Platelet Hyperreactivity: Predictive and intrinsic properties

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Abstract

Platelet thrombi are central to the development of most vascular ischemic events. There is marked inter-individual variation in platelet responsiveness, with some subjects displaying platelet hyperreactivity. An increasing number of reports indicate there are laboratory measures of platelet function that predict clinical thrombotic events. Some, but not all measures of platelet function are reproducible. Platelet hyperreactivity can be assessed with multiple stimuli in multiple assays, and is more likely to be present in females and in subjects with elevated fibrinogen levels.

Keywords

platelets; platelet reactivity; platelet aggregation; coronary heart disease

Introduction

Occlusive vascular diseases, such as myocardial infarction (MI), are the major causes of mortality and morbidity in the U.S. MI and other acute coronary syndromes develop when a platelet thrombus forms at the site of a ruptured or eroded unstable coronary atherosclerotic plaque. Pathologic platelet thrombi also cause ischemia in the brain (stroke) and extremities (peripheral arterial disease). There is increasing evidence that enhanced platelet reactivity can *prospectively* identify subjects at risk for these conditions. Increased platelet reactivity may also mitigate hemorrhage in inherited or acquired bleed conditions. There is a well-known inter-individual variation in platelet reactivity, which may contribute to the risk of thrombosis in one individual, but protection in another. On the other hand, reduced platelet function may also contribute to hemorrhagic disorders, usually in very different clinical settings. This review will consider inherent differences in platelet function and the clinical relevance of platelet hyperreactivity. The first section will examine patient studies linking ex vivo platelet functional assays to clinical events. The second section will consider those variables and subject characteristics associated with platelet hyperreactivity.

Platelets in arterial thrombosis

The pathophysiology of arterial thrombosis is complex, with many genes dictating physiologic and biochemical traits that modify one another to increase or decrease the risk for developing adverse clinical outcomes. Environmental factors also impinge on the risk, as do age and gender. Figure 1 emphasizes the central role of platelets in the pathogenesis of clinical thrombosis in arterial disease. The position of platelets in acute coronary syndromes is well

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established and is grounded in physiologic, pathologic, and clinical studies [1-3]. Platelet deposition onto the subendothelium is proportional to the shear rate [4], such that platelets play a particularly important role in arterial thrombosis. Upon arterial plaque rupture, von Willebrand factor (VWF) molecules are rapidly localized to the subendothelium and the initial platelet contact with the wound is a tethering of platelets to this insoluble form of VWF. The glycoprotein (GP) GPIbα subunit of the GPIb-IX-V complex [5,6] mediates this tethering to VWF, resulting in a slower platelet velocity, which in turn permits platelet GPVI to bind to collagen [7]. Signaling between and through GPIbα and GPVI causes platelet activation, resulting in secretion, firm platelet adhesion through activated integrins: α2β1 binding to exposed collagen and GPIIb-IIIa (integrin αIIbβ3) binding to VWF and fibrinogen [8]. An expanding thrombus ensues when platelets aggregate via the intercellular bridging of fibrinogen and VWF binding to the activated conformation of GPIIb-IIIa. Blood flow ceases when an occlusive platelet plug forms. The importance of GPIIb-IIIa, GPIbα and α2β1 is underscored by the moderate to severe bleeding seen in patients with inherited defects in these genes.

Platelet assays as predictors of CHD events

Substantial evidence has accumulated linking platelet hyperreactivity with vascular thrombotic diseases. Table 1 lists conditions in which enhanced platelet reactivity have been demonstrated. Other than heparin-induce thrombocytopenia, the mechanisms responsible for the hyperreactivity are generally poorly understood. Most studies in this area have included patients with acute coronary events, and have measured ex vivo markers of platelet activation. For example, patients admitted to the coronary care unit have increased levels of circulating P-selectin [9]. However, cross-sectional studies cannot distinguish the cart from the horse; i.e., did underlying platelet hyperreactivity contribute to the thrombotic event? Or did the stress of the acute event induce platelet activation? The plethora of cross-sectional studies will not address these questions. Prospective studies are required to appropriately test the hypothesis that ex vivo platelet reactivity predicts coronary heart disease (CHD) outcomes. In fact, a modest body of literature exists supporting this hypothesis. The design of most prospective studies is as follows: 1) high-risk patients are identified, 2) any of a variety of platelet functional assays are performed, and 3) patients are followed for variable periods of time for a variety of different outcomes. Table 2 lists the different platelet assays that have been utilized in these types of clinical studies.

