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Platelet reactivity and cardiovascular events

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GENERAL DISCUSSION
AND SUMMARY

INTRODUCTION

Cardiovascular disease, particularly arterial thrombosis, is one of the leading causes of mortality and morbidity in the Western world. Platelets play an important role in the development of arterial thrombosis, not only in the acute onset of thrombosis after atherosclerotic plaque rupture but also in the initiation and progression of atherosclerosis and plaque formation.^{1,2} In recent years, the awareness has grown that platelet function may vary among individuals and that high platelet reactivity may increase the risk of cardiovascular events.³⁻⁶ This thesis addresses variation in platelet reactivity in relation to occurrence of cardiovascular events. The aim of this thesis was to provide insight into the extent, the causes and the clinical consequences of interindividual variation in platelet reactivity. This has been set out in further detail in chapter 1.

HIGH ON-TREATMENT PLATELET REACTIVITY

High on-aspirin platelet reactivity

In the chapters 2, 3, 5 and 6, we studied high on-aspirin platelet reactivity, i.e., platelet reactivity in subjects treated with aspirin. In chapter 2, we set out to systematically review and quantify all available evidence on the prevalence of high on-aspirin platelet reactivity in subjects with cardiovascular disease. We included 42 studies in our meta-analysis. Many different tests were used to quantify platelet reactivity. The mean prevalence of high platelet reactivity was 24% (95% confidence interval (95%CI) 20 to 28%). However, there was considerable heterogeneity among prevalence estimates in the different studies. In order to explain this heterogeneity, we examined whether the prevalence depended on the study population, the aspirin dose, and differences in definitions of high platelet reactivity (i.e., the used test). The prevalence decreased with increasing dose of aspirin: The overall weighted mean prevalence was 36% (95%CI 28 to 43) in subjects using 100 mg aspirin per day or less, 28% (95%CI 21 to 36) in subjects using 100 to 300 mg, and 19% (95%CI 11 to 26) in subjects using 300 mg or higher. Furthermore, it appeared that the prevalence was highly assay-dependent: in studies using arachidonic-acid induced light transmission aggregometry (LTA-AA), which is a relatively specific test for the effect of aspirin, the prevalence was 6% (95%CI 0 to 12), whereas in less specific tests the prevalence was much higher (26%, 95%CI 21 to 31).

An important question is whether high on-aspirin platelet reactivity has clinical relevance, i.e., is associated with increased cardiovascular risk, or merely is an *in vitro* phenomenon in the laboratory. Therefore, in chapter 3 we studied the association of high on-aspirin platelet reactivity with recurrent cardiovascular events in subjects with manifest cardiovascular disease. To this end, we again systematically reviewed the available evidence in literature. We could include 16 studies, of which 15 reported an adverse association between high platelet reactivity and cardiovascular outcome. The pooled odds ratio (OR) was 3.8 (95%CI 2.3 to 6.1), indicating a nearly fourfold

increased risk of cardiovascular events in subjects with high on-aspirin platelet reactivity. Later, this has been corroborated by other meta-analyses which could also include recent studies.^{7,8} However, those meta-analyses could not answer the important question which definition is most informative from a clinical point of view, i.e., which test and which cut-off point best indicate which subjects are at high risk of recurrent cardiovascular events. As mentioned above, the prevalence of high platelet reactivity depends on the test used, which implies that at least the magnitude of the risk associated with high platelet reactivity according to the different tests should vary.

