Clarifying the importance of CYP2C19 and PON1 in the mechanism of clopidogrel bioactivation and in vivo antiplatelet response

Inna Y. Gong1,2, Natalie Crown2, Colin M. Suen2, Ute I. Schwarz1,2, George K. Dresser1, Michael J. Knauer1,2, Daisuke Sugiyama1, Marianne K. DeGorter1,2, Sarah Woolsey1,2, Rommel G. Tirona1,2, and Richard B. Kim1,2*

1Division of Clinical Pharmacology, Department of Medicine, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario, Canada; and 2Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario, Canada

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Aims
It is thought that clopidogrel bioactivation and antiplatelet response are related to cytochrome P450 2C19 (CYP2C19). However, a recent study challenged this notion by proposing CYP2C19 as wholly irrelevant, while identifying paraoxonase-1 (PON1) and its Q192R polymorphism as the major driver of clopidogrel bioactivation and efficacy. The aim of this study was to systematically elucidate the mechanism and relative contribution of PON1 in comparison to CYP2C19 to clopidogrel bioactivation and antiplatelet response.

Methods and results
First, the influence of CYP2C19 and PON1 polymorphisms and plasma paraoxonase activity on clopidogrel active metabolite (H4) levels and antiplatelet response was assessed in a cohort of healthy subjects (n = 21) after administration of a single 75 mg dose of clopidogrel. There was a remarkably good correlation between H4 AUC (0–8 h) and antiplatelet response (r² = 0.78). Furthermore, CYP2C19 but not PON1 genotype was predictive of H4 levels and antiplatelet response. There was no correlation between plasma paraoxonase activity and H4 levels. Secondly, metabolic profiling of clopidogrel in vitro confirmed the role of CYP2C19 in bioactivating clopidogrel to H4. However, heterologous expression of PON1 in cell-based systems revealed that PON1 cannot generate H4, but mediates the formation of another thiol metabolite, termed Endo. Importantly, Endo plasma levels in humans are nearly 20-fold lower than H4 and was not associated with any antiplatelet response.

Conclusion
Our results demonstrate that PON1 does not mediate clopidogrel active metabolite formation or antiplatelet action, while CYP2C19 activity and genotype remains a predictor of clopidogrel pharmacokinetics and antiplatelet response.

Keywords
CYP2C19 • PON1 • Clopidogrel • Pharmacokinetics • Pharmacogenetics • Antiplatelet response

Introduction
Antiplatelet therapy is an important therapeutic intervention for prevention of ischaemic events in patients with high-risk cardiovascular disease, particularly for those who undergo percutaneous coronary intervention (PCI).1 Currently, the standard of care for managing such patients is dual antiplatelet therapy with a P2Y₁₂ receptor antagonist and the cyclooxygenase 1 inhibitor aspirin. Clopidogrel is the most widely prescribed thienopyridine that exerts its pharmacological effect by irreversibly binding to P2Y₁₂ receptors on platelets, thereby diminishing adenosine diphosphate (ADP)-mediated platelet aggregation.2 Although benefits from clopidogrel have been widely documented in large clinical trials, marked interpatient variation in platelet responsiveness has meant that 21% of the patients remain at risk for coronary artery and stent thrombosis.3

Clopidogrel is a prodrug and its clinical efficacy appears to be a function of the amount of enzymatically derived active thiol
Previous in vitro studies have delineated that this bioactivation is a two-step process, catalysed by several cytochrome P450 (CYP) isozymes. Clopidogrel is first metabolized to the intermediate metabolite, 2-oxo-clopidogrel, followed by metabolism to a number of thiol metabolite stereoisomers, only one of which (H4) is active in vivo. Notably, both metabolic steps leading to H4 formation have been shown to be predominantly dependent on CYP2C19 and, to a lesser extent, CYP3A4. Importantly, in large clinical trials, CYP2C19*2 or *3 loss-of-function single-nucleotide polymorphisms (SNPs) have been associated with lower platelet inhibition and, consequently, an increased risk of major cardiovascular events.