The vast majority of research linking platelet reactivity to CHD events has utilized three routine clinical measures of platelet reactivity: standard lumiaggregometry with platelet rich plasma (PRP), the PFA-100® and the mean platelet volume (MPV). As summarized in Table 3A, spontaneous aggregation, and aggregation induced by arachidonic acid, epinephrine, ADP and collagen have all been found to predict clinical CHD outcomes. On the other hand, most studies have not found platelet aggregation predicts re-stenosis at angiography (Table 3B), the exception being one of the two studies by Torres et al. [12]. Both the PFA-100® and the MPV have been found to predict CHD outcomes (Table 3C). Of note, the study by Frossard et al. [19] utilized an intermediate outcome of troponin T levels. The largest study to test whether a platelet parameter predicted a CHD outcome was that of Martin et al. [23]. These investigators identified 2,033 male MI patients, and obtained hematologic studies on 1,716 patients six months post-event. After a follow-up of approximately 24 months 126 patients had experienced a CHD death or recurrent MI. The average MPV was significantly higher in those patients who experienced a recurrent ischemic event compared to those who did not (P<0.001). Only one or two prospective studies have been performed that tested the value of the last nine measures of platelet function listed in Table 2, and these will not be reviewed in detail. However, the report by Eikelboom et al. is noteworthy for its sample size [26]. These investigators found

that high levels of urinary thromboxane in patients on aspirin predicted recurrent vascular events.

It must be emphasized that there are limitations in drawing conclusions from these prospective studies (Table 4). The patients comprising the data summarized in Tables 3A-3C are quite heterogeneous. Differences include acute versus chronic disease, coronary versus peripheral vessels, different lengths of follow-up, different endpoints, etc. Most of these studies were rather small – many with fewer than 30 CHD events. There were technical differences as well – the timing of phlebotomy from the acute event, as well as different assays and different agonist concentrations. This type of research also lends itself to publication bias, since a study finding no predictive value for a give platelet assay has less impact. Finally, since most study populations included patients with vascular ischemia, nearly all subjects were on anti-platelet medications. Many of these agents are known to affect the results of in vitro assays. In and of itself, this does not undermine the conclusions from these studies because patients biologically resistant to the platelet inhibitory effects of these agents might be expected to have both worse clinical outcomes and enhanced ex vivo measures of platelet reactivity. However, only a small number of these studies were designed specifically to consider the effect of anti-platelet agents, with the vast majority not considering this variable in the analysis. Having acknowledged these limitations, it is nevertheless fair to conclude that there is a substantial body of evidence – both cross sectional and prospective – supporting the hypothesis that ex vivo platelet reactivity predicts CHD outcomes.

Are these platelet assays clinically useful? This is quite a separate issue, and the answer is largely related to the failure of anti-platelet therapy and whether there are alternative approaches to "rescue" an inadequate ex vivo response to anti-platelet therapy. For example, if a compliant patient taking aspirin for secondary prevention of MI is found to have enhanced epinephrine-induced platelet aggregation and an MPV in the upper quartile of the normal distribution, will they benefit from the addition of clopidogrel? These are important and unresolved questions that need additional clinical research.

Intrinsic platelet function variation

There is a common belief that substantial inter- and intra-individual variation exists in platelet responsiveness. This variation persistently challenges the interpretation of clinical research involving platelet assays. To more rigorously characterize this variation and to determine the factors contributing to this variation, we recruited a large number of healthy subjects to donate blood samples. The design of this study has been previously detailed [36,37] and Table 5 lists the assays that were performed. Figure 2 illustrates the variability observed amongst the first 280 subjects studied, using collagen as the agonist in platelet lumiaggregometry. Similar variability was observed with all other agonists (not shown). Note the striking variation in a healthy population on the "steep" portion of the dose-response curve. At the two extremes (very little stimulation and maximal stimulation) there is considerably less variability, as indicated by the smaller standard deviations. From the point of view of platelet risk factors for arterial thrombosis, the subjects with maximal aggregation at low concentrations of agonist might be considered to have a hyperreactive platelet phenotype. It is this population that is especially interesting. Knowing the characteristics of this group could provide valuable clinical information and provide insights into the molecular basis of platelet reactivity.

