Comparison of Immature Platelet Count to Established Predictors of Platelet Reactivity During Thienopyridine Therapy

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ABSTRACT

BACKGROUND Previous data suggest that reticulated platelets significantly affect antiplatelet response to thienopyridines. It is unknown whether parameters describing reticulated platelets can predict antiplatelet response to thienopyridines.

OBJECTIVES The authors sought to determine the extent to which parameters describing reticulated platelets can predict antiplatelet response to thienopyridine loading compared with established predictors.

METHODS This study randomized 300 patients undergoing elective coronary stenting to loading with clopidogrel 600 mg, prasugrel 30 mg, or prasugrel 60 mg. Adenosine diphosphate (ADP)-induced platelet reactivity was assessed by impedance aggregometry before loading (intrinsic platelet reactivity) and again on day 1 after loading. Multiple parameters of reticulated platelets were assessed by automated whole blood flow cytometry: absolute immature platelet count (IPC), immature platelet fraction, and highly fluorescent immature platelet fraction.

RESULTS Each parameter of reticulated platelets correlated significantly with ADP-induced platelet reactivity (p < 0.01 for all 3 parameters). In a multivariable model including all 3 parameters, only IPC remained a significant predictor of platelet reactivity (p < 0.001). In models adjusting each of the 3 parameters for known predictors of on-treatment platelet reactivity including cytochrome P450 2C19 (CYP2C19) polymorphisms, age, body mass index, diabetes, and intrinsic platelet reactivity, only IPC prevailed as an independent predictor (p = 0.001). In this model, IPC was the strongest predictor of on-treatment platelet reactivity followed by intrinsic platelet reactivity.

CONCLUSIONS IPC is the strongest independent platelet count-derived predictor of antiplatelet response to thienopyridine treatment. Given its easy availability, together with its even stronger association with on-treatment platelet reactivity compared with known predictors, including the CYP2C19*2 polymorphism, IPC may become the preferred predictor of antiplatelet response to thienopyridine treatment. (Impact of Extent of Clopidogrel-Induced Platelet Inhibition During Elective Stent Implantation on Clinical Event Rate—Advanced Loading Strategies [ExcelsiorLOAD]; DRKS00006102) (J Am Coll Cardiol 2016;68:286-93) © 2016 by the American College of Cardiology Foundation.
Antiplatelet response to adenosine diphosphate (ADP)-receptor inhibitors, clopidogrel in particular, is associated with significant interindividual variability (1-3). Multiple studies have demonstrated that patients with high on-clopidogrel platelet reactivity are at risk for major ischemic events such as myocardial infarction, stent thrombosis, or death (4-7). Several factors have been associated with this variability, including demographic and clinical variables, as well as genetic polymorphisms (8-11). However, all these factors predict only a minor proportion of the observed variability in response to clopidogrel and are therefore no substitute for platelet function testing (8,10,12,13).

Recently, several studies have shown that immature or “reticulated” platelets may be a novel predictor of impaired response to P2Y12 receptor inhibitors (14-17). Further studies demonstrated an association between levels of reticulated platelets and adverse cardiovascular events in patients on P2Y12 receptor inhibitors (18-20). This platelet subpopulation presumably reflects platelet turnover and has initially been defined by a specific staining pattern of ribonucleic acids in light microscopy (21,22).

Given the improvement of laboratory techniques, reticulated platelet parameters are widely available for routine clinical use. Contemporary, fully automated cell analyzers providing blood count parameters can also determine reticulated platelets (23). These analyzers provide multiple parameters describing reticulated platelets such as the absolute count (immature platelet count [IPC]), the fraction of the total platelet pool (immature platelet fraction [IPF]), and the fraction of highly reticulated platelets (highly fluorescent immature platelet fraction [hiPf]). Previous studies have used different parameters to assess the impact of reticulated platelets on response to antiplatelet drugs (24). We studied whether any of these parameters can independently predict on-treatment platelet reactivity compared with established predictors in patients treated with P2Y12 receptor antagonists.

METHODS

This study represented a pre-specified subanalysis of the ExcelsiorLOAD (Impact of Extent of Clopidogrel-Induced Platelet Inhibition during Elective Stent Implantation on Clinical Event Rate—Advanced Loading Strategies) trial. Details of this randomized, controlled phase IIIb trial have been recently published (25). In brief, the ExcelsiorLOAD trial compared the peri-interventional pharmacodynamic effectiveness of loading with clopidogrel 600 mg, prasugrel 30 mg, or prasugrel 60 mg administered immediately before elective coronary intervention in stable patients pretreated with aspirin. Key exclusion criteria were acute myocardial infarction; treatment with ticagrelor, prasugrel, fibrinolytic agents, or glycoprotein IIb/IIIa inhibitors within 7 days before enrollment; contraindication for aspirin or any of the study medications; oral anticoagulation; or any severe disorder of the coagulation system. Following coronary intervention, all patients were treated with a daily maintenance dose of aspirin 100 mg and clopidogrel 75 mg for at least 30 days as recommended by current guidelines (26,27). All patients gave written informed consent before angiography or any study procedure.

