Platelet reactivity and cardiovascular events

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PLATELET REACTIVITY IN PATIENTS WITH STABLE CARDIOVASCULAR DISEASE USING ASPIRIN: CORRELATION BETWEEN DIFFERENT TESTS AND ASSOCIATION WITH SUBJECT CHARACTERISTICS

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ABSTRACT

Background

Platelet reactivity among subjects with cardiovascular disease 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 correlation between a variety of COX-1-dependent and COX-1-independent tests as well as the determinants of the various tests.

Methods

Platelet reactivity was assessed in 252 male subjects with stable cardiovascular disease using serum thromboxane B_2, urinary 11-dehydro-thromboxane B_2, arachidonic acid-induced light transmission aggregometry, the VerifyNow Aspirin assay, the PFA-100 collagen-epinephrine and collagen-adenosine-diphosphate cartridges, and adenosine-diphosphate and cross-linked collagen-related peptide induced P-selectin expression by platelets.

Results

On-aspirin platelet reactivity varied markedly according to the different tests. The VerifyNow Aspirin assay correlated with serum thromboxane B_2 levels: $r^2 = 0.49$, agreement highest quintiles 85%, $\kappa = 0.53$ (95%CI 0.38 to 0.67). The correlation between all other tests was weaker or even absent. Similarly, the determinants of the tests differed between the tests, although several factors seemed to be of more general importance, including aspirin dose below 80 mg, obesity, dyslipidemia, high platelet count, high mean platelet volume, decreased renal function, low hemoglobin and low hematocrit.

Conclusion

We showed that among male subjects with established cardiovascular disease chronically using aspirin, high on-aspirin platelet reactivity was not a uniform finding using several COX-1-dependent and COX-1-independent tests. We identified several determinants of platelet reactivity according to the various tests.

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]. It is still not clear which test is most useful from a clinical point of view.

Reflecting the different definitions of high platelet reactivity and the different tests available, the prevalence of high platelet reactivity varies highly over studies from 1-2 percent using a highly specific method for the pharmacological effect of aspirin up to 65 percent when more global tests are used.[7,10,13] Furthermore, several studies have showed poor correlations between the different tests, including several COX-1-dependent assays.[14-17] In the present study we present a further evaluation of the relationship between a variety of COX-1-dependent and COX-1-independent tests of platelet reactivity among subjects with stable cardiovascular disease chronically using aspirin. Furthermore, we examined potential determinants of on-aspirin platelet reactivity according to the different tests.

METHODS

Study design and subjects

We evaluated the correlation between platelet function tests and the determinants of platelet reactivity 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)[18] and 542 of them were followed for recurrent events in a cohort study.[19] In 2008/2009 all living patients were recruited again to provide a blood and urine sample to test platelet reactivity.[20] 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.

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. All subjects also completed a detailed questionnaire about recurrent cardiovascular events, cardiovascular risk factors and medication use. 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.

Laboratory measurements

Included tests of platelet reactivity were 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 (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. 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.

To measure S-TxB_2, blood was collected in a Becton Dickinson Vacutainer® glass tube without anticoagulant (BD Biosciences, San Jose, CA, USA), incubated for 1 hour at 37°C, and centrifuged at 2500 *g* for 10 min. The supernatant serum was stored at −80°C until analysis.[21] TXB_2 was measured by a previously validated enzyme immunoassay.[16,22]

Urine for measurement of U-TxB_2 was stabilized in Becton Dickinson C&S tubes with preservative and stored at -80°C until analysis. U-TxB_2 was measured by the AspirinWorks® Test Kit (Corgenix Inc., Westminster, CO, USA) according to the manufacturer's instructions. In the same urine samples creatinine levels were measured. U-TxB_2 levels were expressed as pg/mg creatinine.

To measure platelet function with the VerifyNow® Aspirin assay, blood was collected in a VerifyNow Greiner Bio-One 3.2% citrate tubes (Greiner Bio-One GmbH, Kremsmünster, Austria). The assay was performed according to the manufacturer's instructions. Results are expressed as Aspirin Responsiveness Units (ARU).

LTA, PFA-100 and P-selectin expression measurements were performed in 3.2% citrate Sarstedt S-Monovette® tubes. LTA was performed using a Chrono-Log 490 aggregometer (Chrono-Log Corp., Havertown, PA, USA) as previously described.[23] Platelet-rich plasma was prepared by centrifugation for 15 min at 126 *g* and platelet-poor plasma was prepared by centrifugation for 15 min at 2700 *g*. Arachidonic acid 1.6 mM was used to induce aggregation. The maximum extent of aggregation during 15 min was recorded as a percentage of light transmission. Light transmission using platelet poor plasma was set as 100% of transmission.

PFA-100 measurements were performed using the Col/Epi and the Col/ADP cartridge. The outcome measure is the time required to occlude a microscopic aperture or closure time (CT).

P-selectin expression was measured in citrated whole blood by flow cytometry using a Beckman Coulter® EPICS® XL-MCL (Miami, FL, USA) flow cytometer, as previously described.[20] Antibodies used were FITC-anti-CD62p (anti-P-selectin) and PE-anti-CD61 (anti-glycoprotein IIIa). Platelet activation was measured in response to no agonist, ADP (10^{-6}M, 10^{-5}M, 10^{-4}M) and CRP-XL (0.1, 1, 10 µg/mL). CRP-XL was kindly provided by Dr R.W. Farndale (University of Cambridge, Cambridge, UK). Results were expressed as the percentage of platelets (CD61-positive cells) expressing P-selectin (CD62p). To obtain single effect measures for ADP- and CRP-XL-induced P-selectin expression, we calculated the area under the curve (AUC) which can be drawn through the individual values of P-selectin expression for the different agonist concentrations.

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 assess the association between aspirin use and platelet function according to the different tests, we calculated the median difference with 95% confidence intervals (CI) in platelet reactivity according to the different tests between subjects using and not using aspirin 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. Associations with aspirin use were estimated both including and excluding subjects using vitamin-K antagonists.

The r^2 for correlation between the different tests was calculated for all subjects (including those not using aspirin) and also restricted to aspirin-using subjects, assuming a linear relationship. Correlations of S-TxB_2 and U-TxB_2 with other tests appeared to be non-linear, as also previously demonstrated.[16] Hence, second order polynomial models were used.

All further analyses regarding correlation between the tests and determinants of the tests were performed within the aspirin-using subjects only since those tests usually will be performed only in subjects using aspirin. We calculated κ statistics for agreement between the tests in assessing high on-aspirin platelet reactivity. Values in the highest quintile were set as high and values in the lower four quintiles as normal platelet reactivity (lowest versus higher quintiles for PFA-100).