Reproducibility of ex vivo platelet assays

Table 6 lists some of the potential sources of variation in platelet functional assays. If one wanted to characterize the contribution of any one of the factors listed in Table 6, then the extent of variation contributed by the other factors must either be minimal or must be known and considered in the analyses. In practical terms, knowing the reproducibility of the platelet

assays becomes critical. Surprisingly little information exists on the reproducibility of platelet function. This is especially important for studies with small numbers of subjects, typical of many clinical studies and virtually all basic research studies. Because of our interest in identifying genes that modify platelet function, we performed a formal reproducibility study of the assays listed in Table 5. Twenty seven subjects were studied on a weekly basis for four weeks. Subjects were studied weekly because of concerns that 1) a longer time interval might introduce new variables, such as age, and 2) a shorter time interval would not account for the effects of new platelet production. For agonist concentrations selected to identify a hyperreactive phenotype, poor reproducibility was observed for collagen and ristocetin, but there was excellent reproducibility for epinephrine and a collagen-related-peptide (CRP) [36]. Reproducibility analyses with submaximal concentrations ADP were inconclusive. Fifteen of the 27 subjects had had platelet function assays 2-25 months prior to the reproducibility study. Fourteen of these 15 showed remarkably consistent results over this time period in response to 0.4 μM epinephrine [36]. This study demonstrated that submaximal concentrations of epinephrine and CRP can be used to assess in vitro platelet hyperreactivity. We recommend that such a classification be limited to subjects whose platelets demonstrate more than 60% aggregation to epinephrine or more than 50% aggregation to CRP on at least 2 occasions.

Platelet hyperreactivity is global

Subjects with hyperreactivity to epinephrine are more likely to exhibit hyperfunction in each major aspect of platelet activity, including adhesion (response to ristocetin), activation (surface P-selectin expression and PAC-1 binding after stimulation) and aggregation to applied shear stress (PFA-100 and cone-and-plate viscometer) [37]. Figure 3 shows that hyperreactivity to epinephrine was strongly associated with hyperreactivity to submaximal concentrations of other agonists. The consistent results using different platelet function assays show that platelet hyperreactivity is not specific to epinephrine mediated aggregation, but generalizes to multiple forms of platelet stimulation. This indicates underlying mechanisms involved in the early aspects of platelet activation and that are shared by multiple pathways affecting distinct aspects of platelet function.

Factors associated with platelet hyperreactivity

Factors associated with platelet hyperreactivity are listed in Table 7. Healthy subjects demonstrating platelet hyperreactivity did not differ significantly from subjects without hyperreactive platelets for age, race, body mass index, smoking status, or the presence of hypertension. The predilection of platelet hyperreactivity for female gender has been noted in other studies in both humans [38-40] and mice [41]. The lack of association between platelet hyperreactivity and oral contraceptive use, menopausal status, or phase of the menstrual cycle at the time of testing among female subjects suggests an underlying genetic basis (XX vs. XY) for this gender difference. Feng et al also found that higher fibrinogen levels were associated with increased aggregation to epinephrine, but other factors must also contribute [37]. It seems likely that no single factor will induce platelet hyperreactivity in most subjects, but it remains to be determined whether combinations of the factors listed in Table 7 increase the risk of this platelet phenotype.

Platelet genes regulating platelet variation

The genetic contributions to coronary artery disease (CAD) and arterial thrombosis are well established [42]. We have observed strong heritability (h^2) in platelet function studies on extended family structures in whites and African Americans [43]. There are well-known examples of inherited gene variations affecting platelet function, the best understood being

mutations that lead to a clinical hemorrhagic phenotype (e.g., Glanzmann thrombasthenia and storage pool diseases).

In 1990 we reported the first genetic variation in a platelet-mediated inherited bleeding disorder [44], and since that time many other platelet gene defects have been described that produce a clinical hemorrhagic phenotype due to platelet hypofunction. Only in the last decade have investigators begun to consider inherited variations that enhance platelet reactivity. In 1996 we reported the first platelet genetic variation associated with acute myocardial infarction (MI) [45]. Since then many platelet genetic epidemiology studies have been performed, but the results have often been inconsistent (for review, see reference [46]). These inconsistencies should not be interpreted to mean platelet genes are not involved in the pathogenesis of these thrombotic disorders. Since thrombosis is one of several components involved in the pathophysiology of pathologic thrombi (such as MI), the effect of a prothrombotic variations are "diluted" by the effects of genes affecting other components of the pathogenesis of the clinical phenotype. Ten of 11 polymorphisms in 8 platelet genes were not associated with platelet hyperreactivity, including functional polymorphisms in genes encoding the α_2 adrenergic receptor, integrins $\beta3$ and $\alpha2$, and GPIbα and GP VI [37]. However, platelet hyperreactivity was associated with the C825T polymorphism in the gene encoding the $\beta3$ subunit of G-proteins.

