On platelet biology and cardiovascular disease

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Content

Introduction

Atherosclerosis and cardiovascular disease

Cardiovascular disease is one of the major causes of death, being responsible for 30% of the deaths worldwide in 2010 [1]. Cardiovascular disease is mainly caused by atherosclerosis. Clinical complications occur when a plaque suddenly ruptures and platelets form a thrombus on the plaque and the coronary artery becomes occluded. Atherosclerotic plaque rupture is the cause of approximately 70% of myocardial infarctions [2].

In 1879 it was recognized by Ludwig Hektoen that myocardial infarction was caused by atherothrombosis and that this was secondary to sclerotic changes of the coronary arteries [3]. It was long believed that the process of atherosclerotic development was mainly concerned with the passive deposition of lipids in the vascular wall. It is now recognized that atherosclerosis is a chronic inflammatory disease, which is characterized by deposition of lipids, mainly low density lipoprotein (LDL) and the infiltration of leukocytes [1]. Blood platelets play a pivotal role in cardiovascular disease, as aggregate formation of blood platelets in response to an unstable atherosclerotic plaque causes acute blockade of blood flow, causing ischemia and infarction [2].

In addition to the role of platelets in arterial thrombosis, it is becoming clearer that platelets also play an important role in the progression of atherosclerosis. Atherosclerosis is an inflammatory process that is characterized by leukocyte infiltration and participation of other components of the (innate) immune response [4]. Platelets contain numerous cytokines, chemokines and growth factors in their granules and through interaction with leukocytes and endothelial cells platelets can promote inflammation and atherosclerosis [5]. It has been described that inflamed endothelial cells have the ability to bind platelets and red blood cells, despite that the endothelial cell layer is intact [6,7]. Mouse models have shown that platelets enter the site of early atherosclerotic lesion far before the first white blood cell is detected [7,8]. This has given rise to the notion that platelets are involved in early atherogenesis by being able to stimulate inflammation and mediate leukocyte infiltration into the vascular wall [7].

Cardiovascular disease risk prediction

Assessment of the cardiovascular disease risk is important to guide clinical decision-making and to choose therapeutic strategy [9]. The Framingham study was one of the first large studies aimed to identify risk factors associated with cardiovascular

disease. Their findings resulted in a score consisting of LDL cholesterol, high density lipoprotein (HDL) cholesterol, elevated blood pressure, diabetes, smoking, age, and sex, all predicting a risk of ischemic coronary artery disease and myocardial infarction in individual patients. Large trials thereafter showed that risk reduction for coronary artery disease could be achieved by intervention for example with cholesterol levels and blood pressure [3]. In 1988 it was shown that therapeutic treatment with aspirin improved outcome in patients with suspected myocardial infarction [10]. This illustrated the importance of platelet reactivity in coronary artery disease.

Until now, cardiovascular disease risk studies have had a strong focus on evaluating levels of serological markers, but over the last decades no leads of novel markers with clinical utility have been identified (Chapter 1, this thesis). Therefore, a multicentre initiative was started to study functionality of circulating cells as biomarkers of coronary artery disease severity and future outcome in a cohort of patients scheduled for coronary angioplasty. Platelet responsiveness is a promising circulating cell biomarker because anti-platelet medication belongs to the most potent inhibitors of cardiovascular disease risk [11]. In addition, as platelets circulate through the body they interact with the vessel wall, which may influence the biology of the platelets.

There are a number of platelet function assays that are used in clinical laboratories, which have been evaluated in relation to cardiovascular disease. These platelet function tests can be categorized into agonist induced aggregation assays such as the classical light transmission aggregometry (LTA), PlateletWorks assay (Helena Laboratories), the VerifyNow (Accumetrics), the Multiplate Analyzer (Dynabite), and the Chronlog device (Chronolog); shear-stress-based assays such as the platelet function analyzer (PFA; Siemens) and the cone platelet analyzer (Impact-R); or flowcytometric assays such as the vasodilator stimulated phosphoporteins (VASP) assay (PLVASP/P2Y12) [12]. Platelet aggregation measurements, such as LTA, the newer Multiplate® assay, and Platelet function analysis (PFA) have proven to be reliable platforms for diagnosis of bleeding disorders, but they lack precision and reproducibility to study platelet function in arterial thrombosis [13-17]. The VerifyNow® is an established platform to monitor aspirin and $P2Y_{12}$ inhibitor therapy, but does not provide specific insight in the major platelet activation pathways. Above all, these assays mainly examine the ability of platelets to aggregate, but specifically granule release of platelets is not assessed by any commercial assay. This despite that granule release provides platelets with diverging functions other than thrombus formation, one of which is mediating inflammation [5,7]. In addition to this, it has been shown that expression of

P-selectin measured with flow-cytometry provides a broader advantage to discriminate individuals on platelet responsiveness than aggregation.

Aspects of platelet function

The formation of platelet aggregates is favourable to prevent lethal blood loss, but it also causes occlusive atherothrombosis [5,7]. Healthy and intact endothelium controls platelet reactivity by the release of prostacyclin, nitrous oxide (NO) and by the presence of ADP degrading enzymes like CD39 [18]. But these mechanisms fail after rupturing or erosion of an atherosclerotic plaque, where the extracellular matrix components become exposed to the circulating blood [19,20]. Platelet thrombus formation can cause ischemia at the site of plaque rupture, or further downstream when a clump is released and embolizes. Firm evidence for a central role of platelets in occlusive cardiovascular disease has been provided with the effective risk reduction of cardiovascular disease by treatment with anti-platelet medication with aspirin and clopidogrel [11].

Platelets are relatively small anucleate cells that arise from megakaryocytes in the bone marrow. In response to injury of the vasculature, platelets aggregate in a process that originally was called viscous metamorphosis [21,22]. Platelets trigger a repair process by responding to the extracellular matrix that becomes exposed when the endothelial layer of a blood vessel becomes disrupted [21,22]. Blood rheology plays an important role in this process. Blood flows with a higher rate at the center of a vessel than at the wall, which gives rise to layers of blood that flow with different speeds. So-called "shear forces" develop between these layers, which are dependent on the flow rate and vascular radius [23]. There is a broad variation in blood flow rates in the human circulation that range from 500/sec in small veins to 5000/sec in small arteries, and 40000/sec in stenosed arteries [22]. Platelet function depends on the shear conditions [24,25]. Injury in vessels with high shear rates requires von Willebrand Factor (vWF) to slow down platelets to establish firm adhesion [26]. During high shear, vWF attaches to exposed collagen the extracellular matrix, causing it to unroll and to subsequently tether platelets via their glycoprotein (GP)Ib receptors to the surface [23]. In vessels with low shear, interaction of vWF with GPIb is less important [27].

When platelets are tethered to the surface of the wound, they become activated to establish firm adhesion to the extracellular matrix. Collagen activates platelets via GPVI and $\alpha_2\beta_1$ [28], which causes the release of secondary activators, such as the $P2Y_{12}$ receptor agonist adenosine diphosphate (ADP), the TP agonist thromboxane (TXA2), and serotonin [29-31]. Parallel to this, tissue factor exposed by the endothelium triggers formation of thrombin, which further activates platelets via the protease activated

receptors (PAR)-1 and PAR-4 [32]. Firm adhesion of platelets to the extracellular matrix is mediated by integrins, including the collagen adhesion receptor $\alpha_2\beta_1$, and $\alpha_{IIb}\beta_3$. Upon platelet activation, $\alpha_{IIb}\beta_3$ undergoes a conformational change that is associated with an increase of affinity for fibrinogen, fibrin, and vWF [23]. This latter step enables platelets to form an aggregate that seals the injury of the vessel wall.

Content of this thesis

The central goal of this thesis is to investigate the responsiveness of platelets by means of α-granule release in relation to cardiovascular disease severity, and to future cardiovascular disease events. In addition to platelet response in relation to cardiovascular disease, we have identified novel conditions and aspects that regulate platelet function.

Chapter 1 addresses a systematic review to the current state of art of cardiovascular disease risk prediction with circulating markers. Chapter 2 describes a study to the relation between platelet responsiveness and cardiovascular disease state and future cardiovascular disease events, which are assessed in the CTMM Circulating Cells cohort. The effect of ischemic coronary artery narrowing on platelet responsiveness is investigated in Chapter 3. The influence of selective serotonin re-uptake inhibitors on platelet granule release is addressed in chapter 4. In chapter 5 we describe that platelets can release their granules independently of an aggregatory response, which is the result of selective $\alpha_{IIb}\beta_3$ inhibition. In chapter 6, we describe the discovery and characterisation of a novel intracellular signalling molecule that belongs to the class of regulators of the RhoGTPase family. And lastly, in Chapter 7 the composition of the platelet releasate in response to stimulation of either the thrombin receptor PAR-1, or PAR-4 is described with the use of novel quantitative mass spectrometry techniques.

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Chapter 1

Circulating biomarkers for predicting cardiovascular disease risk; a systematic review and comprehensive overview of meta-analyses

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Abstract

Cardiovascular disease is one of the major causes of death worldwide. Assessing the risk for cardiovascular disease is an important aspect in clinical decision making and setting a therapeutic strategy, and the use of serological biomarkers may improve this. Despite an overwhelming number of studies and meta-analyses on biomarkers and cardiovascular disease, there are no comprehensive studies comparing the relevance of each biomarker. We performed a systematic review of meta-analyses on levels of serological biomarkers for atherothrombosis to compare the relevance of the most commonly studied biomarkers.

Medline and Embase were screened on search terms that were related to "arterial ischemic events" and "meta-analyses". The meta-analyses were sorted by patient groups without pre-existing cardiovascular disease, with cardiovascular disease and heterogeneous groups concerning general populations, groups with and without cardiovascular disease, or miscellaneous. These were subsequently sorted by end-point for cardiovascular disease or stroke and summarized in tables. We have identified 85 relevant full text articles, with 214 meta-analyses. Markers for primary cardiovascular events include fibrinogen, C-reactive protein, apolipoprotein B, the apolipoprotein A/apolipoprotein B ratio, cholesterol, high density lipoprotein, and vitamin D. Markers for secondary cardiovascular events include cardiac troponins I and T, C-reactive protein, serum creatinine, and cystatin C. For primary stroke, fibrinogen and serum uric acid are strong risk markers. Limitations reside in heterogenous reporting of the meta-analysis results (HR, OR, RR, RRR), which are not interchangeable and therefore difficult to compare with each other. In addition, there is no acknowledged search strategy for prognostic studies or meta-analyses.

In conclusion, for primary cardiovascular events, markers with strong predictive potential are mainly associated with lipids. For secondary cardiovascular events, markers are more associated with ischemia. Fibrinogen is a strong predictor for primary stroke.

Introduction

Atherothrombosis is one of the major causes of death worldwide [1]. Upon rupture of an atherosclerotic plaque, a hemostatic response is initiated that could lead to infarction causing ischemia downstream. Assessment of cardiovascular disease risk that can be supported by biomarker analysis is a primary requirement to stratify those at high-risk and for optimized treatment of patients.

Large cohort studies are crucial for cardiovascular disease risk estimation with the use of biomarkers, and confirmation of results in independent populations is desirable. Many results from different studies have become available over time, which makes it challenging to assess those markers that consistently keep a predictive value. Meta-analyses combine the results from different studies and present one aggregate score for a risk marker in question, but these studies have also been performed in large numbers. This systematic review presents a comprehensive overview of serological biomarkers for primary and secondary cardiovascular disease and stroke investigated in meta-analyses of the past 24 years [2]. It compares the relevance of the most commonly studied biomarkers used to assess the risk of ischemic cardiovascular event and stroke. The selection of meta-analyses was restricted to prospective studies only, as pooled results from cross-sectional and retrospective case-control studies overestimate the risk for the marker in question. To our knowledge, this is the first systematic review on meta-analyses for biomarkers of atherothrombosis.

Materials and methods

A literature search on published studies from 1988 to 2011 has been performed in Medline (using the advanced search option in Pubmed), and Embase (see flow diagram in Figure 1). A protocol for the search and data abstraction was set up and discussed with one skilled epidemiologist, and three established investigators for consensus. The search terms were related to “arterial ischemic events” and “meta-analyses”, and were set up broadly to reduce the possibility that publications that use trivial nomenclature would be missed (see Supplementary Methods). We have taken the PRISMA statements as a framework for reporting the systematic review [3].

Titles and abstracts were screened on “meta-analyses of prospective studies”, “arterial ischemic disease”, and “levels of circulating markers” (Figure 1, Step 1.). Duplicates were removed from the search results (Flowchart in Figure 1, Step 2.).

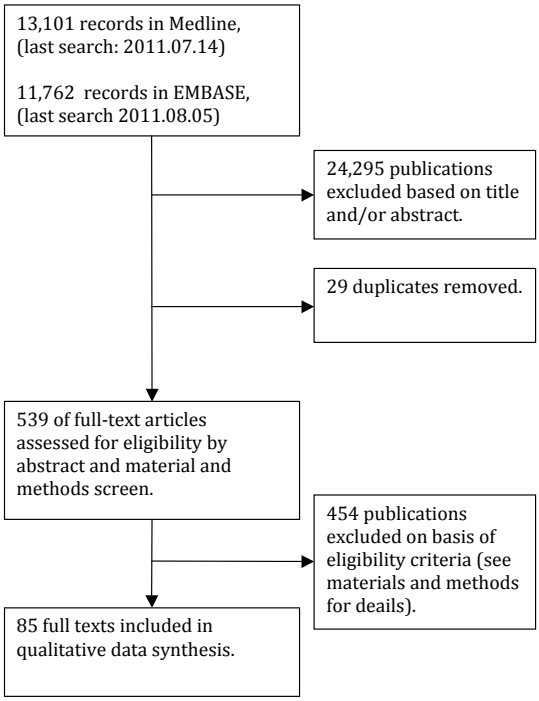

Figure 1. PRISMA flow diagram.

Eligibility of the papers was assessed by reading the abstracts and material and methods section of the publications (Flowchart in Figure 1, Step 3.). Studies were considered as "not eligible" if they were not prospective (e.g. cross-sectional, retrospective studies), reported other than levels of circulating markers (e.g. alleles, or prediction models), investigated the risk relating to all cause death or hemorrhagic stroke, did not explicitly report the pooled results in the text, figures, or tables; did not pool data from 2 prospective studies or more, did not report risk in relation to levels of the investigated marker (e.g. comparison of marker levels in case and control group), were unpublished reports (e.g. abstracts, posters), or that were not available online via either Medline or Embase.

After the first selection round, manuscripts were selected for a full text screen. The result of the meta-analysis was extracted, which was reported either by odds ratio (OR), relative risk (RR), relative risk ratio (RRR), or hazard ration (HR), together with the follow up period. In addition, the following parameters were abstracted: the type of investigated end-point(s), to what group the risk applied and how this group was defined (e.g. tertiles, cut-offs), nature of pooled studies (e.g. individual patient data

(IPD), cohorts), whether there had been adjustment for other risk factors, presence of statistical heterogeneity or heterogeneity mentioned by the authors, how the pooling was performed (either by regression, Cox-regression, random effects model, fixed effects model, or inverse variance weighted combined risks), number of patients (the amount of cases within the pooled cohorts was preferred but if this was not present the total cohort size was given), number of pooled cohorts, and which population was represented in the results (a population with or without pre-existing cardiovascular disease, a specific subgroup population, or the general population).

If both unadjusted and adjusted results were reported, the adjusted results were abstracted. If risks for more quantiles were reported, only the most extreme was used. If subgroup meta-analyses were reported in one publication (e.g. different age groups, sex), these were abstracted unless these were excluded according to the earlier specified criteria. If the amount of cases was not explicitly reported, these were calculated by hand whenever possible. If heterogeneity between cohorts was reported and both random effects and fixed effects analysis was performed, the random effects results were abstracted. Stratification of the meta-analyses was performed on population based studies, cohorts without pre-existing cardiovascular disease, cohorts with pre-existing cardiovascular disease, pooled results from populations with and without pre-existing cardiovascular disease, and specific subgroups.

The evaluation criteria for novel risk markers as described by Hlatky et al. 2009 were used as guide to set up evaluation parameters for meta-analyses [4]. We consider the following parameters indicative for the clinical value and quality of the different meta-analyses:

- There should be relevant stratification of the researched individuals. Groups with, or without previous cardiovascular disease are clinically more relevant than groups representing the general population, or meta-analyses where cohorts with and without cardiovascular disease were pooled for one result.
- The prediction is preferred to be expressed as a hazard ratio, rather than an odds ratio, relative risk, or relative risk ratio as it considers the event rate, and not the difference in number of events at one specific time point.
- Pooled end-points should not be too diverse, or at least clinically relevant. Pooling of diverse end-points complicates the interpretation of the results.
- The novel risk marker should be able to predict risk beyond the established risk markers, and therefore it should add statistical value in a model where other risk factors are included.

- The result of a meta-analysis becomes more reliable with increasing number of events and is even more convincing when a large number of cohorts are used, especially when in absence of heterogeneity between the pooled cohorts.
- If heterogeneity of the results is present, this should be addressed by conservative pooling of the results, using a random effects model (see http://www.cochrane.org/). Statistical power of risk assessment depends on the number of outcome events, and therefore reporting of the number of events rather than total study size is preferred.

Results

A total of 24.863 publications were screened, which were available online in the period June the 10th of 2011 to August the 5th of 2011. Twenty-nine duplicates were removed and after a screen on title and abstract on studies evaluating risk markers for cardiovascular disease and ischemic stroke, a total of 539 publications remained (see Figure 1 for flow diagram). After monitoring the abstracts and material and methods, 85 publications remained with 214 meta-analyses. On basis of cohort characteristics and end point 9 different types of meta-analyses were identified, which are summarized in Supplementary Tables 1-9. Meta-analyses for cardiovascular disease events that were performed with studies from groups without pre-existing cardiovascular disease are presented in Supplementary Table 1. Meta-analyses for cardiovascular disease events that were performed with studies from groups with pre-existing cardiovascular disease are presented in Supplementary Table 2. Meta-analyses reported for stroke events in populations without cardiovascular disease are presented in Supplementary Table 3. Pooled results for stroke events in populations with pre-existing cardiovascular disease are provided in Supplementary Table 4. Results from studies with heterogeneous populations being general populations, populations with and without pre-existing disease, and miscellaneous groups for either cardiovascular or stroke events are summarized in the Supplementary Tables 5-9. The tables are organized into categories of markers (e.g. markers related to hemostasis), and per category in descending order of result. The studies reporting on populations only without pre-existing cardiovascular disease, reporting on populations only with pre-existing cardiovascular disease, for either cardiovascular disease or stroke (Supplementary Table 1-3) are considered most clinically relevant, and therefore are discussed in this review. Meta-analyses reporting on stroke in populations only with pre-existing cardiovascular disease are not discussed

in this review, as only two meta-analyses were found in this category and are too few to draw any conclusions upon.

In total, 61 meta-analyses were found for cardiovascular disease events in populations without pre-existing cardiovascular disease. In these populations, the highest risk for cardiovascular disease is reported for markers associated with hemostasis, inflammation and lipids. These include fibrinogen, C-reactive protein (CRP), apolipoprotein (Apo) B, cholesterol, and Vitamin D [5-9] (Supplementary Table 1).

For populations with pre-existing cardiovascular disease, 43 meta-analyses were found reporting on markers for cardiovascular disease events. Markers with high prognostic value were associated with ischemia, inflammation and kidney function. These include cardiac troponin (cTn) I and T, high sensitivity (hs) CRP, serum creatinine, and cystatin C [10-14] (Supplementary Table 2).

For ischemic stroke events in individuals without pre-existing cardiovascular disease, 18 meta-analyses were found. These were related to hemostasis and kidney function, being fibrinogen, and serum uric acid [5,15] (Supplementary Table 3).

Discussion

This systematic review of meta-analyses on levels of serological biomarkers for atherothrombosis was performed to provide a comprehensive overview of the state of art, and to compare the relevance of the most commonly studied biomarkers. For primary cardiovascular events, markers with strong predictive potential are mainly associated with lipids. For secondary cardiovascular events, markers with strong predictive potential are associated with ischemia. Fibrinogen has strong predictive potential for primary stroke.

The clinical relevance of a marker depends not only on its risk prediction strength, but also the setup of the investigations (e.g. case-control versus cohort study). The quality of the reporting of results (e.g. reporting of adjustment for other risk factors) is another important aspect. It is attractive to use a score to assess the quality and clinical value of meta-analyses as it gives means to rank the reports. Conversely, a score to assess the quality and clinical value requires assigning weight to different factors that influence the results, which is difficult and hard to motivate. Therefore, we have abstracted aspects of the meta-analyses that may have influenced their results without assigning scores. These aspects, summarized in the methods, were adapted from Hlatky et al., 2009 and are reported in the columns of the tables. These rankings provide the reader with insight into the quality and clinical relevance of the markers.

Many of the markers listed in the tables are well known, and some are established risk markers that are applied in the clinic as a risk marker for cardiovascular disease. Of special interest are markers that are associated with high risk, but which are without clinical application in cardiovascular disease risk prediction as of yet. For such a marker to have clinical utility, the biomarker should be able to predict risk independently of other established risk markers. In addition, there should be an established assay that is specific and sensitive in measuring the marker [16,17]. The possibility to intervene therapeutically based on the levels of risk marker, associated with a reduced risk for cardiovascular disease enables the option to use it to evaluate the efficacy of a therapeutic intervention. We will discuss the clinical utility of three selected markers with strong risk prediction that do not have a clinical application in cardiovascular disease risk prediction to date.

Fibrinogen is one of the strongest markers for both predicting stroke and cardiovascular disease in populations without pre-existing cardiovascular disease. It is involved in hemostasis and blood viscosity. Moreover it is known as an acute phase reactant [18]. Age, sex and cohort corrected results remained significant for cardiovascular disease events and stroke [5].

There are 40 different assays to measure fibrinogen, and although they are reported to be relatively accurate, there is much to gain on assay standardization for overall comparability of measurements. In addition, there is great variation in results between different laboratories, with concentrations ranging from 121 to 437 mg/dL for one specific sample [19]. Improvement in assay standardization would make fibrinogen an interesting biomarker.

Specific members of the fibrate class bezafibrate and clofibrate are able to lower fibrinogen levels besides improving high density lipoprotein and triglyceride levels [20]. However, they have not been shown to be of any benefit in reducing cardiovascular disease risk in relation to their fibrinogen lowering levels [21]. Lowering fibrinogen with bezafibrate also has no effect on occurrence of secondary stroke [22]. A causal relationship of high fibrinogen levels and increased cardiovascular disease risk is unclear, as only some of the polymorphisms that influence the level of fibrinogen are associated with increased cardiovascular disease risk [18]. Two genetic variants that affect the levels of fibrinogen are related to the risk for ischemic stroke, but not for myocardial infarction [23].

Low levels of vitamin D are an independent risk factor for cardiovascular death in populations without pre-existing cardiovascular disease [9]. Systematic reviews on interventional vitamin D supplementation and cardiovascular disease risk reported

that vitamin D supplementation had no effect on cardiovascular disease risk, indicating a lack of a causal relationship [24,25].

Serum Vitamin D level is widely measured in diagnostic laboratories, to assess vitamin D status in a number of clinical conditions such as rickets, osteomalacia, osteoporosis, hyperparathyroidism, chronic kidney disease or pregnancy [26]. The main type of assays are either competitive immunoassays, or direct detection methods with high performance liquid chromatography or liquid chromatography combined with tandem mass spectrometry [26]. There is considerable variation between the results obtained with the various methods, as well as between laboratories [26]. A standard for vitamin D measurements (SRM 972) is available to increase comparability across laboratories, but as of yet it is unclear how comparability has improved. Immunoassays are less sensitive and specific for vitamin D measurements than high performance liquid chromatography, and liquid chromatography combined with tandem mass spectrometry. The latter two techniques are less attractive in aspects of high throughput and required training of staff [26].

For secondary cardiovascular events, cystatin C is one of the strongest risk predictors. Plasma cystatin C is a marker for chronic kidney disease, a disease strongly associated with an increased risk for cardiovascular disease [27,28]. The contribution of cystatin C in a multivariate model remains significant, which indicates its added value to established risk factors [11]. The reason for the incremental prognostic information given by cystatin C is still unknown, but it is likely to be related to the sensitivity of cystatin C to detect preclinical kidney dysfunction [28].

Because of the association of renal dysfunction with cardiovascular disease, it is unclear whether cystatin C is a direct marker of cardiovascular disease or merely a marker for renal failure, which has implications for therapeutic intervention. In addition, no therapy has been evaluated to date that aimed to treat patients for cardiovascular disease on stratification by cystatin C values [28]. Cystatin C is measured by immunoassays, using particles coated with cystatin-C specific antibodies, and subsequent turbidometry or nephelometry [29]. The assays are precise, as both detection methods provide coeffients of variation ranging from 2 to 8% [30].

As cardiovascular disease is still one of the major causes of death worldwide, there is an ongoing need for new biomarkers that are able to assist in clinical decision making. Other emerging types of biomarkers for cardiovascular disease risk prediction may prove their value in the future. A novel initiative in cardiovascular risk prediction is the Circulating Cells Consortium that investigates the information present in circulating cells such as platelets and leukocytes in relation to cardiovascular disease events.

As these cells interact with the vessel wall, their responsiveness may convey clinical relevant information on cardiovascular disease risk.

This systematic review is subject to some limitations. This review has included only meta-analyses, so the novelty of reported markers is limited. Also, risk markers are absent in this review when they have not been included in a meta-analysis. Some of the meta-analyses are smaller in size than some single cohort studies. The advantage of a meta-analysis compared with a single large cohort study is that the results represent the ability of a marker to predict events in different cohorts, which increases reliability. The meta-analyses in this review are heterogeneous in the reported results (OR, HR, RR, RRR), which are not interchangeable and therefore not comparable with each other one-to-one. Heterogeneity among the meta-analyses exists also in the adjustment for other prognostic factors, and in the methods used to pool the results. This limits the comparability of the different risk markers. Lastly, there is no widely acknowledged search strategy, neither for prognostic studies, nor for meta-analyses. We therefore have applied a broad search strategy, but still some meta-analyses may have been missed.

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Supplementary Methods

Terms used for search in databases. The "Outcome" terms were combined with the "Determinant" terms both separated with "[ ]" brackets, using the command "AND". Grey marked sections apply only for Embase searches, to exclude results from medline.

Outcome: "arterial" OR "arterial ischaemia" OR "arterial ischemia" OR "ischaemic event" OR "ischemic event" OR "arterial occlusion" OR "occlusive event" OR "arterial thrombosis" OR "atherothrombosis" OR "athero thrombosis" OR "athero" OR "vascular" OR "cardiovascular disease" OR "cardio vascular disease" OR "cardiovascular ischaemia" OR "cardiovascular ischemia" OR "cardio vascular ischaemia" OR "cardio vascular ischemia" OR "stroke" OR "ischaemic stroke" OR "ischemic stroke" OR "brain infarction" OR "brain ischaemia" OR "brain ischemia" OR "transient ischaemic attack" OR "transient ischemic attack" OR "heart infarction" OR "myocardial infarction" OR "myo-cardial infarction" OR "myo cardial infarction" OR "myocardial" OR "myo-cardial" OR "myo cardial" OR "coronary artery occlusion" OR "coronary artery ischaemia" OR "coronary artery ischemia" OR "coronary heart disease" OR "coronary artery" OR "atherosclerosis" OR "athero-sclerosis" OR "athero sclerosis" AND [embase]/lim NOT [medline]/lim AND [1988-2012]/py

Determinant: "meta analysis" OR "meta-analysis" OR "metaanalysis" OR "systematic review" OR "systematic analysis" OR "quantitative review" OR "quantitative analysis" OR "comparing published studies" OR "combining results" OR "combined results" OR "combining data" OR "combined data" OR "pooling results" OR "pooled results" OR "pooling data" OR "pooled data" AND [embase]/lim NOT [medline]/lim AND [1988-2012]/py

Supplementary Table 1. Meta-analyses of cohorts without pre-existing cardiovascular disease on markers for cardiovascular disease risk.

Marker	Outcome	Risk Applies To	Risk	Results	95% ci[1]	N Patients	N Cohorts	Het.[2]	Pooling Method	Adj.[3]	Patient group	IPD[4]	Follow-up Period	Publi-cation
Diabetes related														
Glucose (post load)	Fatal, non-fatal CVD[5]	Above: 7.8 mmol/L	RR	1.58	1.19 - 2.10	1,467 cases	7	no	Random effects model	n.a.[6]	No pre-existing disease	no	5 -15.6 yr	[1]
Glycated hemoglobine (HBA(1c))	CVD death	HbA1c level: 0.7	RR	1.58	1.22 - 2.06	1,366 cases	7	no	Random effects model	yes	No pre-existing disease	no	9.2 yr mean	[2]
Glycated hemoglobine (HBA(1c))	CVD death	HbA1c level: 0.6	RR	1.34	1.13 - 1.58	1,366 cases	7	no	Random effects model	yes	No pre-existing disease	no	9.2 yr mean	[2]
Glucose (fasting)	Fatal, non-fatal CVD	Above 6.1 mmol/L	RR	1.33	1.06 - 1.67	1,053 cases	6	no	Random effects model	n.a.	No pre-existing disease	no	4 -14 yr	[1]
Glycated hemoglobine (HBA(1c))	CHD[7] death, non-fatal MI[8]	1 SD[9] increase	RR	1.20	1.10 - 1.31	1,639 cases	9	yes	Random effects model	yes	No pre-existing CVD, Western	no	> 1 yr	[3]
Glycated hemoglobine (HBA(1c))	CVD death	HbA1c level: 0.5	RR	1.13	1.05 - 1.21	1,366 cases	7	no	Random effects model	yes	No pre-existing disease	no	9.2 yr mean	[2]
Glucose (fasting)	CHD death, non-fatal MI	1mmol/L increase	RR	1.06	1.00 - 1.12	10,808 cases	23	yes	Random effects model	yes	No pre-existing CVD, Western	no	> 1 yr	[3]
Glucose (non fasting)	CHD death, non-fatal MI	1mmol/L increase	RR	1.05	1.03 - 1.07	12,652 cases	27	yes	Random effects model	yes	No pre-existing CVD, Western	no	> 1 yr	[3]

1 95% ci: 95% confidence interval
2 Het.: heterogeneity between cohorts.
3 Adj.: adjustment performed with other risk factors.
4 IPD: individual patient data.
5 CVD: cardiovascular disease
6 n.a.: not available.
7 CHD: coronary heart disease
8 MI: myocardial infarction
9 SD: standard deviation

Marker	Outcome	Risk Applies To	Risk	Results	95% ci[1]	N Patients	N Cohorts	Het.[2]	Pooling Method	Adj.[3]	Patient group	IPD[4]	Follow-up Period	Publi-cation
Hemostasis														
Fibrinogen	Other vascu-lar death	1 g/L increase	HR	2.33	1.91 - 2.84	992 cases	31	n.a.	Cox regression	yes	No pre-existing CHD	yes	> 1 yr	[4]
Fibrinogen	CHD	1 g/L increase	HR	1.93	1.79 - 2.08	7,118 cases	31	yes	Cox regression	yes	No pre-existing CHD	yes	>1 yr	[4]
Von Willebrand Factor	Cardiac death, non-fatal MI	Top vs bottom tertile	OR	1.23	1.14 - 1.33	3,969 cases	n.a.	n.a.	Inverse variance weighted mean	yes	No pre-existing CVD	no	n.a.	[5]
Hormones														
Vitamin D (serum 25-OH D)	CVD death	Decrease in different predefined categories	HR	1.83	1.19 - 2.80	2,007 cases	5	yes	Random effects model	yes	No pre-existing CVD	no	6.2 - 27.1 yr	[6]
Vitamin D (serum 25-OH D)	CVD	Decrease in different predefined categories	HR	1.54	1.22 - 1.95	756 cases	4	no	Random effects model	yes	No pre-existing CVD	no	5 - 10 yr	[6]
Thyroid stimulating hormone	CHD	Above 4-5 mU/L	RR	1.18	1.02 - 1.38	8,071 total	3	n.a.	Fixed effects model	yes	No pre-existing CVD	no	4 - 20 yr	[7]
Testosteron	MI, IHD[10], CVD, ather-osclerosis, stroke, death	1 SD increase	RR	0.89	0.83 - 0.96	4,598 cases	18	yes	Inverse variance weighted mean	yes	No pre-existing vascular disease	no	3 - 15.3 yr	[8]
Testosteron	MI, IHD, stroke, ath-erosclerosis, death	1 SD increase	RR	0.88	0.78 - 1.00	1,709 cases	7	yes	Inverse variance weighted mean	yes	No pre-existing vascular disease	no	3 - 15.3 yr	[8]
Inflammation														
CRP[11]	CHD	Top vs bottom tertile	RR	2.43	2.10 - 2.83	3,181 cases	12	n.a.	Random effects model	n.a.	Healthy individuals	no	2.9 - 20 yr	[9]

10 IHD: ischemic heart disease
11 CRP: C-reactive protein

Marker	Outcome	Risk Applies To	Risk	Results	95% ci[1]	N Patients	N Cohorts	Het.[2]	Pooling Method	Adj.[3]	Patient group	IPD[4]	Follow-up Period	Publi-cation
CRP	Cardiac de-ath, non-fatal MI	Top vs bottom tertile	OR	1.58	1.48 - 1.68	7,068 cases	22	yes	Inverse variance weighted mean	yes	No pre-existing CVD	no	12 yr mean	[5]
CRP	CHD death, MI	1 SD increase	RR	1.37	1.27 - 1.48	5,373 cases	31	yes	Random effects model	yes	No pre-existing CVD	yes	> 1yr	[10]
IL-6	CHD death, MI	1 SD increase	OR	1.26	1.19 - 1.35	5,730 cases	17	yes	Random effects model	yes	No pre-existing vascular disease	no	6 yr mean	[11]
Kidney function														
Serum creatine (eGFR)[12]	CHD death, non-fatal MI	Below 60mL/min/ 1.73m2	RR	1.32	1.19 - 1.47	4,720 cases	7	no	Fixed effects model	yes	No pre-existing vascular disease	no	>1 yr	[12]
Serum uric acid	CHD death	Above 4.7 - 7.7 mg/dL	RR	1.16	1.01 - 1.30	4,038 cases	8	yes	Random effects model	yes	No pre-existing CVD	no	8.2 - 24.9 yr	[13]
Serum uric acid	CHD	Above 4.7 - 7.7 mg/dL	RR	1.09	1.03 - 1.16	5,198 cases	9	yes	Random effects model	yes	No pre-existing CVD	no	6.5 - 17.5 yr	[13]
Lipids														
Apo[13]B	CHD	Top vs bottom tertile	RR	1.99	1.65 - 2.39	6,920 cases	19	yes	Random effects model	yes	No pre-exsting CVD	no	9 yr mean	[14]
ApoB/ApoAI ratio	CHD	Top vs bottom tertile	RR	1.86	1.55 - 2.22	3.730 cases	7	yes	Random effects model	yes	No pre-existing CVD	no	10 yr mean	[14]
HDL[14]	IHD death	0.33 mmol/L decrease	HR	1.83	1.65 - 2.03	1,198 cases	23	no	Cox regression	no	No pre-existing CVD (age 60-69)	yes	8 yr mean	[15]
Triglycerides	CHD death, non-fatal MI	Top vs bottom tertile	OR	1.72	1.56 - 1.90	10,158 cases	29	yes	Inverse variance weighted mean	yes	No pre-existing disease	no	3.2 - 20 yr	[16]

12 eGFR: estimated glomerular filtration rate.
13 Apo: apolipoprotein
14 HDL: high density lipoprotein

Marker	Outcome	Risk Applies To	Risk	Results	95% ci[1]	N Patients	N Cohorts	Het.[2]	Pooling Method	Adj.[3]	Patient group	IPD[4]	Follow-up Period	Publi-cation
HDL	IHD death	0.33 mmol/L decrease	HR	1.63	1.44 - 1.85	764 cases	23	yes	Cox regression	no	No pre-existing CVD (age 40-59)	yes	8 yr mean	[15]
ApoAI	CHD	Bottom vs top tertile	RR	1.62	1.43 - 1.83	6,333 cases	21	yes	Random effects model	yes	No pre-exsting CVD	no	9 yr mean	[14]
Non-HDL cholesterol	CHD	43 mg/dL increase	HR	1.59	1.36 - 1.85	12,785 cases	68	yes	Random effects model	yes	No pre-existing vascular disease	yes	8.1 yr median	[17]
ApoB	CHD	29 mg/dL increase	HR	1.58	1.39 - 1.79	4,499 cases	22	yes	Random effects model	yes	No pre-existing vascular disease	yes	8.1 yr median	[17]
Non-HDL cholesterol	CHD	1.53 unit increase	HR	1.50	1.38 - 1.62	4,499 cases	22	yes	Random effects model	yes	No pre-existing vascular disease	yes	8.1 yr median	[17]
ApoB/ApoAI ratio	CHD	0.27 unit increase	HR	1.49	1.39 - 1.60	4,499 cases	22	yes	Random effects model	yes	No pre-existing vascular disease	yes	8.1 yr median	[17]
Cholesterol	CVD death	Fourth vs other quartiles	HR	1.49	1.05 - 2.10	201 cases	5	no	Cox regression	yes	No pre-existing CVD	yes	4.5 - 10.6 yr	[18]
Low densitty lipoprotein	CHD	33 mg/dL increase	HR	1.38	1.09 - 1.73	2,076 cases	8	yes	Cox regression	yes	No pre-existing vascular disease	yes	8.1 yr median	[17]
HDL	IHD death	0.33 mmol/L decrease	HR	1.35	1.22 - 1.49	1,058 cases	23	no	Cox regression	no	No pre-existing CVD (age 70-89)	yes	8 yr mean	[15]
Cholesterol	CHD death	1 mmol/L increase	RR	1.22	1.18 - 1.27	18,244 total	6	n.a.	Fixed effects model	yes	Not selected on any disease, Men; middle-age and above	no	3 - 32 yr	[19]
Cholesterol	CHD death	1 mmol/L increase	RR	1.22	1.15 - 1.28	13,486 total	11	n.a.	Fixed effects model	yes	Not selected on any disease, Men: >65 yr	no	3 - 32 yr	[19]
Lipopro-tein(a)	CHD	1 SD increase	RR	1.10	1.02 - 1.18	106,645 total	30	yes	Random effects model	yes	No pre-exiting CHD	no	>1 yr	[20]
Cholesterol	CHD death	1 mmol/L increase	RR	1.04	0.85 - 1.23	9.342 total	6	n.a.	Fixed effects model	yes	Not selected on any disease, Women: >65 yr	no	3 - 32 yr	[19]

Marker	Outcome	Risk Applies To	Risk	Results	95% ci[1]	N Patients	N Cohorts	Het.[2]	Pooling Method	Adj.[3]	Patient group	IPD[4]	Follow-up Period	Publi-cation
Triglycerides	CHD	Per 68% increase	HR	0.99	0.94 - 1.05	12,785 cases	68	yes	Random effects model	yes	No pre-existing vascular disease	yes	8.1 yr median	[17]
Cholesterol	IHD death	1 mmol/L decrease	HR	0.85	0.82 - 0.89	5,626 cases	61	no	Cox regression	no	No pre-existing CVD, age 80-89	yes	13 yr mean	[15]
Cholesterol	IHD death	1 mmol/L decrease	HR	0.82	0.80 - 0.85	10,829 cases	61	no	Cox regression	no	No pre-existing CVD, age 70-79	yes	13 yr mean	[15]
ApoAI	CHD	29 mg/dL increase	HR	0.78	0.72 - 0.86	4,499 cases	22	yes	Random effects model	yes	No pre-existing vascular disease	yes	8.1 yr median	[17]
HDL	CHD	15 mg/dL increase	HR	0.77	0.72 - 0.83	12,785 cases	68	yes	Random effects model	yes	No pre-existing vascular disease	yes	8.1 yr median	[17]
Non-HDL cholesterol	IHD death	1 mmol/L decrease	HR	0.73	0.67 - 0.79	1,058 cases	23	no	Cox regression	no	No pre-existing CVD (age 70-89)	yes	8 yr mean	[15]
Cholesterol	IHD death	1 mmol/L decrease	HR	0.72	0.69 - 0.74	10,419 cases	61	no	Cox regression	no	No pre-existing CVD, age 60-69	yes	13 yr mean SD:6	[15]
Cholesterol/ HDL ratio	IHD death	1.33 units decrease	HR	0.69	0.63 - 0.74	1,058 cases	23	no	Cox regression	no	No pre-existing CVD (age 70-89)	yes	8 yr mean	[15]
Non-HDL cholesterol	IHD death	1 mmol/L decrease	HR	0.66	0.61 - 0.71	1,198 cases	23	no	Cox regression	no	No pre-existing CVD (age 60-69)	yes	8 yr mean	[15]
Cholesterol/ HDL ratio	IHD death	1.33 units decrease	HR	0.60	0.56 - 0.64	1,198 cases	23	yes	Cox regression	no	No pre-existing CVD (age 60-69)	yes	8 yr mean	[15]
Cholesterol	IHD death	1 mmol/L decrease	HR	0.58	0.56 - 0.61	5,561 cases	61	yes	Cox regression	no	No pre-existing CVD, age 50-59	yes	13 yr mean SD:6	[15]
Non-HDL cholesterol	IHD death	1 mmol/L decrease	HR	0.57	0.52 - 0.62	764 cases	23	no	Cox regression	no	No pre-existing CVD (age 40-59)	yes	8 yr mean	[15]
Cholesterol/ HDL ratio	IHD death	1.33 units decrease	HR	0.56	0.51 - 0.60	764 cases	23	no	Cox regression	no	No pre-existing CVD (age 40-59)	yes	8 yr mean	[15]
Cholesterol	IHD death	1 mmol/L decrease	HR	0.44	0.42 - 0.48	1,309 cases	61	yes	Cox regression	no	No pre-existing CVD, (age 40-49)	yes	13 yr mean	[15]

Marker	Outcome	Risk Applies To	Risk	Results	95% ci[1]	N Patients	N Cohorts	Het.[2]	Pooling Method	Adj.[3]	Patient group	IPD[4]	Follow-up Period	Publi-cation
Miscellaneous														
Homocys-teine	Cardiac events	Homocys-teine levels increase	RR	1.38	1.16 - 1.63	2,529 cases	15	yes	Random effects model	yes	No pre-existing CVD	no	3 - 12.8 yr	[21]
Erythrocyte sedimentati-on rate	Cardiac de-ath, non-fatal MI	Top vs bottom tertile	OR	1.33	1.22 - 1.44	4,386 cases	6	n.a.	Inverse variance weighted mean	yes	No pre-existing CVD	no	n.a.	[5]
Homocys-teine	CHD	5 mmol/L increase	OR	1.23	1.06 -1.41	1,943 cases	10	yes	Random effects model	no	No pre-existing CVD	no	n.a.	[22]
Homocys-teine	CHD	5 mmol/L increase	RR	1.18	1.10 - 1.26	22,652 total	21	no	Random effects model	yes	No pre-existing vascular disease	no	2.7 - 24 yr	[23]
Homocys-teine	CHD	5 mmol/L increase	OR	1.06	0.99 - 1.13	269 cases	2	no	Fixed effects model	no	No pre-existing CVD, males	no	n.a.	[22]
Homocys-teine	IHD	25% lower usual ho-mocysteine level	OR	0.89	0.83 - 0.96	1,855 cases	11	yes	Regression	yes	No pre-existing cerbrovascular disease	yes	n.a.	[24]
Selenium	CHD, MI	Top vs bottom quantiles	RR	0.85	0.74 - 0.99	1,366 cases	14	no	Random effects model	yes	No pre-existing CVD	no	3 - 25 yr	[25]

Supplementary Table 2. Meta-analyses of cohorts with pre-existing cardiovascular disease on markers for cardiovascular disease risk.

Marker	Outcome	Risk Applies to	Risk	Results	95% ci	N Patients	N Cohorts	Het.	Pooling Methods	Adj.	Patient group, general population	IPD	follow-up period	Publication
Hemostasis														
Von Willebrand Factor	CHD death, non-fatal MI	Top vs bottom tertile	OR	1.6	1.0 - 2.5	723 cases	8	no	Inverse variance weighted mean	yes	Pre-existing CVD	no	7 yr mean	[26]
Fibrinogen	Acute coronary events	Above median fibrinogen levels	HR	1.42	1.18 - 1.70	477 cases	3	no	Cox regression	yes	TIA or ischemic stroke patients	yes	2.6 - 10 yr	[27]
Tissue plasminogen activator	CHD death, non-fatal MI	Top vs bottom tertile	OR	1.32	0.70 - 2.50	450 cases	6	yes	Inverse variance weighted mean	yes	Pre-existing CVD	no	8 yr mean	[28]
Fibrinogen	Acute ischemic vascular events	Above median fibrinogen levels	HR	1.31	1.15 - 1.49	1,005 cases	3	no	Cox regression	yes	TIA or ischemic stroke patients	yes	2.6 - 10 yr	[27]
Inflammation														
hs[15]-CRP	Cardiac death > 180 days after vascular surgery	1 mg/L > hs-CRP > 3 mg/L	OR	5.65	1.71 - 18.73	477 total	4	no	Fixed effects model	n.a.	Vascular surgery patients	no	>180 days	[29]
hs-CRP	MACE[16] > 180 days after vascular surgery	1 mg/L > hs-CRP > 3 mg/L	OR	2.76	1.38 - 5.55	386 total	3	no	Fixed effects model	n.a.	Vascular surgery patients	no	>180 days	[29]
hs-CRP/CRP	MACE <30 days after vascular surgery	High risk vs low risk concentration	OR	2.58	0.42 - 16.01	85 total	2	no	Fixed effects model	n.a.	Vascular surgery patients	no	<30 days	[29]

15 hs: high sensitivity

16 MACE: major adverse cardiac events.

Marker	Outcome	Risk Applies to	Risk	Results	95% ci	N Patients	N Cohorts	Het.	Pooling Methods	Adj.	Patient group, general population	IPD	follow-up period	Publication
CRP	Death, non-fatal cardiovascular events	Top vs bottom tertile	RR	1.97	1.78 - 2.17	6,485 cases	83	yes	Random effects model	yes	Stable coronary disease patients	no	0.014 - 12 yr	[30]
CRP	CHD	Top vs bottom tertile	RR	1.5	1.1 - 2.1	604 cases	3	no	Inverse variance weighted mean	yes	Pre-existing CVD	no	8 yr mean	[31]
hs-CRP	Non-fatal MI > 180 days after vascular surgery	1 mg/L > hs-CRP > 3 mg/L	OR	1.38	0.63 - 3.03	386 total	3	no	Fixed effects model	n.a.	Vascular surgery patients	no	>180 days	[29]
IL-6	Death after stroke, CVD, cancer, other	Unit increase	OR	1.07	1.04 - 1.10	1,184 total	3	yes	Fixed effects model	yes	Stroke patients	no	0 - 6 monts	[32]
Leukocyte count	MI	1 E6/L increase	HR	1.02	0.99 - 1.05	582 cases	3	n.a.	Cox regression	no	PCI[17] patients	no	1 yr	[33]
Ischemia														
cTn[18]T + cTnI	MI, death	cTnT above 0.1-0.2 ng/mL, cTnI above 0.1-3.1 ng/mL	OR	9.39	6.46 - 13.67	160 cases	10	no	Fixed effects model	n.a.	Unstable angina patients	no	30 days	[34]
BNP + NT-pro BNP[19]	CHD death, non-fatal MI	BNP above 116 gp/mL, NT-proBNP above 227.5 pg/mL	OR	7.9	4.7 - 13.3	75 cases	5	yes	Random effects model	yes	Vascular surgery patients	yes	30 months	[35]
cTnI	MI, death	Above unknown level	RR	5.7	1.8 - 19	882 cases	4	yes	Fixed effects model	n.a.	Unstable angina pectoris patients	no	4 weeks	[36]

17 PCI: percutaneous coronary intervention.
18 cTn: cardiac troponin
19 (NT-pro)BNP: (N-terminal prohormone of) brain natriuretic peptide.