In contrast, Bouman et al. recently demonstrated that a non-CYP enzyme, paraoxonase-1 (PON1), was the key determinant of clopidogrel active metabolite formation. Importantly, they showed that plasma PON1 activity as well as the Q192R SNP (rs662) in PON1, but not CYP2C19 SNPs, was predictive of antiplatelet response and risk for stent thrombosis in clopidogrel-treated patients. These findings fundamentally challenged our prior understanding of clopidogrel metabolism and efficacy. In the report, we set out to define a mechanistic link between clopidogrel metabolism and antiplatelet action to clarify the clinical relevance of PON1 and CYP2C19 to clopidogrel response.

Methods

Clinical study design

The study was approved by the Research Ethics Board at the University of Western Ontario. All subjects (age 18–65) were non-smokers, not taking concomitant medications, without previous exposure to clopidogrel, and deemed healthy per medical exam. Healthy volunteers who met the eligibility criteria were enrolled upon provision of written informed consent (n = 21; see Supplementary material online, Table S1).

Overnight fasted subjects received a single oral dose of clopidogrel (75 mg). In addition, 100 µg of midazolam was administered orally as an in vivo probe for CYP3A4 activity. For pharmacokinetic analysis, blood samples were collected over an 8 h period. Clopidogrel thiol metabolites were stabilized for analysis using EDTA tubes containing 50 µL of 125 mM 2-bromo-3-methoxyacetophenone (MPB) (Sigma-Aldrich, Oakville, Canada).

To determine antiplatelet response, blood was collected using a 1.8 mL sodium citrate (3.2%) tube at baseline and 4 h post-clopidogrel dose and subjected to the VerifyNow P2Y12 assay (Accumetrics, San Diego, CA, USA), as per the manufacturer’s protocol.

Genotype analysis and plasma paraoxonase activity were determined as described in Supplementary material online.

Clopidogrel bioactivation

The in vitro metabolic profiling of clopidogrel metabolism was conducted in microsomes (see Supplementary material online).

Liquid chromatography tandem mass spectrometry (LC–MS/MS) analysis

Quantitation of midazolam and clopidogrel metabolites (in vivo and in vitro) was performed as described in Supplementary material online.

Data analysis

All data analyses are as described in Supplementary material online.

Results

Influence of CYP2C19, PON1, and CYP3A4 on clopidogrel kinetics and response

Following a 75 mg dose, carriers of at least one reduced function CYP2C19 allele [CYP2C19*2 or *3 allele, reduced metabolizers (RMs); 38% of the study population] had significantly decreased total plasma exposure (area under the plasma concentration curve, AUC) of the H4 active metabolite when compared with the non-carrier extensive metabolizers (EMs) (Figure 1A; see Supplementary material online, Figure S1A). Similarly, the maximum plasma concentration (Cmax) was higher in EMs than RMs (Table 1). Prior to administration of clopidogrel, the mean platelet responsiveness [platelet reactive units (PRU)] induced by 20 µmol/L ADP and 22 nM prostaglandin E1 (PGE1) was similar between EMs and RMs (VerifyNow P2Y12 assay; Table 2). Four hours following clopidogrel administration, the absolute percentage change in PRU was significantly lower in RMs when compared with EMs (Figure 1B). In fact, we observed a strong correlation between H4 plasma exposure and platelet inhibition, demonstrating that individuals with highest exposure to H4 active metabolite have the greatest antiplatelet response (Figure 1C). Interestingly, we did not observe any correlation between gain-of-function PON1 Q192R polymorphism and clopidogrel pharmacokinetics or response, despite the fact that PON1 plasma activity, assessed ex vivo using paraoxon as the prototypical substrate, in the same healthy volunteers correlated well with the PON1 Q192R genotype (Figure 1D and E; see Supplementary material online, Figure S1B). In addition, no significant correlation was found between paraoxonase plasma activity and antiplatelet response (Figure 1F). Of note, exclusion of non-Caucasian participants in these analyses does not modify the above findings (data not shown).

