Platelet reactivity and cardiovascular events

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THE T13254C-ALLELE OF *GP6* IS ASSOCIATED WITH DECREASED PLATELET ACTIVATION AND A REDUCED RISK OF RECURRENT CARDIOVASCULAR EVENTS AND MORTALITY

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ABSTRACT

Background

Contradictory results have been published on the effects of T13254C (rs1613662), which distinguishes the two major isoforms of *GP6*, the gene encoding the platelet receptor glycoprotein VI, on platelet function and the risk of cardiovascular disease.

Methods

We performed a population-based case-control study, the Study of Myocardial Infarctions in LEiden (SMILE), among 547 male patients with a first myocardial infarction (MI) and 646 control subjects, as well as a prospective cohort study in which the same MI patients were followed for recurrent events (fatal and non-fatal MI and unstable angina) and mortality (median follow-up 12 years). P-selectin expression by platelets induced by crosslinked collagen-related peptide (CRP-XL) was measured by whole blood flow cytometry in 274 MI patients.

Results

T13254C was not associated with a first MI, but seemed to be associated with a reduced incidence of recurrent events (per allele hazard ratio 0.77, 95% confidence interval (CI) 0.56 to 1.06) and mortality (hazard ratio 0.57, 95%CI 0.37 to 0.89). Pooling with the Heart and Estrogen/progestin Replacement Study (HERS) revealed hazard ratios of 0.81 (95%CI 0.66 to 0.99) and 0.73 (95%CI 0.55 to 0.96). The minor C-allele was also strongly associated with a reduced percentage of P-selectin expressing platelets. The reduction per C-allele was 23% (95%CI 18 to 28%). In an independent study of 219 healthy volunteers, the per-allele reduction of CRP-XL-induced aggregation was 10% (95%CI 2 to 18%).

Conclusion

The minor allele of *GP6* T13254C that reduced platelet activation and aggregation also seemed to be associated with a reduced incidence of recurrent cardiovascular events and mortality, but was not associated with first MI.

INTRODUCTION

Platelets play an essential role in the development of cardiovascular disease. Clinically, the importance of platelet function has been established by large trials, which have shown the efficacy of antiplatelet therapy in the prevention of ischemic heart disease.[1] Moreover, studies have shown that variation in platelet function is an important determinant of atherothrombotic events.[2-5] Platelets can be activated by multiple agonists via specific receptors located on the platelet membrane. The receptor glycoprotein (GP) VI has a major role in collagen-induced platelet signaling.[6] Therefore, variations in *GP6*, the gene encoding GPVI, may influence platelet reactivity toward collagen and hence influence platelet function and the risk of cardiovascular disease.

Studies have shown that *GP6* contains two common haplotypes with relative frequencies of 85.1 and 15.9 percent (*GP6a* and *GP6b*); the remaining haplotypes are rare.[7,8] Another study identified 18 single nucleotide polymorphisms (SNPs) in *GP6* and confirmed the existence of two common isoforms in Caucasians.[9] It has been consistently shown that the minor isoform is associated with decreased collagen-induced platelet function, most likely caused by impaired signal transduction.[8,10-13] Equivocal results were obtained when platelet function was quantified with the Platelet Function Analyzer-100.[10,14,15] Studies on the effect of variation in *GP6* on the risk of cardiovascular disease provided conflicting results, which might be the result of heterogeneity with respect to study populations and endpoints.[7,16-22] In contrast to the inconclusive results in the field of arterial thrombosis, presence of the minor allele has been consistently associated with a decreased risk of venous thrombosis in large, genome-wide association studies.[23,24]

Given this inconclusive evidence, we set out to study the association between polymorphic variation in *GP6* and the risk of a first myocardial infarction (MI), recurrent cardiovascular events and mortality. Furthermore, we studied the association with crosslinked collagen-related peptide (CRP-XL)-induced platelet function. As a marker of variation in *GP6*, we studied the effects of the *GP6* T13254C polymorphism (rs 1613662). This polymorphism predicts a Ser219 ⇨ Pro substitution and identifies the aforementioned two common haplotypes in *GP6* (*GP6a* and *GP6b*).[7,9-12]

