CYP2C19 But Not PON1 Genetic Variants Influence Clopidogrel Pharmacokinetics, Pharmacodynamics, and Clinical Efficacy in Post–Myocardial Infarction Patients

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Background—Reduced concentrations of clopidogrel active metabolite have been associated with diminished platelet inhibition and higher rates of adverse cardiovascular events. Paraoxonase-1 (PON1) has recently been proposed as a key enzyme for clopidogrel metabolic activation. We tested the effects of PON1 polymorphisms on clopidogrel pharmacokinetics and pharmacodynamics and the occurrence of cardiovascular outcomes in young post–myocardial infarction (MI) patients treated with clopidogrel.

Methods and Results—We genotyped PON1 (Q192R and L55M) and CYP2C19 variants in 106 patients enrolled in the PK/PD CLOVIS-2 trial. Patients were randomly exposed to a 300-mg or 900-mg clopidogrel loading dose in a crossover study design. Clopidogrel active metabolite isomer H4 (clopi-H4) and platelet function testing were measured serially after loading dose. There was no significant association between PON1 Q192R or L55M and clopi-H4 formation or antiplatelet response to clopidogrel after either loading dose. Using multivariable linear regression analyses, the CYP2C19*2 allele was the only predictor of clopi-H4 generation and platelet response irrespective of the platelet function assay. CYP2C19 loss-of-function but not PON1 variants were significantly associated with increased risk of major cardiovascular events (death, MI, and urgent coronary revascularization) occurring during long-term clopidogrel exposure in 371 young post-MI patients (age <45 years) enrolled in the AFJII cohort (CYP2C19 loss-of-function allele carrier versus noncarrier: hazard ratio, 2.26; 95% confidence interval, 1.15–4.41, P=0.02; PON1 QQ192 versus QR/RR192: hazard ratio, 1.03; 95% confidence interval, 0.50–2.11, P=0.93; PON1 LL55 versus LM/MM55: hazard ratio, 1.52; 95% confidence interval, 0.75–3.08, P=0.24).

Conclusions—Our study does not confirm that PON1 Q192R or L55M can influence clopidogrel pharmacokinetics or pharmacodynamics in post-MI patients.

Clinical Trial Registration—URL: http://www.clinicaltrials.gov. Unique identifier: NCT00822666.

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Key Words: clopidogrel | genetic polymorphisms | pharmacokinetics | cytochrome P450 | paraoxonase-1

| coronary artery disease | platelets | coronary stenting | myocardial infarction |

The pharmacological and clinical responses to clopidogrel are highly variable between patients.1,2 Lesser degrees of platelet inhibition and/or persistent high-on-treatment residual platelet reactivity are associated with a higher risk of acute thrombotic events.3-5 Clopidogrel is a prodrug that requires metabolic activation to generate its active thiol metabolite.6,7

Previous pharmacokinetic (PK) and pharmacodynamic (PD) analyses suggest that a significant portion of the variability in platelet response is explained by variability in plasma concentrations of the clopidogrel active metabolite.8-10 The genetic component of such variability is important.11 To date, the reduced-function genetic variants in the hepatic cytochrome P450–2C19 (CYP2C19) gene have been identified as the most prominent contributors to this variability.12 Carriers of CYP2C19 loss-of-function variants exhibit reduced concentrations of active drug metabolite,9,13 diminished platelet inhibition,9,13,14 and higher rates of adverse cardiovascular events.15,16 However, CYP2C19 genetic variants only account for a limited proportion of this response variability.11

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Paraoxonase-1 (PON1) is a hepatic esterase that binds circulating high-density lipoproteins to prevent oxidative modification of low-density lipoproteins and has been recently proposed as an important factor in the transformation of the intermediate metabolite 2-oxo-clopidogrel into the active thiol metabolite.\textsuperscript{17} Two common nonsynonymous single-nucleotide polymorphisms (Q192R and L55M) have been associated with significant changes in serum PON1 activity and level of expression.\textsuperscript{18,19} Using in vitro liver microsomal assays, Bouman et al found that PON1 QQ192 was associated with a lower rate of clopidogrel metabolic activation. The PON1 Q192 allele was also associated with a decrease in both maximal plasma concentrations of clopidogrel active metabolite and platelet inhibition after a single 600-mg clopidogrel loading dose (LD). In the same study, PON1 Q192 carriers had a higher risk for adverse cardiovascular outcomes; yet, no effect from CYP2C19 was identified.\textsuperscript{17}