Summary

Platelets play a central role in ischemic arterial occlusions and enhanced platelet reactivity is a risk factor for clinical CHD events. The use of state-of-the-art genomic, proteomic and RNA expression studies for gene discovery will require rigorously defined platelet phenotypes. Fortunately, platelet hyperreactivity can be easily and reproducibly detected in healthy subjects in the absence of anti-platelet therapy. Formal reproducibility studies are needed in patients taking aspirin and clopidogrel. Platelet hyperreactivity appears to be a global phenomenon, observed with multiple stimuli, suggesting mechanisms that involve the early pathways of platelet activation. There is marked inter-individual variation in platelet reactivity, and this variation is associated with female gender, high plasma fibrinogen and genetic variation. The future identification of novel genetic variants associated with platelet reactivity may be useful predictors of clinical thrombotic events, as well as modifiers of bleeding disorders or pharmacologic effects.

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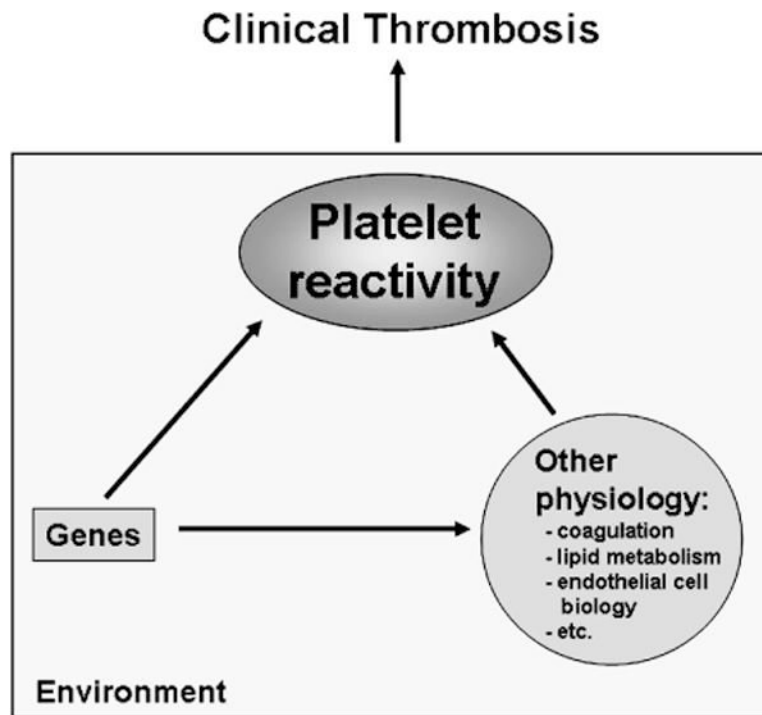
Figure 1. Schema indicating the multifactorial nature of thrombosis
The central role of platelets is emphasized.
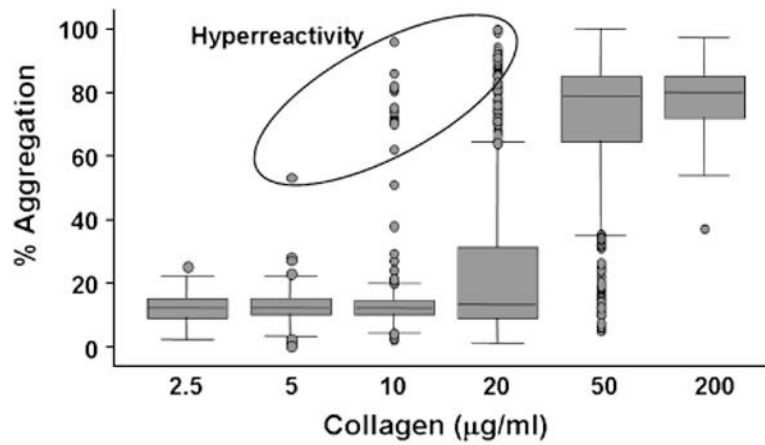
Figure 2. Variation in platelet reactivity
The lumiaggregation response in plateletrich plasma in response to increasing concentrations of type I acid soluble collagen is shown. The box indicates the 25th-75th percentiles, the line in the box indicates the median, the lines outside the box indicate the 10th and 90th percentiles, and the dots are the outliers below the 10th percentile and above the 90th percentile. An arbitrary group of hyperreactive outliers is circled.