METHODS

The Study of Myocardial Infarctions in LEiden (SMILE)–Platelets project comprised three studies within a single study population to assess the association between T13254C and (1) first MI, (2) recurrent cardiovascular events and mortality, and (3) platelet function. The relation with recurrent events and mortality was also studied in the Heart and Estrogen/progestin Replacement Study (HERS). The relation with platelet function was also studied among healthy volunteers. All studies were approved by the Medical Ethics Committees of the participating centers, and performed in accordance with the Declaration of Helsinki. All subjects gave written informed consent before participation in the studies.

First myocardial infarction

We examined the association between *GP6* and first MI in the SMILE case-control study. Details of the study design have been published previously.[25] Briefly, cases were 560 consecutive male patients who presented at the Leiden University Medical Center and the Diaconessen Hospital Leiden, the Netherlands, with a first MI between 1990 and 1996. Thirteen patients were excluded because their presumed first event appeared to be a recurrent event, leaving 547 eligible cases. Control subjects consisted of 646 men without a history of MI who had undergone a minor orthopedic intervention between 1990 and 1996 and were identified in the records of the Leiden Anticoagulation Clinic. All subjects completed a questionnaire concerning presence of cardiovascular risk factors and medication use.

Recurrent cardiovascular events and mortality

The MI patients were prospectively followed until September 2004 after their inclusion in SMILE, to assess the occurrence of recurrent cardiovascular events and mortality.[26] Five of the 547 patients did not consent to participate in the follow-up study, so our study population consisted of 542 male patients who survived a first MI. The median follow-up was 12 years (5th to 95th percentile 9 to 14 years). Only four patients were lost to follow-up. They were censored at the last date on which they were known to have not reached one of the study endpoints.

Our primary study endpoints were recurrent cardiovascular events (defined as fatal and non-fatal MI and unstable angina requiring hospitalization) and all-cause mortality. For further analysis of effects on mortality, death certificates were requested from the Central Bureau of Statistics for all deceased patients to obtain causes of death. Details regarding data collection and endpoint classification have been published before.[26]

To obtain more robust effect estimates of the effect of T13254C on recurrent cardiovascular events and mortality, we pooled the findings from SMILE and the placebo-group of HERS, a randomized trial among postmenopausal women (median follow-up 7 years).[22,27,28] To our knowledge, HERS is the only other study evaluating long-term outcome associated with T13254C among patients with established cardiovascular disease. We did not include women allocated to hormone therapy, because of potential interaction between treatment and *GP6*.

Platelet function

We invited all living MI patients who participated in SMILE to come for a single visit to our centre between May 2008 and March 2009, because fresh blood samples were required for the platelet function tests. Two hundred and seventy-four patients provided a blood sample and completed an additional questionnaire on the presence of cardiovascular risk factors and medication use at time of blood sampling. Platelet activation was quantified by flow cytometric measurement of CRP-XL-induced P-selectin expression.

To further characterize functional variation in *GP6*, we recruited 219 healthy volunteers who did not use any drugs known to affect platelet function. Platelet

function was studied using CRP-XL-induced light-transmittance aggregometry in platelet-rich plasma. Details about the study population and the platelet function tests have been published previously.[29,30]

Laboratory analyses in SMILE

DNA was extracted from blood samples collected at baseline as described previously.[25] The status of the *GP6* T13254C polymorphism (rs1613662) was determined with 5'-exonuclease (Taqman) technology, using a commercially available assay (Applied Biosystems, Carlsbad, CA, USA; C_8717873_10). Genotyping was successful in more than 99.5% of patients, control subjects and patients who underwent platelet function testing. The very few subjects with missing genotype data (respectively two, three and one), the absence of which was assumed to be completely random, were excluded from the analyses.