To further replicate these important new findings, we interrogated CYP2C19 and PON1 (Q192R and L55M) among patients enrolled in the CLOVIS-2 randomized clinical trial (NCT 00822666) in whom clopidogrel active metabolite isomer H4 and platelet inhibition were measured in response to a 300 mg or 900 mg clopidogrel LD.\textsuperscript{8} We also compared the PD responses to maintenance dose (MD) of clopidogrel according to PON1 genetic variants in a larger cohort of post–myocardial infarction (MI) patients\textsuperscript{20} and assessed the association between PON1 and adverse cardiovascular outcomes.

WHAT IS KNOWN

- A significant portion of the variability in platelet response to clopidogrel is explained by the variable generation of its active metabolite.
- CYP2C19 has been identified as key determinant of clopidogrel activation but explains only a limited portion of the overall variability.
- Paraoxonase-1 (PON1) has recently been proposed as a more crucial enzyme than CYP2C19 for clopidogrel metabolic activation.

WHAT THE STUDY ADDS

- In contrast to CYP2C19, PON1 is not involved in clopidogrel active metabolite generation as measured in vivo in patients with coronary artery disease.
- PON1 does not influence platelet responsiveness to clopidogrel loading and maintenance doses, nor does it modify the risk of cardiovascular outcomes in clopidogrel-treated young post–myocardial infarction patients.

Methods

Patients

The present data correspond to secondary analyses of the CLOVIS-2 study (ClinicalTrials.gov number NCT00822666)\textsuperscript{8} and the AFJII multicenter registry (Appraisal of risk Factors in young Ischemic patients Justifying aggressive Intervention).\textsuperscript{20}

The CLOVIS-2 study was designed to investigate clopidogrel PK/PD according to CYP2C19 genetic variants. A total of 106 young post-MI patients of the AFJII multicenter registry\textsuperscript{20} treated with a MD of 75 mg of aspirin and/or 75 mg of clopidogrel (n=86) for at least 3 months were randomly assigned to an open label LD of 300 mg or 900 mg clopidogrel in a 2-period crossover design. An enrichment strategy was used where homozygous and heterozygous CYP2C19*2 patients were matched according to age and sex to CYP2C19 *1/*1 patients (noncarriers) from the same AFJII program with a 1:1 ratio for each heterozygous and a 2:1 ratio for each homozygous patient.\textsuperscript{8} Blood sampling was performed at sequential time points (ie, baseline, 1, 2, 4, and 6 hours after loading) during the 6 hours after drug intake for clopidogrel active metabolite isomer H4 (clopi-H4) quantification. Platelet function testing was performed before loading and 6 hours after drug loading.

The AFJII prospective multicenter registry has been designed to investigate genetic and nongenetic factors in patients ages >18 and <45 years and who have an established coronary disease defined by an episode of MI (ST-elevation or non–ST-elevation–MI). The influence of CYP2C19*2 carriage on clinical outcomes in 259 of these patients (included until April 1, 2008) has been previously reported.\textsuperscript{20} The present investigation reports on 371 patients on clopidogrel MD (75 mg/d) and for whom long-term clinical follow-up was available. Furthermore, platelet function monitoring using the VerifyNow P2Y\textsubscript{12} assay (Accumetrics Corporation, San Diego, CA) was available for 194 of these patients. Both research programs were approved by the Pitié-Salpêtrière University Hospital Ethics Committee. A written informed consent form for study participation and genetic analyses was obtained from each patient.

