Sandwich Immunoassay for Soluble Glycoprotein VI in Patients with Symptomatic Coronary Artery Disease

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BACKGROUND: Platelet glycoprotein VI (pGPVI) expression is increased in acute coronary syndrome (ACS), reflecting platelet activation. There is no reliable method available to measure pGPVI. Our aim was to develop a bead-based sandwich immunoassay to measure soluble GPVI (sGPVI).

METHODS: Based on antibodies for sGPVI developed earlier, we established and validated a bead-based sandwich immunoassay in 2438 consecutive patients with stable angina pectoris (SAP; n = 1371), non–ST-elevation myocardial infarction (NSTEMI; n = 724), and ST-elevation MI (STEMI; n = 343). In a subgroup (n = 1011), we measured surface expression of pGPVI using flow cytometry.

RESULTS: The assay revealed a working range of 8–500 ng/L. Intra- and interassay imprecision was <7% and <14%, respectively. Patients with NSTEMI and STEMI showed significantly lower mean sGPVI concentrations than patients with SAP [mean (SD), 8.4 (3.6) μg/L and 8.6 (4.1) μg/L vs 9.8 (4.8) μg/L; $P = 0.002$], whereas subgroup analysis revealed significantly enhanced pGPVI in NSTEMI (n = 276) and STEMI (n = 80) patients compared with SAP (n = 655) [mean fluorescence intensity (SD), 21.2 (8.1) and 19.8 (6.8) vs 18.5 (7.7); $P = 0.002$ and $P = 0.018$]. pGPVI and sGPVI were inversely correlated ($r = -0.076$; $P = 0.023$). Area under the ROC curve was 0.716, 95% CI 0.681–0.751, for sGPVI, distinguishing patients with SAP from those with ACS, and was superior ($P = 0.044$) to the curve of subgroup analysis for pGPVI (0.624, 95% CI 0.586–0.662). sGPVI ($P = 0.023$) and pGPVI ($P = 0.028$) had better association with the development of ACS than troponin I ($P = 0.055$) in the very early stage of disease, based on logistic regression analysis.

CONCLUSIONS: This sandwich immunoassay reliably measures sGPVI and may help to identify patients with ACS earlier than other laboratory markers.

Increased platelet activation plays a considerable role in the development of acute coronary syndrome (ACS).[6] Platelet collagen receptor glycoprotein VI (pGPVI) is the key regulator of platelet activation and aggregation at sites of vascular injury where collagen is exposed *(1, 2)*. In previous studies, we and others found that enhanced surface expression of pGPVI is associated with patients with ACS *(3, 4)*. Based on the results of our pilot study, we were able to confirm the previous findings in a prospective study that concentrations of pGPVI are significantly increased in ACS patients compared with patients with stable angina pectoris (SAP) *(5)*. Subsequent studies showed that pGPVI may also be helpful as a biomarker for the early identification of an imminent myocardial infarction in patients with ambiguous electrocardiogram (ECG) and in patients with chest pain *(6, 7)*. Further research analyzed differences in pGPVI expression in atrial fibrillation as well as in platelet count *(8, 9)*. Apart from its predictive value, pGPVI may be used for the prediction of clinical outcome *(10)*; moreover, we have shown that pGPVI may help to identify patients with transient ischemic attack or ischemic stroke *(11)*.

Despite promising results, the ROC curves for pGPVI have provided a rather low diagnostic sensitiv-

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[6] Nonstandard abbreviations: ACS, acute coronary syndrome; pGPVI, platelet collagen receptor glycoprotein VI; SAP, stable angina pectoris; ECG, electrocardiogram; sGPVI, soluble GPVI; CAD, coronary artery disease; NSTEMI, non–ST-elevation myocardial infarction; STEMI, ST-elevation MI; CRP, C-reactive protein; mAb, monoclonal antibody; MFI, mean fluorescence intensity; AUC, area under the curve; S/N, signal-to-noise ratio.

