Platelet reactivity and cardiovascular events

Snoep, J.D.

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PLATELET REACTIVITY AND RECURRENT CARDIOVASCULAR EVENTS IN PATIENTS WITH STABLE CARDIOVASCULAR DISEASE USING ASPIRIN: A HEAD-TO-HEAD COMPARISON OF DIFFERENT TESTS

J.D. Snoep, J.C.J. Eikenboom, J.J. Zwaginga, M. Roest,
C. Patrono, B. Rocca, G. Petrucci, F.R. Rosendaal, J.G. van der Bom

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ABSTRACT

Background

Platelet reactivity among subjects using aspirin can be quantified using multiple methods, either specific or non-specific to the effect of aspirin on cyclooxygenase (COX)-1. We assessed the association between high on-aspirin platelet reactivity according to a variety of COX-1-dependent and COX-1-independent tests and recurrence of major adverse cardiac events (MACE).

Methods

On-aspirin platelet reactivity was assessed in 179 male subjects with stable cardiovascular disease by serum thromboxane B_2 (S-TxB_2), urinary 11-dehydro-thromboxane B_2 (U-TxB_2), arachidonic acid-induced light transmission aggregometry (LTA-AA), the VerifyNow Aspirin assay, PFA-100 collagen-epinephrine (Col/Epi) and collagen-adenosine-diphosphate (Col/ADP) cartridges and ADP- and cross-linked collagen-related peptide (CRP-XL)-induced P-selectin expression by platelets.

Results

Ninety-one subjects had a MACE during a median follow-up of 15 years while 88 remained event-free after their first myocardial infarction. When we compared the highest quintile of platelet reactivity with lower platelet reactivity, the odds ratios with 95% confidence intervals for MACE were 0.36, 0.16 to 0.79 (S-TxB_2); 1.19, 0.57 to 2.49 (U-TxB_2); 0.45, 0.21 to 0.97 (LTA-AA); 0.36, 0.16 to 0.78 (VerifyNow); 1.47, 0.69 to 3.13 (PFA-100 Col/Epi); 1.52, 0.70 to 3.31 (PFA-100 Col/ADP); 2.51, 1.14 to 5.51 (P-selectin, ADP); and 1.20, 0.57 to 2.53 (P-selectin, CRP-XL). Depending on the cut-off point, PFA-100 Col/ADP (2.05, 1.12 to 3.74) and CRP-XL-induced P-selectin expression (2.08, 1.12 to 3.85) were also associated with MACE.

Conclusion

In our data, several COX-1-dependent methods were inversely associated with MACE while COX-1-independent methods, particularly ADP-induced P-selectin expression, were positively associated with MACE in male subjects on long-term aspirin. We put forward several potential explanations for this disparity. Future studies are needed to further evaluate clinical consequences of high on-aspirin COX-1-dependent and COX-1-independent platelet reactivity.

INTRODUCTION

Platelets play an essential role in the development of cardiovascular disease.[1,2] Therefore, inhibition of platelet function is an important therapeutic goal in cardiovascular prevention. Low-dose aspirin irreversibly inhibits thromboxane (Tx) A_2 production by platelets by permanent blockade of platelet cyclooxygenase (COX)-1, and is a cornerstone in the prevention of cardiovascular events in subjects with manifest cardiovascular disease.[3-6] During the last decades, the concept of interindividual variability of platelet function in subjects using aspirin has gained a lot of attention in literature.[6-9] The definition of high platelet reactivity and the most suitable test to detect high platelet reactivity have been widely and still inconclusively debated.[10-12] Inability of aspirin to reach its pharmacological target, i.e., COX-1, which requires specific tests to detect, has been distinguished from high residual on-treatment platelet reactivity, in which more global tests of platelet function could also be used.[10-12]

In line with previous findings,[13-16] we showed that there is indeed wide variability in high on-aspirin platelet reactivity within individuals according to several COX-1-dependent and COX-1-independent assays.[17] In this respect, it is not known which test is most useful from a clinical point of view, i.e., which test best identifies patients at risk of recurrent cardiovascular events. Several meta-analyses recapitulated an increased risk of recurrent cardiovascular events associated with high on-aspirin platelet reactivity, but could not differentiate between the many different definitions of high platelet reactivity that are being used.[18-20] At present, direct evidence from head-to-head comparisons regarding the relation between high on-aspirin platelet reactivity according to the various tests and clinical outcomes is still surprisingly scarce.[21,22] Therefore, we set out to assess the association of high on-aspirin platelet reactivity according to a variety of COX-1-dependent and COX-1-independent tests with recurrence of major adverse cardiac events (MACE) in male subjects with stable cardiovascular disease using aspirin.

METHODS

Study design and subjects

We evaluated the association between platelet function tests and MACE within the SMILE-Platelets project. Briefly, 560 male subjects with a first myocardial infarction between 1990 and 1996 were included as cases in the Study of Myocardial Infarctions in Leiden (SMILE)[23] and 542 of them were followed for MACE in a cohort study.[24] In 2008/2009, all living patients were recruited again to provide a blood and urine sample to test platelet reactivity.[25] Of the 542 subjects, 171 subjects died, 12 subjects were lost to follow-up, and 85 subjects did not want to participate. Therefore, 274 subjects were included in the platelet function study. Furthermore, we excluded, 12 subjects using clopidogrel, 4 subjects using dipyridamole, 4 subjects using non-steroidal anti-inflammatory drugs other than aspirin, and 2 subjects without suitable blood samples, leaving 252 subjects for the present analyses.