The study was approved by the ethics committee of the University of Freiburg (Germany) and the regulatory agency (Federal Institute for Drugs and Medical Devices, Bonn, Germany). The trial has been registered at the German Clinical Trial Register (DRKS00006102).

One of the key results of the ExcelsiorLOAD trial was that from 30 min onward, prasugrel 60 mg was associated with significantly stronger platelet inhibition than the clopidogrel 600-mg loading dose; indeed, prasugrel 60 mg roughly halved the time to onset of platelet inhibition compared with loading with clopidogrel 600 mg.

PLATELET FUNCTION TESTING AND ANALYSIS. Blood samples for platelet function testing were taken after randomization, immediately before intake of study medication and start of coronary intervention, and on day 1 following the procedure just before intake of the first maintenance dose of clopidogrel 75 mg. Blood was collected into 2.7-ml tubes containing r-hirudin (45 μg/ml, Sarstedt AG & Co., Nuembrecht, Germany). ADP (6.4 μmol/l)-induced platelet aggregation was assessed in whole blood by impedance aggregometry according to the manufacturer’s instructions (Multiplate Analyzer, Roche Diagnostics, Mannheim, Germany). The area under the curve of aggregation units (AU) was recorded over 6 min, and results were described as AU × min. High on-treatment platelet reactivity for the ADP test was defined as ≥468 AU × min according to previously published data and consensus recommendations (28).

For the determination of reticulated platelets, a fully automated flow cytometer assay was used (Sysmex 2100, Sysmex, Norderstedt, Germany). The following reticulated platelet parameters were analyzed: IPC, representing the absolute count of reticulated platelets (10^3/μl); IPF, representing the...
fraction of reticulated platelets of the total platelet count (%); and hIPF, representing the fraction of very reticulated platelets of the total platelet count (%). The hIPF represents a sub-fraction of the IPC. Both are differentiated by fluorescence intensity of staining with polymethine/oxazine. All reticulated platelet parameters were determined in blood samples drawn at the time of randomization.

Subjects were genotyped for *CYP2C19*<sup>2</sup> loss-of-function polymorphism (c.681G>A; rs4244285) and *CYP2C19*<sup>17</sup> gain-of-function polymorphism (c.806C>T; rs12248560) using a commercially available validated Drug Metabolism Genotyping Assay as previously reported (Applied Biosystems, Frankfurt, Germany) (9). The concordance rate was 100% for all duplicate samples throughout the different genotype assessments. The distribution of the *CYP2C19* genetic variants did not deviate significantly from Hardy-Weinberg equilibrium (*CYP2C19*<sup>2</sup> chi-square = 0.570; *p* = 0.45; *CYP2C19*<sup>17</sup> chi-square = 0.025; *p* = 0.87).

**STATISTICAL ANALYSIS.** Spearman rank correlation coefficient (r) was used to describe the relationship between platelet aggregation and immature platelet parameters. The 2-tailed Student *t* test was used to compare continuous variables or the Kruskal-Wallis test with Dunn multiple comparisons post-test was utilized for non-Gaussian variables. Multivariable linear regression models were used to test whether reticulated platelet parameters are independently associated with levels of platelet reactivity. The strength of association of reticulated platelet parameters with levels of platelet reactivity was determined by calculating partial η<sup>2</sup>, which describes the proportion of variance in platelet reactivity explained by a specific variable (e.g., a partial η<sup>2</sup> of 0.04 indicates that in this model, 4% of variability in platelet reactivity can be explained by the variable). In these models, levels of on-thienopyridine platelet reactivity were entered as dependent variables, and dichotomous variables as fixed factors. For multivariable analyses, the following parameters, which have previously been shown to be strongly associated with antiplatelet response to thienopyridines, were used for adjustment: age, sex, body mass index, renal function, left ventricular function, smoking status, diabetes mellitus, intrinsic platelet reactivity, platelet count, *CYP2C19*<sup>2</sup> 681G>A polymorphism, and *CYP2C19*<sup>17</sup> 806C>T polymorphism (8,10,29–32). Predictive Analytics SoftWare package, version 18 (IBM Corporation, Armonk, New York) was used for all analyses.