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ity and specificity, with limited assessment for clinical decision making processed by flow cytometry. Moreover, the availability of this technical method is limited to a few academic health centers that offer a suitable infrastructure with around-the-clock laboratory facilities. Therefore, the development and validation of an ELISA or sandwich immunoassay would be desirable to reliably detect platelet-specific plasmatic soluble GPVI (sGPVI). Thus, we and others began to determine the concentration of sGPVI in smaller groups of patients *(8, 9, 12, 13)*.

The aim of this project was to develop a sandwich immunoassay to reliably measure sGPVI and validate the novel assay in a large group of patients with symptomatic coronary artery disease (CAD).

Materials and Methods

STUDY POPULATION

We evaluated the concentration of sGPVI in 2438 consecutive patients with symptomatic CAD who underwent coronary angiography; 1371 patients presented with SAP, 724 with non–ST-elevation myocardial infarction (NSTEMI), and 343 with ST-elevation MI (STEMI). Categorization of ACS was according to the American Heart Association/American College of Cardiology guidelines *(14)*. In a subgroup of 1011 patients, we measured the surface expression of pGPVI by flow cytometry in 655 patients with SAP, 276 with NSTEMI, and 80 with STEMI. Exclusion criteria were age <18 years, inability to give or lack of informed consent, and noncoronary cause of chest pain. The study was approved by the local ethics committee of the University Hospital Tübingen and performed in accordance with the Declaration of Helsinki.

SAMPLE COLLECTION AND PLASMA PREPARATION

Blood samples were drawn from the antecubital vein and collected at the time of admission to the hospital. We measured troponin I immediately by use of the TnI-Ultra assay measured on an Advia-Centaur System (both from Siemens Healthcare Diagnostics) and C-reactive protein (CRP) by use of Siemens hsCRP Advia2120 (Siemens Medical, Solutions Diagnostics GmbH). Blood for the platelet biomarker assessment in 5-mL heparinized vials (for sGPVI determination) and 5-mL citrate phosphate dextrose adenine vials (for pGPVI determination) was processed and analyzed immediately by flow cytometry to determine the surface expression of platelet receptors (pGPVI and GPIb) as described *(4, 5)*. For analysis of sGPVI, heparin-anticoagulated blood was centrifuged at 3000*g* for 15 min at 4 °C. Two thirds of the supernatant were placed in aliquots, immediately frozen in liquid nitrogen, and stored at −78 °C until analysis. After thawing, samples were spun at 13 000*g* for 10 min at 4 °C. We used heparin-plasma samples for the measurement of sGPVI throughout assay development and study.

GENERATION OF MONOCLONAL ANTIBODY AGAINST HUMAN GPVI

Rat monoclonal antibodies (mAbs) against human GPVI were developed and generated as described *(15)*. Seven positive clones of antibodies have been identified against sGPVI (5C4, 5D4, 6C2, 7E11, 8A2, 8C3, and 8E9).

PREPARATION OF sGPVI-FREE PLASMA

We prepared a HiTrap NHS-activated HP 1-mL column (GE Healthcare) as specified by the manufacturer using 1 mg mAb (5C4) reactive with human sGPVI. We applied 800 μL plasma to the column and depleted sGPVI and used the sGPVI-depleted plasma as matrix for the determination of assay imprecision, assay recovery, and analyte stability.