The association between several patient-related characteristics and platelet reactivity was assessed in univariable and multivariable regression models. First, for all tests mean values of platelet reactivity with standard errors are given in absence and presence of dichotomous variables and per quartile for continuous variables. Differences in platelet reactivity with 95%CI were estimated by linear regression analysis. For continuous variable, those values are the mean difference per quartile increase of the variable. Second, we performed multivariable regression models

including age (≥70y vs. <70y), smoking (current smokers vs. others), obesity (body mass index ≥30 kg/m^2 vs. <30 kg/m^2), diabetes (yes vs. no), hypertension (yes vs. no, defined as ≥140 mmHg systolic or ≥90 mmHg diastolic blood pressure), aspirin dose (<80 mg versus 80-100 mg), use of lipid-lowering drugs and 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) to assess which characteristics were independently related with platelet reactivity. Hematocrit and red blood cell count were not included in the multivariable models simultaneously with hemoglobin to prevent multicollinearity because those factors are highly correlated. We also performed models including hematocrit but excluding hemoglobin. Furthermore, we performed multivariable analyses including the estimated glomerular filtration rate according to the MDRD-formula[24] rather than serum creatinine.

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

RESULTS

We included 252 male subjects with a mean age of 69 years (range 50 to 89 years) at time of the platelet function study. At time of their first myocardial infarction, the mean age was 53 years (range 46 to 60 years). At time of the platelet function study, a total of 189 subjects (75%) were using aspirin, while 63 subjects did not use aspirin. Most of the latter patients used vitamin-K antagonists (n=49). Most subjects on aspirin used 80 to 100 mg; 9 subjects (5%) used 30 to 40 mg. The median duration of aspirin use was 15 years (interquartile range 14 to 16 years). The majority of our study population used lipid-lowering (n=219, 87%) and antihypertensive (n=212, 84%) drugs. 45 subjects were current smokers (22%), 69 subjects were obese (27%) and 38 subjects had type 2 diabetes (15%).

Association between aspirin use and platelet reactivity

The association between aspirin use and platelet reactivity according to the different tests is shown in Figure 1. Aspirin use was clearly associated with lower platelet reactivity when measured by COX-1-dependent tests as compared with subjects not using aspirin: Median differences with 95%CIs according to aspirin use were -178 ng/mL (-207 to -139) for S-TxB_2, -3432 pg/mg creatinine (-3901to -3048) for U-TxB_2, -79% (-81 to -75) for LTA-AA, -187 ARU (-198 to -177) for VerifyNow. Furthermore, aspirin use was associated with prolonged PFA-100 Col/Epi (52 sec; 95%CI 43 to 62) and, to some extent, Col/ADP (6 sec; 95%CI 2 to 11) closure times. Aspirin use was not associated with P-selectin expression according to both agonists: ADP 1 (95%CI -6 to 3); CRP-XL 3 (95%CI -5 to 10). When subjects using vitamin-K antagonists were excluded to examine the effect of aspirin on platelet reactivity more accurately, the differences using COX-1-dependent tests were somewhat more pronounced: -273 ng/

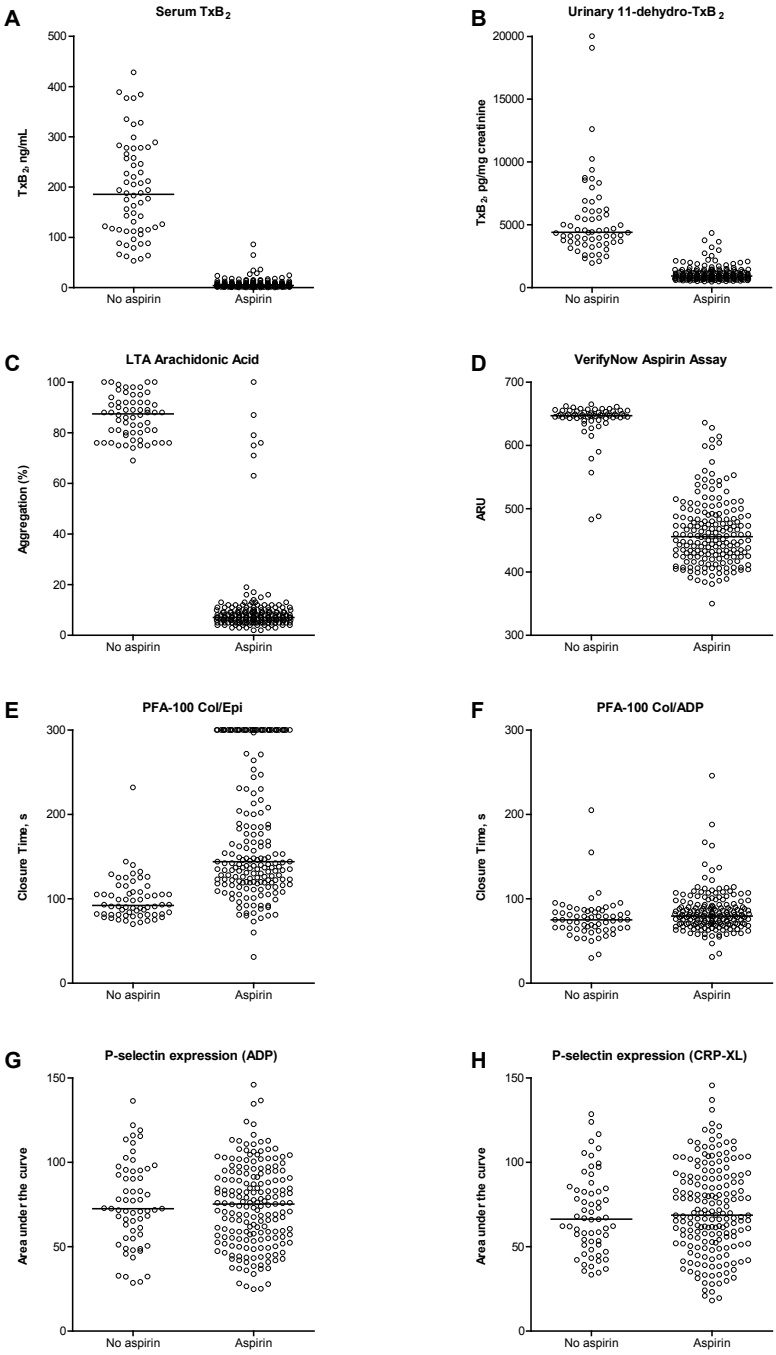


Figure 1 – Association between aspirin use and platelet reactivity. Distribution of platelet reactivity in subjects using and not using aspirin. The lines indicate median platelet reactivity. TxB_2, thromboxane; LTA, light transmission aggregometry; Col/Epi, collagen/epinephrine; Col/ADP, collagen/adenosine diphosphate; CRP-XL, cross-linked collagen-related peptide

mL (95%CI -322 to -118) for S-TxB_2, -5056 pg/mg creatinine (95%CI -7296 to -3040) for U-TxB_2, -80% (95%CI -85 to -75) for LTA-AA, and -194 ARU (95%CI -208 to -181) for VerifyNow.

Correlation between the tests

Table 1 shows the correlation between the different tests. In the whole study population, including subjects using and not using aspirin (Table 1a), the correlation between COX-1-dependent tests was good, whereas there was poor correlation between COX-1-dependent and COX-1-independent tests. Among subjects using aspirin (Table 1b), the VerifyNow Aspirin assay correlated reasonably well with S-TxB_2 levels (r^2 = 0.49). The correlations of LTA-AA (r^2 = 0.35) and U-TxB_2 (r^2 = 0.28) with S-TxB_2 were weaker. The closure times using the two cartridges of the PFA-100 were positively, although poorly, correlated (r^2 = 0.13). The two tests of P-selectin expression were also positively correlated (r^2 = 0.29). All other correlations between tests were lower than these values. Interestingly, the correlations between S-TxB_2 and functional COX-1-dependent tests appeared to be non-linear (Tables 1a and 1b). As an example, the correlation between S-TxB_2 levels and VerifyNow ARUs in aspirin users is presented in Figure 2.

