The C50T polymorphism of the cyclooxygenase-1 gene and the risk of thrombotic events during low-dose therapy with acetyl salicylic acid

Nick Clappers1, Martijn G. H. van Oijen2, Santosh Sundaresan3, Marc A. Brouwer1, Rene H. M. te Morsche2, Wessel Keuper1, Wilbert H. M. Peters2, Joost P. H. Drenth2, Freek W. A. Verheugt1
1Radboud University Medical Centre, Department of Cardiology; 2Department of Gastro-enterology; Nijmegen, The Netherlands; 3Christian Medical College and Hospital, Department of Gastrointestinal Sciences, Vellore, India

Summary
Aspirin prevents thrombotic events by inhibiting platelet cyclooxygenase-1 (COX-1), thus reducing thromboxane A2 formation and platelet aggregation. The C50T polymorphism of COX-1 is associated with an impaired inhibition of both thromboxane production and in-vitro platelet aggregation by aspirin. We studied whether this polymorphism is also associated with the risk of clinical thrombotic events in patients using aspirin. We included 496 patients admitted to our Coronary Care Unit for various indications treated with aspirin 80 mg daily. Genotyping for the C50T polymorphism demonstrated that 86.7% of the patients had the common genotype, and 13.3% had the variant (12.5% heterozygous, 0.8% homozygous). Baseline variables were well balanced, except that patients with the common genotype more frequently used aspirin prior to admission compared to those patients with the variant genotype. The composite primary endpoint of myocardial infarction, stroke, and/or cardiovascular death occurred in 98 patients (19.8%). Myocardial infarction occurred in 9.6% of patients, stroke in 1.6%, and cardiovascular death in 12.1%. The unadjusted hazard ratio (95% CI) for the primary endpoint for patients with the variant versus the common genotype was 1.07 (0.62–1.85), p=0.8. The adjusted hazard ratio was 0.86 (0.49–1.50), p=0.6. In prior laboratory studies the COX-1 C50T polymorphism was associated with an impaired inhibitory effect of aspirin on thromboxane production and platelet function. However, in this cohort of patients using low-dose aspirin for secondary prevention the polymorphism was not associated with a higher risk of atherothrombotic events.

Keywords
Aspirin, antiplatelet therapy, clinical follow-up, cyclooxygenase 1, pharmacogenetics

Introduction
Aspirin is widely used for secondary prevention after coronary thrombotic events, resulting in a relative reduction of recurrent events of about 25% (1). The main mechanism of action is the irreversible inactivation of cyclooxygenase-1 (COX-1) in platelets, which in turn leads to a strong suppression of the prothrombotic substance thromboxane A2 (TxA2) (2). Since COX-1 is the target of aspirin, genetic variation in this enzyme may affect its activity as well as modulate the effect of aspirin. Many single nucleotide polymorphisms of the COX-1 gene have been identified (3, 4). One of these, the C50T polymorphism (NCBI accession number rs3842787) is of particular interest as several studies have consistently demonstrated a functional effect of this variant. Specifically, the presence of this polymorphism reduces the effect of 80–160 mg aspirin on TxA2 production in vitro (5) and in vivo (6), and on platelet aggregation in vitro (3, 5, 6). In patients chronically using aspirin, the odds of being “aspirin resistant” as measured with arachidonic acid-stimulated light transmittance aggregometry were four to seven times higher among patients who were hetero- or homozygous for the variant (3, 5).

The C50T polymorphism is located in exon-2 of the COX-1 gene, and causes a proline to leucine amino acid substitution in
position 17 of the signal peptide of COX-1. The C50T polymorphism is in complete linkage disequilibrium with the A-842G polymorphism, which is located in the promoter region of the gene. Studies in patients with coronary artery disease performed so far mainly included Caucasians, and 12–19% were carriers of the C50T polymorphism (3, 5, 7). The percentage of carriers is probably similar among African Americans (8), but the polymorphism was not found in a Chinese population (9).

Although several studies have investigated the C50T polymorphism in relation to the laboratory antiplatelet effects of aspirin, to the best of our knowledge, this study is the first to investigate the polymorphism in relation to the risk of clinical thrombotic events in patients using low-dose aspirin. Therefore, we hypothesized that among the patients using 80 mg aspirin daily for secondary prevention, those who are hetero- or homozygous for the C50T variant would have a higher risk of new clinical thrombotic events compared to the patients with the most common COX-1 genotype.