Marker	Outcome	Risk Applies to	Risk	Results	95% ci	N Patients	N Cohorts	Het.	Pooling Methods	Adj.	Patient group, general population	IPD	follow-up period	Publication
cTnI	MI, death	Above different level per study	OR	4.94	3.9 - 6.2	1,168 cases	13	n.a.	Regression	n.a.	ACS[20], nSTEMI [21]patients	no	4 days - 6 months	[37]
cTnT + cTnI	MI, death	cTnT above 0.1-0.2 ng/mL, cTnI above 0.1-0.6 ng/mL	OR	4.93	3.77 - 6.45	1,602 cases	16	yes	Fixed effects model	n.a.	nSTEMI patients	no	30 days	[34]
cTnT	MI, death	Above 0.1-0.2 ng/mL	OR	4.58	3.8 - 5.5	1,965 cases	16	n.a.	Regression	n.a.	ACS, nSTEMI patients	no	4 days - 6 months	[37]
cTnT	Need for revascularization	Above 0.1-0.2 mg/L	OR	4.4	3.0 - 6.5	163 cases	4	n.a.	Regression	n.a.	Non-AMI[22] patients	no	hospitalization - 34 months	[38]
cTnT	Cardiac death, AMI	Above 0.1-0.2 mg/L	OR	4.3	2.8 - 6.8	96 cases	7	n.a.	Regression	n.a.	Non-AMI patients	no	hospitalization - 34 months	[38]
cTnI	Cardiac death, non-fatal MI	Above 0.03-3.1 mg/L	RR	4.2	2.7 - 6.4	n.a.	9	no	Regression	n.a.	Unstable angina pectoris patients	no	42 days median	[39]
cTnT	MI, death	Above unknown level	RR	3.8	2.6 - 5.5	1,292 cases	12	yes	Fixed effects model	n.a.	Unstable angina pectoris patients	no	1 -147 weeks	[36]
cTnT + cTnI	MI, death	cTnT above 0.1-0.2 ng/mL, cTnI above 0.1-3.1 ng/mL	OR	3.11	2.59 - 3.74	201 cases	21	no	Fixed effects model	n.a.	Unstable angina patients	no	5 months -3 yr	[34]
cTnT	MI, death	Above 0.1-0.2 ng/ml	OR	2.86	2.35 - 3.47	1,330 cases	3	no	Fixed effects model	n.a.	STEMI patients	no	30 days	[34]
cTnT + cTnI	MI, death	cTnT above 0.1-0.2 ng/mL, cTnI above 0.6 ng/mL	OR	2.79	2.17 - 3.58	322 cases	5	no	Fixed effects model	n.a.	nSTEMI patients	no	5 months -3 yr	[34]

20 ACS: acute coronary syndrome.
21 (n)STEMI: (non)-ST elevated myocardial infarction.
22 AMI: acute myocardial infarction.

Marker	Outcome	Risk Applies to	Risk	Results	95% ci	N Patients	N Cohorts	Het.	Pooling Methods	Adj.	Patient group, general population	IPD	follow-up period	Publication
cTnT	Cardiac death, non-fatal MI	Above 0.1-0.25 mg/L	RR	2.7	2.1 - 3.4	n.a.	12	n.a.	Regression	n.a.	Unstable angina pectoris patients	no	30 days median	[39]
cTnT + cTnI	MI, death	cTnT above 0.1-0.2 ng/mL, cTnI above unknown level	OR	2.5	2.0 - 3.1	241 cases	10	n.a.	Random effects model	no	ACS, non-ST elevated patients	no	1- 147 weeks	[40]
cTnT + cTnI	MI	Above 0.1-1.5 ng/ml	OR	2.27	1.62 - 3.16	2,401 total	3	n.a.	Regression	n.a.	Patients undergoing PCI	no	1.5 - 68 months	[41]
cTnI	Death, nSTEMI	Above 2.3-0.026 ng/mL	OR	1.77	1.36 - 2.30	1,174 cases	16	no	Random effects model	n.a.	Post elective PCI patients	no	3 - 50 months	[42]
cTnT	Death, nSTEMI	Above 0.1-0.03 ng/ml	OR	1.77	1.29 - 2.45	293 cases	6	no	Random effects model	n.a.	Post elective PCI patients	no	6 - 67 months	[42]
cTnT + cTnI	MI, death	cTnT above 0.03-0.1 ng/mL, cTnI above 2.3-0.08 ng/mL	OR	1.59	1.29 - 1.95	6,885 total	15	n.a.	Fixed effects model	n.a.	Post elective PCI patients	no	3 - 67 months	[43]
cTnT + cTnI	Death, MI, revascularization, angina	Above 0.1-1.5 ng/ml	OR	1.03	0.84 - 1.26	1,831 cases	7	n.a.	Regression	n.a.	Patients undergoing PCI	no	1.5 - 77 months	[41]
Kidney function														
Serum creatine (eGFR)	CVD death	Reference value vs 15-29ml/min/1.73m2	HR	3.98	3.02 - 5.24	266,975 total	6	n.a.	Random effects model	yes	Hypertension, pre-exist CVD, diabetes	no	4.1 yr mean	[44]
Cystatin C	CVD	Top vs bottom quintile	RR	2.62	2.05 - 3.37	2,321 cases	13	yes	Random effects model	yes	High CVD risk population, elderly, CVD patients	no	1 - 12.8 yr	[45]

Marker	Outcome	Risk Applies to	Risk	Results	95% ci	N Patients	N Cohorts	Het.	Pooling Methods	Adj.	Patient group, general population	IPD	follow-up period	Publication
Serum creatine (eGFR)	CVD death	Reference value vs 30-44ml/min/1.73m2	HR	2.50	2.10 - 2.97	266,975 total	6	n.a.	Random effects model	yes	High risk CKD[23] (hypertension, pre-exist CVD, diabetes)	no	4.1 yr mean	[44]
Cystatin C	CHD	Top vs bottom tertile	RR	1.72	1.27 - 2.34	741 cases	4	yes	Random effects model	yes	High CVD risk population, elderly, CVD patients	no	3 - 7.4 yr	[45]
Serum creatine (eGFR)	CVD death	Reference value vs 45-59ml/min/1.73m2	HR	1.63	1.22 - 2.18	266,975 total	6	n.a.	Random effects model	yes	High risk CKD (hypertension, pre-exist CVD, diabetes)	no	4.1 yr mean	[44]
Serum creatine (eGFR)	CVD death	Reference value vs 60-74ml/min/1.73m2	HR	1.01	0.80 - 1.28	266,975 total	6	n.a.	Random effects model	yes	High risk CKD (hypertension, pre-exist CVD, diabetes)	no	4.1 yr mean	[44]
Serum creatine (eGFR)	CVD death	Reference value vs 75-89ml/min/1.73m2	HR	0.98	0.85 - 1.13	266,975 total	6	n.a.	Random effects model	yes	High risk CKD (hypertension, pre-exist CVD, diabetes)	no	4.1 yr mean	[44]
Lipids														
Lipoprotein(a)	CHD death, non-fatal MI	Top vs bottom tertile	RR	1.3	1.1 - 1.6	1,392 cases	9	no	Inverse variance weighted mean	yes	Pre-existing CHD	no	> 1 yr	[46]
Lp-PLA2[24]	Vascular death	1 SD increase	RR	1.10	0.98 - 1.25	186 cases	5	n.a.	Random effects model	yes	Ischemic event patients	no	1.1 yr median	[47]
Lp-PLA2	CHD	1 SD increase	RR	1.01	0.92 - 1.11	708 cases	6	n.a.	Random effects model	yes	Ischemic event patients	no	1.1 yr median	[47]
Miscellaneous														
Hematocrit	CHD death, non-fatal MI	Top vs bottom tertile	RR	1.81	1.19 - 2.76	1,162 cases	3	no	Inverse variance weighted mean	yes	Pre-existing CVD	no	16 yr mean	[48]

23 CKD: chronic kidney disease.

24 Lp-PLA2: lipoprotein associated phospholipase A2.

Supplementary Table 3. Meta-analyses of cohorts without pre-existing cardiovascular disease on markers for stroke.

Marker	Outcome	Risk Applies To	Risk	Results	95% ci	N Patients	N Cohorts	Het.	Pooling Method	Adj.	Patient group	IPD	Follow-up Period	Publi-cation
Hemostasis														
Fibrinogen	Ischemic Stroke	1 g/L increase	HR	1.75	1.55 - 1.98	2,775 cases	31	yes	Cox regression	yes	No pre-existing CHD	yes	> 1yr	[4]
Inflammation														
CRP	Ischemic stroke	Per 1 SD increase	RR	1.27	1.15 - 1.40	1,931 cases	15	no	Random effects model	yes	No pre-existing CVD	yes	> 1yr	[10]
Kidney function														
Serum uric acid	Stroke	Above unknown level	RR	1.47	1.19 - 1.76	1,031 cases	4	no	Random effects model	yes	No previous stroke	no	7.2 - 12.6 yr	[49]
Serum uric acid	Stroke death	Above unknown level	RR	1.26	1.12 - 1.39	2,059 cases	6	no	Random effects model	yes	No previous stroke	no	12.4 - 23 yr	[49]
Lipids														
ApoB	Ischemic stroke	29 mg/dL increase	HR	1.19	1.05 - 1.34	1,192 cases	8	yes	Random effects model	yes	No pre-existing vascular disease	yes	8.1 yr median	[17]
non-HDL cholesterol/ HDL	Ischemic stroke	1.53 unit increase	HR	1.14	1.05 - 1.24	1,192 cases	8	yes	Random effects model	yes	No pre-existing vascular disease	yes	8.1 yr median	[17]
ApoB/ApoAI ratio	Ischemic stroke	0.27 unit increase	HR	1.13	1.05 - 1.21	1,192 cases	8	yes	Random effects model	yes	No pre-existing vascular disease	yes	8.1 yr median	[17]
Cholesterol	ischemic stroke	1 mmol/L decrease	HR	1.09	0.95 - 1.26	225 cases	61	yes	Cox regression	no	No pre-existing CVD, age 80-89	yes	13 yr mean	[15]
Lipopro-tein(a)	Ischemic stroke	1 SD increase	RR	1.08	1.01 - 1.16	69,539 total	13	no	Fixed effects model	yes	No pre-existing CHD	no	>1 yr	[20]
non-HDL cholesterol	Ischemic stroke	43 mg/dL increase	HR	1.08	0.97 - 1.20	2,534 cases	68	yes	Random effects model	yes	No pre-existing vascular disease	yes	8.1 yr median	[17]
Cholesterol	Ischemic stroke	1 mmol/L decrease	HR	1.06	0.95 - 1.17	540 cases	61	yes	Cox regression	no	No pre-existing CVD, age 70-79	yes	13 yr mean	[15]

Marker	Outcome	Risk Applies To	Risk	Results	95% ci	N Patients	N Cohorts	Het.	Pooling Method	Adj.	Patient group	IPD	Follow-up Period	Publi-cation
Triglycerides	Ischemic stroke	Per 68% increase	HR	1.02	0.94 - 1.11	2,534 cases	68	yes	Random effects model	yes	No pre-existing vascular disease	yes	8.1 yr median	[17]
ApoAI	Ischemic stroke	29 mg/dL increase	HR	0.97	0.88 - 1.08	1,192 cases	8	yes	Random effects model	yes	No pre-existing vascular disease	yes	8.1 yr median	[17]
HDL	Ischemic stroke	15 mg/dL increase	HR	0.96	0.90 - 1.02	2,534 cases	68	yes	Random effects model	yes	No pre-existing vascular disease	yes	8.1 yr median	[17]
Cholesterol	Ischemic stroke	1 mmol/L decrease	HR	0.89	0.79 - 1.01	850 cases	61	yes	Cox regression	no	No pre-existing CVD, age 60-69	yes	13 yr mean	[15]
Cholesterol	Ischemic stroke	1 mmol/L decrease	HR	0.73	0.61 - 0.87	225 cases	61	yes	Cox regression	no	No pre-existing CVD, age 40-59	yes	13 yr mean	[15]
Miscellaneous														
Homocys-teine	Ischemic stroke	Homocys-teine levels increase	RR	1.37	0.99 - 1.91	314 cases	3	no	Fixed effects model	yes	No pre-existing cerbrovascular disease	no	5 -12.8 yr	[21]
Homocys-teine	Stroke	25% lower usual ho-mocysteine level	OR	0.81	0.69 - 0.95	435 cases	9	no	Regression	yes	No pre-existing cerbrovascular disease	yes	n.a.	[24]

Supplementary Table 4. Meta-analyses of cohorts with pre-existing cardiovascular disease for stroke.

Marker	Outcome	Risk Applies To	Risk	Results	95% ci	N Patients	N Cohorts	Het.	Pooling Method	Adj.	Patient group	IPD	Follow-up Period	Publication
Hemostasis														
Fibrinogen	Ischemic Stroke	Above median fibrinogen levels (per study)	HR	1.21	1.01 - 1.44	512 cases	3	no	Cox regres-sion	yes	TIA[25] or ischemic stroke patients	yes	2.6 - 10 yr	[27]
Lipids														
Lp-PLA2	Stroke	1 SD increase	RR	1.02	0.82 - 1.27	111 cases	4	n.a.	Random effects model	yes	Ischemic event patients	no	1.1 yr median	[47]

25 TIA: transient ischemic attack.

Supplementary Table 5. Meta-analyses of cohorts representing the general population on markers for cardiovascular disease.

Marker	Outcome	Risk Applies To	Risk	Results	95% ci	N Patients	N Cohorts	Het.	Pooling method	Adj.	Patient group	IPD	Follow-up period	Publication
Diabetes related														
Insulin fasting	CVD death	Top vs bottom quantile	HR	2.66	1.45 - 4.90	68 cases	7	no	Fixed effects model	yes	Population based, no diabetes, women Europe	yes	6.3 - 11.8 yr	[50]
Pro-insulin	CHD death, non-fatal MI	Top vs bottom tertile	OR	2.23	1.65 - 3.00	413 cases	3	no	Fixed effects model	yes	Population based	no	9.5 yr mean	[51]
Insulin fasting	CVD death	Top vs bottom quantile	HR	1.54	1.16 - 2.03	362 cases	10	no	Fixed effects model	yes	Population based, no diabetes, men Europe	yes	4.7 - 12 yr	[50]
Insulin post glucose load	CVD death	Top vs bottom quartile	HR	1.36	0.53 - 3.45	33 cases	7	no	Fixed effects model	yes	Population based, no diabetes, women Europe	yes	6.3 - 11.8 yr	[50]
Insulin non-fasting	CHD death, non-fatal MI	Top vs bottom tertile	OR	1.35	1.14 - 1.60	1,980 cases	8	no	Fixed effects model	yes	Population based	no	13.7 yr mean	[51]
Insulin non-fating	CHD death, MI, HF[26]	250 pmol/L increase	RR	1.25	1.03 - 1.51	907 cases	17	yes	Random effects model	yes	General population	no	3.5 - 17 yr	[52]
Insulin fasting	CHD death, MI, HF	50 pmol/L increase	RR	1.17	1.09 - 1.26	731 cases	17	no	Fixed effects model	yes	General population	no	3.5 - 17 yr	[52]
Insulin fasting	CHD death, non-fatal MI	Top vs bottom tertile	OR	1.12	0.98 - 1.28	2,649 cases	14	yes	Fixed effects model	yes	Population based	no	9.1 yr mean	[51]
Insulin post glucose load	CVD death	Top vs bottom quartile	HR	0.85	0.60 - 1.21	295 cases	10	no	Fixed effects model	yes	Population based, no diabetes, men Europe	yes	4.7 - 12 yr	[50]
Hemostasis														
Fibrinogen	CVD	Top vs bottom tertile	OR	2.46	2.22 - 2.72	1,910 cases	8	no	Inverse variance weighted mean	no	General population	no	0.5 - 13.5 yr	[53]

26 HF: heart failure.

Marker	Outcome	Risk Applies To	Risk	Results	95% ci	N Patients	N Cohorts	Het.	Pooling method	Adj.	Patient group	IPD	Follow-up period	Publication
Fibrinogen	CVD	Top vs bottom tertile	OR	2.3	1.9 - 2.8	15,688 total	6	n.a.	Fixed effects model	yes	General population, men mainly	no	[3]2yr	[54]
Tissue plasminogen activator	CHD death, non-fatal MI	Top vs bottom tertile	OR	1.47	1.19 - 1.81	1,669 cases	7	yes	Inverse variance weighted mean	yes	Population based	no	8 yr mean	[28]
Von Wille-brand Factor	CHD death, non-fatal MI	Top vs bottom tertile	OR	1.2	0.8 - 1.9	899 cases	6	no	Inverse variance weighted mean	yes	Population based	no	7 yr mean	[26]
Plasminogen activator inhibitor-1	CHD death, non-fatal MI	Top vs bottom tertile	OR	0.98	0.53 - 1.81	833 cases	5	no	Inverse variance weighted mean	yes	Population based	no	5 yr mean	[28]
Hormones														
Thyroid stimulating hormone	IHD	Below: 0.1–0.6 (unit n.a.)	OR	1.27	0.95 - 1.69	9,627 total	5	yes	Random effects model	yes	Population based	no	4 -20 yr	[55]
Thyroid stimulating hormone	CHD	Above: 4.5 mU/L	RR	1.21	0.88 - 1.68	1,392 cases	5	no	Random effects model	yes	Population based	no	2 - 20 yr	[56]
Thyroid stimulating hormone	CHD	Below: 4.5 mU/L	RR	1.20	0.97 - 1.49	2,134 cases	10	no	Random effects model	yes	Population based	no	2 - 20 yr	[56]
Thyroid stimulating hormone	CVD death	Above: 4.5 mU/L	RR	1.19	0.81 - 1.76	911 cases	5	no	Random effects model	yes	Population based	no	2 - 20 yr	[56]
Thyroid stimulating hormone	CVD death	Below: 4.5 mU/L	RR	1.18	0.98 - 1.42	1,167 cases	8	no	Random effects model	yes	Population based	no	2 - 20 yr	[56]
Thyroid stimulating hormone	CVD death	Below: 0.1–0.6 (unit n.a.)	OR	1.09	0.84 - 1.41	14,719 total	8	yes	Random effects model	yes	Population based	no	4 -20 yr	[55]

Marker	Outcome	Risk Applies To	Risk	Results	95% ci	N Patients	N Cohorts	Het.	Pooling method	Adj.	Patient group	IPD	Follow-up period	Publication
Inflammation														
CRP	CHD	Top vs bottom tertile	RR	2.0	1.6 - 2.5	1,953 total	11	no	Inverse variance weighted mean	yes	Population based	no	8 yr mean	[31]
Albumin	CHD	Bottom vs top tertile	RR	1.5	1.3 - 1.7	3,770 cases	7	no	Inverse variance weighted mean	yes	General population	no	12 yr mean	[57]
Lipids														
Lipopro-tein(a)	CHD death, non-fatal MI	Top vs bottom tertile	RR	1.7	1.4 - 1.9	4,044 cases	18	no	Inverse variance weighted mean	yes	Population based	no	> 1 yr	[46]
Triglycerides	CHD	1 SD increase	HR	1.56	1.20 - 2.03	368 cases	11	no	Cox regres-sion	yes	Asia-Pacific population	yes	2.5 - 19.7 yr	[58]
Triglycerides	CHD death	Increase: <0.8, 0.8-1.0, 1.1-1.3, 1.4-1.8, >1.8 mmol/L	HR	1.38	1.18 - 1.62	2,082 cases	30	yes	Cox regres-sion	yes	Asia-Pacific population	yes	2.5 - 25.1 yr	[59]
Triglycerides	CVD	1 mmol/L increase	RR	1.37	1.13 - 1.66	439 cases	5	n.a.	Regression	yes	Population based, women	no	11.4 yr mean	[60]
Cholesterol	CHD death	Increase: <4.2 , 4.2-4.6, 4.7-5.1, 5.2-5.8, >5.8 mmol/L	HR	1.31	1.23 - 1.39	2,082 cases	30	no	Cox regres-sion	yes	Asia-Pacific population	yes	2.5 - 25.1 yr	[59]
Cholesterol	CHD death	0.7 mmol/L increase in usual cholesterol level	HR	1.23	1.18 - 1.29	4,841 cases	25	no	Cox regres-sion	yes	Not selected on any disease, Asia Pacific region	yes	2.5 - 24.6 yr	[61]
Triglycerides	CVD	1 mmol/L increase	RR	1.14	1.05 - 1.28	2,445 cases	16	n.a.	Regression	yes	Population based, men	no	8.4 yr mean	[60]

Marker	Outcome	Risk Applies To	Risk	Results	95% ci	N Patients	N Cohorts	Het.	Pooling method	Adj.	Patient group	IPD	Follow-up period	Publication
Miscellaneous														
Erythrocyte sedimentation rate	CHD death	Top vs bottom tertile	RR	1.33	1.15 - 1.54	1,703 cases	4	no	Inverse variance weighted mean	yes	Population based	no	14 yr mean	[48]
Hematocrit	CHD death, non-fatal MI	Top vs bottom tertile	RR	1.16	1.05 - 1.29	8,020 cases	16	no	Inverse variance weighted mean	yes	Population based	no	16 yr mean	[48]

Supplementary Table 6. Meta-analyses of cohorts with and without cardiovascular disease on markers for cardiovascular disease.

Marker	Outcome	Risk Applies To	Risk	Results	95% ci	N Patients	N Cohorts	Het.	Pooling Method	Adj.	Patient group	IPD	follow-up period	Publication
Diabetes related														
Glucose (non fasting)	Fatal, non-fatal CVD	Top vs bottom category	RR	1.84	1.45 - 2.33	37,117 total	5	n.a.	Random effects model	no	Non-diabetic patients, no exclusion on disease	no	4.8 - 20 yr	[62]
Glycated hemoglobine (HBA(1c))	Fatal, non-fatal CVD	Top vs bottom category	RR	1.70	0.99 - 2.94	3,602 total	3	n.a.	Random effects model	yes	Non-diabetic patients, no exclusion on disease	no	8 yr	[62]
Glucose (fasting)	Fatal, non-fatal CVD	Top vs bottom category	RR	1.27	1.13 - 1.43	127,617 total	18	n.a.	Random effects model	yes	Non-diabetic patients, no exclusion on disease	no	4 - 22 yr	[62]
Glucose post load	Fatal, non-fatal CVD	Top vs bottom category	RR	1.27	1.09 - 1.48	61,773 total	13	n.a.	Random effects model	yes	Non-diabetic patients, no exclusion on disease	no	5 -22 yr	[62]
Hemostasis														
Fibrinogen	CVD	Top vs bottom tertile	OR	2.35	2.14 - 2.57	2,581 cases	13	no	Inverse variance weighted mean	no	General population + pre-existing CVD	no	0.5 - 13.5 yr	[53]
Fibrinogen	CHD	Top vs bottom tertile	RR	1.8	1.6 - 2.0	4,018 cases	18	no	Inverse variance weighted mean	yes	General population + pre-existing CVD	no	8 yr mean	[57]
D-dimer fibrinogen	CHD	Top vs bottom tertile	OR	1.7	1.3 - 2.2	1,535 cases	7	no	Regression	yes	Population based + pre-existing CVD	no	5 yr mean	[63]
Hormone														
Adiponectin	CHD death, non-fatal MI	Top vs bottom tertile	OR	0.84	0.70 - 1.01	1,313 cases	7	no	Inverse variance weighted mean	n.a.	Population based + pre-existing CVD	no	9.7 yr mean	[64]

Marker	Outcome	Risk Applies To	Risk	Results	95% ci	N Patients	N Cohorts	Het.	Pooling Method	Adj.	Patient group	IPD	follow-up period	Publication
Infection														
Chlamydia Pneumoniae IgA titers	CHD	Top vs bottom tertile	OR	1.25	1.03 - 1.53	2,283 cases	10	no	Inverse variance weighted mean	yes	Population based and pre-existing CVD	no	11 yr mean	[65]
Chlamydia Pneumoniae IgG titers	CHD	Top vs bottom tertile	OR	1.15	0.97 - 1.36	3,169 cases	15	no	Regression	yes	Population based + pre-existing CVD	no	10 yr mean	[66]
Inflammation														
CRP	CHD	Top vs bottom tertile	RR	1.7	1.4 - 2.1	1,053 cases	7	no	Inverse variance weighted mean	yes	General population + pre-existing CVD	no	6 yr mean	[57]
Serum ameloid A	CHD	Top vs bottom tertile	RR	1.6	1.1 - 2.2	1,057 cases	4	no	Inverse variance weighted mean	yes	Population based + pre-existing CVD	no	10 yr mean	[31]
CRP	CHD death, non-fatal MI	1.0 mg/mL > CRP > 3.0 mg/mL	RR	1.58	1.37 - 1.83	45,563 total	10	no	Random effects model	yes	No pre-existing CVD, pre-existing CVD	no	2.9 - 13 yr	[67]
Granulocyte count	CHD death, non-fatal MI	Top vs bottom tertile	RR	1.51	0.99 - 2.30	1,643 cases	5	yes	Random effects model	yes	Pre-existing CVD, no pre-existing CVD	no	3 - 18 yr	[68]
Leukocyte count	CHD	Top vs bottom tertile	RR	1.5	1.4 - 1.6	7229 cases	19	yes	Inverse variance weighted mean	yes	General population+ Pre-existing CVD	no	8 yr mean	[57]
Neutrophil count	CHD death, non-fatal MI	Top vs bottom tertile	RR	1.48	1.02 - 2.15	1,562 cases	5	yes	Random effects model	yes	Pre-existing CVD, no pre-existing CVD	no	3 - 18 yr	[68]
Soluble ICAM[27]1	CHD death, non-fatal MI	Top vs bottom tertile	OR	1.39	1.11 - 1.73	1396 cases	5	yes	Inverse variance weighted mean	yes	Population based + pre-existing CVD	no	11 yr mean	[69]

27 ICAM: intercellular adhesion molecule

Marker	Outcome	Risk Applies To	Risk	Results	95% ci	N Patients	N Cohorts	Het.	Pooling Method	Adj.	Patient group	IPD	follow-up period	Publication
IL-18	CHD, stroke	Top vs bottom tertile	RR	1.35	1.25 - 1.51	3,047 cases	12	no	Fixed effects model	yes	Population based, CAD[28] patients, high risk CHD, men	no	1.58 - 20 yr	[70]
Soluble E-Selectin	CHD death, non-fatal MI	Top vs bottom tertile	OR	1.16	0.87 - 1.55	832 cases	2	no	Inverse variance weighted mean	yes	Population based + pre-existing CVD	no	13 yr mean	[69]
Lymphocyte count	CHD death, non-fatal MI	Top vs bottom tertile	RR	1.11	0.99 - 1.25	1,755 cases	7	no	Fixed effects model	yes	Pre-existing CVD, no pre-existing CVD	no	3 - 18 yr	[68]
Monocyte count	CHD death, non-fatal MI	Top vs bottom tertile	RR	1.10	0.98 - 1.24	1,750 cases	7	no	Fixed effects model	yes	Pre-existing CVD, no pre-existing CVD	no	3 - 18 yr	[68]
Soluble VCAM[29]1	CHD death, non-fatal MI	Top vs bottom tertile	OR	1.02	0.81 - 1.29	1307 cases	4	no	Inverse variance weighted mean	yes	Population based + pre-existing CVD	no	11 yr mean	[69]
Ischemia														
BNP + NT-proBNP	CHD death, non-fatal MI	Top vs bottom tertile	RR	2.03	1.54 - 2.66	4,301 cases	19	yes	Random effects model	yes	Population based, high CVD risk, pre-existing CVD	no	5 yr mean	[71]
Kidney function														
Serum uric acid	CHD	Top vs bottom tertile	OR	1.22	1.05 - 1.40	1,645 cases	8	yes	Fixed effects model	yes	(No) pre-existing CVD, women	no	10.5 yr mean	[72]
Serum uric acid	CHD	Top vs bottom tertile	OR	1.12	1.05 - 1.19	7,813 cases	15	yes	Fixed effects model	yes	(No) pre-existing CVD, men	no	10.5 yr mean	[72]
Lipids														
Lp-PLA2	CVD	Top vs bottom quantile	OR	1.60	1.27 - 2.00	12,098 total	8	yes	Random effects model	yes	No exclusion on disease	no	1 - 14 yr	[73]

28 CAD: coronary artery disease.
29 VCAM: vascular cell adhesion molecule.

Marker	Outcome	Risk Applies To	Risk	Results	95% ci	N Patients	N Cohorts	Het.	Pooling Method	Adj.	Patient group	IPD	follow-up period	Publication
ApoB	Fatal, non-fatal CVD	1 SD increase	RRR	1.43	1.35 - 1.51	22,950 cases	12	yes	Random effects model	yes	No specified population	no	n.a.	[74]
Non-HDL cholesterol	Fatal, non-fatal CVD	1 SD increase	RRR	1.34	1.24 - 1.44	22,950 cases	12	yes	Random effects model	yes	No specified population	no	n.a.	[74]
LDL[30]	Fatal, non-fatal CVD	1 SD increase	RRR	1.25	1.18 - 1.33	22,950 cases	11	yes	Random effects model	yes	No specified population	no	n.a.	[74]
Lp-PLA2	Vascular death	1 SD increase	RR	1.13	1.05 - 1.22	2,887 cases	11	no	Random effects model	yes	No pre-existing CVD, stable CHD	no	5.8 yr median	[47]
Lp-PLA2	CHD	1 SD increase	RR	1.11	1.08 - 1.15	4,361 cases	12	no	Fixed effects model	yes	No pre-existing CVD, stable CHD	no	5.8 yr median	[47]
Miscellaneous														
Homocys-teine	CVD and stroke	Above: 14.5-23.9 mmol/L	OR	3.74	2.53 - 5.54	317 cases	4	yes	Fixed effects model	no	No exclusion on disease	no	2.7 - 9 (2 studies n.a.)	[75]
Homocys-teine	CHD	5 mmol/L increase	RR	1.3	1.1 - 1.5	1,041 cases	5	yes	Inverse variance weighted mean	yes	No pre-existing CVD, pre-existing CVD	no	8 yr mean	[76]
Homocys-teine	Cardiac death, non-fatal MI	5 mmol/L increase	OR	1.23	1.14 - 1.32	3,144 cases	16	n.a.	Random effects model	yes	No renal disease	no	3 - 13 yr	[77]
Serum ferritin	CHD	Above: 200 mg/L	RR	1.03	0.83 - 1.29	570 cases	5	yes	Inverse variance weighted mean	yes	Population based + pre-existing CVD	no	8 yr mean	[78]
Iron-binding capacity	CHD	Top vs bottom tertile	RR	0.98	0.66 - 1.46	2755 cases	4	no	Inverse variance weighted mean	yes	Population based + pre-existing CVD	no	13 yr mean	[78]

30 LDL: low density lipoprotein.

Marker	Outcome	Risk Applies To	Risk	Results	95% ci	N Patients	N Cohorts	Het.	Pooling Method	Adj.	Patient group	IPD	follow-up period	Publication
Transferrin saturation	CHD	Top vs bottom tertile	RR	0.92	0.74 - 1.14	6194 cases	5	no	Inverse variance weighted mean	yes	Population based + pre-existing CVD	no	14 yr mean	[78]
Serum ferritin	CHD	Top vs bottom tertile	RR	0.83	0.67 - 1.03	2848 cases	3	yes	Inverse variance weighted mean	yes	Population based + pre-existing CVD	no	14 yr mean	[78]

Supplementary Table 7. Meta-analyses of miscellaneous cohorts on markers for cardiovascular disease.

Marker	Outcome	Risk Applies To	Risk	Results	95% ci	N Patients	N Cohorts	Het.	Pooling Method	Adj.	Patient group, general population	IPD	Follow-up Period	Publication
Ischemia														
BNP + NT-proBNP	Non-fatal MI	NT-proBNP above 280-533 pg/mL, BNP above 40-100 pg/mL	OR	6.24	1.82 - 21.40	33 cases	3	no	Fixed effects model	n.a.	Vascular and non cardiac surgery patients	no	<30 days	[79]
BNP + NT-proBNP	MACE	NT-proBNP above 280-319 pg/mL, BNP above 35 pg/mL	OR	3.31	2.10 - 5.24	95 cases	2	yes	Random effects model	n.a.	Vascular and non cardiac surgery patients	no	<180 days	[79]
BNP	MACE	Above 35-255 pg/mL	OR	25.45	12.46 - 51.97	148 casss	6	no	Random effects model	no	Vascular and non-cardiac surgery patients	no	up to 43 days	[80]
BNP + NT-proBNP	Cardiac death	NT-proBNP above 201-791 pg/mL, BNP above 35-255 pg/mL	OR	23.88	9.43 - 60.43	45 cases	7	no	Random effects model	no	Vascular and non-cardiac surgery patients	no	up to 43 days	[80]
BNP + NT-proBNP	MACE	NT-proBNP above 280-533 pg/mL, BNP above 40–100 pg/mL	OR	17.37	3.31 - 91.15	52 cases	4	yes	Random effects model	n.a.	Vascular and non cardiac surgery patients	no	<30days	[79]
NT-proBNP	MACE	Above 201-791 pg/mL	OR	15.65	10.39 - 25.37	135 cases	4	no	Random effects model	no	Vascular and non-cardiac surgery patients	no	up to 43 days	[80]
Kidney function														
Serum creatine (eGFR)	CVD death	30 mmol/L decrease	HR	1.27	1.11 - 1.46	1,784 cases	8	no	Cox regression	yes	Hypertensive patients	yes	5 yr mean	[81]
Serum Uric Acid	CVD death	Above 75 mmol/L	HR	1.13	1.05 - 1.22	1,784 cases	8	yes	Cox regression	yes	Hypertensive patients	yes	5 yr mean	[81]
Lipids														
Cholesterol	CVD death	1 mmol/L increase	HR	1.11	1.05 - 1.17	1,784 cases	8	no	Cox regression	yes	Hypertensive patients	yes	5 yr mean	[81]

Supplementary Table 8. Meta-analyses of cohorts representing the general population for stroke.

Marker	Outcome	Risk Applies To	Risk	Results	95% ci	N Patients	N Cohorts	Het.	Pooling Method	Adj.	Patient group	IPD	Follow-up Period	Publication
Inflammation														
CRP	Stroke	Top vs bottom tertile	RR	1.68	1.40 - 2.01	9,572 total	4	no	Fixed effects model	yes	General population	no	> 8 yr	[82]
Lipids														
Triglycerides	Ischemic stroke	1 SD increase	HR	1.35	1.00 - 1.83	273 cases	13	no	Cox regression	yes	Asia-Pacific population	yes	2.5 - 19.7 yr	[58]
Cholesterol	Ischemic stroke	Increase: <4.2 , 4.2-4.6, 4.7-5.1, 5.2-5.8, >5.8 mmol/L	HR	1.15	0.99 - 1.34	420 cases	24	no	Cox regression	yes	Asia-Pacific population	yes	2.7 - 25.1 yr	[59]
Triglycerides	Ischemic stroke	Increase: <0.8, 0.8-1.0, 1.1-1.3, 1.4-1.8, >1.8 mmol/L	HR	1.03	0.74 - 1.45	420 cases	24	no	Cox regression	yes	Asia-Pacific population	yes	2.7 - 25.1 yr	[59]

Supplementary Table 9. Meta-analyses of cohorts with and without pre-existing cardiovascular disease for stroke.

Marker	Outcome	Risk Applies To	Risk	Results	95% ci	N Patients	N Cohorts	Het.	Pooling Method	Adj.	Patient group, general population	IPD	Follow-up Period	Publication
Ischemia														
BNP + NT-proBNP	Stroke	Top vs bottom tertile	RR	1.93	1.58 - 2.37	2,063 cases	13	yes	Random effects model	yes	Population based, high CVD risk, pre-existing CVD	no	5 yr mean	[71]
Kidney function														
Cystatin C	Stroke	Top vs bottom quintile	RR	1.83	1.12 - 3.00	828 cases	4	yes	Random effects model	yes	Population based, elderly, CVD patients	no	3.1 - 7.4 yr	[45]
Lipids														
Cholesterol	Ischemic stroke	Top vs bottom quintile	HR	1.5	1.3 - 1.8	749 cases	15	yes	Cox regression	yes	Not selected on any disease, Asia Pacific region	yes	2.7 - 24.6 yr	[61]
Lipoprotein(a)	Stroke	Top vs tertile	RR	1.21	1.04 - 1.41	2,009 cases	8	no	Random effects model	yes	Stroke and non-stroke patients	no	3 - 14 yr	[83]
Triglycerides	Ischemic stroke, incl. TIA	1 SD increase	RR	1.11	1.08 - 1.15	2,046 cases	5	no	Inverse variance weighted mean	yes	Population based, CHD patients free of stroke	no	3 - 18 yr	[84]
Lp-PLA2	Ischemic stroke	1 SD increase	RR	1.10	1.04 - 1.16	2,097 cases	5	no	Fixed effects model	yes	No pre-existing CVD, stable CHD	no	5.8 yr median	[47]
Triglycerides	Stroke	10 mg/dL increase	RR	1.05	1.03 - 1.07	3,348 cases	29	n.a.	Regression	yes	Primary and secondary events	no	>1yr	[85]
Miscellaneous														
Homocysteine	Stroke	5 mmol/L increase	OR	1.42	1.21 - 1.66	676 cases	8	n.a.	Random effects model	yes	No renal disease	no	3 - 12 yr	[77]

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Chapter 2

Severe coronary artery disease symptoms is associated with reduced platelet responsiveness, but not with future adverse events

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Abstract

Platelets play a multifaceted role in atherothrombosis. High platelet reactivity increases the risk of ischemic occlusion after a rupture of atherosclerotic plaques, while a myriad of platelet derived factors regulate atherosclerosis progression. Conventional platelet function assays that have been used to study cardiovascular disease severity and risk prediction rely on the ability of platelets to form aggregates. We have investigated platelet granule release in response to specific activation routes in relation with cardiovascular disease severity and the risk on future adverse events in a multicenter cohort study.

A total of 714 patients scheduled for percutaneous coronary intervention were included at 4 different hospitals. Amongst them, the platelet response was measured in 484 patients. Patients that were diagnosed with unstable angina or non-ST elevation myocardial infarction had a strong reduced response to ADP, and iloprost with suboptimal ADP stimulation (OR: 0.40, 95% confidence interval (ci) 0.25 – 0.66, and OR: 0.36, 95% ci 0.22 – 0.60 respectively) compared with patients with stable angina. Platelet response via the $P2Y_{12}$, IP receptor with suboptimal $P2Y_{12}$ stimulation, GPVI, or PAR-1 receptor was not associated with future adverse events.

In conclusion, we find that reduced ADP response is associated with unstable angina independently of the use of ADP antagonists.

Introduction

Predicting cardiovascular disease risk is crucial for clinical decision-making. Biomarkers belong to the most important equipment to predict cardiovascular events. Until recently, the main focus of biomarker research was on plasma proteins. We hypothesized that functionality of circulating cells may provide additional benefit to cardiovascular disease risk prediction. Hyper reactivity of blood platelets, in particular despite anti-platelet treatment, belongs to the most important risk factors for of ischemia and infarction [1,2].

Platelets can be activated via different receptors, that all act in concert during a hemostatic response [2]. The initial platelet activation step after platelet binding to collagen exposed on the rupture plaque is via $\alpha_2\beta_1$ and glycoprotein (GP)VI, which initiates $\alpha_{IIb}\beta_3$ activation and granules release. As collagens are insoluble proteins, to activate newly arriving platelets that adhere to the first platelet layer, additional activation routes are essential. An important secondary activation step is triggered by adenosine diphosphate (ADP) release from the dense granules of platelets. ADP activates the platelets via the purinergic receptors $P2Y_1$ and $P2Y_{12}$ receptors. Parallel to the platelet response, the coagulation cascade and hence thrombin generation is initiated. Thrombin is another potent platelet activator via the protease activated receptors (PAR)-1 and PAR-4. Activation of platelets is damped by the endothelium, for example via release of prostacyclin. Prostacylin inhibits platelets via stimulation of the prostacyclin receptor (IP), which results in decrease of intracellular Ca^{2+} levels [3].

Platelets are appreciated for their essential role in clot formation to stop bleeding. Besides this, there is growing evidence that platelets also mediate inflammatory reactions. Atherosclerosis depends on a myriad of inflammatory processes, which are mediated by the releasate of activated platelets [4]. Alpha granules contain high concentrations of cytokines, chemokines, growth factors and factors that influence angiogenesis [2,4]. Upon granule release platelets express P-selectin, which mediates binding to monocytes and neutrophils. Therefore, platelet activation could affect atherosclerotic plaque formation, and their ability to release their granule content could be associated with cardiovascular disease severity and risk.

The main focus of studies associating platelet function with cardiovascular disease has been focused on a poor response to anti-platelet therapy, which is associated with increased risk on future cardiac adverse events. Platelet aggregation measurements, such as the classical light transmission aggregometry (LTA), the newer Multiplate® assay, and Platelet function analysis (PFA) have proven to be reliable

platforms for diagnosis of bleeding disorders, but they lack precision and reproducibility to study platelet function in relation to arterial thrombosis [5-9]. The VerifyNow® is an established platform to monitor aspirin and $P2Y_{12}$ inhibitor therapy, but does not provide insight in the major platelet activation pathways.

We have used a novel assay based on flow-cytometric analysis of P-selectin expression in response to increasing agonist concentrations. This assay has been shown to better establish platelet responses in healthy individuals [9]. The test is based on platelet activation induced by addition of increasing concentrations of a specific agonist to whole blood. Activation state of platelets is quantified on a flow cytometer by P-selectin expression on the outer membrane of platelets. The most important pathways to activate platelets include the thrombin activation pathway via PAR1, the collagen activation pathway via GPVI and the ADP activation pathway via $P2Y_{12}$, and the inhibitor pathway via IP. We have used this platelet activation assay to evaluate platelet function in relation to coronary artery disease (CAD) severity. In addition, we prospectively investigated specific platelet responses in relation to future cardiac events. We found that reduced platelet response to ADP is associated with more severe CAD symptoms, being unstable angina or non-ST elevation myocardial infarction (nSTEMI). In addition, we assessed the ability of the assay to identify patients at risk for future cardiac events, and its ability to identify patients with high on treatment platelet reactivity.

Materials and methods

Materials

Blood was collected in 3.2% tri-sodium citrate and EDTA Vacutainers tubes, obtained from Becton Dickinson. Adenosine diphosphate (ADP) was obtained from Roche, protease activated receptor (PAR)-1 agonist SFLLRN was obtained from Bachem (H-2936), GPVI agonist cross linked collagen related peptide (CRP-XL) was a kind gift from R. Farndale at Cambridge University, IP receptor agonist iloprost was a kind gift from Schering AG, phycoerythrin (PE) labeled mouse anti-human P-selectin antibodies were obtained from BD Biosciences, fluorescein isothiocyanate (FITC) labeled mouse anti-human GPIb antibodies were obtained from BD Biosciences, FC 500 flow cytometers were obtained from Beckman Coulter, using Flow-Check and Flow-Set Fluorospheres were obtained from Beckman Coulter, data acquisition was performed with CXP SYSTEM software from Beckman Coulter, graphs and area under the curves were made with Prism 5.01 software from Graphpad Software. Coating and biotin

coupled detection antibodies for enzyme linked immunosorbent assay (ELISA) for high sensitivity C-reactive protein (HS-CRP) was obtained from R&D (DY1707 Duoset), coating anti-bodies for N-terminal prohormone of brain type natriuretic peptide (NT-proBNP) were obtained from Hytest (15C4(4NT1)), normal pool plasma was prepared in-house, Liquicheck Cardiac Plus Control LT Level 3 was obtained from BioRad, horse radish peroxidase (HRP) conjugated detection antibodies for NT-proBNP was obtained from Hytest (13G12(4NT1)), streptavidin-HRP was obtained from DAKO (P0397), SuperSignal West Pico Chemiluminescent was obtained from Thermo Scientific, a Spectramax L was obtained from Molecular Devices. Statistics were performed using IBM SPSS 20 software.

Study Population

The current study was performed as part of the Circulating Cells study. A cohort of 714 patients was included and platelet responsiveness was measured in 484 patients. At the Catharina Hospital in Eindhoven, 113 patients were included, 123 patients were included at the University Medical Center Maastricht, 83 patients were included at the University Medical Center Leiden, and 159 patients at the University Medical Center Utrecht. All patients provided written informed consent, and approval from the local medical ethics committee was obtained for every inclusion hospital. Patients scheduled for coronary angiography were included unless diagnosed with ST-elevation myocardial infarction, when they were younger than 18 years, were unable to give informed consent, were suspected of drug or alcohol abuse, suffered from serious concomitant disease, suffered from serious recent infectious disease in six weeks prior inclusion, had a suspected elevated state of the immune disease, or did not cooperate. Patients were followed-up for 9 months for the composite end-point major adverse cardiovascular events (MACE): myocardial infarction (MI), PTCA, coronary artery bypass graft (CABG), death by cardiovascular disease, and cardiovascular accident. The follow-up period for MACE risk in relation to platelet responsiveness was 180 days, as this is the time-period where patients receive intensive anti-platelet treatment. Treatment and periprocedural medication with $P2Y_{12}$ inhibitors and $\alpha_{IIb}\beta_3$ inhibitors were left at the discretion of the operator.

Platelet responsiveness measurements

Blood was collected from the arterial sheath into 3.2% tri-sodium citrate tubes, before administration of intravenous anticoagulants. Platelet responsiveness was determined with agonist concentration series of ADP, CRP-XL, PAR-1 receptor agonist, and IP agonist

with substimulatory ADP. Serial dilutions of ADP (125 μM, 31.25 μM, 7.8 μM, 1.95 μM, 488 nM, 122 nM, 31 nM, 8 nM) were prepared in 50 μL HEPES buffered saline (HBS; 10 mM HEPES, 150 mM NaCl, 1 mM $MgSO_4$, 5 mM KCl, pH 7.4), with 2 μL PE labeled mouse anti-human P-selectin antibodies and 2 μL FITC labeled mouse anti-human GPIb antibodies. Similarly, serial dilutions of CRP-XL (2.5 μgr/mL, 625 ng/mL, 156.3 ng/mL, 39.1 ng/mL, 9.8 ng/mL, 2.4 ng/mL, 600 pg/mL, 153 pg/mL), PAR-1 agonist (625 μM, 156.3 μM, 39.1 μM, 9.8 μM, 2.4 μM, 610 nM, 153 nM, 38 nM), and iloprost (1250 ng/mL, 312.5 ng/mL, 78 ng/mL, 19.5 ng/mL, 4.9 ng/mL, 1.2 ng/mL, 0.31 ng/mL, 0.076 ng/mL) with 5 μM ADP were prepared in 50 μL HBS with 2 μL mouse anti-human P-selectin antibodies and 2 μL mouse anti human GPIb antibodies. The platelet responsiveness assay was initiated by adding 5 μL whole blood to each sample of serial dilutions. Samples were incubated for 20 minutes and subsequently added with 500 μL fixative (0.2% formaldehyde and 0.9% NaCl). All samples were analyzed with a flow cytometer on the same day of processing. Flow set and flow check calibration of the flow cytometers was performed every day of patient inclusion. Data acquisition was performed with the CXP SYSTEM software. Single platelets were gated based on scatter properties and FITC signal intensity. The median fluorescence intensity for PE was measured. Dose-response graphs for P-selectin expression were constructed and area under the curves (AUC) in arbitrary units was calculated.

Laboratory measurement and, ELISA's

Patient platelet poor plasma's were obtained from EDTA anti-coagulated blood by centrifugation at 156 g for 15 minutes, and centrifugation of the supernatant at 330 g for 15 minutes. High density lipoprotein (HDL) and low density lipoprotein (LDL) levels were extracted from the pre-interventional screening. Plasma samples were stored at -80ºC until further processing. Maxisorb plates were coated with mouse anti-human NT-proBNP, or mouse anti-human HS-CRP. Plates were blocked with 1% bovine serum albumin, and incubated with patient plasma, or standards prepared with normal pool plasma for HS-CRP, or Liquicheck cardiac Plus Control LT for NT-proBNP. Plates were washed with phosphate buffered saline (PBS) pH 7.4 with 0.05% Tween 20. Bound factors were detected with HRP conjugated monoclonal mouse anti human NT-proBNP, or with biotynilated mouse anti-human HS-CRP. Plates were washed with PBS pH 7.4 with 0.05% Tween 20. Biotin coupled antibodies were bound with streptavidin-HRP. Detection was performed with SuperSignal West Pico Chemiluminescent substrate, and read with a Spectramax Luminometer.

Statistical analysis

Platelet response by AUC, platelet maximum response, and ELISA measurements were normalized by the median of medians method, to guarantee results were equal for every center of inclusion. From these numbers the natural logarithm was taken. Differences in platelet response between patients with stable angina versus unstable angina patients and patients with nSTEMI, were tested with student's T-test. For binary logistic regression modeling for describing severity of disease with platelet responses, individuals with unstable angina and nSTEMI were grouped together as individuals with unstable angina. Receiver operating characteristic (ROC) analysis was used to determine a cut-off value for platelet responses that were significantly associated with disease severity. The dichotomized platelet response was included in a multivariable binary regression model with established cardiovascular disease risk factors, i.e. age, sex, smoking status, high density lipoprotein (HDL), low density lipoprotein (LDL), and anti-platelet medication being aspirin, and anti-$P2Y_{12}$ treatment. Hypertension was negatively associated with clinical outcome in a univariate model, which disappeared when included in a multivariable model including hypertension treatment. Therefore hypertension treatment was included in the multivariable model for clinical outcome. Association of platelet response with MACE at 180 days of follow-up was investigated with Cox proportional hazard regression using the platelet response as a continuous variable. There was too little power to determine a cut-off value by ROC analysis for platelet responses that were associated with MACE.

