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Paraoxonase-1 is a major determinant of clopidogrel efficacy

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Clinical efficacy of the antiplatelet drug clopidogrel is hampered by its variable biotransformation into the active metabolite[1,2]. The variability in the clinical response to clopidogrel treatment has been attributed to genetic factors, but the specific genes and mechanisms underlying clopidogrel bioactivation remain unclear. Using *in vitro* metabolomic profiling techniques, we identified paraoxonase-1 (PON1) as the crucial enzyme for clopidogrel bioactivation, with its common Q192R polymorphism determining the rate of active metabolite formation. We tested the clinical relevance of the *PON1* Q192R genotype in a population of individuals with coronary artery disease who underwent stent implantation and received clopidogrel therapy. *PON1* QQ192 homozygous individuals showed a considerably higher risk than RR192 homozygous individuals of stent thrombosis, lower PON1 plasma activity, lower plasma concentrations of active metabolite and lower platelet inhibition. Thus, we identified PON1 as a key factor for the bioactivation and clinical activity of clopidogrel. These findings have therapeutic implications and may be exploited to prospectively assess the clinical efficacy of clopidogrel.

Antiplatelet therapy with the ADP P2Y12 receptor antagonist clopidogrel is recommended by current clinical practice guidelines to prevent atherothrombotic events in patients with coronary artery disease (CAD) undergoing percutaneous coronary intervention (PCI) with stent implantation. The main drawback of clopidogrel is a high interindividual variability in antiplatelet response[3]. Low platelet responsiveness to clopidogrel has been found to translate into a high incidence of atherothrombotic events, including stent thrombosis as the most serious and often fatal clinical event[4]. However, the biological mechanisms underlying the response variability remain largely unclear. In a recent genome-wide association analysis, it was estimated that 83% of individual variance in response to clopidogrel was attributable to genetic effects[5], but the gene variants investigated thus far explain only a minor proportion of the response variability[6,7].

Clopidogrel is a thienopyridine prodrug that requires enzymatic conversion into its active thiol metabolite. Pharmacokinetic-pharmacodynamic analyses suggest that the majority of the variability in platelet response is explained by the variability in plasma concentrations of the active metabolite[8,9].

We postulated that genetic variants of drug-metabolizing enzymes would affect the response to clopidogrel. Using a validated microsomal expression system of metabolizing enzymes, we identified PON1, an esterase synthesized in the liver and associated with HDL in blood, as the rate-determining enzyme for the formation of the thiol active metabolite from clopidogrel. We found that the common functional *PON1* Q192R gene polymorphism resulted in a more efficient clopidogrel bioactivation. On the basis of this mechanistic information, we performed a case-cohort study in individuals with CAD who underwent PCI and received clopidogrel therapy to comprehensively assess the relationships between the *PON1* Q192R polymorphism, PON1 enzyme activity, clopidogrel pharmacokinetics, platelet response and the risk of stent thrombosis. Subsequently, we conducted an independent replication study evaluating the reliability and validity of the association between PON1 and clinical end points.

We functionally expressed cytochrome P450 oxidoreductase isozymes (CYPs) and esterases in a human embryonic kidney cell line and monitored conversion of clopidogrel or potential metabolic clopidogrel intermediates in microsome preparations (**Fig. 1** and **Supplementary Fig. 1**). We identified bioactivation of clopidogrel as a two-step process. The first step was an oxidation of clopidogrel to 2-oxo-clopidogrel catalyzed by CYP3A4, CY3A5, CYP2B6, CYP1A2, CYP1A1, CYP2E1 and CYP2A6 at enzymatic efficiencies (V_{max} / K_m) of 16.02, 8.67, 2.61, 1.87, 0.88, 0.72 and 0.70 µl mg^{-1} min^{-1}, respectively. The second step was a hydrolytic cleavage of the γ-thiobutyrolactone ring of 2-oxo-clopidogrel to the pharmacologically active thiol metabolite catalyzed by the esterases PON1 and PON3 at enzymatic efficiencies of 0.36 and 0.030 µl mg^{-1} min^{-1}, respectively. This bioactivation step competed with the hydrolysis of the methyl ester in clopidogrel, 2-oxo-clopidogrel and the thiol metabolite to form inactive carboxylic acid metabolites at considerably higher conversion rates catalyzed by the esterases carboxylesterase-1, carboxylesterase-2 and butyrylcholinesterase (**Supplementary Table 1** and **Supplementary Fig. 2**).

To further exclude that oxidative biotransformation was involved in the second step of active metabolite formation, as suggested previously[10–12], we incubated 2-oxo-clopidogrel with human liver microsomes or with human serum, with equal paraoxonase activity in both preparations. There were no differences in the conversion rates to the thiol metabolite (**Supplementary Fig. 3**). Moreover, pharmacological

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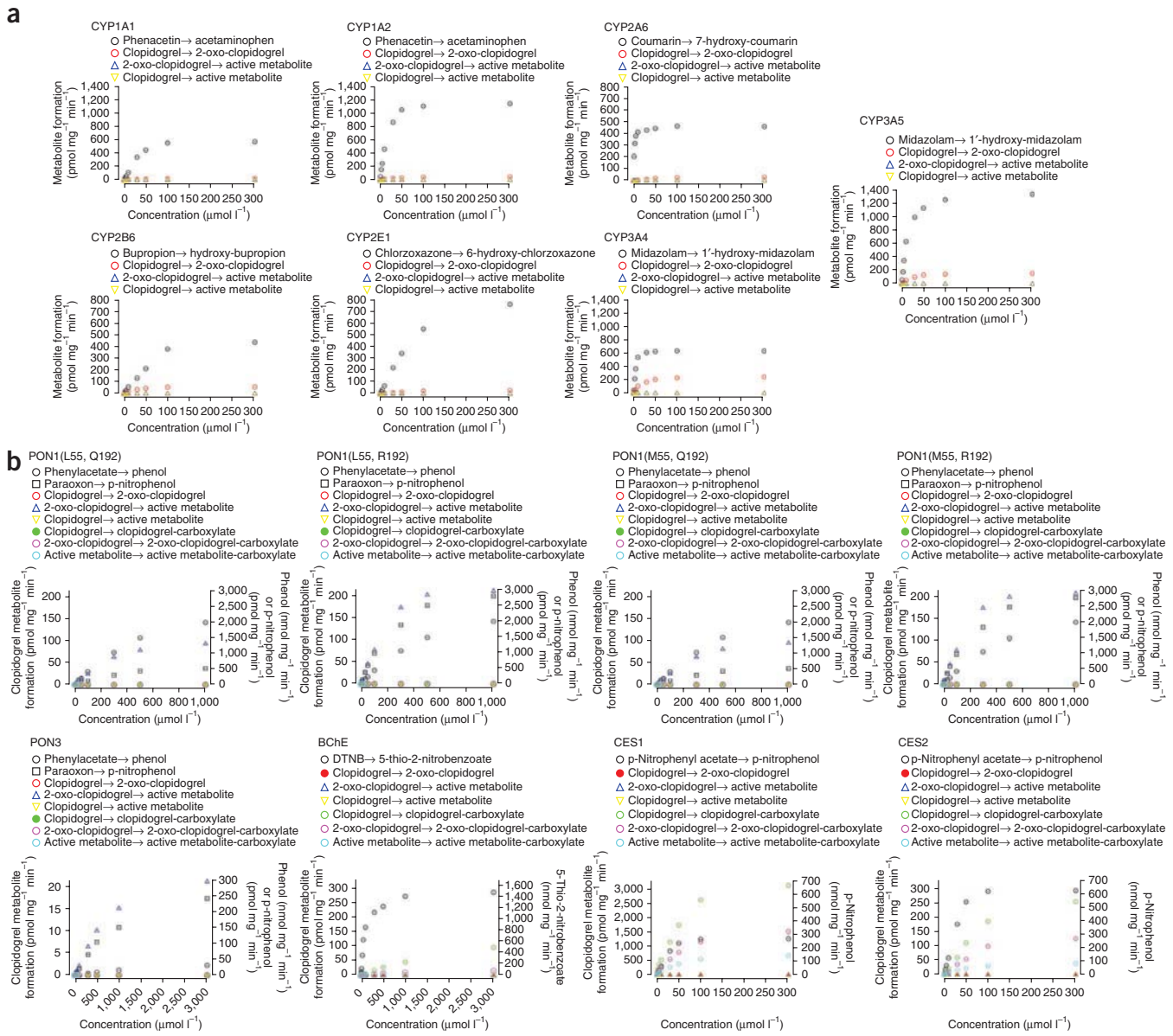

Figure 1 Kinetics of clopidogrel-metabolizing enzymes. (**a**,**b**) Substrate saturation kinetics for microsomal preparations of human cytochrome P450 isozymes (**a**) and esterases (**b**) involved in clopidogrel metabolism. Microsomes were obtained from stably transfected human embryonic kidney cells (HEK 293). Conversion of clopidogrel to 2-oxo-clopidogrel, of 2-oxo-clopidogrel to the active thiol metabolite and of clopidogrel to the thiol metabolite was assayed by incubation with increasing concentrations of substrate for 5 min at 37 °C. For esterases, the conversion of clopidogrel to clopidogrel-carboxylate, of 2-oxo-clopidogrel to 2-oxo-clopidogrel-carboxylate, and of the thiol metabolite to thiol metabolite-carboxylate was also assayed. Kinetics of the PON1 allozymes with the L55M and Q192R polymorphisms were determined. For each enzyme a specific probe reaction (positive control) was performed (black circles). Symbols and error bars represent means ± s.e.m. of three independent incubation experiments each. **Supplementary Figure 1** shows similar kinetic measurements for enzymes not involved in clopidogrel metabolism. BChE, butyrylcholinesterase; CES1, carboxylesterase-1; CES2, carboxylesterase-2. DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

inhibition of PON1 in liver microsomes suppressed formation of the thiol metabolite from both clopidogrel and 2-oxo-clopidogrel substrates, whereas inhibition of CYP3A activity reduced thiol metabolite formation only from clopidogrel (**Supplementary Fig. 3**).

The low conversion rate of 2-oxo-clopidogrel to the thiol compound indicated that this reaction was the rate-limiting step of bioactivation, with PON1 representing the key enzyme. This was supported by our observation that the rates of thiol metabolite formation were not different after incubation of liver microsomes with clopidogrel or 2-oxo-clopidogrel (**Supplementary Fig. 3**). Two common coding polymorphisms of the *PON1* gene are known: Q192R and L55M[13]. The Arg192 allozyme compared with the Gln192 allozyme showed a higher hydrolysis efficiency for 2-oxo-clopidogrel (V_{max} / K_m (s.e.m.), 1.36 (0.21) versus 0.36 (0.045) μl mg^{-1} min^{-1}, $P < 0.001$) and for the probe substrate paraoxon (13.9 (1.1) versus 1.69 (0.25) μl mg^{-1} min^{-1}, $P < 0.001$), but not for phenylacetate (5.19 (0.23) versus 5.09 (0.40) ml mg^{-1} min^{-1}, $P = 0.850$), whereas the Met55 allozyme compared with the Leu55 allozyme showed no differences in the hydrolysis rates toward 2-oxo-clopidogrel or the probe substrates (**Fig. 1** and **Supplementary Table 1**).

Similar effects of the Q192R and L55M polymorphisms on the hydrolysis efficiency of paraoxon and phenylacetate have been suggested in previous reports[14,15]. Corroborating the enzyme activity data, analysis of the crystal structure of PON1 indicates that position 192 (but not position 55) constitutes part the active histidine dyad[16]; therefore alteration of the pK_a value at position 192 by the Q192R substitution would be expected to affect substrate affinity.

We next conducted a case-cohort study[17] in individuals with CAD who underwent PCI and received clopidogrel for 6–12 months in accordance with consensus guidelines[18]. We compared 41 incident cases with nonfatal stent thrombosis and 71 randomly selected subjects without stent thrombosis who accrued in a cohort of 7,719 eligible subjects during 18 months of follow-up. Subsequently, the clopidogrel-free subjects received a single 600-mg test dose of clopidogrel to assess pharmacokinetic and pharmacodynamic responses (**Supplementary Fig. 4**).

Cases and noncases were well balanced with respect to demographic, clinical and procedural characteristics, minimizing the influence of confounding variables on outcome (**Supplementary Tables 2** and **3** and **Supplementary Fig. 5**).

Compared with individuals with either the *PON1* RR192 or the QR192 genotype, QQ192 homozygous individuals were more frequent in the stent thrombosis group than in the control group (odds ratio, 3.6; 95% confidence interval, 1.6–7.9; $P = 0.003$; false-positive reporting probability, 3.7%). In addition, early stent thrombosis (within 30 d, $n = 30$) tended to be more frequent than late or very late stent thrombosis (>30 d, $n = 11$) in QQ192 subjects as compared with QR192 heterozygous and RR192 homozygous individuals (odds ratio, 3.3; 95% confidence interval, 0.82–13.3; $P = 0.095$). The distributions of variant genotypes of *CYP2C19*, *CYP2C9*, *CYP3A4*, *CYP3A5* and *ABCB1*, which have been associated with clopidogrel response in previous studies[19–22], were not significantly different between the stent thrombosis group and the control group (**Table 1**). The genotype distributions were in agreement with the distributions reported from other populations of European descent[19], indicating an absence of population stratification.

Considering not only the occurrence of events but also the time of event occurrences after index PCI, the hazard ratio of stent thrombosis for QR192 heterozygous individuals was considerably higher compared with RR192 homozygous individuals and increased in an approximately log-additive fashion in QQ192 homozygous individuals (**Table 2** and **Fig. 2a**,**b**). The *PON1* Q192R genotype was the only significant factor that was independently associated with the occurrence of stent thrombosis in univariate and multivariate stepwise Cox regression analyses ($P < 0.001$). Adjustment for other potentially explanatory covariates did not change the hazard ratios (**Table 2**).

After administration of the 600-mg clopidogrel test dose, the individuals who had experienced nonfatal stent thrombosis during follow-up showed a lower plasma concentration of the active clopidogrel metabolite, a higher concentration of the 2-oxo-clopidogrel metabolite, a higher ratio of 2-oxo-clopidogrel to active metabolite and a lower inhibition of platelet aggregation compared with participants without incident stent thrombosis (**Fig. 2c** and **Supplementary Table 4**). Concentrations of parent clopidogrel and of the inactive carboxylic acid metabolite and baseline platelet aggregation were not different between the groups (**Fig. 2c** and **Supplementary Table 4**).

Parameters reflecting clopidogrel activity—paraoxonase plasma activity, maximal plasma concentration of the active metabolite and maximal platelet inhibition—were equally strong predictors as the *PON1* Q192R genotype for the risk of stent thrombosis in univariate and multivariate Cox regression analyses (**Table 2** and **Fig. 2a**,**b**). The hazard estimates for the *PON1* Q192R genotype lost statistical significance after individual adjustment for variables reflecting clopidogrel activity as listed above, indicating a strong interaction between the *PON1* genotype and the clopidogrel activity markers (**Supplementary Table 5**). Consistently, the *PON1* Q192R genotype

Table 1 Distribution of variant genotypes among case-cohort subjects

Variant genotype SNP (star allele or variant protein, if applicable)	Number with stent thrombosis (%) ($n = 41$)	Number without stent thrombosis (%) ($n = 71$)	*P* value
CYP2C9			
C430T (CYP2C9*2)			0.84 [0.52]
CC (*1/*1)	30 (75.0)	57 (80.3)	
CT (*1/*2)	10 (25.0)	14 (19.7)	
A1075C (CYP2C9*3)			0.50 [0.69]
AA (*1/*1)	32 (80.0)	59 (83.1)	
AC (*1/*3)	8 (20.0)	12 (16.9)	
CYP2C19			
G681A (CYP2C19*2)			0.31 [0.53]
GG (*1/*1)	26 (63.4)	49 (69.0)	
GA (*1/*2)	12 (29.3)	20 (28.2)	
AA (*2/*2)	3 (7.3)	2 (2.8)	
CYP3A4			
A(–392)G (CYP3A4*1B)			0.63 [0.85]
AA (*1/*1)	38 (95.0)	68 (95.8)	
AG (*1/*1B)	2 (5.0)	3 (4.2)	
IVS10+G12A (CYP3A4*1G)			0.88 [0.80]
GG (*1/*1)	31 (77.5)	58 (81.7)	
GA (*1/*1G)	7 (17.5)	11 (15.5)	
AA (*1G/*1G)	2 (5.0)	2 (2.8)	
CYP3A5			
A6986G (CYP3A5*3)			0.69 [0.90]
AA (*1/*1)	1 (2.5)	1 (1.4)	
AG (*1/*3)	5 (12.5)	10 (14.1)	
GG (*3/*3)	34 (85.0)	60 (84.5)	
PON1			
A576G (Q192R)			< 0.001 [0.001]
AA (QQ)	27 (65.9)	25 (35.2)	
AG (QR)	13 (31.7)	33 (46.5)	
GG (RR)	1 (2.4)	13 (18.3)	
ABCB1			
C3435T			0.71 [0.59]
CC	7 (17.5)	16 (22.5)	
CT	20 (50.0)	38 (53.5)	
TT	13 (32.5)	17 (24.0)	

All variant alleles were designated by their nucleotide substitution (relative to position 1 of the coding DNA reference sequence); for *CYP* variants the common harmonized star allele designation, and for the *PON1* variant the one-letter amino acid substitution (relative to the peptide reference sequence), are given in parentheses. The variant alleles *CYP2C19*3*, *CYP2C19*4*, *CYP2C19*5*, *CYP3A4*2* and *CYP3A4*3* were not present in the study population. For one subject in the stent thrombosis group, genotype information was missing for *CYP2C9*, *CYP3A4*, *CYP3A5* and *ABCB1*. *P* values were determined by univariate Cox regression with weighting of subcohort noncases with the inverse of the sampling fraction according to a previously described method[33] and additionally by binary logistic regression (values are presented in square brackets). *P* values were not corrected for multiple testing. Comparison between Cox regression and logistic regression indicates that the significance of the genetic associations did not depend on the timing of the stent thrombosis events. $P < 0.05$ was considered a statistically significant difference. IVS, intervening sequence; SNP, single nucleotide polymorphism.

