Platelet reactivity and cardiovascular events

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HIGH BASAL PLATELET REACTIVITY IS ASSOCIATED WITH MYOCARDIAL INFARCTION IN PREMENOPAUSAL WOMEN: A POPULATION-BASED CASE-CONTROL STUDY

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ABSTRACT

Background

Platelets are involved in the occlusion of coronary arteries after rupture of an atherosclerotic plaque. Furthermore, activated platelets release large quantities of growth factors, chemokines and interleukines that regulate inflammatory reactions. Therefore, we hypothesized that high basal platelet reactivity may contribute to an increased risk of myocardial infarction in premenopausal women.

Methods

We assessed the relation between high platelet reactivity and myocardial infarction in a population-based case-control study among premenopausal women (aged <50 years). We used multivariable logistic regression to quantify the effect of high platelet reactivity, adjusted for potential confounders. Platelet reactivity was estimated by plasma levels of neutrophil activating peptide 2 (NAP-2), CXC chemokine ligand (CXCL)4, soluble glycoprotein Ib (sGPIb) and soluble P-selectin.

Results

High platelet reactivity (i.e., levels ≥90th percentile control subjects) was associated with a two- to threefold increased incidence of myocardial infarction: adjusted odds ratios were 3.0 (95% confidence interval 1.4 to 6.4) for NAP-2, 2.2 (0.9 to 5.1) for CXCL4, 1.9 (0.7 to 4.6) for sP-selectin and 2.5 (1.1 to 5.7) for sGPIb. The incidence of myocardial infarction dose-dependently increased when more markers were elevated. High platelet reactivity according to both NAP-2 and sGPIb was associated with an up to ten-fold increased incidence (odds ratio 9.9, 95% confidence interval 2.0 to 48.3).

Conclusions

High basal platelet reactivity was associated with a two to three-fold higher incidence of myocardial infarction compared with normal platelet reactivity in premenopausal women. Our results suggest that high basal platelet reactivity may contribute to a higher risk of myocardial infarction.

INTRODUCTION

Platelets are involved in the occlusion of the coronary arteries after rupture of an atherosclerotic plaque. Clinically, the importance of platelet aggregation has been firmly established by large trials which have shown that antiplatelet therapy attenuates the risk of ischemic heart disease.[1] Moreover, recent studies have shown that variation in platelet function in patients using antiplatelet therapy is an important determinant of atherothrombotic events.[2,3] Platelets are not only key players of the primary haemostatic system but also seem to be involved in the regulation of inflammatory reactions.[4] Platelet activation is accompanied by the release of large quantities of growth factors, cytokines, chemokines and interleukins, which play a role in the interaction with white blood cells and endothelial cells.[4,5]

Upon activation, platelets release their granule contents as soluble proteins into the circulation. The most abundant chemokines in the α-granules are neutrophil activating peptide 2 (NAP-2), which is the active end product of a series of products collectively called β-thromboglobulins, and CXC chemokine ligand (CXCL)4 , also known as platelet factor 4.[6-8] There is extensive evidence that both NAP-2[6-8] and CXCL-4[9-14] play important roles in hemostasis and regulation of inflammation. P-selectin (CD62P) plays a role in platelet-leukocyte interactions and adhesion of platelets and leukocytes to the endothelium.[15] Upon platelet activation, P-selectin migrates from the α-granule membrane to the outer cell membrane and is released as a soluble protein (sP-selectin) into the circulation, which has been proposed as a marker of in vivo platelet activation.[15,16] Soluble glycoprotein Ib (sGPIb, CD42b), a platelet receptor that is released into the circulation upon platelet activation,[17] is another attractive platelet activation marker, because GPIb is platelet-specific and highly abundant on the platelet wall.

Because of the major role of platelets in hemostasis and inflammation, high platelet reactivity according to those platelet activation markers may be detrimental for ischemic heart disease. Several studies have been performed to assess the relation between plasma markers of platelet reactivity and the risk of cardiovascular disease, but they did not reveal consistent results.[15,18-27] A disadvantage of those studies is that they generally 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. The risk associated with high platelet reactivity has never been studied in premenopausal women, who form an ideal study population to study the effect of platelet hyperreactivity, since classical risk factors are unlikely to entirely explain cardiovascular disease in this low-risk group. Therefore, we have studied whether high basal platelet reactivity represented by high levels of NAP-2, CXCL4, sGPIb and sP-selectin is associated with an increased risk of myocardial infarction in premenopausal women.

