Stability of the high on-treatment platelet reactivity phenotype over time in clopidogrel-treated patients

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Summary
Interindividual response variability to clopidogrel treatment is a well established phenomenon. In recent studies and ongoing large-scale trials where patients with high on-treatment platelet reactivity (HPR) to clopidogrel are being randomised to an intensified antiplatelet treatment, confirmation of the HPR phenotype is based on one single platelet function assessment. The stability of the HPR phenotype over time has never been investigated but should be considered crucial for justification of intensified antiplatelet treatment regimens beyond clinical trials. The goal of this study was to test for the stability of the HPR phenotype over time in clopidogrel-treated patients. Patients (n=31) under chronic clopidogrel treatment (75 mg/day) were investigated by serial adenosine diphosphate (ADP)-induced platelet aggregation assessment with multiple electrode aggregometry (MEA) on a Multiplate analyser and light transmission aggregometry (LTA) at three different time points (once per week) during monitored antiplatelet treatment. On the basis of a cut-off level approach (468 AU*min for MEA, 53% for LTA) patients were classified into patients with (n=27) or without (n=4) HPR. For MEA, the phenotype was stable in 93.5% (n=29) of patients whereas 6.5% (n=2) crossed the cut-off level. For LTA, the phenotype was stable in 68% (n=21) of patients whereas 32% (n=10) patients crossed the cut-off level (chi-square P=0.01 for comparison of phenotype stability between both assays). In conclusion, the HPR phenotype is stable over time in the majority of clopidogrel-treated patients. Comparative assessment of phenotype stability across available platelet function assays warrants further investigation.

Keywords
Clopidogrel, platelet aggregation, high on-treatment platelet reactivity

Introduction
Response to clopidogrel is not uniform and interindividual response variability is a well established phenomenon (1–3). Despite the fact that dual antiplatelet therapy with aspirin and clopidogrel has resulted in a significant improvement of the outcomes in patients with acute coronary syndrome (ACS) and in those undergoing percutaneous coronary intervention (PCI) (4), a considerable number of cardiovascular events continue to occur (5, 6) and clopidogrel low-responsiveness or high on-treatment platelet reactivity (HPR) has emerged to an own clinical entity (1, 2, 7–10). In recent studies that investigated the predictive value of platelet function measurements on clinical outcome of patients (3, 7–14) and also in ongoing clinical trials that currently randomise patients with HPR to an intensified antiplatelet treatment (9), confirmation or exclusion of HPR is based on a single platelet function assessment.

Recently, the importance of genotyping of specific genetic variants within the cytochrome P450 (CYP) system that alter clopidogrel metabolism was emphasised and it was argued that platelet function testing may provide inconsistent and dynamic results measured at different time points in one and the same patient (15). However, prior studies using light transmission aggregometry (LTA) have shown relatively stable measurements over time (16, 17). Recently, a consensus has been achieved on the definition of HPR with various assays including LTA and the Multiplate® analyser (18). These consensus cut-off values have never been tested for their stability in patients over time and measurements obtained with the Multiplate device have never been analysed in this setting as well. Indeed, the stability of the HPR phenotype should be considered crucial for justification of an intensified antiplatelet treatment and this especially for future treatment of patients beyond ongoing clinical trials. The goal of this study was to test for the stability of the HPR phenotype over time in clopidogrel-treated patients.

Materials and methods
Study population and study design
Between March 2008 and October 2008 patients were enrolled in the setting of a double-blind randomised trial at the Deutsches
Herzzentrum München (Technische Universität München, Germany) that aimed to explore the possible existence and prevention of a rebound phenomenon of platelets after clopidogrel cessation (19). Details of this study population including inclusion and exclusion criteria have been reported previously (19). For the present post-hoc analysis, we included patients (n=34) that have been randomised to the so-called “off-group” during the primary trial. As these patients continued to take clopidogrel for four more weeks before stopping it completely, we were able to assess their on-treatment platelet reactivity at three different time points (once per week) under steady-state and monitored conditions.

Figure 1 shows the flow chart for the present study. For the time period relevant for the present analysis (=28 days), patients uniformly continued treatment with 100 mg aspirin (twice/day) and 75 mg clopidogrel (once/day). The study was approved by the institutional ethics committee, complies with the Declaration of Helsinki and all patients gave written informed consent for it.

**Blood sampling and platelet function testing**

Peripheral venous blood samples for platelet function testing were drawn during outpatient visits in a fasting state through a short venous catheter inserted into a forearm vein. Platelet function was measured once per week at three different time points by using two different methods simultaneously: First, in whole blood with MEA on a Multiplate analyser and also in platelet-rich plasma (PRP). Details of the study protocol with regard to platelet function testing have been reported previously (19). In brief, platelet aggregation was measured by MEA in response to 6.4 μM ADP and values are expressed as area under the curve (AUC=AU*min) of aggregation units. For LTA, the platelet aggregation profiler (PAP 8) aggregometer (Moelab, Berlin, Germany) was used to assess the maximal platelet aggregation (in %) in citrated PRP. As agonist, 5 μM ADP (Moelab) was used.

