidogrel, e.g., *CYP2C19*, are located on other chromosomes than *GUCY1A3*. As an example for poor clopidogrel metabolizer status, distribution of *CYP2C19*2* carriers was not different between the investigated *GUCY1A3* genotypes (**Supplemental Table 5**). Non-risk allele carriers displayed a trend towards lower risk of *definite or probable stent thrombosis within 30 days* (OR 0.77 [95% CI 0.56-1.06], p=0.11; **Supplemental Figure 4**).

3.4 *GUCY1A3* genotype and risk of bleeding after PCI

To assess whether *GUCY1A3* genotype influences risk of bleeding, we performed a meta-analysis of the cohorts. *Non-CABG major or minor bleeding within 30 days* was analyzed as primary outcome. We did not detect a genotype-dependent effect on risk of *non-CABG major or minor bleeding within 30 days* (OR 0.95 [95% CI 0.74-1.23], p=0.71; **Figure 3**) or *non-CABG major bleeding within 30 days* after PCI (OR 1.00 [95% CI 0.69-1.45], p=0.98; **Supplemental Figure 5**).

4. Discussion

To the best of our knowledge this is the first report of association of a CAD risk allele with on-aspirin platelet reactivity. Consequently, *GUCY1A3* genotype was also associated with adverse outcome, i.e., the combination of cardiovascular death and stent thrombosis after PCI. Interestingly, the risk allele was not accompanied by an altered risk of bleeding as assessed by non-CABG-related major and minor bleeding after PCI.

NO-cGMP-signaling has been known for a long time to influence platelet aggregation. For instance, activation of platelet sGC by sodium nitroprusside *in vitro* leads to an increase in platelet cGMP levels [20] which activates cGMP-dependent protein kinase I leading to the phosphorylation of several intracellular targets like inositol-1,4,5-trisphosphate receptor-associated cGMP kinase substrate (IRAG) and vasodilator-stimulated phosphoprotein (VASP). Phosphorylation of both IRAG and VASP has been shown to be involved in cGMP-dependent inhibition of platelet aggregation [21,22]. ADP alone did not lead to alterations of intracellular platelet cGMP levels [20]. Arachidonic acid, however, has been demonstrated to significantly reduce platelet cGMP levels in a dose-dependent manner, an effect that could be reversed by indomethacin-mediated inhibition of cyclooxygenase 1 and 2 [23]. Hence, a reduction of intracellular cGMP levels secondary to arachidonic acid exposure could be more pronounced in homozygous *GUCY1A3* risk allele carriers who have been reported to show reduced levels of α_1-sGC [14]. This is in line with the data presented here that revealed an association of *GUCY1A3* genotype with arachidonic acid-induced platelet aggregation on aspirin therapy but not ADP-induced platelet aggregation. This pathway which is reviewed and graphically summarized in **Figure 4** illustrates how platelet cGMP homeostasis and VASP phosphorylation can be influenced by arachidonic acid. Aspirin might compensate for these effects to some extent. In the situation of reduced sGC availability and activity, as in homozygous *GUCY1A3* risk allele carriers [14], the effect of aspirin on this pathway, however, could be insufficient.

Ischemic events secondary to PCI remain serious complications of treatment of atherosclerotic lesions in coronary arteries. High on-aspirin platelet reactivity has been identified as a

biomarker for ischemic events after PCI [5]. Interestingly, these data also suggested that "aspirin resistance" or high on-aspirin platelet reactivity are indeed not only caused by noncompliance as it had been postulated before [24,25]. A study performed in healthy volunteers rather reported delayed and reduced absorption due to different coatings as a cause of pseudoresistance whereas "aspirin resistance" was rare [26]. In addition to "aspirin resistance" or pseudoresistance, optimal dosing of aspirin as well as dosing intervals are subject of discussion [6]. Here, we provide first evidence that a genetic CAD risk variant at the *GUCY1A3* locus which is not directly involved in arachidonic acid metabolism but in downstream NO-cGMP-signaling in platelets, influences platelet aggregation under aspirin therapy in CAD patients and relates to adverse outcomes after aspirin administration and PCI.