METHODS

Study design

The Risk of Arterial Thrombosis In relation to Oral contraceptives (RATIO) study is a multicenter population-based case-control study among premenopausal women. The study was initiated to evaluate the risk of arterial thrombosis due to oral contraceptives of different generations (1990 to 1995).[28] Blood was collected during the second phase of the study, in order to study prothrombotic conditions of the coagulation system (1998 to 2002).[29] The median time between the index event and blood sampling was 5 years (range 3 to 9 years). All participants gave written informed consent and the study was approved by the Medical Ethics Committees of the participating centers and performed in accordance with the Declaration of Helsinki.

Participants

Patient selection has been described in detail previously.[28] Briefly, we included 248 women aged 18 to 49 years who presented with myocardial infarction to one of the 16 participating hospitals in the Netherlands between 1990 and 1995. These included all academic hospitals in the Netherlands and eight surrounding hospitals. Myocardial infarction was diagnosed by the presence of clinical symptoms, elevated cardiac enzyme levels, and electrocardiographic changes. A population-based control group was approached by random digit dialing. Eligible women were aged 18 to 49 years and had no history of arterial thrombosis; women were frequency matched on age, area of residence, and year of the index event. In total, 925 women were included in the control group. All participants completed a standardized questionnaire on personal and familial medical history, use of oral contraceptives, smoking habits and general participant characteristics. Blood samples were drawn in 203 cases and 628 control subjects from the antecubital vein into two Sarstedt Monovette® (SARSTEDT AG & Co, Nümbrecht, Germany) tubes containing 0.106 mol L^{-1} trisodium citrate after discarding the first milliliters and centrifuged for 10 min at 2500 g at room temperature. Plasma was stored at -70°C until analysis. Non-participation in this second stage of the study was predominantly due to lack of subject motivation. More specifically, only 1% of the patients died before blood sampling and no recurrent events occurred.

Materials

Antibodies, raised against human NAP-2 (MAB393, BAF393), human CXCL4 (MAB7951, AF795) and human soluble P-selectin (DYE137) were all purchased form R&D systems, Abbington, UK. A monoclonal antibody against GPIb (6.30) was raised in our own lab, whereas a biotin-labeled secondary anti-GPIb antibody (M1852) was purchased from Sanquin, Amsterdam, the Netherlands. Bovine serum albumin (BSA) was purchased from Sigma (A7906, Zwijndrecht, the Netherlands) and Amplex red reagent from Invitrogen (A36006, Breda, the Netherlands).

ELISA procedure

Levels of NAP-2, CXCL4, P-selectin and GPIb were successfully determined in plasma samples of 181 to 200 of the 203 patients and 585 to 610 of the 628 control subjects using semi-automated ELISA on a TECAN RSP150 robot. Failure of the measurements for some participants was assumed to be completely at random and hence subjects with a missing value for platelet reactivity were excluded from the analyses concerning that marker. Each antigen was measured on a separate Nunc maxisorb ELISA plate (Thermofischer Scientific, Roskilde, Denmark). Capture antibodies, MAB393 (1 µg/mL), MAB7951 (1 µg/mL), DYE137 capture antiboody (360 µg/mL) and 6.30 (0.9 ng/mL) were coated on four different plates for two hours. Unbound antibodies were washed with five steps PBS/0.5%Tween. Plasma samples were diluted 1/80, 1/75, 1/10 and 1/25, for NAP-2, CXCl4, P-selectin and GPIb measurements, respectively and then added to the plate with the corresponding capture antibody. Each plate contained two calibration curves (duplex) consisting of dilution ranges of a standard serum sample with known NAP-2, CXCl4, P-selectin and GPIb concentrations. Dilutions were made in PBS/1% BSA for two hours. Unbound antigens were removed with five wash steps with PBS/0.5% Tween. Detection antibodies, BAF393 (50 ng/mL), AF795 (50 ng/mL), DY137 detection antibody (3.6 µg/mL) and M1852 (0.25 µg/mL) were added to the corresponding plates. After five washing steps with PBS/0.5% Tween, streptavidin/horseradish peroxidase (HRP) was added for 2 hours to bind the biotin on the detection antibody. After five other washing steps, Amplex red reagent was added and fluorescence intensity was measured, after 1- and 3-h incubation, with a fluorstar galaxy fluorimeter (emission: 490; excitation: 520).