For high on platelet treatment reactivity, the natural logarithm of platelet response to the ADP assay of patients on anti-$P2Y_{12}$ treatment and not on anti-$P2Y_{12}$ treatment was compared with Students T-test. A ROC analysis was performed of ADP response in relation to anti-$P2Y_{12}$ medication use, to determine the cut-off value for identification of patients with high response on ADP despite anti-$P2Y_{12}$ treatment. High on anti-$P2Y_{12}$ treatment versus non-high on anti-$P2Y_{12}$ treatment reactivity was compared in a Cox regression analysis.

Results

Study population

Patients were included at medical centers participating in the CTMM Circulating Cells consortium. A total of 714 patients were included, of which 484 patients we were able to measure platelet responsiveness. Patients not included were mainly due irregularities

in platelet assay supply at the start of the study, or due infrastructural reasons. Baseline characteristics are described in Table 1. Baseline characteristics of patients with stable angina, unstable angina, and with nSTEMI are described in Supplementary Table 1. Baseline characteristics for individuals with MACE during follow-up, and patients with high on treatment platelet reactivity are described in Supplementary Table 2.

Table 1. Baseline characteristics of total included population.

Clinical parameters	**Total population**
Male gender	325/478 (68%)
Age yrs (median ± IQR[31])	62 ± 14
Hypercholesterolemia	286/478 (60%)
Diabetes Mellitus	89/478 (17%)
Hypertension	291/478 (61%)
Current smoker	98/478 (21%)
Renal insufficiency	8/478 (2%)
Previous MI[32]	143/478 (30%)
Previous PTCA[33]	172/478 (36%)
Previous CABG[34]	33/478 (7%)
Stable angina	383/478 (81%)
Unstable angina	52/383 (14%)
nSTEMI	43/478 (9%)
Medication	
Aspirin	397/478 (83%)
Anti-$P2Y_{12}$ treatment	246/478 (51%)
Hypertension treatment	439/478 (92%)
Statins	372/478 (78%)
Laboratory measurements	
LDL[35] (median ± IQR)	2.4 ± 1.3
HDL[36] (median ± IQR)	1.1 ± 0.4
NT-proBNP[37] ng/mL (median ± IQR)	22.7 ± 27.5
HS-CRP[38] mg/mL (median ± IQR)	2.7 ± 4.7

31 IQR: interquartile range
32 MI: myocardial infarction
33 PTCA: percutaneous transluminal coronary angioplasty
34 CABG; coronary artery bypass graft
35 LDL: low density lipoprotein
36 HDL: high density lipoprotein
37 NT-proBNP: N-terminal prohormone of brain natriuretic peptide
38 HS-CRP: high sensitivity C-reactive protein

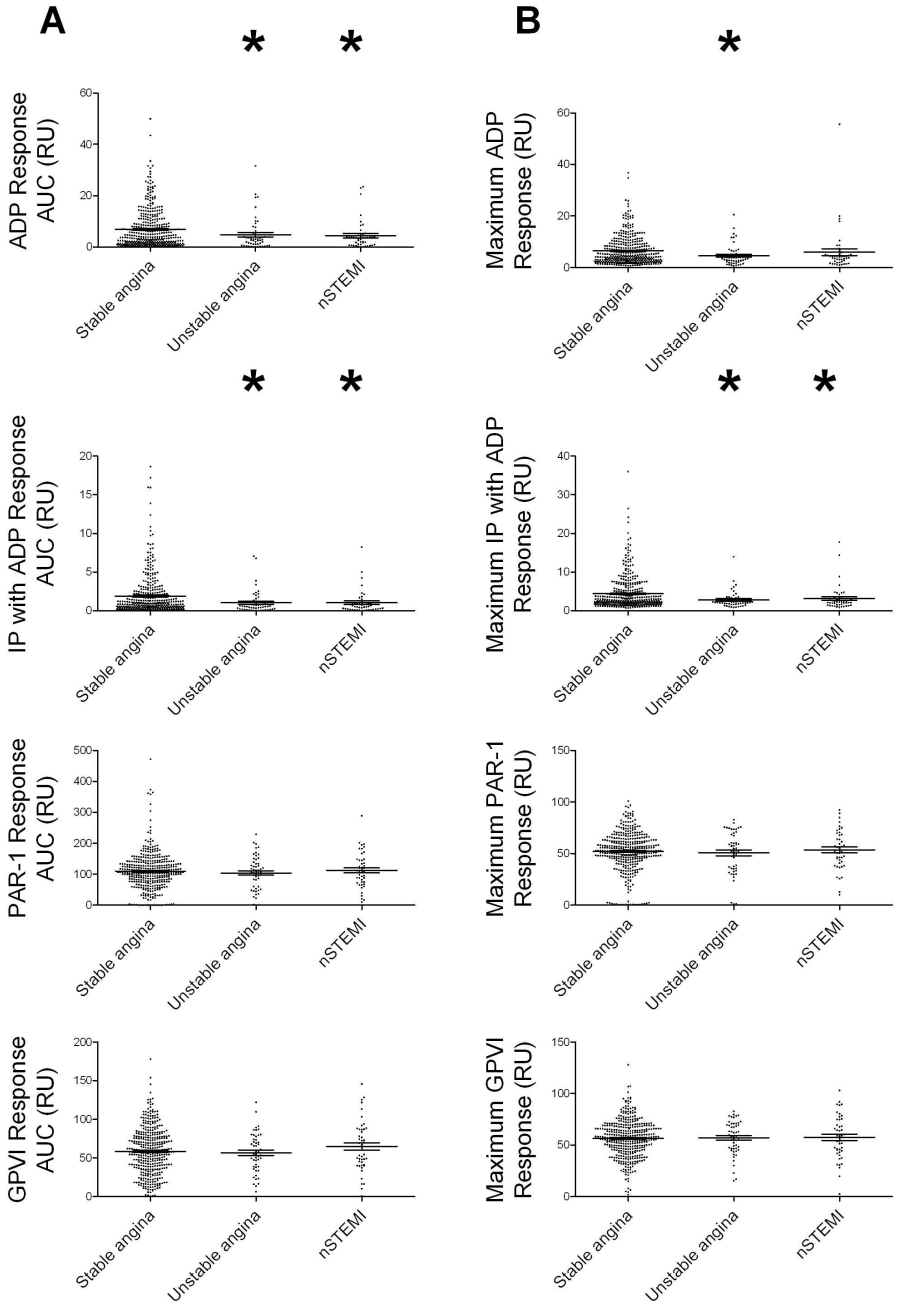

Figure 1. Platelet response in relation to disease severity. A) The means of area under the curve (AUC) for dose-response for ADP, prostacyclin receptor (IP) with suboptimal ADP, PAR-1 agonist, or GPVI agonist of patients with stable angina, unstable angina, and nSTEMI were compared with Students t-test (*P<0.05). B) As in A) but for the maximum response for either ADP, Iloprost with suboptimal ADP, PAR-1 agonist, or GPVI agonist.

Cardiovascular disease severity is associated with a reduced platelet ADP response, either with or without inhibitory iloprost

Maximum platelet response and the AUC of the response to increasing concentrations of ADP were both reduced in patients suffering from unstable angina, and in patients suffering from nSTEMI, when compared with patients with stable angina (Figure 1A and B). Similarly, the maximum platelet response, and the AUC for increasing concentrations of inhibitory IP agonist with suboptimal stimulatory ADP concentrations were also reduced in patients with unstable angina or nSTEMI, when compared with patients with stable angina (Figure 1A and B).

Cut-off values for platelet ADP response, and IP agonist with suboptimal ADP response were determined by ROC analysis. Patients were dichotomized according to this cut-off value, and this status was introduced in a multivariable binary regression model to investigate the association of patient platelet response with disease severity, in context of other factors associated with CAD. The maximum response and the AUC to ADP remained associated with disease severity after adjustment for age, and sex. Adjustment for anti-$P2Y_{12}$ treatment, aspirin use, smoking status, hypertension treatment, HDL levels, and LDL levels seemed to further reduce the OR with disease

Table 2. Odds ratio's with 95% confidence intervals of binary logistic regression on disease symptom severity and area under the curve of platelet responsiveness.

	OR[39] for AUC ADP	**OR for AUC ILO[40]**
Platelet Response only	0.40 (0.25 - 0.66) **[41]	0.36 (0.22 - 0.60) **
Plus Sex and Age	0.39 (0.24 - 0.65) **	0.36 (0.22 - 0.59) **
Plus anti-$P2Y_{12}$ treatment and aspirin	0.32 (0.19 - 0.53) **	0.27 (0.16 - 0.47) **
Plus Smoking status, hypertension treatment, HDL, LDL	0.26 (0.13 - 0.53) **	0.25 (0.12 - 0.51) **

Table 3. Odds ratio's with 95% confidence intervals of binary logistic regression on disease symptom severity and maximum platelet response.

	OR for MAX ADP	**OR for MAX ILO**
Platelet Response only	0.38 (0.23 - 0.63) **	0.41 (0.24 - 0.70) **
Plus Sex and Age	0.38 (0.23 - 0.63) **	0.40 (0.24 - 0.68) **
Plus anti-$P2Y_{12}$ treatment and aspirin	0.32 (0.19 - 0.54) **	0.30 (0.17 - 0.53) **
Plus Smoking status, hypertension treatment, HDL, LDL	0.27 (0.13 - 0.55) **	0.26 (0.12 - 0.55) **

39 OR: odds ratio
40 ILO: iloprost with suboptimal ADP stimulation.
41 **: Result was significant on P<0.01.

severity (Table 2 and 3). Maximum response and the AUC for increasing concentrations of inhibitory IP agonist with suboptimal ADP were both significantly associated with reduced disease severity. Adjustment for sex and age had no effect on this relationship. Further adjustment for anti-$P2Y_{12}$ treatment, aspirin use, smoking status, hypertension treatment, HDL levels, and LDL levels did not affect the relation between platelet response and disease severity (Table 2 and 3). The response to stimulation of PAR-1 or GPVI did not show association with disease severity (Figure 1A and B).

MACE is not associated with platelet responsiveness or high on treatment platelet reactivity

Cox proportional hazards regression analysis was performed with the AUC and the maximum platelet response at 180 days of follow-up to predict the risk of MACE. There was no risk difference for MACE for the AUC, or with maximum response to increasing concentrations of ADP, IP agonist with suboptimal ADP concentration, PAR-1 agonist, or GPVI agonist (Table 4 and 5).

As expected, the ADP response was different between patients on anti-$P2Y_{12}$ treatment, and patients not on anti-$P2Y_{12}$ treatment (Figure 2). A ROC curve was made to determine a cut-off value to identify high on platelet treatment reactivity for the maximum and the AUC of the ADP response, which nearly gave the same results (Supplementary table 2). Cox proportional hazards regression analysis on adverse outcome for patients with high on treatment platelet reactivity determined with either

Table 4. Hazard ratio's with 95% confidence intervals for association of MACE with the area under the curve of platelet responsiveness.

	HR[42] for AUC ADP	**HR for AUC ILO**	**HR for AUC TRAP**	**HR for AUC CRP-XL**
Platelet Response only	1.03 (0.78 - 1.35)	0.94 (0.73 - 1.21)	0.88 (0.70 - 1.11)	1.10 (0.66 - 1.85)

Table 5. Hazard ratio's with 95% confidence intervals for association of MACE with maximum of platelet response.

	HR for MAX ADP	**HR for MAX ILO**	**HR for MAX TRAP**	**HR for MAX CRP-XL**
Platelet Response only	0.95 (0.62 - 1.47)	1.07 (0.66 - 1.72)	0.82 (0.62 - 1.08)	1.09 (0.47 - 2.54)

42 HR: Hazard ratio

the maximum or with the AUC of the ADP response did not show a significant risk difference compared to patients with no high on treatment platelet reactivity (Table 6).

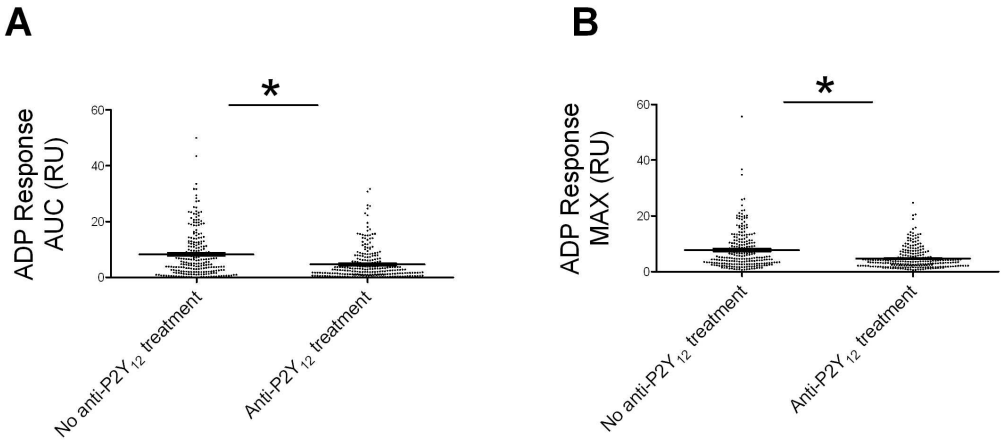

Figure 2. ADP response of patients on anti-$P2Y_{12}$ treatment and not on anti-$P2Y_{12}$ treatment. A) The ADP response expressed as AUC was compared between patients with anti-$P2Y_{12}$ treatment and patients not on anti-$P2Y_{12}$ treatment (*:P<0.0001 using Student's t-test). B) As described in A), but then with maximum ADP response.

Table 6. Hazard ratio'swith 95% confidence intervals for association of MACE with high platelet response despite anti-$P2Y_{12}$ treatment.

	HR for HPR[43]
HPR by AUC ADP	2.11 (0.79 - 5.63)
HPR by MAX ADP	1.71 (0.66 - 4.42)

43 HPR: high platelet response despite anti-$P2Y_{12}$ treatment

Discussion

The current cohort study on circulating cell function investigated the relation between platelet responsiveness and presence of unstable angina and nSTEMI. In addition we studied whether platelet responsiveness to specific agonists predicts future major cardiovascular events. Conventional techniques to measure platelet function have issues relating to sensitivity, specificity, and reproducibility [5-9]. In order to evaluate multiple platelet responsiveness pathways, we measured P-selectin expression in response to increasing concentrations of specific agonists and one antagonist. This platelet activation measurement is superior in discriminating individuals on their platelet response, when compared with LTA [9]. Furthermore, P-selectin is a direct measurement of granule release, which is thought to have an important role in atherosclerosis disease initiation and progression [2,4].

Unexpectedly, we found a reduced platelet ADP response to be associated with the diagnosis unstable angina or nSTEMI. This relationship was independent of other CAD risk factors or anti-platelet therapeutics. Similarly, low platelet response for the IP agonist assay with suboptimal stimulatory ADP was associated with unstable angina or nSTEMI. This relationship was also independent of other CAD risk factors or therapeutics. Anti-$P2Y_{12}$ medication did not influence the relation between platelet response and unstable diagnosis, as the use of the platelet inhibitor was equal or even reduced in the group with nSTEMI. The same was observed for aspirin use. Remarkably, introduction of anti-platelet medication in a multivariable model enhanced the relation between ADP induced platelet activation, and the iloprost inhibited ADP activation with severity of disease.

Intra-plaque hemorrhage is a marker for plaque vulnerability, and contributes to the progression of the atherosclerotic lesion [10]. We hypothesize that a weak response to ADP may reflect the relative inability of platelets to restore plaque rupture in time, causing more extensive bleeding in the plaque and therefore contributing to the development of an unstable atherosclerotic lesion. Anti-platelet medication may aggravate this relative inability, causing it to interact with the platelet ADP response in relation to disease severity. Alternatively, the process leading to unstable CAD symptoms may initiate a compensatory mechanism that reduces platelet responsiveness, but all this is speculation.

To investigate the association of platelet response for future cardiovascular events, we assessed the platelet response at 180 days of MACE. A follow-up of 180 days was taken, as this is the time-period where patient receive intensive anti-platelet

treatment. We found that none of the activation pathways were associated with increased risk for future adverse events. Besides overall platelet response, we also investigated high on treatment platelet reactivity. High on treatment platelet reactivity is an important topic in cardiovascular disease management, as a poor response to anti-platelet treatment pre-disposes patients to future adverse events [1,11]. With the assay used in this manuscript we discriminated between patients on anti-$P2Y_{12}$ treatment, and patients not treated with anti-$P2Y_{12}$ medication. The general accepted strategy to identify patients with high on platelet treatment reactivity is by identifying a cut-off value by means of a ROC analysis [12]. By AUC measurements, we identified 217 patients on anti-$P2Y_{12}$ treatment with an increased platelet response. By maximum response, we identified 229 patients on anti-$P2Y_{12}$ treatment with an increased platelet response. Remarkably, these patients were not at an increased risk for MACE at the end of follow-up. It has been described that patients with high platelet reactivity despite being on anti-platelet treatment diagnosed with unstable angina or nSTEMI are at relative low risk for future adverse events compared with STEMI patients [1]. Our data are in line with this observation.

At the start of the study it was assumed that P-selectin expression gives the same information with regard to platelet response as $\alpha_{IIb}\beta_3$ activation [9]. However, it is now known that interfering with the $P2Y_{12}$ response results in inhibition of platelet aggregate formation without inhibiting the platelet release reaction and thus P-selectin expression. In this respect it is not surprising that we did not observe a relationship between platelet activation status and future MACE because we did not measure the ability of platelets to aggregate but the ability of platelets to release the content of their α-granules. We assume that the expression of P-selectin is predominately a measurement of the platelet involvement in immunological processes (= plaque stability) while $\alpha_{IIb}\beta_3$ activation is a direct measurement of the ability of platelets to aggregate (= thrombus formation). We did see a correlation between reduced P-selectin expression and plaque instability but not with an increased ability to aggregate. Given these recent developments (Roest et al 2013, chapter 5 this thesis), it would have been very interesting to compare the P-selectin response with $\alpha_{IIb}\beta_3$ activation on platelets.

In conclusion, we find that reduced ADP response is associated with unstable angina, which interacts with anti-platelet treatment. Although the assay presented in this manuscript is useful in discriminating between patients responding to anti-$P2Y_{12}$ treatment and not responding to anti-$P2Y_{12}$ treatment, we did not find an association with major adverse cardiac events and platelet response, or with high on treatment platelet reactivity.

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Supplementary Tables

Supplementary Table 1. Baseline characteristics of patients with stable angina, unstable angina and nSTEMI.

	Stable angina	Unstable angina	nSTEMI
Clinical parameters			
Male gender	263/383 (69%)	30/52 (58%)	32/43 (74%)
Age yrs (median ± IQR[44])	62 ± 15	61 ± 11	62 ± 18
Hypercholesterolemia	233/383 (61%)	31/52 (60%)	22/43 (51%)
Diabetes Mellitus	75/383 (20%)	11/52 (21%)	3/43 (7%)
Hypertension	242/383 (63%)	29/52 (56%)	20/43 (47%)
Current smoker	67/383 (17%)	16/52 (31%)	15/43 (35%)
Renal insufficiency	8/383 (2%)	0/52 (0%)	0/43 (0%)
Previous MI[45]	117/383 (31%)	19/52 (37%)	7/43 (16%)
Previous PTCA[46]	142/383 (37%)	22/52 (42%)	8/43 (19%)
Previous CABG[47]	27/383 (7%)	2/52 (4%)	4/43 (9%)
Medication			
Aspirin	330/383 (86%)	43/52 (83%)	24/43 (56%)
Anti $P2Y_{12}$ treatment	202/383 (53%)	30/52 (58%)	14/43 (33%)
Hypertension treatment	366/383 (96%)	47/52 (90%)	26/43 (60%)
Statins	311/383 (81%)	38/52 (73%)	23/43 (53%)
Laboratory measurements			
LDL[48] (median ± IQR)	2.3 ± 1.2	2.9 ± 1.5	3.3 ± 1.6
HDL[49] (median ± IQR)	1.1 ± 0.3	1.1 ± 0.4	1.0 ± 0.4
NT-proBNP[50] ng/mL (median ± IQR)	20.8 ± 24.4	24.7 ± 36.2	37.5 ± 63.5
HS-CRP[51] mg/mL (median ± IQR)	2.4 ± 0.3	4.2 ± 6.2	6.0 ± 24.1

44 IQR: interquartile range
45 MI: myocardial infarction
46 PTCA: percutaneous transluminal coronary angioplasty
47 CABG: coronary artery bypass graft
48 LDL: low density lipoprotein
49 HDL: high density lipoprotein
50 NT-proBNP: N-terminal prohormone of brain natriuretic peptide
51 HS-CRP: high sensitivity C-reactive protein

Supplementary Table 2. Baseline characteristics of patients without an adverse event. or MACE at different time-points within follow-up.

	No event	MACE
Clinical parameters		
Male gender	305/448 (68%)	20/30 (67%)
Age yrs (median ± IQR)	62 ± 14	60 ± 19
Hypercholesterolemia	269/448 (60%)	17/30 (57%)
Diabetes Mellitus	81/448 (18%)	8/30 (27%)
Hypertension	271/448 (60%)	20/30 (67%)
Current smoker	93/448 (21%)	5/30 (17%)
Renal insufficiency	7/448 (2%)	1/30 3%)
Previous MI	136/448 (30%)	7/30 (23%)
Previous PTCA	163/448 (36%)	9/30 (30%)
Previous CABG	28/448 (6%)	5/30 (17%)
Unstable diagosis	87/448 (19%)	8/30 (27%)
Medication		
Aspirin	370/448 (83%)	27/30 (90%)
Anti $P2Y_{12}$ treatment	229/448 (51%)	17/30 (57%)
Hypertension treatment	411/448 (92%)	28/30 (93%)
Statins	347/448 (77%)	25/30 (83%)
Laboratory measurements		
LDL (median ± IQR)	2.4 ± 1.3	2.0 ± 1.0
HDL (median ± IQR)	1.1 ± 0.4	1.0 ± 0.2
NT-proBNP ng/mL (median ± IQR)	22.0 ± 26.8	37.4 ± 58.2
HS-CRP μg/mL (median ± IQR)	2.8 ± 4.7	2.1 ± 1.9
HPR[52] by AUC ADP	203/448 (45%)	14/30 (47%)
HPR by MAX ADP	214/448 (48%)	15/30 (50%)

52 HPR: high platelet response despite anti $P2Y_{12}$ treatment

Chapter 3

The relationship between fractional flow reserve, platelet reactivity and platelet leukocyte complexes in stable coronary artery disease

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Submitted

Abstract

The presence of stenoses that significantly impair blood flow and cause myocardial ischemia negatively affects prognosis of patients with stable coronary artery disease. Altered platelet reactivity has been associated with impaired prognosis of stable coronary artery disease. Platelets are activated and form complexes with leukocytes in response to microshear gradients caused by friction forces on the arterial wall or flow separation. This is associated with high pressure gradients and decreased coronary flow reserve. We hypothesized that the presence of significantly flow-limiting stenoses is associated with altered platelet reactivity and formation of platelet-leukocyte complexes.

One hundred patients with stable angina were studied. Hemodynamic significance of all coronary stenoses was assessed with Fractional Flow Reserve (FFR). Patients were classified FFR positive (at least one lesion with FFR ≤ 0.75) or FFR negative (all lesions FFR > 0.80). Platelet P-selectin was measured after whole blood stimulation with increasing concentrations of ADP, TRAP, CRP-XL and iloprost with substimulatory ADP. Expression of P-selectin, and platelet-leukocyte complexes were measured by flowcytometry. Patients were stratified on clopidogrel. FFR positive and negative patient groups were compared on platelet reactivity and platelet-leukocyte complexes.

Platelet reactivity between FFR positive patients and FFR negative patients did not differ. A significantly lower percentage of circulating platelet-neutrophil complexes in FFR positive patients and a similar non-significant decrease in percentage of circulating platelet-monocyte complexes in FFR positive patients was observed.

In conclusion, the presence of hemodynamically significant coronary stenoses does not alter platelet reactivity but is associated with reduced platelet-neutrophil complexes in peripheral blood of patients with stable coronary artery disease.

Introduction

For patients with stable coronary artery disease, the presence and extent of myocardial ischemia are the most important prognostic factors for myocardial infarction and death (1;2). On the other hand, patients who have coronary artery stenoses that do not significantly obstruct blood flow and consequently do not cause ischemia have a good prognosis with annual event rates being lower than 1% (3). The biological mechanisms that mediate the increased risk in patients with inducible myocardial ischemia are not clear. Increased platelet reactivity is associated with increased risk of myocardial infarction (MI) in patients with stable coronary artery disease (4) and antithrombotic therapy has been shown to be effective in reducing the risk of future MI (5). Furthermore, variation in response to antithrombotic therapy is associated with increased occurrence of atherothrombotic events in patients treated with percutaneous intervention (6;7). Increased levels of circulating platelet-monocyte complexes and increased platelet reactivity have been found in patients with stable coronary artery disease compared to healthy control subjects (8) and in acute coronary syndromes (9-11).

Platelets are functionally affected by conditions of high shear stress (12) and platelets form larger aggregates in response to increasing microshear gradients, independent of soluble agonists (13). Previous reports have shown that lesion severity and calculated shear stress correlate with increased platelet-monocyte complexes distal to a stenosis and further in the coronary sinus, as compared with samples from the proximal coronary artery (14). Also, experimental evidence suggests myocardial ischemia itself as a factor in platelet behavior by secretion of proaggregratory substances (15).

Apart from thrombosis, it has become increasingly clear that platelets are actively involved in all stages of atherosclerosis. Platelets have been shown to interact with both endothelial cells as well as circulating leukocytes to promote atherogenesis (16). Alpha granule fusion with the platelet membrane causes exposure of P-selectin which by interaction with P-selectin glycoprotein ligand – 1 (PSGL-1) mediates the formation of inflammatory platelet-leukocyte complexes. This facilitates a leukocyte influx into the endothelium, thereby presumably assisting in lesion development (17). Fractional Flow Reserve (FFR) is an invasive index of myocardial ischemia due to epicardial coronary stenosis. FFR is an invasive, lesion specific index of myocardial ischemia and is considered the gold standard for the assessment of ischemic potential of coronary lesions. FFR has a well defined cut-off value for ischemic lesions and has shown excellent reproducibility. FFR measures a lesion's ability to cause myocardial ischemia by measuring the pressure gradient across a stenosis during maximum

induced hyperemia (18). We hypothesized that in patients with stable coronary artery disease the presence of ischemia causing, flow limiting coronary lesions as measured by FFR is associated with altered platelet reactivity. Furthermore, we hypothesized that the presence of hemodynamically significant coronary lesions is associated with altered fractions of platelet-leukocyte complexes.

Methods

Interventional procedure

116 patients with stable angina that underwent FFR measurement and platelet reactivity were included from 3 Dutch hospitals: Catharina Hospital Eindhoven, University Medical Center Utrecht and the Maastricht University Medical Centre. Exclusion criteria were active inflammatory state, auto-immune disease or malignancy.

Fractional Flow Reserve of all coronary stenoses was performed *lege artis* under conditions of maximal hyperemia. Evidence of myocardial ischemia was defined by the presence of at least one coronary stenosis with FFR ≤ 0.75. Conversely ischemia was considered absent if none of the measured lesions had an FFR > 0.80. These cut off values have been extensively validated (19). To clearly demarcate the presence or absence of ischemia, we did not include 16 patients with intermediate FFR-values of 0.76 to 0.80, leaving 100 patients for analysis. Decision on treatment of the coronary lesions was left at the discretion of the operator. Depending on whether PCI was expected to be performed on the basis of a previously performed angiography, patients received a loading dose of clopidogrel 600 mg on the day before the procedure, according to local protocol. The study was approved by the local ethics committee of all participating centres and all patients gave written informed consent for participation.

Laboratory methods

Blood was collected from the arterial sheath into 3,2% tri-sodium citrate tubes (Greiner Bio-One), before administration of intravenous anticoagulants. Platelet reactivity was determined with agonist concentration series of adenosine diphosphate (ADP; $P2Y_{12}$ receptor agonist), cross linked collagen related peptide (CRP-XL; GPVI receptor agonist), thrombin receptor activating peptide (TRAP; PAR-1 receptor agonist), and iloprost (IP receptor agonist) with substimulatory ADP. Serial dilutions of ADP (125μM, 31.25μM, 7.8 μM, 1.95 μM, 488 nM, 122 nM, 31 nM, 8 nM) were prepared in 50 μL HEPES buffered saline (HBS; 10 mM HEPES, 150 mM NaCl, 1 mM $MgSO_4$, 5 mM KCl, pH 7.4), with 2 μL

phycoerythrin (PE) labeled mouse anti-human P-selectin antibodies (BD Biosciences, Breda, the Netherlands) and 2 μL fluorescein isothiocyanate (FITC) labeled mouse anti-human GPIb antibodies (BD Biosciences, Breda, the Netherlands). Similarly, serial dilutions of CRP-XL (2.5 μgr/mL, 625 ng/mL, 156.3 ng/mL, 39.1 ng/mL, 9.8 ng/mL, 2.4 ng/mL, 600 pg/mL, 153 pg/mL), TRAP (625 μM, 156.3 μM, 39.1 μM, 9.8 μM, 2.4 μM, 610 nM, 153 nM, 38 nM), and iloprost (1250 ng/mL, 312.5 ng/mL, 78 ng/mL, 19.5 ng/mL, 4.9 ng/mL, 1.2 ng/mL, 0.31 ng/mL, 0.076 ng/mL) with 5 μM ADP were prepared in 50 μL HBS with 2 μL mouse anti-human P-selectin antibodies and 2 μL mouse anti human GPIb antibodies. The platelet reactivity assay was initiated by adding 5 μL whole blood to each sample of serial dilutions. Samples were incubated for 20 minutes and subsequently added with 500 μL fixative (0.2% formaldehyde and 0.9% NaCl). All samples were analyzed on a FC 500 flow cytometer (Beckman Coulter, FL, USA) on the same day of processing. Data acquisition was performed with the SXP SYSTEM software (Beckman Coulter, FL, USA). Single platelets were gated based on FITC signal intensity. The median fluorescence intensity (MFI) for PE was measured. Dose-response graphs for P-selectin expression were constructed and the maximum response and area under the curves (AUC) in arbitrary units was calculated.

For assessment of platelet-leukocyte complexes, 50 μl of citrate anti-coagulated whole blood was diluted with 45 uL phosphate buffered saline (PBS) and platelets were labeled by incubation with 5 μL FITC labelled mouse anti-human GPIb antibodies (BD Biosciences) for 30 minutes at room temperature (20). Triplicate samples were fixed for 10 minutes with 80 μL of Optilyse B (Beckman Coulter), containing 3.4% paraformaldehyde, after which hypotonic red blood cell lysis was achieved by the addition of 820 μL of demineralized water. Monocytes and neutrophils were identified by forward and sideward scatter gating. Platelet – monocyte complexes (PMCs), and platelet – neutrophil complexes (PNC's) were determined by calculating the percentage of cells in these gates that were positive for the platelet marker GPIb. All samples were analyzed on a FC 500 flow cytometer (Beckman Coulter, FL, USA) on the same day of processing. Fifteen thousand cells were counted, and data acquisition was performed with the SXP SYSTEM software (Beckman Coulter, FL, USA).

Statistical Analysis:

Comparison of categorical variables was done with Chi-square testing. Continuous variables were compared with student's T or Mann Whitney test as appropriate, a P-value < 0.05 was considered statistically significant. Statistical analysis was done with SPSS 18 (SPSS inc, Chicago, Ill, USA).

Results

Baseline characteristics.

The baseline characteristics of the included patients are presented in table 1.

Table 1. Baseline characteristics of FFR positive(≤0.75) and FFR negative (FFR>0.80) patients. Continuous values are presented as means ± SD. Categorical values are presented as number (percentages). Non-significant indicates no lesion with stenosis > 50%. MI=myocardial infarction, PCI= percutaneous coronary intervention, CABG= coronary artery bypass grafting, DM= diabetes mellitus, CAD= coronary artery disease.

	FFR ≤ 0.75 (N=57)	FFR > 0.80 (N=43)
Age mean ± SD	61.8 ± 9.8	63.1 ± 9.8
Male gender - N (%)	36 (63.2)	27 (62.8)
History		
Previous MI - N (%)	13 (22.8)	13 (27.7)
Previous PCIN - N (%)	18 (31.6)	20 (46.5)
Previous CABG - N (%)	1 (1.8)	4 (9.3)
Risk factors		
DM - N (%)	13 (22.8)	5 (30.2)
Hypertension - N (%)	27 (47.4)	22 (51.1)
Hyperlipidemia - N (%)	29 (50.9)	21 (48.8)
Smoking - N (%)	13 (22.8)	14 (32.6)
Family history of CAD - N (%)	42 (73.7)	23 (53.5)
Medication at inclusion		
Aspirin - N (%)	53 (93.0)	37 (86.0)
Beta-blockers - N (%)	50 (87.7)	23 (53.5)
Statins - N (%)	50 (87.7)	38 (88.4)
ACE-inihibitors - N (%)	16 (28.1)	13 (30.2)
Clopidogrel (chronic use) - N (%)	21 (36.8)	17 (39.5)
Clopidogrel (loading dose) - N (%)	28 (49.1)	1 (2.3)
Oral anti-coagulation - N (%)	2 (3.5)	3 (7.0)
Angiographic severity		
Non-siginificant - N (%)	0 (0.0)	5 (11.6)
1-vessel disease - N (%)	31 (54.4)	21 (48.8)
2-vessel disease - N (%)	18 (31.6)	11 (25.6)
3-vessel disease - N (%)	8 (14.0)	6 (14.0)
Mean lowest FFR-value - mean ± SD	0.55 ± 0.14	0.87 ± 0.04

As shown in Table 1, age, sex, previous coronary history and traditional cardiovascular risk factors and angiographic extent of coronary disease were similar in both groups, except for a positive family history which was more prevalent in the FFR positive group (P=0.04).

In the FFR negative group, 3 patients used neither ASA nor clopidogrel, 2 of which used oral anticoagulation. In the FFR positive group, all patients used either ASA or clopidogrel (chronically or loading dose). FFR positive patients were more likely to receive treatment with clopidogrel.

Previous research has demonstrated inhibition of clopidogrel on both platelet reactivity and platelet leukocyte complex formation (21;22). To omit a confounding effect of clopidogrel use, we stratified our patients according to clopidogrel use (either chronic or loading dose) yielding 4 groups: FFR negative without clopidogrel (25 patients), FFR positive without clopidogrel (9 patients), FFR negative with clopidogrel (18 patients) and FFR positive with clopidogrel (48 patients).

Platelet reactivity

Platelet reactivity was determined by the maximal expression of P-selectin after stimulation, and cumulative reactivity by the area under the curve (AUC) calculations determined from the dose-response curves. The use of clopidogrel resulted in a markedly lower response to ADP, and iloprost with sub-optimal ADP stimulation for both the maximal response and the AUC. Clopidogrel was not able to significantly inhibit activation by TRAP or CRP-XL as was observed in both the maximal response and the AUC values (Figure 1 and 2). No significant difference was observed between FFR positive and FFR negative patients for all activation responses by either platelet reactivity measurements.

Platelet-leukocyte complexes

Percentages of PMC's and PNC's in whole blood were available for 67 included patients. Patients were again stratified according to clopidogrel use and percentages of complexes were compared between FFR positive and negative patients. The number of platelets per leukocyte as measured by the MFI of anti-CD42b labelling did not significantly differ between groups, as shown in Figure 3. We observed no significant difference in percentage of PMCs or PNCs between the FFR positive and FFR negative patients that were not treated with clopidogrel, although comparison is hampered by the small sample size (n=15 vs n=5) (Figure 4). There was a significant decrease in the percentage of PNC's (p=0.03; Figure 4B) and a trend towards a decrease in the percentage of PMC's

(p=0.08; Figure 4A) in the clopidogrel treated FFR positive patients compared to FFR negative patients.

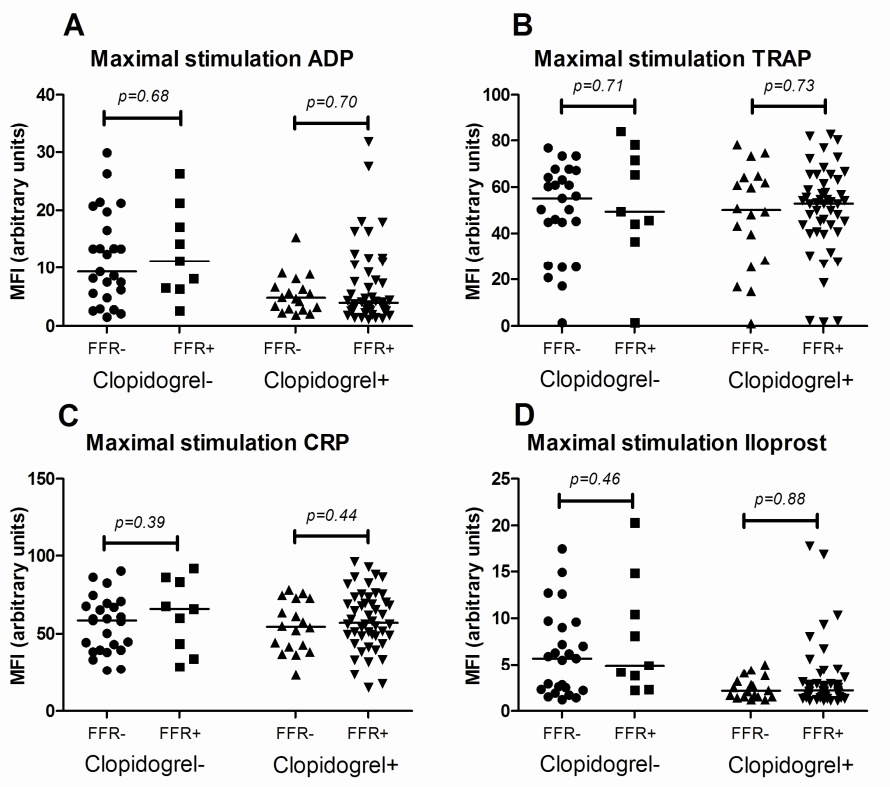

Figure 1. Maximal expression of P-selectin after stimulation with ADP (A), TRAP (B), CRP-XL (C) and Iloprost with substimulatory ADP (D). Lines indicate median values.

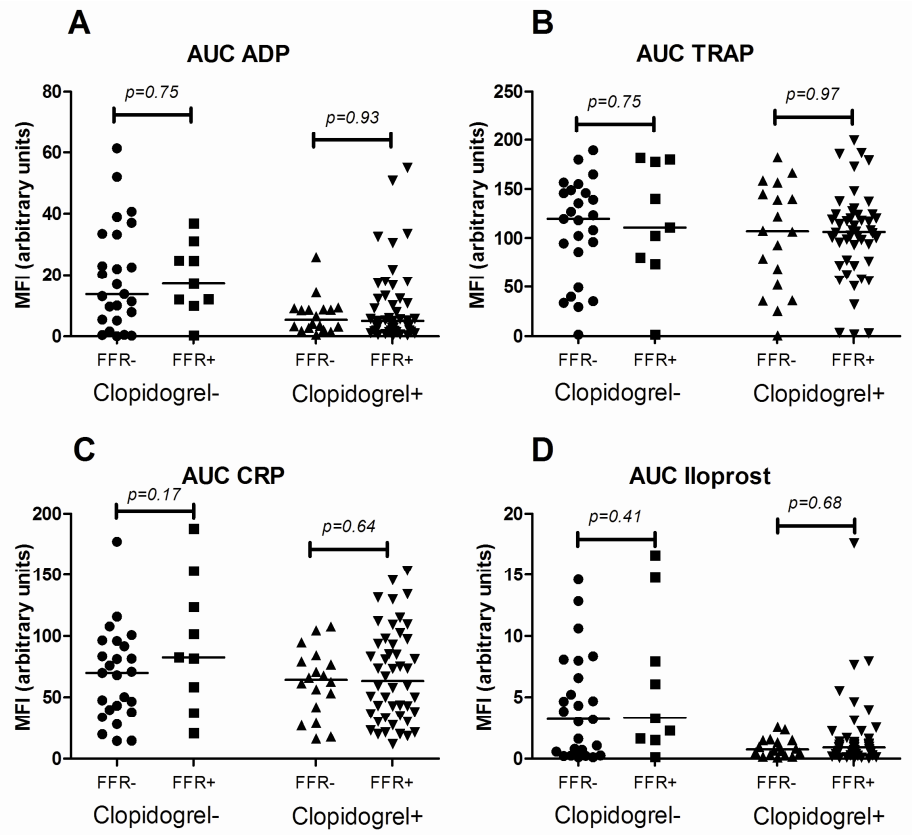

Figure 2. Cumulative expression of P-selectin after stimulation with ADP (A), TRAP (B), CRP-XL (C) and Iloprost with substimulatory ADP (D). Lines indicate median values.

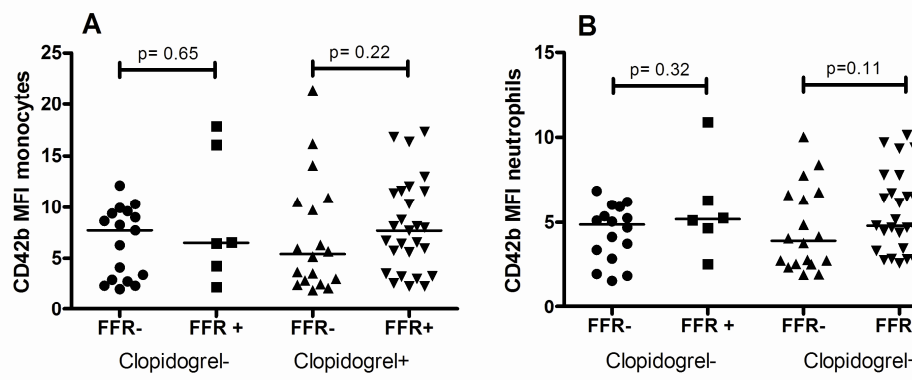

Figure 3. Platelets per monocyte (A) and neutrophil (B) measured by MFI of CD42b. Lines indicate median values.

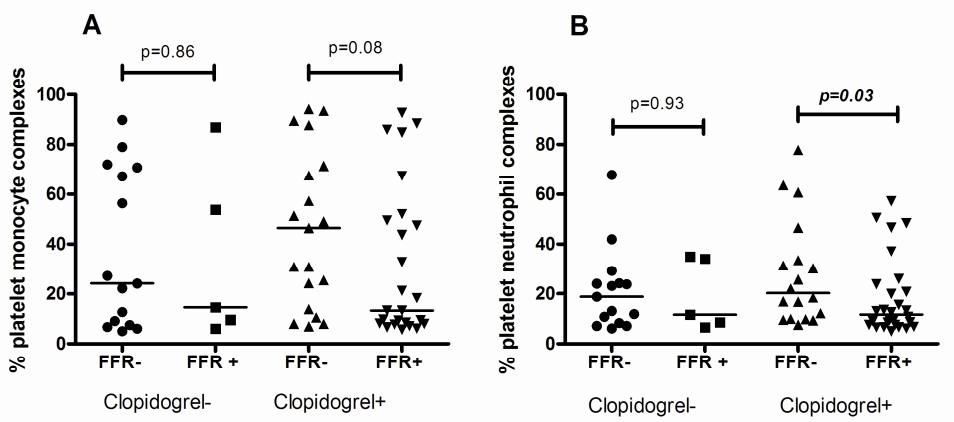

Figure 4. Percentages of platelet-monocyte (A) and platelet-neutrophile (B) complexes in FFR-negative and FFR-positive patients, identified by presence of platelet marker CD42b on monocytes and neutrophils, stratified by clopidogrel use. Lines indicate median values.

Discussion

In this observational study we observed no differences in both maximal and cumulative in vitro platelet reactivity between patients with a positive FFR and a negative FFR. However, we did observe a significant decrease in the percentage PNC's in patients with a positive FFR compared to a negative FFR in the clopidogrel treated group (p=0.03), and a trend towards lower percentages of PMC's in patients with a positive FFR compared to a negative FFR in the clopidogrel treated group (p=0.08).

Previous clinical studies have shown diverging results with respect to the relationship between platelet reactivity, platelet-leukocyte complexes and coronary luminal obstruction or inducible myocardial ischemia. Increased systemic platelet reactivity was described in patients with documented coronary artery disease immediately after peak exercise (23;24), however no relationship with ischemia was found (23). In critical limb ischemia, increased PMC's and expression of P-selectin was observed, implying an ischemic mechanism of platelet activation (25). Conversely, other investigators found an inverse relationship between coronary obstruction and platelet reactivity (26), although all patients investigated had severe single-vessel disease. Others found experimental evidence that platelet reactivity might be reduced by ischemic pre-conditioning (27), which may point to the possibility of down regulation of platelet reactivity by repeated, short-acting bouts of ischemia as occurs in stable coronary disease. We found no evidence for this.

The lower percentage of platelet-leukocyte complexes observed in the FFR positive group may be explained by increased activation of leukocytes due to significantly flow-limiting stenoses (14) and subsequent increased clearance of the complexes from the circulation. Da Costa et al. showed that attachment of monocytes to platelets leads to enhanced transmigration of monocytes into the subendothelium (28). Also, Huo et al. showed that the interaction of infused activated platelets with leukocytes resulted in increased adherence to the endothelium. Subsequent transmigration of the complexes led to absence of detectable levels of platelet-leukocyte complexes in a time frame of 3-4 hours (29). Besides lower systemic percentages of platelet-leukocyte complexes due to increased transmigration of these complexes, neutrophils have been shown to phagocytose activated platelets in vivo (30). Hence, lower percentages of circulating neutrophils with platelets adhered to the cell membrane might reflect leukocyte populations that effectively phagocytosed activated platelets.

Limitations

This study has several limitations. In our population, platelet reactivity and platelet-leukocyte complexes were assessed in stable, non-ischemic conditions, as opposed to the majority of previously mentioned research in which platelets and platelet–leukocyte complexes were mostly investigated during or shortly after ischemia or exercise. Thus, we cannot discount the possibility that acute changes in platelet reactivity occur during ischemic episodes.

Use of clopidogrel before angiography differed significantly between patient groups. The reason for this is that the majority of these patients were referred for PCI in which loading of clopidogrel is mandatory, in contrast to coronary angiography with FFR alone, in which this is not required. This introduces a bias between the FFR positive and FFR negative groups, since patients with severe lesions are more likely to be referred for PCI and thus be treated with clopidogrel upfront while at the same time these are more likely to have a FFR ≤ 0.75. Given the observational nature of this study, however, we could not interfere with local regimens. We therefore chose to stratify patients, which resulted in relatively small groups and reduced statistical power.

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Chapter 4

Citalopram is a more potent platelet function inhibitor than paroxetine in a case-control study

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Serotonin is a major component of platelet dense-granules [1]. It mediates vasoconstriction and supports platelet activation via platelet 5-HT receptors [2, 3]. Platelets take up serotonin via serotonin transporters in their membranes. This uptake is inhibited by selective serotonin reuptake inhibitors (SSRI's) [4], thereby affecting platelet function [4 - 6]. Some studies have shown that SSRI's reduce the risk of cardiovascular disease events [7 - 10], although there is still some controversy on the subject [11, 12]. In line with this, numerous studies have shown an increased bleeding risk with SSRI use, although not all studies could confirm this (for overview of literature see [13]). Studies on the influence of SSRI's on platelet function have been performed mainly ex-vivo [14 -16] or in patient groups administered with different SSRI's, but analyzed together [5, 6, 17], while different types of SSRI's have been reported to differ in therapeutic efficacy [18]. We compared the effects of paroxetine and citalopram on platelet responsiveness in a case-control study of thirteen patients with a platelet function analyzer, and a platelet responsiveness assay to investigate the hypothesis that these SSRI's have different effects on platelet function.

Thirteen patients and twenty controls were enrolled in the present study from September to January 2011. Venous blood was collected from patient and healthy volunteers after obtaining informed consent using vacuum tubes with 3,2% tri-sodium citrate (Greiner Bio-One B.V., Alphen a/d Rijn, the Netherlands). Patients were included at GGNet Mental Health in Apeldoorn, the Netherlands. The control group was slightly younger and contained more women than the patient group (see table 1).

Patients were taking either paroxetine or citalopram on therapeutic basis, but no other anti-depressants or any anti-platelet medication (aspirin or clopidogrel). Drug levels (citalopram, desmethylcitalopram and paroxetine) were measured using ultra performance liquid chromatography (ACQUITY UPLC) and tandem mass-spectrometry (ACQUITY TQD, Waters Chromatography B.V., Etten-Leur, the Netherlands), which on average were within the therapeutic range.