In Figure 3, the values in the highest quintile of S-TxB_2 (>9.0 ng/mL), the most specific test for the pharmacological effect of aspirin,[10] among aspirin users have been marked in black. For the other tests the corresponding values of the same subjects have also been marked. This figure illustrates that the VerifyNow Aspirin assay correlated best with S-TxB_2 levels. Subjects with high aggregation according to LTA-AA have also high S-TxB_2 levels, but the other subjects with high S-TxB_2 levels seemed to be randomly distributed with respect to LTA-AA values. The correlations between S-TxB_2 and other tests are even poorer (Figure 3).

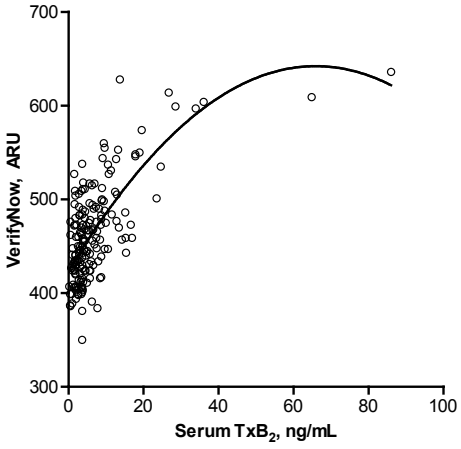
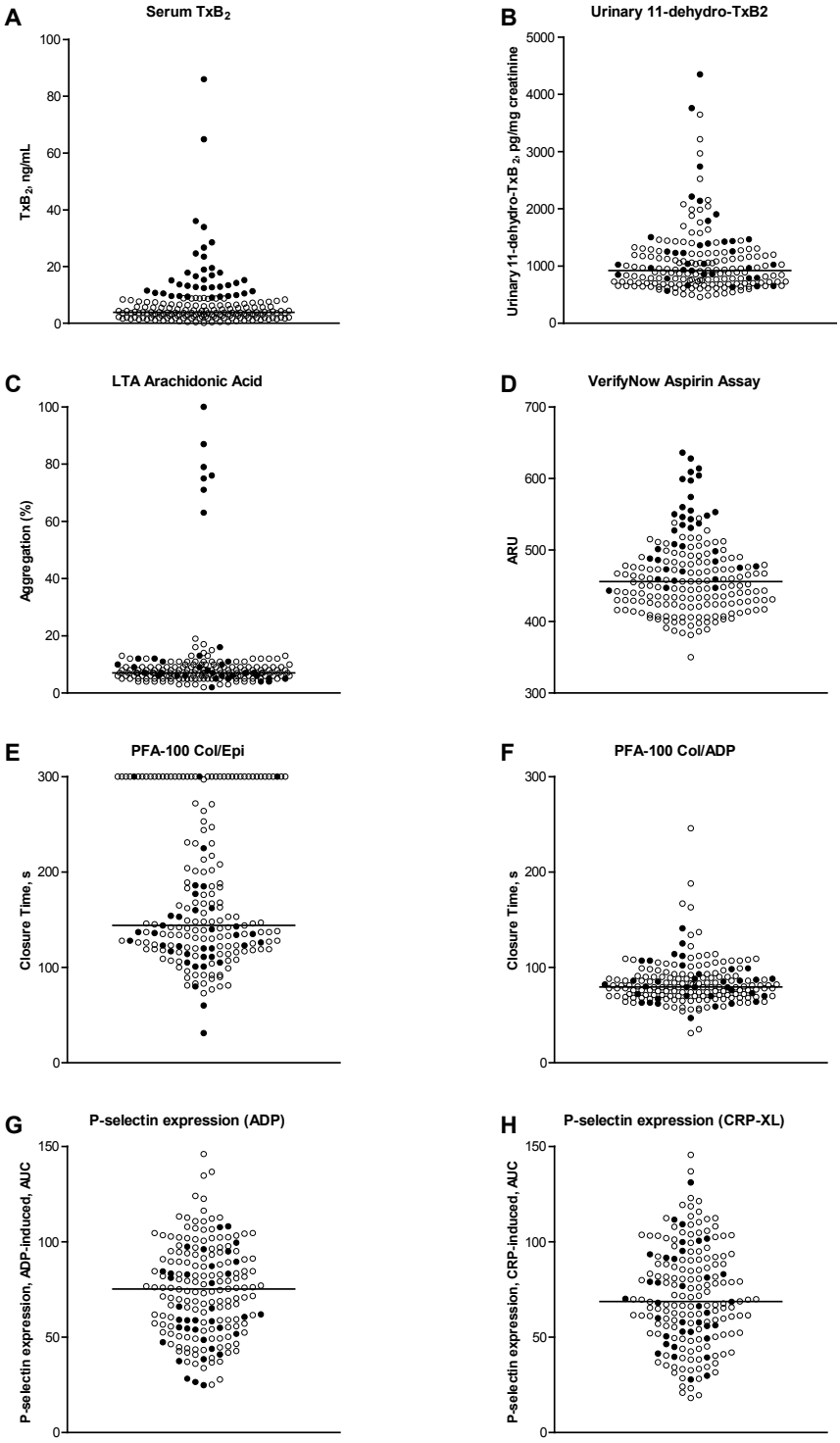


Figure 2 – Non-linear correlation between serum thromboxane B_2 levels and the VerifyNow Aspirin assay in subjects using aspirin

When platelet reactivity in aspirin users according to all tests was

Figure 3 – Correlation between high levels of serum thromboxane B_2 and platelet reactivity ▷ according to the other tests. Subjects with high platelet reactivity according to serum thromboxane B_2 (highest quintile, >9.0 ng/mL) have been marked with black dots for all assays. Similar to Figure 1, the lines indicate median platelet reactivity for all tests. The data refer to aspirin-using subjects only. TxB_2, thromboxane; LTA, light transmission aggregometry; Col/Epi, collagen/epinephrine; Col/ADP, collagen/adenosine diphosphate; CRP-XL, cross-linked collagen-related peptide