### Methods

#### Subjects

Consecutive patients admitted to the Coronary Care Unit (CCU) of the Radboud University Nijmegen Medical Centre during office hours between April 2002 and January 2004 were eligible for inclusion in the cohort. The cohort consisted of all comers to the CCU, and the part of the population that was included between April 2002 and October 2002 has been described previously (10). Patients were asked for their consent to draw and store a blood sample for future analyses and for clinical follow-up. The present analysis is restricted to those patients who were treated with aspirin 80 mg daily during the initial hospitalization and during follow-up after discharge. Demographic variables and baseline characteristics were retrieved from the admission forms of the patients’ medical files. The main diagnosis at discharge was derived from the discharge summary.

<table>
<thead>
<tr>
<th>Table 1: Baseline characteristics.</th>
<th>Common genotype (n=430)</th>
<th>Variant genotype (n=66)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (SD) years</td>
<td>63 (12)</td>
<td>65 (13)</td>
<td>ns</td>
</tr>
<tr>
<td>Median serum creatinine (IQR) µM</td>
<td>90 (79–105)</td>
<td>94 (79–107)</td>
<td>ns</td>
</tr>
<tr>
<td>Male gender (%)</td>
<td>281 (65)</td>
<td>43 (65)</td>
<td>ns</td>
</tr>
<tr>
<td>Prior aspirin use (%)</td>
<td>237 (55)</td>
<td>27 (41)</td>
<td>0.03</td>
</tr>
<tr>
<td>History of atherothrombotic events (%)</td>
<td>171 (40)</td>
<td>21 (32)</td>
<td>ns</td>
</tr>
<tr>
<td>History of stable angina pectoris (%)</td>
<td>69 (16)</td>
<td>12 (18)</td>
<td>ns</td>
</tr>
</tbody>
</table>

#### Cardiovascular risk factors

- Smoking (%): 167 (39) vs 23 (35), ns
- Hypertension (%): 207 (48) vs 28 (42), ns
- Family history of cardiovascular events (%): 141 (33) vs 28 (42), ns
- Diabetes mellitus (%): 81 (19) vs 14 (21), ns
- Hypercholesterolemia (%): 163 (38) vs 23 (35), ns

#### Main reason for admission

- ST elevation myocardial infarction (%): 111 (26) vs 14 (21), ns
- Non-ST elevation myocardial infarction (%): 104 (24) vs 22 (33), ns
- Unstable angina (%): 93 (22) vs 17 (26), ns
- Decompensated heart failure (%): 20 (5) vs 3 (5), ns
- PCI for stable coronary artery disease (%): 23 (5) vs 2 (3), ns
- Atrial fibrillation/flutter (%): 10 (2) vs 0 (0), ns
- Other (%): 69 (16) vs 8 (12), ns

#### Additional antithrombotics at discharge

- Clopidogrel (%): 127 (30) vs 18 (27), ns
- Oral anticoagulation (%): 43 (10) vs 6 (9), ns

SD, standard deviation; IQR, interquartile range; PCI, percutaneous coronary intervention; Ns, not significant.
Clappers et al. ASA, the COX-1 C50T polymorphism, and the risk of thrombotic events

Isolation of DNA and genotyping
Isolation of DNA and genotyping using polymerase chain reaction, followed by restriction fragment length polymorphism (RFLP) was performed as has previously been described in detail (11).

Endpoints and clinical follow-up
The primary study endpoint was the composite of cardiovascular death, myocardial infarction (ST-elevation and non-ST-elevation myocardial infarction) and/or stroke. Secondary endpoints were the individual components of the primary composite endpoint, and all cause mortality. Cardiovascular death was classified according to the criteria given in the study of Lonn et al., and death from uncertain causes was also classified as cardiovascular mortality (12). ST-elevation myocardial infarction (STEMI) was defined as ST-elevation on an electrocardiogram, in combination with a typical rise and fall of a specific cardiac marker. Non-STEMI was defined as typical anginal pain, a typical rise and fall of a specific cardiac marker, but no (persisting) ST-elevation on the electrocardiogram. Stroke was defined as persisting neurological deficit (at least 24 hours) with computed tomography or magnetic resonance imaging confirmation of ischaemic brain damage.