The study was approved by the Medical Ethics Committees of the Leiden University Medical Center, and performed in accordance with the Declaration of Helsinki. All subjects gave written informed consent before participation.

Clinical endpoints

We compared subjects with MACE during follow-up with those who remained event-free after their first myocardial infarction regarding platelet function. MACE was defined as recurrent non-fatal myocardial infarction, unstable angina requiring hospitalization or revascularization. Details about endpoint classification have been published before.[24] All subjects completed a detailed questionnaire about recurrent cardiovascular events, cardiovascular risk factors and medication use, which was extensively verified using hospital files and information requested from primary care physicians of the patients. In case of MACE, special efforts were focused on medication use, particularly antiplatelet therapy, at the moment of the event in relation to the moment of blood sampling for platelet function testing.

Laboratory measurements

Subjects provided morning fasting blood and urine samples for platelet reactivity testing and assessment of chemical and hematological parameters. Blood was collected in sitting position after half an hour of rest using the Sarstedt S-Monovette® blood collection system (Sarstedt AG & Co., Nümbrecht, Germany). The first 10 mL were not used for platelet function measurements. Included tests of platelet reactivity were serum thromboxane B_2 (S-TxB_2),[15] urinary 11-dehydro-thromboxane B_2 (U-TxB_2),[26,27] arachidonic acid-induced light transmission aggregometry (LTA-AA),[28] the VerifyNow® Aspirin assay (Accumetrics, San Diego, CA, USA), the Platelet function analyzer (PFA-100®) (Siemens Healthcare Diagnostics, Deerfield, IL, USA) collagen-epinephrine (Col/Epi) and collagen-adenosine diphosphate (Col/ADP) cartridge and ADP and cross-linked collagen-related peptide (CRP-XL)-induced P-selectin expression measured by whole blood flow cytometry.[25] S-TxB_2, U-TxB_2, LTA-AA, VerifyNow Aspirin are considered COX-1-dependent, whereas the PFA-100 cartridges and ADP- and CRP-XL-induced P-selectin expression are COX-1-independent. Details about the different tests of platelet reactivity that were performed are described elsewhere.[17]

LDL-cholesterol, HDL-cholesterol, triglycerides, glucose and creatinine were measured in serum and hematological cell counts were measured in EDTA-anticoagulated plasma according to routine procedures.

Statistical analyses

To evaluate the association between platelet reactivity and MACE, we first calculated the median difference with 95% confidence intervals (CI) in platelet reactivity according to the different tests between patients with and without a MACE during the follow-up using the CENDIF procedure in Stata 10.1, which estimates robust CIs for median differences. As the upper limit of the PFA-100 is arbitrarily set at 300s by the manufacturer, PFA-100 data mimic survival data with censoring of subjects with a

closure time of 300 s. Therefore, the median difference with 95%CI for the PFA-100 Col/Epi cartridge (using the Col/ADP cartridge, the closure time was never 300s) was estimated using the CENSLOPE procedure, which allows censored data.

Secondly, we divided platelet reactivity into quintiles for each test. We calculated odds ratios (OR) with 95% confidence intervals (95%CI) for the relation with MACE for each quintile relative to the lowest quintile with logistic regression analysis. As smaller PFA-100 closure times indicate higher platelet reactivity, the upper quintile was used as reference category. We also estimated the OR for trend, which indicates the increase in odds per one quintile increase in platelet reactivity.

Thirdly, we calculated ORs for the relation between high platelet reactivity and MACE. High platelet reactivity was defined as the highest quintile of platelet reactivity, lower values being classified as normal platelet reactivity. Furthermore, we applied existing cut-off points from literature to our data. Used cut-off points were 3.1 ng/mL for S-TxB_2,[21] 1500 pg/mg creatinine for U-TxB2 (supplied by manufacturer), 20% for LTA-AA,[28,29] 550 ARU for VerifyNow (supplied by publisher), 193 s for PFA-100 Col/Epi,[21,29] and 65 s for PFA-100 Col/ADP.[21] As no cut-off points exist for ADP- and CRP-XL-induced P-selectin expression in the literature, we used receiver-operator-characteristic (ROC) analysis to evaluate which cut-off point discriminated best between subjects with and without MACE in our data. The cut-off point with the maximal sum of sensitivity and specificity was used. All ORs for the relation between high platelet reactivity and MACE were additionally adjusted for potential confounders, i.e. factors associated with both platelet reactivity[17] and MACE (Table 1) and not in the causal pathway, which were smoking (current smokers vs. others), obesity (defined as a BMI of 30 kg/m^2 or higher vs. <30 kg/m^2), diabetes (yes vs. no), aspirin dose (<80 mg or 80-100 mg) and use of cholesterol-lowering drugs. We also tried to include more potential confounders in our models (age (≥70y vs. <70y), use of blood pressure lowering drugs (yes vs. no), serum glucose (≥7.0 mmol/L vs. <7.0 mmol/L), LDL-cholesterol (≥2.5 mmol/L vs. <2.5mmol/L), HDL-cholesterol (≥0.9 mmol/L vs. <0.9 mmol/L), triglycerides (≥1.7 mmol/L vs. <1.7 mmol/L) and creatinine, platelet count, mean platelet volume, white blood cell count and hemoglobin (all per quartile)), but this did not influence the results.