Statistical analyses

Given 200 cases, 600 controls and an alpha-level of 0.05, we had 93% power to detect associations with odds ratios as low as 2.0.

Continuous data are summarized as medians with interquartile ranges, whereas categorical data are presented as counts and percentages. We used unconditional logistic regression to calculate odds ratios (OR) with accompanying 95% confidence intervals (CI) for the relationship between high platelet reactivity and occurrence of myocardial infarction, which approximate incidence rate ratios.

All ORs were adjusted for the three matching variables: age (as a continuous variable), area of residence, and year of the index event. Use of age as a categorical variable did not influence the results. Since platelet reactivity at time of measurement is not necessarily the same as before the myocardial infarction, spurious associations could arise both by conventional confounders (i.e., common causes of platelet reactivity *before* the myocardial infarction and the myocardial infarction itself) and factors influencing platelet reactivity that emerged *after* the index event (e.g. use of antiplatelet therapy). In different regression models we adjusted for various combinations of the following putative confounding factors: hypertension, hypercholesterolemia, diabetes mellitus and smoking in the year before the index event; use of aspirin, statins and glucose-lowering medication (including oral drugs and insulin), smoking, systolic blood pressure, serum cholesterol, glucose and C-reactive

protein at time of blood sampling. Hypertension, hypercholesterolemia and diabetes mellitus in the index year were self-reported. Some authors have argued that levels of at least β-thromboglobulin, a precursor of NAP-2, depend on serum creatinine levels.[30] We therefore also performed the analyses conditional on serum creatinine.

High levels of platelet reactivity were defined as levels above the 90th percentile of the levels in the control subjects. As a sensitivity analysis, we also performed the analyses with the 75th percentile as cut-off point to assess whether there was a dose-response relationship. Furthermore, because use of preventive medication (i.e. aspirin, statins and glucose-lowering drugs) was rare in control subjects, which potentially might have influenced the results of our multivariable analyses adjusted for therapy, we performed a sensitivity analyses including only patients and control subjects not using any preventive medication.

RESULTS

Participants

Our study comprised 203 female patients with myocardial infarction and 628 female control subjects aged below 50 years. Participant characteristics are summarized in Table 1. As expected, cardiovascular risk factors were more common in patients than in control subjects. At time of blood sampling, differences were less obvious, probably as a result of treatment and lifestyle changes of the patients. Patients used aspirin, statins and glucose-lowering medication more commonly than control women did.

Platelet reactivity

The associations between the different platelet reactivity markers are depicted in Figure 1. NAP-2 and CXCL4 were most strongly associated with each other. Other combinations of markers hardly showed any association. In the patients with myocardial infarction, median levels of the platelet reactivity markers with corresponding interquartile ranges were 48.0 (36.2 to 66.2) ng/mL (NAP-2), 10.3 (8.4 to 12.2) ng/mL (CXCL4), 72.4 (59.0 to 90.5) ng/mL (sP-selectin) and 1.97 (1.44 to 2.50) μg/mL (sGPIb). In the control subjects, those values were 46.5 (35.7 to 58.4) ng/mL (NAP-2), 9.6 (7.7 to 1.7) ng/mL (CXCL4), 71.2 (58.1 to 86.5) ng/mL (sP-selectin) and 2.02 (1.63 to 2.47) μg/mL (sGPIb). The cut-off points for high platelet reactivity were set at 69.7 ng/mL (NAP-2), 14.0 ng/mL (CXCL4), 108.8 ng/mL (sP-selectin) and 2.98 μg/mL (sGPIb), corresponding to the 90th percentile of the control subjects.

Detailed information about the relation between platelet reactivity and subject characteristics is provided in Table 2. Briefly, patients with normal platelet reactivity (i.e. below 90th percentile of the control subjects) used statins and to a less extent aspirin more frequently than patients with high platelet reactivity, most likely indicating effects of treatment started after the myocardial infarction. Subjects with high platelet reactivity often were smokers, both before the event and at blood sampling. Patients with high platelet reactivity (presumably high NAP-2 levels) seemed to have higher