Long-term clinical follow-up was started at hospital admission and was obtained by medical chart review, and by checking the municipal administration for survival status. Patients who were still alive were sent questionnaires to obtain information on hospital admissions for thrombotic events (myocardial infarction or stroke). Additional information was obtained from general practitioners, and/or from treating specialists. When a patient had deceased, the general practitioner was requested for information on re-admissions for thrombotic events prior to death and on the cause of death. Additional information was requested from the treating specialist when necessary. The follow-up was completed in July 2007.

The outcome events have been adjudicated by two clinically experienced physicians who were blinded for the patients’ genotypic information. In the case of disagreement the opinion of a third physician was decisive.

Statistics
Statistical analysis was performed with SPSS statistical software, version 14.0 (SPSS Inc., Chicago, IL, USA). Frequency tables were provided describing patient baseline characteristics. The Chi-square method, or the Fisher exact test, were used to compare categorical variables. Continuous variables were compared with the Student t-test or the Mann-Whitney U method where appropriate. Univariate and multivariate analyses of event-free survival were performed with Cox regression. Covariates were used in the multivariate analysis when they significantly differed between patients bearing the most common or variant genotypes, or when they were associated with the primary endpoint at a level of p<0.1 in the univariate analysis. Using forward selection, a covariate was allowed into the multivariate model if it influenced the model with a likelihood ratio significance level of p<0.05, and was removed again if its significance level exceeded p=0.1 during any of the following steps. The C50T genotype was forced into the model. A Kaplan-Meier plot was made for visual comparison of event-free survival between patients with the common and the variant genotype. Two-sided p-values of <0.05 were considered statistically significant.

Table 2: Univariate analysis of the primary and secondary study endpoints.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Common genotype (N=430)</th>
<th>Variant genotype (N=66)</th>
<th>HR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary composite endpoint (%)</td>
<td>83 (19.3)</td>
<td>15 (22.7)</td>
<td>1.07 (0.62 – 1.85)</td>
<td>0.8</td>
</tr>
<tr>
<td>Cardiovascular mortality (%)</td>
<td>51 (11.9)</td>
<td>9 (13.6)</td>
<td>1.04 (0.51 – 2.11)</td>
<td>0.9</td>
</tr>
<tr>
<td>STEMI (%)</td>
<td>18 (4.2)</td>
<td>5 (7.6)</td>
<td>1.73 (0.64 – 4.67)</td>
<td>0.3</td>
</tr>
<tr>
<td>Non STEMI (%)</td>
<td>23 (5.3)</td>
<td>4 (6.1)</td>
<td>0.99 (0.34 – 2.88)</td>
<td>1.0</td>
</tr>
<tr>
<td>Stroke (%)</td>
<td>8 (1.9)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All cause mortality (%)</td>
<td>86 (20.0)</td>
<td>13 (19.7)</td>
<td>0.88 (0.49 – 1.57)</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Variant genotype: hetero-, or homozygous for the C50T cyclo-oxygenase 1 polymorphism; HR, hazard ratio for variant patients compared to patients with the common genotype; 95% CI, 95% confidence interval; STEMI, ST elevation myocardial infarction.
Results

In total, we included 578 patients in the cohort, of whom 502 (87%) were treated with aspirin 80 mg daily during the index admission and during follow-up. Clinical follow-up was available for 497 patients. Genotyping for the C50T COX-1 polymorphism was successfully performed for 496 patients. These 496 patients were included in the analysis. The subgroup of 430 (86.7%) patients homozygous for the most common allele, was compared to the subgroup of 66 (13.3%) patients with the variant genotype. In the subgroup with the variant genotype 62 (12.5%) were heterozygous, and 4 (0.8%) were homozygous. The genotype distribution was in Hardy-Weinberg equilibrium. Baseline characteristics were well balanced between the genetic subgroups, except aspirin use prior to the index admission was less frequent in patients with the variant genotype compared to patients homozygous for the most common genotype, respectively (41% vs. 55%, p=0.03) (Table 1).

Clinical outcomes

The median duration of clinical follow-up was 2.5 years (interquartile range 1.9 – 3.2). During a total of 1,258 patient-years of follow-up the composite primary endpoint of cardiovascular death, STEMI, non-STEMI, and/or stroke was found in 98 patients (19.8% of the patients) which is consistent with an annual risk of 7.8%. The risk for the primary endpoint did not differ between patients with the variant genotype compared to those with the most common genotype: HR 1.07 (95% CI 0.62 – 1.85), p=0.8 (Fig. 1 and Table 2). Covariates that were significantly associated with the primary endpoint in univariate analysis were: increasing age, increasing serum creatinine, the use of aspirin prior to the index admission, a history of cardiovascular events, hypertension, diabetes mellitus, and a positive family history for premature cardiovascular events (Table 3). These covariates were used in the Cox regression multivariate model. In the multivariate analysis the following covariates were independently associated with an increased risk for the primary endpoint: increasing age, increasing serum creatinine, and diabetes mellitus (Table 3). When the C50T COX-1 polymorphism was entered into the multivariate model, the hazard ratio (HR) for patients with the variant genotype compared to those with the most common genotype was 0.86 (95% CI 0.49 – 1.50), p=0.6.