Platelet P-selectin expression in whole blood was determined in response to serial dilutions of adenosine-diphosphate (ADP) ranging from 0.008 μM to 125 μM, cross-linked collagen related peptide (CRP-XL) ranging from 0.0002 μg/mL to 2.5 μg/mL, thromboxane analogue U-46619 ranging from 0.8 μg/mL to 12500 μg/mL, and thrombin receptor agonist SFLLRN (TRAP) ranging from 0.038 μM to 625 μM (see also [19]). All samples were analyzed on a FACSCalibur flow cytometer (Beckton Dickinson) on the same day of processing. Single platelets were gated based on forward- and side-scatter properties, and their median fluorescence intensity was measured. Dose-response graphs and area under the curves expressed in arbitrary units (AU) were

Table 1. Patient characteristics and results. The platelet responsiveness to ADP, TRAP, and CRP-XL were significantly reduced in patients administered with citalopram, compared with the paroxetine administered patients and healthy controls.

	Citalopram (n=6)	Paroxetine (n=7)	Controls (n=20)
Mean age (yr; range)	60 (28-75)	58 (48-73)	49 (25-61)
Male gender (%)	3 (50%)	4 (57.1%)	4 (20%)
Other anti-depressants	0 (0%)	0 (0%)	0 (0%)
Mean SSRI blood levels (µg/L; ±SEM)	78.6 ± 25.0	51.3 ± 20.1	-
	(ref: 50 - 160)	(ref: 10 - 75)	-
Desmethylcitalopram	37.5 ± 11.9	-	-
(µg/L; mean, ±SEM)	(ref: 25 – 80)	-	-
History of bleeding	None	None	None
Mean PFA-100 Collag. Epi (sec.; ±SEM)	117 ± 11	125 ± 13	117 ± 7
Mean PFA-100 Collag. ADP (sec.; ±SEM)	97 ± 6	96 ± 7	91 ± 3
Mean response ADP (AU; ±SEM)	81 ± 17 **, #	138 ± 21	140 ± 12
Mean response TRAP (AU; ±SEM)	447 ± 52 **, ##	762 ± 77	772 ± 28
Mean response CRP-XL (AU; ±SEM)	337 ± 62 *	486 ± 63	518 ± 32
Mean response U-46619 (AU; mean, ±SEM)	16 ± 5	25 ± 9	26 ± 4

SEM: standard error of the mean, AU: arbitrary units, PFA-100: Platelet function analyzer, ref: reference range. Groups were compared with the Wilcoxon test: citalopram vs. paroxtine ($^{\#}$:P<0.05, $^{\#\#}$:P<0.01), and citalopram vs. healthy controls (*:P<0.05, **:P<0.01).

produced using Prism 5.01 software (Graphpad Software). Non-parametric tests were used to test the difference between the different groups using SPSS 18.0 (PASW Statistics). Platelet P-selectin expression was significantly reduced in patients using citalopram for the $P2Y_{12}$ receptor agonist ADP, the GPVI receptor agonist CRP-XL, and the PAR-1 agonist TRAP, while paroxetine use did not affect platelet reactivity in regard to the control group (see table 1). There were no significant differences between the groups in the area under the curve of the thromboxane receptor agonist U-46619. The platelet function analyzer tests (PFA-100; Siemens Diagnostics, Breda, the Netherlands), performed according to the manufacturer's directions, did not show any differences between any of the groups (see table 1).

For whole blood serotonin measurements, citrated blood stored at -80 °C was thawed and perchloric acid was added up to 0.6 M, vortexed and centrifuged at 13,000 x g for 5 minutes. The supernatant was aspirated and HCl was added up to 3 M. Fluorescence of serotonin was excited at 310 nm and registered at 510 nm. Fluorescence was expressed as relative fluorescence units (RFU) with standard error of mean. Lower serotonin levels were measured in citalopram-administered patients (14.9 ± 2.4 RFU,

P=0.07) and patients using paroxetine (22.1 ± 4.0 RFU, P=0.3) compared with healthy controls (24.1 ± 2.3 RFU), although this was not statistically significant.

Previous studies have studied the effect of SSRI on platelets by adding SSRI's to platelets ex-vivo [14-16,20,21], thereby neither taking physiological and pharmacological conditions into account, nor the effects of platelet serotonin reduction. Other studies on platelet function in SSRI administered patients investigated the effects of SSRI's without regarding the possible differences between distinct SSRI's [5,6,17]. We found that citalopram but not paroxetine significantly reduces P-selectin expression on platelets after activation with ADP, TRAP and CRP-XL compared with healthy controls. We did not observe any effects of the SSRI's with the platelet function analyzer using either the collagen ADP cartridges, or collagen epineprine cartridges. We propose that this assay is not sensitive enough. This is in line with results obtained by others [6,17].

In a direct comparison, citalopram was a more effective SSRI than paroxetine in treating depressions [18], and we show that this is reflected in the platelet responsiveness. Clinical depression is associated with increased platelet reactivity as was shown with aggregation experiments [22]. The absence of inhibition of paroxetine in our platelet responsiveness assay may be caused due to the relatively mild therapeutic efficacy of paroxetine, which relates to its ability to inhibit serotonin uptake. The reduced platelet responsiveness in the citalopram-administered patients coincided with reduced blood levels of serotonin, although this reduction was not significant. Altogether our results show that citalopram has a stronger inhibiting effect on platelet P-selectin expression compared with paroxetine.

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Chapter 5

Differential platelet activation: granule release without $\alpha_{IIb}\beta_3$ activation indicates platelet functions independent of aggregation

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Submitted

Abstract

Platelets are appreciated for their crucial role in maintaining adequate hemostasis, but they are also involved in wound healing, inflammation, angiogenesis, and atherogenesis via the release of cytokines, chemokines, and growth factors from their α-granules. Here we show that in addition to $P2Y_{12}$ antagonism, the prostacyclin analogue iloprost selectively inhibited PAR-1 or PAR-4 initiated $\alpha_{IIb}\beta_3$ activation, while granule release remained on full strength. The same differential activation was observed in-vivo with platelets from patients on clopidogrel treatment. Inhibition of PI3 kinase or increasing cAMP levels, which intervenes with two cellular responses of $P2Y_{12}$ activation, resulted in reduced $\alpha_{IIb}\beta_3$ activation, while the release of α-granules stayed almost completely intact. We propose that selective granule release in the absence of $\alpha_{IIb}\beta_3$ activation is an important feature of platelet function in inflammation, angiogenesis and atherogenesis. The selective inhibition of $\alpha_{IIb}\beta_3$ activation by $P2Y_{12}$ inhibitors may explain the success of clopidogrel as platelet inhibitor.

Introduction

Platelets play a central role in the regulation of an undisturbed blood circulation. On the one hand, platelets are designed to immediately repair vascular injury to prevent lethal blood loss. On the other hand, platelet hyperreactivity is detrimental for blood circulation, as blood clot formation inside a blood vessel can cause downstream ischemia. Therefore, platelet inhibitors are standard therapy for patients with an increased risk of arterial thrombosis.

Besides their role in hemostasis, platelets have additional physiological functions. There is growing evidence for involvement of platelets in inflammatory reactions. On the cellular level, platelets commonly interact with leukocytes to support transport of leukocytes from the circulation to extravascular tissue [1,2]. Furthermore, pro-inflammatory factors are stored in platelet α-granules and are locally released in high quantities upon platelet activation [1]. Besides inflammation, activation of the protease activated receptors (PAR's) is important for the control of angiogenesis and therefore could contribute to tumor growth [3].

Platelet activation is regulated by different receptors on the membrane. Upon activation, granule release and $\alpha_{IIb}\beta_3$ activation take place. The PAR's belong to the most potent platelet activation pathways [4]. Proteolytic cleavage of PAR-1 and PAR-4 results in liberation of N-terminal activator peptides that auto-activate the PAR-1 and PAR-4 receptor and hence activation of phospholipase-Cβ, which is considered a critical event in platelet activation [5]. Activity of phospholipase-C generates second messengers that raise cytosolic calcium concentrations and activate protein kinase C isoforms [5].

Platelet activation induces release of adenosine diphosphate (ADP) from the dense granules. On its own, ADP is a weak platelet agonist, but in concert with other agonists such as collagen or thrombin, it potentiates platelet activation via auto- and paracrine stimulation of the $P2Y_{12}$ and $P2Y_1$ receptors [6]. $P2Y_{12}$ is a G_i-protein coupled receptor that downregulates adenylatecyclase activity and stimulates PI3-kinase activity [5,6]. Adenylatecyclase activity results in production of cyclic adenosine monophosphate (cAMP) and inhibits platelet activation. Activation of PI3-kinase results in the production of phosphatidyl inositol di-and triphosphates and activation of Akt [5,6]. Both the inhibition of cAMP production and activation of PI3-kinase are critical events in platelet activation [5,6]. The physiological importance of ADP in platelet activation is firmly established by the successful prevention of arterial thrombosis with $P2Y_{12}$ antagonists [7]. Further evidence that supports the importance of $P2Y_{12}$ as platelet receptor is the bleeding tendency in patients with $P2Y_{12}$ deficiency [8-10].

The activation of platelets is controlled by the release of inhibitors from the endothelium. One of these inhibitors is prostacyclin, which interacts with the IP receptor. This results in activation of adenylatecyclase and increased intracellular levels of cAMP [5].

In this manuscript we show that inhibition of the $P2Y_{12}$ receptor during PAR activation results in a strong inhibition of $\alpha_{IIb}\beta_3$ activation, while the release of α-granules is maintained approximately normal. We hypothesize that selective inhibition of $\alpha_{IIb}\beta_3$ activation by $P2Y_{12}$ antagonists and IP agonism may be an important (patho)physiological property of platelets to regulate inflammation, angiogenesis and atherogenesis while platelet aggregation is prevented.

Materials and Methods

Healthy blood donors and patients

Blood was obtained from healthy donors and critical limb ischemia (CLI) patients, either using clopidogrel or not using clopidogrel, after approval of the local ethics committee and informed consents were obtained.

Materials

Vacuum 3.2% tri-sodium citrate tubes were obtained from Greiner Bio-one (455322), phycoerythrin (PE) labelled mouse-anti human P-selectin antibodies were obtained from Becton Dickinson (BD; 555524), fluorescein isothiocyanate (FITC) labelled rabbit-anti human fibrinogen antibody was obtained from Dako (F 0111), PE labelled mouse anti-human CD42b was obtained from BD (555473), adenosine diphosphate (ADP) was obtained from Roche (127507), cross-linked collagen related peptide (CRP-XL) was a kind gift from R. Farndale at University of Cambridge, PAR-1 activating peptide SFLLRN was obtained from Bachem (H-2936), PAR-4 activating peptide AYPGKV was obtained from the NKI-AVL in Amsterdam, AR-C69931MX was a kind gift from Astra Zeneca, apyrase was obtained from Sigma (A-6132), Optilyse B was obtained from Beckman Coulter (PN IM 1400). Primary antibodies for enzyme linked immunosorbent assay (ELISA) raised against platelet factor 4 (PF-4; MAB7951), beta-thromboglobulin (β-TG; MAB393), regulated and normal T-cell expressed and secreted (RANTES/CCL5; MAB278), and platelet derived growth factor (PDGF)-AB (DY222) were all obtained from R&D. Biotin coupled antibodies for ELISA raised against PF-4 (AF795), β-TG (BAF393), RANTES (AB-278-NA), and PDGF-AA (DY222) were obtained from R&D. Streptavidin-

horse radish peroxidase (HRP; P0397) was obtained from Dako, Super Signal West Pico Chemiluminescent substrate was obtained from Thermo Scientific (34080), cAMP enzyme immunoassay kit (581001) was obtained from Cayman Chemical, iloprost was a kind gift from Schering AG, wortmannin was obtained from Sigma (W-1628), U73122 was obtained from Sigma (U6756). Flow cytometry measurements were performed with a FACSCanto II flow cytometer from Beckton Dickinson. Aggregometry experiments were performed on a Model 700 aggregometer from Chrono-Log Cooperation. Dose-response graphs and area under the curves were produced using Prism 5.01 software from Graphpad Software. Statistics were performed with SPSS 20 from IBM.

Platelet responsiveness

Venous blood was collected from the healthy volunteers and CLI patients using vacuum tubes. Platelet P-selectin expression and $\alpha_{IIb}\beta_3$ activation were measured using PE labelled anti-P-selectin antibody diluted 1:25, and FITC labelled anti-fibrinogen antibody diluted 1:100. P-selectin expression and fibrinogen binding by platelets in whole blood was determined in response to serial dilutions of ADP ranging from 125 μM to 8 nM, CRP-XL ranging from 2.5 μg/mL to 0.2 ng/mL, and PAR-1 agonist ranging from 625 μM to 38.1 nM, and PAR-4 agonist from 4 mM to 0.2 μM. Assays were performed in the presence or absence of 1 μM AR-C69931MX, or 0.2 U/mL apyrase. All samples were analyzed by flow cytometry on the same day of processing. Single platelets were gated based on forward and side scatter properties and their median fluorescence intensity was measured. Dose-response graphs and area under the curves expressed in arbitrary units (AU) were produced. Areas under the curve (AUC's) were normalized to measurements of P-selectin expression and $\alpha_{IIb}\beta_3$ activation in controls, being either healthy donors, or CLI patients not using clopidogrel.

Aggregation experiments

Platelet rich plasma (PRP) was prepared by centrifugation of whole blood at 160 *g* for 15 minutes at room temperature, no brake. Washed platelets were prepared by adding acid citrate dextrose (8.5 mM tri-sodium citrate, 7.1 mM citric acid, 5.5 mM D-glucose; final concentration) to PRP and centrifugation for 15 minutes with 340 g at room temperature, no brake. The platelet pellet was resuspended in Hepes-Tyrode (HT) buffer (145 mM NaCl, 5 mM KCl, 0.5 mM NaH_2PO_4, 1 mM $MgSO_4$, 10 mM Hepes, 5.5 mM D-glucose, pH 6.5) with 10 ng/mL prostacyclin. Platelets were centrifuged for 15 minutes at 340 g at room temperature and resuspended to 200 x 10^9/L in HT-buffer (pH 7.4). Before the experiments, platelets were allowed to rest for 30 minutes after

isolation. Washed platelets were stimulated with 60 μM ADP, 40 μM PAR-1 agonist peptide, or 250 μM PAR-4 agonist peptide, either with or without 2 μM AR-C69931MX.

Platelet-leukocyte complexes

PE labeled anti-CD42b was added 1:20 together with FITC labeled anti-fibrinogen antibody 1:100 in HEPES buffered saline (HBS; 10 mM HEPES, 150 mM NaCl, 1 mM $MgSO_4$, 5 mM KCl, pH 7.4). PAR-1 agonist was added to a final concentration of 156 μM, either with or without 1 μM AR-C69931MX. Whole blood was added 1:5 and incubated for 20 minutes. The samples were fixed with Optilyse for 10 minutes, and then water was added to lyse red blood cells. Monocytes and granulocytes were gated on forward and side scatter properties, and their median PE and FITC fluorescence was measured and expressed in AU.

ELISA's

Isolated platelets were stimulated with 156 μM PAR-1, either with or without 1 μM AR-C69931MX for 5 minutes. Platelets were centrifuged at 4000 g for 2 minutes and the supernatant was collected. Nunc maxisorb 96-well plates were coated with antibodies against PF-4, β-TG, or RANTES/CCL5 dissolved in coating buffer. PDGF-AB antibody was coated in phosphate buffered saline (PBS: 25 mM Na_2HPO_4, 2.3 mM NaH_2PO_4, 140 mM NaCl, pH 7.4) on Nunc maxisorb plates. Coated plates were blocked with 1% bovine serum albumin (BSA) in PBS. Samples were diluted and incubated on the ELISA plates. Standard curves were obtained with normal pooled serum. Plates were washed 5 times with 0.05% Tween 20 in PBS pH 7.4. Bound factors were detected with biotin coupled antibodies raised against PF-4, β-TG, RANTES, PDGF-AA in PBS with 1% BSA. Plates were washed with PBS pH 7.4 with 0.05% Tween 20. Biotin coupled antibodies were bound with streptavidin-HRP, and detected with Super Signal West Pico Chemiluminescent substrate, and read with a Spectramax Luminometer.

cAMP measurements

Washed platelets were stimulated with 0.02 μg/mL iloprost or HT buffer pH 7.4 for 10 minutes and then activated with 625 μM PAR-1 activating peptide, 156 μM PAR-1 activating peptide, 4 mM PAR-4 activating peptide, or 1 mM PAR-4 activating peptide for 10 minutes. ACD was added to all samples and centrifuged for 1 minute at 6800 g, soft brake. The pellet was resuspended in 0.1 M HCl and incubated for 20 minutes. The supernatant was collected by centrifugation for 10 minutes at 1000 g, and stored at

-80ºC until further processed. The concentration of cAMP was measured with a cAMP enzyme immunoassay kit.

cAMP, PI3-kinase and phospholipase-C inhibitor experiments.

Whole blood was incubated with 0.02 μg/mL iloprost and stimulated with 625 μM PAR-1 activating peptide, 150 μM PAR-1 activating peptide, 4 mM PAR-4 activating peptide, 1 mM PAR-4 activating peptide, or mock treated. For phospholipase-C inhibitor experiments, washed platelets were incubated with 15 μM, 10 μM, 7.5 μM, 5 μM U73122, or mock treated and stimulated with 150 μM PAR-1 activating peptide or 1 mM PAR-4 activating peptide. For PI3-kinase inhibitor experiments, washed platelets were incubated with 50 μM, 10 μM, 5 μM wortmannin, or mock treated. The response of the treated platelets on 150 μM PAR-1 stimulating peptide or 1 mM PAR-4 stimulating peptide was measured with PE-labelled antibodies against P-selectin, or FITC-labelled antibodies against fibrinogen by flow cytometry as described earlier.

Results

PAR-1 and PAR-4 mediated $\alpha_{IIb}\beta_3$ activation but not P-selectin expression depends on $P2Y_{12}$ activation

To establish the effects of $P2Y_{12}$ antagonism on both $\alpha_{IIb}\beta_3$ and P-selectin expression, activation of $\alpha_{IIb}\beta_3$ and P-selectin expression was measured simultaneously by flow cytometry in response to increasing concentrations of ADP, PAR-1 receptor agonist, or PAR-4 receptor agonist in presence or absence of the $P2Y_{12}$ blocker AR-C69931MX, or the ADP degrading enzyme apyrase. Addition of the $P2Y_{12}$ blocker AR-C69931MX or the ADP degrading enzyme apyrase to platelets stimulated via PAR-1 showed a strong inhibitory effect on activation of $\alpha_{IIb}\beta_3$, while it had only a mild inhibitory effect on P-selectin expression (Figure 1Ai, ii). This selective inhibitory effect of AR-C69931MX on PAR-1 activation was also observed with aggregometry (Figure 1B). The area under the curve of $\alpha_{IIb}\beta_3$ activation showed significant reduction in comparison with P-selectin expression in the presence of either AR-C69931MX or apyrase during PAR-1 stimulation (Figure 1C). Similar differential activation was observed for PAR-4 mediated platelet activation (Figure 1D-F). Addition of AR-C69931MX or apyrase to ADP stimulated platelets almost completely inhibited the activation of $\alpha_{IIb}\beta_3$ and P-selecting expression (Figure 1Gi, ii). The strong inhibition of activation of $\alpha_{IIb}\beta_3$ by AR-C69931MX was confirmed with aggregometry (Figure 1H). The areas under the curve of the dose-

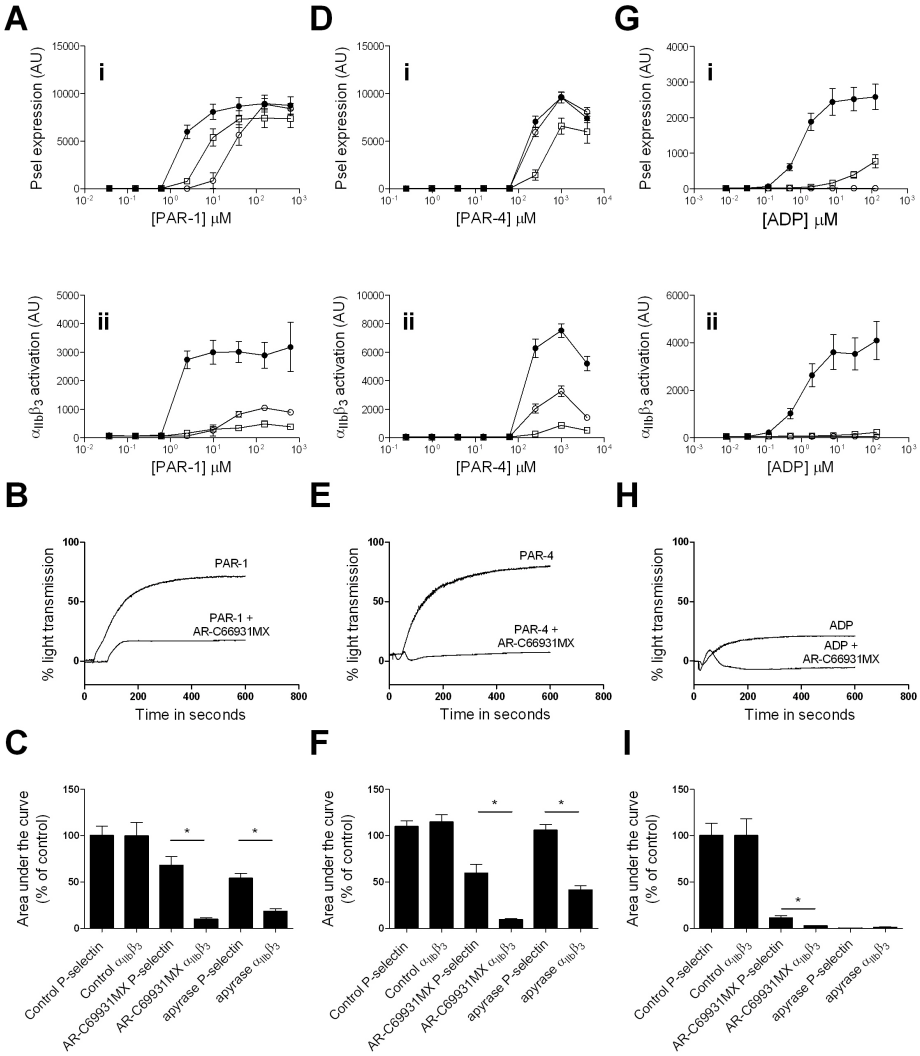

Figure 1. Platelet response after PAR stimulation in the presence of $P2Y_{12}$ antagonism. A) Platelets of healthy donors (n=5) were stimulated with increasing concentrations of PAR-1 agonist in presence of 1 μM AR-C69931MX (white squares), 0.2 U/mL apyrase (white circles, or mock treated (black rounds). Activation of platelets was determined by P-selectin expression (i) and $\alpha_{IIb}\beta_3$ activation (ii) by flow cytometry and expressed in arbitrary units (AU). Mean and standard error of the mean are shown. B) Aggregation experiment was performed with PAR-1 stimulation either in presence or absence of 1 μM AR-C69931MX. Representative aggregation is shown of 3 independent experiments. C) Area under the curve of graphs depicted in Ai,ii were calculated, and are expressed as a percentage of mock treated (Control) area under the curves for either P-selectin expression, or $\alpha_{IIb}\beta_3$ activation. Differences were non-parametrically tested between P-selectin expression and $\alpha_{IIb}\beta_3$ activation (*: P<0.05). Di,ii) As described in Ai,ii), but with increasing concentrations of PAR-4 agonist. E) and F) As described in B) and C), but with PAR-4 agonist. Gi,ii) As in Ai,ii), but with increasing concentrations of ADP. H) and I) As described in B) and C), but with ADP.

response experiments did not strongly differ between P-selectin expression and $\alpha_{IIb}\beta_3$ activation (Figure 1I).

To study whether these differences in expression of platelet activation markers also occurs in vivo, we investigated the effect of clopidogrel use on PAR-1 activation in platelets from patients on clopidogrel treatment and compared it with controls not using clopidogrel. Similar to the in vitro findings, we found that clopidogrel use resulted in a strong inhibition of $\alpha_{IIb}\beta_3$ activation ($\alpha_{IIb}\beta_3$ 25% of controls), but near to normal P-selectin expression (P-selectin 78% of controls; Figure 2Ai,ii and B) after activation with the PAR-1 peptide. Blocking the $P2Y_{12}$ pathway with clopidogrel strongly inhibited the response to ADP with regard to both $\alpha_{IIb}\beta_3$ activation and to P-selectin expression (Figure 2Ci,ii and D).

$P2Y_{12}$ antagonism does not inhibit PAR-1 induced platelet inflammatory responses

To investigate the inflammatory aspect of selective inhibition of $\alpha_{IIb}\beta_3$, the ability of platelets to form platelet-monocyte complexes and platelet-granulocyte complexes was used as read-out for the effects of $P2Y_{12}$ inhibition on PAR-1 mediated platelet activation. Binding of platelets to monocytes and neutrophils is not inhibited in the presence of AR-C69931MX (Figure 3Ai, Bi). In contrast to platelet-monocyte complex formation and platelet-granulocyte complex formation, fibrinogen binding by platelets attached to monocytes is completely blocked by AR-C69931MX during PAR-1 stimulation, compared with PAR1 stimulation without $P2Y_{12}$ pathway inhibitors (Figure 3Aii, Bii).

To further support the lack of effect of $P2Y_{12}$ inhibition on platelet α-granule release, we measured the release of inflammatory and growth factors in response to PAR-1 stimulation in presence or absence of AR-C69931MX. Upon PAR-1 stimulation, the release of soluble PF-4, β-TG, RANTES/CCL5, PDGF-AB were not inhibited when $P2Y_{12}$ was inhibited with AR-C69931MX, whereas a significant increase was measured with regard to unstimulated platelets (Figure 3C).

Stimulation of cAMP levels or inhibition of PI3-kinase impairs $\alpha_{IIb}\beta_3$ activation but not P-selectin expression during PAR-1 activation

One of the consequences of $P2Y_{12}$ activation is inhibition of adenylatecyclase. To assess whether high cAMP levels cause differential expression of platelet activation markers after PAR stimulation, platelets were treated with a concentration of iloprost that increased cAMP levels six-fold (Figure 4A). Stimulation with either PAR-1 or PAR-

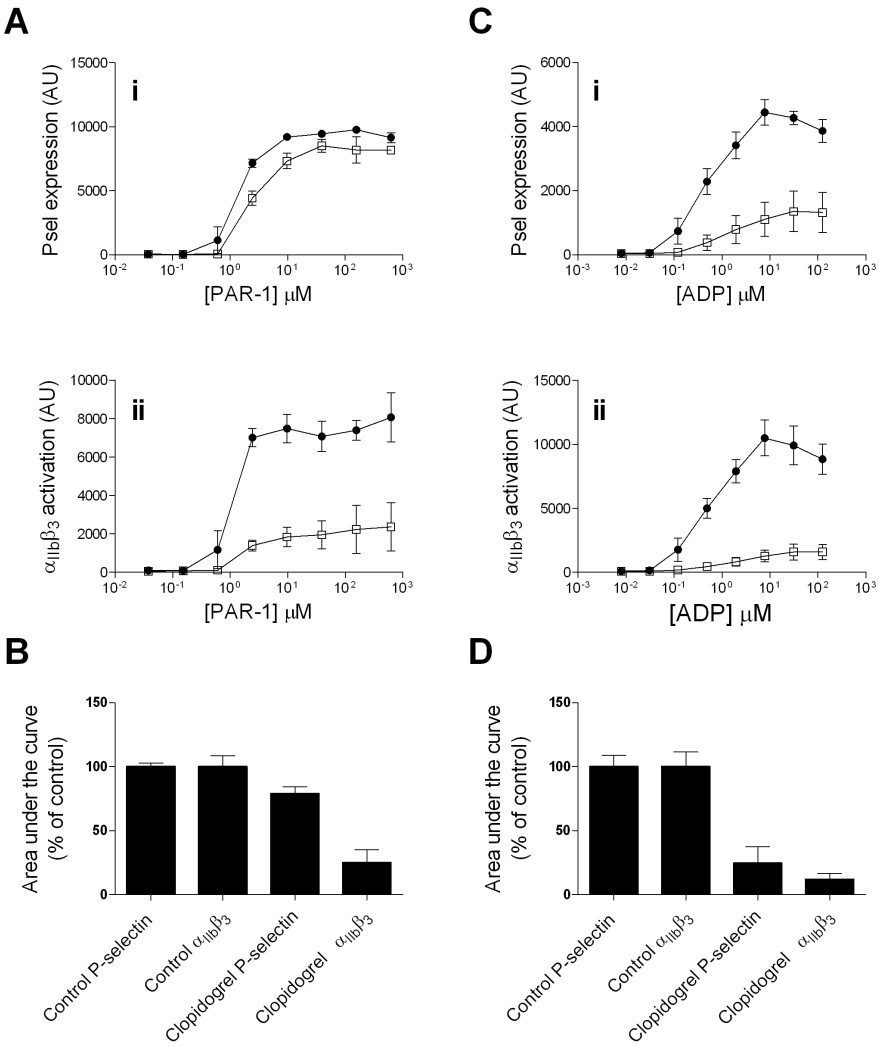

Figure 2. Activation of platelets with PAR-1 agonist or ADP of platelets from individuals treated with clopidogrel. A) Platelets of healthy controls (n=3; black circles) and patients using clopidogrel (n=3; white squares) were stimulated with increasing concentrations of PAR-1 agonist. Activation of platelets was determined by P-selectin (i) expression and $\alpha_{IIb}\beta_3$ activation (ii) by flow cytometry and expressed in arbitrary units (AU). B) Area under the curve of graphs depicted in Ai,ii was calculated, and is expressed as a percentage of the area under the curves of controls for either P-selectin expression, or $\alpha_{IIb}\beta_3$ activation. C) As in Ai,ii, but with increasing concentrations of ADP. D) As in B), but with ADP.

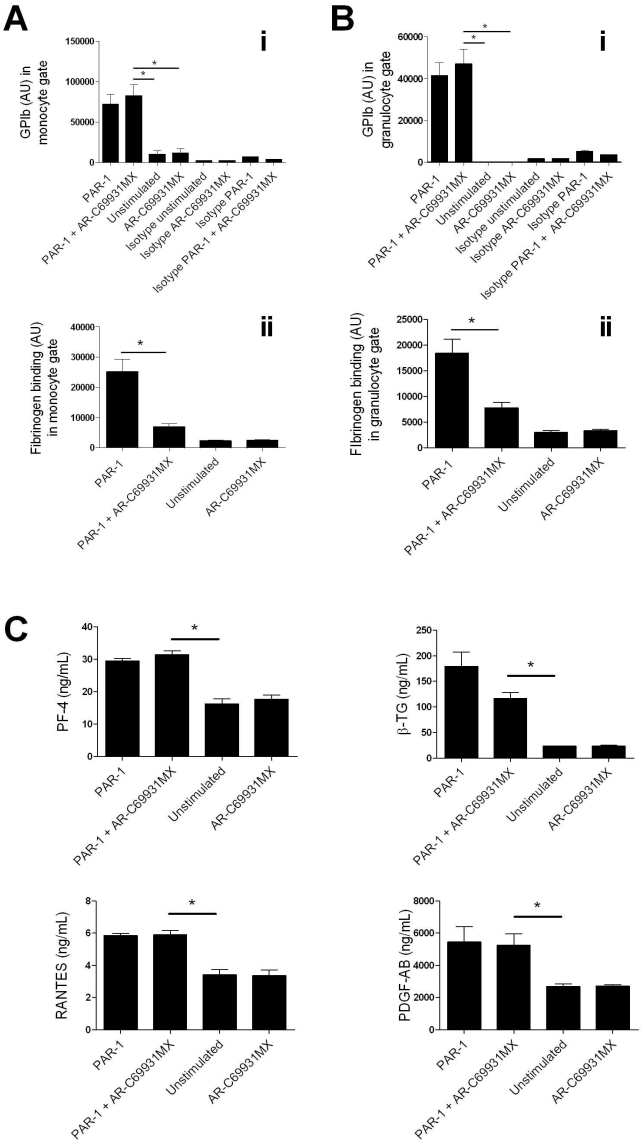

Figure 3. Formation of platelet-monocyte and platelet-granulocyte complexes, and analysis of platelet releasate in the presence and absence of $P2Y_{12}$ antagonist. A) Platelet-monocyte complexes in whole blood of healthy donors (n=5) were measured by flow cytometry after stimulation with 150 μM PAR-1 agonist, either in presence of 1 μM AR-C69931MX or mock treated. The amount of platelets boud to monocytes was measured by GPIb-labeling and expressed in AU (i). Platelet-monocyte complexes were measured for $\alpha_{IIb}\beta_3$ activation, to control for effectiveness of $P2Y_{12}$ inhibition (ii). B) Platelet-granulocyte complexes in whole blood of healthy donors (n=5) were measured by flow cytometry after stimulation with 150 μM PAR-1 agonist, either in presence of 1 μM AR-C69931MX or mock treated. The amount of platelets bound to granulocytes was measured by GPIb-labeling and expressed in arbitrary units (i). Isotype labeling for GPIb was taken as control for all stimulations. Platelet-granulocyte complexes were measured for $\alpha_{IIb}\beta_3$ activation, to control for effectiveness of $P2Y_{12}$ inhibition (ii). C) Platelet releasates of healthy donors (n=5) after stimulation with 150 μM PAR-1 agonist, or stimulation with 150 μM PAR-1 agonist with 1 μM AR-C69931MX were measured with ELISA for concentrations of PF-4, β-TG, RANTES/CCL5, and PDGF-AB. In all graphs, mean and standard error of the mean are shown. Differences were tested non-parametrically (*: $P<0.05$).

4 reduced cAMP levels, but not below levels of resting platelets (Figure 4A). When platelets were stimulated with different concentrations of PAR-1 activating peptide in the presence of iloprost, $\alpha_{IIb}\beta_3$ activation was completely inhibited, while P-selectin expression maintained 80% of activation without iloprost (Figure 4Bi,ii). When platelets were stimulated via PAR-4 in presence of full $\alpha_{IIb}\beta_3$ inhibitory iloprost concentrations, P-selectin expression reached 46% of maximum PAR-4 stimulation without iloprost (Figure 4Ci,ii).

Besides adenylatecyclase inhibition, $P2Y_{12}$ stimulation results in activation of PI3-kinase. Next we investigated whether inhibition of PI3-kinase also leads to the differential expression of platelet activation markers during PAR stimulation. After

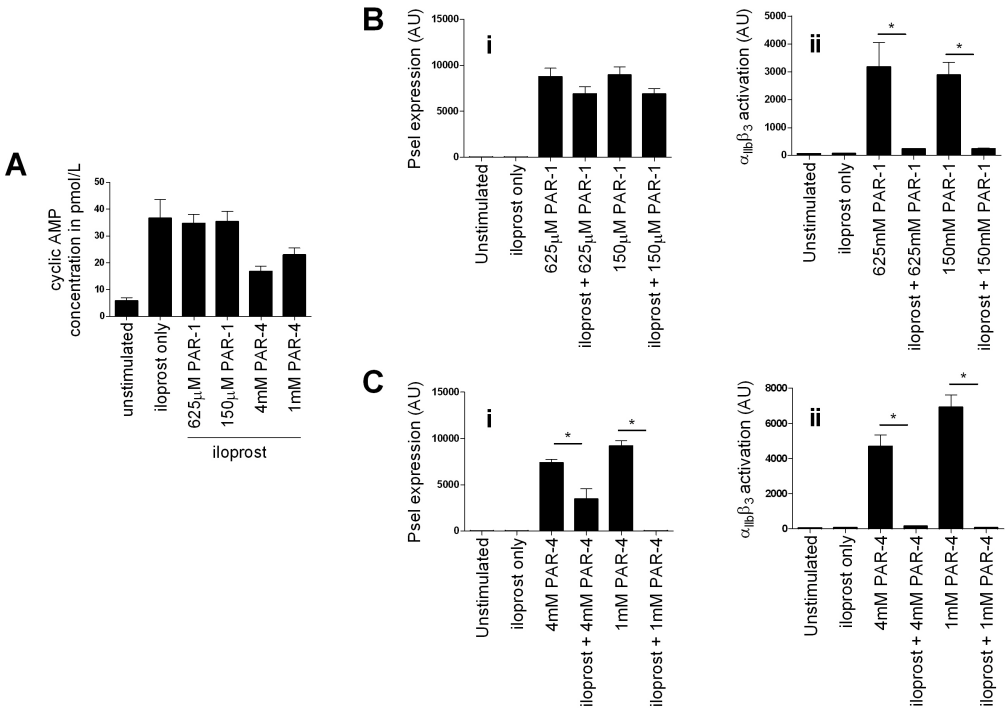

Figure 4. Platelet activation by PAR-1 or PAR-4 in the presence of iloprost. A). cAMP levels in platelets of healthy donors (n=5) were measured in response to 0.02 μg/mL iloprost only, and different concentrations of PAR-1 agonist and PAR-4 agonist in combination with 0.02 μg/mL iloprost. B) Expression of P-selectin (i) and activation of $\alpha_{IIb}\beta_3$ (ii) of platelets from healthy donors (n=5), expressed in arbitrary units (AU), was measured by flow cytometry in response to different concentrations of PAR-1 agonist, either in presence or absence of 0.02 μg/mL iloprost. C) As described in Bi,ii, only with different concentrations of PAR-4 agonist. For all graphs, mean and standard error of the mean are shown. For B) and C), differences were tested non-parametrically (*: $P<0.05$).

PAR-1 stimulation, in presence of different concentrations of the PI3-kinase inhibitor wortmannin $\alpha_{IIb}\beta_3$ activation was strongly inhibited, while P-selectin expression was 68% of PAR-1 stimulated platelets in the absence of wortmannin (Figure 5Ai,ii). Inhibition of PI3-kinase during PAR-4 stimulation resulted in strong $\alpha_{IIb}\beta_3$ inhibition, whereas P-selectin expression was 81% of that with PAR-4 stimulation only (Figure 5Bi,ii). Addition of AR-C69931MX had only a minor additive inhibitory effect on P-selectin expression and none on $\alpha_{IIb}\beta_3$ activation.

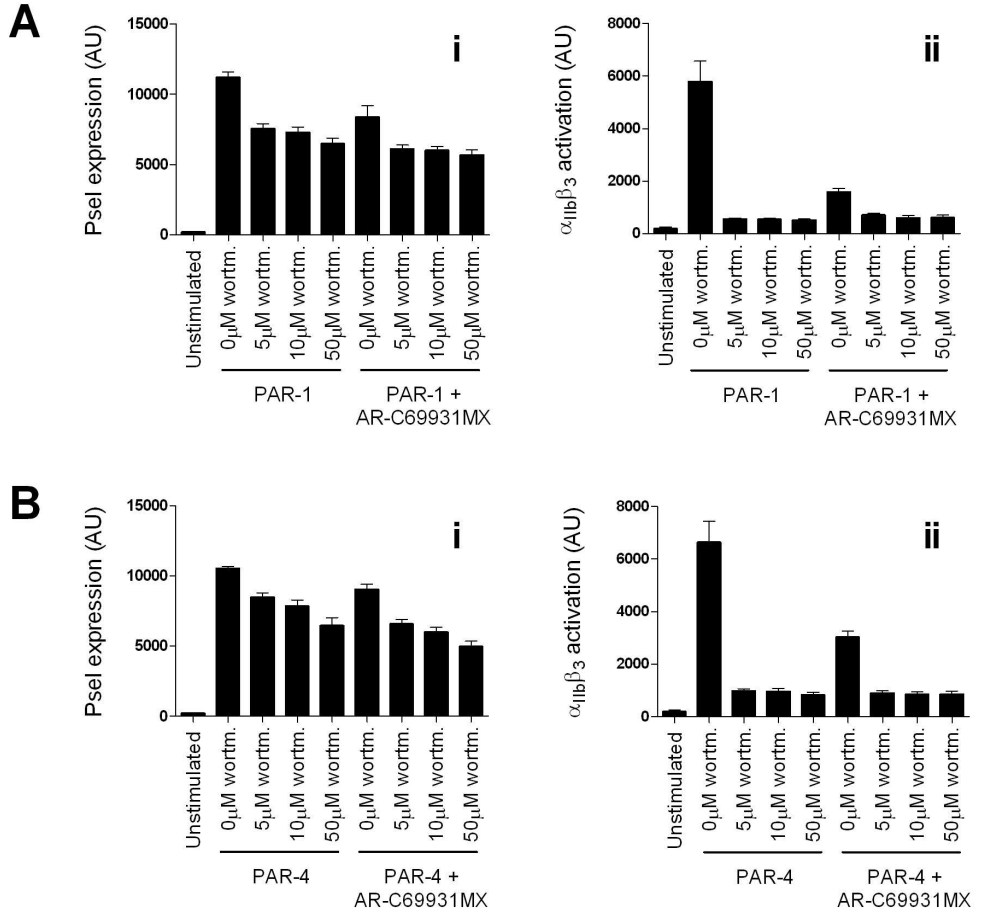

Figure 5. Platelet activation by PAR-1 or PAR-4 in the presence of the PI3-kinase inhibitor wortmannin. A) Platelets of healthy donors (n=5) incubated with increasing concentrations of wortmannin (wortm.) were stimulated with 150 μM PAR-1 agonist, or with 150 μM PAR-1 agonist with 1 μM AR-C69931MX. Expression of P-selectin (i) and activation of $\alpha_{IIb}\beta_3$ (ii) expressed in arbitrary units (AU) was measured by flow cytometry. Mean and standard error of the mean are shown. B) As in Ai,ii, but with 1 mM PAR-4 agonist instead of PAR-1 agonist.

Inhibition of phospholipase-C inhibits both $\alpha_{IIb}\beta_3$ activation and P-selectin expression

Central in PAR induced platelet activation is the activation of phospholipase-C. Inhibition of phospholipase-C with increasing concentrations of U-73122 resulted in a dose dependent inhibition of $\alpha_{IIb}\beta_3$ activation as well as P-selectin expression during stimulation via either PAR-1 or PAR-4 (Figure 6Ai,ii,Bi,ii). Addition of AR-C69931MX to

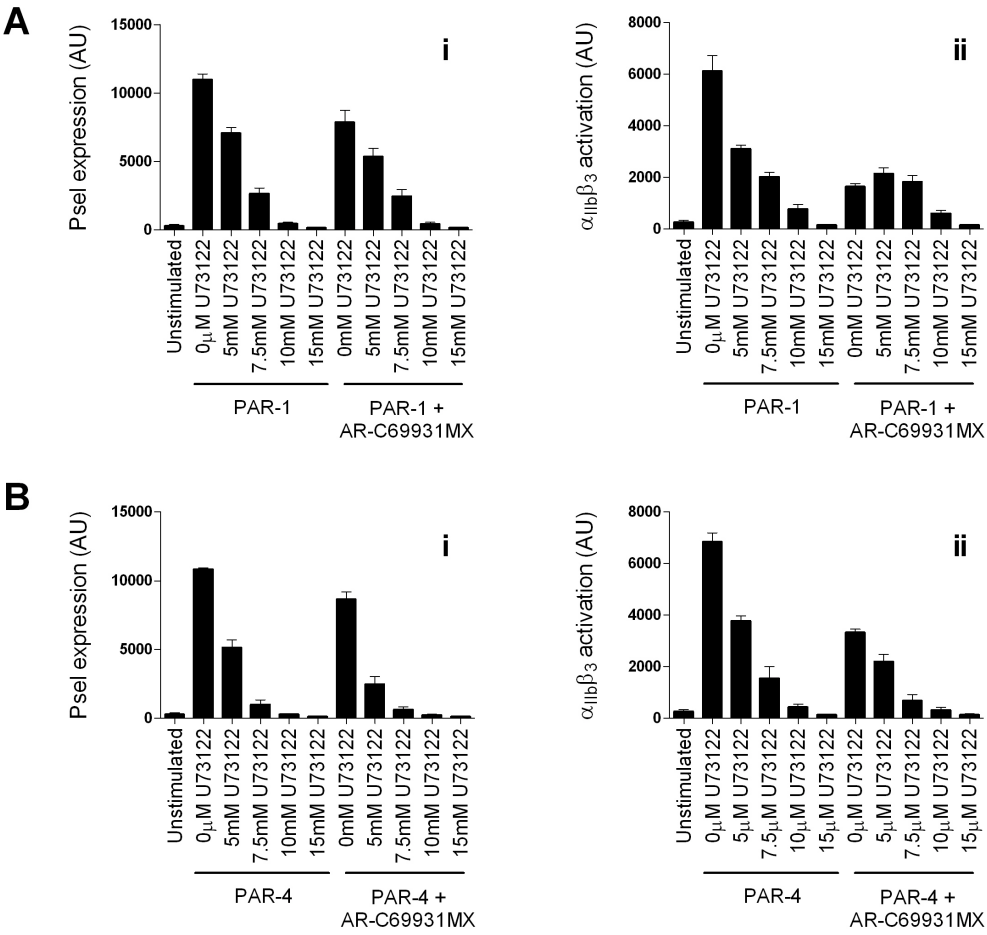

Figure 6. Platelet activation by PAR-1 or PAR-4 in the presence of phospholipase C inhibitor U-73122. A) Platelets of healthy donors (n=5) were incubated with increasing concentrations of U-73122 and were stimulated with 150 μM PAR-1 agonist, or with 150 μM PAR-1 agonist with 1 μM AR-C69931MX. Expression of P-selectin (i) and activation of $\alpha_{IIb}\beta_3$ (ii) expressed in arbitrary units (AU) was measured with flow cytometry. Mean and standard error of the mean are shown. B) As described in Ai,ii, but with 1 mM PAR-4 agonist instead of PAR-1 agonist.

the increasing U-73122 concentrations had only a minor additive inhibitory effect on P-selectin expression and $\alpha_{IIb}\beta_3$ activation.

Discussion

In this manuscript we show that PAR-1 or PAR-4 initiated platelet $\alpha_{IIb}\beta_3$ activation is inhibited with $P2Y_{12}$ antagonism or with the prostacyclin analogue iloprost, while granule release remains on almost full strength [11]. Addition of antagonists of $P2Y_{12}$ had also a strong inhibitory effect on platelet aggregate formation, while we observed normal platelet monocyte and platelet granulocyte complex formation and normal α-granule release. The differential effects of the $P2Y_{12}$ pathway inhibitors on platelet activation was not only observed *in-vitro*, but also in CLI patients on clopidogrel treatment.

The α-granules contain high concentrations of cytokines, chemokines and growth factors that are released from activated platelets. In addition to soluble markers, α-granules contain several membrane proteins, including P-selectin and CD40L, which become expressed on the outer membrane of platelets upon activation. Both CD40L and P-selectin can bind CD40 and PSGL-1, which are their counter receptors on leukocytes. Hence, platelets are not exclusively important for their hemostatic function, but have pleiotropic roles in human (patho)physiology. We therefore hypothesize that selective granule release of platelets without $\alpha_{IIb}\beta_3$ activation is an important physiological process in the blood circulation. This is supported by the fact that endothelial cells continuously produce and release prostacyclin, a selective aggregation inhibitor. Moreover, endothelial cell membranes contain CD39, which is a potent ectopeptidase with a similar capacity as apyrase to degrade ADP [12]. The endothelium hereby creates an environment that inhibits platelet aggregation, while still allowing α-granule release and the formation of platelet leukocyte complexes. Activation via PAR receptors can occur via an array of enzymes such as thrombin, plasmin, activated protein C (APC), thrombocytin, PA-BJ, Factor Xa, Factor VIIa, kallikreins, cathepsin G, trypsin, matriptase, tryptase, and matrix metalloproteinase 1 and 13 [13-17]. This may be detrimental, as platelet cytokines and chemokines may initiate inflammation and accelerate the formation of an atherosclerotic lesion, while PDGF can stimulate smooth muscle cell proliferation and plaque angiogenesis [1,2,18,19]. On the other hand, platelet granule release without aggregation may provide a mechanism to maintain the endothelium without causing ischemia [20].

So far, landmark reviews on platelet function have considered activation of $\alpha_{IIb}\beta_3$ and granule release as equally matched expressions of platelet activation,

suggesting that the hemostatic function of platelets and the inflammatory capacity of platelets are intertwined, although selective inhibition of $\alpha_{IIb}\beta_3$ by $P2Y_{12}$ antagonism has been described [1,2,21,22]. We show that besides $P2Y_{12}$ antagonism, stimulation of the IP receptor allows for PAR-dependent granule release without $\alpha_{IIb}\beta_3$ activation, which has implications for the physiological functions of platelets. In addition, this selective inhibition of platelet activation reveals a specific beneficial effect of anti-$P2Y_{12}$ therapeutics such as clopidogrel, since they inhibit platelet thrombus formation, without interfering with other functions of platelets.