A
Serum TxB2
TxB2, ng/mL
100
80
60
40
20
0
B
Urinary 11-dehydro-TxB2
Urinary 11-dehydro-TxB2, pg/mg creatinine
5000
4000
3000
2000
1000
0
C
LTA Arachidonic Acid
Aggregation (%)
100
80
60
40
20
0
D
VerifyNow Aspirin Assay
ARU
700
600
500
400
300
E
PFA-100 Col/Epi
Closure Time, s
300
200
100
0
F
PFA-100 Col/ADP
Closure Time, s
300
200
100
0
G
P-selectin expression (ADP)
P-selectin expression, ADP-induced, AUC
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P-selectin expression (CRP-XL)
P-selectin expression, CRP-induced, AUC
150
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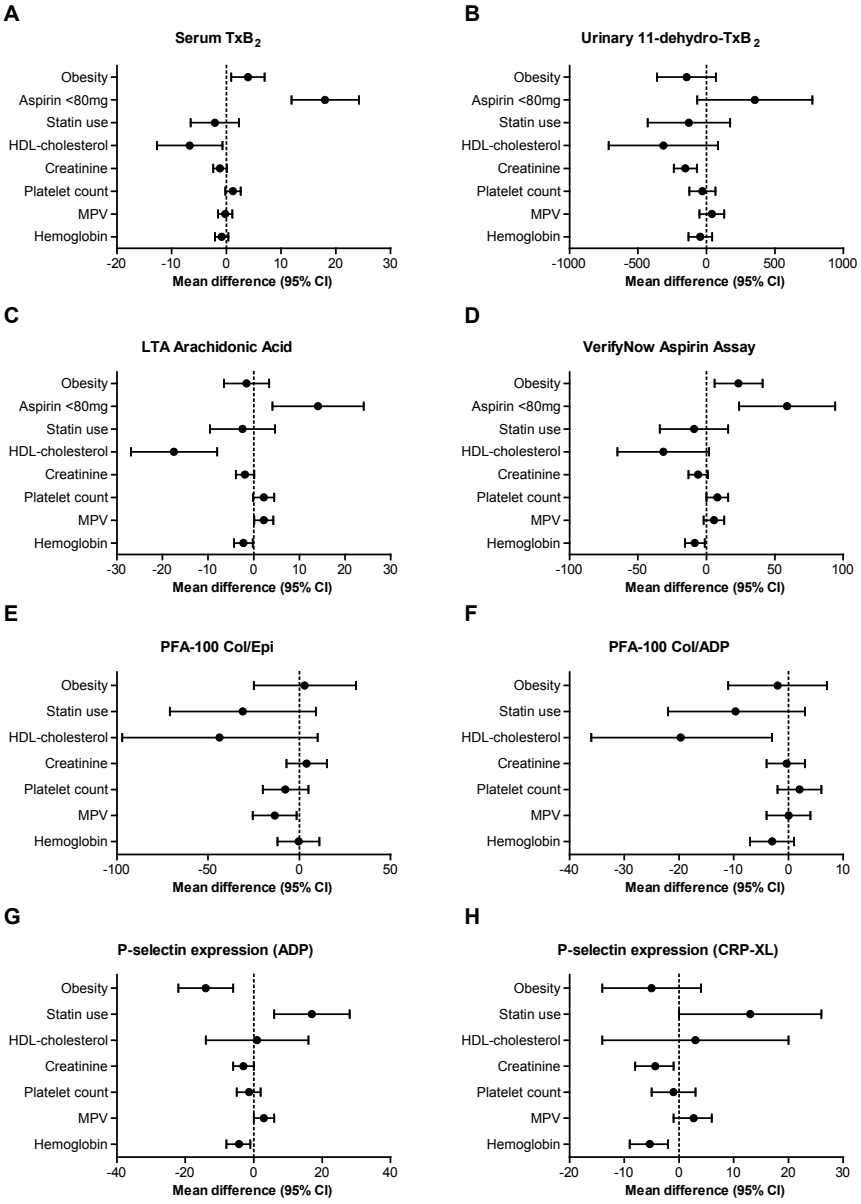

Figure 4 – Determinants of platelet reactivity – multivariate analysis. Relation between patient-related factors and platelet reactivity according to the various tests in multivariate analysis including all factors included in table 1. A selection of factors of interest is presented in the figure. The data refer to aspirin-using subjects. The same cut-off points are used as in Table 1. Age ≥70 years; HDL-cholesterol ≥ 0.9 mmol/L. Serum creatinine, platelet count, MPV, and hemoglobin are divided in quartiles. Regression coefficients and 95% confidence intervals are provided for the association between patient-related factors and platelet reactivity, conditional on all other factors (age, smoking, obesity, diabetes, hypertension, aspirin dose, use of lipid-lowering drugs and blood pressure lowering drugs, serum glucose, LDL-cholesterol, HDL-cholesterol, triglycerides, ▷

Table 1 – Correlation between platelet function tests

A – All subjects							
S-TxB_2							
r^2 = 0.59* *P* < 0.0001	U-TxB_2						
r^2 = 0.83* *P* < 0.0001	r^2 = 0.73* *P* < 0.0001	LTA-AA					
r^2 = 0.75* *P* < 0.0001	r^2 = 0.59* *P* < 0.0001	r^2 = 0.75 *P* < 0.0001	VerifyNow				
r^2 = 0.20* *P* < 0.0001	r^2 = 0.14* *P* < 0.0001	r^2 = 0.19 *P* < 0.0001	r^2 = 0.22 *P* < 0.0001	PFA-100 Col/Epi			
r^2 = 0.01 *P* = 0.10	r^2 = 0.01 *P* = 0.07	r^2 = 0.01 *P* = 0.08	r^2 = 0.01 *P* = 0.11	r^2 = 0.15 *P* < 0.0001	PFA-100 Col/ADP		
r^2 = 0.00 *P* = 0.57	r^2 = 0.00 *P* = 0.82	r^2 = 0.00 *P* = 0.87	r^2 = 0.00 *P* = 0.53	r^2 = 0.01 *P* = 0.07	r^2 = 0.00 *P* = 0.29	P-selectin ADP	
r^2 = 0.00 *P* = 0.39	r^2 = 0.00 *P* = 0.86	r^2 = 0.00 *P* = 0.76	r^2 = 0.01 *P* = 0.18	r^2 = 0.01 *P* = 0.25	r^2 = 0.00 *P* = 0.90	r^2 = 0.23 *P* < 0.0001	P-selectin CRP-XL
B – Subjects using aspirin							
S-TxB_2							
r^2 = 0.28* *p* < 0.0001	U-TxB_2						
r^2 = 0.35* *P* < 0.0001	r^2 = 0.22* *P* < 0.0001	LTA-AA					
r^2 = 0.49* *P* < 0.0001	r^2 = 0.07 *P* = 0.0002	r^2 = 0.21 *P* < 0.0001	VerifyNow				
r^2 = 0.03 *P* = 0.03	r^2 = 0.00 *P* = 0.77	r^2 = 0.00 *P* = 0.49	r^2 = 0.05 *P* = 0.002	PFA-100 Col/Epi			
r^2 = 0.00 *P* = 0.46	r^2 = 0.00 *P* = 0.76	r^2 = 0.01 *P* = 0.31	r^2 = 0.00 *P* = 0.85	r^2 = 0.13 *P* < 0.0001	PFA-100 Col/ADP		
r^2 = 0.04 *P* = 0.007	r^2 = 0.01 *P* = 0.25	r^2 = 0.00 *P* = 0.79	r^2 = 0.02 *P* = 0.08	r^2 = 0.02 *P* = 0.05	r^2 = 0.00 *P* = 0.39	P-selectin ADP	
r^2 = 0.00 *P* = 0.68	r^2 = 0.02 *P* = 0.049	r^2 = 0.00 *P* = 0.39	r^2 = 0.01 *P* = 0.29	r^2 = 0.01 *P* = 0.16	r^2 = 0.00 *P* = 0.76	r^2 = 0.29 *P* < 0.0001	P-selectin CRP-XL