To further evaluate high on-aspirin platelet reactivity according to different tests and the relation with recurrent cardiovascular events, we designed the Study of Myocardial Infarction in LEiden (SMILE)-Platelets project, of which the results are presented in **chapter 5** and **6**. Platelet reactivity was measured in 252 male subjects with stable cardiovascular disease with eight tests: serum thromboxane B₂ (S-TxB₂), urinary 11-dehydro-thromboxane B₂ (U-TxB₂), LTA-AA, the VerifyNow Aspirin assay, the PFA-100 collagen/epinephrine (Col/Epi) and collagen/adenosine diphosphate (Col/ADP) cartridges, and ADP- and cross-linked collagen-related peptide (CRP-XL) P-selectin expression. S-TxB₂ is the most specific test of COX-1-dependent platelet reactivity, the pharmacological target of aspirin.⁹ U-TxB₂, LTA-AA, the VerifyNow Aspirin assay and the PFA-100 Col/Epi cartridge are also COX-1-dependent, whereas the other tests are COX-1-independent. In **chapter 5** we have studied the correlation between the different tests as well as the association between several subject characteristics and platelet reactivity. On-aspirin platelet reactivity varied markedly according to the different tests. The point-of-care VerifyNow Aspirin assay correlated fairly well with serum thromboxane B₂ levels: $r^2=0.49$, agreement highest quintiles 85%, kappa 0.53 (95%CI 0.38 to 0.67). The correlation between other tests was weaker or even absent. Similarly, the determinants of the tests also differed between the tests, although several factors seemed to be of more general importance, including aspirin dose below 80 mg (only COX-1-dependent tests), obesity, dyslipidemia, high platelet count, high mean platelet volume, decreased renal function and low hemoglobin and hematocrit. We can conclude that most tests, even those that are COX-1-dependent, identify different subjects as having high on-aspirin platelet reactivity, which underscores the need for studies comparing different tests of platelet reactivity in relation to clinical outcome.

The analysis of the relationship of the different tests of high on-aspirin platelet reactivity with recurrent cardiovascular events in SMILE-Platelets is presented in **chapter 6**. 179 patients were available for this analysis. 91 of them had a major adverse cardiac event (MACE) during a median follow-up of 15 years prior to platelet function testing, whereas 88 remained event-free after their first myocardial infarction. Remarkably, in our data several COX-1-dependent tests were inversely associated with MACE, whereas the COX-1-independent tests, particularly ADP-induced P-selectin expression, were positively associated with MACE. Comparing the highest quintile with lower levels of platelet reactivity, ORs with 95%CIs for MACE were 0.36, 0.16 to 0.79 (S-TxB₂); 1.19, 0.57 to 2.49 (U-TxB₂); 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 (OR 2.05, 95%CI 1.12 to 3.74) and CRP-XL-induced P-selectin expression (OR 2.08, 95%CI 1.12 to 3.85) were also associated with MACE. In the discussion of **chapter 6** we discussed several potential explanations of this remarkable difference between COX-1-dependent and COX-1-independent tests. Several biases may have been introduced because platelet reactivity was measured in a selected group of subjects many years after surviving a first myocardial infarction, including survival bias, better compliance in subjects with a MACE after the event than in subjects without MACE, and increased medication use (e.g., statins) in subjects with MACE after the event. However, these potential biases are unlikely to entirely explain the discrepancy between the COX-1-dependent and COX-1-independent tests. Therefore, as an alternative explanation, we also discussed the possibility that the associations reflect a real effect. Several lines of evidence suggest that it is possible that long-term aspirin use has adverse effects on clinical outcome, which would explain the inverse association between the COX-1-dependent tests and MACE. Further study is needed to address this striking hypothesis.

High on-clopidogrel platelet reactivity

In **chapter 4** we systematically reviewed and quantified all available evidence regarding the prevalence and clinical consequences of high on-clopidogrel platelet reactivity in patients undergoing coronary stent implantation. We again applied a systematic search strategy and could eventually include 25 studies in our analysis. Most studies used ADP-induced LTA to evaluate platelet reactivity, but the concentration of ADP and the used cut-off point differed over studies. The mean prevalence of high platelet reactivity was 21% (95%CI 17 to 25) and the occurrence of high platelet reactivity was inversely associated with the used loading dose of clopidogrel and the time between loading and platelet reactivity testing. The mean prevalence was 22% (95%CI 15 to 29) when a loading dose of 300 mg was used, compared with 7% (95%CI 0 to 15) with 600 mg. The prevalence was 36% (95%CI 28 to 44) when assessed within 24 hours after loading, 13% (95%CI 5 to 21) 24 to 48 hours after loading, 10% (95%CI 2 to 18) 2 to 7 days and 0% (95%CI 0 to 7) when assessed more than 7 days after loading. When the analysis was restricted to studies using 600 mg, there was no effect of time between loading and measurement of platelet reactivity. This led us to the hypothesis that a higher loading dose or earlier loading before the intervention could reduce high on-clopidogrel platelet reactivity. The recently presented CURRENT OASIS 7 trial indeed showed that a loading dose of 600 mg decreased the risk of stent thrombosis and other cardiovascular events in subjects undergoing percutaneous coronary intervention compared with a loading dose of 300 mg.¹⁰