P-selectin expression was measured in citrated whole blood using flow cytometry, according to previously described methods.[11,31] Briefly, within 15 minutes of blood sampling, hirudin was added to 500 µL of citrated whole blood (final concentration 10 U/mL). After 5 minutes of incubation, 5 µL blood was added to 50 µL of HEPES-buffered saline containing apyrase (4 U/mL) and co-incubated for 20 minutes at room temperature with either cross-linked collagen-related peptide (CRP-XL) (0.1, 1, 10 µg/mL) or no agonist, and both FITC-anti-CD62P (anti-P-selectin) and PE-anti-CD61 (anti-GPIIIa). CRP-XL is a specific GPVI agonist, and was kindly provided by Dr R.W. Farndale (University of Cambridge, Cambridge, UK). A single batch of CRP-XL was used for all experiments. Corresponding isotypes were used as negative controls. We stopped incubation by a 100-fold dilution of the samples using formyl saline (0.2% formaldehyde in 0.9% NaCl).

Flow cytometric analysis was performed using a Beckman Coulter® EPICS® XL-MCL flow cytometer (Beckman Coulter, Miami, FL, USA). The platelet population was identified using a forward and side scatter gate, which was verified with the anti-CD61 antibody.

Statistical analyses

We used logistic regression to estimate odds ratios (OR) and 95%CIs for the relation between the *GP6* variant and the risk of a first MI. We calculated hazard ratios (HR) for the relation with recurrent events and mortality using Cox proportional hazards regression. As some cardiovascular risk factors seemed to be not equally distributed over the three genotypes in the MI patients (Online appendix S1, available from http://bit.ly/hfKwzX), we also performed multivariable Cox regression models to adjust for those potentially confounding factors, including age (as a continuous variable), smoking, obesity (defined as a BMI higher than 30 kg/m^2), and use of glucose-lowering drugs, lipid-lowering drugs, antihypertensive drugs, aspirin, and vitamin K antagonists. Analyses regarding potential interaction between T13254C and use of aspirin were also adjusted for those factors. In HERS, multivariable models included age, race, presence of hypertension and diabetes, creatinine clearance, waist-hip ratio, and use of aspirin and statins.[22,27,28] We pooled the findings from SMILE and

HERS using conventional meta-analysis in which overall results of studies are pooled. We used fixed effects models as there was no evidence of statistical heterogeneity among the two studies.

We used linear mixed models to quantify the effect of the *GP6* polymorphism on P-selectin expression. Such models account for the repeated measurements of P-selectin expression per subject, because we used various concentrations of CRP-XL in each subject. We specified a unique identifier for each subject as random effect, and included the *GP6* variant and concentration of CRP-XL as fixed effects. Furthermore, basal P-selectin expression without using CRP-XL as agonist was added as covariate. Effects on aggregation were analyzed similarly. All analyses in the study among healthy volunteers, which included men and women from various races, were adjusted for sex and race.

Unless otherwise stated, additive models were used for the analyses, indicating the effect per copy of the minor allele. The analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC, USA) or SPSS version 17.0 (SPSS, Chicago, IL, USA).

RESULTS

Characteristics of the study populations

We included 547 male Caucasian MI patients and 646 male Caucasian controls in the SMILE case-control study. Five-hundred and forty-two of the patients consented to be included in the follow-up study and 274 patients participated in the platelet function study. The characteristics of the SMILE participants are summarized in Tables 1 and 2. At time of blood sampling, the platelet function study population was older, used more frequently preventive drugs and smoked less than all MI patients at baseline, indicating the progress of time since inclusion in the SMILE study.

As expected, established cardiovascular risk factors were equally distributed among control subjects with respect to the three different *GP6* genotypes (online appendix S1, available from http://bit.ly/hfKwzX). Among MI patients, risk factors were not completely equally distributed over the genotypes, which prompted us to use multivariable models to adjust for potential confounding. In the SMILE platelet function study, characteristics were equally distributed over the three genotypes (online appendix S2, http://bit.ly/hfKwzX).

