Association of a Functional Polymorphism in the Clopidogrel Target Receptor Gene, P2Y12, and the Risk for Ischemic Cerebrovascular Events in Patients With Peripheral Artery Disease

Sophie Ziegler, MD; Martin Schillinger, MD; Marion Funk, MD; Katharina Felber, MD; Markus Exner, MD; Wolfgang Mlekusch, MD; Schila Sabeti, MD; Jasmin Amighi, MD; Erich Minar, MD; Martin Brunner, MD; Markus Müller, MD; Christine Mannhalter, PhD

Background and Purpose—There is considerable variability in the antiplatelet effects of the thienopyridine agent “clopidogrel.” We tested for an association of gene sequence variations in P2Y12 and occurrence of neurological adverse events in patients with symptomatic peripheral artery disease (PAD) during clopidogrel treatment.

Methods—We studied 137 patients undergoing antiplatelet therapy with clopidogrel and 336 patients with aspirin for the occurrence of neurological events (ischemic stroke and/or carotid revascularization). Prevalence of 2 previously described exonic polymorphisms of the P2Y12 gene, 34C>T and 52G>T, was determined by polymerase chain reaction.

Results—Genotype frequencies for mutated, heterozygous, and wild-type alleles for the 34C>T and the 52G>T polymorphisms were 9% (n=40), 44% (n=210), and 47% (n=223), and 4% (n=17), 27% (n=127), and 70% (n=329), respectively. During the median follow-up of 21 months, neurological events occurred in 8% of patients. In patients with aspirin therapy, neither polymorphism was associated with neurological events. However, in clopidogrel patients, carriers of at least one 34T allele had a 4.02-fold increased adjusted risk for neurological events compared with carriers of only 34C alleles (95% confidence interval, 1.08 to 14.9). Neither polymorphism was associated with all-cause mortality.

Conclusions—in PAD patients, clopidogrel response variability exists, which may result in increased risk for cerebrovascular events. Sequence alterations of the target receptor gene represent one possible mechanism for clopidogrel failure. Whether identification of the 34C>T polymorphism as a contributor to this process could serve as risk stratification tool, an indicator for higher clopidogrel doses, or the use of alternate agents warrants further investigation. (Stroke. 2005;36:1394-1399.)

Key Words: cerebrovascular accident ■ clopidogrel ■ ischemia ■ polymorphism

The recently cloned platelet ADP receptor P2Y12 has been shown to be involved in thrombus growth and the formation of emboli on the downstream side of the initial thrombus. It has been found to be the target of a widely used group of antiplatelet drugs, ie, the thienopyridines ticlopidine and clopidogrel.1 Clopidogrel was demonstrated to prevent cerebrovascular events in patients with known peripheral artery disease (PAD).2

However, a marked inter-individual variability in antiplatelet drug response to clopidogrel has been reported and the issue “failure of clopidogrel therapy” has been raised.3–6 So far, data on this issue are limited and available results differ according to pathogenesis and methodology. Most results have been obtained from either ex vivo investigations or investigations in healthy human volunteers. Recently, however, ex vivo clopidogrel resistance, measured by a platelet function analyzer, was described to be associated with recurrent cardiovascular events in patients with acute coronary syndrome.7

For a number of substances, inter-patient variability in drug response has been shown to be the result of polymorphisms in genes encoding drug targets, such as receptors or enzymes.8 Recent investigations of the functional impact of platelet receptor polymorphisms are inconsistent. Fontana et al re-
ported subjects with sequence variations of the clopidogrel target receptor, “P2Y12,” to display increased ADP-induced platelet aggregation. Carriers of the receptor gene variants were thought to be at increased risk for the presence of PAD, because the receptor alterations alter the function of protein.

In contrast, Hetherington et al could not find any significant effect of any polymorphism in the P2Y12 on platelet response to ADP.

In the present investigation, we studied whether genetic alterations in the P2Y12 receptor gene are associated with inter-individual variability of response to clopidogrel in the secondary prevention of neurological events in patients with PAD. For this purpose, we tested 137 patients receiving clopidogrel and 336 patients receiving aspirin. In the P2Y12 gene, 5 single-nucleotide polymorphisms have been described. Three of these polymorphisms are located in intronic regions of the gene and are in complete linkage disequilibrium with 52G>T in exon 2. For these 4 polymorphisms, haplotypes could be generated, which were studied regarding ADP-induced platelet aggregation. The 34C>T polymorphism in exon 2 is not linked to the 4 polymorphisms and conclusive functional data are not available. We selected the 34C>T and 52G>T, both located in the coding region of the P2Y12 gene, to study the effect on the response to clopidogrel treatment.