Midazolam plasma AUC was not related to H4 AUC or antiplatelet response (P = 0.91, 0.65) (Figure 2).

Identification of other clopidogrel thiol metabolites in plasma

Using an ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) method, we were able to detect and quantify other clopidogrel thiol metabolites isobaric to H4 in the plasma of study subjects (Figure 3A). One metabolite, known to be inactive is termed H3, is stereochemically similar to H4 except being diastereomeric at the carbon 4 position. Another observed thiol metabolite is termed Endo, which differs from H3/H4, in that the carbon double bond is in the endocyclic position. In addition to chromatographic separation of H3, H4, and Endo (Figure 3C and F), the MS fragmentation signatures of the H3/H4 and Endo thiol metabolite were distinct (Figure 3D and E), ensuring analytical specificity of the isomers being analysed.
Biotransformation of clopidogrel to 2-oxo-clopidogrel

In the first step of the bioactivation process, the intrinsic clearances (CL_{int}) calculated from estimated \( k_m \) and \( V_{max} \) values show that CYP2C19 is more efficient in forming the intermediate metabolite than CYP3A4 in vitro (Figure 4A; see Supplementary material online, Table S2).

Biotransformation of 2-oxo-clopidogrel to H3 and H4 thiol metabolites

In the second step of the bioactivation process, the CL_{int} for H4 formation from 2-oxo-clopidogrel by CYP2C19 was greater than that with CYP3A4 (Figure 4B; see Supplementary material online, Table S2). Notably, the formation of H4 from 2-oxo-clopidogrel was dependent on the presence of 5 mM reduced glutathione (GSH) (data not shown). Based on lower H4 formation compared with 2-oxo-clopidogrel formation, it appears that the second reaction is the rate-limiting step of the overall clopidogrel bioactivation. We observed that the inactive metabolite, H3, was also formed from 2-oxo-clopidogrel by CYP2C19 at a relatively similar efficiency as H4 (see Supplementary material online, Table S2).

Biotransformation of 2-oxo-clopidogrel to Endo thiol metabolite

We incubated a range of 2-oxo-clopidogrel concentrations with baculovirus microsomes heterologously expressing CYP2C19 and HeLa cell-derived microsomes constitutively expressing PON1 while lacking any drug-metabolizing CYP enzymes. In the baculovirus microsomes, Endo metabolite formation was greater with CYP2C19 than in control baculovirus microsomes (Figure 4C; see Supplementary material online, Table S3). In addition, HeLa cell microsomes constitutively expressing PON1 were capable of forming Endo metabolite but not H4 (Figures 3B and 4D; see Supplementary material online, Table S3). We further confirmed the ability of PON1 to hydrolyse 2-oxo-clopidogrel using an adenovirus overexpressing system in HeLa cells, where Endo formation was 3.5-fold higher than vector control (LacZ) and no H4 was detected (Figure 4E). Moreover, Endo formation by PON1 was attenuated by the specific PON1 inhibitor 2-hydroxyquinoline (Figure 4E). We note that PON1-mediated Endo formation was not dependent on GSH (data not shown). Overall, our data suggests that PON1 can hydrolyse 2-oxo-clopidogrel to form Endo metabolite while unable to mediate H4 formation and that CYP2C19 also catalyses Endo formation. Analysis of total Endo

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Figure 1 The role of CYP2C19 and PON1 genetic polymorphisms in clopidogrel pharmacokinetic and pharmacodynamic responses. (A and B) Box-and-whisker plots of total active metabolite H4 plasma exposure (AUC) and antiplatelet response according to CYP2C19 genotype. (C) Scatter plot of H4 AUC and antiplatelet response (\( r^2 = 0.78 \)). (D and E) Box-and-whisker plots of H4 AUC, paraoxonase plasma activity, and antiplatelet response according to the PON1 genotype. (F) Scatter plot of plasma paraoxonase activity and antiplatelet response. Boxes indicate 25th and 75th percentile, whiskers denote the 95% confidence interval, and ‘+’ represents the mean. *\( P \), 0.05; **\( P \), 0.01.
plasma exposure in healthy volunteers demonstrated that its levels were more than 20-fold lower than H4 (data not shown), and unlike H4 (Figure 1C), Endo AUC did not correlate with antiplatelet response (Figure 4F).