The present analysis of the placebo group of HERS included 1074 women, of whom most were Caucasian (approximately 90%).[22] The platelet function study among healthy volunteers consisted of 219 individuals (54% female) of various races (43% Caucasian, 23% African American, 16% Hispanic, 11% Asian-Indian, and 7% Asian).[29,30]

First myocardial infarction

T13254C was equally distributed among patients with a first MI and control subjects. The OR for MI was 1.07 (95%CI 0.86 to 1.32) per copy of the C-allele, indicating no effects of T13254C on the risk of a first MI (Table 3).

Table 1 – Characteristics of participants in the SMILE case-control and cohort study

	Patients with first myocardial infarction (n=547)	Control subjects (n=646)
Age, years	57 (49 to 64)	59 (50 to 66)
Smoking	341 (62%)	206 (32%)
Obesity	93 (17%)	106 (16%)
Glucose-lowering drugs	25 (5%)	22 (3%)
Lipid-lowering drugs	12 (2%)	11 (2%)
Antihypertensive drugs	97 (18%)	107 (17%)
Aspirin use after event	384 (70%)	
Clopidogrel use after event	0 (0%)	
Oral anticoagulation use after event	128 (23%)	

Data are *n* (%) or medians (interquartile ranges), where appropriate. Data refer to the year before inclusion in SMILE, unless otherwise specified. Persons were classified as obese when their body mass index was higher than 30 kg/m^2. Patients with first myocardial infarction are the cases of the SMILE case-control study and are the subjects who were followed-up in the SMILE cohort study.

Recurrent cardiovascular events and mortality

During the follow-up of the 542 SMILE MI patients, 149 patients developed recurrent cardiovascular disease, while 97 subjects died. Two hundred and seventy-four of the 1074 women included in HERS developed recurrent cardiovascular disease, while 123 patients died. In both SMILE and HERS, presence of the C-allele was associated in a dose-dependent manner with a decreased incidence of recurrent cardiovascular events and mortality (Table 4). In SMILE (Figure 1), the HR for the association between the C-allele and recurrent events was 0.77 (95%CI 0.56 to 1.06) assuming an additive model. Regarding all-cause mortality, the HR was 0.57 (95%CI 0.37 to -0.89). Adjustment for potential confounders did not influence effect estimates substantially: adjusted HRs were 0.82 (95%CI 0.59 to 1.14) and 0.61 (95%CI 0.39 to 0.95), respectively. In HERS, the HR was 0.84 (95%CI 0.64 to 1.09) for recurrent cardiovascular events and 0.85 (95%CI 0.60 to 1.20) for all-cause mortality. Adjusted HRs were 0.86 (95%CI

Table 2 – Characteristics of participants in the SMILE platelet function study

	Patients (*n*=274)
Age, years	68 (62 to 75)
Smoking	59 (22%)
Obesity	77 (28%)
Glucose-lowering drugs	40 (15%)
Lipid-lowering drugs	241 (88%)
Antihypertensive drugs	232 (85%)
Aspirin	206 (75%)
Clopidogrel	12 (4%)
Oral anticoagulation	36 (21%)

Data are *n* (%) or medians (interquartile ranges), where appropriate. Data refer to the time of blood sampling for the platelet function study. Persons were classified as obese when their body mass index was higher than 30 kg/m^2.

Table 3 – *GP6* T13254C and the risk of a first myocardial infarction

Genotype	Cases		Controls		OR	(95%CI)
	n	(%)	n	(%)		
TT	366	(67%)	446	(70%)	1	[ref]
TC	163	(30%)	176	(27%)	1.13	(0.88 to 1.46)
CC	16	(3%)	21	(3%)	0.93	(0.48 to 1.81)
Additive		(18%)		(17%)	1.07	(0.87 to 1.32)

In the additive model, the increase in risk per copy of the C allele is calculated. For this model, only the (minor) allele frequency is presented, not the count.
OR: odds ratio; CI: confidence interval.