Patients and Methods

Patient Selection
We prospectively enrolled all consecutive patients with advanced PAD, who were admitted to the Angiology Department of a tertiary care university hospital from March 1, 2000 to March 1, 2001 in our cohort study. Patients with symptomatic PAD in terms of intermittent claudication or critical limb ischemia as well as patients with asymptomatic PAD and a history of surgical or endovascular lower limb revascularization were eligible for the study. The study was approved by the local ethics committee, and all patients gave their written informed consent. The study design has been published previously.

Laboratory Parameters
Antecubital venous blood samples for DNA analysis and routine laboratory measurements were taken on admission.

P2Y12 Genotype Assessment
Genomic DNA was isolated from whole blood according to standard procedures. The 2 polymorphisms 34C>T and 52G>T of the P2Y12 gene were determined by a mutagenically separated polymerase chain reaction assay essentially as described by Rust et al. Polymerase chain reaction amplifications were performed in an Eppendorf Cycler using 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. The allele-specific polymerase chain reaction products were separated on Spreadex EL 600 S-50 Wide Mini Gels (Elchrom Scientific).

Study Endpoints
The primary study endpoint was the occurrence of neurological events during follow-up defined as the composite of major or minor ischemic stroke, carotid endarterectomy (CEA), or carotid stenting (CAS). Mandatory cranial computed tomography was used for confirmation of the diagnosis. An independent neurologist who was not aware of the study protocol or the ADP genotypes assessed diagnosis of stroke. Based on the neurologist’s judgment and after confirmation by cranial computed tomography, the study endpoints were adjudicated by 2 of the authors independently. The authors were blinded with respect to medication and genotypes. Furthermore, death from any cause was considered as secondary endpoint.

Medication
All patients were on antplatelet therapy, either with clopidogrel or with acetylsalicylic acid. None of the patients received a combination therapy of clopidogrel and aspirin. Patients were divided according to use or nonuse of clopidogrel, defined as continuous administration of the drug during the follow-up period. The terminology “clopidogrel resistance,” used in the literature is confusing, alternatively meaning failure to achieve a pharmacologic effect or failure of therapy for which a drug is prescribed. For the purpose of reporting the present findings, we use this term to indicate a failure to achieve adequate platelet inhibition by clopidogrel.

Follow-up
Patients were clinically re-evaluated routinely 3, 6, and 12 months after hospital discharge and thereafter annually at the outpatient ward of our department for 2 years.

Statistical Methods
Continuous data are presented as the median and the interquartile range (range from the 25th to the 75th percentile). We used χ² tests to compare proportions and Mann–Whitney U tests or Kruskal–Wallis tests for univariate comparisons, as appropriate. For all analyses of the P2Y12 genotypes in clopidogrel users and nonusers, we considered carriers versus noncarriers of the mutant alleles (homozygous and heterozygous versus wild-type patients). Because of small numbers within the subgroups, no separate analysis for mutated, heterozygous, and wild-type clopidogrel users and nonusers was performed. Event-free survival rates (freedom from stroke, CAS, or CEA) according to patients’ genotype and use or nonuse of clopidogrel are presented as a Kaplan–Meier curve and compared by means of the log rank test. Multivariate Cox proportional hazards analysis was applied to assess the effect of the P2Y12 genotype in clopidogrel users and nonusers on event-free survival. Baseline variables were entered as possible predictor variables into the model to adjust for confounding effects if they: (1) were imbalanced between carriers and noncarriers of the 34C>T polymorphism who were or were not treated with clopidogrel indicated by P<0.2; or (2) were established risk factors for stroke. We tested for interactions between baseline variables by multiplicative interaction terms and log-likelihood χ² tests. Results of the Cox proportional hazards model are presented as the hazard ratio and the 95% confidence interval. We assessed the overall model fit using Cox–Snell residuals. Furthermore, we tested the proportional hazard assumption for all covariates using Schoenfeld residuals (overall test) and the scaled Schoenfeld residuals (variable-by-variable testing). According to the tests, the proportional hazards assumption was not violated. A 2-sided P<0.05 was considered as statistically significant. Calculations were performed with SPSS for Windows (Version 10.0; SPSS Inc) and Stata (release 8.0).

Results
Patients
We included 473 of 535 patients (88%) who were admitted with symptomatic PAD during the study period. 62 patients (12%) had to be excluded because of incomplete follow-up data or missing samples for DNA analysis. Patients with incomplete or missing data compared well to patients included in the study with respect to baseline clinical characteristics, without significant differences (data not shown). The median age of the 473 eligible patients was 69 years (interquartile range, 60 to 76 years), and 267 patients were male (56%). Antiplatelet therapy included clopidogrel (75 mg daily) in 137 patients (29%) and aspirin (100 mg daily) in 336...
patients (71%). Of 336 patients receiving aspirin, 41 patients were additionally administered vitamin K antagonists.

