Variable extent of clopidogrel responsiveness in patients after coronary stenting

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Summary
Clopidogrel is an effective and specific inhibitor of ADP-induced platelet aggregation. After metabolic activation, the active clopidogrel metabolite irreversibly impairs the human platelet P2Y12 ADP receptor. Gialpha-protein activation and inhibition of vasodilator-stimulated phosphoprotein (VASP) phosphorylation are two key elements of the P2Y12 receptor pathway suitable for quantitation of clopidogrel effects. So far, only limited data exist about a diminished responsiveness to clopidogrel and underlying possible mechanisms. We investigated clopidogrel effects in 57 patients after percutaneous coronary intervention and stent implantation by flow cytometry for the analysis of intracellular VASP phosphorylation. Patients were treated with a 300 mg clopidogrel loading dose, followed by 75 mg/day clopidogrel in combination with 100 mg/day aspirin. Samples were drawn after a median of 5 days of clopidogrel treatment.

Considerable differences in the responsiveness to clopidogrel could be observed and it was shown that 17.5% (10/57) of the patients revealed an inadequate responsiveness to clopidogrel despite continuation of clopidogrel intake. Comparable amounts of Gialpha and VASP were found in two clopidogrel low-responding patients as well as in two responding patients. To exclude a molecular defect of P2Y12 ADP receptor, the P2Y12 receptor gene of eight clopidogrel treated patients (seven patients with inadequate responsiveness, one responder) was sequenced. We only found a single silent mutation in exon 2 at position 1828 (GA). We suggest that individual differences in clopidogrel metabolization could cause relevant variations in clopidogrel responsiveness despite the use of a 300 mg clopidogrel loading dose.

Keywords
Clopidogrel, clopidogrel responsiveness, antiplatelet therapy

Introduction
Platelet activation and aggregation play an important role in the pathogenesis of cardiovascular diseases. Thienopyridines (ticlopidine and clopidogrel) are potent inhibitors of ADP-induced platelet aggregation (1). ADP is a platelet activator which is secreted from platelets upon activation and is released from damaged vessels and from red blood cells (2, 3). Three ADP-receptors have been described on platelets, P2X1 (a calcium channel), P2Y1 (coupled to Gq, responsible for mobilization of intracellular Ca2+) and P2Y12, which leads to inhibition of adenylyl cyclase via Gi-protein (4-7). The P2Y12 receptor has been identified as target of clopidogrel (8, 9) and its molecular structure has been recently clarified (10-12). After metabolic activation by the hepatic cytochrome P450-1A or P450-3A4 systems, the active clopidogrel metabolite irre-
versibly impairs the human platelet P2Y12 receptor and the subsequent fibrinogen binding to the GP IIb/IIIa complex (9, 13-16).

Though clopidogrel was shown to be very effective for prevention of atherothrombotic events, there are still patients who suffer vascular ischemic events despite treatment. Adverse cardiac events could be seen in 1.2-1.5% of patients under a 28-day combination therapy of clopidogrel and aspirin after stent replacement (17). In addition to these clinical observations, studies examining platelet inhibition after clopidogrel treatment have demonstrated individual variations in its anti-aggregating effects (9, 18, 19). Based on these observations, a concept of “low-responsiveness to clopidogrel” was propagated, comparable to the widely accepted aspirin resistance. The objective of our study was to identify patients with low-responsiveness to clopidogrel and possible mechanisms. We examined patients after percutaneous coronary intervention (PCI) and stenting, treated with clopidogrel in combination with aspirin. To investigate the inhibitory effects of clopidogrel, determination of VASP (vasodilator-stimulated-phosphoprotein) phosphorylation by flow cytometry was used: blockade of the P2Y12 receptor by clopidogrel should diminish the inhibiting effect of ADP on prostaglandin E1-stimulated VASP phosphorylation (18). In patients showing a decreased responsiveness to clopidogrel, Gialpha and VASP, which are two key elements of the P2Y12 receptor pathway, were analysed by Western blot, and the P2Y12 gene was sequenced to evaluate the possible molecular basis of clopidogrel low-responsiveness.