BEAD-BASED SANDWICH IMMUNOASSAY FOR sGPVI

Plasma concentrations of sGPVI were determined by use of a bead-based sandwich immunoassay and mAb 8E9 reactive with human GPVI as capture antibody. The antibody was covalently bound to color-coded magnetic polystyrene microspheres (Luminex) as described *(16)*. Dilutions of plasma or standards were incubated together with 2000 antibody-coated microspheres in a 96-well PCR plate (ThermoFisher) in a magnetic particle handler (KingFisher96, ThermoFisher) for 1 h at 25 °C, mixing with medium frequency. BSA-coated microspheres were used as a negative control. After the capturing step, microspheres were transferred to another PCR plate containing a biotinylated GPVI-specific detection antibody (1 mg/L, mAb 8A2) using a magnetic particle handler (KingFisher96, ThermoFisher). Incubation with the detection antibody was carried out for 1 h at 25 °C. The final detection step was performed by transferring the microspheres into a third PCR plate containing a streptavidin-phycoerythrin conjugate solution (2.5 mg/L, Prozyme) for 45 min at 25 °C. We transferred the microtiter plate into a Luminex 100 instrument and acquired data according to the manufacturer's instructions. We obtained background data of the assay using microspheres incubated with 10% mouse plasma in Roche Blocking Buffer for ELISA (Roche) containing 0.1% Tween. Recombinant sGPVI was generated as described and used as reference *(15)*. GPVI standard was diluted in Roche Blocking Buffer for ELISA plus 10% mouse serum (Sigma). We calculated plasma concentrations of sGPVI according to a 4-parametric-logarithmic fit of the standard fluorescene. AQ: A

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For imprecision analysis, 3 different amounts recombinant sGPVI were spiked into 2% sGPVI-depleted human plasma (25, 6.25, and 1.55 μg/L) reflecting high, medium, and low plasma concentration. These 3 samples were diluted 50-fold to match the working range of the sandwich immunoassay. We measured intraassay imprecision by 5 determinations per concentration and interassay precision from 5 independent assays. Aliquots of the samples were prepared, frozen, and assayed in 5 replicates on 5 days.

FLOW CYTOMETRY

We measured the surface expression of pGPVI and GPIb by 2-color whole-blood flow cytometry, as described *(4, 5)*, using mean fluorescence intensity (MFI) as the index of receptor expression. Fluorescein-isothiocyanate–conjugated anti-GPVI mAb 4C9 was generated and characterized as described *(4, 5)*, and phycoerythrin-conjugated anti-CD42b (clone SZ2) mAbs were purchased from Immunotec (Beckman Coulter).

STATISTICAL ANALYSIS

A P value <0.05 was considered statistical significant after evaluation with appropriate nonparametric tests. Values are presented as mean (SD). Thus, for pairwise comparisons of SAP, NSTEMI, and STEMI, we applied a Kruskal–Wallis test. For correction of multiple testing of the pairwise comparisons, we performed a Bonferroni–Holm correction. Adjustment by possible confounders, such as medical treatment at the time of admission, classic cardiovascular risk factors, and laboratory markers, was performed by the multifactorial ANOVA for the decadic logarithm of sGPVI. We assessed predictive values of sGPVI and pGPVI for the development of ACS by applying binary logistic regression analysis. Using ROC curves, we determined the optimum cutoff value of sGPVI and pGPVI for ACS. To assess a statistical difference between the areas under the curves (AUCs) of sGPVI and pGPVI in ROC analysis, we applied a DeLong test using Analyse-it for Microsoft Excel (version 2.20) http://www.analyse-it.com/. All other statistical analyses were performed using PASW Statistics software for Windows version 18.0, 2009 (IBM SPSS).

Results

Our study population comprised 2438 consecutive patients with symptomatic CAD who underwent coronary angiography. Thus, patients showed either SAP (n = 1371) or ACS (n = 1067); of the ACS patients, 724 presented with NSTEMI and 343 with STEMI. Details of the demographic data are presented in Table 1. T1

Table 1. Patient characteristics and medical treatment at hospital admission.[a]