Table 2 Hazard ratios of incident nonfatal definite stent thrombosis among case-cohort subjects in relation to *PON1* Q192R genotype, paraoxonase activity, plasma concentration of active clopidogrel metabolite and platelet inhibition

Predictor variable	Unadjusted hazard ratio (95% confidence interval)	*P* value	Adjusted hazard ratio (95% confidence interval)	*P* value
***PON1* genotype**				
RR192	1 (reference)		1 (reference)	
QR192	4.41 (1.89–10.20)	0.001	4.52 (1.81–11.24)	0.001
QQ192	12.82 (4.74–90.91)	< 0.001	12.90 (4.54–95.48)	< 0.001
Paraoxonase plasma activity (nmol min^{-1} ml^{-1})				
Highest tertile (≥180.4)	1 (reference)		1 (reference)	
Middle tertile (<180.4–104.9)	6.29 (2.79–22.07)	< 0.001	7.14 (2.93–19.23)	< 0.001
Lowest tertile (<104.9)	22.03 (6.62–73.38)	< 0.001	22.22 (6.67–76.92)	< 0.001
Active clopidogrel metabolite (c_{max}, ng ml^{-1})				
Highest tertile (≥22.85)	1 (reference)		1 (reference)	
Middle tertile (<22.85–11.57)	6.25 (2.34–16.67)	< 0.001	6.33 (2.57–15.63)	< 0.001
Lowest tertile (<11.57)	16.13 (5.56–45.45)	< 0.001	17.24 (5.08–58.82)	< 0.001
Platelet inhibition (20 μM ADP, Δ%)				
Highest tertile (≥40.6)	1 (reference)		1 (reference)	
Middle tertile (<40.6–19.0)	3.97 (1.64–9.52)	0.002	4.13 (1.74–9.80)	0.001
Lowest tertile (<19.0)	11.23 (3.29–38.46)	< 0.001	11.24 (3.29–38.34)	< 0.001

Hazard ratios were calculated by univariate and multivariate Cox regression with weighting of subcohort noncases with the inverse of the sampling fraction according to a previously described method[33]. Multivariate adjustments were done for baseline demographic (sex, age, body-mass index and smoking) and clinical characteristics (indication of percutaneous coronary intervention, number of treated vessels, type of stent, clopidogrel loading dose, hypertension, hypercholesterolemia, diabetes mellitus, previous myocardial infarction or coronary artery disease, family history of coronary artery disease and left ventricular ejection fraction <45%), and variant genotypes of *CYP2C9*2* and *CYP2C9*3*, *CYP2C19*2*, *CYP3A4*1B*, *CYP3A4*1G*, *CYP3A5*3* and *ABCB1* C3435T. Additional adjustment for the use of aspirin and statins did not change the hazard ratios (data not shown). Genotype distribution and tertile limits in the subcohort sample fraction were extrapolated to the total cohort. Maximal plasma concentration of the active metabolite (c_{max}) and platelet inhibition (maximal predose versus maximal 6-h postdose aggregation induced by 20 μM ADP, Δ%) were determined after administration of a single 600-mg clopidogrel dose. $P < 0.05$ was considered a statistically significant difference.

was the only factor that significantly correlated with paraoxonase plasma activity (unadjusted $R^2 = 0.805$, $P < 0.001$), maximal plasma concentration of the active clopidogrel metabolite ($R^2 = 0.809$, $P < 0.001$) and inhibition of platelet aggregation ($R^2 = 0.725$, $P < 0.001$) in univariate and multivariate stepwise linear regression models. There were also close correlations between paraoxonase activity and both active metabolite concentration ($R^2 = 0.939$, $P < 0.001$) and platelet inhibition ($R^2 = 0.738$, $P < 0.001$). Accordingly, the predictive diagnostic power of the clopidogrel activity markers for stent thrombosis, as determined by receiver operating characteristic curves, did not substantially increase on inclusion of the *PON1* genotype, but the combined predictive power of demographic, clinical and other genetic factors was considerably improved by inclusion of the *PON1* genotype (**Supplementary Fig. 6**). The distribution of the clopidogrel activity markers was independent of other gene variants and was also independent of treatment with potentially interacting drugs (**Supplementary Tables 6** and **7**).

For further evaluation of the genotypic and phenotypic association of PON1 with the clinical response to clopidogrel, we conducted a replication study in a prospective cohort of 1,982 individuals with acute coronary syndromes over a 12-month follow-up period. The results confirmed and extended the findings of the case-cohort study. The unadjusted hazard ratio of fatal and nonfatal definite stent thrombosis in *PON1* QQ192 versus RR192 homozygous individuals was 10.20 (95% confidence interval, 4.39–71.43; $P < 0.001$). Lower but significant risk associations were also found with the etiologically more diverse secondary end points myocardial infarction (hazard ratio, 4.93; 95% confidence interval, 2.16–11.24; $P < 0.001$) and the composite of vascular death, nonfatal myocardial infarction and nonfatal ischemic stroke (hazard ratio, 3.89; 95% confidence interval, 2.10–7.19; $P < 0.001$), suggesting that unmeasured covariates diluted the association with the *PON1* genotype. The *PON1* genotype was not associated with the risk of ischemic stroke (hazard ratio, 2.38; 95% confidence interval, 0.54–10.42; $P = 0.250$) or nonvascular death (hazard ratio, 0.98; 95% confidence interval, 0.20–4.72; $P = 0.981$) (**Supplementary Table 8**), consistent with the lack of efficacy of clopidogrel for stroke and nonvascular death in clinical trials[23]. QQ192 homozygous subjects showed a lower risk of major bleeding (hazard ratio, 0.36; 95% confidence interval, 0.18–0.75; $P = 0.006$) that could be related to a less effective platelet inhibition. Similar risk estimates were obtained for paraoxonase plasma activity (**Supplementary Table 9**). The subject groups with and without the respective clinical outcome did not differ in demographic and clinical characteristics; the characteristics of subjects with and without stent thrombosis are shown in **Supplementary Table 10.** Multivariate adjustments failed to alter the risk estimates (**Supplementary Tables 8** and **9**).

Taken together, these biochemical, clinical and epidemiological studies indicate that the functional *PON1* Q192R gene variant is a major determinant of the pharmacokinetic and pharmacodynamic responsiveness to clopidogrel and thus its clinical efficacy.

Previous studies have suggested associations of several variant genes, such as *CYP3A4*1G* (ref. 20), *CYP3A5*3* (ref. 21), *CYP2C9*2* or *CYP2C9*3* (ref. 22) and the *ABCB1* drug transporter C3435T polymorphism[19], with pharmacokinetic, antiplatelet or clinical responses to clopidogrel. In particular, the common loss-of-function allele *CYP2C19*2* or concomitant administration of CYP2C19-inhibiting drugs have repeatedly been associated with lower responsiveness to clopidogrel[6,7]. However, even in a genetically homogenous population, the *CYP2C19*2* allele was reported to account for only 12% of the variability in ADP-stimulated platelet response to clopidogrel[5]. The high heritability of clopidogrel response but relatively weak prediction by existing proposed genetic markers argues for the involvement of an as yet undiscovered genetic factor. The *PON1* Q192R polymorphism explained 72.5% (as derived from the R^2 statistic of linear regression) of

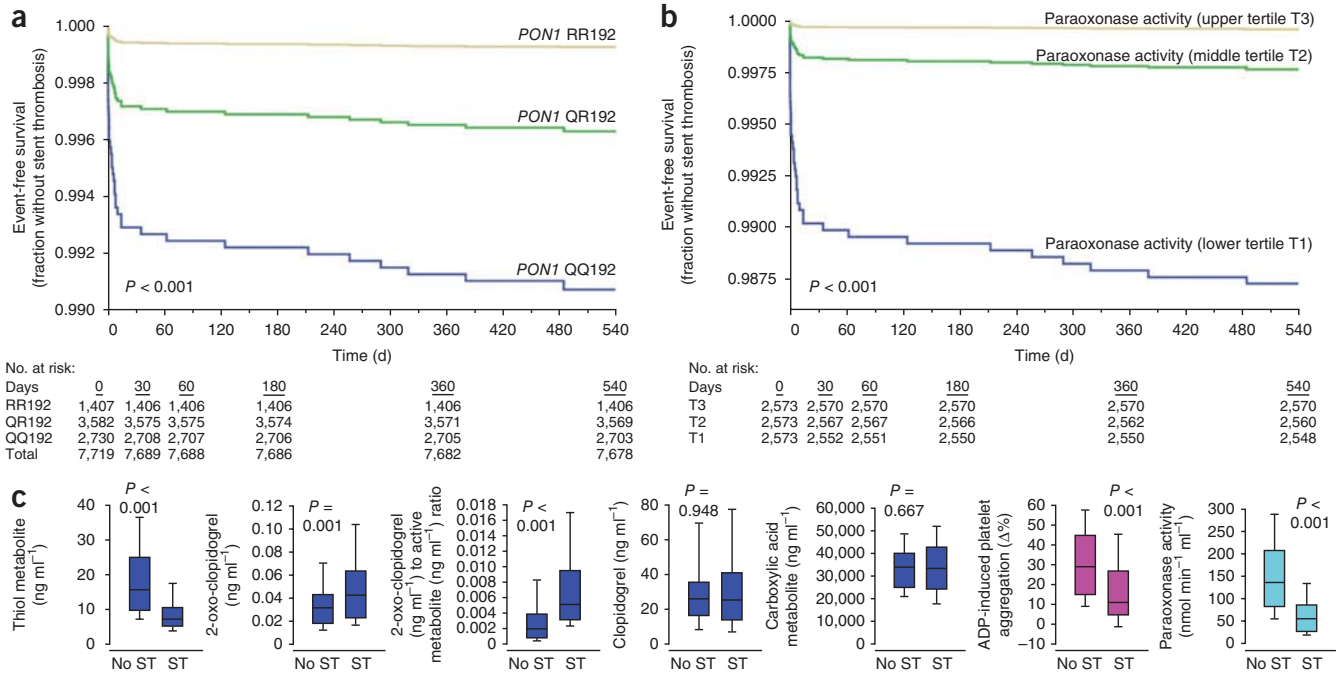

Figure 2 Kaplan-Meier curves for individuals with coronary stent implantation and their pharmacokinetic and pharmacodynamic responses to clopidogrel. (**a**,**b**) Kaplan-Meier survival curves showing cumulative probabilities of case-cohort subjects without incident nonfatal definite stent thrombosis over 18 months of follow-up according to *PON1* Q192R gene polymorphism (**a**) and according to tertiles (T1, T2 and T3) of paraoxonase plasma activity, with T1 < 104.9, T2 < 180.4–104.9 and T3 ≥ 180.4 nmol min^{-1} ml^{-1} (**b**). Genotype distribution and tertile limits were extrapolated to the total cohort, and subcohort noncases were weighted with the inverse of the sampling fraction according to a previously described method[33]. Numbers of individuals at risk at the indicated time points are shown. *P* values for the total model coefficients were calculated by univariate Cox regression. (**c**) Box-and-whisker plots showing median response values with twenty-fifth and seventy-fifth percentiles (box) and tenth and ninetieth percentiles (whisker). After the 18-month follow-up, the clopidogrel-free cases with nonfatal stent thrombosis (ST, $n = 41$) and the subcohort noncases without stent thrombosis (No ST, $n = 71$) were given a single 600-mg clopidogrel dose, and pharmacokinetic and platelet responses were compared by univariate Cox regression. Pharmacokinetic responses are indicated as maximum plasma concentrations (c_{max}, ng ml^{-1}) of active thiol metabolite, 2-oxo-clopidogrel, parent clopidogrel and carboxylic acid metabolite and as ratio of the maximum plasma concentrations of 2-oxo-clopidogrel to thiol metabolite. Platelet response is indicated as percentage of maximal predose versus maximal 6-h postdose aggregation (Δ% induced by 20 μM ADP), Paraoxonase and arylesterase plasma activities are indicated as the velocity of transformation (nmol min^{-1} ml^{-1}) of paraoxon to *p*-nitrophenol and of phenylacetate to phenol, respectively. $P < 0.05$ was considered a statistically significant difference.

the variability in ADP-stimulated platelet aggregation after clopidogrel administration and thus seems likely to be the primary predictor of clopidogrel response. However, a previous genome-wide association study in a cohort of related healthy subjects of Amish descent provided no evidence for an association of the *PON1* gene region with the platelet response to clopidogrel[5]. The basis for this discrepancy with our findings is unclear, but it might be explained by differences in the populations treated with clopidogrel or in the methodology of platelet function testing. Instead of measuring platelet inhibition after a single loading dose, the authors determined platelet inhibition values after multiple doses of clopidogrel[5], which in our validation studies correlated insufficiently with active metabolite formation (**Supplementary Methods**). Likewise, a lack of consistency between *ex vivo*–tested platelet function and clinical response to clopidogrel has been reported in previous studies, and lower magnitudes of the risk estimates to predict clinical events on the basis of platelet function as compared with our studies were found[24], which could be related to differences in the methodology of platelet function testing or to the heterogeneity of the assessed clinical outcomes. In accordance with our findings, the degree of platelet inhibition after a single 600-mg clopidogrel loading dose showed a high predictive ability for definite stent thrombosis in a large prospective study[25].

The genetic association of clopidogrel response with *PON1* genotype was consistent with results from quantitative metabolomic profiling of clopidogrel bioactivation. Two previous studies have quantified the contribution of CYP isozymes to oxidative clopidogrel metabolism but were only in partial agreement with each other[12,26]. Our data are consistent with one of these studies[26], including the lack of involvement of CYP2C19 in clopidogrel oxidation. Our finding that 2-oxo-clopidogrel, the product of CYP-catalyzed oxidation, was opened to the active thiol by hydrolysis is in agreement with the maintenance of the formal oxidation states at sulfur and carbon atoms upon the conversion of 2-oxo-clopidogrel to the thiol metabolite (**Supplementary Fig. 2**). The basis for the discrepancies between our findings and reports of an oxidative CYP-dependent ring cleavage[10–12] remains unclear. As we have shown, the ring opening observed in incubation experiments with liver microsomes results from microsomal PON activity but not from CYP activity. The suggested formation of sulfenic acid intermediates[11] may also result from CYP-dependent oxidation of the free thiol, similarly to the metabolism of spironolactone[27]. In support of our findings, PON1 has been shown to possess thiolactonase activity toward differently substituted γ-thiobutyrolactone compounds[14,28,29].

Associations of specific *CYP* genotypes with a higher atherothrombotic risk might be independent of clopidogrel metabolism. For example, carriers of the *CYP2C19*2* allele showed higher plasma concentrations of inflammatory markers that could have contributed to an increased baseline cardiovascular risk associated with *CYP2C19*2* (ref. 30). Similarly, the *PON1* Gln192-encoding allele has been associated with elevated markers of systemic oxidative stress and a higher risk of major cardiovascular events (hazard ratio, 1.32; 95% confidence interval, 1.04–1.69)[31]. However, a systematic meta-analysis showed an association of the *PON1* Arg192-encoding allele with a higher risk of CAD (relative risk, 1.12; 95% confidence interval, 1.02–1.16)[13]. It is unlikely that the small magnitude of either potentially harmful or protective effects of *PON1* Q192R on baseline cardiovascular risk could substantially bias the large and consistent effects of the *PON1* genotype on cardiovascular risk related to clopidogrel efficacy. Examination of the HapMap-CEU (Caucasian Europeans from Utah) population showed that the Q192R polymorphism is located within a linkage disequilibrium block of *PON1* that, with the exception of Q192R, covers only polymorphisms not affecting paraoxonase activity (L55M and intronic single nucleotide polymorphisms without putative *cis*-regulatory function) and does not extend to the downstream *PON3* and *PON2* genes or other genes, inconsistent with the possibility that Q192R is a tag for the true causal gene variant. Aside from the Q192R genotype, the activity of PON1 has been suggested to be modified by environmental factors such as age, smoking, plasma lipids, ischemic or inflammatory conditions or medication[32]. However, in accordance with previous studies[31,32] we found a strong correlation between the *PON1* Q192R genotype and paraoxonase activity ($R^2 > 0.80$) that was not altered by adjustment for environmental covariates. In particular, the proportion of the phenotypic variance of paraoxonase plasma activity explained by the *PON1* genotype was not different between measurements at nonischemic conditions in the case-cohort study (80.5%) and measurements conducted during the acute ischemic disease state in the replication study (80.7%).

Our studies were adequately powered to discover strong risk predictors with an odds ratio of ≥3.0 for the homozygous risk genotype or the highest risk tertile, but weaker predictors conferring smaller risk increases may have remained undiscovered, yielding no significant associations with stent thrombosis or other investigated disease outcomes ($P > 0.05$). Consequently, the *PON1* Gln192–encoding risk allele arose as the only significant independent predictor of stent thrombosis, and the magnitudes of the risk estimates were considerably higher compared with those previously reported for any other common single allele.

Our findings may have clinical utility in that *PON1* genotyping or PON1 activity measurements may provide prognostic information of clopidogrel efficacy beyond common clinical and laboratory risk markers.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

J.W.v.W. and D.T. conceived the project. E.S., J.M.t.B. and D.T. supervised the project. H.J.B., J.W.v.W., J.V., C.M.H., C.H., C.W., H.-G.S. and D.T. conducted or directed bioanalytics of clopidogrel and its metabolites, metabolomic profiling, blood collection and sample preparation, aggregometry, genotyping and PON1 phenotyping. H.J.B., E.S., J.W.v.W., C.M.H., J.M.t.B. and D.T. conducted or directed recruitment of subjects, disease assessment and follow-up assessments. H.J.B., J.W.v.W. and D.T. did the computational and statistical data analyses. H.J.B. and D.T. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Bioanalytics of clopidogrel and its metabolites. For quantification of clopidogrel and its metabolites, we developed a validated liquid chromatography tandem mass spectrometry method. This included synthesis and pharmacological testing of the active metabolite of clopidogrel and conducting a full validation procedure in accordance with regulatory standards for drug approval (**Supplementary Methods** and **Supplementary Figs. 7** and **8**).

Metabolomic profiling. We generated human embryonic kidney cell (HEK 293) clones of metabolizing enzymes (including common genetic variants) by stable transfection with their respective cDNAs. We obtained microsomal preparations for each enzyme (cytochrome P450 isozymes and esterases) from HEK 293 transfectants and determined enzymatic activity by a specific probe reaction. We incubated HEK 293 cell microsomes, human liver microsomes or serum with synthesized primary reference standards of clopidogrel base or clopidogrel metabolites at various concentrations and determined the rates of biotransformation by Michaelis-Menten analysis (**Supplementary Methods**).

Human studies. From a population of European descent of 7,719 individuals with a clinical presentation of stable angina pectoris or acute coronary syndrome who underwent PCI and received clopidogrel maintenance therapy for 6–12 months, we compared 41 incident cases involving nonfatal definite stent thrombosis that accrued over an 18-month follow-up and a random sample of 71 noncases in a case-cohort design by genotyping, pharmacokinetic and pharmacodynamic testing to evaluate the determinants of clopidogrel response (**Supplementary Fig. 4**). To further assess the association of the *PON1* genotype and phenotype with clinical endpoints, we conducted a replication study in prospective cohort design in a population of European descent comprising 1,982 subjects with acute coronary syndrome, who underwent PCI and were monitored during a 12-month follow-up on clopidogrel maintenance therapy. The Institutional Review Boards of the St. Antonius Hospital Nieuwegein and the University Hospital of Cologne approved the protocols of the studies, and all participants gave written informed consent.

We established an optimized assay of platelet function measurement by light transmission aggregometry for assessment of mechanistic pharmacokinetic-pharmacodynamic relationships in response to clopidogrel (**Supplementary Fig. 9**).

Details of study populations, study designs and clinical laboratory testing (including blood processing, pharmacokinetic analysis, aggregometry, determination of PON1 plasma activity and genotyping) are presented in the **Supplementary Methods**.

Statistical analyses. Details of the statistical analysis (including prospective sample size calculation, Cox regression models, determination of false-positive reporting probability and assessment of effectiveness of diagnostic variables by receiver operating characteristic curves) are presented in the **Supplementary Methods**.

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

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SUPPLEMENTARY INFORMATION

Paraoxonase-1 is a Major Determinant of Clopidogrel Efficacy

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This supplementary appendix includes additional results (**Supplementary Tables 1–10** and **Supplementary Figures 1–6**), additional methods (**Supplementary Methods** and **Supplementary Figures 7–9**), and additional references (**Supplementary References**).

Supplementary Table 1. Enzyme kinetic parameters of clopidogrel metabolism

Catalyzed reaction and involved enzyme	K_m, µmol l^{-1} (s.e.m.)	V_{max}, pmol mg^{-1} min^{-1} (s.e.m.)
Clopidogrel → 2-oxo-clopidogrel:		
- CYP3A4	16.6 (1.5)	266 (6.6)
- CYP3A5	20.3 (2.3)	176 (5.7)
- CYP2B6	25.1 (3.6)	65.4 (2.8)
- CYP1A2	29.0 (4.7)	54.3 (2.8)
- CYP1A1	33.1 (5.0)	29.1 (1.4)
- CYP2E1	44.8 (6.3)	32.6 (1.6)
- CYP2A6	53.9 (12.0)	37.5 (3.1)
2-Oxo-clopidogrel → thiol metabolite:		
- PON1 (L55, Q192)	368 (42.6)	132 (6.5)
- PON1 (L55, R192)	215 (33.3)	270 (14.8)
- PON1 (M55, Q192)	360 (38.6)	133 (6.0)
- PON1 (M55, R192)	208 (33.6)	267 (15.2)
- PON3	926 (58.3)	27.8 (0.75)
Clopidogrel → carboxylic acid metabolite:		
- CES1	59.3 (7.0)	3,842 (173)
- CES2	113 (17.8)	357 (25.5)
- BChE	3,265 (130)	200 (4.9)
2-Oxo-clopidogrel → carboxylic acid metabolite:		
- CES1	64.4 (5.1)	1,852 (57.1)
- CES2	99.5 (18.4)	173 (13.9)
- BChE	1,652 (81.3)	24.9 (0.60)
Thiol metabolite → carboxylic acid metabolite:		
- CES1	52.8 (4.6)	808 (25.9)
- CES2	64.5 (4.3)	48.0 (1.3)

K_m and V_{max} values were derived from fitting individual data by Michaelis-Menten equations. Carboxylic acid metabolite refers to the hydrolyzed methyl ester of the respective starting compound. Arithmetic mean and standard error (s.e.m.) of three individual experiments each are reported.
BChE denotes butyrylcholinesterase; CES1,2 carboxylesterase-1,-2; CYP cytochrome P450 isozyme; PON1,3 paraoxonase-1,-3. Amino acid exchanges L55M and Q192R denote allozymes of PON1.