Discussion

The impact of CYP2C19 genetic variations on clopidogrel antiplatelet response has been documented in a number of studies.\textsuperscript{11,14} In fact, a recent meta-analysis reported that carriers of CYP2C19*2 allele had a higher risk for major adverse cardiovascular events, increased mortality, and stent thrombosis compared with non-carriers, independent of baseline cardiovascular risk.\textsuperscript{14} We note that in contrast to these studies, one trial (CURE) reported similar clopidogrel efficacy irrespective of the CYP2C19 genotype.\textsuperscript{15} A potential explanation for the disparate findings is the difference in rate of PCI with stenting, where only 14.5% of the population underwent stenting in the CURE trial while majority of patients underwent stenting in other CYP2C19-supportive trials. Indeed, it has been consistently shown that the greatest clinical benefit with clopidogrel use is the reduction in stent thrombosis rate.\textsuperscript{16} Moreover, several prospective trials have restored diminished H4 exposure and poor antiplatelet response in CYP2C19 variant carriers by increasing clopidogrel dose, including one recently published multicentre double-blinded randomized clinical trial.\textsuperscript{17–19} However, our understanding of clopidogrel response in the context of pharmacogenomics was further complicated when Bouman et al.\textsuperscript{13} challenged the aforementioned findings by identifying the PON1 Q192R polymorphism as the only genetic marker associated with stent thrombosis, accounting for 72.5% of the response variation.

In the present study, we aimed to determine the influence of CYP2C19 and PON1 on clopidogrel metabolism and antiplatelet response. In contrast to Bouman et al., our results support the notion that decreased CYP2C19 function reduces the formation of clopidogrel active thiol metabolite, while PON1 showed no effect on active metabolite exposure. It should be noted that there are several challenges to accurate quantification of the active metabolite, H4. First, H4 is one of several diastereomeric thiol metabolites of clopidogrel observed during in vitro

### Table 1 H4 active metabolite pharmacokinetic parameters following administration of a single 75 mg oral dose of clopidogrel

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CYP2C19 EM</th>
<th>CYP2C19 RM</th>
<th>PON1 Q192Q</th>
<th>PON1 Q192R</th>
<th>PON1 R192R</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C\textsubscript{max} (ng/mL)</td>
<td>33.9 (21.0)</td>
<td>40.2 (23.3)</td>
<td>25.5 (13.7)</td>
<td>30.34 (19.80)</td>
<td>41.84 (30.67)</td>
<td>27.70 (6.08)</td>
</tr>
<tr>
<td>(t\textsubscript{max} (h))</td>
<td>0.73 (0.18)</td>
<td>0.77 (0.16)</td>
<td>0.67 (0.17)</td>
<td>0.78 (0.16)</td>
<td>0.68 (0.19)</td>
<td>0.65 (0.13)</td>
</tr>
<tr>
<td>(t\textsubscript{1/2} (h))</td>
<td>0.67 (0.19)</td>
<td>0.67 (0.20)</td>
<td>0.68 (0.19)</td>
<td>0.63 (0.25)</td>
<td>0.69 (0.20)</td>
<td>0.67 (0.16)</td>
</tr>
<tr>
<td>AUC\textsubscript{0–8 h} (ng h/mL)</td>
<td>37.40 (21.97)</td>
<td>44.79 (25.09)</td>
<td>27.54 (12.27)</td>
<td>35.31 (21.57)</td>
<td>43.27 (28.33)</td>
<td>27.47 (6.64)</td>
</tr>
</tbody>
</table>