Based on sample size and distribution of platelet reactivity in the analyzed cohort, this analysis has a power of >90% to detect a difference of 20 AU × min

![FIGURE 1 Correlation of Reticulated Platelet Parameters With Platelet Reactivity](image)

On day 1 after loading, every reticulated platelet parameter was significantly correlated with platelet reactivity, with immature platelet count (IPC) demonstrating the numerically highest Spearman correlation coefficient. Solid orange lines = best-fit linear regression line with 95% prediction intervals (dashed orange lines). AU × min = aggregation units × minute; hIPF = highly fluorescent immature platelet fraction; IPF = immature platelet fraction.
RESULTS

Baseline characteristics of the 300 randomized patients were well balanced between cohorts of different loading regimens as previously published (25). One patient in the prasugrel 60 mg group was excluded from further analyses due to missing immature platelet results. A flow chart of the study is shown in Online Figure 1. The median time between loading and blood sampling on day 1 was 18.1 h (interquartile range: 16.6 to 19.9 h). Single absolute values and distribution of immature platelet parameters are depicted in Online Figure 2. The proportion of patients with high on-treatment platelet reactivity at day 1 following loading was low (clopidogrel 600 mg, n = 1; prasugrel 30 mg, n = 1; and prasugrel 60 mg, n = 2).

CORRELATION AND LINEAR REGRESSION ANALYSES.
Platelet reactivity increased with rising levels of all 3 immature platelet parameters (Figure 1, Online Figure 3). There was a modest, but significant, correlation of all 3 analyzed immature platelet parameters and ADP-induced platelet reactivity on day 1 after loading. IPC demonstrated the numerically highest Spearman correlation coefficients ($r = 0.26; p < 0.001$).

In unadjusted linear regression analyses of the entire cohort, IPC and IPF were significantly associated with on-treatment platelet reactivity, but in a multivariable model, IPC was the strongest predictor of platelet reactivity. Results are shown as regression coefficient with 95% confidence intervals. *Adjusted for immature platelet parameters including IPC, IPF, and hIPF. **Adjusted for all parameters including age, sex, body mass index, diabetes mellitus, intrinsic platelet reactivity, platelet count, renal function (glomerular filtration rate), left ventricular function, smoking status, cytochrome P450 CYP2C19*2 681G>A polymorphism, and cytochrome P450 CYP2C19*17 806C>T polymorphism. Abbreviations as in Figure 1.
Megakaryocytes in Bone Marrow

Immature Platelets
- 1.1%-6.1% of all platelets
- More dense granules
- More RNA content
- Different reactivity
- Association to cardiovascular disease

Immature Platelet Count
- Strongest independent platelet count-derived predictor of antiplatelet response to thienopyridines
- Very easy availability, low costs
(partial $\eta^2 = 0.01; p = 0.06$). In a multivariable model including all 3 immature platelet parameters, IPC was the strongest predictor of platelet reactivity (Figure 2). IPF and hIPF were not significantly associated with on-treatment platelet reactivity anymore. In this model, IPC could explain 8% of the variability in platelet reactivity. When analyzing each of the 3 parameters in a multivariable linear regression model, adjusted for selected predictors of antiplatelet response to thienopyridines, only IPC was independently associated with on-treatment platelet reactivity (Figure 2). In this model, IPC showed the highest association with on-treatment platelet reactivity, followed by intrinsic platelet reactivity, even after adjustment for strong predictors of clopidogrel response, such as the CYP2C19$^*$2 polymorphism. In this model, 4% of the observed variability in platelet reactivity could be attributed to IPC.

There was a significant interaction of the allocated P2Y$_{12}$ receptor antagonist treatment regimen with the effect of IPC on on-treatment platelet reactivity ($p$ for interaction = 0.02). The association of IPC and platelet reactivity weakened with rising antiplatelet potency of the loading regimens investigated (Figure 3, Online Figure 4). Following adjustment for all clinical and genetic parameters, the strongest effect was observed in the clopidogrel 600 mg group (unadjusted $\eta^2 = 0.18; p < 0.001$; adjusted partial $\eta^2 = 0.07; p < 0.001$) indicating that 7% of on-clopidogrel platelet reactivity can be explained by IPC. For prasugrel 30 mg, the association of IPC remained significant (unadjusted $\eta^2 = 0.04; p = 0.05$; adjusted partial $\eta^2 = 0.05; p = 0.04$). For prasugrel 60 mg, no significant association was found.

**DISCUSSION**

Recent studies have indicated an association of reticulated platelets with impaired response to thienopyridine treatment. The present study supported the previous data and extended these results by 3 key findings: First, compared with all other parameters of reticulated platelets, IPC is the only independent predictor of antiplatelet response to thienopyridines after adjustment for a panel of clinical and genetic variables. Our data indicated that IPC might be the preferable parameter characterizing the pool of reticulated platelets. Second, IPC had the strongest association with on-treatment platelet reactivity compared with other predictors of impaired response to thienopyridines, including the CYP2C19$^*$2 polymorphism (Central Illustration). Finally, the association of IPC and other variables with on-treatment platelet reactivity appeared to depend on the antiplatelet potency of the loading regimen used and weakened with higher antiplatelet potency.