Supplementary Table 2. Demographic, clinical, and procedural characteristics of case-cohort patients at the time of index percutaneous coronary intervention

Characteristics	With stent thrombosis (*n* = 41)	Without stent thrombosis (*n* = 71)	*P* value
Men – no. (%)	33 (80)	56 (79)	0.93
Age – yrs, mean (s.d.)	61.0 (9.8)	61.3 (7.7)	0.88
Body-mass index – kg m^{-2}, mean (s.d.)	26.9 (3.8)	27.1 (2.9)	0.34
Smoking – no. (%)	14 (34)	25 (35)	0.56
PCI indication:			
- Stable angina – no. (%)	17 (41)	38 (54)	0.32
- Unstable angina or NSTEMI – no. (%)	6 (15)	10 (14)	0.82
- STEMI – no. (%)	18 (44)	23 (32)	0.27
Vessels treated:			
- 1 Vessel – no. (%)	32 (78)	50 (70)	0.35
- 2 Vessel – no. (%)	9 (22)	21 (30)	0.35
Type of stent:			
- Bare metal stent – no. (%)	25 (61)	42 (59)	0.96
- Drug-eluting stent – no. (%)	16 (39)	29 (41)	0.96
Clopidogrel loading dose:			
- 600 mg – no. (%)	23 (56)	39 (55)	0.86
- 300 mg – no. (%)	15 (37)	26 (37)	0.54
- No loading – no. (%)	3 (7)	6 (8)	0.31
Cardiovascular risk factors:			
Hypertension – no. (%)	22 (54)	40 (56)	0.76
Hypercholesterolemia – no. (%)	21 (51)	37 (52)	0.67
Diabetes mellitus – no. (%)	10 (24)	19 (27)	0.61
Previous PCI or CABG – no. (%)	16 (40)	31 (44)	0.61
Previous MI, CAD or PAD – no. (%)	20 (49)	36 (51)	0.80
Previous stroke or TIA – no. (%)	4 (10)	7 (10)	0.93
Family history of CAD – no. (%)	20 (49)	31 (44)	0.64
LVEF <45% – no. (%)	13 (32)	19 (27)	0.36
COPD – no. (%)	2 (5)	3 (4)	0.78
Renal insufficiency – no. (%)	3 (7)	7 (10)	0.72

Body-mass index is calculated as weight in kilograms divided by the square of the height in meters. Smoking is defined as smoking of at least one cigarette per day, actually or within the previous 6 months. No loading denotes patients who were pretreated with 75 mg clopidogrel for at least 5 days before the index procedure and received no additional loading dose. Median exposure time to clopidogrel maintenance treatment (75 mg per day post-PCI) was 12 months in both groups.

P values were calculated by univariate Cox regression with weighting of subcohort noncases with the inverse of the sampling fraction according to the method of Barlow *et al.*[1]. $P < 0.05$ was considered a statistically significant difference.

CABG denotes coronary artery bypass graft, CAD coronary artery disease, COPD chronic obstructive pulmonary disease, LVEF left ventricular ejection fraction, MI myocardial infarction, NSTEMI non ST-elevation MI, PAD peripheral arterial disease, PCI percutaneous coronary intervention, STEMI ST-elevation MI, TIA transient ischemic attack.

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Supplementary Table 3. Clinical laboratory characteristics and medication among case-cohort patients at the time of clopidogrel response test

Characteristics	With stent thrombosis (*n* = 41)	Without stent thrombosis (*n* = 71)	*P* value
Total plasma cholesterol – mg dl^{-1}, mean (s.d.)	186 (33)	183 (35)	0.69
LDL-cholesterol – mg dl^{-1}, mean (s.d.)	119 (27)	114 (32)	0.43
HDL-cholesterol – mg dl^{-1}, mean (s.d.)	49 (10)	48 (12)	0.47
Plasma triglycerides – mg dl^{-1}, mean (s.d.)	148 (44)	147 (57)	0.93
C-reactive protein – mg l^{-1}, median (i.q.r.)	2.6 (1.0-4.1)	2.6 (1.2-3.5)	0.44
Leukocyte count – $x10^9$ l^{-1}, mean (s.d.)	7.68 (2.16)	7.36 (1.82)	0.58
Platelet count – $x10^9$ l^{-1}, mean (s.d.)	244 (84)	241 (50)	0.85
Erythrocyte count – $x10^{12}$ l^{-1}, mean (s.d.)	4.70 (0.62)	4.71 (0.37)	0.48
Hemoglobin – g dl^{-1}, mean (s.d.)	14.4 (1.7)	14.4 (0.78)	0.38
Plasma creatinine – mg dl^{-1}, mean (s.d.)	0.94 (0.24)	0.94 (0.25)	0.71
Platelet aggregation – %, mean (s.d.)	73.7 (9.9)	75.9 (6.4)	0.29
Paraoxonase – nmol min^{-1} ml^{-1}, mean (s.d.)	64 (44)	154 (93)	< 0.001
Arylesterase – nmol min^{-1} ml^{-1}, mean (s.d.)	1,942 (952)	1,915 (934)	0.77
Medication – no. (%):			
Aspirin	37 (90)	65 (92)	0.86
Vitamin K antagonist	5 (12)	8 (11)	0.84
ACE inhibitor or ARB	24 (59)	38 (54)	0.50
Beta blocker	26 (63)	46 (65)	0.67
Calcium-channel blocker	9 (22)	17 (24)	0.90
Diuretics	18 (44)	28 (39)	0.60
Nitrates	11 (27)	23 (32)	0.58
Statins	34 (83)	52 (73)	0.22
Omeprazole or esomeprazole	7 (17)	12 (17)	0.86
Other proton-pump inhibitor	15 (37)	22 (31)	0.75

Continuous, normally distributed data are presented as arithmetic mean and standard deviation (s.d.), non-normally distributed data as median and interquartile range (i.q.r.). Platelet aggregation denotes maximal baseline aggregation before clopidogrel administration induced by 20 μM adenosine diphosphate. Paraoxonase and arylesterase refer to the enzymatic plasma activity using paraoxon or phenylacetate as substrates.

P values were calculated by univariate Cox regression with weighting of subcohort noncases with the inverse of the sampling fraction according to the method of Barlow *et al.*[1]. $P < 0.05$ was considered a statistically significant difference.

ACE denotes angiotensin-converting enzyme, ARB angiotensin-receptor blocker, HDL high-density lipoprotein, LDL low-density lipoprotein. Other proton-pump inhibitors are pantoprazole or lansoprazole.

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Supplementary Table 4. Pharmacokinetic plasma parameters in case-cohort patients after administration of a single 600-mg clopidogrel dose

Parent clopidogrel or metabolite: - Pharmacokinetic parameter	**With stent thrombosis (*n* = 41)**	**Without stent thrombosis (*n* = 71)**	***P* value**
Clopidogrel:			
- c_{max} – ng ml^{-1}, mean (s.d.)	31.4 (25.5)	30.8 (23.7)	0.76
- t_{max} – min, mean (s.d.)	67.8 (28.7)	62.1 (25.4)	0.37
- $AUC_{0\rightarrow\infty}$ – ng min ml^{-1}, mean (s.d.)	4,072 (2,778)	4,000 (3,072)	0.98
2-Oxo-clopidogrel:			
- c_{max} – ng ml^{-1}, mean (s.d.)	0.0488 (0.0337)	0.0352 (0.0209)	< 0.001
- t_{max} – min, mean (s.d.)	56.6 (19.1)	53.5 (19.2)	0.74
- $AUC_{0\rightarrow\infty}$ – ng min ml^{-1}, mean (s.d.)	7.93 (7.31)	5.64 (3.89)	< 0.001
Thiol metabolite:			
- c_{max} – ng ml^{-1}, mean (s.d.)	8.70 (5.56)	18.7 (10.5)	< 0.001
- t_{max} – min, mean (s.d.)	58.5 (22.2)	56.5 (22.7)	0.93
- $AUC_{0\rightarrow\infty}$ – ng min ml^{-1}, mean (s.d.)	1,151 (935)	2,695 (1,637)	< 0.001
Carboxylic acid metabolite:			
- c_{max} – ng ml^{-1}, mean (s.d.)	34,394 (13,074)	34,185 (10,978)	0.67
- t_{max} – min, mean (s.d.)	73.3 (31.3)	69.7 (25.4)	0.84
- $AUC_{0\rightarrow\infty}$ – ng min ml^{-1}, mean (s.d.)	6,175,270 (2,558,783)	5,610,182 (2,335,298)	0.26

C_{max} denotes maximum plasma concentration, t_{max} time to maximum plasma concentration, and $AUC_{0\rightarrow\infty}$ area under the concentration-time curve extrapolated to infinity. The parameters were obtained from fitting individual data by a linear one-compartment lag-time model. Arithmetic means and standard deviations (s.d.) are presented.

P values were calculated by univariate Cox regression with weighting of subcohort noncases with the inverse of the sampling fraction according to the method of Barlow *et al.*[1]. $P < 0.05$ was considered a statistically significant difference.

Supplementary Table 5. Hazard ratios of incident non-fatal definite stent thrombosis in relation to *PON1* Q192R genotype in case-cohort patients after adjustment for paraoxonase activity, plasma concentration of active metabolite or platelet inhibition

Predictor variable	Hazard ratio (95% CI)	*P* value
PON1 genotype (unadjusted):		
- RR192	1 (reference)	
- QR192	4.41 (1.89–10.20)	0.001
- QQ192	12.82 (4.74–90.91)	< 0.001
PON1 genotype with adjustment for tertiles of paraoxonase plasma activity:		
- RR192	1 (reference)	
- QR192	1.04 (0.26–4.18)	0.972
- QQ192	1.17 (0.11–12.94)	0.896
PON1 genotype with adjustment for tertiles of plasma concentration of active clopidogrel metabolite:		
- RR192	1 (reference)	
- QR192	1.46 (0.39–6.95)	0.780
- QQ192	1.76 (0.18–16.91)	0.691
PON1 genotype with adjustment for tertiles of platelet inhibition:		
- RR192	1 (reference)	
- QR192	1.39 (0.59–3.28)	0.720
- QQ192	3.85 (0.46–31.97)	0.213

Hazard ratios were calculated by including tertiles of paraoxonase plasma activity, tertiles of maximal plasma concentration of active metabolite, or tertiles of platelet inhibition as covariates in univariate Cox regression models with *PON1* Q192R as predictor variable and weighting of subcohort noncases with the inverse of the sampling fraction according to the method of Barlow *et al*.[1]. Genotype distribution and tertile limits in the sample fraction were extrapolated to the total cohort. Tertile limits are shown in **Table 2**. Maximal plasma concentrations of the active metabolite and predose versus 6-h postdose platelet inhibition were determined after administration of a single 600-mg clopidogrel dose.
Significant interactions ($P < 0.001$) of *PON1* Q192R with activity markers were detected by including the interaction terms (*PON1* Q192R) x (marker tertiles) in Cox regression models. Collinearity diagnostics showed strong pairwise correlations ($\rho \geq 0.80$) between *PON1* Q192R and the activity variables.
$P < 0.05$ was considered a statistically significant difference. CI denotes confidence interval.

Supplementary Table 6. Paraoxonase plasma activity, plasma concentration of active metabolite and platelet Inhibition in case-cohort patients after administration of a single 600-mg clopidogrel dose according to variant genotype distribution

Gene polymorphism	Homozygous wild-type	Heterozygous	Homozygous mutant	*P* value
Paraoxonase activity – nmol min^{-1} ml^{-1}, mean (s.d.):				
- *CYP2C9*2*	128.6 (97.0)	94.4 (50.5)	n.d.	0.100
- *CYP2C9*3*	121.2 (93.3)	121.0 (75.3)	n.d.	0.991
- *CYP2C19*2*	130.6 (94.3)	107.3 (81.3)	71.8 (24.0)	0.212
- *CYP3A4*1B*	121.3 (90.2)	118.2 (94.5)	n.d.	0.941
- *CYP3A4*1G*	122.7 (92.4)	126.4 (84.3)	63.3 (38.6)	0.421
- *CYP3A5*3*	119.8 (88.8)	119.6 (99.4)	n.d.	0.993
- *PON1* Q192R	53.7 (27.3)	143.3 (42.6)	300.0 (70.1)	< 0.001
- *ABCB1* C3435T	145.9 (104.3)	117.0 (88.9)	110.4 (79.3)	0.320
C_{max} thiol metabolite – ng ml^{-1}, mean (s.d.):				
- *CYP2C9*2*	15.8 (10.9)	12.1 (6.8)	n.d.	0.307
- *CYP2C9*3*	14.9 (10.4)	15.3 (9.7)	n.d.	0.862
- *CYP2C19*2*	15.8 (10.7)	14.0 (9.7)	9.3 (3.0)	0.311
- *CYP3A4*1B*	15.0 (10.2)	15.4 (11.8)	n.d.	0.932
- *CYP3A4*1G*	15.2 (10.3)	15.9 (10.8)	8.9 (5.2)	0.465
- *CYP3A5*3*	14.8 (10.0)	14.5 (10.7)	n.d.	0.918
- *PON1* Q192R	7.3 (2.8)	17.6 (6.0)	35.2 (5.8)	< 0.001
- *ABCB1* C3435T	17.8 (10.7)	15.0 (10.5)	12.8 (9.1)	0.236
Platelet inhibition – Δ%, mean (s.d.):				
- *CYP2C9*2*	27.2 (19.5)	20.5 (13.8)	n.d.	0.220
- *CYP2C9*3*	24.9 (19.5)	29.8 (19.9)	n.d.	0.335
- *CYP2C19*2*	26.9 (20.4)	25.7 (18.0)	13.7 (16.4)	0.348
- *CYP3A4*1B*	25.2 (19.5)	27.4 (19.7)	n.d.	0.713
- *CYP3A4*1G*	25.4 (20.0)	30.9 (19.5)	18.4 (13.0)	0.427
- *CYP3A5*3*	25.1 (19.2)	25.5 (19.4)	n.d.	0.944
- *PON1* Q192R	11.3 (10.5)	32.8 (13.1)	58.3 (9.1)	< 0.001
- *ABCB1* C3435T	31.6 (18.9)	25.1 (19.8)	22.7 (19.4)	0.259

Paraoxonase activity refers to the enzymatic plasma activity derived from the hydrolysis velocity of paraoxon to *p*-nitrophenol. Maximal plasma concentrations c_{max} of the active clopidogrel metabolite were derived from fitting individual data by a linear one-compartment lag-time model. Platelet inhibition indicates percentage of maximal predose versus maximal 6-h postdose platelet aggregation induced by 20 μM adenosine diphosphate.

The comparative genotype analysis indicates a significant interaction between clopidogrel activity markers and the *PON1* Q192R genotype, but the absence of interactions with other gene variants.

Arithmetic means and standard deviations (s.d.) are presented. *P* values were calculated by two-tailed unpaired t-test or one-way analysis of variance as applicable. $P < 0.05$ was considered a statistically significant difference.

n.d. denotes not determined if less than three homozygous mutant individuals.

Supplementary Table 7. Paraoxonase plasma activity, plasma concentration of active metabolite and platelet inhibition in case-cohort patients after administration of a single 600-mg clopidogrel dose according to concomitant drug use

Drugs	Patients with use of drug	Patients without use of drug	*P* value
Paraoxonase activity – nmol min^{-1} ml^{-1}, mean (s.d.):			
- Aspirin	117.8 (83.4)	157.0 (138.9)	0.188
- Vitamin K antagonist	141.2 (125.5)	118.7 (84.3)	0.397
- ACE inhibitor or ARB	123.6 (100.4)	118.5 (74.9)	0.768
- Beta blocker	125.3 (95.3)	114.1 (78.8)	0.529
- Calcium-channel blocker	112.8 (100.6)	123.9 (86.4)	0.585
- Diuretics	125.8 (105.7)	118.2 (77.1)	0.658
- Nitrates	132.5 (94.9)	116.4 (87.3)	0.384
- Statins	115.4 (80.4)	140.7 (114.6)	0.209
- Omeprazole or esomeprazole	115.9 (99.9)	122.4 (87.9)	0.773
- Other proton-pump inhibitor	127.5 (91.1)	118.2 (89.3)	0.610
C_{max} thiol metabolite – ng ml^{-1}, mean (s.d.):			
- Aspirin	14.5 (9.8)	19.7 (13.6)	0.231
- Vitamin K antagonist	17.4 (12.8)	14.7 (9.9)	0.555
- ACE inhibitor or ARB	15.2 (11.0)	14.8 (9.2)	0.829
- Beta blocker	15.8 (11.0)	13.6 (8.5)	0.518
- Calcium-channel blocker	14.1 (11.1)	15.3 (10.0)	0.601
- Diuretics	16.0 (11.9)	14.4 (8.9)	0.716
- Nitrates	16.6 (11.2)	14.3 (9.7)	0.351
- Statins	14.5 (9.6)	16.7 (12.0)	0.547
- Omeprazole or esomeprazole	14.8 (11.6)	14.8 (9.6)	0.994
- Other proton-pump inhibitor	15.4 (10.7)	14.8 (9.6)	0.809
Platelet inhibition – Δ%, mean (s.d.):			
- Aspirin	25.1 (18.6)	34.8 (27.5)	0.166
- Vitamin K antagonist	30.7 (25.2)	25.3 (18.8)	0.419
- ACE inhibitor or ARB	26.9 (20.2)	24.8 (19.1)	0.587
- Beta blocker	28.6 (20.7)	21.4 (17.0)	0.211
- Calcium-channel blocker	21.8 (18.4)	27.3 (19.9)	0.214
- Diuretics	28.2 (21.6)	24.5 (18.2)	0.549
- Nitrates	26.5 (20.5)	25.8 (19.4)	0.858
- Statins	25.6 (19.2)	27.1 (21.3)	0.753
- Omeprazole or esomeprazole	28.1 (21.3)	24..2 (19.9)	0.474
- Other proton-pump inhibitor	27.7 (18.6)	24.2 (19.9)	0.413

Paraoxonase activity refers to the enzymatic plasma activity derived from the hydrolysis velocity of paraoxon to *p*-nitrophenol. Maximal plasma concentration c_{max} of the active clopidogrel metabolite was derived from fitting individual data by a linear one-compartment lag-time model. Platelet inhibition indicates maximal predose versus maximal 6-h postdose platelet aggregation induced by 20 μM adenosine diphosphate.

Supplementary Table 7 continued.

The comparative analysis indicates the absence of significant interactions between clopidogrel activity markers and concomitantly used drugs. Drug interactions with enzymes and transporters potentially involved in clopidogrel response have been reported previously, but were not consistently replicated. For example, aspirin or statins may increase PON1 activity[2-4], statins or dihydropyridine calcium-channel blockers may inhibit CYP3A isozymes[5,6], omeprazole may inhibit CYP2C19, and statins, omeprazole or calcium-channel blockers may inhibit the ABCB1 transporter[8].

Arithmetic means and standard deviations (s.d.) are presented. *P* values were calculated by two-tailed unpaired t-test. $P < 0.05$ was considered a statistically significant difference.

ACE denotes angiotensin-converting enzyme, ARB angiotensin-receptor blocker. Other proton-pump inhibitors are pantoprazole or lansoprazole.