We performed several sensitivity analyses to test the robustness of our data. First, as platelet reactivity was measured after occurrence of the event, high platelet reactivity might be a consequence rather than a cause of MACE (reverse causation). In the presence of reverse causation, one would expect that the association is larger when the event occurred shortly before platelet function testing than when there is a large time interval between the event and platelet function testing. Therefore, we analyzed the relation between platelet reactivity and MACE in three time intervals: platelet function testing within 5 years, 5 to 10 years and 10 years or longer after occurrence of MACE. Furthermore, we found an inverse relationship between high platelet reactivity according to several COX-1-dependent assays and MACE. Therefore, we estimated how many additional subjects who died before platelet function testing or did not want to participate, would have had MACE and high platelet reactivity in order to

explain the inverse relationship when, in reality, there would be a positive association, i.e. whether our findings could be explained by survival bias.

All analyses were performed using Stata 10.1 (Stata Corp LP, College Station, TX, USA).

RESULTS

Subject characteristics

We included 252 male subjects in our study of whom 142 (56%) experienced a MACE during a mean follow-up of 16 years (range 13 to 19 years). 189 participants (75%) used low-dose aspirin at time of platelet function testing, while 63 subjects did not use aspirin. Most of the latter patients used vitamin-K antagonists (n=49). One subject used both aspirin and a vitamin-K antagonist. All analyses are restricted to the subjects using aspirin as single antithrombotic drug, of whom 100 subjects had a MACE while 88 subjects remained event-free after their first myocardial infarction. Nine aspirin-using subjects with MACE did not use aspirin at time of the MACE and were also excluded from further analyses, leaving 91 subjects with and 88 subjects without MACE (in total 179 subjects).

The characteristics of the 179 subjects at time of platelet function testing are presented in Table 1. Cardiovascular risk factors were generally well-controlled in

Table 1 – Subject characteristics

	MACE (*n* = 91)	No MACE (*n* = 88)
Age, years	68 (61, 76)	68 (62, 75)
Smoking	19 (21%)	21 (24%)
Obesity	24 (26%)	25 (28%)
Diabetes	18 (20%)	11 (13%)
Lipid-lowering drugs	86 (95%)	73 (83%)
Blood pressure lowering drugs	78 (86%)	71 (81%)
Systolic blood pressure, mmHg	147 (131, 158)	145 (132, 158)
Diastolic blood pressure, mmHg	85 (78, 94)	85 (78, 94)
LDL-cholesterol, mmol/L	2.2 (1.9, 2.5)	2.6 (2.1, 3.1)
HDL-cholesterol, mmol/L	1.3 (1.1, 1.6)	1.3 (1.1, 1.5)
Triglycerides, mmol/L	1.3 (1.0, 1.8)	1.3 (0.9, 1.9)
Glucose, mmol/L	5.8 (5.4, 6.7)	5.9 (5.4, 6.6)
Creatinine µmol/L	85 (76, 97)	87 (77, 100)

Characteristics at time of platelet function testing of subjects with and without MACE. Data are counts with frequencies or medians with interquartile ranges
LDL, low-density lipoprotein; HDL, high-density lipoprotein.

subjects both with and without MACE during the follow-up, but subjects with MACE used preventive drugs slightly more frequently (Table 1). Most subjects used 80 or 100 mg aspirin. Two subjects with MACE and seven without MACE used 30 or 40 mg aspirin. The median duration of aspirin use was 15 years (interquartile range 14 to 16 years) and was similar in subjects with and without MACE.

Platelet reactivity and MACE

Figure 1 displays the distribution of platelet reactivity according to the different tests for subjects with and without MACE. Subjects with MACE seemed to have lower S-TxB_2 (median difference -1.7 ng/mL, 95%CI -2.8 to -0.5) and VerifyNow (-15 ARU, 95%CI -29 to 0) levels but a higher platelet reactivity according to PFA-100 Col/ADP (-4s, 95%CI -8 to 1), and ADP- (11, 95%CI 4 to 18) and CRP-XL-induced P-selectin expression (7, 95%CI -1 to 15). Median platelet reactivity as measured by U-TxB_2 (median difference 29 pg/mg creatinine, 95%CI -65 to 126), LTA-AA (0%, 95%CI -1 to 1), and PFA-100 Col/Epi (-6s, 95%CI -23 to 12) were similar for subjects with and without MACE. Notably, although median LTA-AA levels were similar for subjects with and without MACE, six of the seven subjects with very high aggregation despite use of aspirin did not have a MACE during follow-up (Figure 1).

In Figure 2, platelet reactivity is divided into quintiles. For each quintile, ORs with 95%CIs for MACE are presented relative to the lowest (PFA-100 highest) quintile of platelet reactivity. The OR for trend, indicating the increase in odds of MACE per 1 quintile increase in platelet function relative to the lowest quintile, is also presented in Figure 2. Both S-TxB_2 and ADP-induced P-selectin expression were dose-dependently related with MACE, although in the opposite direction (S-TxB_2: OR 0.69, 95%CI 0.55 to 0.86; ADP-induced P-selectin expression: OR 1.39, 95%CI 1.12 to 1.73). For LTA-AA and VerifyNow, particularly the highest quintiles showed an inverse association with MACE. For CRP-XL-induced platelet activation, the odds of MACE were most increased in the fourth quintile versus the lowest quintile.