	All	ACS	SAP
n	2438	1067	1371
Mean age, years (SD)	67.4 (11.2)	67.6 (11)	67.3 (11.4)
Sex			
Female	687 (28.2)	277 (26)	410 (29.9)
Male	1751 (71.8)	790 (74)	961 (70.1)
Cardiovascular risk factors			
Arterial hypertension	1921 (78.8)	822 (77)	1099 (80.2)
Hyperlipidemia	1705 (69.9)	770 (72.2)	935 (68.2)
Diabetes	781 (32)	325 (30.5)	456 (33.3)
Family history of CAD	469 (19.2)	196 (18.4)	273 (19.9)
Smoking	998 (40.9)	448 (42)	550 (40.1)
Coronary artery disease			
One vessel	783 (32.1)	366 (34.3)	417 (30.4)
Two vessels	802 (32.9)	303 (28.4)	499 (36.4)
Three vessels	853 (35)	398 (37.3)	455 (33.2)
Left ventricular ejection fraction			
Normal	1287 (52.8)	565 (53)	722 (52.7)
Slightly reduced	552 (22.7)	239 (22.4)	313 (22.8)
Moderate	323 (13.2)	132 (12.4)	191 (13.9)
Low	276 (11.3)	131 (12.2)	145 (10.6)
Medication			
ACE inhibitors	1372 (56.3)	654 (61.3)	718 (52.4)
Angiotensin receptor blockers	284 (11.7)	114 (10.7)	170 (12.4)
Beta blockers	1609 (66)	687 (64.4)	922 (67.3)
Statins	1299 (53.3)	596 (55.9)	703 (51.3)
Aspirin	1270 (52.1)	545 (51.1)	725 (52.9)
Clopidogrel	215 (8.8)	62 (5.8)	153 (11.2)
Vitamin K antagonist	236 (9.7)	97 (9.1)	139 (10.1)

[a] Data are n (%) unless noted otherwise.

To develop a sandwich immunoassay for determination of sGPVI in plasma, we screened 7 mAbs against each other for their capability to serve as capture or detector molecules. The assay was developed on a magnetic suspension-bead array platform (Luminex, L100) *(16)*. The best antibody pair was selected according to the best signal-to-noise ratio (S/N) by dividing the fluorescence signal gained at 125 ng/L sGPVI standard and no standard (Fig. 1A). F1 Additionally, we tested the antibodies for cross-reactivity to mouse sGPVI to identify a suitable dilution matrix for recombinant sGPVI (data not

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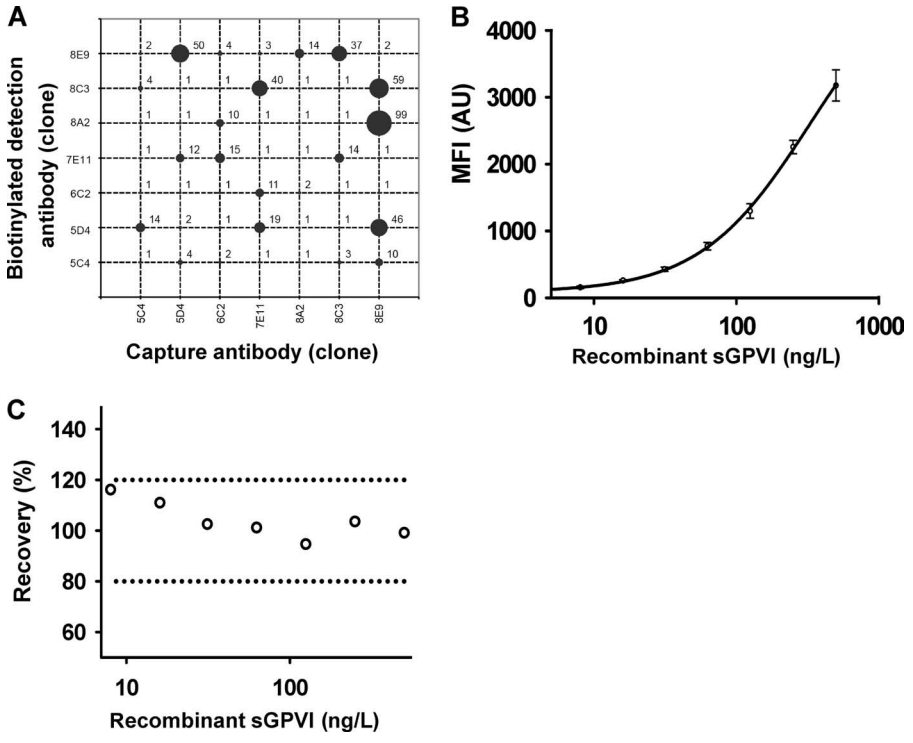

Fig. 1. sGPVI-specific bead-based sandwich immunoassay.