0.69 to 1.09) and 0.84 (95%CI 0.60 to 1.19), respectively. After pooling the results from SMILE and HERS, the HR for recurrent cardiovascular events was 0.81 (95%CI 0.66 to 0.99). The pooled HR for all-cause mortality was 0.73 (95%CI 0.55 to 0.96). Pooling of adjusted effect estimates from SMILE and HERS revealed similar HRs and 95%CIs.

To further explore the association with mortality we calculated the associations with cardiovascular, non-cardiovascular and cancer mortality (Table 4). Pooled per-allele HRs were 0.75 (95%CI 0.50 to 1.12) for cardiovascular mortality, 0.72 (95%CI 0.50 to 1.04) for non-cardiovascular mortality and 0.65 (95%CI 0.37 to 1.13) for mortality from cancer.

Interestingly, the relation between *GP6* and clinical endpoints seemed to be particularly pronounced in patients using aspirin (Table 5). Regarding recurrent events, the pooled HRs were 0.74 (95%CI 0.58 to 0.93) in aspirin users and 1.19 (95%CI

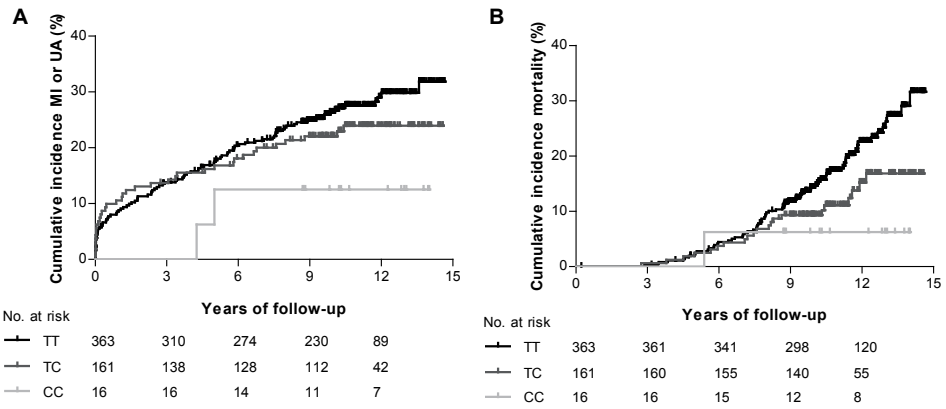

Figure 1: *GP6* T13254C and recurrent cardiovascular events and mortality. **A**: Fatal and non-fatal myocardial infarction (MI) and unstable angina (UA) requiring hospitalization. Hazard ratios relative to the TT genotype are 0.82 (95%CI 0.57 to 1.18) for the TC and 0.38 (95%CI 0.09 to 1.53) for the CC genotype. The HR for the effect per copy of the C-allele is 0.77 (95%CI 0.56 to 1.06). **B**: All-cause mortality. Hazard ratios relative to the TT genotype are 0.60 (95%CI 0.37 to 0.97) for the TC and 0.25 (95%CI 0.04 to 1.80) for the CC genotype. The HR for the effect per copy of the C-allele is 0.57 (95%CI 0.37 to 0.89).

Table 4 – *GP6* T13254C and the incidence of recurrent cardiovascular events and mortality

Endpoint	SMILE (*n*=542)		HERS (*n*=1074)		Pooled (*n*=1616)	
	HR	(95%CI)	HR	(95%CI)	HR	(95%CI)
MI or UA	0.77	(0.56 to 1.06)	0.84	(0.64 to 1.09)	0.81	(0.66 to 0.99)
Mortality						
All-cause	0.57	(0.37 to 0.89)	0.85	(0.60 to 1.20)	0.73	(0.55 to 0.96)
Cardiovascular	0.66	(0.34 to 1.27)	0.81	(0.50 to 1.33)	0.75	(0.50 to 1.12)
Non-cardiovascular	0.56	(0.32 to 0.98)	0.88	(0.53 to 1.44)	0.72	(0.50 to 1.04)
Cancer	0.65	(0.32 to 1.31)	0.64	(0.26 to 1.62)	0.65	(0.37 to 1.13)

HRs and 95%CIs are calculated with additive models and indicate the decrease in risk per copy of the C-allele.
MI: myocardial infarction; UA: unstable angina; HR: hazard ratio; CI: confidence interval.