**P2Y12 Receptor Genotype**

Genotype frequencies for mutated, heterozygous, and wild-type patients for the 34C>T and the 52G>T polymorphism were 9% (n=40), 44% (n=210), and 47% (n=223), and 4% (n=17), 27% (n=127), and 70% (n=329), respectively. Because the number of homozygous mutation carriers was small, homozygous and heterozygous patients were combined for statistical analyses. Both genotype distributions followed the Hardy–Weinberg equilibrium and corresponded well with frequencies described previously.9

**Follow-up for Neurological Events**

During the median follow-up period of 21 months (interquartile range, 13 to 25), neurological events occurred in 38 patients (8%); of these, 22 patients had an ischemic stroke, 19 patients underwent CAS, and 4 patients had CEA. Indications for these 23 revascularization procedures were strokes in 7 patients (which are counted in the number of 22 overall strokes), ipsilateral transient ischemic attacks in 10 patients, and rapidly progressive carotid stenosis from a degree of stenosis of <70% to >90% within 6 months in 6 patients.

Fifty-eight patients (12%) died during follow-up; 51 of 58 case fatalities were caused by cardiovascular events. Of the 22 stroke patients, 7 died (4 fatal stroke, 3 late deaths).

**P2Y12 Receptor Genotype, Clopidogrel, and Neurological Outcome**

Cumulative rates of neurological events in clopidogrel users and nonusers according to the 34C>T and the 52G>T polymorphisms are presented in the Figure. Among those patients treated with clopidogrel, carriers of the 34C>T allele had a significantly higher rate of neurological events than noncarriers. In patients with aspirin therapy, the 34C>T polymorphism did not seem to affect neurological outcome. No significant interaction between the 52G>T polymorphism, use of clopidogrel, and neurological events was found (Figure). Being aware of a potential bias introduced by the combined endpoint of stroke and revascularization, we performed a sensitivity analysis calculating the multivariable models for stroke as the study endpoint. Although this resulted in wider confidence intervals because of the smaller number of events (n=22 instead of 38), comparable results were obtained. In clopidogrel nonusers, no significant association between the 34C>T carrier status and stroke was observed (adjusted hazard ratio, 1.44; 95% CI, 0.51 to 6.08; P=0.51), whereas in clopidogrel users 34C>T carriers had a higher risk for stroke than patients with aspirin (adjusted hazard ratio, 3.96; 95% CI, 1.02 to 17.84; P=0.048). Similarly, including the 10 patients who had a transient ischemic attack and subsequent CAS or CEA into a combined endpoint with stroke (overall number of events n=32), a significant association between 34C>T carrier status and neurological outcome was only observed in clopidogrel users. We also applied multivariate Cox proportional hazards models to account for baseline imbalances. To identify possible confounders, demographic data and clinical characteristics of carriers and noncarriers of the 34C>T polymorphism who were users of clopidogrel or aspirin were compared (Table 1). Acknowledging a potential interaction between clopidogrel and statins,17 we formally tested for interaction between these variables, and the ADP genotype with respect to patient outcome, by multiplicative interaction terms and log-likelihood ratio tests, without detecting a significant effect modification. The variables hyperlipidemia, critical limb ischemia, history of stroke, and medication with statins were differently distributed between the 4 groups. We included these and other potential confounders into multivariable Cox models (Table 2). In aspirin users, the 34C>T polymorphism was not associated with neurological events in univariate and multivariate analyses (P=0.27 and P=0.30, respectively). However, in patients receiving clopidogrel therapy, carriers of the 34C>T polymorphism had a 4.02-fold increased adjusted risk for neurological events compared with 34C>T noncarriers (95% CI, 1.08 to 14.9) (Table 2).