(A), Signal-to-noise ratios of the antibody pairs. Size of the bubbles is determined by the S/N values. (B), Typical calibration curve showing recombinant sGPVI standard in a range from 8 to 500 ng/L. AU, arbitrary units. (C), Analyte recovery of sGPVI.

shown). Results revealed no cross-reactivity of the antibodies for mouse sGPVI. Therefore, commercially available mouse serum was used as matrix for preparing standard dilutions. A typical calibration curve in 10% mouse serum is shown in Fig. 1B. Recovery of recombinant sGPVI was between 80% and 120% across the range of the dose–response curve from 8 to 500 ng/L (Fig. 1C). Intraassay CVs did not exceed 7%, and interassay CVs did not exceed 14% (Table 2). Values for assay recovery are identical with intraassay imprecision because no certified standard is available.

The same samples were used for studying analyte stability. For this purpose, we subjected aliquots to 0, 1, 2, 3, or 4 freeze–thaw cycles. For each cycle, samples were frozen in liquid nitrogen and thawed at 37 °C. Results revealed no effects of the freeze–thaw procedure (Table 3). Variation of the results were close to the determined intraassay imprecision (Tables 2 and 3).

Patients with NSTEMI and STEMI had significantly lower sGPVI concentrations than patients with SAP [8.4 (3.6) μg/L and 8.6 (4.1) μg/L vs 9.8 (4.8) μg/L; $P = 0.002$] (Fig. 2A), whereas the subgroup analysis showed a significantly increased surface expression of pGPVI in NSTEMI (n = 276) and STEMI (n = 80) compared with SAP (n = 655) [MFI 21.2 (8.1) and 19.8 (6.8) vs 18.5 (7.7); $P = 0.002$ and $P = 0.018$] (Fig. 2B). Comparing the itemized ACS groups of NSTEMI and STEMI, neither sGPVI ($P = 0.424$) nor pGPVI ($P = 0.652$) showed any significant difference between these groups.

Table 2. Assay imprecision.

	Intraassay variability (n = 5)			Interassay variability (n = 5)		
Expected value	sGPVI, ng/L	SD, ng/L	CV, %	sGPVI, ng/L	SD, ng/L	CV, %
500 ng/L	438	4	1	462	51	11
125 ng/L	108	3	3	118	16	14
31 ng/L	34	2	7	34	5	14

Intra- and interassay precision were determined by assaying 3 samples containing high, medium, and low amounts of recombinant sGPVI spiked into 2% GPVI-depleted human plasma, frozen, and stored at −20 °C. Samples were measured independently 5 times in 5 replicates.

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Table 3. Analyte stability after 4 freeze–thaw cycles.

Expected value	sGPVI, ng/L	SD, ng/L	CV, %
500 ng/L	516	43	8
125 ng/L	118	7	6
31 ng/L	33	2	6

Analyte stability was determined from assaying 3 samples containing high, medium, and low amounts of recombinant sGPVI after 0, 1, 2, 3, or 4 freeze–thaw cycles.

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Table 4. Associations of sGPVI and pGPVI with development of ACS.

	P	Odds ratio	95% CI
sGPVI, μg/L	0.023	0.959	0.925–0.994
pGPVI, MFI	0.028	1.083	1.008–1.163
Troponin I, μg/L	0.055	1.075	0.998–1.158
Creatine kinase, U/L	0.377	1.001	0.999–1.002
CRP, ×10 mg/L	0.586	1.063	0.853–1.324

We found a poor, inverse correlation of pGPVI and sGPVI ($r = -0.076$; $P = 0.023$) (Fig. 2C). The AUC in ROC analysis was 0.716 and 95% CI 0.681–0.751 for sGPVI, distinguishing patients with SAP from those with ACS and was superior (DeLong test: $P = 0.044$) to the curve of the subgroup analysis for pGPVI (AUC 0.624; 95%CI 0.586–0.662) (Fig. 2D). Applying ROC curves, we determined cutoff values for the identification of ACS, which were 8.75 μg/L for sGPVI and 18.6 MFI for pGPVI, respectively. The cutoff value of 8.75 μg/L yielded a diagnostic sensitivity of 72.6% and a diagnostic specificity of 61.4%.