All available proton-pump inhibitors were reported to inhibit CYP2C19 *in vitro* at pharmacologically relevant concentrations[9], but their effects on *in vivo* activity of CYP2C19 and on metabolism and clinical efficacy of clopidogrel are controversial. Concomitant use of omeprazole has been reported to decrease clopidogrel bioactivation or antiplatelet response to clopidogrel[10]; no or weak drug-drug interactions of clopidogrel have been reported with pantoprazole[11] or lansoprazole[12]. However, there are also conflicting data[13,14], and there is no good evidence that pharmacokinetic or pharmacodynamic proton-pump inhibitor–clopidogrel interactions translate into clinically meaningful outcomes[15]. The here presented data indicate that none of the proton-pump inhibitors affects the pharmacokinetic or pharmacodynamic response to clopidogrel or its clinical efficacy (see **Supplementary Table 10**).

Supplementary Table 8. Hazard ratios for primary and secondary end points in prospective cohort patients at 12 months according to *PON1* Q192R genotype

End point – no. (%): - *PON1* genotype [no. of events]	**Unadjusted hazard ratio (95% CI)**	***P* value**	**Adjusted hazard ratio (95% CI)**	***P* value**
Definite stent thrombosis – 44 (2.2):				
- RR192 [1]	1 (reference)		1 (reference)	
- QR192 [12]	3.06 (1.57–5.95)	0.001	3.04 (1.56–5.95)	0.001
- QQ192 [31]	10.20 (4.39–71.43)	< 0.001	10.10 (4.38–76.92)	< 0.001
Myocardial infarction – 142 (7.2):				
- RR192 [7]	1 (reference)		1 (reference)	
- QR192 [46]	2.31 (1.62–3.29)	< 0.001	2.33 (1.63–3.34)	< 0.001
- QQ192 [89]	4.93 (2.16–11.24)	< 0.001	5.24 (2.28–11.90)	< 0.001
Ischemic stroke – 26 (1.3):				
- RR192 [2]	1 (reference)		1 (reference)	
- QR192 [10]	1.70 (0.75–3.83)	0.201	1.75 (0.78–3.97)	0.187
- QQ192 [14]	2.38 (0.54–10.42)	0.250	2.36 (0.53–10.53)	0.257
Nonfatal myocardial infarction, nonfatal stroke, or vascular death – 216 (10.9):				
- RR192 [13]	1 (reference)		1 (reference)	
- QR192 [76]	2.02 (1.52–2.67)	< 0.001	2.05 (1.54–2.73)	< 0.001
- QQ192 [127]	3.89 (2.10–7.19)	< 0.001	4.05 (2.18–7.52)	< 0.001
Nonvascular death – 15 (0.8):				
- RR192 [2]	1 (reference)		1 (reference)	
- QR192 [7]	1.05 (0.35–3.12)	0.935	1.00 (0.33–2.99)	0.995
- QQ192 [6]	0.98 (0.20–4.72)	0.981	0.90 (0.18–4.42)	0.899
Major bleeding – 57 (2.9):				
- RR192 [14]	1 (reference)		1 (reference)	
- QR192 [28]	0.65 (0.35–1.22)	0.177	0.66 (0.35–1.23)	0.189
- QQ192 [15]	0.36 (0.18–0.75)	0.006	0.34 (0.16–0.70)	0.004

Hazard ratios were calculated by univariate and multivariate Cox regression. Multivariate adjustments were done for baseline demographic (sex, age, body-mass index, smoking) and clinical characteristics (indication of percutaneous coronary intervention, number of treated vessels, type of stent, hypertension, hypercholesterolemia, diabetes mellitus, previous myocardial infarction or peripheral arterial disease, family history of coronary artery disease, congestive heart failure). Further adjustment for medication (heparin, angiotensin-converting enzyme inhibitor or angiotensin-receptor blocker, beta blocker, calcium-channel blocker, nitrate, statin, omeprazole or esomeprazole, other proton-pump inhibitor (pantoprazole or lansoprazole)) did not change hazard ratios (data not shown).
$P < 0.05$ was considered a statistically significant difference. CI denotes confidence interval.

Supplementary Table 9. Hazard ratios for primary and secondary end points in prospective cohort patients at 12 months according to paraoxonase plasma activity

End point – no. (%): - Tertiles of paraoxonase plasma activity (nmol min^{-1} ml^{-1}) [no. of events]	**Unadjusted hazard ratio (95% CI)**	***P* value**	**Adjusted hazard ratio (95% CI)**	***P* value**
Definite stent thrombosis – 44 (2.2):				
- Highest tertile (≥157.0) [2]	1 (reference)		1 (reference)	
- Middle tertile (<157.0–80.9) [8]	4.42 (2.04–9.52)	< 0.001	4.57 (2.10–9.90)	< 0.001
- Lowest tertile (<80.9) [34]	18.18 (4.33–76.90)	< 0.001	18.52 (4.44–76.92)	< 0.001
Myocardial infarction – 142 (7.2):				
- Highest tertile (≥157.0) [14]	1 (reference)		1 (reference)	
- Middle tertile (<157.0–80.9) [46]	2.63 (1.86–6.71)	< 0.001	2.71 (1.92–6.89)	< 0.001
- Lowest tertile (<80.9) [82]	6.76 (3.77–12.20)	< 0.001	7.14 (3.95–12.82)	< 0.001
Ischemic stroke – 26 (1.3):				
- Highest tertile (≥157.0) [7]	1 (reference)		1 (reference)	
- Middle tertile (<157.0–80.9) [8]	1.46 (0.59–3.64)	0.411	1.50 (0.60–3.76)	0.397
- Lowest tertile (<80.9) [11]	1.72 (0.67–4.44)	0.260	1.71 (0.65–4.48)	0.275
Nonfatal myocardial infarction, nonfatal stroke, or vascular death – 216 (10.9):				
- Highest tertile (≥157.0) [30]	1 (reference)		1 (reference)	
- Middle tertile (<157.0–80.9) [70]	2.17 (1.50–3.81)	< 0.001	2.25 (1.55–3.95)	< 0.001
- Lowest tertile (<80.9) [116]	4.32 (2.10–7.19)	< 0.001	4.55 (2.16–7.46)	< 0.001
Nonvascular death – 15 (0.8):				
- Highest tertile (≥157.0) [5]	1 (reference)		1 (reference)	
- Middle tertile (<157.0–80.9) [6]	1.25 (0.38–4.08)	0.717	1.28 (0.34–4.90)	0.714
- Lowest tertile (<80.9) [4]	0.89 (0.24–3.32)	0.863	0.83 (0.25–2.76)	0.765
Major bleeding – 57 (2.9):				
- Highest tertile (≥157.0) [30]	1 (reference)		1 (reference)	
- Middle tertile (<157.0–80.9) [18]	0.53 (0.24–1.18)	0.121	0.54 (0.24–1.22)	0.139
- Lowest tertile (<80.9) [9]	0.33 (0.16–0.69)	0.004	0.32 (0.15–0.67)	0.003

Hazard ratios were calculated by univariate and multivariate Cox regression. Multivariate adjustments were done for baseline demographic (sex, age, body-mass index, smoking) and clinical characteristics (indication of percutaneous coronary intervention, number of treated vessels, type of stent, hypertension, hypercholesterolemia, diabetes mellitus, previous myocardial infarction or peripheral arterial disease, family history of coronary artery disease, congestive heart failure). Further adjustment for medication (heparin, angiotensin-converting enzyme inhibitor or angiotensin-receptor blocker, beta blocker, calcium-channel blocker, nitrate, statin, omeprazole or esomeprazole, other proton-pump inhibitor (pantoprazole or lansoprazole)) did not change hazard ratios (data not shown).
The *PON1* Q192R genotype was closely correlated with the paraoxonase plasma activity (unadjusted R^2 = 0.807, P < 0.001).
P < 0.05 was considered a statistically significant difference. CI denotes confidence interval.

Supplementary Table 10. Baseline demographic, clinical and procedural characteristics of prospective cohort patients at the time of index percutaneous coronary intervention; drug therapy and variant genotypes, according to incident fatal and non-fatal definite stent thrombosis

Characteristics	With stent thrombosis (*n* = 44)	Without stent thrombosis (*n* = 1,938)	*P* value
Men – no. (%)	32 (73)	1,381 (71)	0.82
Age – yrs, mean (s.d.)	60.2 (10.7)	62.2 (10.2)	0.20
Body-mass index – kg m^{-2}, mean (SD)	26.7 (4.0)	27.1 (4.3)	0.59
Smoking – no. (%)	14 (32)	695 (36)	0.57
PCI indication:			
- Unstable angina or NSTEMI – no. (%)	28 (64)	1,188 (61)	0.74
- STEMI – no. (%)	16 (36)	750 (39)	0.74
Vessels treated:			
- 1 Vessel – no. (%)	37 (84)	1,572 (81)	0.60
- ≥2 Vessels – no. (%)	7 (16)	366 (19)	0.60
Type of stent:			
- Bare metal stent only – no. (%)	30 (68)	1,339 (69)	0.88
- ≥1 Drug-eluting stent – no. (%)	14 (32)	599 (31)	0.88
Cardiovascular risk factors:			
Hypertension – no. (%)	28 (64)	1,215 (63)	0.93
Hypercholesterolemia – no. (%)	24 (55)	1,075 (55)	0.92
Diabetes mellitus – no. (%)	9 (20)	473 (24)	0.55
Previous PCI or CABG – no. (%)	8 (18)	370 (19)	0.91
Previous MI or PAD – No. (%)	13 (30)	599 (31)	0.85
Previous stroke or TIA – no. (%)	2 (5)	84 (4)	0.92
Family history of CAD – no. (%)	19 (43)	814 (42)	0.86
Congestive heart failure – no. (%)	3 (7)	124 (6)	0.89
COPD – no. (%)	2 (7)	97 (5)	0.57
Renal insufficiency – no. (%)	2 (5)	81 (4)	0.92
Medication administered in hospital or at discharge – no. (%):			
Heparin (unfractionated or LMWH)	32 (73)	1,430 (74)	0.89
ACE inhibitor or ARB	34 (77)	1,492 (77)	0.96
Beta blocker	37 (84)	1,685 (87)	0.57
Calcium-channel blocker	12 (27)	551 (28)	0.86
Nitrate	32 (73)	1,460 (75)	0.68
Statin	38 (86)	1,729 (89)	0.53
Omeprazole or esomeprazole	5 (11)	305 (16)	0.41
Other proton-pump inhibitor	13 (30)	589 (30)	0.92

Supplementary Table 10 continued.

Characteristics	With stent thrombosis (*n* = 44)	Without stent thrombosis (*n* = 1,938)	*P* value
Variant genotypes:			
PON1 genotype – A576G (Q192R):			< 0.001 [< 0.001]
AA (QQ) – no. (%)	31 (70.4)	777 (40.1)	
AG (QR) – no. (%)	12 (27.3)	908 (46.8)	
GG (QR) – no. (%)	1 (2.3)	253 (13.1)	
CYP2C19 genotype – G681A (CYP2C19*2):			0.85 [0.88]
GG (*1/*1) – no. (%)	28 (63.6)	1,296 (66.9)	
GA (*1/*2) – no. (%)	14 (31.8)	575 (29.7)	
AA (*2/*2) – no. (%)	2 (4.6)	67 (3.4)	
CYP2C19 genotype – C(–806)T (CYP2C19*17):			0.82 [0.82]
CC (*1/*1) – no. (%)	28 (63.6)	1,207 (62.3)	
CT (*1/*17) – no. (%)	15 (34.1)	649 (33.5)	
TT (*17/*17) – no. (%)	1 (2.3)	82 (4.2)	

Continuous, normally distributed data are presented as arithmetic mean and standard deviation (s.d.). Body-mass index is calculated as weight in kilograms divided by the square of the height in meters. Smoking is defined as smoking of at least one cigarette per day, actually or within the previous 6 months. *P* values were calculated by univariate Cox regression analysis and *P* values of genotype distributions additionally by binary logistic regression (values are presented in square brackets). $P < 0.05$ was considered a statistically significant difference.

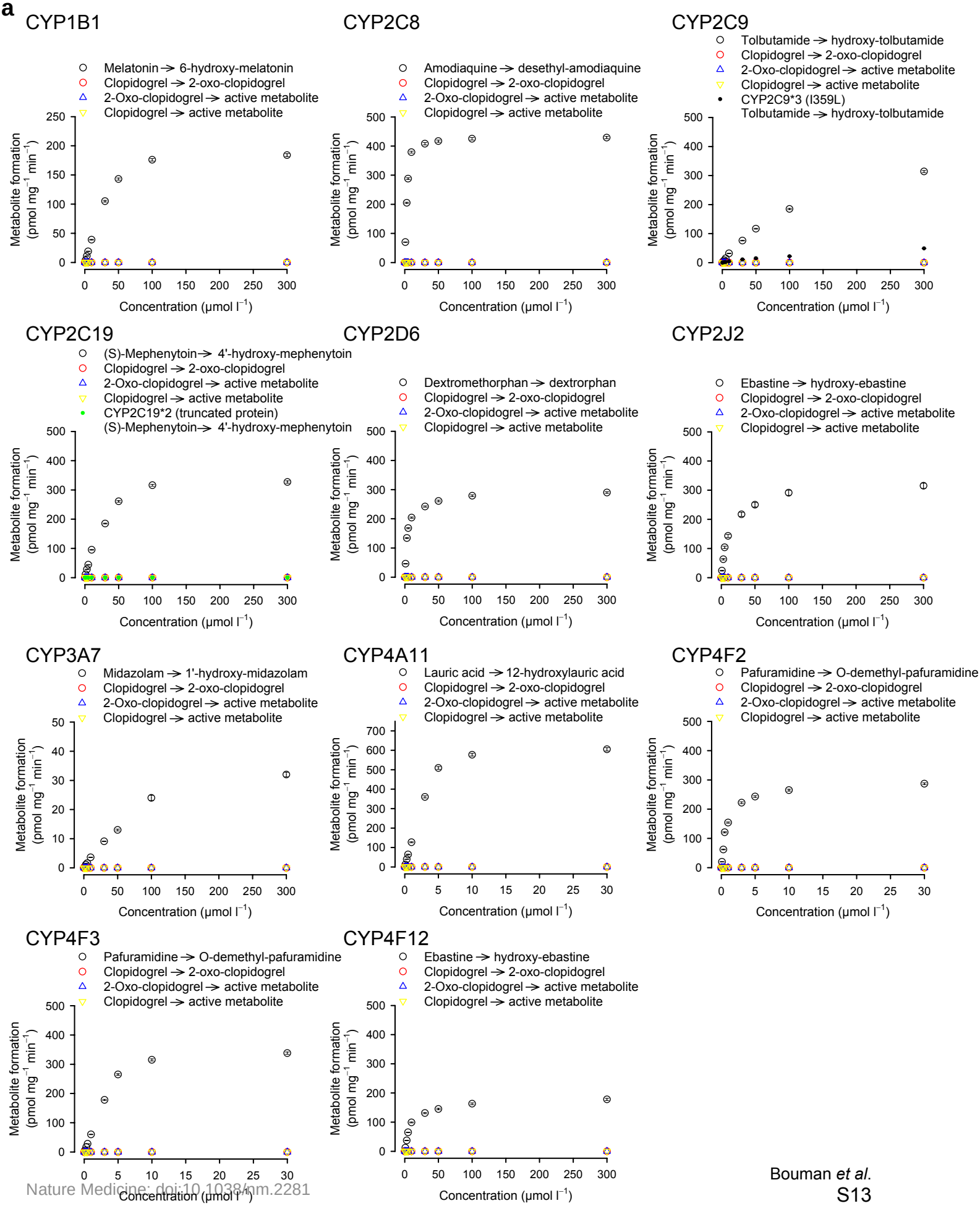
PON1 Q192R genotype distribution was not different from distribution in case-cohort patients: $P = 0.90$ for comparison of stent thrombosis groups and $P = 0.37$ for non stent thrombosis groups (Fisher's exact test). The odds ratio of stent thrombosis in the prospective cohort for QQ192 homozygous individuals versus QR192 heterozygous and RR192 homozygous individuals was 3.6 (95% CI, 1.9–6.8; $P < 0.001$) with a false-positive reporting probability (FPRP) of 0.33%[16]. The combined odds ratio in case-cohort and prospective cohort studies was 3.2 (95% CI, 2.0–6.7; $P < 0.001$), corresponding to a false-positive reporting probability of 0.0016%. In the prospective cohort study, early stent thrombosis (within 30 d, $n = 29$) tended to be more frequent than late stent thrombosis (>30 d, $n = 15$) in the QQ192 subjects as compared with QR192 and RR192 (odds ratio, 3.4; 95% CI, 0.90–12.6; $P = 0.073$). The combined odds ratio from case-cohort and prospective cohort studies of early ($n = 59$) compared with late stent thrombosis ($n = 26$) was significantly higher for QQ192 homozygous individuals versus QR192 and RR192 (3.2; 95% CI, 1.2–8.4; $P = 0.017$).

The distributions of the common loss-of-function variant *CYP2C19*2* and of the common gain-of-function *CYP2C19*17* promoter variant, which has been associated with increased enzyme expression and metabolic activity[17], did not differ between groups. Likewise, the distributions of the haplotype composed of *CYP2C19*2* and *CYP2C19*17* did not differ; frequencies of subjects with at least one **2* and one **17* allele were 6.8% in the stent thrombosis group and 11.7% in the control group ($P = 0.47$, Fisher's exact test). None of the rare loss-of-function alleles **3*–**8* of *CY2C19* was found in patients with stent thrombosis. One copy each of *CYP2C19*3*, **4*, and **8* were detected in three, six, and eleven patients without stent thrombosis, respectively. *CYP2C19*5, *6,* and **7* were not found. **1* refers to the wild-type allele of the respective single nucleotide polymorphism.

CABG denotes coronary artery bypass graft, CAD coronary artery disease, COPD chronic obstructive pulmonary disease, LMWH low-molecular-weight heparin, MI myocardial infarction, NSTEMI non ST-elevation MI, PAD peripheral arterial disease, PCI percutaneous coronary intervention, STEMI ST-elevation MI, TIA transient ischemic attack.