With respect to the secondary endpoints, STEMI occurred in 23 patients (4.6%), non-STEMI in 27 (5.4%), stroke in eight (1.6%), cardiovascular death in 60 (12.1%). The risk for these events was not significantly different between patients with the variant and with the most common genotypes (Table 2). Death of all causes occurred in a total of 99 patients (20.0%). The rate of all cause mortality did not differ either between the genetic subgroups.

Discussion

The present study was performed based on the hypothesis that aspirin-treated patients with the C50T variant of the COX-1 gene would have an increased risk of atherothrombotic events. However, in this cohort study of 496 patients using low-dose aspirin for secondary prevention we found no association between the functional C50T polymorphism and the risk of clinical thrombotic events.

To the best of our knowledge this is the first study to investigate the clinical relevance of the C50T COX-1 polymorphism in patients with coronary artery disease who use aspirin for secondary prevention. In our cardiovascular population the genotype distribution was in excellent agreement with the results of Ulrich et al. who genotyped a population of 621 controls for the C50T polymorphism (13).

Recently several laboratory studies have investigated COX-1 polymorphisms in relation to the antiplatelet effects of aspirin (3, 5, 6). Multiple polymorphisms of COX-1 have been identified, but only the C50T polymorphism was repeatedly shown to be of functional significance in relation to the antiplatelet effect of aspirin as measured in the laboratory. For example, Gonzalez-Conejero et al. demonstrated that aspirin-treated healthy subjects with the C50T polymorphism had an almost two-fold increased in-vivo thromboxane production (6). Lepäntalo et al. showed that platelet reactivity as measured with Platelet Function Analyzer-100 (PFA-100) was higher in cardiovascular patients with the C50T polymorphism (mean PFA-100 closure time 222 compared to 261 seconds). Also, the preferential COX-2 inhibitors celecoxib and rofecoxib usually weakly inhibit COX-1 as well, but this effect is even lower in subjects with the C50T polymorphism (14). In addition, a case-control study on aspirin’s preventive effect on colorectal polyps showed the clinical relevance of the C50T polymor-

Table 3: Results of the univariate and multivariate analyses for the primary composite endpoint.

<table>
<thead>
<tr>
<th>Covariate</th>
<th>HR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age per year increase</td>
<td>1.06 (1.04 – 1.08)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum creatinine per µM increase</td>
<td>1.003 (1.001 – 1.004)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Prior use of aspirin</td>
<td>1.90 (1.25 – 2.91)</td>
<td>0.003</td>
</tr>
<tr>
<td>History of atherothrombotic events</td>
<td>1.81 (1.22 – 2.69)</td>
<td>0.003</td>
</tr>
<tr>
<td>Family history of cardiovascular events</td>
<td>0.42 (0.26 – 0.69)</td>
<td>0.001</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1.55 (1.04 – 2.32)</td>
<td>0.03</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>2.40 (1.57 – 3.67)</td>
<td>0.001</td>
</tr>
<tr>
<td>C50T COX-1 variant</td>
<td>1.07 (0.62 – 1.85)</td>
<td>0.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Covariate</th>
<th>HR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age per year increase</td>
<td>1.06 (1.04 – 1.08)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum creatinine per µM increase</td>
<td>1.003 (1.002 – 1.005)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Prior use of aspirin</td>
<td>1.33 (0.81 – 2.16)</td>
<td>0.26</td>
</tr>
<tr>
<td>History of atherothrombotic events</td>
<td>1.21 (0.76 – 1.91)</td>
<td>0.43</td>
</tr>
<tr>
<td>Family history of cardiovascular events</td>
<td>0.63 (0.37 – 1.06)</td>
<td>0.08</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1.07 (0.70 – 1.62)</td>
<td>0.76</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>2.14 (1.40 – 3.28)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C50T COX-1 variant</td>
<td>0.86 (0.49 – 1.50)</td>
<td>0.6</td>
</tr>
</tbody>
</table>

HR, hazard ratio; CI, confidence interval; COX-1, cyclo-oxygenase 1.
phism (13). In this study, the preventive effect of aspirin and other non-steroid anti-inflammatory drugs (NSAIDs) on colorectal polyps was restricted to patients with the most common COX-1 genotype. Subjects with the C50T variant did not benefit from these drugs. In contrast, the results of our study do not support the hypothesis that the C50T polymorphism would be associated with an increased risk of atherothrombotic events during long-term clinical follow-up in patients using aspirin.