All studies reported an increased risk of cardiovascular events in subjects with high platelet reactivity. The pooled OR was 8.0 (95%CI 3.4 to 19.0). Recently, two updated meta-analyses have been published, in which the ORs were 5.7 (95%CI 3.0 to 10.8) and 3.5 (95%CI 2.4-5.2), respectively.^{11,12} For high on-clopidogrel platelet reactivity, studies directly comparing different tests are also scarce. Recently, the POPular study has shown that high platelet reactivity according to LTA-ADP, the VerifyNow P2Y12 and the PlateletWorks assay was associated with the risk of cardiovascular events,

while several other tests were not.¹³ However, the predictive ability was modest in these assays.

Should we test for high on-treatment platelet reactivity?

Given the results of our studies and other studies in the field an interesting question is whether we should test subjects using aspirin or clopidogrel for high on-treatment platelet reactivity in regular clinical practice. Important issues to consider are: (1) Does high platelet reactivity cause cardiovascular events? (2) Does platelet reactivity testing add in the prediction of adverse clinical events and which test confers the best predictive ability? (3) Is there a therapeutic option for patients with high platelet reactivity? (4) Is tailored antiplatelet therapy effective, in terms of the absolute number of subjects to be screened and additionally treated, adverse effects and costs?

We found an increased risk of cardiovascular events in subjects with high on-aspirin platelet reactivity, reported in **chapter 3**. Since the publication of this study multiple additional, mostly small studies have evaluated the relation between high on-aspirin platelet reactivity and clinical events. The largest studies hitherto have demonstrated an increased risk of cardiovascular events associated with high levels of U-TxB₂,^{14,15} S-TxB₂,¹⁶ LTA-AA,¹⁷ the VerifyNow Aspirin assay,¹⁷ and the PFA-100 Col/ADP cartridge,¹⁶ independent of major cardiovascular risk factors. Furthermore, in **chapter 6**, we report a positive association of COX-independent tests ADP-induced P-selectin expression, and, to a lesser extent, CRP-induced P-selectin expression and the PFA-100 Col/ADP cartridge. However, as mentioned above, we found an inverse association using S-TxB₂, LTA-AA and VerifyNow Aspirin and no association using U-TxB₂ and the PFA-100 Col/Epi cartridge. Given our data, particularly the COX-1-independent tests may provide valuable information in subjects using aspirin for cardiovascular prevention. However, given the results of previous studies, we cannot conclude that high-platelet reactivity according to the COX-1-dependent tests is not associated with cardiovascular risk or even associated with a decreased cardiovascular risk, solely based on our study. This holds especially for relatively short- to medium-term risks, as the follow-up time in previous studies that found a positive association was much shorter (at most 5 years, usually much shorter) than in our study (15 years). Given the paucity of adequately powered studies, additional studies have to be done to establish the association of COX-1-dependent and particularly COX-1-independent platelet reactivity with cardiovascular events, as our study was the first study that examined ADP- and CRP-XL-induced P-selectin expression in this context. Furthermore, tests need to be standardized, optimal cut-off values need to be established, and the improvement in prediction beyond traditional cardiovascular risk factors needs to be studied.

Data about the potential treatment of high on-aspirin platelet reactivity are also scarce. The data presented in **chapter 2** and **5** as well as two small trials indicate that an increase in dose may overcome high platelet reactivity.^{18,19} However, a drawback of dose-escalation could be the decrease in prostacyclin and endothelial progenitor cells associated with a high aspirin dose, which may offset potential clinical benefit.^{20,21} As aspirin use did not affect PFA-100 Col/ADP closure times and P-selectin expression (**chapter 5**), increasing the aspirin dose is not likely to influence platelet reactivity

according to those measures. One small study documented a decrease in COX-1-dependent platelet reactivity in subjects with high platelet reactivity randomized to receive fish oil capsules.¹⁹ The ongoing ASCET study evaluates the effect of changing treatment with aspirin into clopidogrel.²² In summary, although there seems to be a link between high on-aspirin platelet reactivity, both COX-1-dependent and COX-1-independent, and the risk of cardiovascular events, much research has to be done before platelet reactivity testing might be routinely performed in clinical practice.