Procedures

In the CLOVIS-2 trial, platelet measurements were performed immediately after venipuncture in a single laboratory blinded to the assigned LD regimen and to the genetic profile of the study patients. Light transmission aggregometry (Model 490–4D, Chrono-Log Corporation, Kordia, The Netherlands) and the point of care (POC) VerifyNow P2Y\textsubscript{12} (VN-P2Y\textsubscript{12}) assay (Accumetrics Corporation, San Diego, CA) were used to measure platelet aggregation at baseline (before LD) and 6 hours after LD during both phases. The relative change in platelet aggregation was calculated as (aggregation at baseline minus aggregation at 6 hours post-LD)/aggregation at baseline×100. The residual platelet aggregation (RPA) corresponds to the level of aggregation curve (%) measured 6 minutes after 20 μmol/L ADP-induced platelet aggregation. For the VN-P2Y\textsubscript{12} assay, samples were run in the same laboratory according to the device package insert and results expressed as platelet reaction units (PRU).

For the PK analyses, whole blood was immediately stabilized after venipuncture with 500 mmol/L MPBr (3-methoxyphenacyl bromide) and processed to obtain platelet-poor plasma aliquots and stored at –80°C. Plasma concentration of active metabolite isomers stabilized as 3’methoxyacetophenone derivatives was determined by a stereoselective assay using a validated electrospray liquid chromatography tandem mass spectrometry (LC/MS/MS) method.\textsuperscript{21} Data on the active metabolite isomer H4 (clopi-H4) were reported since isomer H3 was inactive and isoforms H1 and H2 were non quantifiable. The area under the plasma concentration (AUC)–time curve from the time of administration of the LD to 6 hours (AUC\textsubscript{0–6}) of active metabolite and maximal plasma concentration (Cmax) were computed by noncompartmental methods of analysis with the use of the log-linear trapezoidal method (Winnonlin software).

In the AFJII cohort, the primary end point was a composite of cardiovascular death, nonfatal MI, and urgent revascularization as previously described.\textsuperscript{20} Definite stent thrombosis was additionally assessed on the basis of definitions from the Academic Research Consortium.\textsuperscript{22} Follow-up was every 6 months, with a detailed report of drug treatment. Clinical events occurring under clopidogrel exposure were retained for this analysis.

PON1 Q192R (rs662) and L55M (rs 854560) were interrogated using commercially available genotyping assays (TaqMan-validated SNP assays, Applied Biosystems, Foster City, CA, USA) and the
Student Briefly, we found that A total of 106 male patients (mean age, 40.1 ± 4.8 years; body mass index, 26.1 ± 3.8 kg/m²) completed the 2 periods of the randomized CLOVIS-2 study. There were no significant differences in baseline characteristics according to PON1 and CYP2C19 genetic variants (data not shown). Primary results of the CLOVIS-2 study have been previously reported.8 Briefly, we found that CYP2C19*2 carriers had significantly reduced responses to clopidogrel with a gene dose-effect and that clopidogrel resistance could be overcome by increasing the LD to 900 mg in heterozygotes but not in homozygous patients.

In the present study, there was no significant association between PON1 Q192R or L55M and either the maximal clopi-H4 concentrations or the AUC0–6 (in ng • h/mL). As shown in Table 1, both clopi-H4 Cmax and AUC0–6 were similar among PON1 Q192R genotype groups after administration of either the 300-mg or the 900-mg clopidogrel LD. After administration of the 300-mg LD, there was a nonsignificant trend for a reduction by ~25% in clopi-H4 Cmax and AUC0–6 in PON1 MM55 individuals as compared with LL55 individuals (P = 0.35 for Cmax; P = 0.18 for AUC0–6); however, this pattern was not observed after the 900-mg LD. In contrast, carriers of the CYP2C19*2 loss-of-function allele had a significant reduction in both clopi-H4 Cmax and AUC0–6 compared with noncarriers (Table 1), which was observed after both 300- and 900-mg LDs. After adjustment for CYP2C19*2, both PON1 variants were still not significantly associated with clopidogrel PK in this population (P = nonsignificant for all analyses on Cmax and AUC0–6 after both LDs). No significant interaction between the tested CYP2C19 and PON1 genetic variants was observed for either LDs.