Table 5 – Interaction between *GP6* T13254C and aspirin use

Endpoint	SMILE (*n*=542)			HERS (*n*=1074)			Pooled (*n*=1616)		
	HR	(95%CI)	*P*	HR	(95%CI)	*P*	HR	(95%CI)	*P*
MI or UA									
Aspirin	0.67	(0.44 to 1.03)	0.12	0.77	(0.58 to 1.02)	0.10	0.74	(0.58 to 0.93)	0.03
No aspirin	1.16	(0.69 to 1.96)		1.21	(0.78 to 1.87)		1.19	(0.85 to 1.66)	
Mortality									
Aspirin	0.50	(0.28 to 0.89)	0.24	0.82	(0.55 to 1.22)	0.67	0.69	(0.50 to 0.97)	0.30
No aspirin	0.89	(0.43 to 1.81)		1.05	(0.52 to 2.12)		0.96	(0.58 to 1.59)	

HRs and 95%CIs are calculated with additive models and indicate the decrease in risk per copy of the C-allele. The *P*-values are *P*-values for interaction between T13254C and aspirin use. In SMILE, 381 (70%) patients used aspirin, whereas 161 (30%) patients did not use aspirin. In HERS, there were 860 (80%) aspirin users and 214 (20%) non-users.
MI: myocardial infarction; UA: unstable angina; HR: hazard ratio; CI: confidence interval.

0.85 to 1.66) in non-users (*P* for interaction 0.03). The effects on mortality were 0.69 (95%CI 0.50 to 0.97) in aspirin users and 0.96 (95%CI 0.58 to 1.59) in non-users (*P* for interaction 0.30).

Platelet function

P-selectin expression by platelets increased dose-dependently upon stimulation by CRP-XL (Figure 2a). The percentage of P-selectin expression decreased dose-dependently per copy of the C-allele. Using various concentrations of CRP-XL, the overall reduction was 23% (95%CI 18 to 28) per copy of the C-allele. Aspirin neither influenced CRP-XL-induced P-selectin expression nor modified the effect of T13254C. The association between *GP6* and P-selectin expression was the same in patients who remained event-free after their first MI and those who experienced recurrent events.