The first columns of Table 2 present ORs for high platelet reactivity versus normal platelet reactivity, i.e., the highest (PFA-100 lowest) quintile of platelet reactivity versus all other quintiles, both crude and adjusted for potential confounders. By definition, prevalence of high platelet reactivity was 20% for all tests. High platelet reactivity according to S-TxB_2 (OR 0.36, 95%CI 0.16 to 0.79), LTA-AA (OR 0.45, 95%CI 0.21 to 0.97) and VerifyNow (OR 0.36, 95%CI 0.16 to 0.78) seemed to be inversely associated with MACE, whereas high platelet reactivity according to ADP-induced P-selectin expression (OR 2.51, 95%CI 1.14 to 5.51) was positively associated with MACE. Adjustment for confounders did not considerably influence the results, although confidence intervals became wider (Table 2).

We also evaluated the relation between high platelet reactivity and MACE using cut-off points that are used in literature or follow from ROC-analysis in absence of cut-off points in literature (Table 2). Using these cut-off points, prevalences of high platelet reactivity were 65% (S-TxB_2), 12% (U-TxB_2), 4% (LTA-AA), 6% (VerifyNow), 69% (PFA-100 Col/Epi), 13% (PFA-100 Col/ADP), 28% (ADP-induced P-selectin expression) and 40% (CRP-XL-induced P-selectin expression). Results using these cut-off points

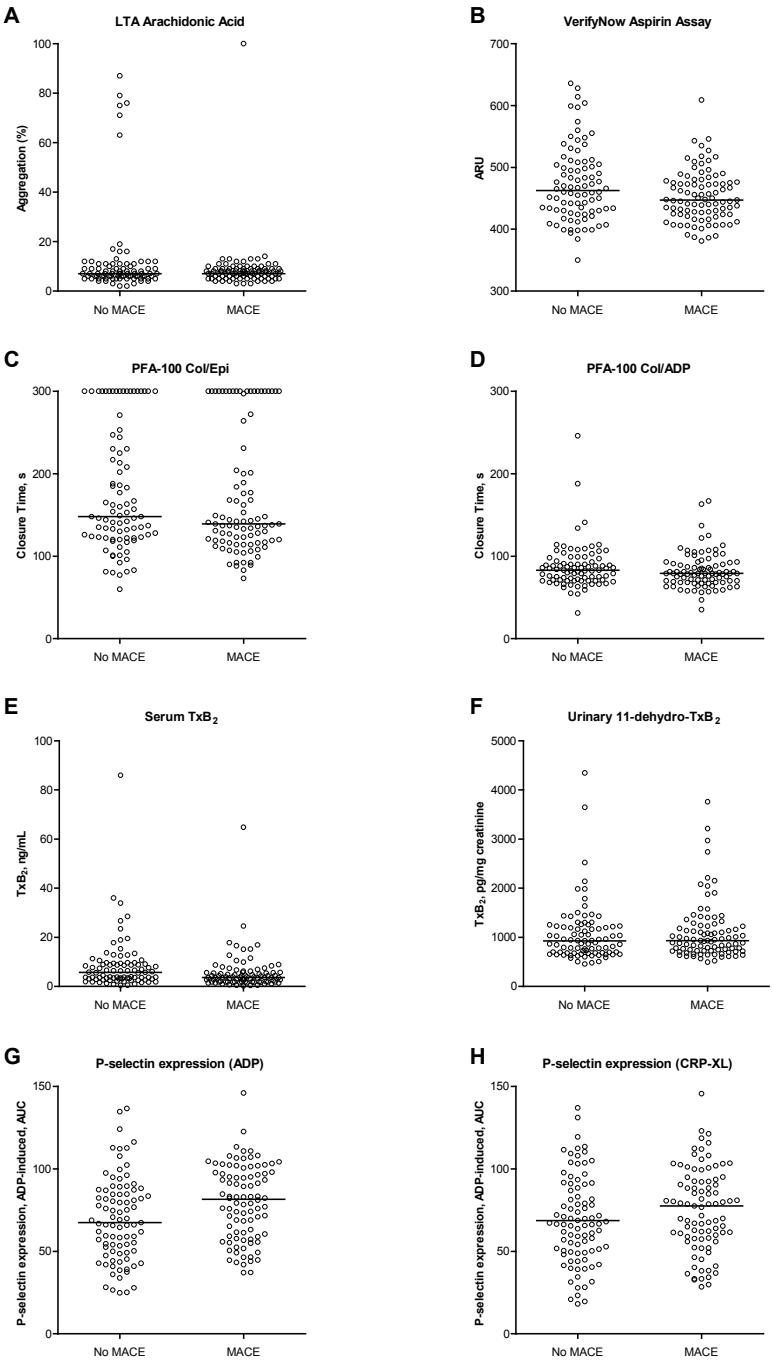

Figure 1 – Association between platelet reactivity and MACE. Distribution of platelet reactivity in subjects with and without MACE. The lines indicate median platelet reactivity. TxB2, thromboxane; LTA, light transmission aggregometry; Col/Epi, collagen/epinephrine; Col/ADP, collagen/adenosine diphosphate; CRP-XL, cross-linked collagen-related peptide.