**Definition of HPR to clopidogrel**

Stratification of patients with or without HPR was based on the platelet function measurements obtained with MEA during the first visit (see Fig. 1). For defining clopidogrel HPR, the consensus cut-off value (18) of 468 AU*min was used, as values ≥468 AU*min have been linked to an increased risk for the occurrence of stent thrombosis in clopidogrel-treated patients undergoing PCI (3, 11). The primary HPR cut-off value that was used for LTA was set with the aim to achieve groups of equal size for both methods (MEA and LTA). This cut-off was determined to be ≥53%, which is similar to previous reports (10, 13, 20). In addition, the consensus cut-off value (18) of >46% for LTA (using 5 μM ADP) was also used to stratify patients into patients with or without HPR and to test for the stability of the HPR phenotype.
Statistical analysis

Determined with Kolomogorov Smirnov test, platelet function data obtained with MEA was not normally distributed and is presented as median [IQR, interquartile range]. Platelet function data obtained with LTA was normally distributed and is presented as mean ± standard deviation (SD). The median coefficient of variation (CV) is shown for both methods. Phenotype stability was compared across the assays used by calculating chi-square test. To test for within-subject differences in measurement over time, repeated-measures analysis of variance (ANOVA) test was used. For all statistical analysis, a p-value < 0.05 was considered significant. Analyses were performed using the software package S-PLUS version 4.5 (TIBCO Software Inc., Palo Alto, CA, USA).

Results

Study population

During the primary trial (19), a total of 34 patients were randomised to the “off-group”. Baseline characteristics of this cohort are shown in Table 1. For 31 of the 34 patients (91%) platelet function measurements were available at all three pre-specified time points (see Fig. 1). The controlling of the medication blister demonstrated full compliance with the study protocol in all patients. Co-medication of patients including aspirin treatment was not changed in any of the patients during the entire study period.

Based on the cut-off value of 468 AU*min (11, 18) for MEA, 27 patients (87%) were defined as patients without HPR based on their first assessment of platelet function during visit 1 (see Fig. 1). The remaining patients (n=4, 13%) were defined as HPR patients. A cut-off value of ≥53% for LTA measurements was found that yielded the same proportion of patients with or without HPR (87% vs. 13%). Using the consensus cut-off value of >46% (for 5 μM ADP) (18), a total of 10 patients (32%) showed a HPR whereas 21 patients (68%) did not.

Platelet aggregation with MEA

The median [IQR] ADP-induced platelet aggregation value was 265 [198–393] AU*min (range = 12 – 849 AU*min). Figure 2 shows the time course of platelet aggregation values in the groups of patients with or without the HPR phenotype during the study period. This phenotype was stable in 93.5% (n=29) of patients whereas 6.5% (n=2) crossed the cut-off level (one per group at day 8). Repeated-measures ANOVA test showed no statistically significant differences for measurements within subjects over time (p=0.92). For the entire cohort, the median value of the coefficient of variation for MEA measurements over time was 22% (range = 1.8% – 115%).

Platelet aggregation with LTA

The mean ± SD value for ADP-induced platelet aggregation measurements was 42 ± 11.3% (range = 11% – 72%). Figure 3 shows the time course of platelet aggregation values in patients with or without the HPR phenotype during the study period. This phenotype was stable in 68% (n=21) of patients whereas 32% (n=10) patients crossed the cut-off value of 53%. In the group of patients without HPR, three patients (11.1%) crossed the cut-off value at day 8 and five patients (25.9%) at day 15. In the group of patients (n=4) with HPR (using the 53% cut-off level), two patients (50%) crossed the cut-off value at day 8. Using the consensus cut-off value of >46%, the phenotype was stable in 58% (n=18) of patients whereas 42% (n=13) patients crossed this cut-off value. In detail, six patients with HPR and seven patients without HPR changed the phenotype over time. Repeated-measures ANOVA test showed no statistically significant differences for measurements within subjects over time (p=0.19). The median value of the coefficient of variation for LTA was 18% (range = 4.1% – 56%).

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Comparison of MEA and LTA measurements

The stability of the HPR phenotype was compared between the two assays used and significantly more patients showed an instable phenotype with the LTA assay as compared to MEA measurements (32% vs. 6.5%, respectively; chi-square $p=0.01$ for comparison of phenotype stability between assays). In addition, the patients that were classified as patients with or without HPR were not identical across the two assays. Only 17 of the 27 patients were patients without HPR according to both assays (MEA and LTA) and only one patient was found to be a patient with HPR according to both assays.