There are some potential explanations for the negative result. First, although the C50T polymorphism is related to higher on-aspirin thromboxane production and platelet aggregation, these laboratory markers need to be established as risk factors for atherothrombosis in large prospective studies. Presently, only a relatively small prospective, and a somewhat larger, retrospective study have indicated associations between these tests and the risk of atherothrombotic events (15, 16). Importantly, compliance to aspirin was not biochemically confirmed in these studies. Therefore, failure to take aspirin could have caused both the high residual platelet aggregation as well as the increased risk of thrombotic events. Second, in subjects with the C50T genotype, aspirin has less effect on thromboxane production and platelet aggregation, but this effect could still be enough to prevent clinical events. Third, most patients with the variant genotype were heterozygous. Potentially, homozygosity for the variant genotype is a risk factor for thrombotic events during aspirin treatment, but homozygosity was too rare to analyse this subgroup. Another factor that could have slightly influenced our findings is the difference in the use of aspirin prior to the index admission. This covariate was included in the multivariate analysis, but was not independently associated with the primary endpoint. Finally, clopidogrel use in addition to aspirin may have obscured potential differences in the antithrombotic efficacy of aspirin between the genetic subgroups. However, only 29% of the patients in the cohort were treated with clopidogrel, since the majority of patients was included before clopidogrel became standard therapy for unstable angina and non-STEMI (17), or STEMI (18, 19). Moreover, the most frequent indication for clopidogrel was bare-metal coronary stent implantation, and the treatment duration for this indication was only 28 days. Thus, most of the follow-up occurred during aspirin monotherapy, and only a few events occurred while the patient was on dual antiplatelet therapy. Additionally, the use of clopidogrel was evenly distributed between the genetic groups, and clopidogrel use was not associated with the primary endpoint in univariate analysis (data not shown).

Limitations
The present study was performed in order to find out whether the presence of the C50T polymorphism would be associated with a large increase in the risk of thrombotic events. High on-aspirin thromboxane production, and high on-aspirin platelet aggregation have been related to a two- or three-fold increase in the risk of thrombotic events, respectively (15, 16). For sample size calculation we assumed that the C50T polymorphism would also be associated with a two-fold increase in risk. Also, we assumed that one out of seven patients would be carriers of the polymorphism. In view of the overall event rate of nearly 20%, a sample size of 430 patients would have sufficed to demonstrate this difference in risk. Data from a recent meta-analysis indicated that aspirin resistance as measured with PFA-100 is associated with a relative risk of ~1.6 (20). We would have needed at least 1,000 patients to demonstrate such a risk difference.

Furthermore, we studied a rather heterogeneous cohort of patients. A more homogeneous patient population would have been preferable, for instance, a cohort consisting of aspirin-naïve patients admitted to the hospital for an STEMI. Moreover, we did not verify compliance to aspirin therapy during follow-up, although compliance is an important determinant of clinical prognosis (21). Nevertheless, differences in compliance between the genetic subgroups are unlikely. Finally, we did not perform platelet aggregation studies or take thromboxane measurements. Performing these tests might have provided mechanistic insight into the reason why the studied C50T polymorphism did not affect clinical outcome in our cohort.

Implications
Presently, determining the C50T COX-1 polymorphism in patients using aspirin as an antithrombotic is not indicated for risk stratification, nor for adjustment of the antithrombotic strategy. Since the effect of the polymorphism on aspirin’s laboratory effects are still intriguing, they deserve further exploration in a large, prospective study on atherothrombotic events, preferably with a substudy addressing thromboxane production and/or platelet function.

Conclusion
Several laboratory studies have shown that the C50T COX-1 polymorphism is associated with diminished platelet inhibition by aspirin. However, in this first clinical study of patients using aspirin we could not confirm that this impaired effect of aspirin on laboratory parameters of platelet function also translates into an increased risk of clinical thrombotic events.

References