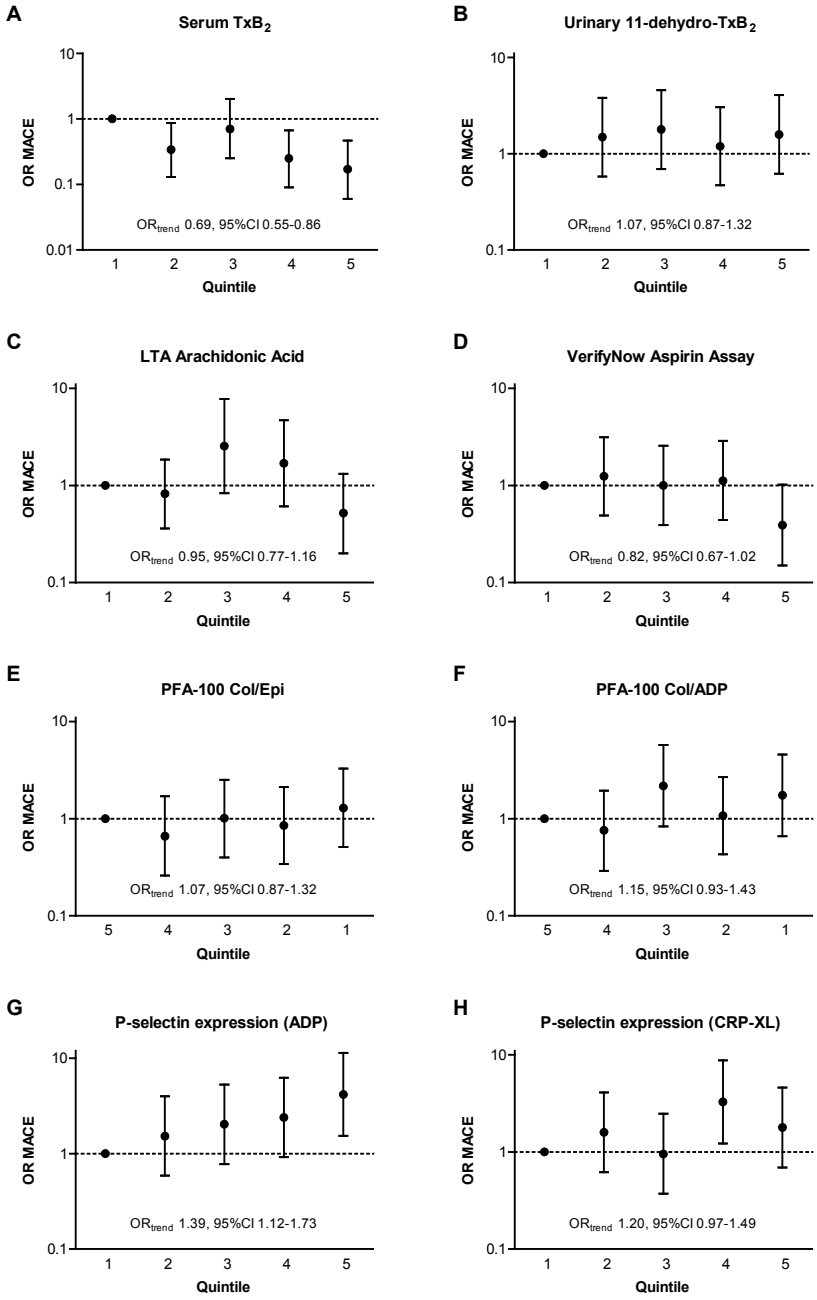

Figure 2 – Association between platelet reactivity in quintiles and MACE. For each quintile the odds ratio for MACE is calculated with the first (PFA-100 last) quintile as reference category. The odds ratio for trend indicates the increase in odds of MACE per quintile. OR, odds ratio; 95%CI, 95% confidence interval; TxB_2, thromboxane; LTA, light transmission aggregometry; Col/Epi, collagen/epinephrine; Col/ADP, collagen/adenosine diphosphate; CRP-XL, cross-linked collagen-related peptide.

Table 2 – High platelet reactivity and MACE

Test	Cut-off point (highest quintile)	MACE *n*	No MACE *n*	OR (95%CI)	OR_{adj} (95%CI)
S-TxB_2	≤9.0 ng/mL	79	62	1 [ref]	1 [ref]
	>9.0 ng/mL	11	24	0.36 (0.16 to 0.79)	0.37 (0.15 to 0.93)
U-TxB_2	≤1300 pg/mg creat	71	71	1 [ref]	1 [ref]
	>1300 pg/mg creat	19	16	1.19 (0.57 to 2.49)	1.26 (0.57 to 2.76)
LTA-AA	≤10%	78	64	1 [ref]	1 [ref]
	>10%	12	22	0.45 (0.21 to 0.97)	0.57 (0.25 to 1.30)
VerifyNow	≤500 ARU	80	62	1 [ref]	1 [ref]
	>500 ARU	11	24	0.36 (0.16 to 0.78)	0.37 (0.16 to 0.86)
PFA-100 Col/Epi	≥116 s	71	73	1 [ref]	1 [ref]
	<116 s	20	14	1.47 (0.69 to 3.13)	1.49 (0.66 to 3.33)
PFA-100 Col/ADP	≥68 s	70	73	1 [ref]	1 [ref]
	<68 s	19	13	1.52 (0.70 to 3.31)	1.68 (0.73 to 3.86)
P-selectin (ADP)	AUC ≤97	66	76	1 [ref]	1 [ref]
	AUC >97	24	11	2.51 (1.14 to 5.51)	2.80 (1.20 to 6.52)
P-selectin (CRP-XL)	AUC ≤95	70	71	1 [ref]	1 [ref]
	AUC >95	19	16	1.20 (0.57 to 2.53)	1.08 (0.49 to 2.35)