**Methods**

**Patients**

57 patients were randomly enrolled in this prospective study. All patients had a history of angina and were treated by conventional PCI and stenting. Patients took a 300 mg clopidogrel loading dose, followed by 75 mg/day clopidogrel in combination with 100 mg/day aspirin. Blood samples were drawn at the last day in the hospital (between day 2 and 53 of treatment, median duration of treatment 5 days). Blood samples were obtained within 4 hours after clopidogrel intake. The study followed the guidelines of the revised declaration of Helsinki and was accepted and approved by the Ethics Committee of the University of Würzburg. Informed consent has been obtained from all subjects.

**Flow cytometry of VASP phosphorylation**

Flow cytometry of platelet VASP phosphorylation was performed as described previously (18). Briefly, platelet-rich plasma (PRP) was isolated from citrate-anticoagulated blood and stimulated with 0.5 μM prostaglandin E1 (PG-E1) and/or 20 μM ADP for 3 min and reaction was stopped by addition of 3% formaldehyde. Then samples were centrifugated for 1 min at 2700g, platelet pellets were dissolved in phosphate-buffered saline (PBS) containing 5.5 mM glucose and 0.5% BSA and were permeabilized in 0.2% Triton X-100 for 10 min at room temperature. After an additional centrifugation step platelets were stained with 16C2 (anti-phospho-VASP Ser239) mono-

![Figure 1: Effect of clopidogrel on ADP-induced inhibition of PG-E1-stimulated platelet VASP phosphorylation.](image-url)
clonal antibody (10 μg/ml = 66 nM) (NanoTools, Lenting, Germany) for 30 min at room temperature and then with 25 μg/ml (= 166 nM) FITC-conjugated goat anti-mouse IgG (Sigma, Deisenhofen, Germany) for 20 min at 4°C in the dark. Mean fluorescence was measured on a Becton Dickinson FACSCalibur and analysed using CELLQuest software, version 3.1f. The extent of clopidogrel effects was expressed by the ratio of VASP serine 239 phosphorylation evoked by the combination of ADP and PG-E1 stimulation relative to that observed with ADP alone (ADP+PG-E1/ADP) (Fig. 1). A ratio of 1.0 shows a complete inhibition of PG-E1-stimulated VASP phosphorylation by ADP, indicating complete absence of clopidogrel effects (i.e. 100% platelet reactivity). Since we know that a high platelet reactivity is associated with low VASP phosphorylation, we determined the percentage of platelet reactivity after ADP stimulation with an inverse correlation between this percentage and the clopidogrel treatment efficacy. A platelet reactivity higher than 50% was defined as “low-responsiveness” according to our former findings (20). This FACS assay is now commercially available for interested investigators from BioCytex, Marseille, France.

**P2Y12 ADP receptor gene analysis**

DNA was isolated from 2 ml of EDTA-anticoagulated whole blood of eight clopidogrel treated patients using standard methods according to the manufacturer instructions (QIAamp DNA Blood Midi Kit, Hilden, Germany). Clopidogrel is supposed to bind to a SH-group of the P2Y12 receptor (16). Therefore, we focussed our genomic sequence analysis on the part of the gene which is translated (Exon 2). Exons 1 and 2 were amplified with primers and conditions as described in Table 2 (Co-medication of patients showing low-responsiveness to clopidogrel).