Table 1 – Characteristics of patients and control subjects

	Patients (n=203)	Control subjects (n=628)
At index event		
Age, years	45 (39-48)	40 (33-46)
Year before index event		
Smoking	167 (82%)	266 (42%)
Hypertension	53 (26%)	39 (6%)
Diabetes mellitus	10 (5%)	10 (2%)
Hypercholesterolemia	20 (10%)	19 (3)
Oral contraceptive use	80 (40%)	209 (33%)
At blood sampling		
Smoking	82 (40%)	210 (33%)
Systolic blood pressure, mmHg	134 (121-149)	128 (115-142)
Diastolic blood pressure, mmHg	84 (76-94)	81 (74-90)
Glucose, mmol/L	4.4 (3.3-5.5)	4.0 (3.3-4.8)
Cholesterol, mmol/L	5.1 (4.5-6.0)	5.5 (4.6-6.1)
HDL-cholesterol, mmol/L	1.1 (1.0-1.4)	1.4 (1.2-1.6)
Triglycerides, mmol/L	1.6 (1.1-2.7)	1.2 (0.9-1.8)
Creatinine, µmol/L	67 (61-74)	68 (61-74)
C-reactive protein, mg/L	2.8 (0.8-10.0)	1.3 (0.5-5.0)
Aspirin use	159 (78%)	11 (2%)
Glucose-lowering drug use	17 (8%)	6 (1%)
Statin use	90 (44%)	14 (2%)

Data are *n* (%) or medians (interquartile ranges), where appropriate. HDL, high-density lipoprotein.

C-reactive protein levels, indicating the relationship between platelet reactivity and inflammation. The other potential confounders specified in the methods section (among others lipid and glucose levels at time of blood sampling) hardly showed any relation to high platelet reactivity.

High platelet reactivity and the risk of myocardial infarction

The relationship between high platelet reactivity and the risk of myocardial infarction has been summarized in Table 3. The "crude" ORs with accompanying 95%CIs (model 1, only adjusted for the matching parameters) for the relation between high platelet reactivity and myocardial infarction were 2.4 (95%CI 1.5 to 3.8) for NAP-2, 1.7 (95%CI 1.0 to 2.9) for CXCL4, 1.1 (95%CI 0.7 to 1.9) for sP-selectin and 1.5 (95%CI 0.9 to 2.6) for sGPIb. In our second model, we adjusted for use of aspirin, statins and glucose-lowering medication and smoking at time of blood sampling, in addition to the matching factors. With this model, high levels of NAP-2, CXCL4, sP-selectin and

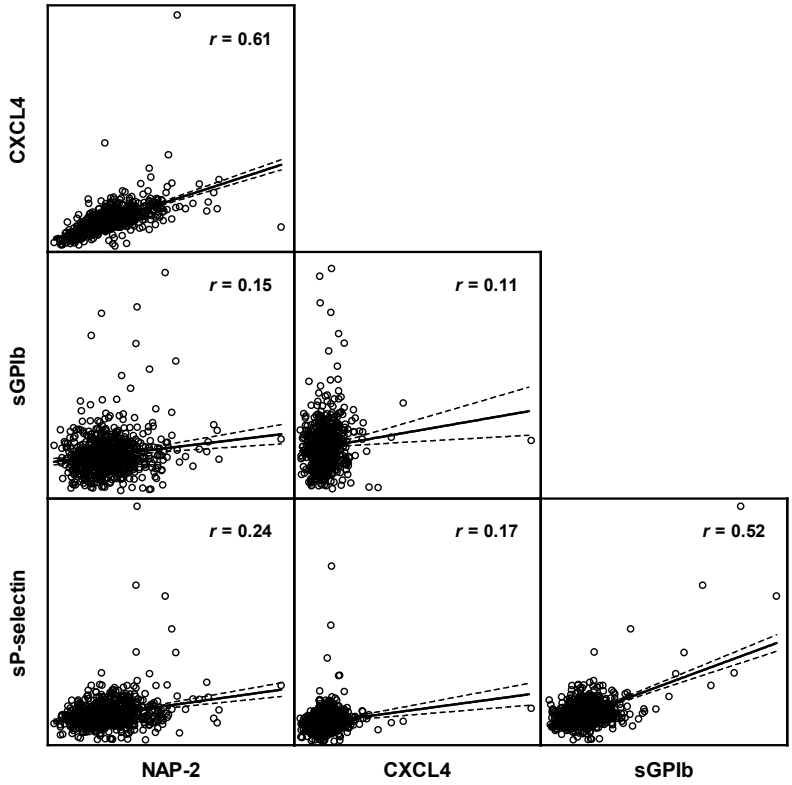

Figure 1 – Correlation between the platelet reactivity markers. The lines are linear regression lines with 95% confidence intervals indicating the strength and precision of the correlation between the markers. The correlation is quantified by *r*, which is Pearson's correlation coefficient. NAP-2: neutrophil activating peptide 2; CXCL4: CXC chemokine ligand 4; sP-selectin: soluble P-selectin; sGPIb: soluble glycoprotein Ib.