* Cut-off points are determined with ROC-analysis since no cut-off points exist in literature.
Odds ratios (OR) and 95% confidence intervals (95%CI) for the relation between high platelet reactivity (i.e. upper quintile, PFA-100 lower quintile) and MACE.
S-TxB2, serum thromboxane B2; U-TxB2, urinary 11-dehydro-thromboxane B2; Creat, creatinine; LTA-AA, arachidonic acid-induced light transmission aggregometry; Col/Epi, collagen/epinephrine; Col/ADP, collagen/adenosine diphosphate; CRP-XL, cross-linked collagen-related peptid

were generally consistent with the other analyses. However, with these definitions high platelet reactivity according to the PFA-100 Col/ADP cartridge (adjusted OR 2.83, 95%CI 1.02 to 7.88) and CRP-XL-induced P-selectin expression (adjusted OR 2.10, 95%CI 1.09 to 4.04) seemed also associated with MACE. When ROC-analysis was applied to the PFA-100 Col/ADP closure times, the optimal cut-off point in our data was 81 s. Using this cut-off point, the crude OR was 2.05 (95%CI 1.12 to 3.74) and the adjusted OR 2.01 (95%CI 1.06 to 3.81) for the PFA-100 Col/ADP cartridge.

Sensitivity analyses

Using the absolute numbers presented in Table 2, we calculated how many of the subjects who died during follow-up or did not want to participate would have needed to have had both high platelet reactivity and MACE in order to explain the inverse association between high platelet reactivity according to several of the COX-1-dependent assays and MACE. To achieve an OR of 3.0, 81 *additional* subjects with MACE and high platelet reactivity (according to the same cut-off points, derived from the current highest quintile) according to S-TxB_2, 69 according to LTA-AA and 82 according to the VerifyNow, keeping other cells constant, would be required to explain the association if caused by post-hoc effects. For example, in our data 11

Cut-off point (literature)	MACE *n*	No MACE *n*	OR (95%CI)	OR_{adj} (95%CI)
≤3.1 ng/mL	38	23	1 [ref]	1 [ref]
>3.1 ng/mL	52	63	0.50 (0.26 to 0.94)	0.52 (0.27 to 1.03)
≤1500 pg/mg creat	78	78	1 [ref]	1 [ref]
>1500 pg/mg creat	12	9	1.33 (0.53 to 3.34)	1.54 (0.58 to 4.08)
≤20%	89	80	1 [ref]	1 [ref]
>20%	1	6	0.15 (0.02 to 1.27)	0.22 (0.02 to 2.03)
≤550 ARU	90	79	1 [ref]	1 [ref]
>550 ARU	1	9	0.10 (0.01 to 0.77)	0.10 (0.01 to 0.87)
≥93 s	27	29	1 [ref]	1 [ref]
<93 s	64	58	1.19 (0.63 to 2.23)	1.18 (0.61 to 2.29)
≥65 s	73	79	1 [ref]	1 [ref]
<65 s	16	7	2.47 (0.96 to 6.35)	2.83 (1.02 to 7.88)
AUC ≤90	55	72	1 [ref]	1 [ref]
AUC >90*	35	15	3.05 (1.52 to 6.15)	3.34 (1.57 to 7.14)
AUC ≤79	46	60	1 [ref]	1 [ref]
AUC >79*	43	27	2.08 (1.12 to 3.85)	2.10 (1.09 to 4.04)

Table 3 – High platelet reactivity and MACE in different time intervals

Test	Cut-off point (highest quintile)	OR (95%CI)		
		MACE <5y	MACE 5-10y	MACE ≥10y
S-TxB_2	>9.0 ng/mL	0.31 (0.09 to 1.12)	0.37 (0.10 to 1.35)	0.39 (0.14 to 1.12)
U-TxB_2	>1300 pg/mg creat	0.53 (0.14 to 1.98)	2.22 (0.81 to 6.07)	1.18 (0.46 to 3.06)
LTA-AA	>10%	0.63 (0.21 to 1.87)	0.42 (0.11 to 1.53)	0.34 (0.11 to 1.07)
VerifyNow	>500 ARU	0.37 (0.10 to 1.35)	0.47 (0.17 to 1.26)	0.40 (0.09 to 1.89)
PFA-100 Col/Epi	<116 s	0.40 (0.09 to 1.89)	1.37 (0.44 to 4.29)	2.61 (1.08 to 6.27)
PFA-100 Col/ADP	<68 s	1.68 (0.57 to 4.99)	1.87 (0.63 to 5.60)	1.23 (0.45 to 3.37)
P-selectin (ADP)	AUC >97	0.83 (0.21 to 3.21)	1.82 (0.56 to 5.86)	5.02 (2.04 to 12.39)
P-selectin (CRP-XL)	AUC >95	0.74 (0.23 to 2.43)	0.93 (0.28 to 3.12)	1.81 (0.75 to 4.39)