Neither the 34C>T polymorphism (P=0.77) nor the 52G>T polymorphism (P=0.66) was significantly associated with all-cause mortality in this patient series.
TABLE 1. Demographic Data and Clinical Characteristics of 473 Patients

<table>
<thead>
<tr>
<th></th>
<th>34C&gt;T Noncarriers</th>
<th>34C&gt;T Noncarriers</th>
<th>34C&gt;T Carriers</th>
<th>34C&gt;T Carriers</th>
<th>34C&gt;T Carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With Aspirin</td>
<td>With Clopidogrel</td>
<td>With Aspirin</td>
<td>With Clopidogrel</td>
<td></td>
</tr>
<tr>
<td>(n=170, 36%)</td>
<td>(n=53, 11%)</td>
<td>(n=166, 35%)</td>
<td>(n=84, 18%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>68 (58 to 76)</td>
<td>69 (63 to 76)</td>
<td>70 (59 to 75)</td>
<td>70 (61 to 77)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>97 (57%)</td>
<td>37 (70%)</td>
<td>86 (52%)</td>
<td>47 (56%)</td>
<td></td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>25.9 (23.6 to 28.5)</td>
<td>25.4 (22.6 to 27.6)</td>
<td>26.0 (23.7 to 29.0)</td>
<td>25.7 (23.3 to 27.8)</td>
<td></td>
</tr>
<tr>
<td>Arterial hypertension</td>
<td>116 (68%)</td>
<td>43 (81%)</td>
<td>130 (78%)</td>
<td>63 (75%)</td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>59 (35%)</td>
<td>17 (32%)</td>
<td>73 (44%)</td>
<td>33 (39%)</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>70 (41%)</td>
<td>20 (38%)</td>
<td>72 (43%)</td>
<td>27 (32%)</td>
<td></td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>125 (74%)</td>
<td>42 (79%)</td>
<td>126 (78%)</td>
<td>76 (91%)</td>
<td></td>
</tr>
<tr>
<td>Serum creatinine, mg/dL</td>
<td>1.07 (0.92 to 1.25)</td>
<td>1.06 (0.94 to 1.32)</td>
<td>1.08 (0.92 to 1.28)</td>
<td>1.04 (0.93 to 1.28)</td>
<td></td>
</tr>
<tr>
<td>Critical limb ischemia*</td>
<td>37 (22%)</td>
<td>4 (8%)</td>
<td>40 (24%)</td>
<td>12 (14%)</td>
<td></td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>75 (44%)</td>
<td>26 (49%)</td>
<td>74 (27%)</td>
<td>39 (46%)</td>
<td></td>
</tr>
<tr>
<td>History of MI</td>
<td>38 (22%)</td>
<td>10 (19%)</td>
<td>43 (26%)</td>
<td>20 (24%)</td>
<td></td>
</tr>
<tr>
<td>History of stroke</td>
<td>15 (9%)</td>
<td>12 (23%)</td>
<td>22 (13%)</td>
<td>9 (11%)</td>
<td></td>
</tr>
<tr>
<td>Statin therapy</td>
<td>74 (44%)</td>
<td>31 (59%)</td>
<td>88 (53%)</td>
<td>57 (68%)</td>
<td></td>
</tr>
</tbody>
</table>

Laboratory data have been obtained at baseline, before clopidogrel therapy had been initiated. Data are given as counts and percentages or median and interquartile range (range from the 25th to the 75th percentile). *Otherwise claudication.

CCS indicates Canadian Cardiovascular Society; MI, myocardial infarction.

We found that subjects undergoing current clopidogrel treatment, who are carriers of the P2Y12 polymorphism 34C>T, had a 4-fold higher risk to have an adverse neurological event, defined as ischemic stroke and/or carotid revascularization within a 2-year observation period than subjects carrying the wild-type genotype. Sequence variation of the P2Y12 is likely to alter affinity of platelets to ADP. Impaired ADP-mediated platelet response in turn may translate to impaired inhibition of ADP-mediated platelet aggregation by clopidogrel, ie, modulation of the receptor binding affinity of clopidogrel. Thromboxane-mediated platelet aggregation, such as efficacy of aspirin therapy, was not affected by sequence variations in P2Y12. These findings suggest that failure of antiplatelet drug therapy with thienopyridines, ie, “clopidogrel resistance,” may be related to genetic variations of the target receptor.

For many drugs, great heterogeneity in individual treatment efficacy has been described. For example, a link between aspirin resistance and recurrent cerebrovascular accidents within 2 years has been reported. Accordingly, inter-individual variability in drug response to other antiplatelet agents, ie, clopidogrel has been suggested, because up to 5% of patients undergoing coronary stenting have thrombotic stent occlusion under clopidogrel treatment. Recently, ex vivo clopidogrel resistance, measured by a platelet function analyzer, was described to account for recurrent cardiovascular events in patients with acute coronary syndrome.