One of the signal transduction steps downstream of $P2Y_{12}$ stimulation is the inhibition of adenylatecyclase, involved in the synthesis of cAMP induced by endothelial cell produced prostacyclin [23]. During stimulation with iloprost, activation of PAR-1 or PAR-4 receptors of platelets leads to reduced cAMP levels although the levels were still higher than the controls. During this iloprost treatment, PAR-1 and PAR-4 dependent platelet stimulation resulted in a similar differential activation pattern as with the inhibition of $P2Y_{12}$, indicating that $\alpha_{IIb}\beta_3$ activation does not occur on high cAMP levels, but α-granule release does.

Our findings are supported by findings of several previous studies. Secondary platelet activation via ADP release, and hence stimulation of $P2Y_{12}$ is required for PAR-1 induced platelet aggregation, which depends on PI3-kinase induced increase of phosphatidylinositol 3,4-bisphosphate [23]. In another study it was observed that in mice, PI3-kinase is not required for granule release [24]. To further investigate the role of PI3-kinase activity in differential activation of platelets, we used the PI3-kinase inhibitor wortmannin. During PAR-1 stimulation, PI3-kinase inhibition with wortmannin strongly inhibited $\alpha_{IIb}\beta_3$ activation, whereas P-selectin expression was only slightly inhibited. Our data further establish that PI3-kinase activity is needed for the activation of $\alpha_{IIb}\beta_3$, but it is not critical in the release of α-granules.

Phospholipase-Cβ is a major downstream effector of PAR receptors [5,25], which is an important pathway for platelet granule-release [24]. Our findings that intracellular inhibition of phospholipase-C activity with U-73122 results in inhibition of both P-selectin expression and $\alpha_{IIb}\beta_3$ activation suggests that the phospholipase-C pathway is required for both α-granule release and $\alpha_{IIb}\beta_3$ activation.

Activity of signal transduction pathways via PI3-kinase, phospholipase-C, and inhibition of adenylatecyclase are crucial for platelet aggregation [5]. We show that all three pathways are required for adequate $\alpha_{IIb}\beta_3$ activation, while the α-granule release is only dependent on phospholipase-Cβ signal transduction pathway. Autocrine activation of platelets with ADP triggers the inhibition of the adenylatecyclase, and activates the

PI3-kinase pathway, which are both required for $\alpha_{IIb}\beta_3$ activation (Figure 7). When the $P2Y_{12}$ pathway is inhibited, ADP induced inhibition of the adenylatecyclase and the activation of the PI3-kinase pathway is prevented. Alternatively, stimulation of the IP receptor increases cAMP levels. Under these conditions there will be α-granule release by PAR stimulation through activity of phospholipase-Cβ, while there will be no $\alpha_{IIb}\beta_3$ activation. A focus on specifically inhibiting granule release may provide more insight on this characteristic of platelets.

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We want to thank Astra Zeneca for providing AR-C69931MX, Schering AG for providing iloprost, and R. Farndale at Cambridge University for supplying XL-CRP.

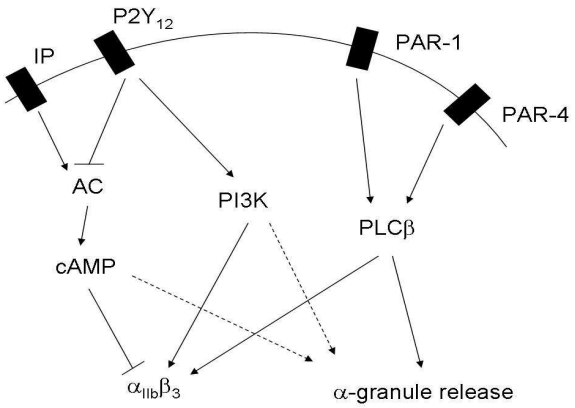

Figure 7. Model for differential activation of platelets during PAR stimulation. Stimulation of $P2Y_{12}$ causes inhibition of adenylatecyclase (AC), being associated with decreased cAMP levels, and activation of PI3-kinase (K). Activation of the PAR-1 and PAR-4 receptor causes activation of phospholipase (PL)Cβ. Activation of phospholipase-Cβ is sufficient to cause release of the α-granules, whereas $\alpha_{IIb}\beta_3$ activation requires the combination of reduced cAMP levels (by inhibition of adenylatecyclase by $P2Y_{12}$ activation or removal of stimulation of the IP), PI3-kinase activity, and phospholipase-Cβ activity. Dotted arrows indicate contribution to α-granule release.

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Chapter 6

Targeted phosphotyrosine profiling of GPVI signaling implicates oligophrenin-1 in platelet filopodia formation

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Abstract

Platelet adhesion to subendothelial collagen is dependent on the integrin $\alpha_2\beta_1$ and glycoprotein (GP) VI receptors. The major signaling routes in collagen-dependent platelet activation are outlined, however crucial detailed knowledge of the actual phosphorylation events mediating them is still limited. Here we explore phosphotyrosine (pTyr) signaling events downstream of GPVI with site-specific detail.

Immunoprecipitations of pTyr-modified peptides from protein digests of GPVI activated and resting human platelets were compared by stable isotope based quantitative mass spectrometry. We surveyed 214 unique pTyr sites over two time points, of which 28 showed a significant increase in phosphorylation upon GPVI activation. Among these was Tyr370 of oligophrenin-1 (OPHN1), a Rho GTPase-activating protein. To elucidate the function of OPHN1 in platelets, we performed an array of functional platelet analyses within a small cohort of rare oligophrenia patients. Due to germline mutations in the *OPHN1* gene locus, these patients lack OPHN1 expression entirely. Our studies revealed that among other unaltered properties, oligophrenia patients show normal P-selectin exposure and $\alpha_{IIb}\beta_3$ activation in response to GPVI, as well as normal aggregate formation on collagen under shear conditions. Finally, the major difference of OPHN1 deficient platelets turned out to be a significantly reduced collagen-induced filopodia formation.

In conclusion, in-depth pTyr screening revealed many novel signaling recipients downstream of GPVI activation uncovering a new level of detail within this important pathway. To illustrate the strength of such data, functional follow-up of OPHN1 in human platelets deficient of this protein showed reduced filopodia formation on collagen, an important parameter of platelet hemostatic function.

Introduction

The response of platelets to vessel injury is essential to prevent bleeding, but hyper-reactivity underlies the pathophysiology of various thrombotic diseases. Exposure of the extracellular matrix to flowing blood induces platelet activation, including the release of the contents of α- and δ-granules. Also, a conformational change of $\alpha_{IIb}\beta_3$ increases its affinity for its ligands (e.g. fibrinogen), and an active reorganization of the actin cytoskeleton accommodates shape change and the formation of filopodia (reviewed in[1]). Collagen, the most abundant matrix protein in the subendothelium, provides a primary activation stimulus and a surface for adhesion[2]. Glycoprotein (GP) VI is considered the predominant receptor responsible for collagen-induced platelet activation[3, 4].

The GPVI-mediated signaling pathway is a promising target for novel antiplatelet therapies because individuals with reduced GPVI expression have a mild increase in bleeding tendencies, while inhibition of the GPVI pathway may reduce thrombosis risk [2, 5-7]. Therefore, it is important to improve our knowledge of the GPVI mediated signaling pathway in platelet activation.

GPVI is a 62-kDa type I transmembrane receptor of the immunoglobulin superfamily of surface receptors, which is exclusively expressed in platelets and megakaryocytes. The signaling capacity of GPVI depends on its association with the Fc receptor (FcR) γ-chain homodimer. Each FcRγ-chain monomer contains a conserved immunoreceptor tyrosine-based activation motif (ITAM), which is characterized by two conserved YXXL motifs separated by 6-12 amino acids [8]. Upon receptor cross-linking by the ligand collagen these two conserved ITAM tyrosine residues are phosphorylated by the Src family tyrosine kinases Fyn and Lyn, which localize to a conserved proline-rich region of GPVI [3, 9]. This phosphorylation then leads to recruitment and activation of the tyrosine kinase Syk, which regulates a complex downstream pathway that involves the adapter proteins LAT, Gads and SLP-76, the Tec family tyrosine kinases Btk and Tec, the GTP exchange factors Vav1 and Vav3, PI3-kinase isoforms and PLCγ2 (for reviews, see [9, 10]).

A handful of proteins that participate in GPVI signaling in human platelets are known, but our understanding of the tyrosine signaling events downstream of GPVI activation is far from complete. This information is considered crucial for understanding the fine molecular details of platelet activation and their clinical implications. Here, we aimed to identify novel GPVI signaling proteins by obtaining site-specific and quantitative information on tyrosine residues being phosphorylated upon stimulation. To this end,

a quantitative analysis of immuno affinity enriched phosphorylated tyrosine peptides [11-13] was performed to compare resting and crosslinked collagen related peptide (CRP-XL) stimulated[14] human platelets. We identified 214 unique phosphotyrosine sites of which 30 showed a more than 2-fold increase in Tyr phosphorylation after stimulation. Next to expected downstream targets of GPVI we also detected three putatively novel ones. One of these, oligophrenin-1 is a Rho GTPase activating protein (RhoGAP). Subsequent characterization of platelets obtained from four patients with X-linked intellectual disability (XLID) caused by germline mutations in the *Oligophrenin-1* (*OPHN1*) gene [OMIM 300486] revealed the specific involvement of *OPHN1* in platelet filopodia formation on collagen, substantiating our data obtained from the targeted phosphotyrosine proteome profiling approach.

Methods

Reagents

Crosslinked collagen related peptide (CRP-XL) and $\alpha_2\beta_1$ binding peptide glycine-phenylalanine-hydroxyproline-glycine-glutamate-arginine (GFOGER) were synthesized and crosslinked as necessary in one of our labs as described [15], but using a CEM Liberty microwave synthesizer. Acetylsalicylic acid was purchased from Sigma; AR-C69931MX was a kind gift from Astra Zeneca.

Patients

Although rare, platelets could be obtained from four male Oligophrenin-1 deficient patients. Their legal representatives had given informed consent for drawing blood samples and performing the experiments described in this paper. None of the patients had a history of abnormal hemostasis or easy bruisability. Moreover, patient 1, a 9 year old boy, underwent several orthodontic and surgical procedures (correction of phymosis, strabism and an adenotomy) without any hemostatic problems. Patients 2 and 3 comprised two affected brothers. The patients had moderate to severe intellectual disability (ID) and in patient 2 and 4 cerebral imaging was performed which revealed vermis hypoplasia and enlarged ventricles. Genetics: Patient 1 showed normal G-banded karyotyping (46) with XY chromosome complement. Array-CGH with the Agilent 105K oligo-array (Oxford design) showed a de novo ~137Kb X:67,249,563-67,386,688(hg18) deletion in Xq12, deleting a large part (exons 6 through 17) of the *OPHN1* gene (total 25 exons). The adult brother pair (patients 2 and 3) had a missense mutation (c.16658A>T;p.

Val533Glu) in *OPHN1*. The clinical and molecular characteristics of adult patient 4 have been published previously [16]; he had a loss-of-function mutation (c.556C>T; p.Gln186X) in *OPHN1*. Controls were healthy volunteers.

Blood sample preparation

Venous blood was collected from the patients and healthy volunteers after obtaining informed consent. Blood for mass spectrometry (MS) analysis was collected with an open system, anti-coagulated with 3.2% tri-sodium citrate (Merck). Blood for other experiments was collected using vacuum tubes with 3.2% tri-sodium citrate (BD). Platelet rich plasma (PRP) was prepared by centrifugation of whole blood at 160 g for 15 minutes at room temperature, no brake. Washed platelets for mass spectrometry analysis were prepared by adding PRP with ACD (8.5 mM tri-sodium citrate, 7.1 mM citric acid, 5.5 mM D-glucose; final concentration) and centrifugation for 15 minutes with 340 g at room temperature, no brake. The platelet pellet was resuspended in Tris-buffer (145 mM NaCl, 5 mM KCl, 260 nM NaH_2PO_4, 1 mM $MgSO_4$, 100 mM Tris, 5.5 mM D-glucose, pH 6.5) with 10 ng/mL prostacyclin. Platelets were centrifuged for 15 minutes with 340 g at room temperature and resuspended up to 200 x 10^9/L in Tris-buffer (pH 7.3), with 100 μM acetylsalicylic acid, and 1 μM AR-C69931MX. Platelets were not used until 30 minutes after isolation. Washed platelets for other experiments were prepared, by adding ACD to PRP and subsequent centrifugation at 340 g at room temperature, no brake. The platelet pellet was resuspended in Hepes-Tyrode (HT) buffer (145 mM NaCl, 5 mM KCl, 0.5 mM NaH_2PO_4, 1mM $MgSO_4$, 10 mM Hepes, 5.5 mM D-glucose, pH 6.5) with 10 ng/mL prostacyclin. Platelets were centrifuged for 15 minutes at 340 g at room temperature and resuspended to 200 x 10^9/L in HT-buffer (pH 7.3). Platelets were not used until 30 minutes after isolation.

GPVI stimulation for mass spectrometry analysis

Platelet suspensions were stimulated with 2.5 μg/mL CRP-XL for 5 and 30 minutes. Unstimulated platelet suspensions were taken as control for the same time points. After the indicated incubation times, platelet suspensions were centrifuged at 4000 g for 2 minutes, the supernatant aspirated and subsequently the pellet was snap frozen in liquid nitrogen.

Protein extraction, digestion and stable isotopic labeling of peptides

Mock- or CRP-XL-treated platelet pellets, stored at -80ºC, were rapidly thawed and subsequently lysed on ice in 8 M urea in 100 mM Tris pH 8.5, 10 mM DTT, 1 mM sodium orthovanadate and 1X PhosSTOP in the presence of protease inhibitors. Efficient lysis was ensured by sonication on ice using a tip sonicator (3 x 30seconds at full power with interval 0.8, followed by 30 seconds continuous sonication) and proteins were further reduced at 56 °C for 20 minutes on a shaker (600 rpm). Protein concentration was determined using a Bradford Assay (BioRad, Veenendaal, The Netherlands). Total protein lysate from each condition (3 mg total protein) was alkylated with iodoacetamide and subsequently taken for proteolytic digestion using the Filter-Aided Sample Preparation (FASP) procedure (protocol 2), essentially as described in [17]. Proteins were digested with LysC (1:100 w/w) for 4 hours at 37°C, followed by overnight digestion with trypsin (1:50 w/w). See 'Supplementary Methods' for further details. Peptides eluted from the FASP-filter unit were desalted and stable isotope dimethyl labeled on a Sep-Pak C18 column (Waters, USA, Massachusetts) as described previously [18, 19]. Control samples were labeled with "light" and CRP-XL-treated samples with "heavy" label (See Figure 1). For both the 5 minutes and the 30 minutes time point, the light and intermediate sample were mixed in a 1:1 ratio, lyophilized and stored at -80ºC until immunoprecipitation.

Immunoprecipitation (IP) of phosphotyrosine peptides.

Immunoprecipitation was performed as described earlier [11, 12, 20]. Differentially labeled peptide mixtures of each time point were reconstituted in IP buffer containing 50 mM Tris (pH7.4), 150 mM NaCl, 1% NOG, and protease inhibitor cocktail (Roche diagnostics). Agarose-conjugated anti-pTyr (PY99) antibodies (Santa Cruz) (prewashed three times with IP buffer) were added to the peptide mixture and incubated overnight at 4°C under constant rotation. After incubation, the beads were washed three times with 1 ml of IP buffer and twice with 1 ml of water, all at 4ºC. Peptides were eluted twice with 0.15% TFA, subsequently desalted and concentrated on stop-and-go extraction (STAGE) tips, dried *in vacuo* and stored at -80ºC until LC-MS analysis.

On-line nanoflow LC-MS

Nanoflow LC-MS/MS was performed by coupling an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) to a LTQ-Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) as described previously [21]. Briefly, dried peptide fractions were reconstituted in 10% formic acid and delivered to a trap column (ReproSil-Pur

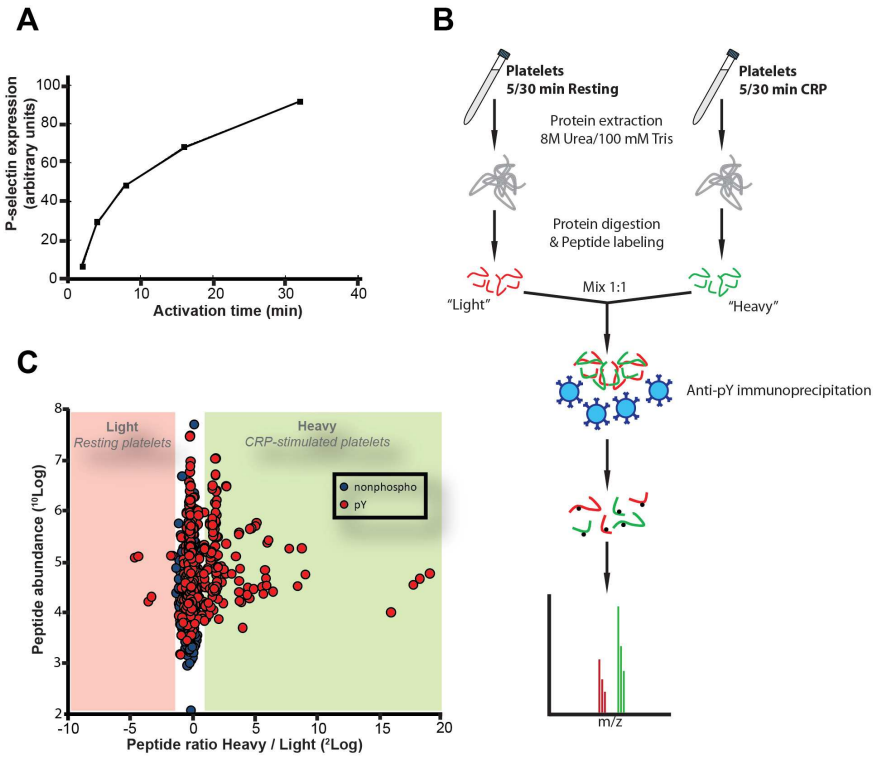

Figure 1. Targeted tyrosine phosphorylation profiling in stimulated platelets.
(A) Platelet stimulation with 2.5 µg/mL CRP-XL as monitored by P-selectin expression on the plasma membrane. (B) Overview of the experimental quantitative proteomics workflow. In parallel, resting (left) and CRP-XL activated (5 and 30 minutes, right) platelets were lysed, proteins extracted and subsequently digested with trypsin. The peptides were differentially labeled using stable isotope dimethyl labeling. For each time point, two differentially labeled digests were combined, followed by enrichment of tyrosine phosphorylated peptides using immobilized phosphotyrosine-specific antibodies. The enriched fraction was analyzed by nano-flow LC-MS/MS. (C) Proteomics data representation (30 minute experiment is shown as an example). Using MSQuant software, the ratio [Heavy/Light] = [CRP-XL/Ctrl] was calculated for immunoprecipitated phosphotyrosine (pY)-containing peptides (red dots) and normalized on the ratio of non-phosphorylated peptides (blue dots), based on the extracted ion chromatograms of the differentially labeled isotopomers of each peptide. Peptide ratios (2Log values) were plotted against peptide abundance (intensity, 10Log values).

C18-AQ, 3 µm, Dr. Maisch GmbH, Ammerbuch, Germany; 20 mm × 100 µm ID, packed in-house) at 5 µL/min in 100% solvent A (0.1 M acetic acid in water). Subsequently, peptides were transferred to an analytical column (ReproSil-Pur C18-AQ, 3 µm, Dr. Maisch GmbH, Ammerbuch, Germany; 40 cm × 50 µm ID, packed in-house) at ~100 nL/min in a 3 hour gradient from 0 to 40% solvent B (0.1 M acetic acid in 80% Acetonitrile).

The mass spectrometer was operated in data dependent mode, automatically switching between MS and MS/MS. Full scan MS spectra (from m/z 300-1500) were acquired in the Orbitrap with a resolution of 60,000 at m/z 400 after accumulation to target value of 500,000. The ten most intense ions at a threshold above 5000 were selected for collision-induced fragmentation in the linear ion trap at normalized collision energy of 35% after accumulation to a target value of 10,000.

Data analysis

All MS2 spectra were converted to single DTA files and mgf files were created using MSQuant 2.0 [22] at default settings. Runs were searched using an in-house licensed MASCOT search engine (Mascot version 2.2) software platform (Matrix Science, London, UK) against the Swissprot human database (version 56.2, 398181 sequences) with carbamidomethyl cysteine as a fixed modification. Light and intermediate dimethylation of peptide N-termini and lysine residues, oxidized methionine and phosphorylation of tyrosine, serine and threonine were set as variable modifications. Trypsin was specified as the proteolytic enzyme and up to one missed cleavage was allowed. The mass tolerance of the precursor ion was set to 5 ppm and for fragment ions to 0.6 Da. The assignment of phosphorylation sites of identified phosphopeptides was performed by the PTM scoring algorithm implemented in MSQuant [22]. Individual MS/MS spectra from phosphopeptides were accepted for a Mascot score ≥ 20. The FDR at this score was estimated to be less than 1% by performing a concatenated decoy database search [11]. All identified phosphopeptides that were found to be differentially phosphorylated were manually scrutinized for site localization. Quantification of peptide doublets was performed using an in-house dimethyl-adapted version of MSQuant [18, 22]. Based on the [CRP-XL/Ctrl] ratio distributions of phosphotyrosine (pTyr) containing and non-phosphorylated peptides, tyrosine phosphorylation was considered significantly regulated over a threshold of 2-fold change (see Figure 2 and Supplementary Figure 1B and C). A list of all quantified phosphopeptides is available as Supplementary Table 1, and all data are available in the PRIDE database [23] under accession numbers 19671 and 19672: http://tinyurl.com/3cx3ppb.

Western blotting

Washed platelets were lysed in SDS sample buffer (1.1 M glycerol, 62 mM Tris-HCl pH 6.8, 70 mM SDS, 29 μM Broom-phenol blue) and heated at 95°C for 5 minutes. Samples were subjected to SDS-PAGE using a NuPAGE 4%-12% gradient Bis-Tris-HCl pH 6.4 gel (Invitrogen) with MOPS running buffer (Invitrogen) and 50 mM dithiothreitol,

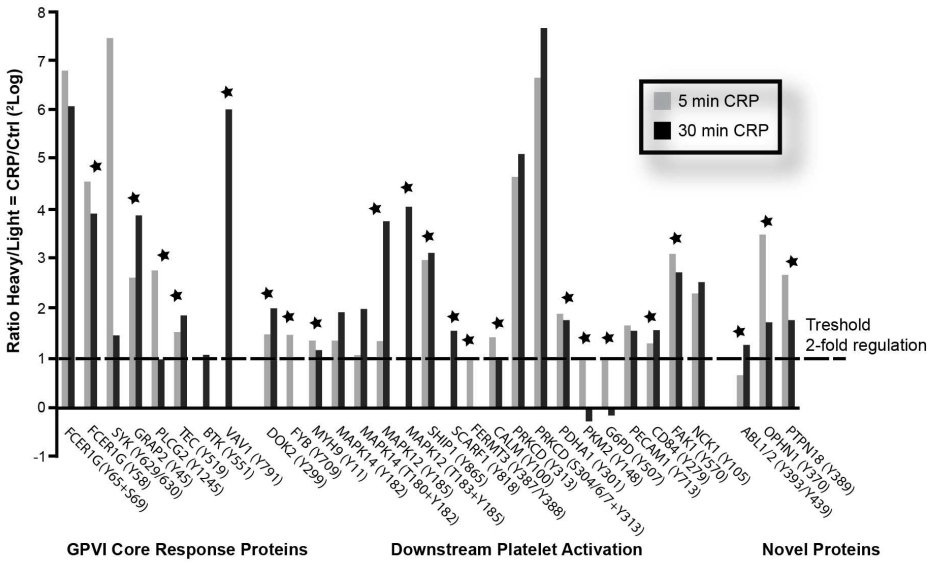

Figure 2. Tyrosine sites undergoing increased phosphorylation downstream of GPVI activation. Tyrosine phosphorylation sites with increased phosphorylation downstream of GPVI in platelets activated with CRP-XL. Twenty-eight pTyr sites on 27 proteins showed at least a 2-fold increase in phosphorylation in response to platelet activation through GPVI after 5 minutes (grey bars), and/or 30 minutes (black bars). A substantial part of these sites belongs to the GPVI core response proteome (Supplementary Figure 2). Novel pTyr sites in platelet activation are marked with black stars.

electrotransferred to PVDF membrane (Millipore), and immunoblotted with goat anti-human oligophrenin-1 polyclonal antibodies (Santa Cruz), or biotin coupled sheep anti-human GPVI polyclonal antibodies (R&D). Blot was probed with donkey anti-goat IRDye 800CW (LI-COR) and streptavidin IRDye 680 (LI-COR) and imaged using a LI-COR Odyssey imager.

Quantification of platelet membrane proteins

Detection of β_1 receptor was performed with 25 μL APC mouse anti-human CD29 (BD) dissolved in 25 μL HEPES buffered saline (HBS; 10 mM HEPES, 150 mM NaCl, 1 mM $MgSO_4$, 5 mM KCl, pH 7.4). Detection of GPIX was performed with 50 uL FITC labeled mouse anti-human CD42a (BD). For GPIb and β_3, 2 μL FITC labeled mouse anti-human CD42b (BD), and 2 μL FITC labeled mouse anti-human CD61 (Sanquin) were dissolved in 48 μL HBS, respectively. Labeling was initiated by adding 5 μL fresh, citrate anti-coagulated whole blood to each sample of antibody dilution. After 20 minutes of incubation, the samples were fixed with 500 μL 0.2% formyl saline (0.2% formaldehyde,

0.9% NaCl) and kept at room temperature until analyses. All samples were analyzed on a FACS Canto II flow cytometer from BD Biosciences on the same day of processing. Single platelets were gated based on forward and side scatter properties. The mean fluorescence intensity (MFI) in the platelet gate was measured with FACS analysis.

Platelet filopodia and lamellipodia formation

Cover glasses 24 x 60 mm (Mariënfeld) were treated with 2% chromosulfaric acid. Cover glasses were coated with CRP-XL dissolved to 100 μg/ml together with GFOGER dissolved to 100 μg/ml in 10 mM acetic acid, or 100 μg/ml fibrinogen in phosphate buffered saline (PBS: 25 mM Na_2HPO_4, 2.3 mM NaH_2PO_4, 140 mM NaCl, pH 7.4) overnight at 4ºC. Coated cover glasses were blocked with 1% HSA for 90 minutes at room temperature. For perfusion with CRP-XL/GFOGER coated coverslips, PRP was added with 0.2 mM RGD. PRP was warmed for 20 minutes at 37ºC, and subsequently perfused at 25 /s for 20 minutes. Pictures were taken every 10 seconds with differential interference contrast microscopy (DIC) using a Carl Zeiss Observer Z1 microscope coupled to an AxioCam MRm camera and AxioVision Rel. 4.8 software (Carl Zeiss B.V., Sliedrecht, The Netherlands). Platelets forming filopodia and lamellipodia were counted and expressed as a percentage of total quantified platelets. Wilcoxon's rank sum test was used to test the difference between the patients and healthy controls.

Results

Tyrosine phosphoproteome analysis of CRP-XL stimulated platelets

Platelets need to respond rapidly to changes in vascular integrity to prevent excessive blood loss. Signaling pathways leading to platelet activation are therefore rapidly activated upon stimulation. To capture most detail, optimal time points of GPVI stimulation for our in-depth targeted and quantitative analysis were evaluated on the kinetics of CRP-XL dependent platelet activation. To this end, quantification of platelet membrane P-selectin expression, a general marker of activation, was used (Figure 1A). Two time points were selected: 5 minutes to represent the onset and 30 minutes to represent maximal activation. The chosen proteomics approach, which utilizes specific immune enrichment of peptides carrying a tyrosine phosphorylation is schematically depicted in Figure 1B [11, 12, 24]. After analysis of both the 5 and 30 minute time point, a total of 214 phosphotyrosine (pTyr) sites on 148 proteins were identified (Supplementary Table 1).

The quantitative data based on stable isotope dimethyl labeling revealed that overall protein abundance levels (reflected in the [CRP-XL/Ctrl] ratios of non-phosphorylated peptides) remained identical when comparing resting and activated platelets at both the 5 and 30 minute time point (Figure 1C and Supplementary Figure 1A and B). In contrast, many tyrosine-containing peptides showed a more than 2-fold increased phosphorylation upon CRP-XL stimulation (28 unique tyrosine sites on 27 proteins), the majority being detected at both time points (Figure 2 and Supplementary Figure 1C). Among these were several expected proteins and Tyr phosphorylation sites belonging to the presumed core GPVI response proteome (Figure 2 and Supplementary Figure 2): one of the FcRγ-chain ITAM domains (FCER1G; Tyr65), SYK (Tyr629/Tyr630), GRAP2 (GADS; Tyr45), and other proteins comprising the 'LAT signalosome'[9]. Twenty-two (80%) of the regulated tyrosine sites with increased phosphorylation upon GPVI activation are novel in platelets (Figure 2, black stars), according to the Uniprot and PhosphoSitePlus human databases and several key references[25-27]. Three particular sites were present on proteins not earlier shown to be involved in platelet collagen signaling: the protein tyrosine kinase ABL1/ABL2 (Tyr393/Tyr439), the non-receptor type protein tyrosinephosphatase 18 (PTPN18; Tyr389), and the Rho-GTPase activating protein (RhoGAP) oligophrenin-1 (Tyr370).

Characterization of oligophrenin-1 deficient platelets

Deficiency of oligophrenin-1 (OPHN1$^{-/y}$) is associated with a rare form of X-linked mental retardation known as oligophrenia, a syndrome characterized by defects in neuronal dendrite formation and synaptic plasticity[28, 29]. Despite that oligophrenia is a rare disorder, we were able to obtain blood from four OPHN1 deficient patients. As far as we know no bleeding disorders are reported in relation to loss of oligophrenin-1. In line with this, the patients did not have a bleeding phenotype, and there were no indications of thrombotic complications. Although each patient had a different gene variant, Western blotting confirmed the absence of oligophrenin-1 in the platelets of each patient (Figure 3A), whereas two control individuals showed robust expression of oligophrenin-1 in their isolated platelet lysates (apparent molecular mass 91 kDa). The mean platelet count (434 ± SD 56 x 10^9/L), mean platelet volume (7.3 ± SD 0.6 fL) and the expression of the platelet surface receptors GPIbα, GPIX, β_1 integrin, and the β_3 integrin were within the normal range in OPHN1$^{-/y}$ platelets (Figure 3B).

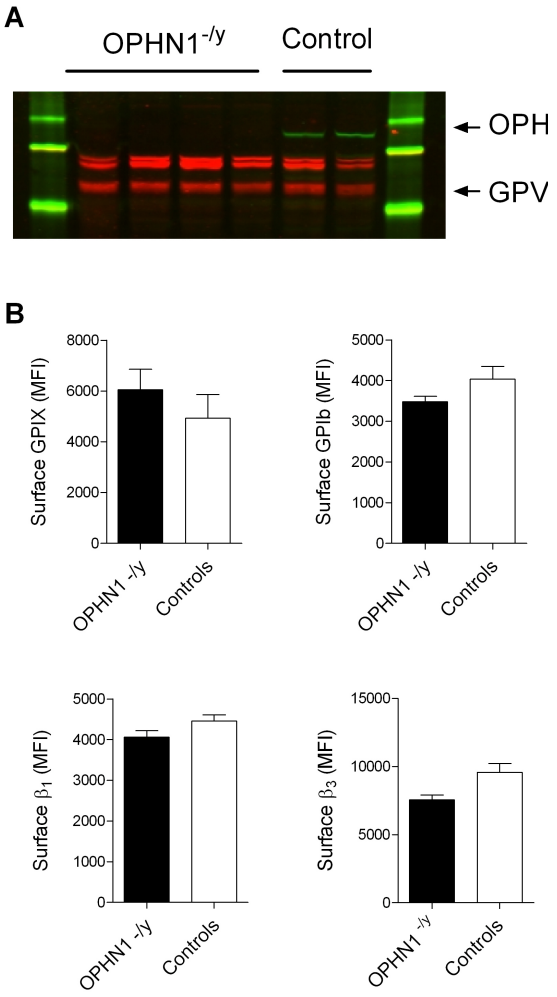

Figure 3. Expression levels of oligophrenin-1 and several platelet receptors in OPHN1$^{y/-}$ patients and controls.

(A) Washed platelets from four OPHN1$^{-/y}$ patients and two healthy controls were lysed and analyzed by western blotting using goat anti-oligophrenin-1 polyclonal antibody (green) and sheep anti-GPVI polyclonal antibody (red). (B) Platelets from four OPHN1$^{-/y}$ patients (black bars), and five controls (white bars) were incubated with antibodies against β_3, α_2, GPIb, and GPIX and expression was analyzed by flow cytometry. Data are expressed as mean median relative fluorescence units (MFI) ± SD.

Oligophrenin-1 deficient platelets are hemostatically normal

To determine whether the absence of oligophrenin-1 affects platelet function, we assessed the response of oligophrenin-1 deficient platelets to stimulation of $P2Y_{12}$, PAR-1, and GPVI (Supplementary Figure 3). Oligophrenin-1 deficient platelets showed no significant differences in P-selectin expression or $\alpha_{IIb}\beta_3$ activation compared with healthy controls. We then assessed the influence of oligophrenin-1 on platelet adhesion to collagen under conditions of high shear flow (1600/s; Supplementary Figure 4) and found that OPHN1$^{-/y}$ platelets adhered and formed aggregates on a collagen-coated surface to a similar extent as healthy controls. Moreover, the absence of OPHN1 did

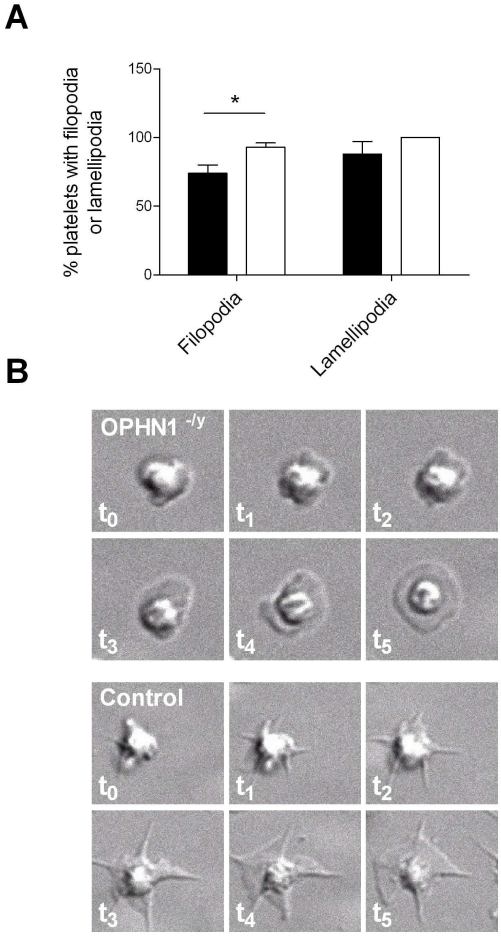

Figure 4. Filopodia formation in platelets of OPHN1$^{y/-}$ patients and controls. Oligophrenin-1 deficiency is associated with decreased filopodia formation prior to spreading on CRP-XL/GFOGER coated coverslips. (A) Platelet-rich plasma of four OPHN1$^{-/y}$ patients (black bars) and five healthy controls (white bars) containing RGD was perfused over CRP-XL/GFOGER coated cover glassesat 25 /s for 20 minutes. Pictures were taken every 10 seconds. Filopodia formation and subsequent lamellipodia formation was counted and expressed as a percentage of total quantified platelets. Data is shown as mean percentage ± 95% confidence interval. Differences between patients and controls were significant (* Wilcoxon's rank $P < 0.05$). (B) Snap shots of a platelet from an OPHN1$^{-/y}$ patient (upper panels) and a healthy control (lower panels) adhering and spreading at increasing time points (t_0 to t_5), perfused over CRP-XL/GFOGER.

not affect clot retraction in thrombin-stimulated platelet rich plasma (Supplementary Figure 5).

Oligophrenin-1 deficient platelets show defective filopodia formation

Since deficiency of oligophrenin-1 is reported to be associated with decreased neuronal dendrite formation[29, 30], we looked into the role of oligophrenin-1 in platelet spreading using real-time microscopy. Because oligophrenin-1 phosphorylation was increased upon stimulation of the collagen-dependent activation pathway, we also studied platelet spreading on a mixture of the collagen peptides that bind GPVI (CRP-XL) and $\alpha_2\beta_1$ (GFOGER)[31]. CRP-XL is a potent activator of platelets and causes rapid aggregate

formation. As this obscures the spreading process, we prevented aggregate formation with 0.2 mM of RGD peptide, thereby blocking $\alpha_{IIb}\beta_3$ ligand interactions. Under these conditions, OPHN1$^{-/y}$ platelets showed equal lamellipodia formation but significantly less filopodia formation during spreading (Figure 4A and B). OPHN1$^{-/y}$platelets form filopodia (OPHN1$^{-/y}$: 100% ±SEM 0%, controls: 99% ±SEM 1%, not significant), and spread normally on fibrinogen (OPHN1$^{-/y}$: 68 ±SEM 11%, controls: 83 ±SEM 7%, not significant), which is mainly $\alpha_{IIb}\beta_3$ dependent.

Discussion

To study the nature of GPVI signaling specifically in human platelets, we employed anti-phosphotyrosine immunoprecipitation of peptides, directly from primary human platelet digests. The quantitative proteomics data show immediately that GPVI signaling was rapidly engaged due to the highly increased phosphorylation of the ITAM domain at Tyr65 after 5 minutes. Also the phosphorylation of other known downstream targets was prominent (Syk, GADS, etc.), confirming the validity of our approach.

Garcia et al.[25] have used phosphotyrosine immunoprecipitation at the protein level to identify several proteins that are implicated in GPVI signaling in human platelets. In our study, immunoprecipitation of tyrosine phosphorylation at the peptide level combined with stable isotope labeling-based quantitation adds much additional detail. For instance, we were able to identify the specific phosphorylation sites on the earlier implicated proteins (DOK2 (Tyr299), MAPK14 (Tyr182) and PTPN6/SHP-1 (Tyr64)), and quantified their relative up-regulation upon GPVI stimulation. The three novel platelet proteins with increased tyrosine phosphorylation downstream of GPVI seem valid novel additions to the downstream GPVI signaling cascades. ABL1 (Tyr393) and ABL2 (Tyr439; the observed tyrosine-phosphorylated peptide is present in both isoforms) regulate cytoskeletal reorganization in several myeloid cell types[32] and known ABL interactors such as Src family kinases, GADS, NCK1 and SLP76 are also found regulated in this study. Given the importance of cytoskeletal rearrangement in platelet activation, the presence of ABL and its phosphorylation in platelets are not unexpected.

PTPN18 is a member of the PEST-family of protein tyrosine phosphatases. Little is known about its biological function, although overexpression studies suggested a role in neurite outgrowth and actin cytoskeleton reorganization[33]. PTPN18 is regulated by tyrosine phosphorylation, including the GPVI downstream target site discovered in the present study (Tyr389).

Our attention was drawn to the potential impact of oligophrenin-1 deficiency on platelet function. In patients with *OPHN1* mutations, loss or dysfunction of oligophrenin-1 is associated with reduced dendritic spine and filopodia length of neurons, the molecular explanation of their neurological phenotype[29, 30]. In neurons, oligophrenin-1 localizes to filopodia, lamellipodia and stress fibers to regulate the actin cytoskeleton [29, 34, 35]. To determine the platelet phenotype, here a thorough functional screen of the platelets of oligophrenia patients was performed. All functional tests were normal with no exception for the clot retraction assay, a measure for actin cytoskeleton contractility in which we expected to see differences due to the strong effects on filopodia length in neurons. The single phenotype we found was reduced filopodia formation of platelets during spreading on a collagen like surface, but not on fibrinogen, indicative of the specific function of oligophrenin-1 in human platelets.

Recently, Elvers *et al.*[36] reported a study on the presence of oligophrenin-1 in human and murine platelets and its Rho-GTPase activity towards RhoA, Cdc42, and Rac1 in an A5-CHO cell culture model system. They showed that upon platelet spreading on fibrinogen, oligophrenin-1 co-localized with actin in filopodia, the actin ring and lamellipodia. In addition, oligophrenin-1 co-localized with Rac1 and Cdc42 in the late phase of platelet spreading on fibrinogen, whereas RhoA co-localization was observed independently of activation and spreading. In our experiments we could not confirm a role for oligophrenin-1 in platelet spreading on fibrinogen. Given the data of Elvers *et al.*, oligophrenin-1 may have a redundant role in platelet spreading on fibrinogen in human platelets, which becomes apparent when overexpressed.

On collagen we found a more pronounced role for the RhoGAP, as its absence leads to reduced filopodia formation prior to spreading. In the experiments of Elvers *et al.*[36], overexpression of oligophrenin-1 in A5-CHO cells inhibited lamellipodia formation. This may be consistent with the observed phenotype in this manuscript (Figure 4), as the balance towards lamellipodia formation by absence of oligophrenin-1 may overrule formation of filopodia, causing platelets to spread without the formation of filopodia.

In conclusion, we have identified 28 phosphotyrosine (pTyr) sites on 27 proteins which undergo a more than 2-fold increase in phosphorylation upon GPVI activation in human platelets. We discovered three novel factors that are involved downstream of GPVI signaling after platelet activation, one of which was oligophrenin-1. In response to GPVI stimulation, oligophrenin-1 becomes phosphorylated at Tyr370 and plays a role in the formation of filopodia during platelet spreading on collagen.

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Conflict-of-interest disclosure

The authors declare no competing financial interests.

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Supplementary Methods and Figures

1. Supplementary Methods

Reagents and antibodies

All chemicals used for proteomics experiments were purchased from commercial sources and were of analytical grade. Trypsin (sequencing grade), Complete Mini protease inhibitor and PhosSTOP phosphatase inhibitor cocktails were purchased from Roche Diagnostics; Lys-C was obtained from Wako Chemicals. Microcon YM-30 spin columns were obtained from Millipore; mouse monoclonal anti-phosphotyrosine agarose (PY99, sc-7020) was obtained from Santa Cruz Biotechnology. Adenosine diphosphate (ADP) was purchased from Roche, SFLLRN-trifluoroacetate salt, thrombin receptor activating peptide (TRAP) specific for PAR-1 was purchased from Bachem.

Protein extraction and digestion

Mock- or CRP-XL-treated platelet pellets, stored at -80˚C, were rapidly thawed and subsequently lysed on ice in 8 M urea in 100 mM Tris pH 8.5, 10 mM DTT, 1 mM sodium orthovanadate and 1X PhosSTOP in the presence of protease inhibitors. Efficient lysis was ensured by sonication and proteins were further reduced at 56˚C for 20 minutes on a shaker (600rpm). Protein concentrations were determined using a Bradford Assay (BioRad, Veenendaal, The Netherlands). The total protein lysate from each condition (3 mg protein) was subsequently taken for proteolytic digestion using the Filter-Aided Sample Preparation (FASP) procedure[1], protocol 2. The concentrated samples were mixed with 0.2 mL of 8 M ureain 0.1 M Tris/HCl pH 8.5 (buffer UA), loaded into Microcon YM30 filtration devices (Millipore), and centrifuged at 14,000 g for 15 minutes. The concentrates were diluted in the devices with 0.2 mL of buffer UA and centrifuged again. After centrifugation, the concentrates were mixed with 0.1 mL of 50 mM iodoacetamide in UA solutionand incubated at room temperature for 30 minutes in the dark. Following centrifugation for 15 minutes, the concentrate was diluted with 0.2 mL of UA solution and concentrated again.This step was repeated twice. Next, the concentrate was diluted with 0.1 mL of 8 M urea in 0.1 M Tris/HCl pH 8.0 (buffer UB). This step was repeated once. Subsequently, LysC (1:100 w/w) in 30 µL UB was added to the filter and the samples were incubated at 37°C for 4 hours. Then 120 µL ammonium bicarbonate with trypsin (1:50 w/w) was added, followed by overnight incubation at 37°C. The peptides

were collectedby centrifugation of the filter units followed by two additional 30 μL-washes with 0.5 M NaCl.

Collagen response under flow

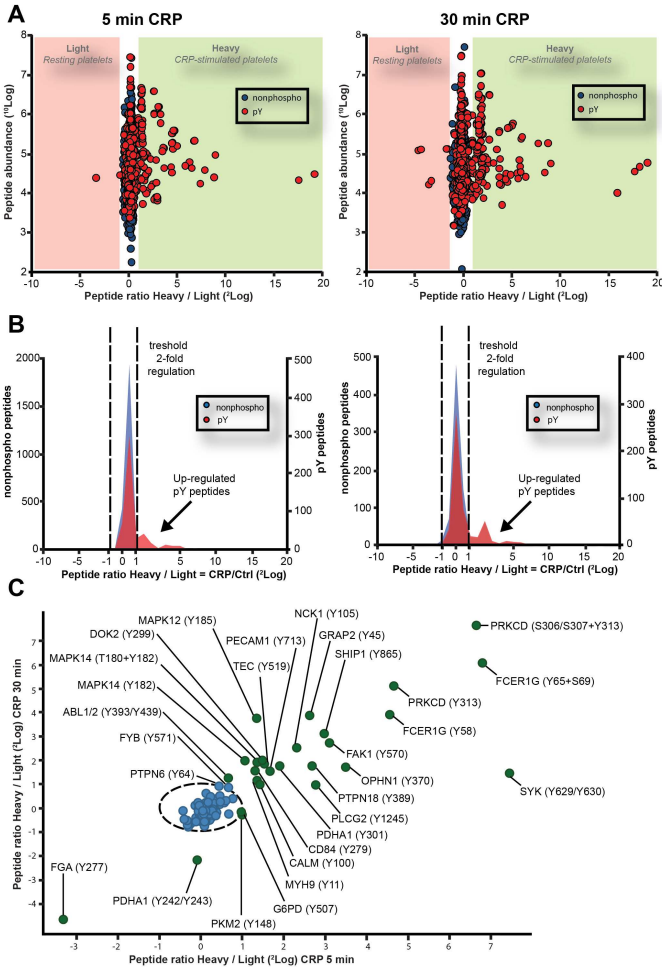
Cover glasses were coated with collagen dissolved to 100 μg/ml in HBS pH 7.4 for 90 minutes at room temperature. Collagen coated cover glasses were blocked with 1% human serum albumin (HSA; MP Biomedicals) overnight at 4ºC. Whole blood was warmed for 20 minutes at 37ºC, and subsequently perfused at 1600/s over collagen coated cover glasses. Pictures were taken every second for 5 minutes. Pictures were taken using a Zeiss Observer.Z1 microscope coupled to an AxioCamMRm camera and Axiovision Rel. 4.8 software. Platelet surface coverage on collagen was determined automatically with MacBiophotonics Image J software.

Platelet activation and responsiveness

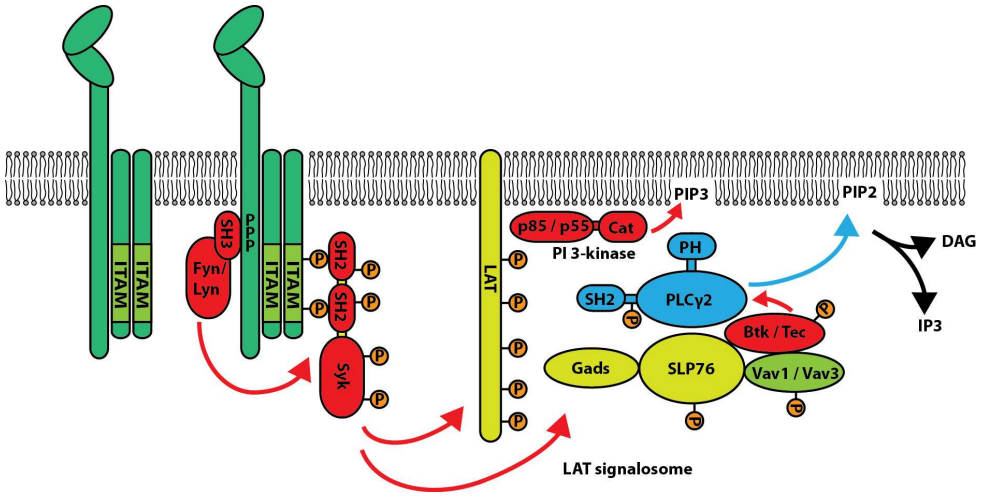
Platelet responsiveness was determined with agonist concentration series for $P2Y_{12}$ (ADP), GPVI (XL-CRP), and PAR-1 (TRAP). Serial dilutions of ADP (125 μM, 31.25 μM, 7.8 μM, 1.95 μM, 488 nM, 122 nM, 31 nM, 8 nM) were prepared in 50 μL, with 2 μL PE labeled mouse anti-human P-selectin (BD) antibodies and 0.5 μL Alexa-488 fibrinogen (Molecular Probes). Similarly serial dilutions of CRP-XL (2.5 μg/mL, 625 ng/mL, 156.3 ng/mL, 39.1 ng/mL, 9.8 ng/mL, 2.4 ng/mL, 600 pg/mL, 153 pg/mL), and TRAP (625 μM, 156.3 μM, 39.1 μM, 9.8 μM, 2.4 μM, 610 nM, 153 nM, 38 nM) were prepared in 50 μL HBS with 2 μL mouse anti-human P-selectin antibodies and 0.5 μL Alexa-488 fibrinogen. The platelet activation assay was initiated by adding 5 μL fresh, citrate anti-coagulated whole blood to each sample of serial dilutions. Sample incubation, fixation and measurement were performed as described in the 'Quantification of platelet membrane proteins' section of the manuscript (Methods section). Platelets were defined positive for P-selectin expression or positive for $\alpha_{IIb}\beta_3$ activation when the MFI exceeded 1% of baseline measurement. Non-parametric tests were used to test the difference in response for each concentration of agonist between the patients and healthy controls.