sGPIb corresponded with adjusted ORs for myocardial infarction of 3.0 (95%CI 1.4 to 6.4), 2.2 (95%CI 0.9 to 5.1), 1.9 (95%CI 0.7 to 4.6) and 2.5 (95%CI 1.1 to 5.7). The effect estimates did not change substantially when additional variables were added to the model, which is consistent with the finding that other putative confounding factors were not associated with high platelet reactivity or myocardial infarction, as mentioned above. Therefore, all further analyses were based on model 2. Adjustment for serum creatinine levels slightly increased all ORs, although the confidence intervals became also wider. To further evaluate the effect of smoking on the relation between platelet reactivity and myocardial infarction, we stratified on smoking status at time of blood sampling. Interestingly, the effect of high platelet reactivity seemed to be particularly present among smokers. ORs were 4.3 (95%CI 1.2 to 16.1) (NAP-2), 5.5 (95%CI 1.6 to 19.4) (CXCL4), 3.9 (95%CI 1.0 to 14.6) (sP-selectin) and 2.9 (95%CI 0.8 to 10.8) (sGPIb) among smokers, whereas in non-smokers effects of 2.6 (95%CI 1.0 to

6.7) (NAP-2), 1.0 (95%CI 0.3 to 3.5) (CXCL4), 1.1 (95%CI 0.3 to 4.1) (sP-selectin) and 2.3 (95%CI 0.8 to 6.8) (sGPIb) were found.

Combinations of platelet reactivity markers and the risk of myocardial infarction

To further evaluate whether the different markers of platelet reactivity represent different aspects of platelet activation, we assessed risk estimates of high platelet reactivity according to individual platelet reactivity markers conditional on the other platelet reactivity markers (in addition to model 2). This revealed ORs for the relation of high platelet reactivity with myocardial infarctions of 3.2 (95%CI 1.3 to 8.2) (NAP-2), 1.1 (95%CI 0.4 to 3.2) (CXCL4), 1.4 (95%CI 0.5 to 3.9) (sP-selectin) and 2.5 (95%CI 1.1 to 6.1) (sGPIb), respectively. In addition, the risk of myocardial infarction increased with the number of markers showing high platelet reactivity. Participants with one positive marker had an OR for myocardial infarction of 2.7 (95%CI 1.3 to 5.9) relative to participants who did not have high platelet reactivity according to any marker. Two positive markers led to an OR of 2.1 (95%CI 0.7 to 7.0), while presence of high platelet reactivity according to three of four markers corresponded to an OR of 8.9 (95%CI 2.6 to 30.1). Subjects with high platelet reactivity according to both NAP-2 and sGPIb had a ten-fold increased incidence of myocardial infarction (OR 9.9, 95%CI 2.0 to 48.3).

Sensitivity analyses

As a sensitivity analysis, we repeated our analyses using the 75th percentile of levels of platelet reactivity of the control subjects as a cut-off point. With this cut-off point, the ORs calculated with model 2 were 2.3 (95%CI 1.2 to 4.3) (NAP-2), 1.9 (95%CI 1.0 to 3.5) (CXCL4), 1.6 (95%CI 0.8 to 3.2) (sP-selectin) and 1.5 (95%CI 0.8 to 3.0) (sGPIb), indicating a dose-response relationship. Furthermore, we performed an analysis restricted to patients (n=28) and control subjects (n=602) not using aspirin, statins or glucose-lowering drugs. This analysis revealed ORs for myocardial infarction of 2.4 (95%CI 0.9 to 6.3) (NAP-2), 2.6 (95%CI 1.0 to 7.1) (CXCL4), 2.1 (95%CI 0.8 to 5.6) (sP-selectin) and 2.5 (95%CI 1.0 to 6.5) (sGPIb).