**Immunoprint analysis of Gialpha and VASP**

PRP obtained from 4 patients (2 patients with low-responsive-ness, 2 responding patients) was centrifuged, and platelet pellet was lysed and resuspended in SDS containing buffer (0.1 M Tris HCl, pH 6.7, 5 mM EDTA, 4% SDS, 7.5% Glycerol, 0.02% bromphenol blue, 250 μg/ml PefablocR (Roche Diagnostics GmbH, Mannheim, Germany)). Platelet lysates containing equal amounts of protein were electrophoresed in 9% SDS-polyacrylamide gels and electrotransferred to nitrocellulose membranes (Schleicher & Schuell GmbH, Würzburg, Germany), which were blocked with 6% dry milk powder (BioRad Laboratories, München, Germany) in PBS with 0.1% Tween. Membranes were incubated with the primary antibody against VASP (M4) (NanoTools, Germany) and Gialpha (1, 2) (Sigma) and then with the corresponding horseradish peroxidase-conjugated IgG antibodies (Bio Rad Laboratories). Antibody concentrations were used as recommended in the respective data sheet. A blocking peptide was used to show the specificity of the P2Y1 receptor antibody, for the analysis of Gialpha a recombinant Gialpha2 subunit was used as positive control. The proteins were visualised using the enhanced chemiluminescence detection kit (ECLTM) (Amersham Pharmacia Biotech, Freiburg, Germany). After exposure to x-ray film (Fuji Super RX 100), the bands corresponding to Gialpha and P-VASP were quantified by scanning densitometry.

### Table 1: Characterization of clopidogrel responding and low-responding patients.

<table>
<thead>
<tr>
<th>Number of patients (n)</th>
<th>responders</th>
<th>low-responders</th>
</tr>
</thead>
<tbody>
<tr>
<td>47</td>
<td>10</td>
<td>37</td>
</tr>
<tr>
<td>Age (mean)</td>
<td>67 years (43-83 years)</td>
<td>65 (36-85 years)</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>38/10 (79/21%)</td>
<td>46/0 (60/40%)</td>
</tr>
<tr>
<td>Duration of clopidogrel intake (median)*</td>
<td>5.0 days (2-52 days)</td>
<td>5.5 (3-53 days)</td>
</tr>
<tr>
<td>Previous myocardial infarction n (%)</td>
<td>18 (38%)</td>
<td>4 (40%)</td>
</tr>
<tr>
<td>Current smokers n (%)</td>
<td>4 (8%)</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>Hypertension n (%)</td>
<td>29 (60%)</td>
<td>6 (60%)</td>
</tr>
<tr>
<td>Diabetes mellitus n (%)</td>
<td>6 (13%)</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>Hyperlipidemia n (%)</td>
<td>27 (56%)</td>
<td>5 (50%)</td>
</tr>
</tbody>
</table>

*data available from 40 responding and 8 low-responding patients. Seven responders and 2 low-responders were on permanent treatment and were unable to report the exact duration of clopidogrel treatment.
were amplified by 25 or 30 PCR cycles, respectively (45 sec at 94°C, 60 sec at 57°C, 90 sec at 72°C), using 500 ng DNA in 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.08% Nonidet P40, 1.5 mM MgCl2, 0.2 mM dNTP, 0.1 U Taq DNA polymerase (MBI Fermentas, St. Leon-Rot, Germany) and 0.2 μM of specific primers each (exon1: 5’: 5’-GAGGCTAA TTAACAA TTGTC-T-CTG–3’ and 3’: 5’-GCCA TCCTCA TCCCACA TTTCCAG-3’; and exon 2: 5’: 5’-GTAAAGAAGA TTA TA TCCAA TTGA TCG-3’ and 3’: 5’-AGCAA TAA TAACTACCTTAGGCGT-3’). Amplification products were purified using QIAquick PCR Purification Kit (QIAGEN). PCR products were analysed by automated DNA sequencing using capillary sequencers ABI 3700 and 3730xl, Amersham Biosciences, Piscataway, USA.