Despite a growing number of randomized clinical trials on clopidogrel efficacy, the molecular mechanism of action and pharmacological properties of this drug have not been fully elucidated yet. Biotransformation of the pro-drug by cytochrome P-450 has been suggested. The active clopidogrel metabolite forms disulfide bridges in its target, the ADP platelet receptor P2Y12, thereby irreversibly inhibiting binding of ADP to platelets. As a result, platelet activation,
degranulation, and aggregation are blocked.\textsuperscript{19,20} For clopidogrel resistance, several possible mechanisms have been proposed, including: (1) inappropriate dosing; (2) drug–drug interactions; (3) inherently increased platelet reactivity; and (4) a low baseline metabolic activity or downregulation of cytochrome P450.\textsuperscript{3,5,17,21,22} The recent discovery of several mutations in platelet surface glycoproteins has stimulated interest in the role of genetic alterations in platelet physiology such as inter-individual differences in response to antiplatelet treatment.\textsuperscript{23}

In the present study, 2 frequent receptor polymorphisms were studied. Of the patients studied, 50% displayed at least 1 mutated allele of the 34C>T, whereas mutations of the 52G>T polymorphism were found in 30%. The prevalences are in good agreement with an earlier study of Fontana et al on a smaller number of patients with PAD.\textsuperscript{10} Neither polymorphism was associated with all-cause mortality. The 52G>T receptor polymorphism did not influence the response to clopidogrel therapy, corroborating earlier findings of a lack of association between the 52G>T polymorphism and history of ischemic events.\textsuperscript{10} However, acknowledging the lower frequency of 52G>T carriers as compared with the 34C>T mutation, the lack of association between 52G>T and ischemic events might also be a problem of sample size and power. In contrast, carriers of the 34C>T sequence variation had a significantly higher risk for cerebrovascular events under clopidogrel therapy.

The molecular mechanism by which the 34C>T polymorphism leads to a poor response to clopidogrel remains to be determined. Possibly, the 34C>T mutation confers a higher mRNA stability followed by a higher receptor density on the platelet surface.\textsuperscript{9} Consequently, in carriers of this polymorphism, the standard dosage of clopidogrel may not modulate the receptor binding sites sufficiently. Increasing the dosage of antiplatelet therapy, such as combination therapies, however, are at considerable risk for the occurrence of bleeding complications.\textsuperscript{24}

Whether patients with PAD and concomitant cerebrovascular lesions carrying the 34C>T mutation would benefit from higher clopidogrel dosage will have to be studied in prospective trials.

Several limitations of the present investigation should be mentioned. First, the use of the term “clopidogrel resistance” to indicate a failure to achieve adequate platelet inhibition by clopidogrel in this study is not supported by clinical efficacy data, because we did not determine platelet aggregation. Second, other mechanisms, already addressed, such as low baseline activity or downregulation of cytochrome-P450\textsuperscript{3,5,17,21,22} or drug–drug interactions of clopidogrel with other medications, ie, statins,\textsuperscript{17} could have potentially been contributing to the observed resistance to clopidogrel therapy. Furthermore, we cannot rule out that other mechanisms, than drug resistance, influenced the reasons for the risk for cerebrovascular events. Because of the lack of the entire gene sequence structure of P2Y12, it cannot be ruled out that additional gene alterations in the P2Y12 gene and further sequence alterations of other platelet receptor genes contributed to the observed hyper-reactivity and thrombosis, ie, by modulating affinity to other receptors. The nonrandomized study design certainly limits the power of our conclusions, particularly because selection bias for clopidogrel cannot be excluded. Patients with more severe disease were probably receiving clopidogrel rather than aspirin, assuming an enhanced vascular-protective effect in patients with advance PAD.\textsuperscript{2}

Conclusion
Clopidogrel response variability exists in healthy subjects and in patients with coronary artery disease. The present analysis suggests that genetic variation of the P2Y12 receptor gene is associated with higher numbers of cerebrovascular events in patients with PAD receiving clopidogrel therapy. Whether determination of the P2Y12 polymorphism (34C>T) could serve as a risk stratification marker to predict possible failure of therapy or to identify patients who are candidates for alternate agents or higher doses of clopidogrel should be the subject of future testing.

References


Association of a Functional Polymorphism in the Clopidogrel Target Receptor Gene, \textit{P2Y12}, and the Risk for Ischemic Cerebrovascular Events in Patients With Peripheral Artery Disease

Sophie Ziegler, Martin Schillinger, Marion Funk, Katharina Felber, Markus Exner, Wolfgang Mlekusch, Schila Sabeti, Jasmin Amighi, Erich Minar, Martin Brunner, Markus Müller and Christine Mannhalter

\textit{Stroke}. 2005;36:1394-1399; originally published online June 2, 2005; doi: 10.1161/01.STR.0000169922.79281.a5

\textit{Stroke} is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/36/7/1394

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in \textit{Stroke} can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to \textit{Stroke} is online at:
http://stroke.ahajournals.org//subscriptions/