### Table 2 Platelet response pre- and 4 h post-clopidogrel administration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CYP2C19 EM</th>
<th>CYP2C19 RM</th>
<th>PON1 Q192Q</th>
<th>PON1 Q192R</th>
<th>PON1 R192R</th>
<th>P-value</th>
</tr>
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<tr>
<td>Pre-dose PRU</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CYP2C19 EM</td>
<td>348 (32)</td>
<td></td>
<td>315 (43)</td>
<td>343 (41)</td>
<td>306 (34)</td>
<td>361 (29)</td>
</tr>
<tr>
<td>CYP2C19 RM</td>
<td>24.5 (19.0)</td>
<td>11.31 (14.82)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PON1 Q192Q</td>
<td>15.4 (13.4)</td>
<td>24.8 (28.2)</td>
<td></td>
<td></td>
<td></td>
<td>0.07</td>
</tr>
<tr>
<td>PON1 Q192R</td>
<td>14.51 (4.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.55</td>
</tr>
</tbody>
</table>

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C\textsubscript{max}, maximum plasma concentration; EM, extensive metabolizer (CYP2C19 *1/*1 or *1/*17); RM, reduced metabolizer (*1/*2, *2/*2 or *1/*3); \(t\textsubscript{max}, time to C\textsubscript{max}; \(t\textsubscript{1/2}, half-life; AUC\textsubscript{0–8 h}, area under the plasma concentration curve. Data are represented as mean with standard deviation.
metabolomic analysis that requires analytical techniques capable of distinguishing the related species.\textsuperscript{5,9} Secondly, due to the reactive nature of the free thiol metabolites, derivatization using alkylating agents, such as MPB, has been required to trap the metabolite for quantitation.\textsuperscript{9} In the current study, we followed a recently published stereoselective UHPLC–MS/MS method to determine plasma concentrations of clopidogrel thiol metabolites (H4, H3, and Endo) in our cohort.\textsuperscript{9} In the study by Bouman et al.,\textsuperscript{13} stereospecific separation of H3, H4, and Endo was not demonstrated, thus resulting in inaccurate clopidogrel pharmacokinetic analysis. Furthermore, they used an alternative method of stabilizing the active metabolite for quantitation, and thus, these technical discrepancies may in part explain the discordant findings with regards to contribution of CYP2C19 and PON1 to clopidogrel pharmacokinetics.

Similar to a number of recent studies that refuted a clinically relevant role of PON1 genotypes to clopidogrel response, we show that CYP2C19 but not PON1 genotype is related to ADP-induced antiplatelet response.\textsuperscript{20–22} This agrees well with a recently published genome-wide association study in a healthy Amish population, showing that the CYP2C19*2 allele had a significant association with clopidogrel platelet aggregation, while the PON1 genotype did not.\textsuperscript{23} The strong correlation between H4 exposure and antiplatelet response ($r^2 = 0.78$) presented here suggests that known genetic variation in CYP2C19 as well as interpatient variation in expression and activity of this enzyme likely account for clopidogrel resistance observed in non-responders.

The role of CYP3A4 in clopidogrel pharmacokinetics and pharmacodynamics remains unclear. While some studies postulate that CYP3A4 genotype and inhibition modulates clopidogrel pharmacokinetics and action,\textsuperscript{24} many others do not.\textsuperscript{25} Midazolam has been well documented as an in vivo probe drug for CYP3A4 activity.\textsuperscript{26} Thus, administration of a microdose of midazolam was used here to measure CYP3A4 phenotype in subjects. To the extent of our knowledge, we demonstrate for the first time that CYP3A4 activity, as measured by midazolam exposure, is not an important driver of clopidogrel pharmacokinetics or antiplatelet response.

Recently, a correspondence from Dansette et al. questioned the validity of the original findings of Bouman and colleagues.\textsuperscript{27} Specifically, they report that the Endo metabolite, but not other thiol metabolites, is formed in human liver microsomes incubated with 2-oxo-clopidogrel in the absence of CYP-requiring NADPH. This Endo formation was attenuated in the presence of the PON1 substrate paraoxon but unaffected by the presence of a CYP inhibitor. Moreover, Dansette and colleagues found that the Endo metabolite was generated in serum (devoid of CYP enzymes) upon ex vivo incubation with 2-oxo-clopidogrel. Those results indicate a role for PON1 in the formation of the Endo metabolite but not in the bioactivation to H4. The results from the current study not only confirm the findings of Dansette et al., but solidify PON1 as the key player in Endo but not H4 generation through systematic and definitive metabolic experiments that include a PON1 overexpression system and recombinant CYP enzymes.