Correlation between the different test in (A) all subjects and (B) restricted to subjects using aspirin. R^2 indicates the proportion of variance in a test explained by the other test. A linear correlation is assumed except in cases marked with an asterisk (*), in which a second order polynomial model fitted better.
S-TxB_2, serum thromboxane B_2; U-TxB_2, urinary 11-dehydro-thromboxane B_2; LTA-AA, arachidonic acid-induced light transmission aggregometry; Col/Epi, collagen/epinephrine; Col/ADP, collagen/adenosine diphosphate; CRP-XL, cross-linked collagen-related peptide

▷ creatinine, platelet count, mean platelet volume, white blood cell count and hemoglobin). The regression coefficients can be interpreted as mean differences in case of dichotomous factors and mean differences per quartile in case of continuous factors. TxB_2, thromboxane; LTA, light transmission aggregometry; Col/Epi, collagen/epinephrine; Col/ADP, collagen/adenosine diphosphate; CRP-XL, cross-linked collagen-related peptide

dichotomized in high and normal, with the highest quintile (for the PFA-100 the lowest quintile) set as high platelet reactivity, the agreement between S-TxB_2 and VerifyNow was 85%, resulting in a κ of 0.53 (95%CI 0.38 to 0.67). The κ was 0.21 (95%CI 0.07 to 0.36) for the relation between U-TxB_2 and VerifyNow, 0.30 (95%CI 0.16 to 0.45) for the two PFA-100 cartridges and 0.27 (95%CI 0.12 to 0.41) for the two tests of P-selectin expression. All other κ values were below 0.20.

Determinants of platelet reactivity

In Table 2 the crude associations between various patient-related factors and the platelet function tests in aspirin-using subjects are presented. Figure 4 shows the results of multivariable regression models for several of those factors. In the multivariable models, obesity was associated with higher S-TxB_2 (4.0 ng/mL, 95%CI 0.9 to 7.0) and VerifyNow (23 ARU, 95%CI 6 to 41) values, but, remarkably, also with lower values of ADP (difference AUC -14, 95%CI -22 to -6) and to a lesser extent with CRP-XL (difference AUC -5, 95%CI -14 to 4) induced P-selectin expression. Aspirin dose was strongly associated with the COX-1-dependent assays, with doses <80mg/day resulting in higher platelet reactivity than daily doses of 80mg and higher (Figure 4). There was no clear effect of statin use on COX-1-dependent assays, but all point estimates were in the direction of less platelet reactivity (Figure 4). Interestingly, statin use seemed to be associated with higher platelet reactivity according to both PFA-100 cartridges (-31s, 95%CI -71 to 9 and -10s, 95%CI -22 to 3) and P-selectin expression induced with both agonists (difference AUC 17, 95%CI 6 to 28 and 13, 95%CI 0 to 26). Notably, when the analysis was performed in subjects *not* using aspirin, statin use was clearly associated with lower S-TxB_2 (-82 ng/mL, 95%CI -141 to -23) and U-TxB_2 levels (-4005 pg/mg creatinine, 95%CI -6003 to -2008). Among aspirin-users high HDL-cholesterol levels were associated with lower levels of S-TxB_2, U-TxB_2, LTA-AA and VerifyNow, but also with shorter closure times (i.e. higher platelet reactivity) according to both PFA-100 assays. Interestingly, higher levels of triglycerides (not shown in Figure 4) seemed also associated with shorter PFA-100 closure times (-14s, 95%CI -42 to 14 and -7s, 95%CI -16 to 2). Higher creatinine levels were dose-dependently related to less platelet reactivity according to all assays, except for the PFA-100 tests (Figure 4). When the estimated clearance was used rather than serum creatinine, a clearance below 90 mL/min/1.73 m^2 was again related to decreased platelet reactivity (S-TxB_2 -3.6 ng/mL, 95%CI 6.6 to -0.7; U-TxB_2 -365 pg/mg creatinine, 95%CI -566 to -163; LTA-AA -5.2%, 95%CI -9.9 to -0.4; VerifyNow -21 ARU, 95%CI -38 to -4, ADP-induced P-selectin expression -6, 95%CI -13 to 2; CRP-XL-induced P-selectin expression -8, 95%CI -17 to 0). When analyzed per quartile, a dose-response relationship was present. Similarly, in multivariable models, higher hemoglobin levels were related to decreased platelet reactivity according to all tests except the PFA-100, especially for LTA-AA and the VerifyNow, as well as both tests of P-selectin expression (Figure 3). When hematocrit was included in the multivariable models instead of hemoglobin, this revealed similar associations. Higher platelet counts were associated with higher levels of S-TxB_2, LTA-AA, and VerifyNow. An increased mean platelet volume was associated

with higher platelet reactivity according to LTA-AA, VerifyNow, PFA-100 Col/Epi, and ADP and CRP-XL-induced P-selectin expression in the multivariable models.

Several observations not shown in Figure 4 are also worth noting. There were no clear associations between diabetes or glucose levels and platelet reactivity in multivariable models, but the point estimates suggest higher levels of platelet reactivity according to S-TxB_2 (1.9 ng/mL, 95%CI -1.9 to 5.8), LTA-AA (5.9%, 95%CI -0.4 to 12.1) and ADP-induced P-selectin expression (difference AUC 5, 95%CI -4 to 15). Higher white blood cell counts seemed to be associated with the PFA-100 Col/ADP assay (-3 *10^9/L per quartile increase, 95%CI -7 to 1), ADP- and CRP-XL-induced P-selectin expression (difference AUC per quartile increase 3, 95%CI 0 to 6 and 4, 95%CI 0 to 8). Age above 70 years was associated with shorter PFA-100 Col/Epi (-15s, 95%CI -42 to 12) and particularly Col/ADP (-10s, 95%CI -18 to -2) closure times, but was not associated with other assays. Duration of aspirin therapy was not associated with platelet reactivity according to any assay.

DISCUSSION

The present study assessed the association between 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 of platelet reactivity as well as the determinants of those tests, in male patients with stable cardiovascular disease using aspirin. The VerifyNow assay showed the best correlation with S-TxB_2, which is the biochemical assay strictly reflecting the enzymatic activity of platelet COX-1 and thus measuring the pharmacologic effect of aspirin based on its mechanism of action.[10] Other correlations with S-TxB_2 were weaker (U-TxB_2, LTA-AA) or absent (other tests). Similarly, the determinants of the tests also differed between the tests, although several factors seemed to be of more general importance.