Prior studies focusing on reticulated platelets used thiazol orange staining as the detection method because fully automated assays were not available at that time. This staining method is prone to considerable interference, a major limitation for interpretation of these early findings (24). More recent studies, including our analysis, applied well-standardized analytical methods. However, most of these studies used IPF but did not include additional immature platelet parameters. In 2012, it was shown for the first time that IPF is associated with impaired response to clopidogrel in healthy volunteers (17). Recently, Bernlochner et al. (14) published data on the impact of reticulated platelets on antiplatelet response to prasugrel and ticagrelor. In this study, platelet reactivity correlated moderately with IPF in patients receiving prasugrel, but not in patients on ticagrelor. The missing correlation of IPF and on-ticagrelor platelet reactivity was confirmed in a subsequent study by Verdoia et al. (33). An association of IPF and hIPF with cardiovascular death was demonstrated for patients with acute coronary syndrome treated with clopidogrel in a subanalysis of the AMI-Florence study (19). The present analysis extended these data by investigating further parameters of reticulated platelets. In agreement with previous findings, these data showed that reticulated platelets are one of the main factors predicting antiplatelet response. However, when comparing the various reticulated platelet parameters, only IPC was independently associated with on-treatment
ADP-induced platelet reactivity. Multivariable analyses demonstrated that IPC could explain up to 4% of the observed total variability of on-thienopyridine platelet reactivity and up to 7% of variability in patients treated with clopidogrel.

Although previous studies investigated the association of IPF with antiplatelet response to P2Y12 receptor antagonists, data on the impact of IPC have been sparse. The predictive value of IPC for ischemic clinical endpoints was recently demonstrated in a prospective cohort study of patients with coronary heart disease (16). A potential role of IPC as predictive tool for antiplatelet response to aspirin has been suggested in 2 studies with limited sample size (34,35).

The stronger association of IPC with antiplatelet response to thienopyridines may be due to a more precise description of the drug metabolite-target cell relation by an absolute cell count. However, further studies are needed to elucidate the mechanisms behind these finding. The finding that the association of IPC and platelet reactivity is weakened with rising antiplatelet potency of the loading regimen is important. This may have clinical impact in settings associated with high IPC, such as acute coronary syndrome. In interpreting this observation, it should be kept in mind that the potency of the active metabolite of prasugrel and clopidogrel is essentially equal. Yet, a higher plasma concentration of active metabolite is achieved than with prasugrel than with clopidogrel. The higher dose of active metabolite with prasugrel as compared with clopidogrel might have overcome a potential resistance of immature platelets to inhibition by thienopyridines. The active metabolite of prasugrel also has a longer half-life than the active metabolite of clopidogrel (36). Thus, prasugrel can inhibit more new-build platelets that are the proposed key fraction of the reticulated platelet population.

STUDY LIMITATIONS. The present study was only powered for pharmacodynamic endpoints. Further studies adequately powered for clinical endpoints are needed to test whether IPC is also the preferable predictor of all reticulated platelet parameters for clinical risk prediction. All 3 evaluated immature platelet parameters are closely related. Thus, there have been significant interactions between parameters in the adjusted analyses that could have biased the results. To reduce this limitation, the multivariable analyses were adjusted for clinical and genetic parameters, and calculated only for the single reticulated platelet parameters. Because this study focused on antiplatelet effects of thienopyridine loading, it cannot answer whether these findings can be translated to thienopyridine maintenance therapy and antiplatelet effects of ticagrelor.

CONCLUSIONS

IPC is the strongest independent platelet-derived predictor of antiplatelet response to thienopyridine treatment. Even compared with established variables linked to impaired response to thienopyridine treatment, including the CYP2C19*2 polymorphism, IPC remains the dominant predictor of antiplatelet response. Given its easy availability and excellent standardization, it may become the preferable risk marker of impaired antiplatelet response to thienopyridines and, in particular, to clopidogrel treatment.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: In stable patients undergoing percutaneous coronary intervention, an elevated immature platelet count is associated with reduced inhibition of platelet aggregation in response to thienopyridine loading.

COMPETENCY IN PATIENT CARE: Prospective studies are needed to assess the value of IPC at the inception of thienopyridine therapy as a predictor of long term clinical outcomes.

REFERENCES


KEY WORDS clopidogrel, percutaneous coronary intervention, prasugrel, reticulated platelets

APPENDIX For supplemental figures, please see the online version of this article.