DISCUSSION

Our study addressed the question whether high basal platelet reactivity contributes to increased risk of myocardial infarction in premenopausal women. The main result of our study is that high platelet reactivity represented by high levels of NAP-2, CXCL4, sP-selectin and sGPIb was associated with a two to three-fold increased incidence of myocardial infarction. Those markers represent different aspects of platelet activation and subsequent regulation of inflammation,[8,31] and indeed, according to our data, joint exposure to high platelet reactivity according to several markers is associated with an up to ten-fold increased incidence, which may reflect complementary effects of the markers.

Table 2 – Subject characteristics in relation to platelet reactivity

	NAP-2	
	< p90	≥ p90
At index event		
Age, years	40 (33-46)	39 (33-46)
Year before index event		
Smoking	234 (43%)	24 (39%)
Hypertension	36 (7%)	2 (3%)
Diabetes mellitus	8 (2%)	2 (3%)
Hypercholesterolemia	16 (3%)	1 (2%)
Oral contraceptive use	182 (33%)	23 (38%)
At blood sampling		
Smoking	66 (42%)	16 (39%)
Systolic blood pressure, mmHg	128 (115 to 142)	130 (114 to 144)
Diastolic blood pressure, mmHg	81 (74 to 90)	80 (74 to 89)
Glucose, mmol/L	4.1 (3.3 to 4.8)	3.9 (3.3 to 4.7)
Cholesterol, mmol/L	5.3 (4.5 to 6.1)	5.4 (4.7 to 6.2)
HDL-cholesterol, mmol/L	1.4 (1.2 to 1.6)	1.4 (1.1 to 1.6)
Triglycerides, mmol/L	1.2 (0.9 to 1.8)	1.3 (0.9 to 1.9)
C-reactive protein, mg/L	1.3 (0.5 to 4.4)	2.1 (0.7 to 5.8)
Creatinine, µmol/L	68 (61 to 74)	67 (61 to 74)
Aspirin use	124 (79%)	32 (78%)
Glucose-lowering drug use	13 (8%)	3 (7%)
Statin use	73 (47%)	14 (34%)

Data are *n* (%) or medians (interquartile ranges), where appropriate. HDL, high-density lipoprotein; NAP-2: neutrophil activating peptide 2; CXCL4: CXC chemokine ligand 4; sP-selectin: soluble P-selectin; sGPIb: soluble glycoprotein Ib. P90: 90th percentile of the control subjects. The distribution of subject characteristics in relation to platelet reactivity has been determined in control subjects, except for use of preventive medication and smoking after the event, which is presented for the patients.

NAP-2 is the active product that is proteolytically cleaved from a series of precursors initially produced by the CXCL7 gene and secreted from the α-granules upon platelet activation. The most proximal (inactive) precursor of NAP-2 is known as β-thromboglobulin.[8] CXCL4 is directly secreted from the α-granules as an active protein and is also known as platelet factor 4.[8] NAP-2 and CXCL4 are currently known to play important roles in regulation of inflammation and formation of atherosclerosis.[4-8] Our data suggest that women with high levels of NAP-2 and CXCL4 have a two to three-fold increased risk of myocardial infarction. Some decades ago, largely unaware of their biologic function, multiple authors addressed the relation between β-thromboglobulin and platelet factor 4 and cardiovascular disease, with conflicting results.[18-26] This is the first study on the relation between platelet reactivity and cardiovascular disease in which a specific antibody against the biologically active NAP-2 has been used. Various