Clot retraction.

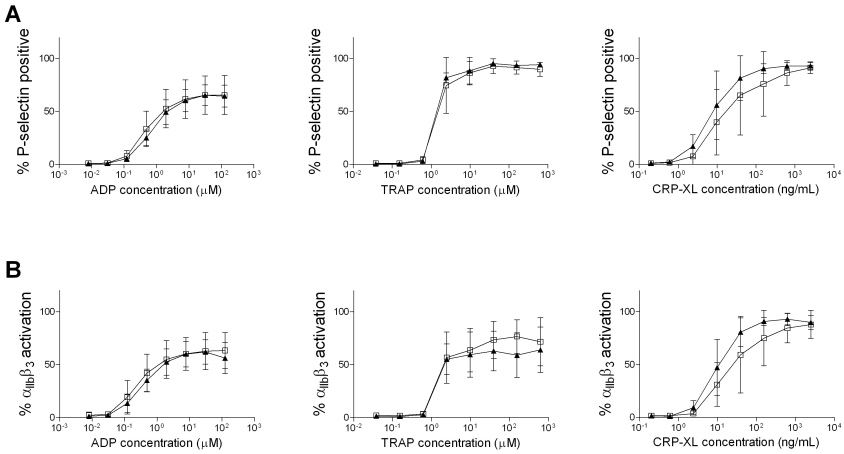
Citrated PRP was recalcified up to 15 mM $CaCl_2$. Clotting was initiated by adding thrombin up to 0.5 U/mL. Pictures were taken every 3 minutes for 2 hours. Clot surface was manually determined using MacBiophotonics Image J software.



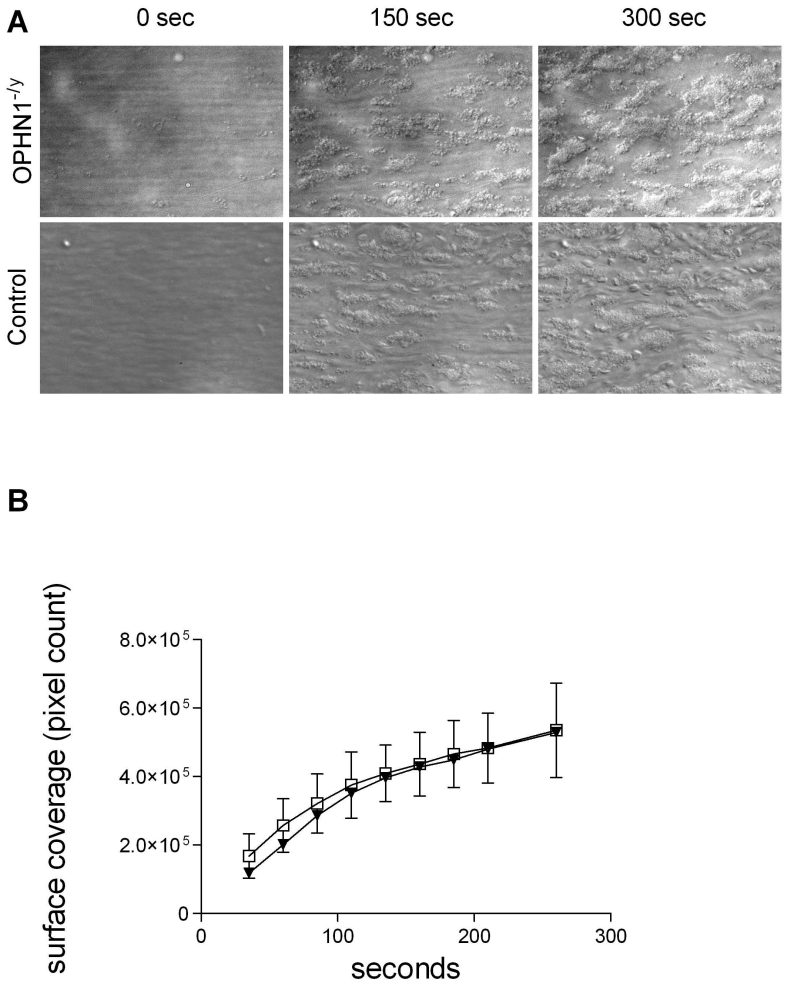
Supplementary Figure 1. Correlation between 5 minutes CRP-XL and 30 minutes CRP-XL experiment. Platelets were kept in a resting state (Ctrl) or stimulated with CRP-XL for 5 or 30 minutes. (A) Using MSQuant software, the ratio [Heavy/Light] = [CRP-XL/Ctrl] was calculated for immunoprecipitated phosphotyrosine (pY)-containing peptides (red dots) and normalized on the ratio of non-phosphorylated peptides (blue dots), based on the extracted ion chromatograms of the differentially labeled isotopomers of each peptide. Peptide ratios (2Log values) were plotted against peptide abundance (intensity, 10Log values). (B) Histogram representation of panel A. Peptides were binned into [CRP-XL/Ctrl] ratio categories and the number of peptides was plotted against the [CRP-XL/Ctrl] ratio. Whereas all non-phosphorylated peptides (blue histogram) exhibit a heavy/light ratio between -1 and +1 (2Log), the pY peptide population (red histogram) clearly contains up-regulated peptides in GPVI-activated platelets. For both time points, pY peptides were considered significantly up-regulated when [CRP-XL/resting] ≥ 2-fold (2Log value of 1). (C) The [CRP-XL/Ctrl] ratio of the 30 minutes CRP-XL experiment was plotted against the [CRP-XL/Ctrl] ratio of the 5 minutes CRP-XL experiment (2Log scale in both cases) for all pY peptides that were quantified in both experiments. Green dots represent peptides with significantly up- or down-regulated pY sites (i.e. 2Log([CRP-XL/Ctrl]) ≤ -1 or 2Log([CRP-XL/Ctrl] ≥ 1, threshold is indicated with dashed circle).



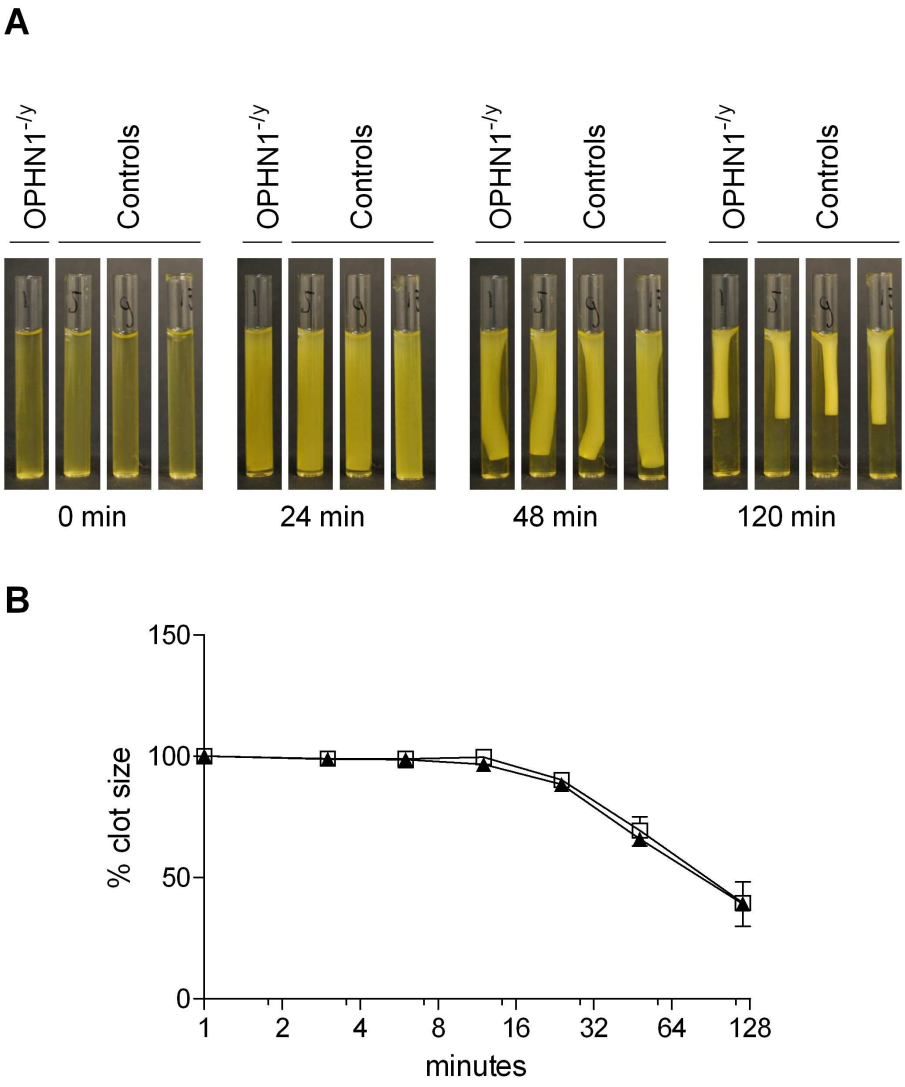
Supplementary Figure 2. GPVI response proteome. Proteins involved in GPVI signaling (adapted from Watson *et al.*[2]). Crosslinking of GPVI by collagen or CRP-XL induces tyrosine phosphorylation of the FcRγ-chain ITAM by the Src family kinases, Fyn and Lyn, which are constitutively bound to the proline-rich region in the GPVI cytosolic tail. This initiates a Syk-dependent signaling cascade that leads to the formation of a LAT signalosome and activation of PLCγ2. PLCγ2 associates directly with LAT, and indirectly via the adapters Gads and SLP-76. PLCγ2 also associates with the membrane via binding of its PH domain to PIP3. Functional homologues from the Tec and Vav families support activation of PLCγ2.



Supplementary Figure 3. Oligophrenin-1 deficient platelets respond normal to activation. Oligophrenin-1 deficiency of four OPHN1$^{-/y}$ patients (black traingles) is not associated with significantly increased (A) P-selectin expression and (B) $\alpha_{IIb}\beta 3$ activation compared to nine controls (white blocks). Platelets were stimulated with increasing concentrations of ADP, TRAP, and CRP-XL. Expression of P-selectin and $\alpha_{IIb}\beta_3$ activation was determined by FACS analysis with mouse-anti P-selectin-PE, and fibrinogen-FITC respectively. The mean of the percentage of P-selectin expression, and $\alpha_{IIb}\beta_3$ activation with ± SD is shown. Differences between patients and controls for each concentration of agonists were non-significant (Wilcoxon's rank $P > 0.05$).



Supplementary Figure 4. Oligophrenin-1 deficient platelets form normal aggregates. (A) Whole blood of an OPHN1$^{-/y}$ patient and three healthy controls was perfused over collagen coated coverslips. Pictures were taken every 10 seconds. Representative pictures are shown. (B) Surface coverage of platelets from an OPHN1$^{-/y}$ patient (black triangles) and three healthy controls (white squares) on collagen coated cover glasses was determined at the indicated time points by pixel count using Image J. Perfusions were performed in duplicate of which the mean was calculated. Data are expressed as mean ± 95% confidence interval of the mean of duplicate experiments.



Supplementary Figure 5. Oligophrenin-1 deficiency is not associated with altered clot retraction compared to healthy controls. (A) PRP of a single OPHN1$^{-/y}$ patient was stimulated with a PAR-1 peptide. Pictures were taken at indicated time points. (B) Clot size of an OPHN1$^{-/y}$ patient (black triangles) and three healthy controls (white squares) was determined by pixel count using Image J. Experiments were performed in quadruplicate and the mean was taken. Data are represented as mean ± 95% confidence interval of the mean of quadruplicate measurements of three healthy controls.

Supplementary Table 1. Phosphotyrosine peptides identified and quantified after CRP-XL treatment. List of all phosphotyrosine peptides identified (manually scrutinized) and quantified after CRP-XL treatment. pY, phosphotyrosine; pT, phosphothreonine; pS, phosphoserine. If MS/MS fragments were not sufficient for phosphosite localization, the alternative is indicated by e.g. Yxxx/Txxx. If multiple phosphorylized residues are present on the identified peptide this is indicated by e.g. Yxxx+Txxx. Phosphorylized sites within the same protein are given in a new row. Light grey rows indicate increased pY upon stimulation with CRP-XL.

Swissprot Accession Number	Gene	Protein name	Phosphotyrosine peptide	Phosphosite	Ratio (CRP/Ctrl) 5 min	Ratio (CRP/Ctrl) 30 min
P21333	FLNA	Filamin-A	VANPSGNLTETpYVQDR	Y1308	1,2	0,9
			VHSPSGALEECpYVTEIDQDK	Y2379	1,1	1,2
			SPFEVpYVDK	Y373		0,9
Q9Y490	TLN1	Talin-1	ALDpYYMLR	Y70/Y71	1,1	0,9
			TMQFEPSTMVpYDACR	Y26	1,0	0,8
			AGALQCSPSDApYTK	Y1945		1,3
			EAApYHPEVAPDVR	Y2224		1,0
			IGITNHDEpYSLVR	Y127		0,9
P35579	MYH9	Myosin-9	ALELDSNLpYR	Y754	1,1	0,9
			VIQpYLAYVASSHK	Y190	1,3	1,0
			VIQYLApYVASSHK	Y193	1,2	
			YLpYVDK	Y11	2,6	2,2
P60709	ACTB	Actin, cytoplasmic 1	DLTDpYLMK	Y188	1,1	1,0
			DSpYVGDEAQSK	Y53	1,3	1,4
			GpYSFTTTAER	Y198	1,4	1,1
			QEpYDESGPSIVHR	Y362	1,5	1,2
			IWHHTFpYNELR	Y91		0,9
P07996	THBS1	Thrombospondin-1	AQGpYSGLSVK	Y1058	1,1	0,7
			DNCPNLPNSGQEDpYDK	Y729	1,1	0,7
			IMADSGPIpYDK	Y1139	1,0	0,7
			CTSpYPDGSWK	Y565		0,6
			DNCQpYVYNVDQR	Y815		0,9
			DNCQYVpYNVDQR	Y817	1,0	0,8
			RPPLCpYHNGVQYR	Y319		0,7
			VVMpYEGKK	Y1126		0,5
P18206	VCL	Vinculin	SFLDSGpYR	Y822	1,1	1,5
P02671	FGA	Fibrinogen alpha chain	GGSTSpYGTGSETESPR	Y277	0,1	0,0
			QFTSSTSpYNR	Y589		0,6
P05106	ITGB3	Integrin beta-3	EATSTFTNITpYR	Y785	1,1	0,9
			FQpYYEDSSGK	Y695	1,1	1,0
			WDTANNPLpYK	Y773	1,1	1,0
P49840	GSK3A	Glycogen synthase kinase-3 alpha	GEPNVSpYICSR	Y279	1,2	0,9
P23528	CFL1	Cofilin-1	HELQANCpYEEVK	Y140	1,0	0,8
			YALYDATpYETK	Y89	1,1	0,7
P07737	PFN1	Profilin-1	CpYEMASHLR	Y129	1,1	0,8
P14618	PKM2	Pyruvate kinase isozymes M1/M2	ITLDNApYMEK	Y148	2,0	0,8
P06241	FYN	Tyrosine-protein kinase Fyn	GApYSLSIR	Y185	1,1	0,9
			LDNGGpYYITTR	Y213	1,1	0,9

Swissprot Accession Number	Gene	Protein name	Phosphotyrosine peptide	Phosphosite	Ratio (CRP/Ctrl) 5 min	Ratio (CRP/Ctrl) 30 min
			LIEDNEpYTAR	Y420	0,9	1,1
			WTAPEAALpYGR	Y440	1,0	1,0
P07948	LYN	Tyrosine-protein kinase Lyn	SLDNGGYpYISPR	Y194	1,0	0,8
			VENCPDELpYDIMK	Y473	0,9	0,8
			VIEDNEpYTAR	Y397	0,7	0,9
Q16539	MAPK14	Mitogen-activated protein kinase 14 (p38 alpha)	HTDDEMTGpYVATR	Y182	2,6	3,8
			HTDDEMpTGpYVATR	T180+Y182	2,1	4,0
P50552	VASP	Vasodilator-stimulated phosphoprotein	ATVMLpYDDGNK	Y16	1,2	0,8
			VQIpYHNPTANSFR	Y39	1,1	0,8
Q86UX7	FERMT3	Fermitin family homolog 3	EKEPEEELpYDLSK	Y162	1,1	1,0
			TASGDpYIDSSWELR	Y11	1,0	1,1
			ETTLSpYYK	Y387/Y388	2,0	
P11142	HSPA8	Heat shock cognate 71 kDa protein	TTPSpYVAFTDTER	Y41	0,8	0,6
			VQVEpYK	Y107	1,4	
P08238	HSP90AB1	Heat shock protein HSP 90-beta	SIpYYITGESK	Y484	1,2	0,8
P62158	CALM1	Calmodulin	DGNGpYISAAELR	Y100	2,7	2,0
Q04837	SSBP1	Single-stranded DNA-binding protein, mitochondrial	SGDSEVpYQLGDVSQK	Y73	1,2	1,0
Q01518	CAP1	Adenylyl cyclase-associated protein 1	QVAYIpYK	Y354	1,1	0,9
			EMNDAAMFpYTNR	Y164		0,8
Q06124	PTPN11	Tyrosine-protein phosphatase non-receptor type 11 (SHP-2)	IQNTGDpYYDLYGGEK	Y62	1,2	0,9
			IQNTGDYYDLpYGGEK	Y66		1,5
P30273	FCER1G	High affinity immunoglobulin epsilon receptor subunit gamma (FcR gamma)	AAITSpYEK	Y58	23,5	15,0
			SDGVpYTGLpSTR	Y65+S69	110,5	67,2
P02775	PPBP	Platelet basic protein	GKEESLDSDLpYAELR	Y58	0,5	
P21291	CSRP1	Cysteine and glycine-rich protein 1	CSQAVpYAAEK	Y127	1,1	0,9
Q05655	PRKCD	Protein kinase C delta type	GRGEpYFAIK	Y374	1,1	0,9
			SDSASSEPVGIpYQGFEK	Y313	88,7	34,5
			SDpSASSEPVGIpYQGFEK	S304/S306/S307 + Y313	>100	>200
			STFDAHIYEGR	Y64	1,2	0,8
Q9HBI1	PARVB	Beta-parvin	QLEEDLpYDGQVLQK	Y116	1,2	0,8
P12931	SRC	Proto-oncogene tyrosine-protein kinase Src	GApYCLSVSDFDNAK	Y187	1,2	0,9
			LDSGGFpYITSR	Y216	1,1	0,9
			LIEDNEpYTAR	Y419		1,1
			WTAPEAALpYGR	Y439		1,0
Q86Z02	HIPK1	Homeodomain-interacting protein kinase 1	AVCSTpYLQSR	Y352	1,2	0,8

Swissprot Accession Number	Gene	Protein name	Phosphotyrosine peptide	Phosphosite	Ratio (CRP/Ctrl) 5 min	Ratio (CRP/Ctrl) 30 min
			AVCSTpYLQpSR	Y352+S355	1,1	0,9
Q13201	MMRN1	Multimerin-1	MTDQVNpYQAMK	Y330	1,1	0,9
P30041	PRDX6	Peroxiredoxin-6	DINApYNCEEPTEK	Y89	1,0	0,8
O75563	SKAP2	Src kinase-associated phosphoprotein 2	RIpYQFTAASPK	Y197	1,1	0,8
			TVFYpYYGSDK	Y151	1,2	0,9
			pYGWWVGEMK	Y331	1,2	1,0
P42680	TEC	Tyrosine-protein kinase Tec	YVLDDQpYTSSSGAK	Y519	2,9	3,6
P08567	PLEK	Pleckstrin	EGpYLVK	Y10	1,6	0,9
Q13627	DYRK1A	Dual specificity tyrosine-phosphorylation-regulated kinase 1A	IYQpYIQSR	Y321	1,2	0,9
P07195	LDHB	L-lactate dehydrogenase B chain	MVVESApYEVIK	Y240	1,1	0,9
P51659	HSD17B4	Peroxisomal multifunctional enzyme type 2	AVANpYDSVEEGEK	Y73	1,3	1,0
O00116	AGPS	Alkyldihydroxyacetonephosphate synthase, peroxisomal	QVpYDIAAK	Y485	1,2	0,9
			IRPVPEpYQK	Y387	1,0	
			ETNISpYSQEADDR	Y175		0,8
			WNGWGpYNDSK	Y98		1,0
P06239	LCK	Tyrosine-protein kinase Lck	NLDNGGFpYISPR	Y192	1,2	1,1
P50851	LRBA	Lipopolysaccharide-responsive and beige-like anchor protein	SIVEEEEDDDpYVELK	Y1110	0,8	0,6
O15117	FYB	FYN-binding protein	TTAVEIDpYDSLK	Y571	1,6	1,9
			VLpYSTK	Y709	2,8	
P00338	LDHA	L-lactate dehydrogenase A chain	QVVESApYEVIK	Y239	1,1	0,9
Q9H422	HIPK3	Homeodomain-interacting protein kinase 3	TVCSTpYLQSR	Y359	1,1	0,8
P08559	PDHA1	Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial	AAASTDpYYK	Y242	0,9	0,2
			AAASTDYpYK	Y243	0,8	0,1
			pYHGHSMSDPGVSYR	Y289		1,4
			YHGHSMSDPGVSpYR	Y301	3,7	3,4
			YHGHpSMSDPGVSpYR	S293+Y301	1,7	1,3
P16284	PECAM1	Platelet endothelial cell adhesion molecule	DTETVpYSEVR	Y713	3,2	2,9
Q99952	PTPN18	Tyrosine-protein phosphatase non-receptor type 18	SAEEAPLpYSK	Y389	6,4	3,4
Q9UBW5	BIN2	Bridging integrator 2	LNHNLpYEVMSK	Y232	1,3	0,9
			DVFpYR	Y221	1,1	
Q9NRY4	GRLF1	Glucocorticoid receptor DNA-binding factor 1	NEEENIpYSVPHDSTQGK	Y1105	1,4	1,4
Q05397	FAK1	Focal adhesion kinase 1	pYMEDSTYYK	Y570	8,6	6,6
P26599	PTBP1	Polypyrimidine tract-binding protein 1	GQPIpYIQFSNHK	Y127	1,2	1,0
Q9Y316	MEMO1	Protein MEMO1	YSYYDESQGEIpYR	Y210	1,1	0,9
P53990	KIAA0174	IST1 homolog	EIADpYLAAGK	Y43	1,0	0,8
P45983	MAPK8	Mitogen-activated protein kinase 8 (c-Jun terminal kinase JNK1)	TAGTSFMMTPpYVVTR	Y185	1,2	1,1

Swissprot Accession Number	Gene	Protein name	Phosphotyrosine peptide	Phosphosite	Ratio (CRP/Ctrl) 5 min	Ratio (CRP/Ctrl) 30 min
P16333	NCK1	Cytoplasmic protein NCK1	ETVpYCIGQR	Y339	1,0	1,0
			LpYDLNMPAYVK	Y105	4,9	5,8
O43639	NCK2	Cytoplasmic protein NCK2	VQLVDNVpYCIGQR	Y342	1,0	1,0
P00519/ P42684	ABL1/ ABL2	Tyrosine-protein kinase ABL1 / ABL2	LMTGDTpYTAHAGAK	Y393(ABL1)/ Y439 (ABL2)	1,6	2,4
Q00610	CLTC	Clathrin heavy chain 1	ENPpYYDSR	Y899	1,2	1,2
P29350	PTPN6	Tyrosine-protein phosphatase non-receptor type 6 (SHP-1)	EDVpYENLHTK	Y564	1,2	1,4
			IQNSGDFYDLpYGGEK	Y64	1,4	1,9
			QPYpYATR	Y214		0,8
P37840	SNCA	Alpha-synuclein	EGVLpYVGSK	Y39	1,2	0,8
O60496	DOK2	Docking protein 2	GQEGEpYAVPFDAVAR	Y299	2,8	4,0
P45984	MAPK9	Mitogen-activated protein kinase 9 (c-Jun terminal kinase JNK2)	TACTNFMMTPpYVVTR	Y185	1,0	1,1
P11413	G6PD	Glucose-6-phosphate 1-dehydrogenase	VGFQYEGTpYK	Y507	2,0	0,9
			VGFQpYEGTYK	Y503	1,3	
			VQPNEAVpYTK	Y401	1,1	0,9
			IDHpYLGK	Y202	1,2	0,7
P11279	LAMP1	Lysosome-associated membrane glycoprotein 1	ALQATVGNSpYK	Y336	1,2	0,9
O00459	PIK3R2	Phosphatidylinositol 3-kinase regulatory subunit beta	EYDQLpYEEYTR	Y464	1,2	0,8
Q15118	PDK1	[Pyruvate dehydrogenase [lipoamide]] kinase isozyme 1, mitochondrial	GVPGQVDFpYAR	Y49	1,2	1,0
P53778	MAPK12	Mitogen-activated protein kinase 12 (p38 gamma)	QADSEMTGpYVVTR	Y185	2,5	13,5
			QADSEMpTGpYVVTR	T183+Y185		16,6
O75791	GRAP2	GRB2-related adapter protein 2 (GADS)	AELGSQEGpYVPK	Y45	6,2	14,6
P29597	TYK2	Non-receptor tyrosine-protein kinase TYK2	LLAQAEGEPCpYIR	Y292	0,8	0,6
P05556	ITGB1	Integrin beta-1	WDTGENPIpYK	Y783	1,0	1,2
P53396	ACLY	ATP-citrate synthase	TTDGVpYEGVAIGGDR	Y682	1,1	0,8
P42229	STAT5A	Signal transducer and activator of transcription 5A	AVDGpYVKPQIK	Y694	1,5	1,2
			LGHpYATQLQK	Y90		0,7
P00558	PGK1	Phosphoglycerate kinase 1	ELNpYFAK	Y196	1,2	
Q7L7X3	TAOK1	Serine/threonine-protein kinase TAO1	ELDNLQpYR	Y309	1,1	0,7
Q99798	ACO2	Aconitate hydratase, mitochondrial	FNPETDpYLTGTDGK	Y513	1,1	0,9
O60890	OPHN1	Oligophrenin-1	EPIpYHSPITK	Y370	11,2	3,3
Q99613	EIF3C	Eukaryotic translation initiation factor 3 subunit C	QGTpYGGYFR	Y881	1,0	0,9
P61006	RAB8A	Ras-related protein Rab-8A	TpYDYLFK or TYDpYLFK	Y5/Y7	1,1	1,0
Q9NP81	SARS2	Seryl-tRNA synthetase, mitochondrial	EGpYSALPQLDIER	Y52	1,1	0,8

Swissprot Accession Number	Gene	Protein name	Phosphotyrosine peptide	Phosphosite	Ratio (CRP/Ctrl) 5 min	Ratio (CRP/Ctrl) 30 min
P11216	PYGB	Glycogen phosphorylase, brain form	DFpYELEPEK	Y473	1,2	0,9
P25787	PSMA2	Proteasome subunit alpha type-2	SILpYDER	Y57	1,1	0,8
			HIGLVpYSGMGPDYR	Y76		0,6
Q14197	ICT1	Immature colon carcinoma transcript 1 protein	LpYPESQGSDTAWR	Y49	1,1	0,9
O75063	FAM20B	Protein FAM20B	DHVVEGEPpYAGYDR	Y138	1,0	0,8
Q14247	CTTN	Src substrate cortactin	LPpSSPVpYEDAASFK	S417/S418 + Y421	0,8	0,7
			SAVGFDpYQGK	Y178	1,0	0,7
Q9UIB8	CD84	SLAM family member 5	TIpYTYIMASR	Y279	2,5	3,0
P22694	PRKACB	cAMP-dependent protein kinase catalytic subunit beta	ATEQpYYAMK	Y69	1,2	0,9
Q9Y624	F11R	Junctional adhesion molecule A	VIpYSQPSAR	Y280	1,0	1,1
P16885	PLCG2	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma-2	EFSVNENQLQLpYQEK	Y1245	6,8	2,0
Q7KZF4	SND1	Staphylococcal nuclease domain-containing protein 1	EpYGMIYLGK	Y109	0,9	0,7
P37802	TAGLN2	Transgelin-2	GPApYGLSR	Y8		0,8
P04075	ALDOA	Fructose-bisphosphate aldolase A	CQpYVTEK	Y204		0,8
P06733	ENO1	Alpha-enolase	AAVPSGASTGIpYEALELR	Y44		0,9
P50395	GDI2	Rab GDP dissociation inhibitor beta	TDDYLDQPCpYETINR	Y203	1,0	0,7
O43665	RGS10	Regulator of G-protein signaling 10	EIpYMTFLSSK	Y86	1,0	0,9
Q9NUM4	TMEM106B	Transmembrane protein 106B	EDApYDGVTSENMR	Y58		1,2
Q06187	BTK	Tyrosine-protein kinase BTK	HYVVCSTPQSQpYYLAEK	Y344/Y345		1,1
			YVLDDEpYTSSVGSK	Y551		2,1
Q7LDG7	RASGRP2	RAS guanyl-releasing protein 2	ALILGIpYK	Y523	0,8	0,7
Q9BSJ8	ESYT1	Extended synaptotagmin-1	HLSPpYATLTVGDSSHK	Y822	1,2	0,9
O15144	ARPC2	Actin-related protein 2/3 complex subunit 2	DDETMpYVESK	Y153		1,4
Q6VY07	PACS1	Phosphofurin acidic cluster sorting protein 1	IpYSLSSQPIDHEGIK	Y251		0,8
O60268	KIAA0513	Uncharacterized protein KIAA0513	AVTApYSPEDEK	Y278		0,8
Q5VY43	PEAR1	Platelet endothelial aggregation receptor 1	DSGTpYEQPSPLIHDR	Y979		0,8
P43405	SYK	Tyrosine-protein kinase SYK	ELNGTpYAIAGGR	Y74		1,0
			LRNpYYYDVVN	Y629/Y630	173,8	2,8
Q16644	MAPKAPK3	MAP kinase-activated protein kinase 3	LLpYDSPK	Y76		1,0
P63167	DYNLL1	Dynein light chain 1, cytoplasmic	NFGSpYVTHETK	Y65		0,9
P15498	VAV1	Proto-oncogene vav	ARpYDFCAR	Y791		64,2
			GEIpYGR	Y826	1,0	
Q9NXR7	BRE	BRCA1-A complex subunit BRE	VQpYVIQGYHK	Y263		0,9

Swissprot Accession Number	Gene	Protein name	Phosphotyrosine peptide	Phosphosite	Ratio (CRP/Ctrl) 5 min	Ratio (CRP/Ctrl) 30 min
Q9Y4D1	DAAM1	Disheveled-associated activator of morphogenesis 1	AVETELEpYQK	Y912		0,8
Q92569	PIK3R3	Phosphatidylinositol 3-kinase regulatory subunit gamma	EYDRLpYEEYTR	Y199		0,9
P14625	HSP90B1	Endoplasmin	GLFDEpYGSK	Y401		1,0
P06493	CDK1	Cell division control protein 2 homolog	IGEGTYGVVpYK	Y19	1,2	0,9
Q92835	SHIP1	Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1	LpYDFVK	Y865	7,9	8,7
Q8N392	ARHGAP18	Rho GTPase-activating protein 18	CLDDDTpYMK	Y643	1,0	0,8
Q5JSL3	DOCK11	Dedicator of cytokinesis protein 11	TQIpYSDPLR	Y57	1,0	
O60674	JAK2	Tyrosine-protein kinase JAK2	IQDpYHILTR	Y221	1,1	0,6
			EVGDpYGQLHETEVLLK	Y570		0,7
P48147	PREP	Prolyl endopeptidase	MTELpYDYPK	Y71	1,1	0,9
Q9NZE8	MRPL35	39S ribosomal protein L35, mitochondrial	NWYVDDPpYQK	Y177	1,0	0,9
Q9C0H2	TTYH3	Protein tweety homolog 3	QAHDSLpYR	Y439	1,1	0,7
O43426	SYNJ1	Synaptojanin-1	VTFAPTpYK	Y784	1,0	0,9
Q8IZP0	ABI1	Abl interactor 1	VVAIpYDYTK	Y455	1,1	1,2
Q92499	DDX1	ATP-dependent RNA helicase DDX1	GEpYAVR	Y496	1,1	
Q13131	PRKAA1	5'-AMP-activated protein kinase catalytic subunit alpha-1	VVNPpYYLR	Y441/Y442	1,2	
Q8IZX4	TAF1L	Transcription initiation factor TFIID 210 kDa subunit	YNGPESQpYTK	Y1608	1,1	
Q02218	OGDH	2-oxoglutarate dehydrogenase E1 component, mitochondrial	YHLGMpYHR	Y354	1,2	
Q9BQP7	C20orf72	Uncharacterized protein C20orf72	NQNIQKPEpYSE	Y342	1,2	
P00367	GLUD1	Glutamate dehydrogenase 1, mitochondrial	NLNHVSpYGR	Y451	1,2	
P40763	STAT3	Signal transducer and activator of transcription 3	YCRPESQEHPEADPGSAAPpYLK	Y705	1,4	1,2
P08758	ANXA5	Annexin A5	LYDApYELK	Y94	1,2	
P36959	GMPR	GMP reductase 1	STCTpYVGAAK	Y318	1,3	0,8
P18433	PTPRA	Receptor-type tyrosine-protein phosphatase alpha	VVQEYIDAFSDpYANFK	Y798		0,9
P05771	PRKCB	Protein kinase C beta type	GTDELpYAVK	Y368		0,9
			NLVPMDPNGLSDPpYVK	Y195		0,8
Q5HYK3	COQ5	Ubiquinone biosynthesis methyltransferase COQ5, mitochondrial	SYQpYLVESIR	Y283		1,0
Q01082	SPTBN1	Spectrin beta chain, brain 1	IVSSSDVGHDEpYSTQSLVK	Y777		0,9
P05023	ATP1A1	Sodium/potassium-transporting ATPase subunit alpha-1	GIVVpYTGDR	Y260		0,8
Q9UIQ6	LNPEP	Leucyl-cystinyl aminopeptidase	GLGEHEMEEDEEDpYESSAK	Y70		0,6
Q15126	PMVK	Phosphomevalonate kinase	LLDTSTpYK	Y68		1,0
P17987	TCP1	T-complex protein 1 subunit alpha	HGSpYEDAVHSGALND	Y545		1,2
O14964	HGS	Hepatocyte growth factor-regulated tyrosine kinase substrate	VVQDTpYQIMK	Y132		0,8
Q07912	TNK2	Activated CDC42 kinase 1	KPTpYDPVSEDQDPLSSDFK	Y518		1,0
Q12959	DLG1	Disks large homolog 1	NTSDFVpYLK	Y399		0,6

Swissprot Accession Number	Gene	Protein name	Phosphotyrosine peptide	Phosphosite	Ratio (CRP/ Ctrl) 5 min	Ratio (CRP/ Ctrl) 30 min
P05141	ANT2	ADP/ATP translocase 2	AApYFGIYDTAK	Y191		1,0
Q9BRJ2	MRPL45	39S ribosomal protein L45, mitochondrial	FTPPIpYQPK	Y46		0,9
Q96CV9	OPTN	Optineurin	QELVpYTNK	Y356		1,4
Q14162	SCARF1	Endothelial cells scavenger receptor	QAEEERQEEPEpYENVVPISRPPEP	Y818		2,9
Q9UDY2	ZO2	Tight junction protein ZO-2	AYDPDpYER	Y261		1,4
Q99426	TBCB	Tubulin folding cofactor B	LGEpYEDVSR	Y98		0,8
P62805	HIST1H4A	Histone H4	ISGLIpYEETR	Y52		0,9
Q9BU61	C3orf60	Uncharacterized protein C3orf60	LSPADDELpYQR	Y42		1,0

Supplementary references

1. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nat Methods*. 2009;6:359-362
2. Watson SP, Auger JM, McCarty OJ, Pearce AC. Gpvi and integrin alphaiib beta3 signaling in platelets. *Journal of thrombosis and haemostasis : JTH*. 2005;3:1752-1762

Chapter 7

Mass spectrometry analysis shows similar release and phosphorylation profiles after PAR-1 or PAR-4 stimulation of platelets

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Abstract

Platelets play a role in the regulation of inflammation, angiogenesis and atherogenesis. Differential release of pro- and anti-angiogenic factors from platelet α-granules by protease activated receptors (PAR)-1 and -4 could be important for the regulation of angiogenesis. We analyzed the releasates of unstimulated and PAR-1 and PAR-4 stimulated platelets by quantitative mass spectrometry. Furthermore, we compared the phosphoproteome of PAR-1 and PAR-4 stimulated platelets. A strong correlation between all the 93 proteins identified in the releasates after both stimuli was observed (Spearman's r 0.93, r^2 0.86, $p<0.0001$), and this was supported with ELISA measurements of different α-granule components in these releasates. Down stream signal transduction measurements after stimulation with PAR-1 and PAR-4 agonist revealed similar phosphorylation patterns on Western blot. Mass spectrometry analysis of phosphorylation events also showed nearly identical regulations. We conclude that there is no differential release for any of the 93 proteins that came out of the proteomics analysis. Furthermore, there is little support for differential signal transduction downstream of PAR-1 and PAR-4

Introduction

Platelets are important to prevent lethal blood loss upon injury. In addition, they play an important role in regulating angiogenesis and inflammation [1,2]. Interestingly, platelet α-granules contain both pro-angiogenic and anti-angiogenic factors, which are released upon platelet activation. This puzzling observation has been addressed in different studies, which argued that there are distinct subsets of α-granules that either contain pro-angiogenic or anti-angiogenic content, and which are preferentially released after either stimulation of protease activating receptor (PAR)-1 or PAR-4 [3-5]. This suggests functional organization of pro-angiogenic and anti-angiogenic proteins. In contrast to this hypothesis, pairwise comparison of angiogenic relevant proteins that reside in α-granules suggested that there are no α-granule subsets with distinct functional cargo [6,7].

The topic of differential release by PAR-1 and PAR-4 stimulation is currently limited to a few target proteins, despite the fact that α-granules are abundantly filled with different proteins [3-5,8]. Therefore a more unbiased, global analysis of the platelet releasate upon these two thrombin receptors could shed light on this conundrum. If differential release is in fact occurring, then there should also be differential signaling cues to establish this. However, no attempt has been made to monitor whether different signaling routes are employed downstream of PAR-1 and PAR-4, which might further support differential release of α-granular content. To address these issues, we compared the released proteome by quantitative mass spectrometry as to identify thematic groups of factors after either PAR-1 or PAR-4 stimulation. In addition, we have investigated protein phosphorylation that drives PAR-1 and PAR-4 stimulated α-granule release.

Materials and methods

Materials

Trisodium citrate (3.2%) for open system blood collection was obtained from Merck, Vacuum 3.2% tri-sodium citrate tubes were obtained from Greiner Bio-one (455322), acetylsalicylic acid was obtained from Sigma, AR-C69931MX was a kind gift from Astra Zeneca, PAR-1 activating peptide SFLLRN was obtained from Bachem (H-2936), PAR-4 activating peptide AYPGKV was obtained from the NKI-AVL in Amsterdam, phycoerythrin (PE) labelled mouse-anti human P-selectin antibodies were obtained from Becton Dickinson (BD; 555524), flow cytometry measurements were performed

with a FACSCanto II flow cytometer from BD, SDS-PAGE was performed with a NuPAGE 4%-12% gradient Bis-Tris-HCl pH 6.4 gel obtained from Invitrogen, MOPS running buffer was obtained from Invitrogen, polyvinylidene difluoride (PVDF) membrane was obtained from Millipore, rabbit anti-human phosphorylated RRxpS/pT motif polyclonal antibodies were obtained from Cell Signaling Technology, biotin coupled sheep anti-human GPVI polyclonal antibody was obtained from R&D, donkey anti-goat IRDye 800CW and streptavidin IRDye 680 were obtained from LI-COR, Western blot imaging was performed on an Odyssey imager obtained from LI-COR, Complete Mini protease inhibitors was obtained from Roche Diagnostics, PhosSTOP phosphatase inhibitor cocktail was obtained from Roche Diagnostics, a 2D-Quant Kit was obtained from GE Healthcare, Lys-C was obtained from Wako, Sep-Pak C18 cartridges were obtained from Waters Corporation, LTQ-Orbitrap Velos instrument was obtained from Thermo Scientific, Agilent 1200 series LC system equipped with a 20 mm ReproSil-Pur C18-AQ was obtained from Dr. Maisch GmbH, trapping column was packed in-house, i.d., 100 μm; resin, 5 μm, 400 mm ReproSil-Pur C18-AQ was obtained from Dr. Maisch GmbH, Proteome Discoverer software package version 1.3.339 was obtained from Thermo Scientific, the Mascot search engine version 2.3 was obtained from Matrix Science. Coating mouse anti-human beta-thromboglobulin (β-TG; MAB393), mouse anti-human platelet factor (PF)4 (MAB7951), mouse anti-human thrombospondin-1 (THBS1; DY3074), mouse anti-human platelet derived growth factor (PDGF) BB (DY222), mouse anti-human regulated and normal T cell expressed and secreted (RANTES/CCL5; MAB278) antibodies were obtained from R&D. Coating rabbit anti-human von Willebrand Factor (vWF; A0082) antibodies were obtained from DAKO. Detection antibodies goat anti-human β-TG (BAF393), goat anti-human PF4 (AF795), goat anti-human THBS1 (DY3074), goat anti-human PDGF-AA (DY222), goat anti-human RANTES/CCL5 (AB278-NA) were obtained from R&D. Peroxidase conjugated rabbit anti-human von Willebrand Factor (P0226) detection antibody was obtained from DAKO. Streptavidin-horse radish peroxidase (HRP; P0397) was obtained from DAKO, rabbit anti-goat HRP (P0449) was obtained from DAKO. SuperSignal West Pico Chemiluminescent substrate was obtained from Thermo Scientific (34080), the Spectamax Luminometer was obtained from Molecular Devices.

Blood sample preparation

Venous blood was collected from healthy volunteers after obtaining informed consent. Blood for mass spectrometry (MS) analysis was collected with an open system, anti-coagulated with 3.2% tri-sodium citrate. Blood for other experiments was collected

using vacuum tubes with 3.2% tri-sodium citrate. Platelet rich plasma (PRP) was prepared by centrifugation of whole blood at 160 g for 15 minutes at room temperature, no brake. Washed platelets for mass spectrometry analysis were prepared by adding PRP with ACD (8.5 mM tri-sodium citrate, 7.1 mM citric acid, 5.5 mM D-glucose; final concentration) and centrifugation for 15 minutes with 340 g at room temperature, no brake. The platelet pellet was resuspended in Tris-buffer (145 mM NaCl, 5 mM KCl, 260 nM NaH_2PO_4, 1 mM $MgSO_4$, 100 mM Tris, 5.5 mM D-glucose, pH 6.5) with 10 ng/mL prostacyclin. Platelets were centrifuged for 15 minutes at 340 g at room temperature and resuspended up to 200 x 10^9/L in Tris-buffer (pH 7.3), with 100 μM acetylsalicylic acid, and 1 μM AR-C69931MX. Platelets were not used until 30 minutes after isolation.

Platelet responsiveness

Platelet responsiveness was determined with agonist concentration series for PAR-1 or PAR-4 agonist. Serial dilutions of PAR-1 agonist ranging from 625 μM to 38 nM were prepared in 50 μL Hepes buffered solution (HBS: 10 mM HEPES, 150 mM NaCl, 1 mM $MgSO_4$, 5 mM KCl, pH 7.4) with 2 μL PE labeled anti P-selectin antibodies antibodies. Similarly, serial dilutions of PAR-4 agonist ranging from 4 mM to 0.2 μM were prepared in 50 μL HBS with 2 μL PE labeled anti P-selectin antibodies. The platelet responsiveness assay was initiated by adding 5 μL fresh, citrate anti-coagulated whole blood to each sample of serial dilutions. After 20 minutes of incubation, the samples were fixed with 500 μL 0.2% formyl saline (0.2% formaldehyde, 0.9% NaCl) and kept at room temperature until flow cytrometry analysis. All samples were analyzed on the same day of processing. Single platelets were gated based on forward and side scatter properties. The median fluorescence intensity (MFI) in the platelet gate was measured with FACS analysis.

Western blot

Washed platelets were stimulated with 625 μM PAR-1 peptide and 4 mM PAR-4 peptide for 5 minutes. Platelet suspensions were centrifuged at 4000 g for 2 minutes, and the pellets were lysed in SDS sample buffer (1.1 M glycerol, 62 mM Tris-HCl, 70 mM SDS, 29 μM bromine-phenol blue, pH 6.8) and heated at 95°C for 5 minutes. Samples were subjected to SDS-PAGE using a NuPAGE 4%-12% gradient Bis-Tris-HCl pH 6.4 gel with MOPS running buffer and 50 mM dithiothreitol , electrotransferred to a polyvinylidene difluoride (PVDF) membrane, and immunoblotted with rabbit anti-human phosphorylated RRxpS/pT motif polyclonal antibodies, or biotin coupled sheep anti-human GPVI polyclonal antibodies for loading control. Blot was probed

with donkey anti-goat IRDye 800CW and streptavidin IRDye 680 and imaged using an Odyssey imager.

Platelet stimulation for proteomics

The platelet protein releasate after PAR-1 or PAR-4 stimulation was determined in three separate experiments, in platelets isolated from different healthy donors. The analysis of phosphorylation events downstream of PAR-1/PAR-4 activation was performed in the first experiment. Platelet suspensions were stimulated with 625 μM PAR-1 peptide and 4 mM PAR-4 peptide for 5 minutes. Unstimulated platelet suspensions were taken as control for the same time point. After stimulation, platelet suspensions were centrifuged at 4000 g for 2 minutes. For mass spectrometry analysis, the supernatant was aspirated and the pellet was snap-frozen in liquid nitrogen and stored at -80^{0}C until analysis. Platelet pellets were reconstituted in a buffer containing 100 mM Tris, 10 mM DTT, 2% SDS at pH 8.0 with Complete Mini protease inhibitors and PhosSTOP phosphatase inhibitor cocktail. Cell suspensions were sonicated and cleared as described in Supplementary Methods and protein concentrations were determined using a 2D-Quant Kit. Lysates were subsequently reduced, alkylated and digested using the filter-aided sample preparation (FASP) approach [9]. Digestion was performed for 4 hours with Lys-C, followed by overnight trypsin digestion. Peptides were desalted using Sep-Pak C18 cartridges and subsequently labeled on-column with stable isotope dimethyl labeling as described previously [10]. Resting platelets were labeled 'light', whereas PAR-1 and PAR-4 stimulated platelets received the 'intermediate' and 'heavy' label, respectively. Labeling efficiency was checked by LC-MS/MS before mixing the pools in a 1:1:1 ratio. The samples were dried *in vacuo* and reconstituted in 10% formic acid prior to strong cation exchange (SCX) chromatography.