Discussion

To the best of our knowledge, this is the first study investigating the stability of the clopidogrel HPR phenotype over time in patients under a steady state phase of clopidogrel treatment and using consensus definitions (18) for HPR. The major finding of this study is that the HPR phenotype is stable over time in the majority of treated patients with both methods used for testing (although to a lesser extent for LTA as compared to MEA). Some patients, however, could be misclassified based on single time point testing and would in fact show an instable phenotype when serial measurements had been conducted. For LTA we used two different cut-off values here: one (53%) to achieve phenotype groups of equal size in comparison to MEA and one (46%) based on the consensus value to define HPR (18). Results tended out to be similar regardless of the value used and add to the knowledge (16, 17) on the previously shown stability of LTA measurements over time that has been shown by Gurbel et al. Present findings may have implications for clinical practice as they show that single platelet function testing draws a representative picture of the HPR phenotype in the majority of stable patients undergoing platelet function testing for assessing antiplatelet drug response. Further on, however, it must be acknowledged that stratification of patients into patients with or without HPR seems to be assay dependent as the phenotype determined by MEA measurements was not identical in all cases with the phenotype based on LTA measurements. This confirms previous observations, where it has been shown that the clopidogrel responder phenotype is an assay dependent phenomenon and may differ in one and the same patient across assays (21–23). As the agreement of measurements across different methods of platelet function testing is only moderate (24), different assays evaluated in this setting are likely to yield different results.

Strengths of the present study include the closely monitored intake of clopidogrel as the daily clopidogrel treatment dose was taken from a specifically prepared blister. Moreover, patients were in a steady state situation as ACS patients or patients with recent coronary stenting were excluded here (19). Both assays used for platelet function testing are widely accepted (3, 7, 10, 25–27), although their principles of testing are obviously different. As enrolment and follow-up of patients was conducted in the setting of a randomised trial, a selection bias seems highly unlikely and the present cohort investigated here may be considered as representative for a cohort of stable coronary artery disease patients with prior coronary stenting and stent related chronic dual antiplatelet treatment.

Concerning the parallel assessment of phenotype stability based on consensus cut-off values in contrast to the assessment of the CV for both assays, we believe that looking at the HPR phenotype is more relevant for clinical practice. Patients may differ over time for their absolute aggregation values and this may cause a high CV for the individual patient. As long, however, as a patient does not change the phenotype over time, this would not influence clinical decision making for the individual patient in terms of tailored antiplatelet treatment. In relation to this, for the most commonly used assays of platelet function testing, a cut-off approach is favoured for both HPR phenotyping and risk prediction (18, 28).

Here, we did not test for the stability of the HPR phenotype with VerifyNow P2Y$_{12}$ assay (Accumetrics, San Diego, CA, USA) measurements. This specific issue warrants further investigation keeping in mind that ongoing clinical trials such as GRAVITAS and
TRIGGER-PCI use this assay for patient stratification and subsequent randomization to intensified antiplatelet treatment regimens. Given the known disagreement between platelet function assays (24), extrapolation of our results to the VerifyNow assay is not possible.

**Study limitations**

The following limitations of the present study merit mention. First, we only used two different assays for platelet function testing and we are therefore unable to say in how far the present results can be extrapolated to other methods not investigated here such as the VerifyNow P2Y12 assay, the PFA-100 assay or the VASP assay. This warrants investigation in separate studies. Second, the cohort under investigation was relatively small, and the results provided here require external confirmation in independent and larger study cohorts. Here, we only focused on the ADP-induced platelet aggregation and we did not assess the aspirin responsiveness over time in the same cohort of patients simultaneously. Finally, adjustment for platelet count in PRP was done here, which may have influenced LTA results.

**Conclusions**

The high on-treatment platelet reactivity phenotype is stable over time in the majority of clopidogrel-treated patients. Comparative assessment of phenotype stability across available platelet function assays warrants further investigation.

**What is known about this topic?**

- Response to clopidogrel is not uniform and inter-individual response variability to clopidogrel treatment is a well established phenomenon described by various studies.
- High on-treatment platelet reactivity to clopidogrel treatment determined with multiple electrode aggregometry and other assays is significantly associated with the occurrence of ischemic events including stent thrombosis.
- A consensus has been achieved on how to define high on-treatment platelet reactivity (HPR).
- For clinical routine and also for the basis of randomisation of patients to more potent antiplatelet treatment regimes in ongoing trials, it is a practical approach to measure platelet function once.

**What does this paper add?**

- The HPR phenotype is stable over time in the majority of patients under chronic clopidogrel treatment.
- Single platelet function testing draws a representative picture of the HPR phenotype in the majority of patients undergoing platelet function testing for assessing antiplatelet drug response.

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**Conflict of interest**

Dr. Sibbing has received speaker fees from the Medicines Company and Dynabyte and fees for advisory board activities from Astra Zeneca and Eli Lilly.

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