Supplementary Figure 1.

a



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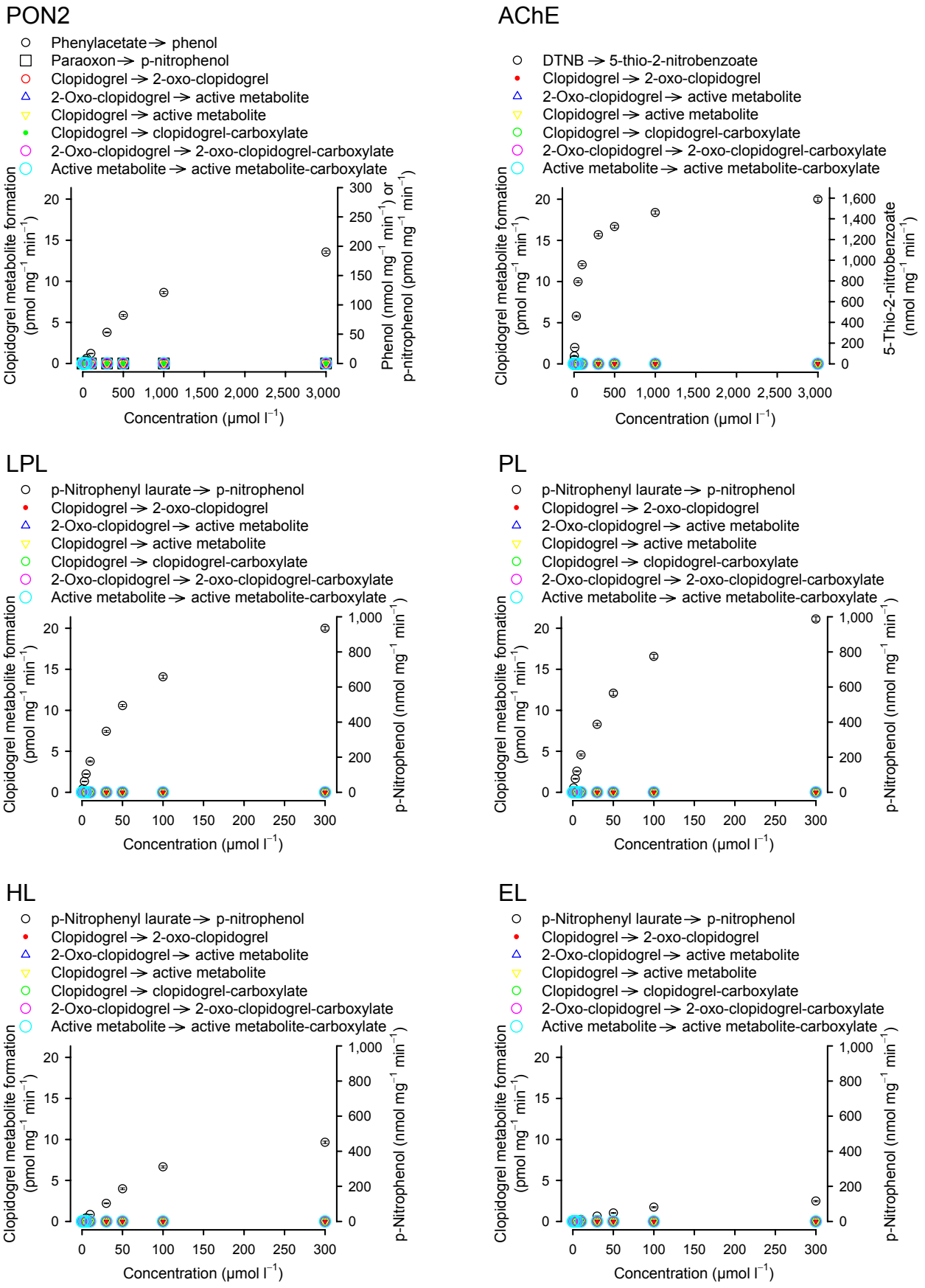
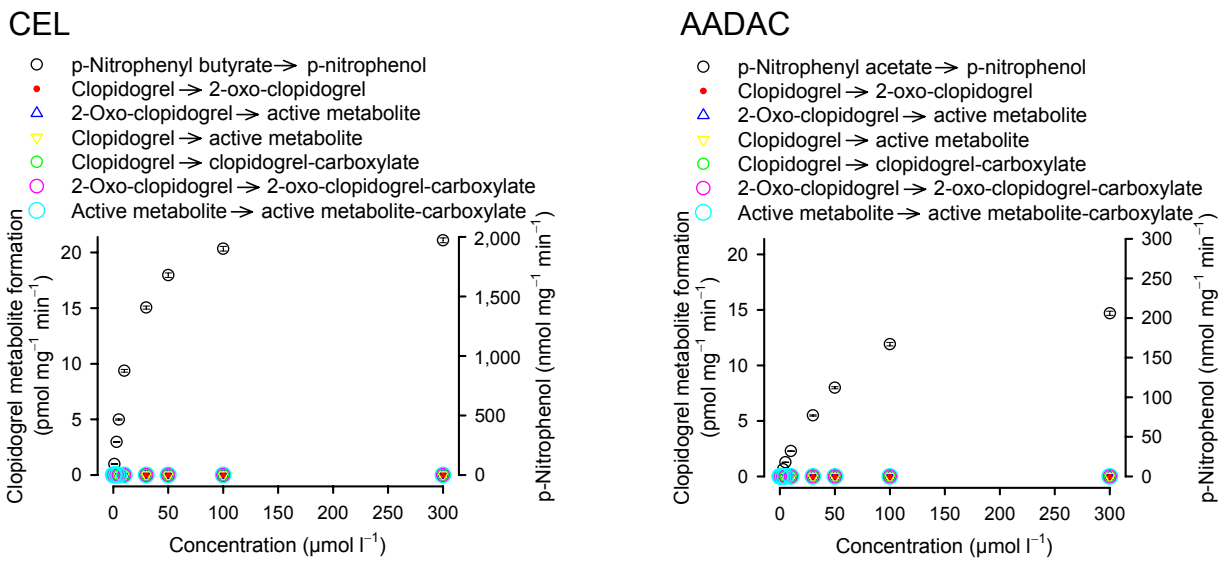
Supplementary Figure 1 continued.

b



Supplementary Figure 1 continued.



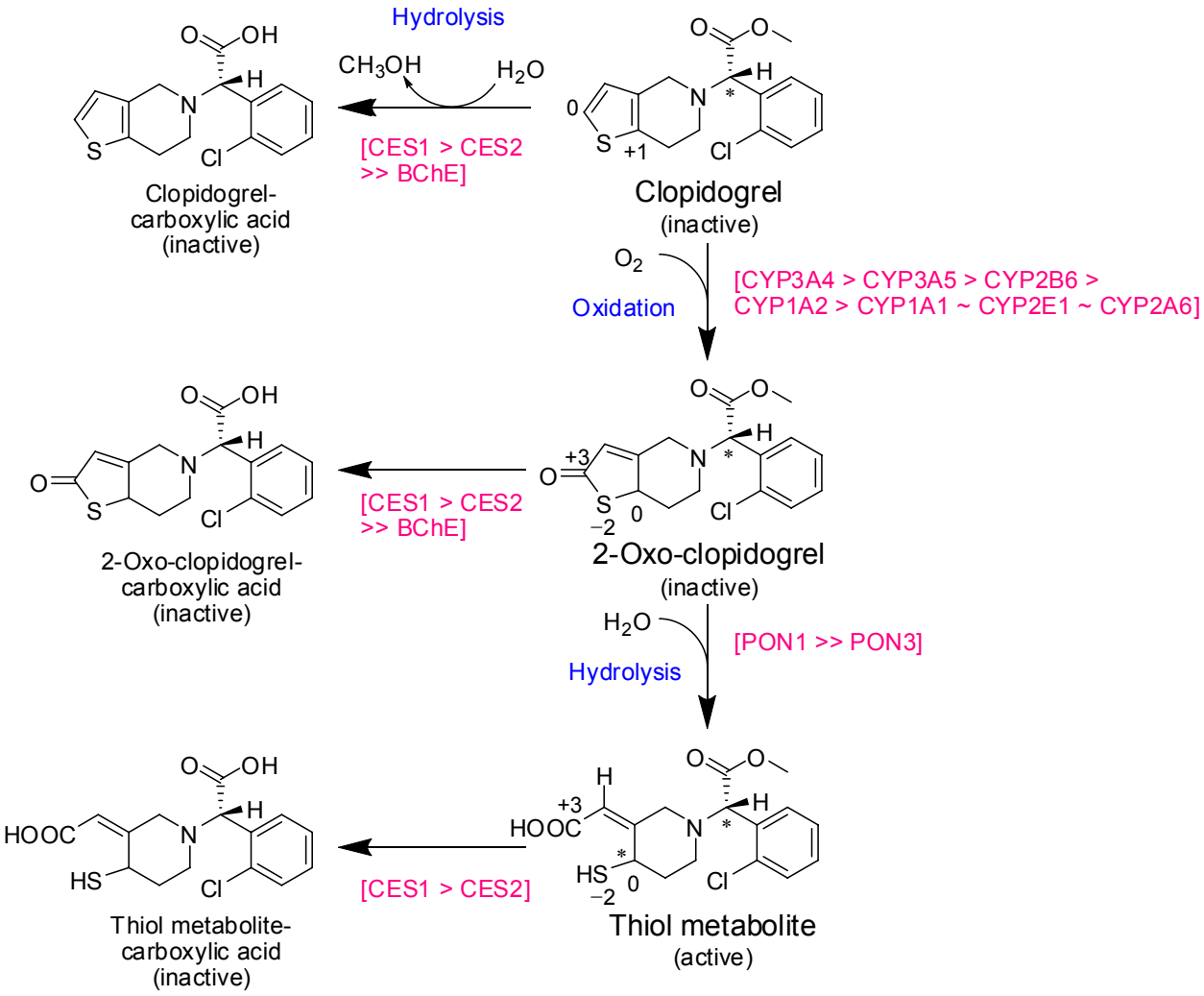
Supplementary Figure 1. Kinetics of drug-metabolizing enzymes not involved in clopidogrel metabolism.

(**a,b**) Substrate saturation kinetics for microsomal preparations of cytochrome P450 isozymes (**a**) and esterases (**b**) showing no catalytic activity for clopidogrel or its intermediate metabolites. Microsomes were obtained from stably transfected human embryonic kidney cells (HEK 293). Conversion of clopidogrel to 2-oxo-clopidogrel, of 2-oxo-clopidogrel to the active thiol metabolite, and of clopidogrel to the thiol metabolite was assayed by incubation with increasing concentrations of substrate for 5 min at 37 °C. For esterases, the conversion of clopidogrel to clopidogrel-carboxylate, of 2-oxo-clopidogrel to 2-oxo-clopidogrel-carboxylate, and of the thiol metabolite to thiol metabolite-carboxylate was also assayed. For each enzyme a specific probe reaction (positive control) was performed (black circles). Symbols and error bars represent arithmetic means and standard errors of three independent incubation experiments each.

To test the possibility that the failure to observe formation of clopidogrel metabolites could be due to a slow rate of enzymatic conversion, we conducted an additional set of experiments. We incubated every enzyme preparation (and HEK 293 control microsomes transfected with empty vector) for 30, 60 and 120 min with 50 μM clopidogrel or 2-oxo-clopidogrel, and then determined the enzyme-specific recovery of the starting compounds by correcting for the recovery rates obtained with control microsomes (n = 3). For none of the enzymes the mean recovery of clopidogrel or 2-oxo-clopidogrel was significantly different from control incubations (P > 0.638 and P > 0.515, respectively). The rates of enzyme-specific recovery for clopidogrel were 96.5–101.4% at 30 min, 97.1–102.6% at 60 min and 97.3–103.0% at 120 min, and for 2-oxo-clopidogrel 96.4–103.4% at 30 min, 96.3–102.9% at 60 min and 95.9–103.8% at 120 min. Control incubations with 50 μM of the respective probe substrates (5 μM of lauric acid and pafuramidine) yielded enzyme-specific recovery rates of less than 10% (data not shown). These data provide further evidence that the CYP isozymes 1B1, 2C8, 2C9, 2C19, 2D6, 2J2, 3A7, 4A11, 4F2, 4F3, 4F12 and the esterases PON2, AChE, LPL, PL, HL, EL, CEL, AADAC do not contribute to the metabolism of clopidogrel.

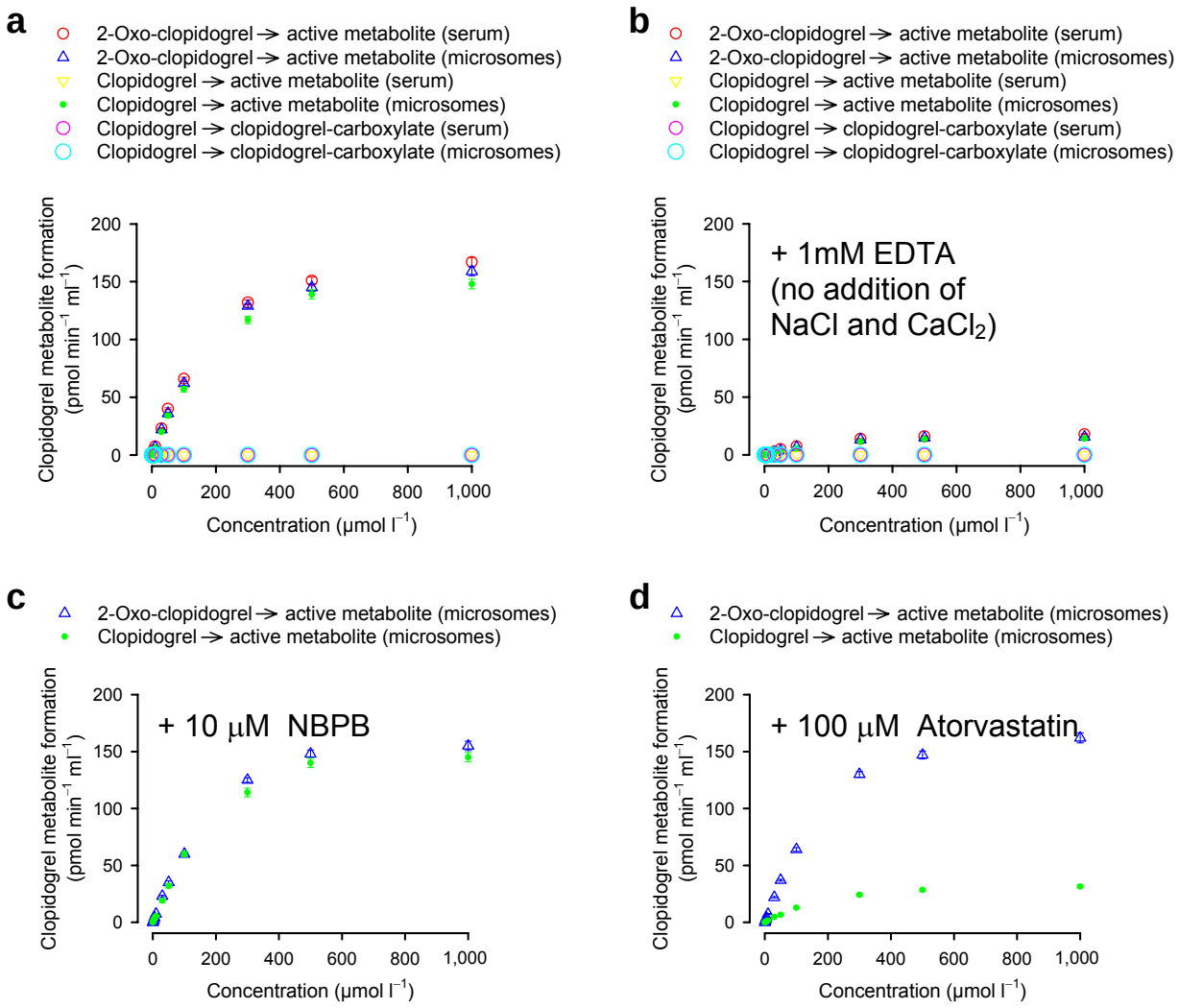
AADAC denotes arylacetamide deacetylase, AChE acetylcholine esterase, CEL carboxyl ester lipase, DTNB 5,5′-dithiobis-(2-nitrobenzoic acid), EL endothelial lipase, HL hepatic lipase, LPL lipoprotein lipase, PL pancreatic lipase, PON2 paraoxonase-2.

Supplementary Figure 2.



Supplementary Figure 2. Scheme of clopidogrel metabolism and involved enzymes.
Numbers in the formulas indicate formal oxidation state of sulfur and carbon atoms. Stars indicate chiral carbon atoms. Absolute configurations of clopidogrel and 2-oxo-clopidogrel with one chiral center at C-7 (7S) and of the active thiol metabolite with chiral centers at C-7 (7S) and C-4 (4S or 4R) and a geometric center at C-3 (ethylene bond, 3Z) are displayed. Relative catalytic efficiencies of enzymes are shown.
BChE denotes butyrylcholinesterase; CES1,2 carboxylesterase-1,-2; CYP cytochrome P450 isozyme; PON1,3 paraoxonase-1,-3.

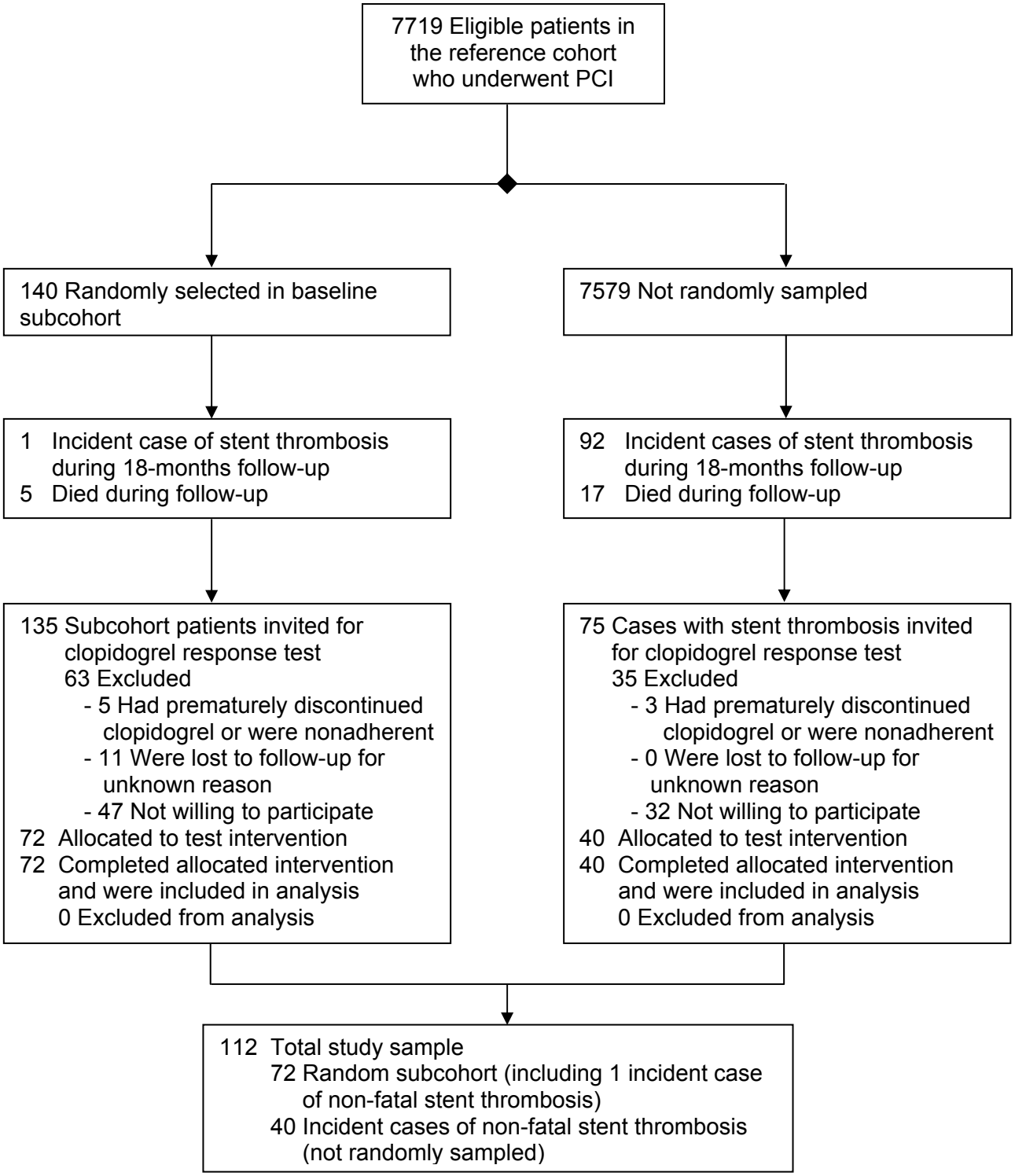
Supplementary Figure 3.



Supplementary Figure 3. Saturation kinetics of clopidogrel metabolite formation in human serum and liver microsomes.