Strong cation exchange chromatography and mass spectrometry

For assessment of the platelet releasate, 250 μg of pooled, labeled digests were subjected to SCX as described previously [11]. A total number of 50 SCX fractions were collected and dried in a vacuum centrifuge. Twenty fractions were reconstituted in 10% formic acid and analyzed by LC-MS/MS on an LTQ-Orbitrap Velos instrument, using a data-dependent decision-tree analysis with the orthogonal fragmentation methods HCD and ETD, as described previously [12,13] (see Supplementary Methods for further details). Nano-LC was achieved on an Agilent 1200 series LC system equipped with a 20 mm ReproSil-Pur C18-AQ trapping column (packed in-house, i.d., 100 μm; resin, 5 μm) and a 400 mm ReproSil-Pur C18-AQ analytical column (packed in-house, i.d., 50 μm; resin,

3 μm), with the flow passively split to 100 nl/min. Fractions were analyzed with a 3 hour elution profile as described [12]. For phosphoproteome profiling, 1 mg of pooled, labeled protein digests was subjected to SCX as described previously [14]. Six fractions enriched in phosphopeptides were then analyzed by LC-MS/MS in duplicate on an LTQ-Orbitrap Velos instrument connected to the nano-LC system described above, using a 3 hour elution profile and HCD fragmentation of the 10 most intense peaks as described previously [15].

Data analysis

Peak lists were generated from the raw data files using the Proteome Discoverer software package version 1.3.339. Peptide identification was performed by searching the individual peak lists (HCD, ETD-IT and ETD-FT) against a concatenated target-decoy database containing the human sequences in the Uniprot database (release 2012_06) supplemented with a common contaminants database using the Mascot search engine version 2.3 via the Proteome Discoverer interface. The search parameters included the use of trypsin as proteolytic enzyme allowing up to a maximum of 2 missed cleavages. Carbamidomethylation of cysteines was set as a fixed modification, whereas oxidation of methionines, phosphorylation and the dimethyl "light", "intermediate" and "heavy" labels on N-termini and lysine residues were set as variable modifications. For phosphoproteome analysis, phosphorylation on serine, threonine and tyrosine residues was set as additional variable modifications. Precursor mass tolerance was initially set at 50 ppm, while fragment mass tolerance was set at 0.6 Da for ETD-IT fragmentation and 0.05 Da for HCD and ETD-FT fragmentation. Subsequently, the peptide identifications were filtered for true mass accuracy <5 ppm and an ion score between 20 and 30 until an FDR <1% at peptide level was achieved. All quantified phosphopeptides were manually scrutinized and phosphosite localization probabilities were assessed with the phosphoRS 2.0 [16] module implemented in Proteome Discoverer. The localization of phosphosites was determined with "high confidence" when 75% <pRS site probability <100%, and with "medium confidence" when 50% <pRS site probability <75%.

Peptide and protein quantification were performed using Proteome Discoverer as described previously [17]. Protein ratios were normalized per replicate based on the average ratio of the top 6 cytoskeletal platelet proteins based on the number of PSMs: Talin-1, Filamin-A, Myosin-9, Vinculin, Alpha-actinin-1 and Actin (cytoplasmic 1). Phosphopeptide ratios were normalized on the above mentioned protein ratios.

Releasate determination

For each biological replicate, a 95% confidence interval was used to determine the 'outliers' [18]. The ratios were binned according to the number of quantification counts in such a fashion that each bin consists of at least 200 proteins and proteins with an identical quantification count can only be contributed to 1 bin. Proteins that were determined as outlier with a down-regulated (Activated / Resting) ratio in at least 2 of 3 biological replicates of the PAR-1 stimulated or PAR-4 stimulated platelets were attributed to the releasate (Supplementary Table 1) [18]. The percentage release was subsequently calculated by (1 – the average ratio of the 3 replicates) x100%. Only proteins with a positive % were considered.

ELISA

Washed platelets were stimulated with 625 μM PAR-1 peptide and 4 mM PAR-4 peptide for 5 minutes. Platelet suspensions were centrifuged at 4000 g for 2 minutes and the supernatant was collected. Maxisorb plates were coated with mouse anti-human β-TG, mouse anti-human PF4, mouse anti-human THBS1, mouse anti-human PDGF-BB, mouse anti-human RANTES/CCL5, or rabbit anti-human vWF. Plates were blocked with 1% bovine serum albumin, and incubated with supernatants. Plates were washed with phosphate buffered saline (PBS) pH 7.4 with 0.05% Tween 20. Bound factors were detected with biotin coupled goat anti-human β-TG, goat anti-human PF4, biotin coupled goat anti-human THBS1, biotin coupled goat anti-human PDGF-AA, goat anti-human RANTES/CCL5, or peroxidase conjugated rabbit anti-human vWF. Plates were washed with PBS pH 7.4 with 0.05% Tween 20. Biotin coupled antibodies were bound with streptavidin-HRP, or goat anti-human antibodies with rabbit anti-goat HRP (DAKO, P0449). Detection was performed with SuperSignal West Pico Chemiluminescent substrate, and read with a luminometer.

Results

Platelet response to PAR-1 and PAR-4

The agonist concentration for optimal PAR-1 or PAR-4 granule release was determined by P-selectin expression of platelets in response to increasing concentrations of both agonists (Figure 1). Platelet activation reached a maximum at 625 μM of PAR-1 agonist, whereas the maximum PAR-4 response was reached at 4 mM agonist, both showing

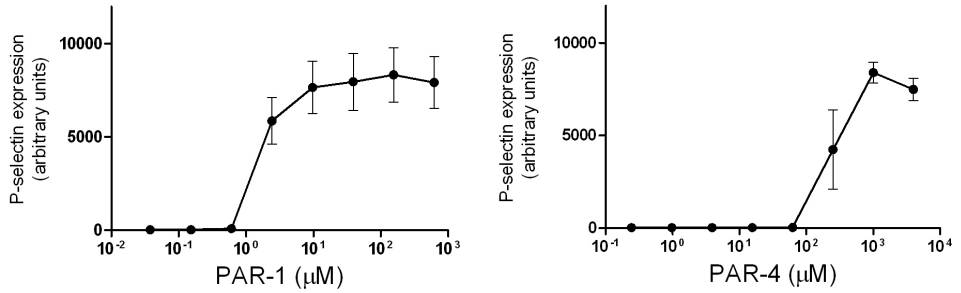

Figure 1. Dose reponse assays to determine the optimal concentrations of PAR-1 and PAR-4 agonist. Granule release by P-selectin measurement was performed in response to increasing concentrations of PAR-1 agonist and PAR-4 agonist with platelets isolated from three individuals.

comparable P-selectin expression. These concentrations were used to further study the releasates of platelets.

Analysis of platelet releasates with mass spectrometry and ELISA

We used quantitative mass spectrometry to identify subsets of factors released by either PAR-1 or PAR-4 stimulation. Employing the 'reversed protein profiling' strategy recently published by Wijten et al. [18], we determined significantly decreased levels of proteins after PAR-1 or PAR-4 stimulation compared with unstimulated platelets. In total 3142 unique proteins were quantified, of which 2296 (73%) could be quantified in at least 2 out of 3 individuals. The remainder of the platelet proteome, which reflects the platelet releasate was evaluated by determination of the intensity ratios between the light (unstimulated), intermediate (PAR-1 stimulated) and heavy (PAR-4 stimulated) stable isotopic labeled peptides. The vast majority of proteins (>95%) had a (Activated / Resting) ratio around 1.0. Using the statistical criteria described in the Materials and Methods section, 93 proteins were determined as being significantly released from platelets activated with PAR-1 and/or PAR-4 (Supplementary Table 1), with release percentages ranging from ca. 20-90%.

We identified most established α-granule proteins, such as vWF, RANTES/CCL5, PDGF, fibrinogen (FGB), and platelet basic protein (PPBP). The release of a few hallmark α-granule proteins from platelets activated with either agonist is displayed in Figure 2A. The majority of the quantified proteins were released from platelets to a similar extent upon stimulation with either PAR-1 or PAR-4 agonist, with the release of PPBP, PF4, Vascular endothelial growth factor C (VEGFC), and THBS1 being slightly more efficient

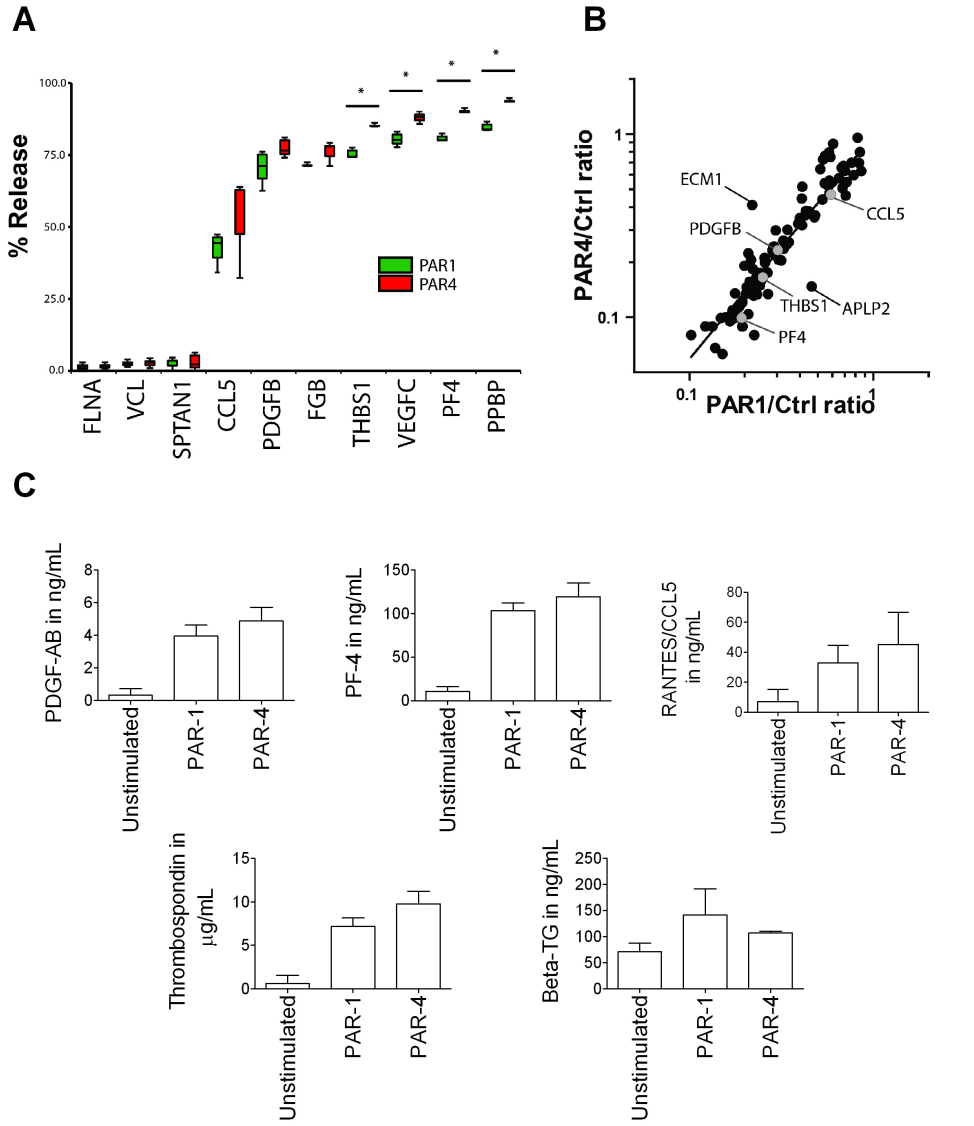

Figure 2. Stimulation with PAR-1 and PAR-4 shows equal release of factors. A) The extent to which several hallmark α-granule proteins are released from PAR-1 (green boxplots) and PAR-4 (red boxplots) activated platelets are displayed and compared with a few non-released matrix proteins. Stars indicate a statistically significantly different extent of release (Student's t-test, $p<0.05$ (see Supplementary Table 1). FLNA, Filamin-A; VCL, Vinculin; SPTAN1, Spectrin alpha chain, non-erythrocytic 1; CCL5, C-C motif chemokine 5; PDGFB, Platelet-derived growth factor beta subunit beta; FGB, Fibrinogen beta chain; THBS1, thrombospondin-1; VEGFC, Vascular endothelial growth factor C; PF4, Platelet factor 4; PPBP, platelet basic protein (*: Students t-test $P<0.05$). B) Correlation of quantities of PAR-1 and PAR-4 induced released factors. C. ELISA measurements of release from platelets of five individuals of PDGF-Ab, PF-4, RANTES/CCL5, Thrombosponding, and beta-TG after stimulation with PAR-1 and PAR-4.

after PAR-4 activation. A correlation analysis of the release of all identified releasate proteins (Supplementary Table 1) upon PAR-1 and PAR-4 stimulation gave a coefficient of 0.93 (r^2: 0.86, Spearman P<0.0001) (Figure 2B, Table 1). The release of granule components after full PAR-4 stimulation is more efficient than after PAR-1 stimulation, causing the correlation to shift to the right indicating more of the granule content remained within the platelet after PAR-1 stimulation. Next, we analyzed the releasate of either PAR-1 or PAR-4 stimulated platelets with ELISA (Figure 2C). The release of PDGF-AB, PF-4, RANTES/CCL5, THBS1, and β-TG after PAR-1 and PAR-4 stimulation were similar.

Phosphorylation analysis

Global serine/threonine phosphorylation downstream of both G-protein coupled receptors was monitored with Western blot in platelets isolated from 4 healthy volunteers. Western blotting was performed using an anti-human phosphorylated RRxS/T motif antibody. The results of the phosphorylation Western blot on platelet lysates stimulated with either PAR-1 or PAR-4 agonist (Figure 3A) show equal patterns of phosphorylated proteins, although PAR-4 activation seems to induce a slightly stronger serine and threonine phosphorylation. Comprehensive analysis of phosphorylation-based signalling downstream of PAR-1 and PAR-4 stimulated platelets compared with unstimulated platelets was performed by global phosphoproteome profiling with mass spectrometry from a healthy individual. In this dedicated screen, 227 phosphopeptides originating from 183 proteins were confidently identified with 1% FDR, which harbored 244 phoshosites (Supplementary Figure 1, and Supplementary Table 2) of which the location was robustly assessed. Seventy-six phosphosites displayed a more than 2-fold increase in phosphorylation upon PAR-4 activation and 102 sites showed a more than 2-fold increase in phosphorylation downstream of PAR-1 (Supplementary Table 2). Fifty-three phosphosites were increasingly phosphorylated downstream of both receptors (Supplementary Table 2, highlighted in grey). Although differences in the amount of phosphorylated sites were found, a similar trend in phosphorylation signalling downstream of both receptors was found, resulting in a correlation coefficient of the observed ratios of 0.49 (r^2: 0.24; P < 0.0001 Figure 3B). We found a strong increase in phosphorylation of factors associated with platelet activation, such as PKCθ (PRKCQ), Ephrin B1 (EFNB1), talin-1 (TLN1), zyxin (ZYX, a VASP targeting protein), Fyn (FYN), Lyn (LYN), cyclic AMP deaminase-2 (AMPD2), and the endothelial binding protein CD226 (Figure 3B). We found that the PAR-1/PAR-4 phosphorylation ratio of protein kinase C (PKC)α was 0.97, of proto-oncogene tyrosine-protein kinase Src 0.86, and of

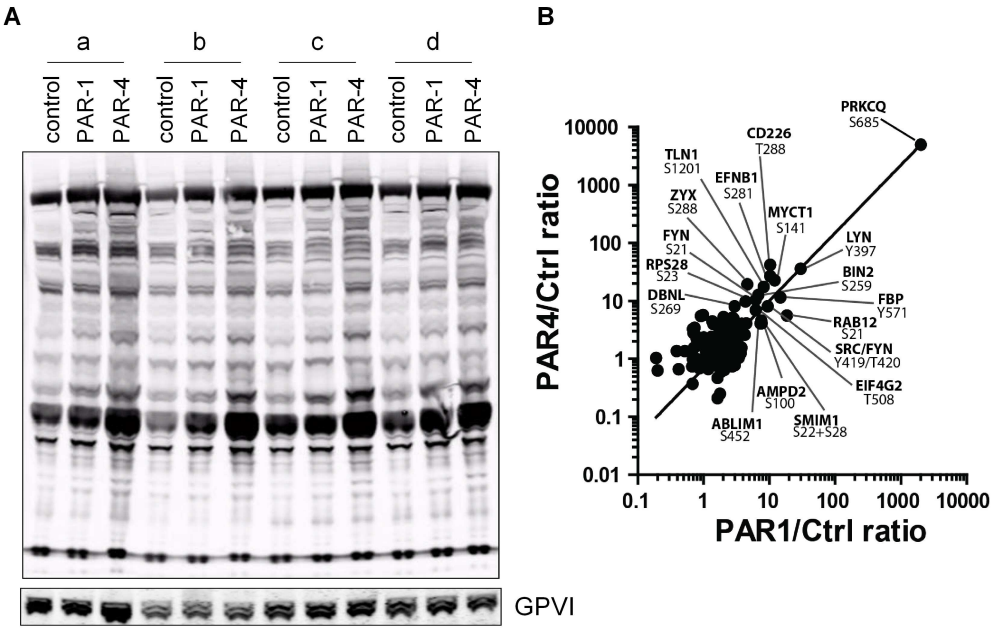

Figure 3. Phosphorylation analysis of intracellular signaling molecules after stimulation with PAR-1 and PAR-4. A). Lysates of platelets from 4 individuals stimulated with either PAR-1 or PAR-4 or unstimulated were analysed by Western blot analysis on phosphorylated serine and threonine residues. GPVI was taken as loading control. B). PAR-4/Ctrl phosphopeptide ratios are plotted against PAR-1/Ctrl phosphopeptide ratios (Supplementary Table 2), showing the correlation between phosphosite (black circles) regulation by PAR-1 or PAR-4 platelet activation. Highlighted are proteins that are strongly regulated (increase in phosphorylation of over 7 compared to control).

mitogen activated protein kinase (MAPK) P38α (MAPK14) 0,92 (Supplementary Table 2). We did not identify uniquely phosphorylated sites proteins for either stimulation with PAR-1 or PAR-4, thereby confirming the results obtained by Western blot.

Discussion

Platelet α-granules are filled with a large amount of proteins with opposing effects on angiogenesis and atherosclerosis, such as vascular endothelial growth factor (VEGF), stromal cell-derived (SDF)-1α, platelet factor (PF)4, and endostatin. It has been suggested that release of these angiogenic factors with opposing functions is controlled by differential release by PAR-1 and PAR-4 stimulation [3-5]. The goal of the present research was to comprehensively identify functional clusters of proteins that are preferentially released after PAR-1 or PAR-4 stimulation, and to identify phosphorylation-signaling events associated with differential release. For this we have utilized a 'reversed protein profiling' strategy to identify platelet releasate components [18]. Lack of differential release was confirmed with ELISA on PF4, β-TG, RANTES/CCL5, PDGF-AB and thrombospondin. In these assay conditions, maximum PAR-4 stimulation, which was obtained from dose response measurements, seemed to be a slightly stronger activator of platelet granule release than maximum PAR-1.

Differential release of proteins upon PAR-1 and PAR-4 should be associated with different intra-cellular signaling events. To have a general overview of differences in phosphorylation events, a Western blot on phosphorylated serine and threonine residues was performed with samples stimulated either with PAR-1 or PAR-4 agonist. With this approach we did not identify differences in the pattern of phosphorylation of proteins, although overall PAR-4 stimulation leads to stronger phosphorylation responses than PAR-1 stimulation.

We also compared differences in downstream signal transduction between PAR-1 and PAR-4 activated platelets, using broad spectrum phospho-motif antibodies and a mass spectrometry based phosphoproteomics approach. Although some subtle differences became apparent, we did not observe major differences in phosphoproteins that would suggest that PAR-1 and PAR-4 relay their effects through different pathways to induce differential release of α-granule content. We found strong phosphorylation of factors associated with platelet activation, such as, PKCθ, ephrin B1 [19], the $\alpha_{IIb}\beta_3$ associated talin-1 [20], the VASP targeting protein zyxin [21], the Scr like kinase Fyn, the tyrosine protein kinase Lyn [20], the cyclic AMP deaminase-2 [22], and the endothelial binding protein CD226 [23]. Other factors such as ABLIM, SMIM1, RAB12, RPS28, DBNL, EIF4G2, and BIN2 we could not link to any previous reports on platelet function. The PAR-1/PAR-4 phosphorylation ratio of PKCα, central in granule release did not show any differences for the two stimuli [24]. Similarly, MAPK p38α, which is an essential component of the MAPK pathway, or proto-oncogene tyrosine-protein kinase Src showed

hardly any difference between stimulation with PAR-1 or PAR-4 (Supplementary Table 2). Chatterjee et al. proposed that protein kinase PKC, and MAPK P38 play a role in PAR-4 induced secretion, whereas Src plays a specific role in PAR-1 induced secretion [3]. Our data does not support this notion. We do realize that phosphoproteomic analysis on one individual is inconclusive, but together with other data we present it provides support one secretion mechanism for both PAR-1 and PAR-4 induced activation.

The topic of differential release by PAR-1 and PAR-4 has been studied with a few factors with a focus on SDF-1α, VEGF, PF4, and endostatin [3-5]. With the exception of PF4, all those factors are low abundant in platelet granules [18]. As determined by us and by others previously, only a few picograms of those factors are released from activated platelet, which makes quantification unreliable. Recently, Jonnagaladda et al. have researched differential release induced by PAR-1 and PAR-4 agonist with micro ELISA's, where they were able to determine release of 17 proteins after PAR-4, and 23 after PAR-1 activation [8]. We took a comprehensive approach with quantitative mass spectrometry, which identified the release of 93 proteins. We hypothesized that with the large palette of proteins being present in α-granules, more proteins would be subjected to differential release. These would have to show as clusters containing either pro- or anti-angiogenic factors in a graph where the relative release after PAR-1 or PAR-4 stimulation is plotted. We were not able to identify such clusters of protein.

The existence of subsets of α-granules that are packed with antagonistic factors has been debated. Van Nispen tot Pannerden et al. identified two types of morphologically different α-granules, but one type was identified in only 16% of the platelets [25]. In addition, Kamykowski et al. argued that there is no functional co-clustering of factors in subsets of α-granules, rather antagonistic factors are stochastically packed into the same large granules [6].

Recently the release kinetics of different α-granule proteins was investigated, which showed that although PAR-1 and PAR-4 induce release of proteins with different kinetics, there are no functional patterns in release kinetics between PAR-1 and PAR-4 stimulation [8]. Our data supports the earlier reported random sorting of α-granule content, and the lack of thematic patterns of release kinetics for PAR-1 or PAR-4 stimulated platelet releasates [8]. The differences in release kinetics and the release in response to different agonist concentrations as reported by Jonnalagadda et al. provide context as into how certain activation conditions could result into what may appear thematically differential α-granule release [6,8].

In conclusion, our findings show that there is no differential release for any of the 93 proteins that came out of the proteomics analysis. Furthermore, there is no differential signal transduction downstream of PAR-1 and PAR-4.

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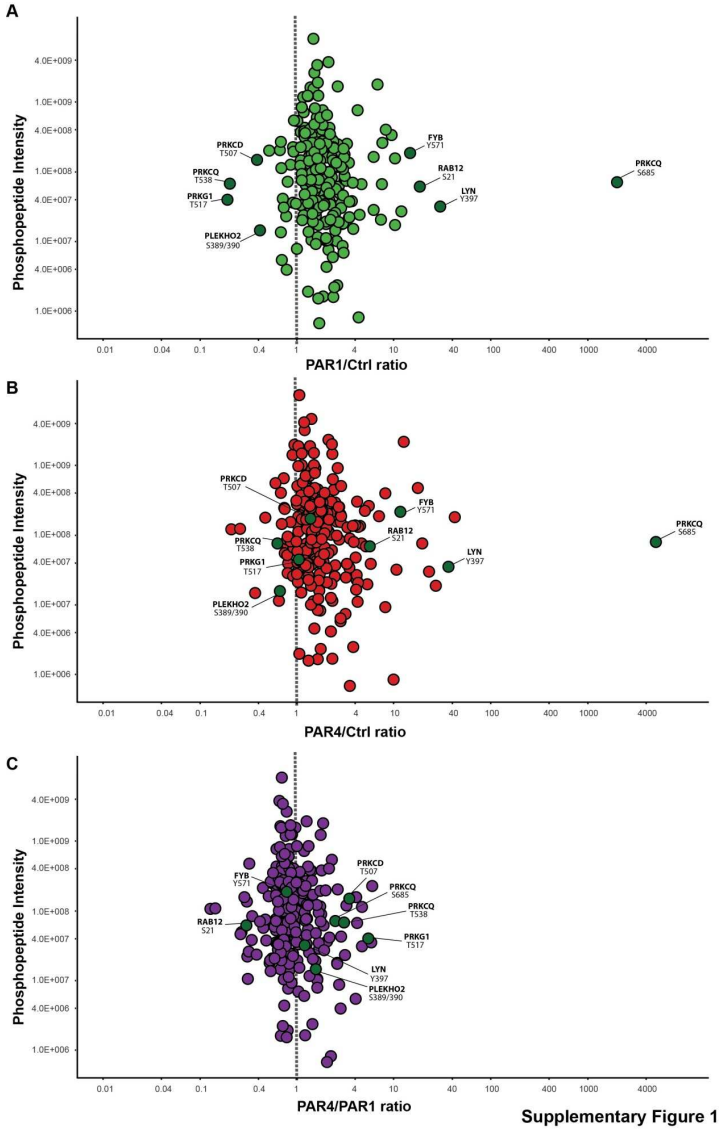
Supplementary Figures



Supplementary Figure 1

Supplementary Figure 1. Phosphosite regulation downstream of PAR-1 and PAR-4. Phosphopeptide ratios were plotted against peptide abundane (10Log scale) for PAR-1 stimulated platelets (panel A) and PAR-4 stimulated platelets (panel B). In both panels, the 4 most highly up- and down-regulated phosphosites in PAR-1 stimulated platelets were highlighted as an example for comparison between the two types of proteinase-activated receptor activation. PRKCQ, protein kinase C theta; LYN, tyrosine-protein kinase Lyn; RAB12, Ras-related protein Rab-12; FYB, Fyn-binding protein; PRKCD, protein kinase C delta; PRKG1, cGMP-dependent protein kinase 1; PLEKHO2,Pleckstrin homology domain-containing family O member 2. A correlation plot comparing PAR-1 and PAR-4-induced phosphosite regulation is displayed in panel C.

Suplementary Table 1. Platelet protein releasate upon stimulation with PAR1 or PAR4 of separate experiments with three individuals. The table includes Uniprot accession number; protein description; gene name; average (Avg) stimulated/resting (Ctrl) protein ratio for both PAR-1 and PAR-4 with standard deviation (StDev); and the percentage released compared with resting. NA: not available.

Uniprot Accession	Description	Gene	PAR1/ Ctrl (Avg)	PAR1/ Ctrl (StDev)	PAR4/ Ctrl (Avg)	PAR4/ Ctrl (StDev)	**Release % PAR1 (Avg)**	**Release % PAR4 (Avg)**
P01009	**Alpha-1-antitrypsin**	SERPINA1	0,241	0,035	0,174	0,013	75,9	82,6
P02765	**Alpha-2-HS-glycoprotein**	AHSG	0,266	0,085	0,175	0,070	73,4	82,5
P05067	**Amyloid beta A4 protein**	APP	0,223	0,046	0,131	0,015	77,7	86,9
Q15389	**Angiopoietin-1**	ANGPT1	0,326	0,058	0,237	0,030	67,4	76,3
P01008	**Antithrombin-III**	SERPINC1	0,223	0,044	0,149	0,016	77,7	85,1
P02647	**Apolipoprotein A-I**	APOA1	0,680	0,101	0,506	0,076	32,0	49,4
P04114	**Apolipoprotein B-100**	APOB	0,528	0,070	0,438	0,096	47,2	56,2
P15291	**Beta-1,4-galactosyltransferase 1**	B4GALT1	0,408	0,081	0,445	0,048	59,2	55,5
O43852	**Calumenin**	CALU	0,409	0,069	0,318	0,049	59,1	68,2
P13501	**C-C motif chemokine 5**	CCL5	0,577	0,073	0,473	0,176	42,3	52,7
P10909	**Clusterin**	CLU	0,672	0,059	0,654	0,038	32,8	34,6
P01024	**Complement C3**	C3	0,176	0,022	0,099	0,011	82,4	90,1
P0C0L4	**Complement C4-A**	C4A	0,257	0,036	0,203	0,078	74,3	79,7
P00751	**Complement factor B**	CFB	0,227	0,058	0,134	0,016	77,3	86,6
P02671	**Fibrinogen alpha chain**	FGA	0,289	0,012	0,243	0,022	71,1	75,7
P02675	**Fibrinogen beta chain**	FGB	0,286	0,002	0,242	0,041	71,4	75,8
P02679	**Fibrinogen gamma chain**	FGG	0,282	0,004	0,232	0,029	71,8	76,8
P02751	**Fibronectin**	FN1	0,429	0,033	0,380	0,059	57,1	62,0
Q12841	**Follistatin-related protein 1**	FSTL1	0,346	0,009	0,257	0,075	65,4	74,3
P07093	**Glia-derived nexin**	SERPINE2	0,324	0,016	0,262	0,054	67,6	73,8
P04196	**Histidine-rich glycoprotein**	HRG	0,221	0,101	0,183	0,064	77,9	81,7
P01876	**Ig alpha-1 chain C region**	IGHA1	0,294	0,087	0,298	0,053	70,6	70,2
P01857	**Ig gamma-1 chain C region**	IGHG1	0,172	0,033	0,100	0,013	82,8	90,0
P01834	**Ig kappa chain C region**	IGKC	0,205	0,008	0,146	0,025	79,5	85,4
P0CG05	**Ig lambda-2 chain C regions**	IGLC2	0,231	0,006	0,165	0,021	76,9	83,5
Q14624	**Inter-alpha-trypsin inhibitor heavy chain H4**	ITIH4	0,216	0,044	0,206	0,025	78,4	79,4
P01042	**Kininogen-1**	KNG1	0,315	0,044	0,204	0,054	68,5	79,6
Q14766	**Latent-transforming growth factor beta-binding protein 1**	LTBP1	0,573	0,097	0,526	0,071	42,7	47,4
P01033	**Metalloproteinase inhibitor 1**	TIMP1	0,193	0,026	0,121	0,008	80,7	87,9
P35625	**Metalloproteinase inhibitor 3**	TIMP3	0,305	0,071	0,206	0,066	69,5	79,4
P14543	**Nidogen-1**	NID1	0,399	0,073	0,349	0,095	60,1	65,1
Q14112	**Nidogen-2**	NID2	0,341	0,005	0,300	0,025	65,9	70,0
Q02818	**Nucleobindin-1**	NUCB1	0,446	0,070	0,379		55,4	62,1
P05121	**Plasminogen activator inhibitor 1**	SERPINE1	0,186	0,009	0,114	0,010	81,4	88,6
P00747	**Plasminogen**	PLG	0,257	0,030	0,198	0,010	74,3	80,2
P02775	**Platelet basic protein**	PPBP	0,151	0,020	0,063	0,003	84,9	93,7
P02776	**Platelet factor 4**	PF4	0,190	0,016	0,099	0,005	81,0	90,1
P10720	**Platelet factor 4 variant**	PF4V1	0,209	0,022	0,104	0,018	79,1	89,6

Uniprot Accession	Description	Gene	PAR1/ Ctrl (Avg)	PAR1/ Ctrl (StDev)	PAR4/ Ctrl (Avg)	PAR4/ Ctrl (StDev)	**Release % PAR1 (Avg)**	**Release % PAR4 (Avg)**
P01127	**Platelet-derived growth factor subunit B**	PDGFB	0,290	0,083	0,219	0,050	71,0	78,1
Q10471	**Polypeptide N-acetylgalactosaminyltransferase 2**	GALNT2	0,574	0,070	0,794	0,265	42,6	20,6
P07602	**Proactivator polypeptide**	PSAP	0,650	0,018	0,575	0,127	35,0	42,5
P29122	**Proprotein convertase subtilisin/kexin type 6**	PCSK6	0,543	0,021	0,539	0,038	45,7	46,1
P10124	**Serglycin**	SRGN	0,233	0,030	0,133	0,007	76,7	86,7
P02787	**Serotransferrin**	TF	0,176	0,023	0,106	0,022	82,4	89,4
P09486	**SPARC**	SPARC	0,165	0,018	0,101	0,008	83,5	89,9
P02768	**Serum albumin**	ALB	0,166	0,030	0,095	0,016	83,4	90,5
P07996	**Thrombospondin-1**	THBS1	0,242	0,026	0,149	0,001	75,8	85,1
P01137	**Transforming growth factor beta-1**	TGFB1	0,596	0,045	0,546	0,048	40,4	45,4
Q15582	**Transforming growth factor-beta-induced protein ig-h3**	TGFBI	0,256	0,019	0,211	0,005	74,4	78,9
P02766	**Transthyretin**	TTR	0,478	0,136	0,351	0,112	52,2	64,9
Q8NDZ4	**UPF0672 protein C3orf58**	C3orf58	0,410	0,068	0,516	0,108	59,0	48,4
P49767	**Vascular endothelial growth factor C**	VEGFC	0,193	0,032	0,120	0,020	80,7	88,0
P02774	**Vitamin D-binding protein**	GC	0,138	0,030	0,068	0,019	86,2	93,2
P07225	**Vitamin K-dependent protein S**	PROS1	0,202	0,013	0,134	0,020	79,8	86,6
P25311	**Zinc-alpha-2-glycoprotein**	AZGP1	0,148	0,064	0,099	0,045	85,2	90,1
P02763	**Alpha-1-acid glycoprotein 1**	ORM1	0,171		0,109		82,9	89,1
P19652	**Alpha-1-acid glycoprotein 2**	ORM2	0,267		0,134	0,000	73,3	86,6
P04217	**Alpha-1B-glycoprotein**	A1BG	0,122		0,089	0,044	87,8	91,1
Q16706	**Alpha-mannosidase 2**	MAN2A1	0,338	0,158	0,250	0,056	66,2	75,0
Q06481	**Amyloid-like protein 2**	APLP2	0,461		0,147		53,9	85,3
Q15057	**Arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein 2**	ACAP2	0,605	0,245	0,885	0,178	39,5	11,5
P15907	**Beta-galactoside alpha-2,6-sialyltransferase 1**	ST6GAL1	0,225		0,080		77,5	92,0
P56202	**Cathepsin W**	CTSW	0,701	0,077	0,630	0,040	29,9	37,0
P12259	**Coagulation factor V**	F5	0,687	0,066	0,670	0,090	31,3	33,0
P08603	**Complement factor H**	CFH	0,177		0,135		82,3	86,5
P29279	**Connective tissue growth factor**	CTGF	0,102		0,080		89,8	92,0
P01034	**Cystatin-C**	CST3	0,199		0,191		80,1	80,9
Q12805	**EGF-containing fibulin-like extracellular matrix protein 1**	EFEMP1	0,424		0,359		57,6	64,1
Q16610	**Extracellular matrix protein 1**	ECM1	0,219		0,410		78,1	59,0
Q92896	**Golgi apparatus protein 1**	GLG1	0,212		0,156		78,8	84,4
P00738	**Haptoglobin**	HP	0,390		0,324		61,0	67,6
P02790	**Hemopexin**	HPX	0,193		0,089		80,7	91,1
Q16775	**Hydroxyacylglutathione hydrolase, mitochondrial**	HAGH	0,821	0,180	0,955	0,088	17,9	4,5
P01860	**Ig gamma-3 chain C region**	IGHG3	0,194		0,089		80,6	91,1
P07942	**Laminin subunit beta-1**	LAMB1	0,515	0,216	0,644	0,142	48,5	35,6
P11047	**Laminin subunit gamma-1**	LAMC1	0,544	0,069	0,760	0,105	45,6	24,0

Uniprot Accession	Description	Gene	PAR1/ Ctrl (Avg)	PAR1/ Ctrl (StDev)	PAR4/ Ctrl (Avg)	PAR4/ Ctrl (StDev)	**Release % PAR1 (Avg)**	**Release % PAR4 (Avg)**
P10619	**Lysosomal protective protein**	CTSA	0,834	0,078	0,696	0,118	16,6	30,4
P58215	**Lysyl oxidase homolog 3**	LOXL3	0,209		0,223		79,1	77,7
Q96QG7	**Myotubularin-related protein 9**	MTMR9	0,533	0,299	0,726	0,228	46,7	27,4
P00734	**Prothrombin**	F2	0,133		0,089		86,7	91,1
P02743	**Serum amyloid P-component**	APCS	0,158		0,100		84,2	90,0
Q9HAT2	**Sialate O-acetylesterase**	SIAE	0,783	0,373	0,596	0,110	21,7	40,4
O43493	**Trans-Golgi network integral membrane protein 2**	TGOLN2	0,584	0,133	0,749	0,054	41,6	25,1
Q9Y274	**Type 2 lactosamine alpha-2,3-sialyltransferase**	ST3GAL6	0,761	0,379	0,698	0,224	23,9	30,2
Q7Z3E2	**Uncharacterized protein C10orf118**	C10orf118	0,481		0,360		51,9	64,0
P04004	**Vitronectin**	VTN	0,577	0,174	0,556	0,283	42,3	44,4
P38571	**Lysosomal acid lipase/ cholesteryl ester hydrolase**	LIPA	0,679	0,196	0,508	0,142	32,1	49,2
P07339	**Cathepsin D**	CTSD	0,846	0,069	0,796	0,157	15,4	20,4
Q8WVC6	**Dephospho-CoA kinase domain-containing protein**	DCAKD	0,859	0,227	0,629	0,365	14,1	37,1
Q92820	**Gamma-glutamyl hydrolase**	GGH	0,715	0,079	0,547	0,071	28,5	45,3
P01859	**Ig gamma-2 chain C region**	IGHG2	0,245		0,158		75,5	84,2
P61626	**Lysozyme C**	LYZ	0,559	0,152	0,529	0,228	44,1	47,1
P49757	**Protein numb homolog**	NUMB	0,706		0,462		29,4	53,8

Supplementary Table 2. Protein phosphorylation upon platelet stimulation with either PAR-1 or PAR-4 from an experiment with one donor. The table includes Uniprot Accession number, protein name, gene name, the phosphosite present in the phosphopeptide being a serine (S), threonie (T) or tyrosine (Y) residue; the stimulated/resting phosphopeptide ratio, and the PAR-4/PAR-1 phosphopeptide ratio. The confidence of phosphosite localization is defined high when probability of identification of the site is between 100% and 75%, and medium if the probability of the identification of the site is between 50% and 75%. If identification of phosphosite was medium, the alternative is indicated by e.g. Yxxx/Txxx. If multiple phosphorylized residues are present on the identified peptide this is indicated by e.g. Yxxx+Txxx. Phosphorylized sites within the same protein are given in a new row.

Uniprot Access-ion	Protein name	Gene	Phospho-site	Confi-dence	PAR1/ Ctrl ratio	PAR4/ Ctrl ratio	PAR4/ PAR1
B2RUZ4	Small integral membrane protein 1	SMIM1	S22+S28	High	7,51	4,59	0,61
P60709	Actin, cytoplasmic 1	ACTB	Y198	High	1,59	1,34	0,84
O00151	PDZ and LIM domain protein 1	PDLIM1	S90	High	1,40	1,35	0,97
O00161	Synaptosomal-associated protein 23	SNAP23	S110	High	1,65	1,34	0,81
O00264	Membrane-associated progesterone receptor component 1	PGRMC1	S57	High	1,36	4,44	3,26
O14545	TRAF-type zinc finger domain-containing protein 1	TRAFD1	S409	High	2,02	1,47	0,73
			T414/ S415	Medium	3,11	2,34	0,76
O14639	Actin-binding LIM protein 1	ABLIM1	S452	High	7,33	4,07	0,56
			T429/ S431	Medium	2,28	2,36	1,04
O15117	FYN-binding protein	FYB	S46	High	1,84	1,53	0,83
			T158	High	2,18	1,67	0,77
			S573	High	1,37	1,07	0,78
			Y571	High	14,64	11,54	0,79
O15231	Zinc finger protein 185	ZNF185	S453	High	2,08	2,09	1,00
O43182	Rho GTPase-activating protein 6	ARHGAP6	S777	High	2,07	1,27	0,61
			S770	High	2,09	1,20	0,57
O43306	Adenylate cyclase type 6	ADCY6	S576	High	2,01	5,26	2,61
			S54	High	1,66	1,30	0,78
O43488	Aflatoxin B1 aldehyde reductase member 2	AKR7A2	S255	High	1,83	1,38	0,75
O43516	WAS/WASL-interacting protein family member 1	WIPF1	S444	High	3,43	1,05	0,31
			S234	High	3,13	2,56	0,82
O43572	A-kinase anchor protein 10, mitochondrial	AKAP10	S187	High	2,38	2,46	1,03
O60229	Kalirin	KALRN	S2236	High	2,63	1,60	0,61
O60271	C-Jun-amino-terminal kinase-interacting protein 4	SPAG9	S730+S732/ S733	Medium	1,42	2,30	1,62
O75396	Vesicle-trafficking protein SEC22b	SEC22B	S137	High	2,70	5,08	1,88
O94804	Serine/threonine-protein kinase 10	STK10	S951/ T952	Medium	1,92	1,20	0,62
			S438	High	2,03	1,26	0,62
O95810	Serum deprivation-response protein	SDPR	S287	High	1,23	0,78	0,64
			S287/ S288+S293	Medium	1,20	1,00	0,83
			S293	High	1,16	0,89	0,77
P02545	Prelamin-A/C	LMNA	S22	High	1,82	1,28	0,70

Uniprot Access-ion	Protein name	Gene	Phospho-site	Confi-dence	PAR1/ Ctrl ratio	PAR4/ Ctrl ratio	PAR4/ PAR1
			S392	High	1,31	1,10	0,84
P02671	Fibrinogen alpha chain	FGA	S274	High	0,91	1,86	2,04
P04075	Fructose-bisphosphate aldolase A	ALDOA	S36	High	1,43	1,25	0,87
P04792	Heat shock protein beta-1	HSPB1	S78+S82	High	1,62	0,21	0,13
			S82	High	2,11	1,40	0,66
P05771	Protein kinase C beta type	PRKCB	T642	High	1,09	1,55	1,41
P06241	Tyrosine-protein kinase Fyn	FYN	S21	High	6,20	10,48	1,69
P07359	Platelet glycoprotein Ib alpha chain	GP1BA	S603+S606	High	1,17	1,39	1,19
P07948	Tyrosine-protein kinase Lyn	LYN	Y397	High	30,09	36,11	1,20
P0C7M8	C-type lectin domain family 2 member L	CLEC2L	S48	High	1,31	2,38	1,81
			S50	High	1,36	1,36	1,00
P11413	Glucose-6-phosphate 1-dehydrogenase	G6PD	Y401	High	1,36	1,29	0,95
P12931	Proto-oncogene tyrosine-protein kinase Src, Tyrosine-protein kinase Fyn	SRC, FYN	Y419/ T420	Medium	9,45	8,11	0,86
P13224	Platelet glycoprotein Ib beta chain	GP1BB	S191	High	1,12	1,48	1,32
			S191+T193	High	1,12	1,11	1,00
P13747	HLA class I histocompatibility antigen, alpha chain E	HLA-E	S355/ S357	Medium	1,26	0,81	0,64
P13861	cAMP-dependent protein kinase type II-alpha regulatory subunit	PRKAR2A	S78+S80	High	1,46	0,99	0,68
P16150	Leukosialin	SPN	S355	High	3,67	1,26	0,34
P16157	Ankyrin-1	ANK1	S856	High	1,99	1,78	0,90
P17252	Protein kinase C alpha type	PRKCA	S226	High	1,32	1,28	0,97
P17612	cAMP-dependent protein kinase catalytic subunit alpha	PRKACA	T198	High	0,52	1,36	2,64
P19105	Myosin regulatory light chain 12A	MYL12A	S19	High	4,24	2,61	0,62
P19634	Sodium/hydrogen exchanger 1	SLC9A1	S703	High	1,59	2,04	1,28
P21291	Cysteine and glycine-rich protein 1	CSRP1	S192	High	1,42	1,26	0,89
P21333	Filamin-A	FLNA	S2338/ S2339	Medium	1,36	0,97	0,71
			S11	High	2,59	4,19	1,62
			S2152+S2158	High	1,40	4,02	2,87
			S1459	High	1,48	1,04	0,70
			S966	High	2,93	1,54	0,53
P21731	Thromboxane A2 receptor	TBXA2R	S329	High	1,78	1,25	0,70
P24534	Elongation factor 1-beta	EEF1B2	S106	High	1,38	0,93	0,68
P27987	Inositol-trisphosphate 3-kinase B	ITPKB	S43	High	1,66	0,74	0,44
			S269	High	1,28	1,70	1,33
P30273	High affinity immunoglobulin epsilon receptor subunit gamma	FCER1G	Y65	High	2,75	0,74	0,27
P30419	Glycylpeptide N-tetradecanoyltransferase 1	NMT1	S47	High	1,57	1,31	0,83
P35236	Tyrosine-protein phosphatase non-receptor type 7	PTPN7	S143	High	1,28	0,78	0,61
P35579	Myosin-9	MYH9	S1943	High	0,78	2,44	3,13
P35611	Alpha-adducin	ADD1	S358	High	1,19	1,62	1,37
P36507	Dual specificity mitogen-activated protein kinase kinase 2	MAP2K2	T394	High	1,35	1,25	0,92
P37802	Transgelin-2	TAGLN2	S163	High	1,90	2,78	1,46

Uniprot Access-ion	Protein name	Gene	Phospho-site	Confi-dence	PAR1/ Ctrl ratio	PAR4/ Ctrl ratio	PAR4/ PAR1
P42566	Epidermal growth factor receptor substrate 15	EPS15	S323/ S324	Medium	1,33	1,07	0,80
P47756	F-actin-capping protein subunit beta	CAPZB	S263	High	3,09	3,46	1,12
P49841	Glycogen synthase kinase-3 beta	GSK3B	Y216	High	1,49	1,02	0,69
P53814	Smoothelin	SMTN	S792	High	3,30	2,46	0,74
P55209	Nucleosome assembly protein 1-like 1	NAP1L1	T62	High	1,63	1,42	0,87
P61006	Ras-related protein Rab-8A	RAB8A	S181+S185	High	1,31	1,05	0,80
P62857	40S ribosomal protein S28	RPS28	S23	High	4,35	9,80	2,25
P78344	Eukaryotic translation initiation factor 4 gamma 2	EIF4G2	T508	High	6,23	6,96	1,12
P78559	Microtubule-associated protein 1A	MAP1A	T504	High	2,98	2,25	0,75
P98082	Disabled homolog 2	DAB2	S723	High	3,13	2,69	0,86
P98172	Ephrin-B1	EFNB1	S281	High	10,29	26,79	2,60
Q01082	Spectrin beta chain, brain 1	SPTBN1	S2164/ S2165	Medium	1,26	2,23	1,77
Q01433	AMP deaminase 2	AMPD2	S76	High	4,46	4,08	0,91
			S100	High	7,67	4,12	0,54
Q04759	Protein kinase C theta type	PRKCQ	S685	High	2004,00	4988,00	2,49
			S695	High	2,02	1,03	0,51
			T538	High	0,20	0,63	3,06
Q05655	Protein kinase C delta type	PRKCD	T507	High	0,39	1,36	3,50
			S664	High	2,52	1,14	0,45
Q08495	Dematin	EPB49	S333	High	1,66	1,48	0,89
			S303	High	2,32	1,42	0,61
			T10/ S11	Medium	2,45	4,25	1,73
			S287	High	1,51	1,19	0,79
			S92	High	1,10	1,10	1,01
Q0ZGT2	Nexilin	NEXN	S357	High	2,87	1,75	0,61
			T363/ S365	High	1,60	1,99	1,24
Q13177	Serine/threonine-protein kinase PAK 2	PAK2	S58	High	3,24	1,53	0,47
			Y139	High	2,20	2,25	1,02
Q13459	Myosin-IXb	MYO9B	T1346	High	3,11	2,55	0,82
Q13501	Sequestosome-1	SQSTM1	S272	High	2,00	2,02	1,01
Q13586	Stromal interaction molecule 1	STIM1	S616/ S618+S620/ S621	Medium	2,02	1,40	0,69
Q13884	Beta-1-syntrophin	SNTB1	S219	High	1,62	1,88	1,16
Q13976	cGMP-dependent protein kinase 1	PRKG1	T517	High	0,19	1,04	5,43
Q14157	Ubiquitin-associated protein 2-like	UBAP2L	S467	High	2,11	1,72	0,81
Q14247	Src substrate cortactin	CTTN	S417/ S418	Medium	1,30	2,29	1,77
			T401	High	0,91	5,48	6,01
Q14432	cGMP-inhibited 3',5'-cyclic ph	PDE3A	T311	High	2,23	1,40	0,63
Q14644	Ras GTPase-activating protein 3	RASA3	S833	High	0,71	1,54	2,16
Q15036	Sorting nexin-17	SNX17	S331/ S333	Medium	1,76	1,57	0,89
Q15052	Rho guanine nucleotide exchange factor 6	ARHGEF6	S488	High	1,62	1,41	0,87