In multivariable linear regression analyses containing the tested PON1 and CYP2C19 polymorphisms, the CYP2C19*2 allele was the only predictor of clopi-H4 Cmax and AUC0–6 after both LDs (AUC0–6 300 mg: P < 0.005; AUC0–6 900 mg: P < 0.01). Adjustment for potential confounders (weight, diabetes and use of proton pump inhibitor) did not alter this result. Of note, CYP2C19*2 genotype accounted for 8% of the variation in clopidogrel PK after both LDs.

Consistent with the PK analyses, platelet inhibition was not significantly associated with the tested PON1 genetic variants among these patients. Figure 1A and 1B show the relative change in platelet aggregation as assessed by the VerifyNow-P2Y12 assay. Similar results were found for residual and maximal platelet aggregation after stimulation with ADP 20 μmol/L (data not shown). The proportion of patients with high on-treatment platelet reactivity (as defined by maximal platelet aggregation >50%) on maintenance therapy was not significantly influenced by PON1 genetic variants: QQ192:
33.3% versus QR192: 34.3% versus RR192: 33.3%, P = 0.98; LL55: 38.3% versus LM55: 31.2% versus MM55: 14.3%, P = 0.50. In multivariate linear regression analyses using residual platelet aggregation after stimulation with ADP 20 μM/L or P2Y12 PRU values, CYP2C19*2 carriage remained the only significant predictor of platelet function response to clopidogrel LD irrespective of the platelet function assay (P < 0.001 for both loading doses). Multivariate analyses using maximal platelet aggregation or high on-treatment platelet reactivity as dependent variables lead to similar results. Again, no significant interactions between the tested CYP2C19 and PON1 genetic variants were observed.

Pharmacodynamic and Clinical Outcomes in the AFIJI Patients
The characteristics of the 371 genotyped patients are presented in Table 2. Interestingly, we found a significant association between the PON1 L55M loss-of-function variant and diabetes. PON1 LL55 patients were more frequently diabetics than PON1 LM55 or MM55 genotype carriers (LL55 18.1% versus LM/MM55 5.6%; P < 0.0001). There were no other significant differences identified according to PON1 L55M or Q192R carriage.

Most of our study population did not carry CYP2C19*2 (wt/wt homozygotes, n = 262; 71.0%). The vast majority of carriers were heterozygotes (wt/*2, n = 94, 25.5%), with a small proportion being homozygous (*2/*2, n = 13, 3.5%). Four patients were carriers of the *4 loss-of-function allele and two of the *3 loss-of-function allele. All of them were also carriers of one copy of the *2 allele. Baseline characteristics according to CYP2C19*2 carriage were well balanced20 (data not shown). The median clopidogrel exposure time was 2.6 years (interquartile range, 1.0–4.8) and did not differ according to PON1 or CYP2C19 genotypes.

Thirty-five patients presented with a major adverse cardiac event during clopidogrel exposure. Eighteen patients presented with a stent thrombosis. The rate of major adverse cardiac events (MACE) did not differ according to PON1 Q192R

Table 2. Baseline Characteristics of Genotyped Patients in the AFIJI Cohort

<table>
<thead>
<tr>
<th></th>
<th>Overall (n=371)</th>
<th>PON1 Q192R</th>
<th>PON1 L55M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QQ192 (n=168)</td>
<td>RR192 (n=148)</td>
<td>RR192 (n=55)</td>
</tr>
<tr>
<td>Age, y</td>
<td>40.3±5.5</td>
<td>39.6±5.4</td>
<td>41.0±5.5</td>
</tr>
<tr>
<td>Male sex, %</td>
<td>84.6</td>
<td>83.3</td>
<td>86.5</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.9±4.0</td>
<td>25.9±4.5</td>
<td>26.1±3.6</td>
</tr>
<tr>
<td>Ancestry, %</td>
<td>94.8</td>
<td>96.4</td>
<td>94.6</td>
</tr>
<tr>
<td>European</td>
<td>2.2</td>
<td>1.2</td>
<td>2.7</td>
</tr>
<tr>
<td>Black</td>
<td>3.0</td>
<td>2.4</td>
<td>2.7</td>
</tr>
<tr>
<td>Risk factors, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>10.5</td>
<td>8.3</td>
<td>10.1</td>
</tr>
<tr>
<td>Familial CAD</td>
<td>41.2</td>
<td>41.7</td>
<td>41.9</td>
</tr>
<tr>
<td>Current smoker</td>
<td>51.5</td>
<td>54.8</td>
<td>50.7</td>
</tr>
<tr>
<td>Hypertension</td>
<td>23.2</td>
<td>23.2</td>
<td>20.9</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>56.1</td>
<td>53.6</td>
<td>56.8</td>
</tr>
<tr>
<td>PCI, %</td>
<td>79.8</td>
<td>78.0</td>
<td>80.4</td>
</tr>
<tr>
<td>CABG, %</td>
<td>8.1</td>
<td>8.3</td>
<td>8.1</td>
</tr>
</tbody>
</table>