For high on-clopidogrel platelet reactivity, much more is known. Unlike aspirin, which inhibits >95% of TxA₂ synthesis²⁰, the effect of clopidogrel is much more variable, most likely because it is a non-active pro-drug which requires metabolization in the liver. The responsiveness to clopidogrel follows a normal distribution.⁹ In addition to the studies included in our analysis in **chapter 4**, several large studies have shown that using several tests prediction of cardiovascular events can be improved beyond classical risk factors and angioplasty-related factors, although modestly.^{13,23-27} Although the negative predictive values were generally high, the positive predictive values were only about 10 to 15%. Furthermore, it has been shown that high on-clopidogrel platelet reactivity can be reduced by the new antiplatelet agents prasugrel and ticagrelor.^{28,29} To get an idea about the potential clinical benefit of platelet reactivity testing, assuming high platelet reactivity in 40% of subjects¹³, a positive predictive value for cardiovascular events within a year of 15%, and a 25% relative risk reduction of cardiovascular events by changing treatment, the number of patients needed to test to prevent one event would be 100 and the number of these patients needing a change of treatment to prevent one event would be 40. Of note, when the effect of changing treatment would be smaller, these numbers will increase, and when a population at higher risk is tested, these numbers may decrease. Currently, several randomized trials evaluate the effect of tailoring antiplatelet therapy on clinical outcome (GRAVITAS (NCT00645918), DANTE (NCT00774475), ARCTIC (NCT00827411), and TRIGGER-PCI (NCT00910299)). At present, testing for high on-clopidogrel platelet reactivity is not advisable.

HIGH BASAL PLATELET REACTIVITY

As platelets are involved in the occlusion of arteries after rupture of an atherosclerotic plaque and also contribute to atherosclerotic plaque formation, we hypothesized that interindividual variation in basal platelet reactivity may influence the risk of cardiovascular events. In **chapter 7**, we have examined this hypothesis in a population-based case-control study among premenopausal women. Platelet reactivity was estimated by plasma levels of neutrophil activating peptide (NAP)-2, CXC chemokine ligand (CXCL)4, soluble glycoprotein Ib and soluble P-selectin in 203 cases with myocardial infarction and 628 control subjects. Interestingly, those molecules are not only markers of platelet reactivity but also directly exert proinflammatory and atherogenic effects. High platelet reactivity was associated with a two- to threefold incidence of myocardial infarction. ORs adjusted for potential confounders were 3.0 (95% CI 1.4 to 6.4) for NAP-2, 2.2 (95% CI 0.9 to 5.1) for CXCL4, 1.9 (95% CI 0.7 to 4.6) for soluble P-selectin and 2.5 (95% CI 1.1 to 5.7) for soluble GPIb. The incidence of myocardial infarction dose-dependently increased

when more markers were elevated. High platelet reactivity according to both NAP-2 and soluble GPIb was associated with an up to tenfold increased incidence (OR 9.9, 95% CI 2.0 to 48.3). So, our results suggest that high basal platelet reactivity contributes to an increased risk of myocardial infarction. Of note, we could not address the question whether those molecules themselves caused a higher risk or that they are a marker of high platelet reactivity which increased the risk. Given their function, it is biologically plausible that those molecules influence the risk directly via their proinflammatory and proatherogenic effects.^{30,31} Of note, as discussed in **chapter 7**, although the association between platelet reactivity and myocardial infarction seemed to very consistent in multiple multivariable analyses and sensitivity analyses, residual bias and confounding could not be excluded. Future studies are needed to confirm our findings and to study the potential clinical applicability of the use of those plasma markers of platelet reactivity in risk stratification.