We believe that lack of analytical specificity, followed by misidentification of the synthesized analytical standard used to quantify clopidogrel active metabolite levels, may have been the critical missteps which led to the conclusion by Bouman et al. that PON1 generates the active metabolites.\textsuperscript{13} First, the conditions for the separation of H4 from other structurally similar, but inactive metabolites requires high-resolution chromatographic techniques such as UHPLC coupled with MS/MS. The liquid chromatographic method used by Bouman et al. was likely insufficient for discriminating between active H4 from the inactive H3 and Endo metabolites. It is also not certain whether the analytical method used could quantify non-Endo thiol metabolites such as H4. Secondly, Bouman et al. obtained their thiol metabolite reference standard from purification of PON1-mediated 2-oxo-clopidogrel hydrolysis.\textsuperscript{13} They suggest that this product was active based on platelet reactivity experiments. However, those
experiments were performed at purified thiol metabolite concentrations of 2 mg/L, which is 100 times greater than the reported plasma thiol metabolite concentration. Since we clearly show that PON1 can only generate Endo and not H4, it is clear that the thiol metabolite they used as analytical standard to represent the active metabolite was in fact the inactive Endo metabolite.

**Figure 3** Representative chromatograms of derivatized H4 and Endo metabolite. (A) Chromatogram of a human plasma sample derivatized with 2-bromo-3-methoxyacetophenone. (B) Chromatogram of a sample derived from PON1-mediated hydrolysis of 2-oxo-clopidogrel. (C and D) Chromatogram of Endo standard and its MS/MS fragmentation signature. (E and F) Chromatogram of H4 standard and its tandem mass spectrometry fragmentation signature.
Bouman and colleagues admit that their analytical methods did not distinguish between different thiol metabolites derived from PON1, human liver microsomes, or human serum in vitro. They argue that the ratio of produced active thiol metabolites to total thiol metabolites was constant between enzyme preparations based on platelet reactivity studies of purified thiols, suggesting...
that PON1 creates a similar degree of active metabolites as human liver microsomes. Again, it is important to note that those platelet incubations were performed at purified thiol metabolite concentrations of 0.5–5 mg/L, values that are 25–250 times greater than their reported concentration of thiol metabolites patient plasma, bringing to question the relevance of such experiments as an argument for not requiring greater analytical specificity. Without identification and quantitation of these unknown PON1-derived active metabolites in relation to the amount of H4 active metabolite known to be found at significant levels in patient/subject plasma, it would seem that Bouman and colleagues have not clarified a role for PON1 in clopidogrel bioactivation.

Here, the in vitro data are consistent with previous studies that demonstrated the importance of CYP isozymes in clopidogrel bioactivation.6,7,27 In addition, the mechanism by which the 2-oxo-clopidogrel ring opens to expose the free thiol in H4 active metabolite known to be found at significant levels in patient/subject plasma, it would seem that Bouman and colleagues have not clarified a role for PON1 in clopidogrel bioactivation.

Overall, we propose that both steps of clopidogrel bioactivation are mainly driven by CYP2C19, ultimately generating active H4, while PON1-mediated hydrolysis of 2-oxo-clopidogrel generates the Endo metabolite (Figure 5). Notably, we show that the estimated enzyme affinity ($K_m$) of 2-oxo-clopidogrel to CYP2C19 is much higher than that of PON1, concordant with the low 2-oxo-clopidogrel affinity for PON1 demonstrated by Bouman et al.13 Accordingly, in hepatocytes, where clopidogrel bioactivation occurs, CYP2C19-mediated oxidation of 2-oxo-clopidogrel would be the preferred pathway at therapeutic concentrations of the drug. Importantly, to the extent of our knowledge, this is the first study to quantify Endo and H4 concentrations simultaneously in humans. We demonstrate that Endo levels are nearly 20-fold lower compared with H4; thus, Endo is unlikely to contribute substantially to clopidogrel antiplatelet response, in addition to the lack of association between Endo levels and antiplatelet response. Moreover, these results are further substantiated by our findings of no association between PON1 plasma activity and genotype with H4 levels or antiplatelet activity.