Odds ratios (OR) and 95% confidence intervals (95%CI) for the relation between high platelet reactivity (i.e. upper quintile, PFA-100 lower quintile) and MACE. ORs are presented for different time intervals relative to platelet function testing.
S-TxB2, serum thromboxane B2; U-TxB2, urinary 11-dehydro-thromboxane B2; creat, creatinine; LTA-AA, arachidonic acid-induced light transmission aggregometry; Col/Epi, collagen/epinephrine; Col/ADP, collagen/adenosine diphosphate; CRP-XL, cross-linked collagen-related peptide

subjects with and 24 subjects without MACE had S-TxB_2 levels above 9.0 ng/mL, while 79 subjects with and 62 subjects without MACE had S-TxB_2 levels below this threshold (Table 2), which led to a crude OR of 0.36 ((11*62)/(24*79)). To obtain an OR of 3.0, 81 additional subjects with MACE and high platelet reactivity would be required ((92*62)/(24*79)). Under this scenario, 45% of subjects would have S-TxB_2 levels of 9.0 ng/mL or higher, 42% 10 percent aggregation according to LTA-AA or higher, and 45% 500 ARU according to the VerifyNow, whereas those percentages are now, by definition, 20%.

We analyzed whether the association between high platelet reactivity defined as the highest quintile and MACE depends on the time between platelet function testing and occurrence of the event (Table 3). 28 events occurred within 5 years before platelet function testing, 24 events occurred between 5 and 10 years, and 39 events occurred 10 years or more before platelet function testing. Generally, the association between high platelet reactivity and MACE seemed to be consistent over the three time intervals, although due to the small numbers of events in each interval there were some outliers.

DISCUSSION

The present study addressed the association between on-aspirin platelet reactivity according to several COX-1-dependent (S-TxB_2, U-TxB_2, LTA-AA, VerifyNow Aspirin) and COX-1-independent (PFA-100 Col/Epi and Col/ADP, and ADP- and CRP-XL-induced P-selectin expression) tests and recurrent cardiovascular events in male patients with stable cardiovascular disease. Our data suggest an inverse association between high on-aspirin platelet reactivity according to S-TxB_2, LTA-AA and VerifyNow Aspirin as COX-1-dependent assays, and MACE. Contrarily, high platelet reactivity according to COX-1-independent assays like ADP-induced P-selectin expression, and, depending on the used cut-off point, also according to the PFA-100 Col/ADP cartridge and CRP-XL-induced P-selectin expression, seemed to be positively associated with MACE.

Several meta-analyses have documented an increased risk of cardiovascular events in subjects with high on-aspirin platelet reactivity.[18-20] However, these studies did not differentiate between the many different definitions of high platelet reactivity used in the underlying studies. Studies directly comparing different tests of on-aspirin platelet reactivity in relation to clinical outcomes are surprisingly scarce[21,22]. Frelinger *et al.* compared S-TxB_2, various AA-induced flow-cytometric assays, and the PFA-100 Col/Epi and Col-ADP cartridge.[21] They found an increased risk of MACE in subjects with high platelet reactivity according to S-TxB_2 and the PFA-100 Col/ADP cartridge during an average follow-up of two years. While, to our knowledge, there are no other studies evaluating the relation between S-TxB_2 and MACE, few small studies also documented an increased cardiovascular risk in association with shorter PFA-100 Col-ADP closure times (indicating higher platelet reactivity) in subjects using aspirin.[30,31] In a one-year follow-up study of subjects treated with dual antiplatelet therapy undergoing an elective percutaneous coronary intervention, Breet *et al.* found an increased risk of cardiovascular

events in subjects with high platelet reactivity according to LTA-AA and the VerifyNow Aspirin assay.[22] In some small previous studies, LTA-AA and the VerifyNow Aspirin assay were also positively associated with adverse clinical outcome.[19,32-35] Multiple small studies evaluating PFA-100 Col/Epi closure times suggest an increased cardiovascular risk in subjects with high platelet reactivity,[36,37] but this could not be confirmed in the larger studies of Frelinger *et al.* and Breet *et al.*[21,22] In two of the largest studies of Eikelboom *et al.*, high levels of U-TxB_2 were associated with recurrent cardiovascular events.[26,27] In conclusion, although sufficiently powered studies are scarce for most methods, in general, high platelet reactivity according to COX-1-dependent assays as well as the COX-1-independent PFA-100 Col-ADP cartridge seems to be associated with an increased risk of cardiovascular events in subjects using aspirin. Studies using the PFA-100 Col/Epi cartridge are inconclusive and ADP- and CRP-XL-induced P-selectin expression have never been studied in this context.

In the present study, we examined the association between several COX-1-dependent and COX-1-independent tests of platelet reactivity and MACE in a comprehensive and direct head-to-head comparison. Remarkably and in contrast to previous studies, high platelet reactivity according to the COX-1-dependent S-TxB_2, LTA-AA and the VerifyNow Aspirin assay were inversely associated with MACE. U-TxB_2 and PFA-100 Col/Epi were not associated with MACE. However, the COX-1-independent tests PFA-100 Col/ADP, CRP-XL-induced P-selectin expression and particularly ADP-induced P-selectin expression were positively associated with MACE. Thus, according to our data, COX-1-dependent and COX-1-independent platelet reactivity may be oppositely associated with recurrent MACE in patients using asprin, which is unexpected given the results of previous studies. There are several potential explanations for this finding that have to be discussed.