AFIJI indicates Appraisal of risk Factors in young Ischemic patients Justifying aggressive Intervention; PON1, Paraoxonase-1; BMI, body mass index; CAD, coronary artery disease; PCI, percutaneous coronary intervention; and CABG, coronary artery bypass graft.
The hazard ratio (HR) for QQ192 versus QR/RR192: HR, 1.07; 95% CI, 0.39–2.89; \( P = 0.89\); \( PON1 \) LL55 versus LM/MM55: HR, 1.94; 95% CI, 0.71–5.27, \( P = 0.19 \); and \( CYP2C19 \) loss-of-function allele carrier versus noncarrier: HR, 2.79; 95% CI, 1.09–7.16, \( P = 0.03 \).

Finally, PD response to a clopidogrel 75 mg/d MD was assessed using the VerifyNow P2Y12 assay in 194 of the 371 patients. \( PON1 \) allelic frequencies were similar between this subgroup of patients and the overall cohort of patients. In line with our findings on the effect of 300/900 mg clopidogrel LDs, there was no detectable effect of \( PON1 \) Q192R in QQ192 versus QR/RR192: HR, 1.03; 95% CI, 0.50–2.11, \( P = 0.93 \); \( PON1 \) LL55 versus LM/MM55: HR, 1.52; 95% CI, 0.75–3.08, \( P = 0.24 \); and \( CYP2C19 \) loss-of-function allele carrier versus noncarrier: HR, 2.26; 95% CI, 1.15–4.41, \( P = 0.02 \). Similar results were found when considering the occurrence of stent thrombosis (\( PON1 \) QQ192 versus QR/RR192: HR, 1.07; 95% CI, 0.39–2.89; \( P = 0.89 \); \( PON1 \) LL55 versus LM/MM55: HR, 1.94; 95% CI, 0.71–5.27, \( P = 0.19 \); and \( CYP2C19 \) loss-of-function allele carrier versus noncarrier: HR, 2.79; 95% CI, 1.09–7.16, \( P = 0.03 \)).

Discussion
The major finding of the present investigation is that \( PON1 \) polymorphisms (Q192R and L55M) are unlikely to be major determinants of the pharmacokinetic and pharmacodynamic responses to clopidogrel, and clinical efficacy of clopidogrel in young post-MI patients. This evidence comes from (1) the lack of difference in clopidogrel active metabolite isomer H4 plasma levels (measured with a stereoselective assay\(^{21}\)) according to \( PON1 \) genotype, (2) the concordant lack of influence of \( PON1 \) on the PD responsiveness to clopidogrel loading and maintenance doses in 2 separate studies, and (3) the absence of significant association between \( PON1 \) and the risk of cardiovascular outcomes in a large cohort of clopidogrel-treated young post-MI patients followed for up to 6 years. Conversely, this study did confirm the influence of \( CYP2C19 \) genetic variants on clopidogrel PK, PD, and clinical efficacy.

Our results are thus in contrast with the previous report from Bouman et al.\(^{17}\) who found that (1) \( PON1 \) is a crucial enzyme for transformation of the intermediate 2-oxo-clopidogrel metabolite to the active thiol metabolite using an in vitro metabolizing enzyme microsomal expression system, and (2) \( PON1 \) QQ192 genotype was associated with a 5-fold
reduction of active thiol metabolite concentration compared with RR192 patients. This discrepancy may be linked to differences in the methodology of active metabolite measurements between studies. Due to its stereochemical structure, the clopidogrel active metabolite belongs to a mixture of 4 different isomers.6–7 Among these, only the inactive H3 and active H4 isomers are detectable in human plasma.21 The existence of several active metabolite isomers and their chemical instability in blood might lead to approximation when measuring active metabolite concentrations with non-selective techniques.21 In our study, the clopidogrel active metabolite isomer H4 was specifically quantified with a recently developed stereoselective assay using chemically synthesized H4 isomer as a calibration standard.21 As such, we cannot exclude the possibility that PON1 participates in the generation of other inactive metabolites.