Results

Flow cytometry of VASP phosphorylation
To ensure the precision of the assay, a serial measurement (n=20) of VASP phosphorylation after stimulation with PG-E1 and PG-E1/ADP rsp. was performed in a sample of one untreated healthy blood donor. Coefficients of variations were calculated to be 10.5% and 16.6%. Results obtained from 57 patients after PCI and stenting showed a considerable variability of responsiveness to clopidogrel as shown in Figure 2. Platelet reactivity varied from 6.0% to about 82%; in 10 patients (17.5%) platelet reactivity was higher than 50% (low-responsiveness). Blood samples from clopidogrel low-responding patients were drawn after a median of 5.5 days (range 3-53 days) of clopidogrel intake (data available from 8 patients) compared to a median of 5.0 days (range 2-52 days) in clopidogrel responding patients (data available from 40 patients) (Table 1). Information about concomitant drug therapy could be obtained from 9 of 10 clopidogrel low-responding patients (Table 2) and 46 responding patients. None of these patients were on medication with cerivastatin or lovastatin. Seven out of 9 low-responding (about 78%) and 31 out of 46 responding (about 67%) patients were medicated with atorvastatin or simvastatin. This difference was not significant.

Immunoprint analysis of Gialpha and VASP
We performed a Western blot analysis of Gialpha and VASP to exclude a lack of these two key elements of the P2Y12 ADP receptor pathway. Bands corresponding to Gialpha and VASP were analysed by scanning densitometry. Platelets of clopidogrel sensitive and clopidogrel low-responding patients demonstrated approximately equal amounts both of Gialpha and P-VASP (Fig. 3).

P2Y12 ADP receptor gene analysis
To exclude a molecular defect of the P2Y12 receptor, which would lead to a decreased binding of the active clopidogrel metabolite, Exon 1 and 2 of the P2Y12 ADP receptor gene were...
sequenced from eight clopidogrel treated patients (seven patients with low-responsiveness, one responder). Two clopidogrel low-responders and one responding patient showed a homozygous substitution of guanine by adenine at position 1828 (corresponding to cytosine at position 92 in mRNA sequence; GenBank accession number AF313449). 3 clopidogrel low-responding patients were heterozygous. This polymorphism was a silent mutation.

Discussion

This paper addresses the question of thienopyridine low-responsiveness in cardiovascular patients. Our major findings are: 1) clopidogrel low-responsiveness was found in 17.5% of the patients (10 out of 57); 2) clopidogrel low-responsiveness was neither due to patients' non-compliance, nor to decreased amounts of Gialpha or VASP or a molecular defect of the P2Y12 ADP receptor.

To exactly quantitate clopidogrel effects in patients we used a flow cytometric approach for detection of ADP-induced inhibition of prostaglandin E1-stimulated VASP phosphorylation at serine 239 with a phosphospecific monoclonal antibody (Fig. 1) (18). In our opinion, this assay seems to be superior to ADP-induced platelet aggregation and also to ADP-evoked platelet fibrinogen binding as it is not influenced by concomitant aspirin treatment (20) or therapy with GPIIb/IIIa antagonists (in-vitro data, not shown). In a clinical study, this assay has been successfully used to identify thienopyridine non-responding patients that are at high risk for coronary thrombosis (20). In our present study, a collection of 57 patients undergoing coronary stenting was investigated. All patients received a 300 mg clopidogrel loading dose, followed by 75 mg/day clopidogrel combined with 100 mg/day aspirin. Inter-individual differences in the degree of platelet inhibition have been observed in volunteers during a 7-day clopidogrel treatment (75 mg/day) (18) and it was shown that maximum inhibition was achieved with wide variations after up to 7 days of repeated dosing. Based on these observations, treatment regimens were introduced comprising clopidogrel loading doses of 300 to 600 mg, showing a rapid effect of clopidogrel with levels of platelet inhibition close to steady-state within 2 hours and favourable clinical outcome (17, 21, 22).