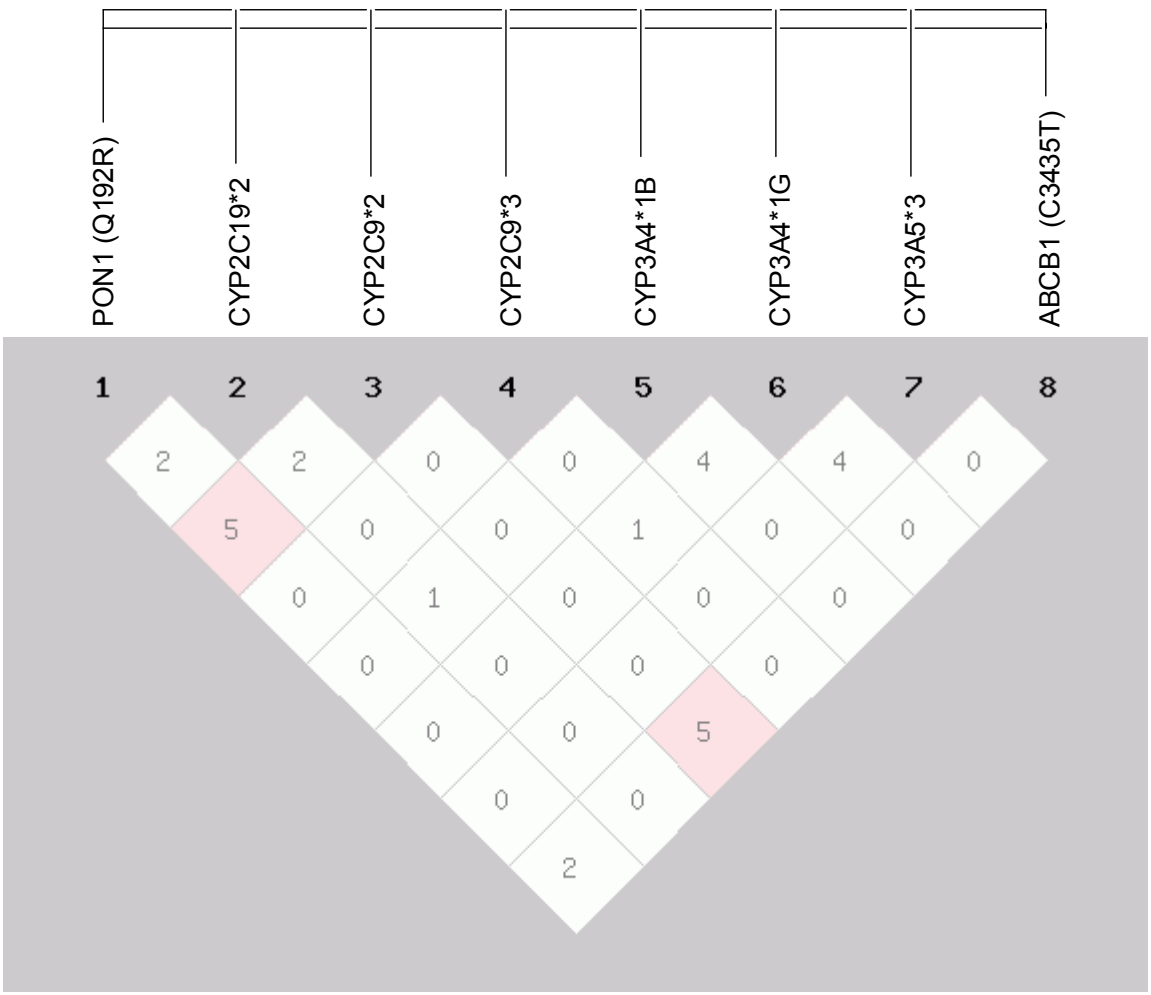
(**a**) The rates of formation of active metabolite from 2-oxo-clopidogrel or clopidogrel and of clopidogrel-carboxylate form clopidogrel are shown. Human serum or liver microsomes (BD Biosciences) were preincubated for 30 min at 25 °C with 100 mM potassium fluoride to inhibit carboxylesterases and acetyl- and butyrcholinesterase. Substrates (at concentrations of 0, 1, 3, 5, 10, 30, 50, 100, 300, 500, and 1,000 μM) were incubated for 5 min at 37 °C with serum or liver microsomes, adjusted to a paraoxonase activity of 100 nmol min^{-1} ml^{-1} in reaction mixtures of 200 μl containing 100 mM sodium phosphate buffer, pH 7.4, 3.3 mM $MgCl_2$, 1 M NaCl, 2 mM $CaCl_2$, NADPH-generating system and 5 mM reduced glutathione as described in **Supplementary Methods**. The rates of active metabolite formation from 2-oxo-clopidogrel were not different when the NADPH-generating system was omitted from the incubations (data not shown). (**b**) Saturation kinetics after inhibition of PON1 paraoxonase activity by addition of 1 mM EDTA to preincubation mixtures and omission of NaCl and $CaCl_2$[18]. (**c**) Saturation kinetics of active metabolite formation after inhibition of CYP2C19 by addition of 10 μM of the selective inhibitor NBPB ((-)-*N*-3-benzyl-phenobarbital))[19]. (**d**) Saturation kinetics of active metabolite formation after inhibition of CYP3A isozymes by addition of 100 μM atorvastatin[20]. Inhibitors were validated by demonstrating ≥90% suppression of metabolite formation from enzyme probe substrates paraoxon, mephenytoin and midazolam, respectively, using HEK 293 cell microsomes of PON1, CYP2C19, CYP3A4 and CYP3A5 (data not shown). Symbols and error bars represent arithmetic means and standard errors of three independent incubation experiments each.

Supplementary Figure 4.



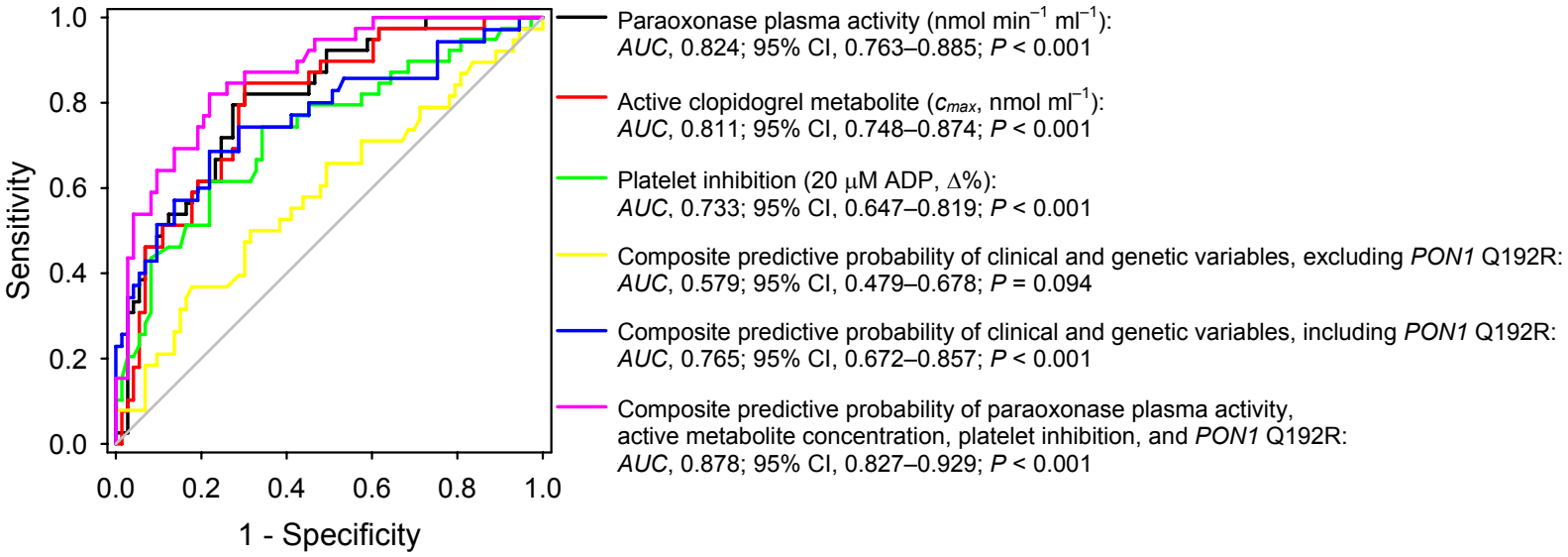
Supplementary Figure 4. Diagram of the case-cohort study design.
PCI denotes percutaneous coronary intervention.

Supplementary Figure 5.



Supplementary Figure 5. Haploview linkage disequilibrium plot of variant genotypes.
The linkage disequilibrium (LD) matrix map of polymorphic genotypes of *PON1*, *CYP2C9*, *CYP2C19*, *CYP3A4*, *CYP3A5* and *ABCB1* in case-cohort patients is shown.
The extent of LD between all possible pairs of single nucleotide polymorphisms was expressed in terms of the square of the coefficient of correlation. $r^2 \leq 0.05$ indicates the absence of significant LD among the investigated gene variants, i.e. the independence of each genetic effect. Distributions of the gene variants did not deviate from expected proportions predicted by the Hardy-Weinberg equilibrium both in cases and noncases. The allele frequencies in the random subcohort were in accordance with those reported for other white populations[21].
To evaluate whether the association of *PON1* with stent thrombosis could be confounded by potential population stratification or unobserved ancestral relationships between cases and noncases ('cryptic relatedness'), the *CYP* and *ABCB1* variants were used as unlinked genetic markers for estimation of the inflation factor λ (by taking the median of the observed Chi-square test statistics for independency of allelic distributions, divided by the median (0.456) of the ideal Chi-square test statistic)[22]. The computed value $\lambda < 1$ indicated the absence of population substructure and thus obviated the need for applying an inflation correction to the association tests between *PON1* and stent thrombosis.

Supplementary Figure 6.



Supplementary Figure 6. Receiver operating characteristic curves for predictor variables of stent thrombosis.

The nonparametric receiver operating characteristic (ROC) curves of diagnostic variables for discriminating non-fatal definite stent thrombosis in case-cohort patients are shown.

The predictive probabilities (plots of sensitivity (true positive fraction) against 1-specifity (true negative fraction) at all the possible cutoff values) for paraoxonase plasma activity, maximal plasma concentration of active clopidogrel metabolite and platelet inhibition (predose versus 6-h postdose aggregation) are displayed. The composite predictive probabilities for baseline demographic (sex, age, body-mass index, smoking) and clinical variables (indication of percutaneous coronary intervention, number of treated vessels, type of stent, clopidogrel loading dose, hypertension, hypercholesterolemia, diabetes mellitus, previous myocardial infarction or coronary artery disease, family history of coronary artery disease, left ventricular ejection fraction <45%), medication use (aspirin, statins) and variant genotypes of *CYP2C9*2* and **3*, *CYP2C19*2*, *CYP3A4*1B*, *CYP3A4*1G*, *CYP3A5*3* and *ABCB1* C3435T, either excluding or including *PON1* Q192R, were calculated by multivariate logistic regression analysis.

The diagnostic effectiveness was evaluated by the area under the ROC curve (*AUC*)[23]. The ROC *AUC* is the probability that a randomly selected noncase will have a higher value of the classifier than a randomly selected case. The discriminatory power in distinguishing between patients at low and high risk for stent thrombosis was slightly higher for paraoxonase activity and active metabolite concentration compared with platelet inhibition (P = 0.04 each for pairwise comparisons of *AUCs* according to the method of DeLong *et al.*[24]). The predictive effectiveness of the composite of demographic, clinical and genetic characteristics did not exceed the random threshold of 0.5 (diagonal line). Adding the *PON1* Q192R genotype to the analysis resulted in a significant increment of the *AUC*. The predictive effectiveness of the composite of *PON1* Q192R genotype, paraoxonase plasma activity, active metabolite concentration and platelet inhibition was not substantially higher compared with the single predictor variables (P = 0.08 for pairwise comparison with paraoxonase activity), indicating a strong interaction between *PON1* and activity variables.

Supplementary Methods

Bioanalytics of clopidogrel and its metabolites

We performed full validation of a high-performance liquid chromatography tandem mass spectrometry (LC-MS-MS) assay for simultaneous determination of clopidogrel, 2-oxo-clopidogrel, carboxylic acid metabolite and thiol metabolite in human plasma according to the 2001 FDA Guidance for Bioanalytical Method Validation[25] and the subsequent 2006 Bioanalytical Methods Validation Workshop white paper[26]. We obtained values for the essential validation parameters selectivity, sensitivity, accuracy, precision, reproducibility and stability. In addition, we determined validation parameters for matrix effects, extraction efficiency, calibration range and response function, positional differences within an analytical run, dilution integrity and carryover effects.

LC-MS-MS conditions

Analyses were carried out on an electron multiplier-modified (sensitivity-enhanced) triple quadrupole mass spectrometry instrument (TSQ Quantum, Thermo Fisher) with a Surveyor LC pump and a Surveyor autosampler (Thermo Fisher). The autosampler was set at 4 °C. 15 µl of the clear supernatants (plasma extracts) were injected onto a 5-µm Kromasil C-8 analytical column (100 mm x 3.0 mm) with a 5-µm Kromasil C-8 guard column (10 mm x 3.0 mm) (Thermo Fisher) maintained at 50 °C. The isocratic mobile phase consisted of acetonitrile and water (90:10, v v^{-1}) both containing 0.1% (v v^{-1}) formic acid. Flow rate was 300 µl min^{-1}. Run time was 4 min. The mass detector was operating in positive electrospray ionization mode (ESI+). Each analyte was identified by selected reaction monitoring (SRM) of at least four characteristic precursor ion $[M+H]^+$ → product ion transitions (including the ^{35}Cl and ^{37}Cl isotopes) as described[27]. Quantification of analytes was performed by monitoring the following $[M+H]^+$ → product ion transitions: clopidogrel (*m/z* 322 → 212 at 22 eV collision energy), 2-oxo-clopidogrel (*m/z* 338 → 183 at 25 eV), carboxylic acid metabolite (*m/z* 308 → 198 at 22 eV), thiol metabolite (*m/z* 356 → 183 at 25 eV), and internal standard (IS) 1-methyl-4-phenylpyridinium (*m/z* 170 → 128 at 36 eV). Scan width was set at 0.5 *m/z*, scan time at 0.2 ms and resolution at 0.7 Dalton FWHM on Q1 and Q3. Ion source spray voltage was 4,500 V. Nitrogen sheath and auxiliary gas pressure were 40 and 4 arbitrary units, respectively. Argon collision gas pressure was 1.0 mTorr, and source CID collision energy was 2 eV. Capillary temperature was 350 °C. LCquan version 2.0 software (Thermo Fisher) was used for peak processing.

Synthesis of primary reference standards

We derived clopidogrel base from clopidogrel besylate salt (Cimex Pharma AG). A solution of clopidogrel besylate (10 g , 20.83 mmol) in 200 ml saturated aqueous sodium bicarbonate (0.1 M, pH 8.3) was stirred for 20 h at 25 °C, extracted with ethyl acetate (2 x 100 ml), combined organic layers were washed with brine (saturated NaCl solution), dried over Na_2SO_4, filtered and concentrated under vacuum. Purification by flash chromatography (cyclohexane : ethyl acetate = 3:1, v v^{-1}) yielded the clopidogrel base (6.632 g, 20.60 mmol, 98.9%) as a yellow oil.

Carboxylic acid metabolite was derived from clopidogrel base. To a solution of clopidogrel base (100 mg, 0.312 mmol) in 3 ml ethanol was added dropwise 1.1 ml aqueous 1 N NaOH, stirred for 20 h at 25 °C, mixed with 10 ml bidest. H_2O, neutralized with aqueous 1 N HCl and extracted with ethyl acetate (2 x 10 ml). Combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated under vacuum. Purification by flash

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chromatography (cyclohexane : ethyl acetate = 2:1, v v^{-1}) and recrystallization in ethyl acetate yielded the product as colorless crystalline solid (88 mg, 0.286 mmol, 91.7%).

We derived 2-oxo-clopidogrel base from clopidogrel base on a gram scale by a newly developed synthesis procedure as described in detail elsewhere[28].

We derived thiol metabolite base from 2-oxo-clopidogrel base on a microgram scale by enzymatic conversion and subsequent purification by semipreparative LC-MS-MS. 2-Oxo-clopidogrel (86 µg, 0.280 µmol) was incubated with microsomes (1 mg ml^{-1} protein) prepared from PON-1 (R192)-transfected HEK 293 cells (see page S28) in 200 µl 10 mM Tris-HCl buffer pH 8 containing 1 M NaCl and 2 mM $CaCl_2$ for 10 min at 37 °C. The reaction was stopped by protein precipitation with 600 µl of –20 °C acetonitrile and the supernatant cleared by centrifugation at 16,100 *g* for 10 min at 4 °C. 50 µl of supernatants were in injected onto a 5-µm Kromasil C-8 HPLC column (100 mm x 3.0 mm) (Thermo Fisher). Isocratic elution was performed at 500 µl min^{-1} flow rate with 90% acetonitrile / 10% water (v v^{-1}) containing 0.1% (v v^{-1}) formic acid and clopidogrel metabolites were monitored by their selective MS-MS transitions. The preparative flow from 1.69–1.81 min containing the thiol metabolite peak (*m/z*: 338 → 183) was directed by a divert valve (Rheodyne 7750E-185) into a collection tube (0 °C), continuously evaporated under a nitrogen stream and recrystallizated in ethyl acetate to yield the product as colorless solid (11 µg, 0.031 µmol, 11%) that was kept under argon atmosphere at –80 °C. Purity of the thiol standard was >99% calculated by peak area-percentage analysis using LC-MS-MS. The ratio of the MS-MS peak of the thiol metabolite dissolved in methanol was determined versus the sum of all detected MS-MS peak areas from other clopidogrel metabolites corrected for the peaks present in the solvent blank (**Supplementary Figure 7**).

Accordingly, we derived the hydrolyzed methyl esters 2-oxo-clopidogrel-carboxylate and thiol metabolite-carboxylate from 2-oxo-clopidogrel base and thiol metabolite base by enzymatic conversion using microsome preparations of CES1-transfected HEK 293 cells and subsequent purification by semipreparative LC-MS-MS (purity of products >98%).

Bioactivity of the thiol metabolite

We confirmed pharmacological activity of the synthesized thiol metabolite by light transmission aggregometry as described on page S34. Native platelet-rich plasma obtained from five healthy volunteers was incubated with 2 mg l^{-1} of the thiol metabolite or buffer for 2 h at 37 °C. Platelet aggregation was initiated with 20 µM adenosine diphosphate. The absolute difference of the maximal platelet aggregation (mean (standard deviation)) on thiol versus buffer was 39.2 (7.5)%. This was in a similar range to the platelet inhibition measured in platelet-rich plasma from healthy volunteers after oral administration of a 600-mg clopidogrel dose (see page S34).