CXCL-4		sP-selectin		sGPIb	
< p90	≥ p90	< p90	≥ p90	< p90	≥ p90
40 (33-46)	39 (30-47)	40 (33-46)	43 (37-47)	40 (33-46)	40 (35-46)
228 (42%)	28 (47%)	229 (42%)	28 (47%)	218 (41%)	26 (45%)
35 (7%)	3 (5%)	35 (6%)	3 (5%)	34 (6%)	4 (7%)
7 (1%)	2 (3%)	8 (2%)	2 (3%)	10 (2%)	0 (0%)
17 (3%)	0 (0%)	16 (3%)	1 (2%)	17 (3%)	0 (0%)
181 (33%)	24 (40%)	184 (34%)	20 (33%)	174 (33%)	16 (28%)
67 (39%)	15 (52%)	67 (38%)	15 (63%)	62 (40%)	11 (41%)
128 (114 to 142)	129 (116 to 145)	128 (114 to 142)	130 (117 to 140)	129 (115 to 142)	128 (119 to 144)
81 (74 to 90)	80 (74 to 90)	81 (74 to 90)	81 (75 to 91)	81 (74 to 90)	81 (75 to 89)
4.0 (3.3 to 4.8)	4.4 (3.5 to 5.1)	4.1 (3.3 to 4.8)	4.1 (3.3 to 4.8)	4.1 (3.4 to 4.8)	3.9 (3.2 to 4.8)
5.3 (4.6 to 6.1)	5.0 (4.2 to 6.2)	5.3 (4.5 to 6.2)	5.5 (4.7 to 5.9)	5.3 (4.6 to 6.1)	5.4 (4.7 to 6.0)
1.4 (1.2 to 1.6)	1.3 (1.1 to 1.5)	1.4 (1.2 to 1.6)	1.5 (1.2 to 1.7)	1.4 (1.2 to 1.6)	1.4 (1.2 to 1.6)
1.2 (0.9 to 1.8)	1.3 (0.9 to 2.2)	1.2 (0.9 to 1.8)	1.3 (0.9 to 1.7)	1.3 (0.9 to 1.8)	1.2 (0.8 to 1.7)
1.3 (0.5 to 4.6)	1.5 (0.7 to 5.1)	1.3 (0.5 to 4.6)	1.6 (0.7 to 4.7)	1.4 (0.5 to 4.6)	0.9 (0.4 to 4.9)
68 (61 to 74)	67 (61 to 73)	68 (61 to 73)	73 (61 to 79)	68 (62 to 74)	66 (60 to 72)
135 (79%)	22 (76%)	139 (79%)	18 (75%)	124 (81%)	18 (67%)
12 (7%)	5 (17%)	13 (7%)	4 (17%)	11 (7%)	3 (11%)
79 (47%)	9 (31%)	79 (45%)	10 (42%)	69 (45%)	9 (33%)

authors have been concerned about the influence of blood sampling procedures and specifically applied stasis on levels of β-thromboglobulin and platelet factor 4, as well as potential effects of serum creatinine as a marker of renal function on β-thromboglobulin.[30,32,33] It is highly unlikely that blood-sampling procedures could have influenced our results. Blood sampling and handling was performed by well-trained and highly skilled technicians, independent of patient or control status. Furthermore, patients and control subjects visited the research centers within the same period and interchangeably. Further evidence that all four markers represent *in vivo* platelet function rather than *in vitro* manipulation is provided by functional studies with a flow cytometry based platelet function assay we performed. High platelet reactivity according to the four markers dose-dependently correlated to higher levels of convulxin-induced platelet activation (data not shown). Renal function could also not have influenced our results. Creatinine levels were not associated with platelet reactivity and adjustment for creatinine in our models only increased the effect estimates.

Table 3 – Platelet reactivity and the risk of myocardial infarction

Platelet reactivity marker	Patients (*n*)	Controls (*n*)	Odds Ratio (95% Confidence Interval)			
	(203)	(628)	Model 1	Model 2	Model 3	Model 4
NAP-2						
< 90th percentile controls	41	62	1 [ref]	1 [ref]	1 [ref]	1 [ref]
≥ 90th percentile controls	157	549	2.4 (1.5 to 3.8)	3.0 (1.4 to 6.4)	3.2 (1.5 to 6.9)	3.3 (1.5 to 7.4)
CXCL4						
< 90th percentile controls	29	60	1 [ref]	1 [ref]	1 [ref]	1 [ref]
≥ 90th percentile controls	170	548	1.7 (1.0 to 2.9)	2.2 (0.9 to 5.1)	2.4 (1.0 to 5.5)	2.4 (1.0 to 5.7)
sP to selectin						
< 90th percentile controls	24	60	1 [ref]	1 [ref]	1 [ref]	1 [ref]
≥ 90th percentile controls	176	549	1.1 (0.7 to 1.9)	1.9 (0.7 to 4.6)	1.8 (0.7 to 4.6)	1.8 (0.7 to 4.6)
sGPIb						
< 90th percentile controls	27	58	1 [ref]	1 [ref]	1 [ref]	1 [ref]
≥ 90th percentile controls	154	527	1.5 (0.9 to 2.6)	2.5 (1.1 to 5.7)	2.7 (1.2 to 6.1)	2.5 (1.1 to 5.8)