We observed high inter-individual variability in the responsiveness to clopidogrel after 5 days of intake (range 2-53 days) and 17.5% of the patients showed low-responsiveness to clopidogrel's antiplatelet action (Fig. 2). In this cohort of patients, duration of clopidogrel intake was rather comparable between clopidogrel responding and low-responding patients. Furthermore, a relevant interaction by nutrition has not been seen in former studies (23, 24). It is unclear at this point whether gender might influence clopidogrel responsiveness. We found a very high proportion of female patients (60 vs. 21%) in the low-responder group. The rate of low-responsiveness in our study was similar to that in a recent study, in which 5 to 11% clopidogrel “non-responders” and 9 to 26% “low-responders” were identified by aggregometry in patients with stable angina (25).

There is evidence that P2Y12 receptor gene sequence variations might influence ADP induced platelet aggregation (26). To identify a possible genomic basis of clopidogrel resistance, the P2Y12 receptor gene was sequenced. P2Y12 ADP receptor has been described to be a 7 transmembrane domain receptor, which is mainly expressed in human brain and platelets. The gene of P2Y12 receptor consists of two exons (64 bp and 1269 bp) and 1 intron (1736 bp) (10). Biological activity of the active clopidogrel metabolite is based on an irreversible disulfide bridging between a reactive thiol group of the metabolite and a cystein residue of the platelet P2Y12 receptor (16). The receptor contains four extracellular cystein residues, which were special points of interest. One polymorphism was found in exon 2 at position 1828 but this mutation was silent. A molecular defect of P2Y12 ADP receptor leading to a decreased binding of active clopidogrel metabolite could not be found. A decreased expression of the P2Y12 receptor-coupled Gialpha signalling protein was excluded by Western blot analysis that revealed normal levels of this protein as well as of the VASP signalling molecule in thienopyridine low-responding patients.

Taken together, considerable variations in the clopidogrel responsiveness were observed in patients after coronary stenting, in 17.5% a low-responsiveness to clopidogrel was found. Comparable levels of Gialpha and VASP could be observed in responding and low-responding patients. In P2Y12 ADP receptor gene no significant mutation was found. The frequency of a low-responsiveness against clopidogrel seems to be comparable to that against aspirin (about 10-20%) (recently reviewed in McKee et al. (27). Based on these data, we calculated that a combined defect should be found in about 1-2 % of patients treated with clopidogrel plus aspirin. This rate is surprisingly similar to the generally accepted rates of stent thrombosis after coronary stenting (17). There is evidence that variations in clopidogrel responsiveness might influence the incidence of coronary stent thrombosis, as a strong correlation was found between clopidogrel effect and the occurrence of coronary stent thrombosis in 16 consecutive patients with stent thrombosis compared to 30 others without thrombosis (20).

We believe that individual differences in clopidogrel metabolism cause considerable variations in clopidogrel responsiveness despite the use of a clopidogrel loading dose. New data about the contribution of hepatic cytochrome P450 3A4 metabolic activity to the phenomenon of clopidogrel resistance support this hypothesis (28). This might also be due to polymorphisms in the genes for cytochrome P450. A reduction of clopidogrel action by atorvastatin, possibly due to a dose-dependent
competitive inhibition of clopidogrel activation by cytochrome P450 3A4 has recently been described (29). Furthermore, it has been hypothesized that cerivastatin, lovastatin and simvastatin could also diminish the activation of clopidogrel (14). However there is now data available supporting the notion that cytochrome P450 3A4 metabolized statins do not interfere with clopidogrel (30, 31). In our study, about 78% of clopidogrel low-responding and 67% of responsive patients were on atorvastatin or simvastatin medication. We conclude from this observation that atorvastatin and simvastatin were not the cause for clopidogrel low-responsiveness in our patients. We would like to mention at this point that patients with low-responsive-
ness received a multi-pharmacological treatment. Whether or not patients at risk for clopidogrel low-responsiveness were candidates for (I) a higher loading dose and/or maintenance dose of clopidogrel, (II) additional antiplatelet therapy or merely an (III) alteration of combined drug therapies, considering potential drug interactions, remains to be determined. Clearly, this topic now needs to be addressed by larger clinical studies and protocols.

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References