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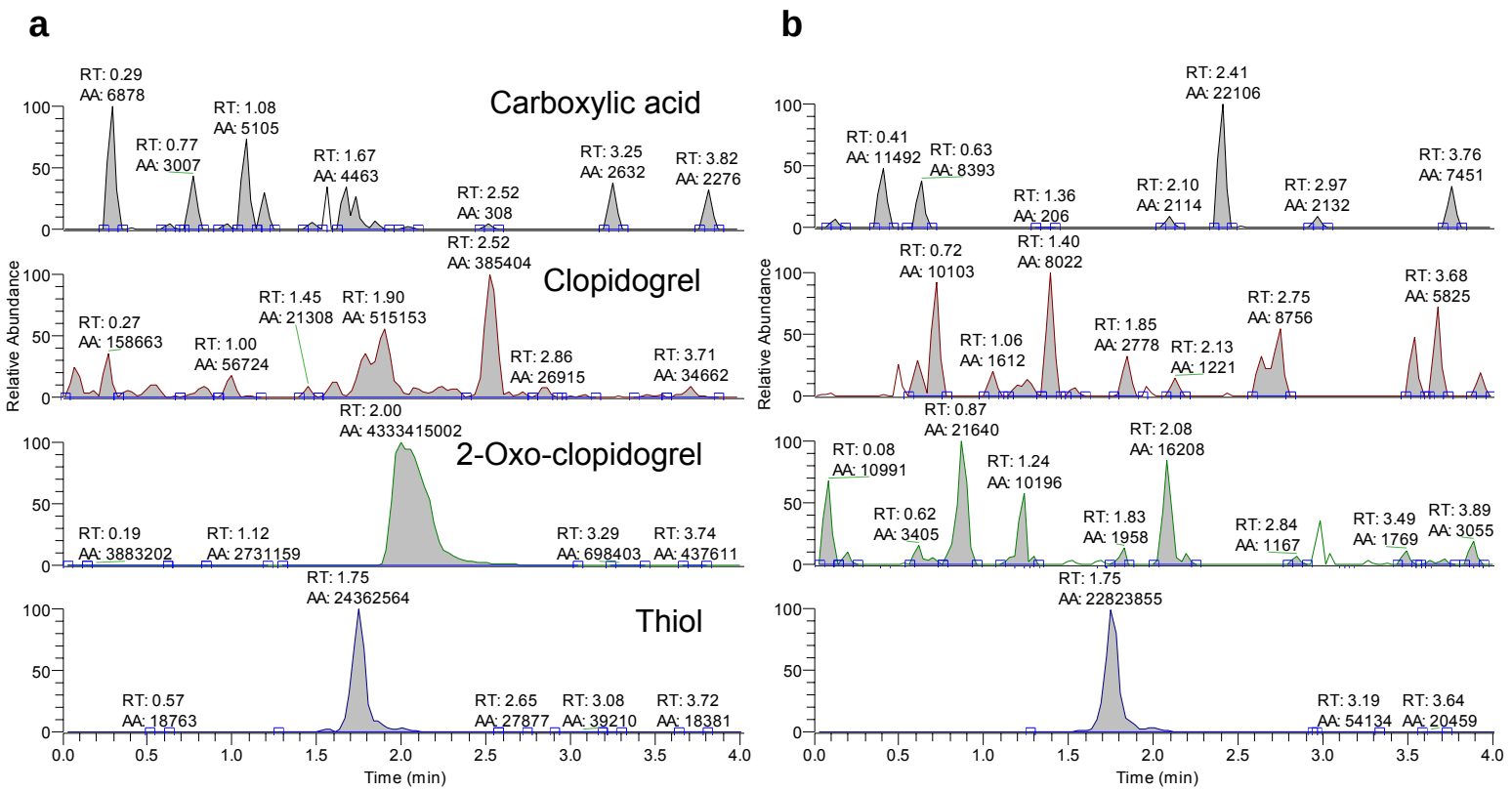

Supplementary Figure 7. Biosynthesis of the active clopidogrel metabolite.
(**a**) Original recording of the LC-MS-MS selective reaction monitoring (SRM) chromatogram of the reaction mixture of 2-oxo-clopidogrel treated with microsomal paraoxonase-1 (10 min, 37 °C). The traces of the MS-MS transitions for the carboxylic acid metabolite (*m/z*: 308 → 198, trace 1), parent clopidogrel (*m/z*: 322 → 212, trace 2), 2-oxo-clopidogrel (*m/z*: 338 →183, trace 3), and the thiol metabolite (*m/z*: 356 → 183, trace 4) are shown. **(b)** Original recording of the SRM chromatogram of the purified thiol metabolite in methanol.
The recordings demonstrate the absence of contaminations (<10 ppm) with parent clopidogrel or other clopidogrel metabolites in purified standards of thiol metabolite. No SRM peaks (with signal-to-noise ratio >3) were detected for the carboxylic acid metabolite (trace 1; retention time: 1.45–1.75 min) and parent clopidogrel (trace 2; retention time: 2.85–3.05 min); residual contamination with 2-oxo-clopidogrel (trace 3; retention time: 1.85–2.75 min) was <1 ppm.
AA denotes area under the SRM peak, RT retention time.

Bioanalytical method validation of clopidogrel and its metabolites

Calibration range, response function and sensitivity. We prepared 8–15 non-zero standards of the analytes in blank human EDTA plasma and analyzed these in duplicate in three analytical runs. SRM area ratios of analyte versus internal standard (1-methyl-4-phenylpyridinium, 50 ng ml^{-1}) were plotted against the nominal concentrations using linear regression. Deviations from linearity were assessed by the Mandel test. Deviations of calculated standard concentrations from nominal concentrations should be within ± 15% and coefficients of variation (CV) ≤15%, with at least two-third of the non-zero standards should meet these criteria. The lowest standard on the calibration curve should be accepted as lower limit of quantification (LLOQ) if the analyte response areas were ≥5 times the blank response areas, and deviations from nominal concentrations were within ± 20% with CV values ≤20%. The calibration curves were linear (without using a weighting factor) over the ranges 0.10–200 ng ml^{-1} for clopidogrel, 0.0005–1.5 ng ml^{-1} for 2-oxo-clopidogrel, 100–150,000 ng ml^{-1} for the carboxylic acid metabolite, and 0.10–50 ng ml^{-1} for the thiol metabolite. Pearson correlation coefficients *r* were ≥0.992. At all standard levels (including the LLOQ), deviations from nominal concentrations were between –7.4% and 6.8% and CV values ≤7.2%. No standard was outside the acceptance criteria.

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. We evaluated the selectivity of the method by analyzing six lots (extractions) of blank human EDTA plasma in comparison with analyte-spiked plasma at the LLOQ level and with IS-spiked plasma. The peak areas of the blanks at the retention time of analytes should be no more than 20% of the LLOQ response and no more than 5% of the IS response. There were no peak areas >1% observed in any of SRM chromatograms of the blank plasma samples at the retention times of the analytes and no peak areas >0.01% at the retention time of the internal standard.

Reproducibility of the method was investigated in terms of accuracy and precision of intra-assay runs, inter-assay runs and dilution integrity. For determination of inter- and intra-assay accuracy and precision, quality control (QC) samples at the LLOQ level and at low, medium and high levels of the calibration range were analyzed: 0.10 - 0.30 - 7 - 150 ng ml^{-1} of clopidogrel, 0.0005 - 0.0015 - 0.04 - 1.125 ng ml^{-1} of 2-oxo-clopidogrel, 100 - 300 – 6,000 – 11,2500 ng ml^{-1} of carboxylic acid metabolite, and 0.10 – 0.30 – 3 – 37.5 ng ml^{-1} of thiol metabolite. Accuracy (relative error) should be within ± 20% at the LLOQ and within ± 15% at the other QC levels. Precision (CV) should be ≤20% at the LLOQ and ≤15% at all other QC levels. At least two-third of the individual QC values should meet these criteria.. Assaying five replicates of each QC level in a single run from one calibration curve revealed an intra-run accuracy between –5.9% and 5.1% and an intra-run precision ≤5.2%. Analyzing five replicates of each QC concentration in five runs from five separate calibration curves revealed an inter-run accuracy between –7.3% and 8.1% and an inter-run precision ≤9.0%. The dilution integrity for analyzing above the upper limit of quantification (ALOQ) samples was investigated by 10-fold dilution of 500 ng ml^{-1} clopidogrel, 4 ng ml^{-1} 2-oxo-clopidogrel, 400,000 ng ml^{-1} carboxylic acid metabolite, and 125 ng ml^{-1} thiol metabolite with blank plasma. Assessment of five replicates of each ALOQ sample in a single run revealed an accuracy of –2.1% to 4.4% and a precision ≤3.7%. No QC value was outside the acceptance criteria.

Matrix effect is the suppression or enhancement of ionization of analytes by the presence of matrix components in the biological samples. We determined the IS-normalized matrix factor (*MF*), defined as the peak area ratio (PAR) of the analyte versus IS in the presence of matrix ions, divided to the PAR in the absence of matrix ions, as quantitative measure of matrix effect. Peak responses of acetonitrile extracts (2:1, v v^{-1}) of five individual lots of blank plasma spiked with

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analyte at low, middle and high QC level were compared with the solutions of QCs and IS in acetonitrile–water (2:1, v v^{-1}). Precision of IS-normalized *MF* should be ≤15%. Maximal CVs were 5.3% (at low QC), 3.3% (at middle QC), and 4.9% (at high QC), respectively, indicating robustness of the matrix ionization effects and effectiveness of the IS to compensate for matrix effects.

Recovery (extraction efficiency) was determined by comparing the peak areas of analytes and IS in acetonitrile extracts (2:1, v v^{-1}) from spiked plasma and whole EDTA blood samples with respective analyte and IS dilutions in acetonitrile extracts (2:1, v v^{-1}) of blank plasma samples, representing 100% recovery. Mean recoveries of five replicates of plasma samples spiked at low, middle and high QC levels with clopidogrel, 2-oxo-clopidogrel, carboxylic acid metabolite, and thiol metabolite and with IS were: 93.3%-92.7%-91.9%-93.1% (at low QC), 96.7%-97.1%-95.0%-95.9% (at middle QC), 95.8%-94.1%-96.7%-96.0% (at high QC), and 96.6% (IS, 50 ng ml^{-1}). Respective mean recoveries of five replicates of spiked whole blood samples were: 92.5%-93.3%-94.1%-92.0% (at low QC), 95.3%-98.0%-94.6%-93.5% (at middle QC), 94.8%-96.3%-94.2%-95.1% (at high QC), and 96.8% (IS, 50 ng ml^{-1}). Extraction efficiency was consistent, precise, and reproducible.

Positional differences and carryover. To determine if the sample position in the chromatographic run sequence had an influence on the observed response, we analyzed calibration standards and QC samples in a predefined order, placing one set of calibration standards at the beginning and one at the end of the run, and the QC samples in the middle of the run. To determine carryover effects we evaluated the peak area response in a blank sample placed after the highest calibration standard in five replicates. The analyte response in this sample should be less than 20% of that of the respective LLOQ sample. No changes in response over the course of the run were observed. The analyte carryover was less than 12.8% of the LLOQ.

Stability tests

Stock solution stability. Stock solutions of analytes were prepared in methanol at concentrations of 1-1-10-0.01 mg ml^{-1} (clopidogrel - 2-oxo-clopidogrel - carboxylic acid metabolite - thiol metabolite) and diluted with water to obtain working solutions of 10-1-10,000-10 ng ml^{-1} for analysis. Mean peak areas derived of working solutions from freshly made stock solutions were compared in five replicates with mean peak areas derived from stock solutions kept for 6 h at –20 °C. The difference in responses (%RE) should be less than 7%. Observed differences were –2.3% - –4.7% - 3.0% - 0.6%.

Long-term storage stability. Long-term stability assessment evaluates the analyte stability in the test matrix covering the length of time from sample collection to sample analysis. Five aliquots of QC samples at the low and high concentration level in blank EDTA plasma were stored for 12 months at –20 °C and analyzed with freshly prepared standard curves. The mean of the observed concentrations should be within ±7% (%RE) of the nominal concentrations and the precision (% CV) should be ≤15%. Accuracy was –4.6% - –4.7% - 5.1% - –3.6% at the low QC levels and –3.2% - 3.9% - 1.1% - 2.8% at the high QC levels. Precision was ≤6.1%.

For assessment of storage stability over very long time, we obtained from patients who underwent PCI five aliquots of 20 plasma samples at 0.5, 2, and 6 h after administration of a loading dose of 600 mg clopidogrel. Samples were stored for 72 months at –20 °C and compared with five historical replicates of the freshly processed samples at day 0. Absolute differences of mean observed analyte concentrations in each of the 20 samples between initial determinations and determinations after 72 months were less than 8.7%, CVs were less than 10.3%.

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Short-term temperature stability. Short-term stability is evaluated to confirm that analyte degradation does not occur during the preparation of samples prior to their analysis. Five aliquots of QC samples at the low and high concentration levels in blank EDTA plasma stored at –20 °C were thawed at room temperature (22 °C) and kept at this temperature for 6 h. At the end of this period, a second set of five aliquots of stability samples stored at –20 °C was thawed at room temperature (22 °C). Immediately after thawing of the second set, both sets of stability samples were extracted and analyzed against freshly prepared calibration standards. Stability was indicated if the absolute differences in the mean calculated concentrations of set 1 versus set 2 and of set 1 versus the nominal values were ≤15%. The observed differences of set 1 versus set 2 were –2.3% - –76.4% - 1.1% - –1.6% (at low QC) and 1.8% - –73.1% - –0.7% - 1.2% (at high QC). The differences of set 1 to the nominal values were –2.7% - –77.8% - 0.6% - –2.0% (at low QC), and –1.0% - –72.6% - 2.4% - –3.1% (at high QC). This demonstrates that 2-oxo-clopidogrel was highly unstable in plasma at room temperature.

Freeze-thaw stability. Five aliquots of stability samples in blank EDTA plasma each at the low and high QC level were subjected to three freeze-thaw cycles. The first cycle consisted of the storage of samples at –20 °C for 24 h, thawing at room temperature (22 °C) and immediate refreezing at –20 °C, followed by two 12-h cycles under the same temperature conditions. These samples were analyzed together with a set of five aliquots of stability samples subjected only to one 24-h freeze-thaw cycle. The differences of the mean calculated concentrations from the nominal concentrations should be within ± 15% for both sets of samples, and the precision (CV, %) should be ≤15%. Differences to the nominal concentrations for the 3-cycle set were 3.9% - –10.2% - –3.1% - 2.5% (at low QC) and –2.4% - –8.7% - –3.0% - –2.5% (at high QC), and for the 1-cycle set 2.6% - –7.6% - 4.2% - –4.0% (at low QC) and –1.1% - –6.3% - 1.7% - 3.2% (at high QC). Precision was ≤5.3%.

Post-preparative stability. The stability of ready-to-inject samples on the instrument is assessed to determine whether re-analysis of processed samples is possible in the event that their initial analysis is interrupted. Following the initial analysis of five replicates of processed QC samples at the low, middle and high concentration level with calibration standards, samples were kept on the autosampler at 4 °C and re-analyzed after 72 h. Mean concentrations of re-analyzed stability samples were calculated using both the standard curve of the initial analysis and the standard curve of the re-analysis. Absolute differences between both values should be ≤7%. Observed differences were –3.0% - –4.1% - 2.7% - –5.0% (at low QC), –1.2% - –2.7% - 0.8% - –3.2% (at middle QC), and –2.8% - –2.0% - –0.5% - –4.3% (at high QC) for clopidogrel, 2-oxo-clopidogrel, carboxylic acid metabolite, and thiol metabolite, respectively. This indicates that processed (protein-precipitated) plasma samples of 2-oxo-clopidogrel were stable at 4 °C.

Pre-extraction stability. 2-Oxo-clopidogrel was unstable in plasma but stable in blank buffer and protein-precipitated plasma extracts. The thiol metabolite was unstable in plasma and buffer solution, but did not degrade in tubes coated with patented stabilizer (patent no. DE 10 2004 046 159.7). At low temperature the half-life of both metabolites substantially increased. Degradation after 60 min storage at 4 °C was less than 7% (**Supplementary Figure 8**).

To evaluate analyte stability within the time period from blood collection to plasma preparation, we compared analyte concentrations in samples obtained from stabilized blood collection tubes with samples from non-stabilized collection tubes, with both tubes continuously kept at 0–4 °C during the preparation process. 4 ml each of venous blood from five clopidogrel-naïve, 12-h fasting individuals was aspirated into standard potassium EDTA Sarstedt S-Monovette collection tubes and into the same tubes coated with patented stabilizer. Blood was spiked with analytes at the low and high QC level. Monovette tubes were stored on ice for 30 min and plasma was obtained by centrifugation at 1,500 *g* for 15 min at 4 °C. Plasma from coated

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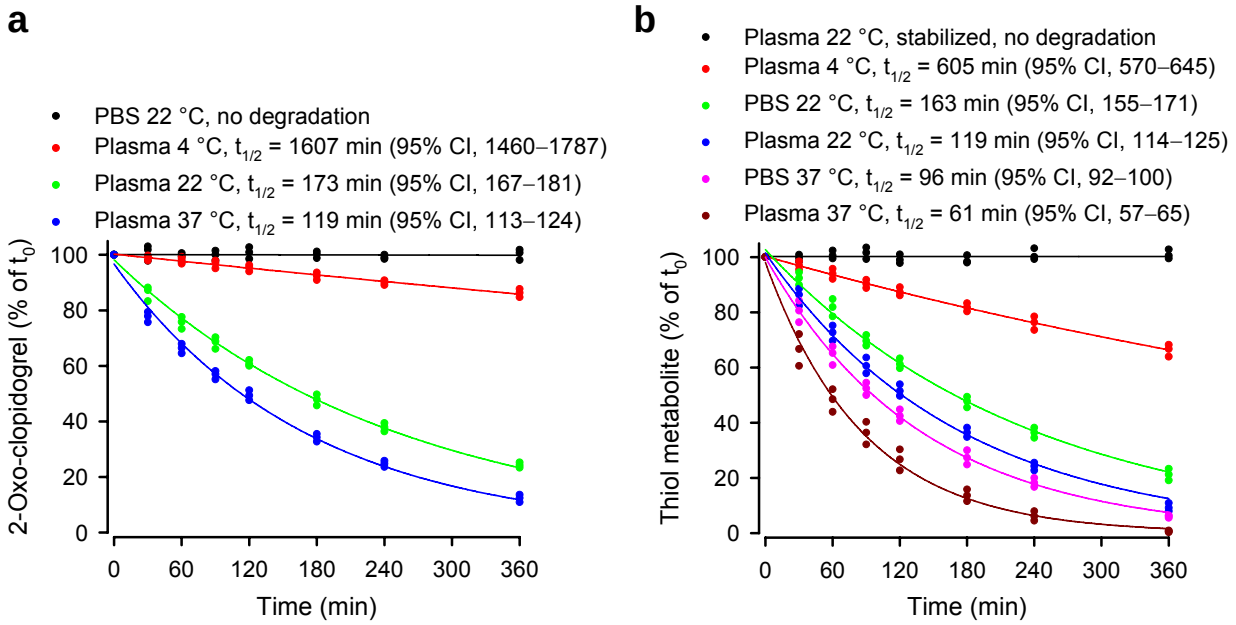
Monovettes was aliquoted into standard propylene tubes, and plasma derived from non-coated Monovettes was aliquoted into 1.5 ml propylene tubes with stabilizer coating. Both sets of extracted plasma samples were analyzed in triplicate for analyte concentrations. The differences of the mean observed concentrations between both sets of samples and to the nominal concentrations should be within ± 7% and the precision should be ≤15%. Differences in mean observed concentrations of set 1 versus set 2 were –1.5% - –1.8% - 1.1% - –2.3% (at low QC levels) and 0.6% - –1.2% - –2.0% - –1.5% (at high QC levels) for clopidogrel - 2-oxo-clopidogrel - carboxylic acid metabolite - thiol metabolite. Differences of set 1 to nominal concentrations were 0.9% - –2.6% - –1.0% - 0.3% (at low QC levels) and –1.5% - –3.1% - –1.0% - –3.3% (at high QC levels), and differences of set 2 to nominal concentrations were 2.4% - –0.8% - –2.1% - 2.6% (at low QC levels) and –2.1% - –1.9% - 3.0% - –1.8% (at high QC levels). Precision was ≤5.2%. These results show that no degradation of analytes occurred during the preparation process.



Supplementary Figure 8. Stability of clopidogrel metabolites.
(**a,b**) Stability of 2-oxo-clopidogrel (**a**) and of thiol metabolite (**b**) in phosphate-buffered saline (PBS, pH 7.40) or human EDTA plasma at different temperatures.
The half-lifes with 95% confidence intervals ($t_{1/2}$, 95% CI) of the metabolites were derived from fits of individual data at 0, 30, 60, 90, 120, 180, 240, and 360 min (n = 3) using a one phase exponential decay function.