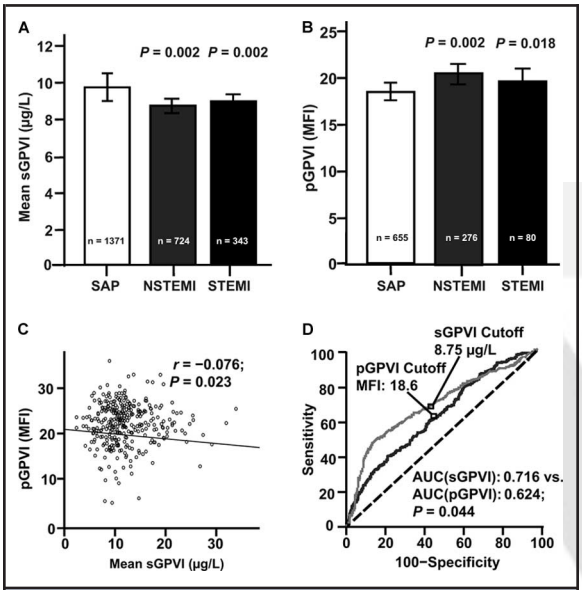

Fig. 2. Concentrations of sGPVI and pGPVI in patients with symptomatic CAD.

(A), Patients with NSTEMI and STEMI had significantly lower sGPVI concentrations than patients with SAP. (B), Subgroup analysis revealed a significantly increased surface expression of pGPVI in NSTEMI and STEMI compared with SAP. (C), Poor, inverse correlation of pGPVI and sGPVI ($r = -0.076$; $P = 0.023$). (D), Area under the ROC curve analysis for sGPVI distinguishing patients with SAP from those with ACS, also superior ($P = 0.044$) to the curve of the subgroup analysis for platelet GPVI.

To test whether sGPVI is influenced by confounders, comparison of the decadic logarithm of GPVI between ACS and SAP was adjusted by possible confounders such as age, sex, cardiovascular risk factors, and medical treatment. Multifactorial analysis of covariance revealed an independent sGPVI concentration between ACS and SAP ($P = 0.040$).

Compared with troponin I ($P = 0.055$), sGPVI ($P = 0.023$) was more negatively and pGPVI ($P = 0.028$) more positively associated with the development of ACS in the very early stage of disease at hospital admission, according to binary logistic regression analysis (Table 4). T4

Discussion

The major findings of this study are that (*a*) we were able to develop a novel bead-based sandwich immunoassay to reliably measure sGPVI and validate the assay in a large group of patients with a symptomatic CAD; (*b*) area under the ROC curve analysis of sGPVI helped distinguish patients with ACS from those with SAP better than that of pGPVI; (*c*) surface expression of pGPVI and plasma concentration of sGPVI showed a poor inverse correlation; and (*d*) sGPVI was more negatively and pGPVI more positively associated with the development of ACS than conventional laboratory markers in the very early stage of the disease.

Although the mechanisms of plaque-mediated arterial thrombosis and their diagnostic and therapeutic implications are understood, some issues need further clarification. Current diagnostic concepts of established biomarkers still focus on a rather late stage of the disease, demonstrating the effects of inflammation, myocardial stretch, and necrosis *(17, 18)*. Thus, preliminary platelet activation is emerging as a promising target. Concentrations of

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P-selectin (CD62P) may reflect increased platelet activation and have been shown to have a predictive and prognostic value in acute ischemic events. Unlike pGPVI, this marker was also found in endothelial cells and, as such, suffers from being nonspecific for platelets *(1, 19, 20)*. Therefore, we attempted to validate pGPVI as a potential diagnostic tool for the identification of acute ischemic events such as ACS and ischemic stroke *(4, 5, 11)*. The diagnostic sensitivity and specificity of flow cytometry, however, were rather too low to allow adequate clinical decision-making, necessitating a different technical method by developing a sandwich immunoassay for the determination of plasmatic sGPVI *(5)*.