GENETIC VARIATION OF PLATELET REACTIVITY

One of the most important determinants of variation in platelet reactivity may be genetic variation, because this would cause a life-long effect on platelet reactivity and may also modify the effects of antiplatelet therapy.³²⁻³⁴ In **chapter 8**, we evaluated the effect of polymorphic variation in *GP6*, the gene encoding the platelet receptor for collagen glycoprotein VI, on platelet function and cardiovascular disease. We studied the association of the polymorphism T13254C (rs1613662), which distinguishes the two major isoforms of *GP6* on platelet activation and aggregation in 274 subjects with cardiovascular disease within the SMILE-Platelets project, and in 219 healthy volunteers. Platelet activation was measured by CRP-XL-induced P-selectin expression and platelet function was measured by CRP-XL-induced light transmission aggregometry. CRP-XL is a specific agonist for glycoprotein VI. The association with first myocardial infarction was studied in the SMILE case-control study, which included 547 male subjects with myocardial infarction and 646 control subjects. The association with recurrent events was studied among the 542 male subjects included in the SMILE follow-up study and in the placebo group of the Heart and Estrogen/progestin Replacement Study (HERS), including 1074 women.

We found that carriership of the minor C-allele was strongly associated with a reduced percentage of platelets expressing P-selectin (reduction per C-allele 23%, 95%CI 18 to 28) in response to CRP-XL, as well as reduced CRP-XL-induced aggregation (reduction per C-allele 10%, 95%CI 2 to 18). This effect on platelet activation and function did not translate in a reduced risk of first myocardial infarction, but the risk of recurrent cardiovascular events (hazard ratio per C-allele 0.81, 95%CI 0.66 to 0.99) and all-cause mortality (0.73, 95%CI 0.55 to 0.96) was reduced in carriers of the C-allele.

These results indicate that polymorphic variation in *GP6* is a strong determinant of collagen-induced platelet reactivity. Furthermore, variation in *GP6* is a risk factor for recurrent cardiovascular disease and mortality, most likely through its effects on platelet reactivity. Thus, we could relate genetic variation in *GP6* to clinical outcome and put

forward a mechanistic explanation. Another conclusion following from these data is that the glycoprotein VI receptor plays a role in the development of cardiovascular events, which could have clinical implications with respect to pharmacological antagonism of this receptor. Currently, several glycoprotein VI receptor antagonists are in the preclinical phase of development, and some promising results in humans have already been shown using one of those molecules.^{35,36} Future studies are needed to study the effects of variation in other genes that may influence platelet reactivity.

FIRST AND RECURRENT CARDIOVASCULAR EVENTS

Interestingly, as reported in **chapter 8**, variation in *GP6* was associated with recurrent events and mortality, but not with first myocardial infarction. Moreover, in clinical studies, the relative risk reduction of first cardiovascular events seemed to be lower compared with the reduction of second cardiovascular events.^{37,38} Contrarily, we did find an association between platelet reactivity according to several plasma markers and first myocardial infarction in **chapter 7**. However, previous studies addressing platelet reactivity according to plasma markers did not reveal a consistent association with first atherothrombotic event (cf. with **chapter 7**).

Given these findings, the effect of platelet reactivity seems to be more pronounced in recurrent cardiovascular events than in first cardiovascular events. As an explanation for this phenomenon we hypothesize that the pathophysiology of first and recurrent cardiovascular events differs. Development of atherosclerosis and underlying classical cardiovascular risk factors are major determinants of first coronary artery events. Although platelets play a role in the acute onset of cardiovascular events and the progression of atherosclerosis and are therefore expected to be involved in the development of first events too, this effect might be a relatively modest effect compared with the effects of classical risk factors and therefore easily overshadowed. Contrarily, in the situation of recurrent events, when progressive atherosclerosis and stenosis is already present, factors influencing thrombus development may be of more importance than other risk factors, which may explain the stronger effects seen in the context of recurrent events in subjects with manifest vascular disease. In other words, the fraction associated with platelet reactivity in sufficient-cause models may be relatively larger in the causation of recurrent events than first events.³⁹