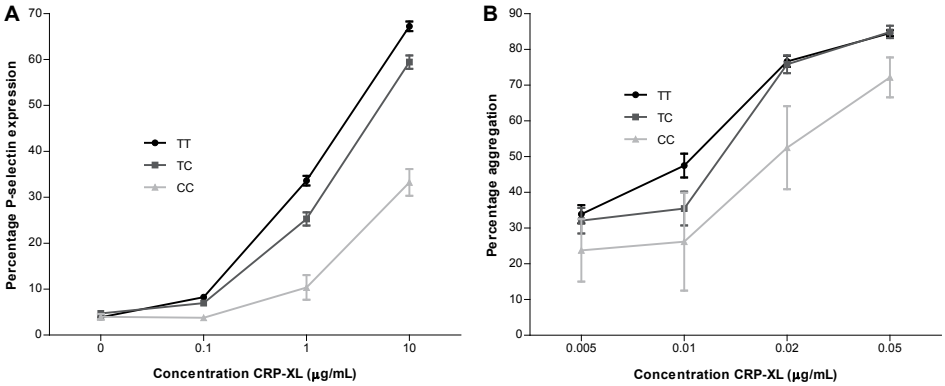

Figure 2: *GP6* T13254C and CRP-XL induced platelet activation and aggregation. Data are means ± standard errors of the mean. A: P-selectin expression in 274 myocardial infarction patients. Averaged over all concentrations of Crosslinked collagen-related peptide (CRP-XL), the mean reduction in platelet activation was 21% (95%CI 14 to 27%) for the TC and 56% (95%CI 40 to 71%) for the CC genotype relative to the TT genotype. The decrease per copy of the C-allele was 23% (95%CI 18 to 28%). B: Platelet aggregation in 219 healthy volunteers. Averaged over all concentrations of CRP-XL, the mean reduction in platelet aggregation was 6% (95%CI -4 to 16%) for the TC and 31% (95%CI 9 to 53%) for the CC genotype relative to the TT genotype. The decrease per copy of the C-allele was 10% (95%CI 2 to 18%). Note that the concentrations of CRP-XL in both figures are dissimilar because different tests of platelet reactivity as well as different preparations of CRP-XL were used in the two studies.

We also measured CRP-XL-induced platelet aggregation in 219 healthy volunteers. One hundred and thirty (59%) subjects had the wild-type TT genotype, 52 (24%) subjects carried on C-allele, and eight (4%) subjects were homozygous for the C-allele, resulting in a minor allele frequency of 16%. Genotype data were missing for the remaining 29 subjects. The C-allele was associated with diminished platelet aggregation (Figure 2b): Using various concentrations of CRP-XL, the overall reduction, adjusted for sex and race, was 10% (95%CI 2 to 18) per copy of the C-allele. After stratification for race, the association seemed to be most pronounced in Caucasians (per C-allele reduction 12%, 95%CI 4 to 20) and Asian-Indians (22%, 95%CI 10 to 34), whereas there was no association or even some suggestion of an inverse association in other races (African Americans -4%, 95%CI -12 to 5; Hispanics -7%, 95%CI -20 to 7; Asians -8%, 95%CI -30 to 13).

DISCUSSION

The present studies addressed the association between the T13254C polymorphism (rs1613662), which identifies two common haplotypes of *GP6*, and risk of a first MI, recurrent cardiovascular events and mortality, together with platelet function. Our main findings are that presence of the minor C-allele was not associated with first MI, but seemed to be associated with a reduced incidence of recurrent cardiovascular events

and mortality, both in a dose-dependent fashion. Pooling of the results of both SMILE and HERS resulted in more robust reduced risk estimates. The pooled HRs per copy of the C-allele for mortality were 0.81 (95%CI 0.66 to 0.99) for recurrent cardiovascular events, and 0.73 (95%CI 0.55 to 0.96) for all-cause mortality. Furthermore, the minor allele was clearly associated with decreased CRP-XL-induced P-selectin expression and, in an independent population, CRP-XL-induced platelet aggregation.

We found strong dose-dependent associations between *GP6* T13254C and CRP-XL-induced P-selectin expression and platelet aggregation in two independent studies. Our results regarding CRP-XL-induced platelet activation and aggregation are corroborated by previous smaller studies using these methods.[10,11] Furthermore, the large study by Jones *et al*. showed that this polymorphism explained almost 40% of variation in CRP-XL-induced P-selectin expression.[12] A recent meta-analysis of genome-wide association studies also confirmed that the response to collagen is strongly diminished in carriers of the minor allele.[8] As demonstrated by Trifiro *et al*., reduced platelet activation associated with the minor C-allele is most likely explained by impaired signal transduction.[13]