Based on promising test series and examination of smaller groups of healthy donors, as well as patients with cerebro- or cardiovascular diseases, by us and other groups *(8, 9, 12, 13)*, we created an assay that proved stable and reproducible (Tables 2 and 3; Fig. 1).

In ROC analysis, the sGPVI assay showed an improved AUC compared with the flow-cytometric results of pGPVI ($P = 0.044$). Moreover, sGPVI ($P = 0.023$) was more negatively and pGPVI ($P = 0.028$) more positively associated with the development of ACS than other conventional laboratory markers such as troponin I ($P = 0.055$) in the very early stage of disease at hospital admission, according to binary logistic regression analysis.

Intriguingly, we found a poor, inverse correlation of an increased surface expression of pGPVI and lower plasma concentrations of sGPVI in patients with ACS. It is well known that pGPVI shows constitutive expression *(1)*. On the one hand, activated platelets show enhanced surface expression of collagen receptor pGPVI in ACS as determined by flow cytometry, in accordance with previous findings *(4, 5)*. On the other hand, platelet activation induces matrix metalloproteinase–dependent pGPVI cleavage, which is followed by an ectodomain shedding of sGPVI *(21, 22)*. This shedding is regulated by at least 3 different types of platelet-expressed proteinases/sheddases *(23)*. Because pGPVI is the key to both types of responses, platelet aggregation and phosphatidylserine expression, loss of pGPVI-mediated phosphatidylserine exposure may result in reduced prothrombinase activity *(24)*. Thus, pGPVI cleavage may protect from thrombosis, and released sGPVI may act as an inhibitor of atherothrombosis *(22)*. Several studies have shown that sGPVI may inhibit platelet adhesion and aggregation to the injured vessel wall in the murine model, and with that may substantially attenuate atheroprogression and endothelial dysfunction *(15, 25, 26)*. These antithrombotic effects of sGPVI described earlier referred to experiments with the dimeric, recombinant form of sGPVI instead of the metalloproteinase-induced shedding of sGPVI, however, a difference that should be taken into consideration. Therefore, it could be speculated that plasma sGPVI binds to sites of vascular injury where collagen is exposed and with that consumed or removed from circulation in patients with ACS, whereas patients with SAP do not present with any substantial plaque rupture.

The pathophysiological mechanism of recombinant sGPVI has been targeted for vascular lesion–directed antiplatelet treatment without systemic effects on circulating platelets in humans. In contrast to the previous idea to apply anti-GPVI antibodies, which has been critically compromised by a prolonged bleeding time (in particular, with a concomitant administration of aspirin *(27)*), administration of sGPVI may offer an alternative, safer therapeutic pathway. To date, a cooperating group has performed studies on this therapeutic principle and accomplished a phase I study that examined safety and pharmacokinetic and -dynamic profiles of an sGPVI called Revacept® in healthy volunteers in a single-center, open-label, dose-escalating study with 5 doses *(28)*. Furthermore, a specially designed bifunctional protein of sGPVI may catch progenitor cells to induce reendothelialization of vascular lesions *(29)*. AQ: B

Although we performed an adjustment for possible confounders, a limitation of this study is that patients had not been examined for genetic polymorphisms and GPVI-related clinical defects *(30, 31)*. However, as Arthur et al. *(31)* have critically observed, although the study by Ollikainen et al. *(30)* found that C-allele carriers were associated with coronary thrombosis, there has been no differentiation between homozygotes and heterozygotes, and only 1.4% presented a homozygote genotype *(30, 31)*. Therefore, the relevance and frequency of innate issues of GPVI remain low.

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