Interestingly, we did find an association between platelet reactivity according to several plasma markers and first myocardial infarction in **chapter 7** while previous studies did not. This might be explained by the difference in study populations included in the various studies. Previous studies were performed in relatively high-risk study populations, in which the potential effects of platelet reactivity may have been overwhelmed by the abundant presence of classical cardiovascular risk factors, as suggested above. However, we studied platelet reactivity in premenopausal women. In this low-risk group classical risk factors are unlikely to entirely explain cardiovascular disease. Thus, in young women, the fraction of platelet reactivity in sufficient-cause could be larger than in older populations in which classical risk factors

have accumulated over time. Of note, since the incidence of cardiovascular disease in young women is low given the relative absence of risk factors in most subjects, the absolute risk associated with high platelet reactivity in this population remains low.

ASPIRIN AND BLOOD PRESSURE

One of the most important risk factors for cardiovascular disease is arterial hypertension. Aspirin is usually thought to have no effect on blood pressure. However, recent studies indicate that aspirin may influence blood pressure, depending on the time of intake.^{40,41} In those studies, use of aspirin decreased blood pressure when taken at bedtime, whereas there was no effect or even an increase of blood pressure when aspirin was taken on awakening. However, a biologically plausible mechanism underlying this phenomenon had not been revealed. In **chapter 9** we have addressed this problem. In a randomized controlled cross-over trial, we compared the effect of aspirin intake on awakening with intake at bedtime with respect to various determinants of blood pressure. Both after intervention with aspirin on awakening and at bedtime, subjects were admitted to our center for 24 hours. Blood was sampled on a regular basis and subjects collected their urine during 24 hours. The main results of our study are that aspirin intake at bedtime compared with on awakening reduced average (24-hour) plasma renin activity by 0.08 $\mu\text{g/L/h}$ (95%CI 0.03 to 0.13), as well as excretion of cortisol (52 nmol/24h, 95%CI 5 to 99), dopamine (0.25 $\mu\text{mol}/24\text{h}$, 95%CI 0.01 to 0.48) and norepinephrine (0.22 $\mu\text{mol}/24\text{h}$, 95%CI -0.03 to 0.46) in 24-hour urine. The reduced activity of these pressor systems associated with aspirin use at bedtime forms a biologically plausible explanation for the finding that aspirin at bedtime may reduce blood pressure, whereas aspirin at morning does not. Future study is needed to reveal the exact mechanism whereby aspirin time-dependently affects those systems.

Given the results of our study and the previous studies, one might consider advising patients to take their aspirin at bedtime rather than on awakening, which is usually current practice. This would be a intervention without any costs which could potentially have a major public health impact, given the high incidence of cardiovascular disease and the prevalence of aspirin use. However, before this strategy may be implemented in clinical practice, future studies are warranted to assess whether the potential blood pressure-lowering effects of aspirin taken at bedtime sustain in this group of patients who have a clinical indication to be treated with aspirin, i.e. patients with established cardiovascular disease. This may be challenging because these patients are likely treated with a variety of antihypertensive drugs which may dilute or interact with the time-dependent effects of aspirin on blood pressure. Another unanswered but important question is whether effects of aspirin on platelet aggregation vary according to time of intake. Ultimately, clinical endpoint studies are needed in order to answer the final question whether aspirin given at bedtime will lead to incremental cardiovascular protection beyond treatment on awakening.

CONCLUSIONS

In summary, according to the data presented in this thesis, interindividual variation in platelet reactivity is an important factor in the development of cardiovascular events. The various chapters showed that high platelet reactivity in subjects using aspirin or clopidogrel, high basal platelet reactivity, and genetic variation in platelet reactivity are related to the risk of cardiovascular events. Moreover, the 110-year old antiplatelet drug aspirin has interesting pleiotropic effects on various pressor systems underlying blood pressure. It has to be determined yet whether those effects are related to COX-1 inhibition, the primary pharmacological target of aspirin, or whether aspirin could influence mechanisms underlying blood pressure independent of COX-1 inhibition. As discussed above, although promising results have been published, routine platelet reactivity testing in daily clinical practice would currently be premature. Future studies are therefore warranted to further investigate the clinical applicability of platelet reactivity testing in subjects at risk for cardiovascular events.

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