Figure 5 Schematic summary of clopidogrel bioactivation.
There are several noteworthy limitations to our study. First, the current clinical study was conducted in a relatively small cohort of study subjects. Therefore, we combined carriers of one or two alleles of CYP2C19*2 or CYP2C19*3 into one group and cannot comment on the differential influence of these alleles or its gene-dose effect on clopidogrel pharmacokinetics and pharmacodynamics. Such pooling of CYP2C19 variant carriers has been done previously for similar reasons.28 It should be noted that in terms of sample size, an 80% power was achieved to detect a 40% difference in antiplatelet response between CYP2C19 EM and RM genotypes with a two-sided significance level of 0.05, and a standard deviation of 20%. With respect to PON1 analysis, the high interindividual variability observed within PON1 genotype groups lead to reduction in power below 80%. However, the lack of PON1 Q192R genetic influence on clopidogrel response is in agreement with recently published studies of large sample sizes. Secondly, this study used the point-of-care VerifyNow P2Y12 assay to measure clopidogrel antiplatelet response. While VerifyNow utilizes ADP and PGE1 to induce and measure global platelet aggregation, it is not as direct a measure of platelet P2Y12 signalling activity as the vasodilator-stimulated phosphoprotein (VASP) assay or even direct receptor occupancy assays using 32P-2MeS-ADP.29 Indeed, clopidogrel H4 metabolite plasma exposure tracks with the VASP phosphorylation platelet reactivity index.17 Whether the H4 metabolite level is associated with VerifyNow PRU response has not been well described until now. Although some studies show high correlation between VASP and VerifyNow measured antiplatelet response,19,30,31 others did not.32 Nevertheless, clopidogrel response as measured by VerifyNow has consistently been shown to predict therapy resistance and clinical outcomes in a number of large trials and thus represents a feasible alternative technique for monitoring clopidogrel response.30,33 Indeed, the more pressing issue to address is delineating relevant cut-off thresholds for distinguishing between responders and non-responders, which will then allow clinicians to increase clopidogrel dose as appropriate. A standardized optimal cut-off value for each P2Y12 activity assay remains to be evaluated and defined. Lastly, since this is a proof-of-principle study in healthy subjects given a single 75 mg dose of clopidogrel, designed to delineate the role of PON1 relative to that of CYP2C19, we cannot directly extrapolate our findings to clopidogrel responsiveness in patients but it should be noted that the influence of CYP2C19 and PON1 on clopidogrel platelet aggregation has been assessed large patient cohorts in multiple studies.20–22 However, a key advantage of our study population and design is the ability to more clearly delineate the effect of pharmacogenetic markers on clopidogrel pharmacokinetics and response and elimination of the potentially confounding effects of concomitant medications and existing disease states. In addition, quantification of the active H4 thiol metabolite here allowed for a more precise assessment of the role of genetics on clopidogrel pharmacokinetics.

In conclusion, we show that PON1, unlike CYP2C19, is incapable of generating the clopidogrel active metabolite H4 in vitro. In human subjects, the CYP2C19 loss-of-function genotype is a major driver of H4 exposure, corresponding to lowered ADP-induced antiplatelet response. Furthermore, we demonstrated that PON1 generates the Endo metabolite; however, no correlation existed between plasma paraoxonase activity and Endo levels to antiplatelet response. Accordingly, although it remains likely that there are other genetic and non-genetic determinants of clopidogrel efficacy, our study suggests that CYP2C19 but not PON1 or CYP3A4 is a mechanistic determinant of inter-patient antiplatelet response variability to clopidogrel therapy.

**Supplementary material**

Supplementary material is available at European Heart Journal online.

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**Conflict of interest:** none declared.

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