Our study has several limitations. First, sGC protein and cGMP levels in platelets were not available which makes it impossible to postulate a causal link between *GUCY1A3* genotype and on-aspirin platelet reactivity. However, a mechanistic study on this genetic variant as well as an association analysis of NO signaling with cardiometabolic phenotypes have shown increased expression of *GUCY1A3* in tissues of non-risk allele carriers [14,27] rendering a causal involvement likely. Second, *GUCY1A3* genotype was only associated with a combined endpoint, i.e., *cardiovascular death or stent thrombosis within 30 days*. Unfortunately, data on ischemic endpoints within 30 days after PCI are sparse and we were not able to retrieve further cohorts to increase sample size and power to investigate the association of *GUCY1A3* genotype and, e.g., definite stent thrombosis. Additionally, due to the relatively small sample sizes the majority of which is derived from one study, i.e., PLATO, as well as the retrospective nature of this study, it is unfortunately not possible to adjust results for some potentially relevant covariates. In particular, there is a chance that the genotypes affect the extent of coronary artery disease or blood pressure which might also have influenced the outcome. Third, we were only able to analyze the clopidogrel arm of the PLATO trial. As 95% of individuals included in the ISAR-ASPI registry were also taking clopidogrel in addition to aspirin [5], we cannot generalize our findings to intake of other ADP-receptor antagonists. Lastly, this study did not take into account all CYP variants that are associated with ischemic events due

to poor clopidogrel metabolism [3]. However, as *GUCY1A3* genotype was not associated with ADP-induced platelet aggregation in this study and as at least distribution of *CYP2C19*2* alleles was equal between the genotypes, this might by negligible.

In summary, we conclude that knowledge of *GUCY1A3* genotype may help identify individuals at risk for ischemic events secondary to PCI taking clopidogrel in addition to aspirin. Whether knowledge of *GUCY1A3* genotype at the time of PCI might help tailor antiplatelet strategies after PCI, e.g., via recommendation of more potent ADP receptor antagonists, remains to be investigated.

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Conflict of Interest

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448 **Figure legends**

449 **Figure 1:** Association between the *GUCY1A3* (rs7692387) genotype and on-aspirin platelet
450 reactivity to arachidonic acid. **A**. Homozygous *GUCY1A3* risk allele carriers (GG, n=1,145)
451 displayed higher on-aspirin platelet reactivity compared to non-risk allele carriers (AA/AG,
452 n=533). Kruskal-Wallis test. **B**. More homozygous risk allele carriers presented with high on-
453 aspirin platelet reactivity (AUC > 203 AU·min). Chi-squared test. *AUC*, area under the curve.
454

455 **Figure 2:** Association of *GUCY1A3* genotype and *cardiovascular death or stent thrombosis*
456 *within 30 days*. Homozygous risk allele carriers (GG) were at increased risk for the endpoint
457 compared to non-risk allele carriers (AA/AG). *CI*, confidence interval; *IV*, inverse variance; *SE*,
458 standard error.
459

460 **Figure 3:** Association of *GUCY1A3* genotype and *non-CABG major or minor bleeding*.
461 *GUCY1A3* genotype was not associated with increased risk of bleeding. *CI*, confidence
462 interval; *IV*, inverse variance; *SE*, standard error.
463

464 **Figure 4 (Graphical Abstract)**: Hypothetical interaction of arachidonic acid metabolism and
465 NO-cGMP-signaling in platelets. Arachidonic acid has been shown to directly reduce NO
466 bioavailability in platelets which could be reversed by COX-1 inhibition [23]. Details see text.
467

Tables

Table 1: Description of the analyzed studies. *PCI*, percutaneous coronary intervention; *$P2Y_{12}RA$*, $P2Y_{12}$-ADP-receptor antagonist.

Study	Design (initial study)	Objective	n (current analysis)	Antiplatelet regimen (current analysis)	Ref.
ISAR-ASPI	prospective	On-aspirin platelet reactivity and outcome after PCI	1,678	aspirin + $P2Y_{12}RA$	[5]
PLATO	prospective	Clopidogrel vs. Ticagrelor	3,236	aspirin + clopidogrel	[15]
UCORBIO	prospective	Major adverse cardiac events after coronary angiography	1,003	aspirin + $P2Y_{12}RA$	[17,18]