* Adjusted for gender, fibrinogen, mean platelet volume, platelet count.
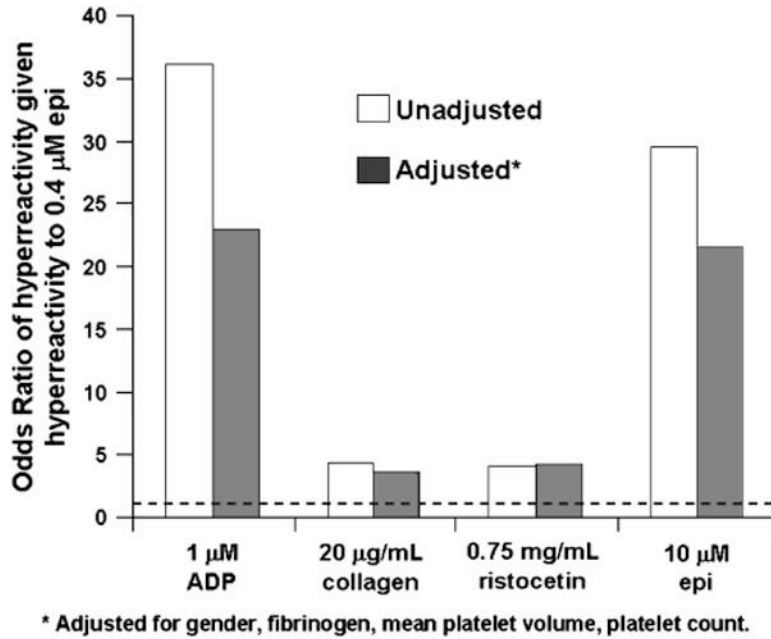
Figure 3. Association between platelet hyperreactivity to epinephrine and hyperreactivity to other agonists
The unadjusted and adjusted Odds Ratios (probability of hyperreactivity to the specified agonist given hyperreactivity to epinephrine/probability of hyperreactivity to the specified agonist given no hyperreactivity to epinephrine) are shown for three different agonists. As expected, hyperreactivity to 0.4 μM epinephrine predicts hyperreactivity to 10 μM epinephrine. The dashed line indicates the position of an OR=1.0. Every bar represents an Odds Ratio that is significant at the <0.001 level.

Table 1

Conditions associated with enhanced platelet reactivity.

Coronary artery disease
Cerebrovascular disease
Peripheral artery disease
Acute coronary syndromes
Percutaneous coronary interventions
Coronary artery bypasses graft surgery
Antiphospholipid syndrome
Myeloproliferative disorders
Heparin-induced thrombocytopenia

Table 2

Platelet measures reported to prospectively predict CHD outcomes.

Assay	Reference
Platelet aggregation	[10-17]
PFA-100®	[18-22]
Mean Platelet Volume	[12,23-25]
Urinary thromboxane	[26]
Platelet count	[11]
Platelet secretion	[27]
Platelet-leukocyte aggregates	[28]
Platelet microaggregation (PA-200®)	[29,30]
Platelet fibrinogen binding	[31,32]
Soluble CD40L	[33]
Platelet nitric oxide responsiveness	[34]
Ultegra RPFA®	[35]

Table 3A

Enhanced platelet aggregation may predict CHD outcomes.

n	Population	n	Outcome	Follow-up (mo)	Predictive	Ref
149	MI survivors	12	Recurrent CHD events	60	Spontaneous	Trip 1990
150	Healthy subjects	11	Fatal events	60	ADP	Thaulow 1991
47	PTCA patients	10	Re-occlusion by angiography	6	epi, ADP, collagen	Terres 1995
326	CAD	34	Recurrent CHD events	3	AA and ADP	Gum 2003[*]
106	NSTEMI → PCI	12	Recurrent CHD events	24	ADP	Cuisset 2006

[*] stratified by aspirin responsiveness

MI, myocardial infarction; CHD, coronary heart disease; PTCA, Percutaneous transluminal coronary angioplasty; NSTEMI, non-ST elevation MI; PCI, Percutaneous coronary intervention; ADP, adenosine diphosphate; epi, epinephrine; AA, arachidonic acid

Table 3B

Enhanced platelet aggregation may not predict re-stenosis.

n	Population	n	Outcome	Follow-up (mo)	Not predictive	Ref
98	PTCA patients	-	Angiography	3	ADP, collagen	Terres 1992
53	CAD	-	Angiography	24	epi, ADP, collagen, PF4; thrombin was predictive	Lam 1994
155	PCTA	-	Angiography	20	Spontaneous	Capanni 1999