In line with previous research,[7] high platelet reactivity despite use of aspirin was also highly assay-dependent in the present study. Of the subjects using aspirin, only two (1%) subjects had S-TxB_2 values and seven (4%) subjects had LTA-AA values overlapping with subjects not using aspirin. For other tests, the overlap between aspirin users and non-users was larger. Including both subjects using and not using aspirin, the COX-1-dependent assays were all associated with each other but not with the COX-1-independent assays. However, within subjects using aspirin, the correlations were much weaker. Apparently, patients with high on-aspirin platelet reactivity according to one test do not necessarily display high platelet reactivity as assessed with other tests. As also demonstrated by previous studies, the VerifyNow Aspirin assay showed the best correlation with S-TxB_2,[15-17] both continuously and dichotomized. In our data, the relation between S-TxB_2 levels and functional COX-1-dependent tests was not linear, which corroborates previous findings[16,25] and has potentially important biological and clinical implications: S-TxB_2 needs to be completely suppressed in order to obtain a complete inhibition of platelet function both *in vivo* (urinary metabolites) and *ex vivo* (LTA-AA, and other COX-1-related functional assays). A clinical consequence may

Table 2 – Determinants of platelet reactivity

	n	S-TxB_2 ng/mL	U-TxB_2 pg/mg creatinine	LTA-AA % aggregation	VerifyNow ARU	PFA-100 Col/Epi Closure time, s	PFA-100 Col/ADP Closure time, s	P-selectin ADP AUC	P-selectin CRP-XL AUC
Age									
<70 y	103	6.8 (0.8)	1100 (56)	10 (1)	459 (6)	179 (7)	86 (3)	73 (2)	70 (2)
≥70 y	85	7.0 (1.1)	1050 (68)	10 (1)	466 (5)	173 (9)	82 (2)	76 (3)	74 (3)
		0.2 (-2.6 to 2.9)	-50 (-221 to 122)	0 (-4 to 4)	7 (-8 to 22)	-6 (-29 to 16)	-5 (-12 to 2)	3 (-4 to 10)	5 (-3 to 12)
Smoking									
No	144	7.1 (0.8)	1020 (47)	10 (1)	465 (4)	181 (7)	85 (2)	72 (2)	70 (2)
Yes	41	6.0 (1.6)	1289 (103)	12 (3)	452 (8)	159 (11)	83 (4)	83 (3)	78 (4)
		-1.1 (-4.5 to 2.2)	269 (65 to 473)	2 (-3 to 7)	-13 (-31 to 5)	-22 (-49 to 5)	-2 (-10 to 6)	11 (3 to 19)	8 (-1 to 17)
Obesity									
No	136	5.8 (0.7)	1077 (52)	10 (1)	457 (4)	175 (7)	84 (2)	78 (2)	73 (2)
Yes	52	9.9 (1.6)	1079 (79)	10 (2)	478 (8)	179 (11)	84 (4)	65 (3)	68 (4)
		4.1 (1.1 to 7.1)	3 (-190 to 196)	0 (-5 to 4)	21 (5 to 38)	4 (-21 to 29)	0 (-8 to 7)	-12 (-20 to -5)	-4 (-13 4)
Diabetes									
No	157	6.8 (0.7)	1049 (46)	10 (1)	462 (4)	174 (6)	84 (2)	74 (2)	71 (2)
Yes	31	7.5 (2.2)	1221 (119)	11 (3)	463 (9)	186 (14)	86 (3)	74 (4)	75 (5)
		0.8 (-2.9 to 4.5)	173 (-55 to 401)	1 (-5 to 6)	0 (-20 to 20)	12 (-18 to 42)	2 (-7 to 12)	0 (-10 to 10)	4 (-7 to 14)
Hypertension									
No	70	6.4 (1.0)	1028 (59)	11 (2)	459 (6)	169 (9)	82 (3)	77 (3)	71 (3)
Yes	118	7.2 (0.9)	1107 to (59)	10 (1)	464 (5)	181 (7)	86 (2)	73 (2)	72 (2)
		0.7 (-2.1 to 3.5)	79 (-98 to 255)	-1 (-5 to 3)	5 (-10 to 21)	12 (-11 to 34)	4 (-4 to 11)	-4 (-12 to 3)	1 (-7 to 8)
Aspirin dose									
80-100 mg	177	6.0 (0.5)	1062 (42)	10 (1)	459 (4)	177 (6)	84 (2)	75 (2)	71 (2)
<80 mg	9	25.0 (9.3)	1381 (382)	23 (9)	521 (30)	179 (29)	96 (9)	70 (11)	82 (6)
		19 (13 to 25)	318 (-79 to 716)	13 (4 to 22)	62 (26 to 97)	2 (-53 to 57)	12 (-5 to 29)	-5 (-21 to 12)	11 (-7 to 28)

Table 2 – Determinants of platelet reactivity

	n	S-TxB_2 ng/mL	U-TxB_2 pg/mg creatinine	LTA-AA % aggregation	VerifyNow ARU	PFA-100 Col/Epi Closure time, s	PFA-100 Col/ADP Closure time, s	P-selectin ADP AUC	P-selectin CRP-XL AUC
Lipid-lowering drugs									
No	20	9.3 (2.2)	1146 (148)	14 (5)	477 (12)	202 (18)	93 (9)	59 (5)	64 (6)
Yes	168	6.6 (0.7)	1069 (44)	10 (1)	461 (4)	173 (6)	83 (2)	76 (2)	73 (2)
		-2.7 (-7.1 to 1.7)	-76 (-352 to 199)	-5 (-11 to 2)	-16 (-40 to 8)	-29 (-65 to 6)	-10 (-21 to 1)	17 (6 to 28)	9 (-3 to 21)
Glucose									
<7.0 mmol/L	153	6.5 (0.7)	1087 (49)	9 (1)	461 (4)	175 (6)	83 (2)	74 (2)	72 (2)
≥7.0 mmol/L	34	8.7 (2.0)	1025 (96)	14 (4)	467 (9)	180 (12)	88 (5)	74 (4)	67 (4)
		2.2 (-1.4 to 5.7)	-62 (-286 to 162)	4 (-1 to 10)	5 (-14 to 25)	5 (-24 to 34)	4 (-5 to 13)	0 (-9 to 9)	-5 (-15 to 5)
LDL-cholesterol									
<2.5 mmol/L	111	5.5 (0.5)	1038 (46)	9 (1)	455 (4)	180 (7)	84 (2)	77 (2)	72 (3)
≥2.5 mmol/L	74	8.2 (1.3)	1096 (77)	11 (2)	472 (7)	171 (9)	85 (3)	70 (3)	71 (3)
		2.7 (0.2 to 5.2)	58 (-108 to 225)	1 (-2 to 5)	17 (2 to 32)	-9 (-32 to 14)	1 (-6 to 9)	-7 (-14 to 0)	-1 (-8 to 7)
HDL-cholesterol									
<0.9 mmol/L	11	10.9 (6.1)	1408 (262)	24 (10)	485 (23)	202 (28)	93 (6)	78 (5)	71 (7)
≥0.9 mmol/L	176	6.7 (0.6)	1055 (43)	9 (1)	461 (4)	174 (6)	84 (2)	74 (2)	72 (2)
		-4.2 (-10.2 to 1.8)	-353 (-712 to 7)	-14 (-23 to -6)	-24 (-56 to 8)	-28 (-75 to 19)	-9 (-24 to 5)	-4 (-19 to 11)	1 (-15 to 17)
Triglycerides									
<1.7 mmol/L	138	6.7 (0.8)	1034 (49)	10 (1)	461 (4)	178 (7)	86 (2)	74 (2)	70 (2)
≥1.7 mmol/L	49	7.5 1.5	1193 (92)	12 (3)	467 (8)	172 (11)	80 (3)	74 (3)	76 (4)
		0.8 (-2.3 to 3.9)	159 (-34 to 352)	3 (-2 to 7)	7 (-10 to 24)	-6 (-31 to 19)	-5 (-13 to 3)	0 (-8 to 8)	6 (-3 to 14)
Serum creatinine									
<77 µmol/L	49	9.8 (2.2)	1332 (107)	13 (3)	471 (8)	179 (10)	88 (3)	78 (4)	75 (3)
77-86 µmol/L	46	6.3 (1.0)	1034 (77)	11 (2)	464 (9)	174 (11)	81 (2)	73 (4)	75 (3)