NAP to 2: neutrophil activating peptide 2; CXCL4: CXC chemokine ligand 4; sP to selectin: soluble P to selectin; sGPIb: soluble glycoprotein Ib
Model 1 = Adjusted for the matching factors: age, index year and area of residence.
Model 2 = Model 1, additionally adjusted for aspirin, statin and glucose to lowering medication use and smoking at time of blood drawing.
Model 3 = Model 2, additionally adjusted for systolic blood pressure, serum cholesterol, glucose and C to reactive protein at time of blood drawing.
Model 4 = Model 3, additionally adjusted for hypertension, hypercholesterolemia, diabetes mellitus and smoking in the year before the index event.

sP-selectin is another marker of *in vivo* platelet activation.[15,16,31] Some, but not all, studies on this molecule have demonstrated that increased levels of sP-selectin may predict cardiovascular disease.[15] We found a two-fold increased risk of myocardial infarction among women with high levels of sP-selectin compared to those with normal levels, although the confidence intervals were rather broad.

We used a unique antibody to study the clinical consequences of high levels of GPIb in plasma. GPIb is a platelet specific glycoprotein that is proteolytically cleaved from the platelet membrane upon platelet activation. The soluble external part of GPIb, also called glycocalicin is a specific plasma marker of platelet reactivity.[17] Although glycocalicin has been proposed as a potential interesting marker of platelet activation to study cardiovascular disease risk,[27] there are no data from large studies addressing the relationship between glycocalicin and cardiovascular disease. Our study shows that subjects with high plasma levels of GPIb have a two-and-a-half-fold increased risk of myocardial infarction.

A limitation of our study is that, by design, we measured platelet reactivity after the myocardial infarction occurred. The myocardial infarction or changes in treatment or lifestyle after the event could have influenced platelet reactivity. In our analyses, we adjusted for changes in treatment by relevant medication (i.e., antiplatelet therapy, lipid-lowering therapy and glucose-lowering therapy) and smoking to get rid of the effects of treatment, as well as lifestyle changes. We also adjusted for serum levels of lipids and glucose at time of blood sampling, which may potentially indicate relevant lifestyle changes influencing platelet reactivity next to effects of treatment. We could not completely rule out residual confounding, although our effects estimates seemed very stable upon multiple adjustments by various multivariable logistic regression models (Table 3). Apart from this, when residual confounding plays a role, the real effects of high platelet reactivity would be even larger compared with our estimates, because preventive changes after suffering a myocardial infarction are not likely to *increase* platelet reactivity. Furthermore, the number of control subjects using preventive cardiovascular medication is very low. Nevertheless, when we restricted our analysis to patients and control subjects not using any preventive medication as a sensitivity analysis, the effect estimates remained quite similar compared with the adjusted models, so our estimates seem to be quite robust.

Another potential limitation is the possibility of reverse causation: theoretically, high platelet reactivity might be the result of the myocardial infarction itself or even of underlying atherosclerosis. We do not expect that a history of myocardial infarction or for example stent implantation has influenced levels of platelet reactivity. The median time between the event and blood collection was five years and such a long-lasting direct effect of a local ischemic event on systemic platelet reactivity is biologically unlikely. Furthermore, high platelet reactivity could be a marker of underlying atherosclerosis, which might form an explanation of our results. The extent of this problem seems to be limited since we studied young women, who most likely will not suffer from extended atherosclerosis. By adjusting for hyperlipidemia, hyperglycemia, and smoking both before and after the myocardial infarction, we further attenuated the possibility that underlying atherosclerosis could explain

the association we found. In addition, it is important to stress that it is biologically plausible that high platelet reactivity via multiple mechanisms leads to inflammation and eventually atherothrombosis.[4-8,15] Nevertheless, despite the biologic plausibility of our findings and our efforts to adjust for potential biases and confounding, the present results should be interpreted with caution because platelet reactivity was measured after occurrence of the MI and residual confounding and reverse causation cannot be completely ruled out. Therefore, our data emphasize the need for large prospective cohort studies to replicate our findings as well as to address the question whether platelet reactivity markers can aid in risk prediction of cardiovascular events in premenopausal women and other populations.

In conclusion, high platelet reactivity according to the markers NAP-2, CXCL4, sP-selectin or sGPIb was associated with a two to three-fold higher incidence of myocardial infarction compared with normal platelet reactivity in premenopausal women. Our results suggest that high basal platelet reactivity may contribute to a higher risk of myocardial infarction.

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