CAD, coronary artery disease; PTCA, Percutaneous transluminal coronary angioplasty; ADP, adenosine diphosphate; epi, epinephrine; PF4, platelet factor 4

Table 3C

PFA-100 and MPV may predict CHD outcomes.

	n	Population	n	Outcome	Follow-up (mo)	Predictive	Ref.
PFA-100®							
	98	PAD → angioplasty	25	Restenosis by Doppler	12	ADP	Ziegler 2002
	212	ACS	-	troponin T	-	ADP, epi associated w levels	Frossard 2004
	100	ACS	5	Deaths	6	epi<138 sec	Sambola 2004
	175	ACS → PCI	12?	Recurrent ACSs	6	ADP, epi	Gianetti 2006
	208	ACS	58	Recurrent ACSs	28	ADP, epi	Fuchs 2006
MPV							
	1716	Male MI survivors	126	Recurrent MI, CHD death	24	MPV	Martin 1991
	47	PTCA	-	Restenosis by angiography	4-8	MPV	Smyth 1993
	47	PCI	10	Re-occlusion by angiography	6	MPV	Terres 1995
	398	STMI → PCI	91/29	Restenosis/mortality	6	MPV	Huczek 2005

PAD, peripheral arterial disease; ACS, acute coronary syndrome; MI, myocardial infarction; CHD, coronary heart disease; PTCA, Percutaneous transluminal coronary angioplasty; STMI, ST elevation MI; PCI, Percutaneous coronary intervention; ADP, adenosine diphosphate; epi, epinephrine; AA, arachidonic acid; MPV, mean platelet volume

Table 4

Study limitations

1.	Different disease groups (healthy, acute MI, PCI, etc.)
2.	Generally small sample sizes (many with 10-30 CHD events) with little or no consideration of confounders
3.	Nearly all on anti-platelet medications and other medications
4.	Different platelet assays, different stimuli
5.	Different lengths of follow-up
6.	Different endpoints (angiographic stenosis, clinical events, myocardial necrosis)
7.	Timing of phlebotomy
8.	Publication bias

Table 5

Platelet functional assays.

Assay	Condition (concentration or shear rate) [*]
PRP aggregation[†]	
Epinephrine	0.1, 0.2, 0.4, 1.5, 3, 10 μM
CRP	0.005, 0.01, 0.02, 0.05 μg/mL
ADP	0.05, 0.5, 1, 4, 20 μM
Collagen	2.5, 5. 10, 20, 50, 200 μg/mL
Arachidonic acid	0.5 mg/mL
Ristocetin	0.1, 0.5, 0.75, 1 mg/mL
Spontaneous	
Whole Blood flow cytometry	
P-selectin expression	0, 1, 10 μM ADP
PAC-1 binding	0, 1, 10 μM ADP
Shear assays[†]	
PFA-100 (ADP)	
PFA-100 (epinephrine)	
SIPE (cone and plate viscometer)	0, 500, 1000, 2000, 5000, 10000 sec^{-1}
SIPA (cone and plate viscometer)	0, 500, 1000, 2000, 5000, 10000 sec^{-1}
Receptor density	
αIIbβ3 (CD41)	resting
GPIbα (CD42b)	resting
α2β1 (CD49)	resting
FcγRIIA (CD32)	resting
Clot retraction[†]	

[*] Blank means only a single condition was used.

[†] Assays also performed in PPACK anticoagulant

CRP, collagen-related peptide; SIPE, shear-induced P-selectin expression; SIPA, shear-induced platelet aggregation

Table 6

Sources of variation in platelet functional assays

Genetic/intrinsic	Environmental	Technical
Genes	Cigarette smoking	Instruments
	Alcohol	Different lots of reagents
	Sex hormones	Phlebotomy techniques
	Heparin	Time of day
	Plasma	Changes in personnel
	Unknown	Processing (e.g., time, temp., transportation)
		Unknown

Table 7

Characteristics of subjects with hyperreactive platelets

- Female (p=0.016)
- Higher fibrinogen levels (p=0.009)
- Higher MPV (p<0.001)
- Hyperreactive to other agonists
- Increased platelet secretion
- Increased reactivity to shear
- Increased levels of integrin αIIbβ3 (GP IIb-IIIa)
- 825 T allele of the *GNB3* exon 10 polymorphism