Table 2 – Determinants of platelet reactivity

	n	S-TxB_2 ng/mL	U-TxB_2 pg/mg creatinine	LTA-AA % aggregation	VerifyNow ARU	PFA-100 Col/Epi Closure time, s	PFA-100 Col/ADP Closure time, s	P-selectin ADP AUC	P-selectin CRP-XL AUC
86-100 µmol/L	47	5.7 (0.7)	957 (74)	8 (0.5)	453 (6)	164 (11)	86 (5)	72 (4)	66 (4)
≥100 µmol/L	45	5.5 (0.8)	963 (69)	9 (1)	461 (6)	188 (13)	82 (4)	73 (3)	70 (5)
		-1.4 (-2.6 to -0.2)	-121 (-196 to -47)	-2 (-3 to 0)	-4 (-11 to 3)	1 (-9 to 11)	-1 (-4 to 2)	-2 (-5 to 2)	-2 (-6 to 1)
Platelet count									
<179 *10^9/L	46	5.2 (0.8)	1118 (79)	11 (2)	457 (7)	190 (12)	84 (3)	75 (4)	72 (4)
179-214 *10^9/L	46	5.6 (0.7)	1098 (78)	9 (1)	455 (7)	169 (12)	81 (2)	76 (4)	75 (4)
214-253 *10^9/L	46	6.5 (1.0)	983 (59)	9 (1)	466 (7)	167 (10)	86 (4)	77 (3)	72 (4)
≥253 *10^9/L	45	9.8 (2.4)	1098 (113)	13 (3)	468 (9)	181 (11)	86 (5)	69 (3)	69 (4)
		1.4 (0.2 to 2.7)	-18 (-96 to 61)	1 (-1 to 3)	4 (-2 to 11)	-3 (-13 to 7)	1 (-2 to 4)	-2 (-5 to 1)	-1 (-5 to 2)
Mean platelet volume									
<7.4 fL	44	8.2 (2.0)	1011 (93)	11 (2)	455 (9)	194 (12)	84 (3)	70 (3)	66 (4)
7.4-7.9 fL	43	7.0 (1.1)	1005 (75)	7 (0.4)	467 (8)	176 (13)	87 (4)	70 (3)	70 (4)
7.9-8.5 fL	47	7.8 (1.5)	1169 (96)	11 (2)	464 (7)	172 (11)	86 (5)	78 (3)	75 (4)
≥8.5 fL	45	4.5 (0.5)	1132 (91)	13 (3)	458 (8)	165 (11)	80 (2)	74 (2)	75 (4)
		-1.0 (-2.3 to 0.2)	53 (-26 to 132)	1 (-1 to 3)	1 (-6 to 8)	-9 (-19 to 1)	-1 (-5 to 2)	4 (1 to 7)	3 (0 to 7)
White blood cell count									
<5.2 *10^9/L	51	6.7 (0.8)	946 (80)	12 (2)	465 (7)	183 (11)	87 (3)	71 (4)	68 (3)
5.2-6.1 *10^9/L	41	7.3 (2.2)	1113 (102)	9 (2)	461 (8)	175 (12)	88 (6)	73 (4)	70 (4)
6.1-7.5 *10^9/L	47	6.8 (0.9)	1017 (71)	10 (2)	465 (7)	187 (12)	84 (3)	71 (3)	70 (4)
≥7.5 *10^9/L	44	6.4 (1.5)	1250 (100)	12 (3)	455 (8)	158 (10)	78 (3)	83 (3)	81 (4)
		-0.1 (-1.4 to 1.1)	82 (5 to 158)	0 (-2 to 2)	-2 (-9 to 4)	-6 (-16 to 4)	-3 (-6 to 0)	3 (0 to 7)	4 (0 to 7)
Red blood cell count									
<4.5 *10^{12}/L	46	4.5 (0.6)	1125 (81)	10 (2)	459 (5)	201 (12)	88 (3)	79 (4)	78 (4)

Table 2 – Determinants of platelet reactivity

	n	S-TxB_2 ng/mL	U-TxB_2 pg/mg creatinine	LTA-AA % aggregation	VerifyNow ARU	PFA-100 Col/Epi Closure time, s	PFA-100 Col/ADP Closure time, s	P-selectin ADP AUC	P-selectin CRP-XL AUC
4.5-4.86 *10^{12}/L	46	9.0 (2.3)	1097 (106)	16 (3)	470 (9)	177 (11)	86 (3)	78 (3)	76 (4)
4.86-5.12 *10^{12}/L	46	6.4 (0.8)	1014 (63)	9 (2)	463 (7)	162 (11)	81 (3)	75 (3)	71 (4)
≥5.12 *10^{12}/L	45	7.3 (1.2)	1058 (99)	7 (0.4)	455 (9)	166 (11)	82 (5)	66 (3)	63 (3)
		0.6 (-0.6 to 1.8)	-28 (-107 to 50)	-2 (-3 to 0)	-2 (-8 to 5)	-12 (-22 to -2)	-2 (-5 to 1)	-4 (-7 to -1)	-5 (-8 to -2)
Hemoglobin									
<8.8 mmol/L	48	8.2 (2.2)	1142 (107)	13 (3)	474 (7)	183 (11)	86 (3)	78 (4)	78 (4)
8.8-9.3 mmol/L	45	6.6 (0.9)	991 (64)	11 (2)	468 (7)	184 (12)	89 (4)	76 (4)	72 (3)
9.3-9.8 mmol/L	50	6.1 (0.7)	1049 (72)	10 (2)	453 (7)	163 (11)	84 (4)	72 (3)	72 (4)
≥9.8 mmol/L	40	6.0 (1.0)	1121 (110)	7 (0.4)	451 (8)	178 (12)	76 (2)	71 (4)	65 (4)
		-0.7 (-2.0 to 0.5)	-5 (-85 to 75)	-2 (-4 to 0)	-9 (-15 to -2)	-4 (-14 to 6)	-3 (-7 to 0)	-3 (-6 to 0)	-4 (-7 to -1)
Hematocrit (fraction)									
<0.42	46	7.8 (2.2)	1130 (110)	13 (3)	471 (7)	194 (11)	88 (3)	75 (4)	76 (4)
0.42-0.44	49	6.4 (0.8)	1004 (61)	12 (2)	468 (7)	171 (11)	83 (4)	76 (4)	71 (4)
0.44-0.47	45	6.7 (1.0)	1060 (85)	9 (2)	457 (8)	171 (11)	88 (5)	75 (4)	76 (4)
≥0.47	43	6.1 (1.0)	1111 (96)	7 (0.4)	450 (7)	171 (12)	71 (3)	71 (3)	66 (4)
		-0.5 (-1.7 to 0.8)	-1 (-81 to 79)	-2 (-4 to 0)	-7 (-14 to -1)	-7 (-17 to 3)	-3 (-6 to 0)	-2 (-5 to 2)	-2 (-6 to 1)