Uniprot Access-ion	Protein name	Gene	Phospho-site	Confi-dence	PAR1/ Ctrl ratio	PAR4/ Ctrl ratio	PAR4/ PAR1
Q15149	Plectin	PLEC	S1435	High	1,73	2,26	1,31
			S4386	High	2,86	4,96	1,73
Q15365	Poly(rC)-binding protein 1	PCBP1	S189/ S190	Medium	2,31	1,48	0,64
Q15746	Myosin light chain kinase, smooth muscle	MYLK	T1778/ S1779	Medium	1,01	2,33	2,30
Q15762	CD226 antigen	CD226	T288	High	10,27	42,09	4,10
Q15942	Zyxin	ZYX	S281	High	1,56	1,87	1,20
			S288	High	4,63	19,39	4,19
			S258/ S259	High	2,79	0,85	0,31
			S344	High	1,64	2,08	1,27
Q16204	Coiled-coil domain-containing protein 6	CCDC6	S244	High	0,67	0,74	1,11
Q16539	Mitogen-activated protein kinase 14	MAPK14	T180+Y182	High	1,51	1,39	0,92
Q2M2I8	AP2-associated protein kinase 1	AAK1	T606	High	2,86	1,99	0,70
Q3ZCW2	Galectin-related protein	GRP	S24+S25	High	1,82	1,52	0,83
			S22+S24/ S25	Medium	2,19	1,59	0,73
Q4KMP7	TBC1 domain family member 10B	TBC1D10B	S678	High	1,18	0,67	0,57
Q5JSH3	WD repeat-containing protein 44	WDR44	T94+S96	High	3,43	2,81	0,82
			S50	High	2,17	2,84	1,31
Q5M775	Cytospin-B	CYTSB	S134	High	1,52	1,27	0,83
Q5T5C0	Syntaxin-binding protein 5	STXBP5	S780	High	0,78	2,21	2,83
			S782	High	1,00	1,62	1,63
			S782+T784/ S785	Medium	2,02	1,37	0,68
Q66K74	Microtubule-associated protein 1S	MAP1S	S759	High	2,99	0,78	0,26
Q684P5	Rap1 GTPase-activating protein 2	RAP1GAP2	S9	High	2,48	0,80	0,32
Q6IAA8	RhoA activator C11orf59	C11orf59	S26/ S27	High	0,93	0,72	0,78
Q6IQ22	Ras-related protein Rab-12	RAB12	S21	High	18,45	5,59	0,30
Q6ZNJ1	Neurobeachin-like protein 2	NBEAL2	T1867	High	1,47	1,69	1,15
Q6ZSS7	Major facilitator superfamily domain-containing protein 6	MFSD6	T10	High	0,73	3,43	4,69
Q7L591	Docking protein 3	DOK3	S330	High	1,63	1,17	0,72
Q7L7X3	Serine/threonine-protein kinase TAO1	TAOK1	S421	High	1,61	1,33	0,83
Q7Z401	C-myc promoter-binding protein	DENND4A	S731	High	1,59	1,02	0,64
Q7Z434	Mitochondrial antiviral-signaling protein	MAVS	S222	High	2,62	1,83	0,70
Q7Z460	CLIP-associating protein 1	CLASP1	S1091	High	1,42	1,02	0,72
Q7Z6Z7	E3 ubiquitin-protein ligase HUWE1	HUWE1	T2889	High	2,57	1,36	0,53
			S1907	High	1,42	1,31	0,92
Q86UX7	Fermitin family homolog 3	FERMT3	S345	High	1,93	3,24	1,68
			S8	High	1,20	1,10	0,91
			T6	High	1,29	1,11	0,86
Q86WR7	Uncharacterized protein C10orf47	C10orf47	S212	High	2,38	1,72	0,72
Q86X10	Ral GTPase-activating protein subunit beta	RALGAPB	S357	High	1,53	1,24	0,81
Q8N4C8	Misshapen-like kinase 1	MINK1	S699	High	2,34	0,90	0,38
			S599	High	1,65	2,23	1,35

Uniprot Access-ion	Protein name	Gene	Phospho-site	Confi-dence	PAR1/ Ctrl ratio	PAR4/ Ctrl ratio	PAR4/ PAR1
			S601	High	1,87	2,29	1,22
Q8N5J2	Protein FAM63A	FAM63A	S103	High	2,38	1,73	0,73
Q8N699	Myc target protein 1	MYCT1	S149	High	2,64	2,26	0,86
			S141	High	12,01	22,83	1,90
			T219	High	1,53	1,02	0,67
Q8ND76	Cyclin-Y	CCNY	S324/ S326	Medium	1,84	0,60	0,32
Q8NF50	Dedicator of cytokinesis protein 8	DOCK8	S451	High	0,86	0,69	0,80
Q8NFH8	RalBP1-associated Eps domain-containing protein 2	REPS2	S492/ S493	Medium	1,24	1,00	0,80
Q8TEA8	D-tyrosyl-tRNA(Tyr) deacylase 1	DTD1	S197	High	2,41	2,69	1,12
Q8TF42	Ubiquitin-associated and SH3 domain-containing protein B	UBASH3B	S377	High	0,70	2,80	4,03
Q8WWA1	Transmembrane protein 40	TMEM40	S137	High	1,10	1,39	1,26
Q8WXF7	Atlastin-1	ATL1	S22+S23	High	1,56	0,91	0,59
Q92619	Minor histocompatibility protein HA-1	HMHA1	S23	High	1,52	1,14	0,75
Q969X1	Transmembrane BAX inhibitor motif-containing protein 1	TMBIM1	S81	High	1,22	1,31	1,08
			S83	High	1,08	1,37	1,27
Q96A00	Protein phosphatase 1 regulatory subunit 14A	PPP1R14A	S26	High	1,40	0,85	0,61
			S128+S136	High	2,02	1,36	0,67
Q96F86	Enhancer of mRNA-decapping protein 3	EDC3	S131	High	2,00	1,46	0,73
Q96MH2	Protein HEXIM2	HEXIM2	S29	High	2,48	1,68	0,68
Q96MK2	Protein FAM65C	FAM65C	S24	High	2,99	2,00	0,67
Q96P48	Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 1	ARAP1	S229	High	1,81	1,39	0,77
Q96RI0	Proteinase-activated receptor 4	F2RL3	S366+S369	High	2,71	4,21	1,56
Q99460	26S proteasome non-ATPase regulatory subunit 1	PSMD1	T273	High	1,13	1,91	1,69
Q99523	Sortilin	SORT1	S825	High	1,72	1,38	0,80
Q99613	Eukaryotic translation initiation factor 3 subunit C	EIF3C	S39	High	0,69	3,27	4,72
Q9C0C9	Ubiquitin-conjugating enzyme E2 O	UBE2O	S399/ S401	Medium	1,70	3,47	2,04
Q9C0I1	Myotubularin-related protein 12	MTMR12	S564	High	2,19	1,90	0,87
Q9GZY8	Mitochondrial fission factor	MFF	S155	High	2,01	1,49	0,74
Q9H4X1	Response gene to complement 32 protein	RGC32	S97	High	2,45	3,95	1,61
Q9HBL0	Tensin-1	TNS1	S1381	High	1,48	1,28	0,87
Q9NQT8	Kinesin-like protein KIF13B	KIF13B	S1379	High	1,78	0,25	0,14
Q9NZQ3	NCK-interacting protein with SH3 domain	NCKIPSD	T181	High	1,43	1,18	0,83
Q9P035	3-hydroxyacyl-CoA dehydratase 3	PTPLAD1	S114	High	1,36	1,72	1,27
Q9P107	GEM-interacting protein	GMIP	S437	High	1,56	1,26	0,81
			S234	High	2,38	1,62	0,68
Q9P266	Junctional protein associated with coronary artery disease	KIAA1462	S1044	High	2,28	0,79	0,35
Q9P270	SLAIN motif-containing protein 2	SLAIN2	S48	High	3,07	2,81	0,92
Q9P2G1	Ankyrin repeat and IBR domain-containing protein 1	ANKIB1	S737	High	1,18	1,02	0,87

Uniprot Access-ion	Protein name	Gene	Phospho-site	Confi-dence	PAR1/ Ctrl ratio	PAR4/ Ctrl ratio	PAR4/ PAR1
Q9UBC2	Epidermal growth factor receptor substrate 15-like 1	EPS15L1	S229	High	0,92	1,83	1,98
Q9UBN7	Histone deacetylase 6	HDAC6	S22	High	1,13	1,66	1,47
Q9UBW5	Bridging integrator 2	BIN2	S259	High	6,79	12,59	1,85
			S263	High	1,74	1,21	0,70
			S273	High	2,17	3,83	1,76
Q9UDT6	CAP-Gly domain-containing linker protein 2	CLIP2	S207	High	2,59	3,77	1,45
Q9UHY1	Nuclear receptor-binding protein	NRBP1	T431	High	2,02	1,91	0,95
Q9UJU6	Drebrin-like protein	DBNL	S269	High	2,96	8,05	2,72
			S275	High	3,03	2,68	0,89
Q9UKE5	TRAF2 and NCK-interacting protein kinase	TNIK	S640	High	2,35	2,12	0,90
Q9UPN4	5-azacytidine-induced protein 1	AZI1	S78	High	2,44	1,74	0,72
Q9UPU5	Ubiquitin carboxyl-terminal hydrolase 24	USP24	S2561	High	1,68	1,45	0,86
			S2047	High	1,26	1,37	1,09
Q9UQ16	Dynamin-3	DNM3	S769+S773	High	1,66	1,38	0,83
Q9Y2G3	Probable phospholipid-transporting ATPase IF	ATP11B	S1152/ S1154	Medium	1,59	1,19	0,75
Q9Y2L6	FERM domain-containing protein 4B	FRMD4B	S604+S608	High	1,66	1,30	0,79
Q9Y2Q0	Probable phospholipid-transporting ATPase IA	ATP8A1	S25+TT28/ S29	Medium	0,98	5,72	5,84
Q9Y2X7	ARF GTPase-activating protein GIT1	GIT1	T480	High	3,78	1,56	0,41
			S362	High	2,16	1,41	0,65
Q9Y490	Talin-1	TLN1	S1201	High	8,24	17,54	2,13
			S2338	High	1,43	1,00	0,70
			T307/ Y308	Medium	3,81	4,05	1,06
Q9Y4D8	Probable E3 ubiquitin-protein ligase C12orf51	C12orf51	S1075	High	1,45	1,07	0,73
			T280	High	1,30	1,28	0,99
Q9Y4G6	Talin-2	TLN2	T1843	High	1,39	1,78	1,28
Q9Y4G8	Rap guanine nucleotide exchange factor 2	RAPGEF2	S1019	High	1,56	1,38	0,88
			S1022	High	1,70	1,30	0,77
Q9Y4H4	G-protein-signaling modulator 3	GPSM3	S35	High	0,80	0,98	1,22
			S39	High	0,69	0,98	1,43
Q9Y570	Protein phosphatase methylesterase 1	PPME1	T242/ S243	Medium	1,46	1,01	0,69
Q9Y608	Leucine-rich repeat flightless-interacting protein 2	LRRFIP2	S320	High	1,74	1,27	0,73
Q9Y613	FH1/FH2 domain-containing protein 1	FHOD1	S523	High	1,52	1,76	1,16
			S486	High	2,84	3,73	1,31
			S367	High	1,75	1,54	0,88
Q9Y6A5	Transforming acidic coiled-coil-containing protein 3	TACC3	S434	High	3,04	3,14	1,03
Q9Y6F6	Protein MRVI1	MRVI1	S462	High	1,09	1,35	1,24
			S367	High	2,04	1,52	0,75
O15400	Syntaxin-7	STX7	T78/ T79	Medium	2,07	0,65	0,31
Q9Y3Q8	TSC22 domain family protein 4	TSC22D4	S62	High	1,44	0,98	0,68
Q53TN4	Cytochrome b reductase 1	CYBRD1	T285	High	3,48	1,69	0,48

Uniprot Access-ion	Protein name	Gene	Phospho-site	Confi-dence	PAR1/ Ctrl ratio	PAR4/ Ctrl ratio	PAR4/ PAR1
Q13131	5'-AMP-activated protein kinase catalytic subunit alpha-1	PRKAA1	S496	High	2,35	2,80	1,19
Q9NR19	Acetyl-coenzyme A synthetase, cytoplasmic	ACSS2	S28	High	1,13	1,40	1,24
Q5T0N5	Formin-binding protein 1-like	FNBP1L	T293	High	2,17	2,10	0,97
P16615	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	ATP2A2	S663	High	1,71	1,70	0,99
P05556	Integrin beta-1	ITGB1	T777	High	1,34	1,23	0,92
P43487	Ran-specific GTPase-activating protein	RANBP1	S60	High	1,88	1,21	0,64
Q9NPQ8	Synembryn-A	RIC8A	Y435/ S436+T441/ T443	Medium	1,20	1,89	1,57
O94929	Actin-binding LIM protein 3	ABLIM3	S280	High	3,47	3,18	0,92
Q9NRW4	Dual specificity protein ph	DUSP22	S58	High	1,99	1,65	0,83
Q8N9U0	Tandem C2 domains nuclear protein	TC2N	S168	High	2,21	1,30	0,59
Q8TDB6	E3 ubiquitin-protein ligase DTX3L	DTX3L	S221	High	0,68	0,37	0,54
Q99501	GAS2-like protein 1	GAS2L1	S489/ S490	High	2,54	1,63	0,64
Q8TD55	Pleckstrin homology domain-containing family O member 2	PLEKHO2	S389/ S390	High	0,42	0,66	1,57
Q9Y6M1	Insulin-like growth factor 2 mRNA-binding protein 2	IGF2BP2	S161/ S162	High	1,62	0,47	0,29

Samenvatting

Hoofdstuk 1: Circulerende biomarkers voor het voorspellen van hart- en vaatziekte: een systematisch literatuuronderzoek en een veelomvattend overzicht van meta-analyses. Dit hoofdstuk beschrijft een systematisch literatuuronderzoek naar serologische biomarkers voor hart- en vaatziekte. Meta-analyses combineren de resultaten van eerder uitgevoerde studies en presenteren één resultaat voor alle studies, om zodoende een overzicht van de resultaten te bieden. Maar ook het aantal meta-analyses is behoorlijk gegroeid in aantal, wat de vraag deed rijzen naar een veelomvattend overzicht waarmee de relevantie van elke biomarker vergeleken kan worden. Daarom hebben we een systematisch literatuuronderzoek gedaan naar meta-analyses die deze vergelijking mogelijk maakt. Een veelomvattende zoekopdracht in de databases Medline en Embase resulteerde na selectie uiteindelijk in 85 artikelen, waarin 214 meta-analyses waren gedaan. Voor een eerste cardiovasculair incident hebben fibrinogeen, C-reactive protein, apolipoprotein B, apolipoprotein A/apolipoprotein B ratio, cholesterol, high density lipoprotein en vitamine D een sterk voorspellende waarde. Voor een cardiovasculair incident bij mensen die al een geschiedenis hebben met hart- en vaatziekte hebben cardiac troponine I en T, C-reactive protein, serum creatine en cystatin C een sterk voorspellende waarde. Voor een beroerte binnen populaties zonder een geschiedenis met hart- en vaatziekte hebben fibrinogeen en serumurinezuur een sterk voorspellende waarde. We concluderen dat markers die een sterk voorspellende waarde voor een eerste cardiovasculair incident hebben vooral geassocieerd zijn met lipides. Voor een cardiovasculair incident bij mensen met een geschiedenis met hart- en vaatziekte zijn biomarkers vooral geassocieerd met ischemie. Fibrinogeen is een sterke voorspeller voor beroerte in een populatie zonder een geschiedenis met hart- en vaatziekte.

Hoofdstuk 2: Verminderde bloedplaatjesrespons is geassocieerd met de mate van coronair vaatlijden en incidentie van secundaire hart- en vaatziekte. In dit hoofdstuk toetsten we de hypothese dat het fuseren van granules met het bloedplaatjesmembraan in respons op stimulatie met verschillende bloedplaatjesagonisten gerelateerd zou zijn aan de mate van de klachten naar aanleiding van hart- en vaatziekte en dat het bovendien een voorspellende waarde zou kunnen hebben voor toekomstige klachten. Daarvoor hebben we de bloedplaatjesrespons getest in een cohort van 484 patiënten die gediagnosticeerd waren met stabiele pijn op de borst, onstabiele pijn op de borst, of klachten hadden door een zogenaamde “non-ST elevation myocardinfarct” (nSTEMI). De bloedplaatjesfunctietest die we gebruikten meet de fusie van granules

met het celmembraan. Dit door expressie van P-selectine in respons tot toenemende concentraties van $P2Y_{12}$-agonist ADP, IP-receptoragonist iloprost samen met een submaximale $P2Y_{12}$-stimulatie, thrombinereceptoragonist (PAR-1 agonist) en agonist voor de collageenreceptor GPVI. We vonden dat bloedplaatjes van patiënten met onstabiele pijn op de borst en met nSTEMI sterk verminderd reageerden op ADP en IP-agonist met submaximale $P2Y_{12}$-stimulatie, in vergelijking met patiënten met stabiele pijn op de borst. De effectiviteit van anti-$P2Y_{12}$-medicatie is een belangrijk onderwerp in behandeling van cardiovasculaire ziekte. Met onze ADP-test konden we goed discrimineren tussen patiënten op anti-$P2Y_{12}$-medicatie en patiënten die niet met anti-$P2Y_{12}$-medicijnen werden behandeld. We hebben een afkapwaarde vastgesteld waarmee we patiënten konden identificeren die ondanks hun anti-$P2Y_{12}$-medicatie nog steeds een hoge ADP-respons hadden. Patiënten met deze zogenaamde "high on treatment platelet reactivity" hadden geen verhoogd risico op een toekomstige cardiovasculair incident. Ook vonden we geen significante associatie tussen toekomstige incidenten en de bloedplaatjesrespons op ADP, IP-agonist met submaximale ADP-stimulatie, PAR-1-agonist of GPVI-agonist.

Hoofdstuk 3: De relatie tussen "fractional flow reserve", bloedplaatjesresponsiviteit en bloedplaatjesleukocytcomplexen in patiënten met stabiele hart- en vaatziekte. Bloedplaatjesfunctie wordt beïnvloed door zogenaamde afschuifkrachten die ontstaan door weerstand tussen het bloed en de vaatwand of vloeistoflagen die met verschillende snelheid langs elkaar stromen. In dit hoofdstuk wilden we de hypothese testen dat de aanwezigheid van significant vernauwende stenoses in de coronair arterie invloed heeft op de bloedplaatjesresponsiviteit en bloedplaatjesleukocytcomplexen. Hiervoor werden de mate van vernauwing van de coronair arterie van 100 patiënten met stabiele pijn op de borst gemeten met behulp van zogenaamde "fractional flow reserve" (FFR), wat een ratio geeft van de druk voor en na een vernauwd bloedvat. Dit hebben we vergeleken met de bloedplaatjesresponsiviteit die gemeten is met dezelfde methode als in hoofdstuk 2, en met de hoeveelheid bloedplaatjesleukocytcomplexen. We vonden geen verschil in de bloedplaatjesresponsiviteit tussen patiënten met een lage FFR en een hoge FFR. Wel vonden we een significant lager percentage circulerende bloedplaatjesneutrofielcomplexen in patiënten met een lage FFR en een vergelijkbare, maar niet significante afname in het percentage circulerende bloedplaatjesmonocytcomplexen. We stellen voor dat deze afnames veroorzaakt worden door het verhoogd uittreden van bloedplaatjesleukocytcomplexen uit de circulatie naar de bloedvaatwand in patiënten met een hevigere ziekte.

Hoofdstuk 4: Citalopram is een sterkere bloedplaatjesfunctieremmer dan paroxetine in een case-control studie. Serotonine is een van de meest talrijke stoffen in de zogenaamde "dense" granules in bloedplaatjes, en autoactiveert bloedplaatjes via hun 5-HT-receptoren. Bloedplaatjes nemen serotonine op uit de bloedstroom met behulp van serotoninetransporters in hun membraan. Dit kan worden geremd door selectieve serotonineheropnameremmers (zogenaamde "selective serotonin reuptake inhibitors; SSRI's"). Eerder uitgevoerde studies op SSRI-gebruik en bloedplaatjesfunctie includeerden individuen die naast hun SSRI's ook bloedplaatjesremmers gebruikten. Andere studies pasten geen stratificatie toe op het type SSRI dat werd gebruikt ondanks dat verschillen in effectiviteit van verschillende SSRI's wel is beschreven. In dit hoofdstuk hebben we bloedplaatjesfunctie gemeten met flowcytometrie en de "platelet function analyzer" (PFA) in 13 patiënten die of paroxetine of citalopram gebruikten, maar geen andere medicatie waarvan bekend is dat ze bloedplaatjesfunctie beïnvloeden. Deze metingen vergeleken we met de resultaten van 20 gezonde controles. We vonden dat patiënten die citalopram gebruikten een sterk verminderde bloedplaatjesrespons hadden in reactie op ADP, PAR-1-agonist en GPVI-agonist vergeleken met controle-individuen en patiënten die paroxetine gebruikten. Deze bevindingen zijn in overeenstemming met de eerder beschreven effectiviteit van elk afzonderlijke SSRI. The metingen met de PFA gaven geen verschil, wat waarschijnlijk wordt veroorzaakt door de eerder gerapporteerde ongevoeligheid van deze test.

Hoofdstuk 5: Selectieve remming van $\alpha_{IIb}\beta_3$ duidt op bloedplaatjesfunctie onafhankelijk van aggregatie. Bloedplaatjes zijn belangrijk voor het behouden van hemostase, maar ze hebben ook een belangrijke rol in wondheling, ontsteking, bloedvatformatie en atherogenese door het lossen van cytokines, chemokines en groeifactoren uit hun granules. In dit hoofdstuk laten we zien dat naast $P2Y_{12}$-antagonisme, stimulatie van de IP-receptor op een selectieve wijze de activatie van $\alpha_{IIb}\beta_3$ remt tijdens PAR-1 en PAR-4 gemedieerde activatie, terwijl hun granules vrijwel ongeremd losgelaten worden. Dit fenomeen is geassocieerd met een ontstekingscapaciteit, want $P2Y_{12}$-antagonisme gedurende PAR-1-activatie leidt tot onverminderde plaatjesleukocytcomplexformatie en onverminderd vrijkomen van cytokines, chemokines en groeifactoren. Door het remmen van PI3-kinase of door het verhogen van cAMP-concentraties, wat intervenieert met twee cellulaire reacties na $P2Y_{12}$-activatie, wordt $\alpha_{IIb}\beta_3$-activatie bijna volledig geremd, maar worden de granules onverminderd gelost. We stellen voor dat het selectief lossen van granules in afwezigheid van activatie van $\alpha_{IIb}\beta_3$ een belangrijke

eigenschap van bloedplaatjes is om hun rol in ontsteking, bloedvatgroei en atherogenese te ondersteunen.

Hoofdstuk 6: Doelgerichte fosfotyrosineproteoomprofilering van GPVI-signalering laat zien dat oligophrenine-1 bij filopodiaformatie van bloedplaatjes betrokken is. Een van de onderdelen van de subendothele matrix is collageen. Via stimulatie van de GPVI-receptor is dit een van de sterkste bloedplaatjesatimulatoren. Bloedplaatjesactivatie na GPVI-stimulatie leidt tot een uitgebreide signaleringscascade binnen de cel, welke resulteert in vormverandering van het bloedplaatje, het lossen van granules en activatie van $\alpha_{IIb}\beta_3$. In dit hoofdstuk verkennen we met kwantitatieve massaspectrometrie in detail de fosfotyrosinesignalering in bloedplaatjes na stimulatie van GPVI. We verkenden 214 unieke fosfotyrosines, waarvan 30 een significante toename in fosforylatie lieten zien na GPVI-stimulatie. Bij deze 30 zat ook oligophrenine-1 (OPHN1), een zogenaamde "Rho-GTPase activating protein" welke nog niet eerder in menselijke bloedplaatjes beschreven was. We konden vervolgens een functionele analyse van de bloedplaatjes van een klein groepje patiënten zonder OPHN1 doen. Dit liet zien dat, in overeenstemming met de rol in neuronen, OPHN1 een rol heeft in filopodiaformatie van bloedplaatjes tijdens het spreiden op een collageenoppervlak. Verdere analyse op de bloedplaatjesfunctie liet zien dat de bloedplaatjes zonder OPHN1 normaal functioneren, met de uitzondering van de verminderde filopodiaformatie.

Hoofdstuk 7: Massaspectrometrieanalyse laat zien dat in reactie op PAR-1 en PAR-4-stimulatie vergelijkbare factoren worden gelost en vergelijkbare fosforylatieprofielen te zien zijn. Bloedplaatjes spelen een rol in de regulatie van ontsteking, angiogenese en atherogenese. Eerdere studies hebben beschreven dat er differentiële lossing van pro-angiogene of anti-angiogene factoren plaatsvindt vanuit de α-granules na PAR-1 of PAR-4-stimulatie en dat dit belangrijk zou zijn voor de regulatie van angiogenese. In dit hoofdstuk hebben we als doel om groepen van pro- of anti-angiogene proteïnes te identificeren die worden gelost in respons op of PAR-1-stimulatie of PAR-4-stimulatie. Daarvoor hebben we met kwantitatieve massaspectrometrie bekeken wat bloedplaatjes lossen na PAR-1-stimulatie en PAR-4-stimulatie en dit vergeleken met ongestimuleerde bloedplaatjes. Om te bepalen of differentiële activatie van bloedplaatjes met een voorkeur voor of pro-angiogenese of anti-angiogenese geassocieerd is met verschillende intracellulaire signaleringsmechanismes, hebben we het fosfoproteoom van PAR-1 en PAR-4 gestimuleerde bloedplaatjes vergeleken. In plaats van verschillen in de lossing van plaatjes na PAR-1 of PAR-4-stimulatie zagen we een sterke correlatie tussen de

mate van lossing van 93 eitwitten die gelost worden na één van beide stimuli. Deze resultaten konden we bevestigen met behulp van ELISA-metingen op verschillende eiwitten die in de α-granules zitten. Stimulatie van bloedplaatjes met PAR-1 of PAR-4-agonist liet ook vergelijkbare fosforylatiepatronen zien op Western blot. Ook massaspectrometrieanalyse liet vergelijkbare fosforylatiepatronen zien na een van beide stimuli. We concluderen dat er geen differentiële lossing is voor de 93 eiwitten die we hebben geïdentificeerd met massaspectrometrie. Bovendien is er weinig steun voor differentiële signaaltransductie na PAR-1 of PAR-4-stimulatie.

Summary

Chapter 1: Circulating biomarkers for predicting cardiovascular disease risk; a systematic review and comprehensive overview of meta-analyses. This chapter provides a systematic review of the literature on serological biomarkers on cardiovascular disease. To provide overview on potential biomarkers, meta-analyses combine the results of previously performed studies to present one result for all studies. The number of meta-analyses has become overpowering, which fed the need for a comprehensive study comparing the relevance of each biomarker. Therefore we performed a systematic review of meta-analyses on levels of serological biomarkers for atherothrombosis to compare the relevance of the most commonly studied biomarkers. After a comprehensive search on the Medline and Embase literature database, we identified 85 relevant full text articles, with 214 meta-analyses. Markers for primary cardiovascular events include fibrinogen, C-reactive protein, apolipoprotein B, the apolipoprotein A/ apolipoprotein B ratio, cholesterol, high density lipoprotein, and vitamin D. Markers for secondary cardiovascular events include cardiac troponins I and T, C-reactive protein, serum creatinine, and cystatin C. For primary stroke, fibrinogen and serum uric acid are strong risk markers. We conclude that for primary cardiovascular events, markers with strong predictive potential are mainly associated with lipids. For secondary cardiovascular events, markers are more associated with ischemia. Fibrinogen is a strong predictor for primary stroke.

Chapter 2: Reduced platelet responsiveness is associated with coronary artery disease severity, and short-term incidence of secondary disease. In this chapter we have tested the hypothesis that platelet granule release in response to stimulation with specific platelet agonists is related with cardiovascular disease severity and the risk on future adverse events. We measured the platelet response of 484 patients that were diagnosed with

stable angina, unstable angina, or non-ST elevation myocardial infarction (nSTEMI). The assay used measures granule release by means of P-selectin expression in response to increasing concentrations of either $P2Y_{12}$ agonist ADP, increasing concentrations of IP receptor agonist iloprost in presence of suboptimal ADP, increasing concentrations protease activated recepting (PAR)-1 agonist, or increasing concentrations of glycoprotein (GP)VI agonist collagen related peptide (CRP-XL). We found that patients with unstable angina and nSTEMI had a strong reduced response to ADP, and IP agonist with suboptimal ADP stimulation compared with patients without unstable coronary artery disease (CAD). Effectiveness of anti-platelet treatment is an important topic in cardiovascular disease treatment. We found that our ADP response assay was able to identify patients on anti-$P2Y_{12}$ treatment and those not on anti-$P2Y_{12}$ treatment. We determined a threshold value for the ADP response to identify patients with high platelet reactivity despite their anti-$P2Y_{12}$ medication. However, this high on treatment platelet reactivity did not show a significant association with future adverse cardiac events. Additionally, no association with future adverse events was found with platelet response to stimulation with ADP, IP agonist with suboptimal ADP stimulation, PAR-1 agonist, or glycoprotein VI agonist.

Chapter 3: The relationship between fractional flow reserve, platelet reactivity and platelet leukocyte complexes in stable coronary artery disease. Platelet function is influenced by microshear gradients caused by friction forces on the arterial wall or flow separation, and form complexes with leukocytes. In this chapter we hypothesized that the presence of significantly flow-limiting stenoses in the coronary artery is associated with altered platelet reactivity and formation of platelet-leukocyte complexes. To test this, the coronary stenoses of 100 patients with stable angina were assessed with Fractional Flow Reserve (FFR), which provides a ratio of pressure before and after a narrowing of the artery. This was compared to their platelet responsiveness, which was measured with the same assay as in chapter 2. In addition we measured the amount of platelet–leukocyte complexes. We found no differences in platelet response between patients with a low FFR indicating a flow-limiting stenosis, and high FFR. We found a significantly lower percentage of circulating platelet-neutrophil complexes in patients with a low FFR, and a similar non-significant decrease in percentage of circulating platelet-monocyte complexes. We propose that these reductions are caused by platelet leukocyte extravasation in patients with more severe disease.

Chapter 4: Citalopram is a more potent platelet function inhibitor than paroxetine in a case-control study. Serotonin is a major component of platelet dense-granules and supports platelet activation via activation of the platelet 5-HT receptors. Platelets take up serotonin via serotonin transporters in their membranes, which can be inhibited by selective serotonin reuptake inhibitors (SSRI's). Previous studies performed on SSRI use and platelet function either included individuals using anti-platelet medication, or did not stratify on the type SSRI although differences in effectiveness of the medication are described. We measured platelet function with flow cytometry and platelet function analyzer (PFA) of 13 patients using either paroxetine or citalopram but no other medication that influences platelet function, and 20 controls. We found that patients using citalopram have a strong reduction in platelet responsiveness in response to ADP, PAR-1 agonist and GPVI agonist compared with controls and patients using paroxetine. These findings are in line with the described effectiveness of the SSRI's. The measurements with the PFA did not show any differences, which is most likely due to the reported insensitivity of the assay.

Chapter 5: Selective $\alpha_{IIb}\beta_3$ inhibition indicates platelet function independent of aggregation. Platelets are known for their crucial role in maintaining adequate haemostasis, but they are also involved in wound healing, inflammation, angiogenesis, and atherogenesis via the release of cytokines, chemokines, and growth factors from their α-granules. In this chapter, we show that in addition to $P2Y_{12}$ antagonism, stimulation of the IP receptor selectively inhibits PAR-1 or PAR-4 initiated $\alpha_{IIb}\beta_3$ activation, while granule release remained on full strength. When platelets were isolated from patients on clopidogrel treatment the same partial activation was observed, these platelets are able to release their alpha granules but fail to aggregate. This phenomenon could be associated with an inflammatory potential, as the $P2Y_{12}$ antagonism still allows for platelet leukocyte complex formation and release of cytokines, chemokines, and growth factors. Inhibition of PI3-kinase or increasing cAMP levels, which intervenes with two cellular responses of $P2Y_{12}$ activation, resulted in a complete inhibition of $\alpha_{IIb}\beta_3$ activation, while the release of α-granules stayed almost completely intact. We propose that selective granule release in the absence of $\alpha_{IIb}\beta_3$ activation is an important feature of platelet function in inflammation, angiogenesis and atherogenesis.

Chapter 6: Targeted phosphotyrosine proteome profiling of GPVI signaling implicates oligophrenin-1 in platelet filopodia formation. Collagen is a component of the subendothelium, and via stimulation of GPVI one of the most potent activators of

platelets. Activation of GPVI induces a myriad of signaling events, resulting in platelet shape change, granule release and $\alpha_{IIb}\beta_3$ activation. In this chapter we explore in fine detail phospho-tyrosine signaling events downstream of GPVI with quantitative mass spectrometry. We surveyed 214 unique phosphotyrosine sites, of which 30 showed a significant increase in phosphorylation upon activation of GPVI. Among these was phosphorylation of oligophrenin-1 (OPHN1), a Rho GTPase-activating protein (RhoGAP), which had not previously been described in human platelets. Subsequent functional analysis of unique platelets from a small group of patients with germline mutations in the *OPHN1* gene (oligophrenia) revealed that in line with the neuronal cell defect observed in patients with oligophrenia, OPHN1 does seem to have a specific role in platelet filopodia formation and spreading on collagen. Further analysis on platelet function showed normal platelet function of patients with oligophrenia.

Chapter 7: Mass spectrometry analysis shows similar release and phosphorylation profiles after PAR-1 or PAR-4 stimulation of platelets. Platelets play a role in the regulation of inflammation, angiogenesis and atherogenesis. Earlier studies have described that differential release of pro- and anti-angiogenic factors from platelet α-granules by PAR-1 and PAR-4 may be important for the regulation of angiogenesis. In this chapter, we aim to identify groups of either pro- or anti-angiogenic proteins released by stimulation with PAR-1 or PAR-4 agonist. For this, we analyzed the releasates of unstimulated and PAR-1 and PAR-4 stimulated platelets by quantitative mass spectrometry. To identify whether differential release of platelet granule content in favor of either pro- or anti-angiogenesis is associated with a different intra-cellular signaling mechanisms, we compared the phosphoproteome of PAR-1 and PAR-4 stimulated platelets. In stead of different release quantities of proteins after PAR-1 or PAR-4 stimulation, we found a strong correlation between the 93 factors that are released after either stimulus. Analysis of the platelet releasate with ELISA measurements on different α-granule components supported these results. Downstream signal transduction measurements after stimulation with PAR-1 or PAR-4 agonist revealed similar phosphorylation patterns on Western blot, and the mass spectrometry identified phosphorylation events also showed nearly identical regulations. We conclude that there is no differential release for any of the 93 proteins that came out of the proteomics analysis. Furthermore, there is little support for differential signal transduction downstream of PAR-1 and PAR-4.

Discussion

Platelets in cardiovascular disease

Cardiovascular disease is one of the major causes of death worldwide, and therefore risk assessment is a major focus in clinical and diagnostic management. In the last decades, major improvements in coronary artery disease risk reduction have been achieved by intervention of cholesterol levels, blood pressure and platelet reactivity [2,3]. The use of biomarkers to identify patients at risk for a cardiovascular event has resulted in stratification of patients for individualized therapy. A major focus on biomarker identification has been on levels of plasma markers, as is discussed in chapter 1 of this thesis. There is strong evidence that circulating cells play an important role in the development and pathology of cardiovascular disease [4,5]. Therefore, one of the goals of the Circulating Cells consortium was to evaluate the value of information present in blood cells for the use of diagnostic purposes. Our part of this collaborative approach was to evaluate the information that could be present in platelets. Therefore we investigated the platelet responsiveness as a potential marker for cardiovascular disease severity and future outcome.

Platelet function assays which are currently used in the clinic suffer from major limitations related to specificity, the sensitivity, and reproducibility [6-10]. This challenged us to develop a novel technique to evaluate platelet function. The assay that was used to measure platelet function in the Circularing Cells cohort relies on a dose-dependent activation of platelets with specific agonists, and measuring the activation status by P-selectin expression. This method of measuring platelet activation has been shown to be powerful in discrimination of individuals by platelet responsiveness [10].

Our original hypothesis was that we would find increased platelet responsiveness in patients with increased risk for coronary artery disease (CAD). In contrast, we found that platelet response to ADP was reduced in patients with unstable CAD and non ST-elevation myocardial infarction (nSTEMI), compared with patients without unstable CAD (Chapter 2, this thesis). In addition, we found that anti-platelet medication enhanced this association. These findings suggest that patients with a reduced ability to respond hemostatically to small bleeding episodes inside plaques are at increased risk to develop instable CAD. This is in line with the notion that intra-plaque hemorrhage contributes to instable plaque development, and the in our study observed interaction with anti-platelet medication [11]. This means that the classic trade-off of anti-platelet medication, prevention of thrombus formation versus increased bleeding risk, should include the risk of development of an instable plaque. This should be

considered when decided to apply an anti-platelet medication strategy. The data presented in this manuscript could have important consequences and therefore they should be confirmed in more studies both clinically and experimentally, before firm conclusions can be drawn.

The assay used in the Circulating Cells cohort showed to be a reliable tool in discriminating patients on anti-$P2Y_{12}$ treatment, and patients not on $P2Y_{12}$ treatment. By means of receiver operating characteristic analysis, we set a threshold value above which patients still have significant platelet reactivity despite being on anti-$P2Y_{12}$ treatment. The amount of high on treatment platelet reactivity patients is comparable to results obtained with other techniques (Chapter 2, this thesis, [12]). In addition to the earlier mentioned advantages on reproducibility and specificity, the assay as presented in this thesis only requires 300 μL of whole blood. The reduction of the volume together with absence of need for centrifugation provides the possibility to draw blood by means of a finger prick. The assay used in the Circulating Cells cohort has made significant improvements on agonists used and on parameters measured to assess platelet activity. This will be discussed furtheron in this chapter. Therefore, the assay as described in this thesis provides a good alternative to the current available diagnostic assays.

Platelet function monitoring

One of the major benefits of platelet function monitoring compared with plasma marker measurement for cardiovascular disease risk stratification is the ability monitor and adjust therapeutical intervention of platelet function. The use of aspirin and anti-$P2Y_{12}$ medication has lead to major improvements in clinical outcome of coronary artery disease patients [2,13]. In addition, monitoring of anti-$P2Y_{12}$ efficacy has indicated that patients with high platelet reactivity despite being on anti-platelet treatment are at increased risk for secondary events. Although in several studies, monitoring of drug efficiency indicated that intensive treatment of these patients resulted in improved results, recent large studies showed the contrary [14-16]. A likely explanation for these findings is that atherosclerosis is a multifaceted disease, in which not only platelet hyperreactivity predisposes patients to increased CAD risk. Introduction of therapeutics aimed at reducing blood pressure, reducing cholesterol levels, and percuteous coronary intervention have lead to major improvements with regard to mortality due to cardiovascular disease [3]. In addition, although in the study of Collet et al. the effectiveness of anti-platelet treatment monitoring was comparable to a group not monitored on anti-platelet treatment effectiveness, on the individual level anti-$P2Y_{12}$ therapy monitoring will work [15]. One of the major drawbacks of this study was the

late availability of prasugrel, which resulted in administration of 3.3% of the patients with high on treatment platelet reactivity with the alternative anti-$P2Y_{12}$ therapeutic. Other alternative therapeutic strategy was administration of $\alpha_{IIb}\beta_3$ inhibitors, which only 30% of the high on treatment platelet reactivity patients received.

Clopidogrel unresponsiveness is partly dependent on conversion in the liver of the anti-$P2Y_{12}$ drug clopidogrel into its active metabolite [14]. Multiple studies have indicated that insufficient conversion of clopidogrel to its active metabolite is associated with increased risk for CAD [14]. Although for certain patients the issue of anti-platelet medication irreponsiveness can be resolved by simply increasing the dose of clopidogrel, use of alternative anti-$P2Y_{12}$ medication such as prasugrel and ticagrelor in patients with a more severe inability to convert clopidogrel may prove to be beneficial [14]. New cohort studies are required for a more adequate evaluation the effectiveness of anti-$P2Y_{12}$ monitoring with use of alternative anti-$P2Y_{12}$ medication.

New opportunities for high on treatment platelet reactivity assessment

Currently, the absolute ADP platelet response is used to assess the anti-$P2Y_{12}$ therapy response. Patients with high on treatment platelet reactivity are classified by a threshold determined with a receiver operating characteristics. Those patients with a response above this threshold are supposed to have an increased cardiovascular disease risk [14]. One of the complications with such an approach is that patients with an overall low ADP response, but a poor response to anti-platelet medication are still considered as low risk patients. An overall low ADP response should not be underestimated as it has a very important role as a co-stimulator [14].

This issue concerns all commercially available platelet function techniques which all measure absolute ADP response, except the Verify Now that uses normalization for the ADP response. The Verify Now relies on aggregation though, on which flow-cytometry has been proven to be superior to in terms of specificity [10]. Measurement of platelet response before and after anti-platelet treatment can account for this, but this is unattractive due to the risk of supposed over- and underestimation [14], and it requires multiple measurements.

The finding that PAR-1 induced $\alpha_{IIb}\beta_3$ activation and not P-selectin expression is affected by clopidogrel treatment gives the opportunity to determine effectiveness of clopidogrel treatment in one assay. The release of granules and P-selectin expression upon PAR stimulation is hardly affected by anti-$P2Y_{12}$ treatment, because this is mainly dependent on activity of phospholipase C. Activation of $\alpha_{IIb}\beta_3$ is highly dependent on co-stimulation via the $P2Y_{12}$ receptor, which is required for activation of PI3-kinase

and reduction of cyclic AMP levels. A ratio of P-selectin activation and $\alpha_{IIb}\beta_3$ activation gives an indication of anti-$P2Y_{12}$ treatment effectiveness and can be obtained in one sample measurement. Whether a reduced clopidogrel response is due to liver enzyme polymorphisms can be determined by a parallel measurement with added $P2Y_{12}$ antagonist included in the assay. This novel approach may identify non-responding patients that otherwise would fall underneath a threshold, but may benefit from alternative medication. Future research is required to establish whether improved discrimination can be obtained using this technique, and whether this may improve personalized anti-platelet medication.

Increased complexity in platelet function with a central role for the PAR's

Historically, platelet function has been dedicated mainly to hemostasis. Over the last decade, platelets are more and more recognized for their role in inflammatory diseases such as atherosclerosis, and rheumatoid arthritis [4,5,17]. In addition, platelets contain factors that are involved in angiogenesis and have therefore been described to have a role in tumor development and in maintaining the vasculature ([18,19], this thesis Chapter 7). Interestingly, platelets contain both pro-and anti-inflammatory and angiogenic proteins. Studies have described differential granule release in response to stimulation with either PAR-1, or PAR-4 as a mechanism to control release of pro- or angiogenic factors [18-21]. Although these publications got a lot of attention when published, it has become apparent that platelets actually lack this feature in vitro, and these findings can be ascribed to flaws in methodological approaches ([22], Chapter 7 this manuscript).

With regard to differential platelet activation, we have described that platelets actually can be selectively activated with regard to their functions release of growth factors and aggregation. So far, landmark reviews on platelet function have considered activation of $\alpha_{IIb}\beta_3$ and granule release as equally matched expressions of platelet activation [4,5,23], suggesting that the hemostatic function of platelets and the inflammatory capacity of platelets are intertwined (Chapter 5 of this manuscript). We have found PAR-1 or PAR-4 stimulated platelets release their granular content despite platelet inhibition via $P2Y_{12}$ antagonism or stimulation of the IP receptor, whereas the activation of $\alpha_{IIb}\beta_3$ is fully inhibited. The endothelium antagonizes $P2Y_{12}$ activation by surface expression of CD39, an ADP degrading enzymes [24], and releases prostacyclin that stimulates the IP receptor. We believe that selective release of the granules may play an important role in human physiology. In addition, P-selectin interactions and release of inflammatory proteins have an important role in atherogenesis [4,5]. This indicates that selective activation of platelets may also have an important role in

atherogenesis. It would be interesting to investigate whether in an atherosclerotic mouse model intervention with the PAR receptors has an effect on the disease progression. Alternatively, it may be interesting to evaluate the activity of thrombin in patients by means of thrombin-antithrombin (TAT) measurements in relation to their disease severity. If an association exists, PAR antagonism may have new therapeutic potential.

Opportunities to investigate novel players in platelet biology

Platelets and neuronal cells have many similarities, and in this thesis some of these similarities have been described. We have shown that the effect of selective serotonin re-uptake inhibitors (SSRI), which are developed as anti-depressant drugs by prohibiting serotonin uptake from the neuronal synapse also inhibit the serotonin uptake of platelets and thereby their activation response. This effect was associated with the therapeutic potential of the SSRI [25]. In addition, we characterized a novel protein oligophrenin-1 (OPHN1) that is involved in the intracellular collagen activation pathway of platelets by analyzing the platelet function from individuals that are deficient for this protein. OPHN1 deficiency has been described in relation to a mental retardation syndrome, where through absence of its effect on Rho-GTPases affects the ability of neurons to polymerize actin and to form dendrites [26-28]. We showed that the platelets of patients with a deficiency in OPHN1 have impaired filopodia formation, which reflects the disability of neurons to form dendrites.

The similarities between platelets and neuronal cells have been acknowledged already in the 1980's [29], where it was proposed that platelets can be used as models to study neuronal cell function. Like neuronal cells, platelets release granules upon activation and this process is also dependent on calcium [30]. Similarities extend also to receptor expression such as glutamate receptors and GABA receptors [30]. In addition, platelet function is affected in disorders such as schizophrenia and autism [30]. Genes that are associated with autism also affect platelet dense granule release [31].

Vice versa, mental disabilities with a genetic background provide new opportunities to reveal unknown players in platelet function. This is illustrated by the results obtained from the study on OPHN1. With this in mind, we investigated the effect of the deficiency of ARHGEF9 on blood platelet function, which is an ongoing study. ARHGEF9, also known as collybistin, is a CDC42 guanine nucleotide exchange factor (GEF) and therefore, like OPHN1, is involved in regulation of Rho-GTPases. It regulates actin cytoskeletal dynamics and it binds gephyrin, thereby being involved in GABAergic and glycinergic neurotransmission [32,33]. These aspects of ARHGEF9 are described to be the cause of the effects on mental development in case of its deficiency

[33]. ARHGEF9 has not been described previously in platelets, but with Western blot we were able to show the presence of ARHGEF9 in platelets from healthy donors, but its absence in a patient with a mental disability suspected to be due to an ARHGEF9 mutation. Responsiveness to different agonists and aggregate formation under flow on a collagen coated surface was impaired with ARHGEF9 deficient platelets. In agreement with its proposed role in neuronal cells, we also found that upon platelet activation actin polymerization into filaments was reduced. These findings once more underline the similarities between platelets and neuronal cells, and the scientific opportunities to further elucidate platelet biology.

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List of publications

Targeted phosphotyrosine profiling of GPVI signaling implicates oligophrenin-1 in platelet filopodia formation. Van Holten TC, Bleijerveld OB, Preisinger C, Van der Smagt JJ, Farndale RW, Kleefstra T, Willemsen MH, Urbanus RT, De Groot PG, Heck AJR, Roest M, Scholten A. *Accepted manuscript in Arteriosclerosis, Thrombosis and Vascular Biology, 2013.*

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Curriculum Vitae

Thijs Cornelis van Holten was born on the 20th of October 1983 in Leidschendam. After completing high school at the Bataafse Kamp in Hengelo, he studied Biomedical Sciences at the University of Utrecht. After an internship at the Cellular Protein Chemistry Laboratory at the University of Utrecht, his interest for clinical orientated and applied science was initiated. This led him to do his master thesis on hematology at the Laboratory for Clinical Chemistry and Hematology at the University Medical Center in Utrecht, and to do an internship for writing a business plan for a university spin-off Cristal Delivery. In addition, he did a 6 month course on Fundamentals of Business and Economics at the University of Utrech, in cooperation with the University of Nyenrode in 2008.

Following graduation, he started to work as a PhD-student at the Laboratory of Clinical Chemistry and Hematology at the University Medical Center in Utrecht under the supervision of prof. dr. Ph.G. de Groot and dr. M. Roest. During his work as PhD-student, Thijs obtained a NVTH Science Prize in 2011. The results of his research performed at the Laboratory of Clinical Chemistry and Hematology at the University Medical Center in Utrecht are described in this thesis.