First, as we measured platelet reactivity after many years of follow-up, we considered that survival bias could have influenced our findings. For example, when high COX-1-dependent platelet reactivity is indeed a risk factor for recurrent cardiovascular events and when events in subjects with high platelet reactivity are more often fatal than in subjects without high platelet reactivity, the MACE group would gradually get rid of subjects with high platelet reactivity, because they died before inclusion in the study. Theoretically, this could lead to inverse associations. However, this could not explain the difference between the COX-1-dependent and -independent tests, particularly ADP-induced P-selectin expression. Furthermore, we estimated the number of additional subjects who would have needed to have had MACE combined with high platelet reactivity according to S-TxB_2, LTA-AA and VerifyNow in order to explain the inverse association, which was unreasonably high given previous literature. Moreover, especially for S-TxB_2, there was a dose-response relationship with the decreased risk already starting at lower values than the highest quintile which was used as cut-off point: the whole distribution of S-TxB_2 values was lower in subjects with than without MACE, which argues against survival bias as explanation for our findings.

Second, subjects with MACE could be more compliant to aspirin after the event than subjects without MACE, which may differentially influence COX-1-dependent and -independent tests. However, although differences in compliance could weaken

the association between COX-1-dependent tests and MACE, it is unlikely that this would reverse the association. Furthermore, one would expect such a phenomenon only when platelet reactivity is measured in subjects with and without MACE at a random day. However, measurement of platelet reactivity was planned and all subjects were urged several times to adhere to their medication very strictly at least in the week before blood sampling. Moreover, only two aspirin-using subjects (1%) had S-TxB_2-levels overlapping with non-users.[17]

Third, subjects with MACE might have received additional treatment which could have reduced platelet reactivity, e.g. a higher aspirin dose, additional antiplatelet therapy, or statins.[38,39] However, subjects with other antiplatelet or antithrombotic drugs were excluded from the study, as were subjects using no aspirin or a different dose of aspirin at time of MACE compared with blood sampling. There was some suggestion that subjects using lipid-lowering drugs had lower S-TxB_2 and VerifyNow values, although confidence intervals were broad[17] and subjects with MACE used lipid-lowering drugs more frequently than subjects without MACE. Therefore, we adjusted for use of lipid-lowering drugs in our analyses, but this did not considerably influence the results (Table 2).

Fourth, an alternative explanation might be that the observed opposite associations with COX-1-dependent and -independent tests are, at least in part, reflecting a true difference. Besides the design of the study, one of the most prominent differences between previous studies and our study is the length of follow-up, which was on average 15 years in our study and at most a few years in all previous studies. It has been suggested that aspirin might be particularly clinically effective at short term rather than at long term.[40] As shown previously, activated platelets are needed for the recruitment of circulating hematopoietic stem cells and their differentiation into endothelial progenitor cells and eventually functional endothelial cells.[41-43] Furthermore, we previously showed that aspirin decreases the number of endothelial progenitor cells.[43] Therefore, one might hypothesize that, platelet inhibition with aspirin may prevent endothelial regeneration after injury, which might eventually have adverse clinical consequences. Furthermore, aspirin is known to inhibit biosynthesis of prostacyclin, which has vasoprotective and antithrombotic effects.[5,44] Given those potential negative effects of aspirin, decreased effectiveness of aspirin might have a beneficial component at long-term. An inverse association between COX-1-dependent platelet reactivity and MACE in subjects who used aspirin for many years is consistent with this hypothesis. As we could currently not exclude this potential explanation of our data, further study is needed to address long-term effects of aspirin use.

The strengths of our study are that we directly compared eight COX-1-dependent and COX-1-independent platelet reactivity tests in relation to clinical outcomes in subjects with stable cardiovascular disease using aspirin. Furthermore, with 91 events our study is among the largest studies published in this field hitherto. For the first time, we studied ADP- and CRP-XL-induced P-selectin expression in relation to recurrent cardiovascular events in subjects using aspirin. Our study also has limitations. As already mentioned, we measured platelet reactivity after a long period of follow-up rather than before follow-up. This has the advantage that we could follow subjects

over a long time and that the event rate was high, but could also have introduced several biases, as mentioned above, including survival bias, changed compliance and co-medication after a MACE compared with before the MACE, and reverse causality. Given reverse causality, i.e., high platelet reactivity as a consequence rather than a cause of MACE, one would expect stronger associations when the event occurred shortly before platelet reactivity testing. Our results did not support this hypothesis. Furthermore, platelet reactivity was measured only once and assumed to be informative also even though measured after many years. It has been shown that platelet reactivity varies within individuals, although the same study showed that at a group level platelet reactivity was consistently inhibited by aspirin over time.[15]

Given these limitations and the fact that our data cannot provide a mechanism for the inverse association between COX-1-dependent tests and MACE, our data should be interpreted cautiously. Nevertheless, we think that our study adds important new information to this field: We report on the potential usefulness of COX-1-independent tests, particularly ADP-induced P-selectin expression, as marker of cardiovascular risk in subjects using aspirin, which warrants further examination in large prospective studies. Furthermore, the striking hypothesis that COX-1 inhibition might potentially also have adverse effects should be acknowledged and requires further study.

In summary, in a head-to-head comparison of eight COX-1-dependendent and COX-1-independent tests, COX-1-independent platelet reactivity, particularly according to ADP-induced P-selectin expression, was positively associated with MACE in male subjects with stable cardiovascular disease using aspirin. However, several COX-1-independent tests seemed to be inversely associated with MACE. We described several biases that may account for this association, but they were all unlikely to fully explain the data. Future study is warranted to further elucidate the relation between COX-1-dependent and COX-1-independent platelet reactivity and MACE in subjects with established cardiovascular disease on long-term aspirin use.

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