Relation between patient-related factors and platelet reactivity according to the various tests. The data refer to aspirin-using subjects. Mean platelet reactivity and corresponding standard errors are given in absence and presence of dichotomous factors and per quartile of continuous factors. Furthermore to regression coefficients and 95% confidence intervals are provided for the association between patient-related factors and platelet reactivity. The regression coefficients can be interpreted as mean differences in case of dichotomous factors and mean differences per quartile in case of continuous factors.
S-TxB_2 to serum thromboxane B_2; U-TxB_2 to urinary 11-dehydro-thromboxane B_2; LTA-AA to arachidonic acid-induced light transmission aggregometry; Col/Epi to collagen/epinephrine; Col/ADP to collagen/adenosine diphosphate; CRP-XL to cross-linked collagen-related peptide; AUC: area under the curve; NSAIDs: non-steroidal anti-inflammatory drugs; LDL: low-density lipoprotein; HDL: high-density lipoprotein.

be that even a missed aspirin or a less-than-complete S-TxB_2 inhibition for another, disease-based, mechanism, would possibly render less functional effect of aspirin and potentially increased vulnerability of the patient to a new cardiovascular event.

To the best of our knowledge, we used ADP- and CRP-XL-induced P-selectin expression, although well-known tests,[26-28] for the first time to study high on-aspirin platelet reactivity. Of note, these tests are COX-1-independent and are not sensitive to the effect of aspirin. There was wide inter-individual variability in agonist-induced P-selectin expression, a marker of platelet activation, which could be of additional clinical importance to explain major cardiovascular events in patients on aspirin.[10] Both tests of P-selectin expression correlated linearly with each other but not with other tests, most likely because those tests assess different aspects and pathways of platelet function.

Several of the observed associations between patient-related characteristics and high on-aspirin platelet reactivity warrant comment. A strong determinant of all COX-dependent assays was the dose of aspirin, with higher levels of platelet reactivity in subjects using <80mg aspirin (30-40mg) compared to those using 80-100mg aspirin, which is in line with previous research.[7,29] Clinical data and meta-analyses showed equal efficacy for low and high doses of aspirin. However among the low-dose range (<150mg/day), the lowest doses (<75mg) may be less effective than doses of 75-150mg[3] and associated with the highest inter-individual variability in response.

The relation between obesity, dyslipidemia and diabetes and thromboxane-dependent platelet activation has well been established, possibly because of low-grade inflammation leading to lipid-peroxidation[23,30-32] or different bioavailability of aspirin associated with increased BMI.[33,34] We found increased S-TxB_2 and VerifyNow levels in aspirin-using patients with obesity in multivariable analyses, in agreement with previous observations.[33,34] Furthermore, high LDL-cholesterol levels were associated with higher S-TxB_2 and VerifyNow levels, while HDL-cholesterol was inversely related with all COX-dependent assays. In multivariable analyses, HDL-cholesterol and the total cholesterol:HDL-cholesterol ratio were related with platelet reactivity according to S-TxB_2, U-TxB_2, LTA-AA and VerifyNow, corroborating these findings. HDL-cholesterol is probably related to decreased platelet reactivity independently of its lipid-transporting properties.[35-37] Statin use has previously been associated with TxA_2 inhibition,[38,39] but was not clearly associated with COX-1-dependent tests among aspirin users in our data, although the effect estimates point into the direction of lower platelet reactivity, but among non-aspirin users there was a clear inverse relation between statin use and S-TxB_2 and U-TxB_2 levels. We found no clear associations of platelet reactivity with diabetes or serum glucose levels, although the total number of subjects with diabetes was low. Remarkably, obesity and statin use were inversely associated with agonist-induced P-selectin expression compared with the COX-dependent tests, i.e. obesity was associated with lower platelet reactivity and statin use with higher platelet reactivity. Potentially, this is result of the selection of patients. Subjects with cardiovascular disease not using statins after many years may represent a relatively healthy subgroup and the combination of obesity and hyperreactive platelets might have been detrimental before inclusion in the platelet function study.

Furthermore, higher serum creatinine levels were consistently and dose-dependently associated with less platelet reactivity according to all tests except the PFA-100. Uremia is known to lead to platelet dysfunction and associated bleeding diathesis.[40] However, in our study, platelet reactivity already increased with still physiological creatinine values when compared with the lowest quartile. Another study found decreased mean platelet volume and platelet counts even in subjects with a mildly decreased estimated creatinine clearance. Indeed, in our data a creatinine clearance below 90 mL/min/1.73 m^2 (which was present in 70% of included subjects given the relatively old age of participants) was consistently associated with decreased platelet reactivity. An analysis per quartile revealed similar results. This observation might have profound implications, e.g. in (older) diabetic subjects with renal complications.[41,42]

We observed several associations between hematologic parameters and platelet reactivity. A higher platelet count and mean platelet volume were positively associated with several tests, indicating increased platelet function in case of more available platelets and increased platelet turnover, which are known determinants of platelet reactivity.[22,43] Remarkably, hemoglobin and hematocrit were *inversely* associated with platelet reactivity, particularly according to LTA-AA, VerifyNow and both tests of P-selectin expression. Our data corroborate findings from some other studies in which hemoglobin or hematocrit were also inversely associated with the VerifyNow system as well as ADP-induced LTA,[43-46] although the underlying mechanism is currently unclear. A recent study showed an inverse association between hemoglobin, hematocrit, red blood cell count and the number of reticulated platelets, which in turn was associated with high platelet reactivity.[47] Therefore, an increased platelet turnover as a compensatory mechanism of the bone marrow associated with anemia may explain the observed inverse relationship between hemoglobin and platelet reactivity according to several tests.[48] Contrarily, relatively increased citrate concentrations associated with higher hematocrit levels may also contribute to an inverse association between hematocrit and ex vivo platelet reactivity.[49]

Our study has some potential limitations. First, platelet function was only measured once and it has been shown that platelet reactivity varies within individuals.[16] However, the same study showed that at a group level platelet reactivity was consistently inhibited by aspirin over time.[16] Therefore, if anything, the reported associations in the present manuscript are underestimations of the real effect because of non-differential misclassification. Second, poor compliance to aspirin could have been a cause of high platelet reactivity. This would not influence the associations between the different tests, but could have influenced the association between patient-related factors and on-aspirin platelet reactivity. Nevertheless, all subjects were urged several times to adhere to their medication very strictly at least in the week before blood sampling and only two aspirin subjects (1%) had S-TxB_2-levels overlapping with non-users. Third, platelet reactivity was measured in a population that survived a first myocardial infarction for many years. We do not expect that this influenced the correlation between the tests and the determinants of the various tests.

In conclusion, we showed that among male subjects with established cardiovascular disease chronically using aspirin, high on-aspirin platelet reactivity was not a uniform

finding using several COX-1-dependent and independent tests. The best correlation was shown between the VerifyNow Aspirin assay and S-TxB_2 levels. Important determinants of platelet reactivity according to the various tests were a lower aspirin dose, obesity, dyslipidemia, higher platelet count, higher mean platelet volume, lower serum creatinine or glomerular filtration rate and lower hemoglobin or hematocrit. Future studies are needed to establish which test best identifies patients at high risk of recurrent cardiovascular disease in patients with stable cardiovascular disease using aspirin.[50]

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