Notwithstanding the clear effect of *GP6* T13254C on platelet activation, several studies suggested no association with or even an increased risk of a first atherothrombotic event.[7,16-20] Our study also indicates that this polymorphism has no bearing on the risk of a first MI, although the 95%CI is compatible with ORs ranging from 0.86 to 1.32. Our results are in line with a large genome-wide association study that found no association between variation in *GP6* and the risk of a first MI.[32] In contrast to what was found for first MI, we found dose-dependently decreased risks of recurrent cardiovascular events and mortality in subjects carrying the C-allele. Our findings suggest that polymorphic variation in *GP6* may be of less importance with respect to first atherothrombotic events than for recurrent events. Development of atherosclerosis and underlying classical cardiovascular risk factors are the major determinants of first coronary artery events. Regarding recurrent events, factors influencing thrombus development may be more important when progressive atherosclerosis and stenosis is already present. Interestingly, two of the studies that found an association between T13254C and first MI suggested that the effect was particularly present in women.[7,20] As we could only study the association with first MI in men, we cannot exclude a potential effect in women. The observation that the association with recurrent events seemed to be particularly present in subjects using aspirin, both in SMILE and HERS, might potentially also explain the difference in findings between first and recurrent events. As thromboxane A_2 pathway inhibition is known to influence the platelet response to collagen, we can hypothesize that aspirin treatment and reduced platelet activation observed in carriers of the 13254C allele act synergistically to reduce the thrombosis risk. However, given the relatively small number of patients not using aspirin (n=161, 30% in SMILE; n=214, 20% in HERS), caution is warranted in the interpretation of this potential interaction. Future studies are needed to replicate our findings.

The association between T13254C and recurrent cardiovascular events and mortality has also been studied in HERS, which provided effect estimates in the

same direction as the results of SMILE, corroborating the validity of our study. SMILE and HERS are both prospective cohort studies including subjects with manifest cardiovascular disease. SMILE included 542 men who were followed for 12 years (median), whereas HERS included 1074 women who were followed for 7 years (median), extending the applicability of our results to women. Because the results of both studies were very consistent, we considered pooling to be justified. Pooling of the results of HERS and SMILE revealed more robust effect estimates, indicating a 20% reduction of cardiovascular events and a 30% reduction of all-cause mortality per copy of the C-allele.

Surprisingly, there was some suggestion that the association between the *GP6* polymorphism and all-cause mortality is not completely attributable to a decrease in cardiovascular mortality, but the small numbers of cause-specific deaths preclude any firm conclusions. Furthermore, because data about causes of death were obtained from routine registry data, misclassification could be present. However, because potential misclassification is assumed to be independent of the *GP6* genotype, this would only have led to underestimation of the real effects. Interestingly, results from both SMILE and HERS tended to suggest that the survival benefit associated with the C-allele might be partly attributable to a decrease of mortality resulting from cancer, although the confidence intervals were very broad. Given the aforementioned limitations, this finding should be interpreted as hypothesis-generating only. Nevertheless, this observation is consistent with accumulating evidence supporting a role for platelets and platelet activation in cancer progression.[33] *Ex vivo* platelet stimulation by collagen and other agonists has been demonstrated to result in release of proangiogenic factors, which might have important bearing on tumor progression and metastasis.[34,35] Moreover, it has recently been shown in a mouse model that GPVI facilitates metastasis.[36] Furthermore, high platelet count has been associated with mortality due to cancer.[37] Therefore, potential effects of *GP6* on cancer warrant replication and elaboration in future studies.

A potential limitation of our study is that we measured platelet function in a subset of MI patients many years after inclusion in SMILE, which is reflected by changed patient characteristics and a higher minor allele frequency. Nevertheless, we could still validly assess platelet function per genotype, since it is unlikely that the effect of T13254C on platelet function is modified by the changed characteristics. Moreover, there were no differences in the association between *GP6* and platelet function between patients who experienced one MI or also recurrent cardiovascular events, arguing against selection bias as an explanation of our findings. Furthermore, we found similar results in an independent study population of healthy volunteers, supporting our results. The major strength of our project is that we were able to assess effects of T13254C both on clinical outcomes and platelet activation, which renders our results biologically highly plausible. Future studies are warranted to address the potential clinical applicability of our findings.

In conclusion, the minor allele of *GP6* T13254C seems to be associated with a reduced risk of recurrent cardiovascular events and mortality, without affecting the incidence of first MI. The reduced risk of recurrent cardiovascular events and mortality

is consistent with decreased GPVI-mediated platelet activation in subjects with the minor C-allele, most likely through impaired signal transduction. Our results provide new data about the functional and clinical consequences of polymorphic variation in *GP6*, and demonstrate the relevance of the GPVI receptor in platelet function and cardiovascular disease.

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