Differences in the study design are another potential explanation. In the CLOVIS-2 study, the PK/PD measurements were performed prospectively in stable coronary patients who were randomly assigned to an open-label 300-mg or 900-mg clopidogrel LD in a 2-period crossover fashion. This design should have limited the introduction of confounding factors as opposed to a post hoc evaluation of clopidogrel PK/PD in a selected case/control cohort.23 Notably, CYP2C19 was a significant predictor of clopidogrel PK and PD in the CLOVIS-2 study; however, it had no impact in the study from Bouman et al.17 The critical role of the CYP2C19 loss-of-function allele on the generation of clopidogrel active metabolite,6,24 platelet inhibition,8,14 and clinical outcome in clopidogrel-treated patients has been replicated in many independent studies and 2 recent meta-analyses15,16 and is further confirmed in the present investigation. However, we failed to observe such a clear continuum with the tested PON1 genetic variants.

The significant association between PON1 L55M carriage and diabetes is of importance given the established correlation between PON1 activity and the occurrence of diabetes,25,26 glucose intolerance,27 and vascular complications in diabetics.28 Moreover, PON1 genetic polymorphisms have also been associated with an overall increase in cardiovascular risk.18–20 Consistent with these findings, we report a trend for higher rates of cardiovascular events among PON1 LL55 patients, a genotype previously associated with glucose intolerance and impaired insulin secretion.27 Of note, this effect was attenuated after adjustment for diabetes status. These results further suggest that PON1 might influence clinical outcomes in clopidogrel-treated patients irrespective of a direct impact on clopidogrel PK or PD. This hypothesis warrants further investigation, however, because PON1 Q192R has been suggested to be the principal determinant of unexplained clopidogrel response variability17 while no data have been reported for PON1 L55M and clopidogrel PK or PD before our current study.

Our study has some limitations. Pharmacological and clinical responses to clopidogrel vary widely between patients. This interindividual variability can lead to spurious findings when analyzing cohorts of limited sample size, thus explaining the crucial need for replication of genetic association studies.23 Our study was conducted on a limited sample of young coronary patients and could have been underpowered to detect the influence of PON1 polymorphisms. Despite this limitation, our results do not support the Bouman et al finding that 73% of clopidogrel response variability is linked to PON1 Q192R whereas CYP2C19 genetic variants have no detectable effect.17 In addition, PON1 activity is also highly variable among patients. It is possible that such variability has not been fully captured by the current PON1 genotyping, further accounting for our overall negative results. However, many studies have reported a consistent link between the PON1 Q192R and L55M polymorphisms and PON1 activity. MM55 and QQ192 homozygotes have the lowest PON1 activity.18,19,25 In particular, Bouman et al reported that PON1 Q192R genotype and paraoxonase plasma activity have similar capacities to predict cardiovascular outcome.17 With respect to additional PON1 polymorphisms, a common promoter allele (eg, −108C/T) has been reported29 but this polymorphism was not specifically tested in the present study. However, a specific effect from this PON1 promoter polymorphism is unlikely, given its strong linkage disequilibrium with the currently tested L55M polymorphism. Finally, we cannot exclude the possibility that other ethnic-specific rare PON1 variants that were not genotyped in our study or the Bouman et al17 study participate in variable clopidogrel responsiveness.

Taken together, these results do not support an important contribution of PON1 Q192R and L55M as major determinants of clopidogrel PK and PD responsiveness, including the clinical efficacy of clopidogrel in young post-MI patients. Rather, these PON1 polymorphisms might be associated with metabolic disorders and/or cardiovascular risk independent of clopidogrel pharmacogenetics.

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References
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