In-vitro assessment of clopidogrel metabolism (metabolomic profiling)

Cell culture and expression cloning

HEK 293 cells generated by transformation of human embryonic kidney cell cultures with sheared adenovirus 5 DNA (CRL-1573, ATCC) were grown in monolayer culture in an atmosphere of 95% humidified air with 5% CO_2 at 37 °C, using Dulbecco's modified Eagle's medium (GIBCO, Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories). Cells (1 x 10^6) were transfected with the mammalian expression vector pDream2.1/MCS (10 μg), carrying human full-length ORF (open reading frame) cDNA of selected enzymes (GenScript) by the use of Tfx-50 reagent (Promega) according to the manufacturer's instructions. Stable transfectants were obtained by maintenance of cells in selective medium, containing G-418 (Invitrogen) at a concentration of 750 μg ml^{-1} for 3 weeks. Cells transfected with empty pDream2.1/MCS vector served as control. We tested the mRNA expression of enzymes in stably transfected HEK 293 cell clones by quantitative real-time PCR relative to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using TaqMan gene expression probes (Applied Biosystems) as described[29]. Relative quantifications were performed by pair-wise fixed reallocation randomization test[30] and corrected for amplification efficiency as evaluated from standard curves generated from serial dilutions of respective cDNAs in pDream2.1/MCS vector. Intra-assay coefficients of variation determined in three repeats of four different copy numbers of plasmids were <15%, and inter-assay coefficients of variation determined on 4 days with four different plasmid copy numbers were <25%. Single nucleotide polymorphisms (SNPs) were introduced in cDNAs with the QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. We generated expression clones of the following cDNAs (NCBI reference sequence accession number): CYP1A1 (NM_000499), CYP1A2 (NM_000761), CYP1B1 (NM_000104), CYP2A6 (NM_000762), CYP2B6 (NM_000767), CYP2C8 (NM_000770), CYP2C9 (NM_000771; rs1057910 SNP), CYP2C19 (NM_000769; rs4244285 SNP, 40-base pair deletion cDNA fragment), CYP2D6 (NM_000106), CYP2E1 (NM_000773), CYP2J2 (NM_000775), CYP3A4 (NM_017460), CYP3A5 (NM_000777), CYP3A7 (NM_000765), CYP4A11 (NM_000778), CYP4F2 (NM_001082), CYP4F3 (NM_000896), CYP4F12 (NM_023944), PON1 (NM_000446; rs662 and rs854560 SNPs), PON2 (NM_000305), PON3 (NM_000940), CES1 (NM_001266), CES2 (NM_003869), AChE (NM_015831), BChE (NM_000055), LPL (NM_000237), PL (NM_000936), HL (NM_000236), EL (NM_006033), CEL (NM_001807), AADAC (NM_001086). For preparation of microsomes and metabolism assays only HEK 293 cell clones with less than 30% difference in GAPDH-normalized expression with respect to the PON1-transfected clone were used. Each clone was checked for the absence of concomitant expression of any other of the investigated CYPs or esterases by quantitative real-time PCR. A crossing-point above 45 was considered to indicate lack of expression.

We used HEK 293 cells for heterologous functional and stable expression of metabolizing enzymes, because they showed a negligible endogenous expression of CYP P450 isozymes and esterases compared with higher differentiated human cell lines (particularly of hepatic (e.g. HepG2) or intestinal origin (e.g. Caco-2)). Specifically, expression of mammalian enzymes in the micro-environment of a human cell line has advantages over the widely used prokaryotic or lower eukaryotic expression systems (e.g. insect or yeast cells), allowing authentic post-translational processing, correct folding, stabilization by endogenous binding partners and specific localization to membranes and intracellular organelles. Compared with HEK 293 cell microsomes, enzymes expressed in non-mammalian microsomes or purified recombinant enzymes may show

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significantly altered enzyme kinetics[31]. Moreover, to achieve a high and constant enzyme expression, stable HEK 293 cell lines were established.

Microsome preparation

We prepared crude microsomes by adopting a previously described method[32]. Briefly, stably transfected HEK 293 cells were harvested from five 750-cm^2 culture flasks, washed three times in PBS, and swollen for 20 min at 4 °C in 10 ml of hypotonic lysis buffer (10 mM Tris-HCl, pH 7.4, supplemented with a protease inhibitor mixture containing 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 1 μM leupeptin and 1 μg ml^{-1} of aprotinin). Cells were homogenized in an all-glass Dounce grinder on ice. The homogenate was diluted to 20 ml in lysis buffer, 50% (w v^{-1}) sucrose was added to a final concentration of 10% (w v^{-1}), and nuclei were removed by centrifugation at 500 *g* for 5 min at 4 °C. The supernatant was recentrifugated at 2,000 *g* for 5 min at 4 °C. To the remaining supernatant 1 M KCl and 1 M HEPES-NaOH buffer, pH 7.4, was added to a final concentration of 50 mM KCl and 10 mM HEPES, and crude microsomes were collected by sedimentation at 100,000 *g* for 60 min at 4 °C. The microsomal pellets were resuspended in 100 μl of 7% (w v^{-1}) sucrose, 25 mM KCl, 1 mM $MgCl_2$, 15 mM HEPES-NaOH, pH 7.4, at a concentration of 12,000 μg protein ml^{-1} and frozen in liquid nitrogen until use. The protein concentration was determined by the bicinchoninic acid (BCA) method (Pierce) according to the manufacturer's instructions.

Cytochrome P450 assays

We incubated microsomal membrane fractions of cytochrome P450 isozymes (1,000 μg protein ml^{-1}) in mixtures of 100 mM sodium phosphate buffer, pH 7.4, 3.3 mM $MgCl_2$, 2 mM NADPH and 5 mM reduced glutathione at eight different substrate concentrations in a final volume of 200 μl. Samples were preincubated for 2 min at 37 °C. Reactions were started by the addition of microsomes (10 μl), supplemented with 20 pmol recombinant human NADPH-cytochrome P450 reductase and 20 pmol recombinant human cytochrome b_5 (Sigma). Reactions were terminated after incubation for 5 min at 37 °C by addition of 600 μl acetonitrile containing internal standard. Protein-precipitated samples were centrifugated at 16,100 *g* for 3 min at 4 °C, and supernatants were subjected to LC-MS-MS analysis.

For determination of the metabolic activity of each CYP isozyme, the following probe reactions (positive controls) were established and metabolite formation was quantified by running ESI+ SRM and ESI– SRM analyses: CYP1A1 and CYP1A2: phenacetin → acetaminophen, *m/z*: 152 → 110 at 20 eV (ESI+), CYP1B1: melatonin → 6-hydroxy-melatonin[33], *m/z*: 249 → 190 at 25 eV (ESI+), CYP2A6, coumarin → 7-hydroxy-coumarin, *m/z*: 161 → 133 at 25 eV (ESI–), CYP2B6: bupropion → hydroxybupropion, *m/z*: 256 → 139 at 30 eV (ESI+), CYP2C8: amodiaquine → desethyl-amodiaquine, *m/z*: 328 → 283 at 25 eV (ESI+), CYP2C9: tolbutamide → 4-hydroxy-tolbutamide, *m/z*: 287 → 171 at 20 eV (ESI+), CYP2C19: (*S*)-mephenytoin → 4'-hydroxy-mephenytoin, *m/z*: 235 → 150 at 20 eV (ESI+), CYP2D6: dextromethorphan → dextrorphan, *m/z*: 258 → 157 at 35 eV (ESI+), CYP2E1: chlorzoxazone → 6-hydroxy-chlorzoxazone, *m/z*: 184 → 120 at 20 eV (ESI–), CYP2J2 and CYP4F12: ebastine → hydroxy-ebastine[34,35], *m/z*: 486.3 → 167 at 38 eV (ESI+), CYP3A4, CYP3A5 and CYP3A7: midazolam → 1'-hydroxy-midazolam, *m/z*: 342 → 324 at 25 eV (ESI+), CYP4A11: lauric acid → 12-hydoxy-lauric acid[36], *m/z*: 215 → 169 at 25 eV (ESI–), CYP4F2 and CYP4F3: pafuramidine → *O*-demethyl-pafuramidine[37], *m/z*: 351 → 320 at 25 eV (ESI+). Internal standard for monitoring ESI+ transitions was 1-methyl-4-phenylpyridinium (50 ng ml^{-1} final concentration, *m/z*: 170 → 128 at 36 eV) and for monitoring ESI– transitions ethylgallate (100 ng ml^{-1} final concentration, *m/z*: 197 → 169 at 25 eV). In ESI+ mode MS-MS analyses were performed with spray voltage set

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at 4,500 V, capillary temperature at 350 °C, nitrogen sheath gas at 40 and nitrogen auxiliary gas at 4 arbitrary units, and argon collision gas at 1.5 mTorr. In ESI– mode analyses were performed with spray voltage set at 3,500 V, capillary temperature at 400 °C, nitrogen sheath and auxiliary gas at 50 and 2 arbitrary units, argon collision gas at 1.5 mTorr. Chromatographic separations were performed on a 5-μm Aquasil C-18 (100 x 3.0 mm) HPLC column (Thermo Fisher). For elution of analytes isocratic mobile phases were used consisting of water / 0.1% (v v^{-1}) formic acid (A) and acetonitrile / 0.1% (v v^{-1}) formic acid (B) at binary compositions comprising 60–95% (v v^{-1}) of solvent B.

Possible baseline formation of metabolites was monitored by incubation of substrates with microsome preparations obtained from empty vector-transfected HEK 293 cells. In no case baseline corrections were required. Each incubation experiment was performed in triplicate.

Esterase assays

We incubated microsomal fractions of PON1, PON2 and PON3 enzymes in 10 mM Tris-HCl buffer, pH 8.0, 1 M NaCl, and 2 mM $CaCl_2$. Microsomes of CES1 and CES2 were incubated in 20 mM Tris-HCl buffer, pH 8.0. Microsomes expressing triacylglycerol lipases (LPL, PL, HL, EL) were incubated in 20 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, and 0.01% (v v^{-1}) Triton-X100. Microsomes of AChE and BChE were incubated in 100 mM potassium phosphate buffer, pH 8.0. Microsomes of AADAC were incubated in 100 mM potassium phosphate buffer, pH 7.25, and 150 mM NaCl. Microsomes of CEL were incubated in 100 mM sodium phosphate buffer, pH 7.0, 100 mM NaCl, and 6 mM sodium taurocholate.

All assays were performed at 8–11 different concentrations of clopidogrel substrates and at a microsome concentration of 1,000 μg protein ml^{-1} in a final volume of 200 μl. After preincubation for 2 min at 37 °C reactions were started by addition of microsomes (10 μl). After incubation for 5 min at 37 °C reactions were terminated with 600 μl acetonitrile containing internal standard (1-methyl-4-phenylpyridinium, 50 ng ml^{-1} final concentration). Protein-precipitated samples were centrifugated at 16,100 *g* for 3 min at 4 °C and supernatants were subjected to LC-MS-MS analysis. The ESI+ SRM transitions of *m/z* 324 → 198 and *m/z* 342 → 198 at 22 eV collision energy were used to quantify the carboxylic acid derivates formed by hydrolysis of the methyl ester group of 2-oxo-clopidogrel and the thiol metabolite, respectively. No hydrolysis of clopidogrel substrates was determined when control incubations with microsomal preparations of empty vector-transfected HEK 293 cells were performed.

Probe reactions (positive controls) for measurement of PON1, PON2 and PON3 enzyme activities were conducted: (a) with paraoxon in 10 mM Tris-HCl buffer, pH 8.0, 1 M NaCl, and 2 mM $CaCl_2$ and spectrophotometric detection of *p*-nitrophenol formation at 405 nm, and (b) with phenylacetate in 9 mM Tris-HCl, pH 8.0, and 0.9 mM $CaCl_2$ and spectrophotometric detection of phenol formation at 270 nm. For probing CES1 and CES2 activities, initial rates of *p*-nitrophenol production from *p*-nitrophenyl acetate were measured at 405 nm. Activity of cholinesterases was determined by a modified Ellman method[38]. Microsomal fractions of AChE and BChE were incubated in mixtures of 100 mM potassium phosphate buffer, pH 8.0, 0.3 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) using acetylthiocholine iodide or butyrylthiocholine iodide as substrates. Formation of 5-thio-2-nitrobenzoate from DTNB was measured at 405 nm.

Activities of triacylglycerol lipases (LPL, PL, HL, EL) were determined according to the method of Lehner & Verger[39] using *p*-nitrophenyl laurate as substrate. Activity of carboxyl ester lipase (CEL) was evaluated according to the method of Pietsch & Gütschow[40] with *p*-nitrophenyl butyrate as substrate. AADAC activity was determined by the procedure described by McLean *et al*.[41] using *p*-nitrophenyl acetate as substrate. All probe assays were performed at 8–11 different

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concentrations of substrate in a final volume of 200 µl. After preincubation for 2 min at 37 °C reactions were started by addition of substrate and initial rates of hydrolysis were determined by recording the linear increase in absorbance of the metabolite over 5 min incubation at 37 °C. Concentrations were obtained from spectrophotometric calibration curves with primary standards of *p*-nitrophenol, 5-thio-2-nitrobenzoate and phenol, respectively. Initial hydrolytic activity was measured under reaction conditions in which less than 25% of the substrate was metabolized. The enzyme-specific hydrolysis rate was determined by correcting the crude hydrolysis rate for the rate obtained with microsomal preparations of empty vector-transfected HEK 293 cells. In all spectrophotometric assays specific hydrolysis rates were >95% of the crude hydrolysis rates.

Analysis of enzyme kinetics

Initial rates of metabolite formation versus substrate concentration revealed saturation kinetics, and individual data were fitted by the Michaelis-Menten equation using SigmaPlot 11.0 software: $V_0 = V_{max} \cdot [S] / (K_m + [S])$, where V_0 and V_{max} denote initial and maximal conversion velocity (nmol (mg protein)$^{-1}$ min^{-1}), respectively, [S] initial substrate concentration (µmol l^{-1}), K_m substrate concentration at half maximal conversion velocity (µmol l^{-1}), and V_{max} / K_m enzyme efficiency. K_m and V_{max} values were expressed as arithmetic means and standard error. The Michaelis-Menten equation was selected as the best fitting model for all analytes ($r \geq 0.95$).

Human studies

Study population

Eligible study participants were between 18 and 80 years old unrelated white, male and female patients with coronary artery disease (CAD) and a clinical presentation of stable angina pectoris or acute coronary syndrome, who had undergone PCI with stent implantation. Inclusion criteria were successful stent placement defined as a residual stenosis of 30% or less within the stented segment, TIMI flow grade 3, no evidence of residual thrombus or dissection; guideline-conform administration of clopidogrel before PCI[42], either as a single loading dose of 300 or 600 mg within 24 h before intervention or as 75 mg per day on at least 5 d before intervention; a daily maintenance dose of 75 mg clopidogrel for 6–12 months after PCI; daily 80–100 mg aspirin continued indefinitely (unless contraindicated). Exclusion criteria were previous stent thrombosis, active bleeding and bleeding diathesis, platelet count <150 x 10^9 l^{-1}, severe renal or hepatic disorder, hematologic disorder, chronic inflammatory or autoimmune disorder, active malignancy, a body-mass index below 18.5 or above 40 kg m^{-2}, use of vitamin or mineral supplements, hormone replacement therapy or use of contraceptives, premature clopidogrel or aspirin cessation or nonadherence. We identified eligible participants in a population of patients who were admitted to coronary care units or cardiac catheterization laboratories for PCI between June 2003 and December 2007. The disease status was classified by investigators blind to the genetic information of patients.

Classification of European ancestry was based on self-reports. Ethnicity was not ascertained by ancestry informative markers. However, we recruited the entire patient cohort among the white population of the Netherlands and Northwest Germany, representing a homogenous group with low genetic variability according to genome-wide association studies[43,44]. This excluded a potential bias from population stratification that could have been observed in multiethnic or admixed populations due to a marked interethnic variance in distribution of the assessed genotypes[45,46] and in event rates of stent thrombosis[47,48]. Specifically, the *PON1* Q192 allele is more common in European and white U.S. populations (allele

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frequencies 60–75%), whereas the R192 allele is more common in Asian and African American populations (allele frequency around 60%)[46,49]. However, the *CYP* and *ABCB1* gene variants evaluated in this study represented a panel of markers that were unlinked to *PON1* and not related to stent thrombosis, and their distributions were similar to those in other populations of European descent[21], which further indicated the absence of population stratification (**Supplementary Figure 5**). Moreover, it has been suggested that even ignoring ethnicity would not affect the validity of genetic associations in multiethnic populations; under the premise of common gene variants and no extreme interethnic variations of disease rates, population stratification was estimated to bias relative risk measures by less than 10%[50].

Study design

We conducted a case-cohort study[51] in surviving incident cases with definite stent thrombosis and surviving patients without stent thrombosis, who accrued in a cohort of 7719 eligible patients over a follow-up period of 18 months after the index PCI. We identified 93 patients with definite stent thrombosis adjudicated according to the Academic Research Consortium definition[52]. The definition of definite stent thrombosis required angiographic confirmation of a thrombus that originated in the stent or in the segment 5 mm distal or proximal to the stent and the presence of at least one additional clinical criterion within 48 hours: acute onset of ischemic symptoms at rest, new ischemic electrocardiographic changes that suggest acute ischemia, ischemic cardiac biomarkers, occlusive or nonocclusive thrombus. Assessments were performed independently by two investigators. All cohort patients were followed by scheduled clinical visits at 6 weeks, 3, 6, 12, and 18 months and on the basis of emergency hospital admissions during the 18-months follow up. Patients with clinical signs of suspected stent thrombosis (sudden onset of chest pain persisting for more than 15 min; ST segment elevation or depression of at least 1 mm if present in at least 2 contiguous leads in the distribution of the target vessel; rise of the levels of troponin or creatine kinase-MB to >3 times the upper limit of normal) underwent coronary angiography to confirm the diagnosis followed by recanalization of the occluded target vessel by PCI or coronary artery bypass grafting in the case of unsuccessful recanalization. Antiplatelet therapy with clopidogrel was continued after the revascularization procedure for the scheduled duration of 6–12 months. If the stent thrombosis occurred after regular cessation of clopidogrel use or within the last month before the planned cessation, patients received an additional course of clopidogrel therapy for at least 1 month after implantation of a bare metal stent or revascularization without stent implantation and for at least 3 months after implantation of a drug-eluting stent. The total duration of clopidogrel treatment during the 18-months follow-up did not exceed 12 months. None of the patients with a definite first stent thrombosis suffered from a definite recurrent non-fatal stent thrombosis event until the end of follow-up.

We selected a subcohort of 140 patients by non-stratified randomization. Every member entering the open reference cohort was assigned a computer-generated random number u uniformly distributed on the interval (0,1) with the subcohort designated by those with u below the sampling fraction[1]. We preferred this case-cohort design over a traditional case-control study, because it was less subject to potential biases, particularly by accounting for the dynamic nature of the source population and by random selection of control patients yielding a sample representative for the total cohort. The case-cohort design is an unmatched variant of the nested case-control design in which controls are selected by matching on time. The statistical efficiency of the case-cohort design is not lower compared to the nested case-control design, but it is more flexible due to the complete random selection of controls[53,54].

During follow-up 22 patients died, 8 discontinued clopidogrel treatment prematurely or were nonadherent, 11 were lost to follow-up for unknown reason and 79 were not willing to

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participate. Finally, 41 cases and 71 noncases were enrolled (**Supplementary Figure 4**). The study protocol was approved by the institutional reviewing boards (clinical trial registration, clinicaltrials.gov no. NCT01012544). All participants gave written informed consent.

Primary outcome measure was an association of the *PON1* Q192R gene variant or phenotypic paraoxonase activity with incident definite non-fatal stent thrombosis. Secondary outcome measures were associations of clopidogrel metabolite concentrations and platelet response with stent thrombosis and relationships of *PON1* and paraoxonase activity with pharmacokinetic and pharmacodynamic response to clopidogrel. We focused on definite in-stent thrombosis rather than probable or possible stent thrombosis or overall cardiovascular events as primary outcome to avoid possible genetic or pathophysiological heterogeneity in disease outcomes, difficulties in interpreting composite outcomes, misclassification or consideration of outcomes with no direct pathophysiological link to platelet aggregation that could have diluted the true association.

Patients were monitored by standardized interview, physical examination and laboratory testing at hospital discharge and at 6 weeks, 3, 6, 12, and 18 months.

After the 18-months follow-up with completion of the protocol-scheduled 6–12 months course of clopidogrel therapy after the index PCI we invited the clopidogrel-free study patients to our outpatient clinic. All subjects received a single 600-mg loading dose of clopidogrel to assess pharmacokinetics and platelet inhibition. Patients were excluded, if they had suffered from an ischemic or hemorrhagic cardiovascular condition after the end of follow-up, had acute infection, or had used any antiplatelet drug within the preceding 4 weeks (except for aspirin).

Median time from index PCI to the clopidogrel response study was 609 d (interquartil range (i.q.r.), 547–702) in stent thrombosis survivors and 607 d (i.q.r., 575–638) in control patients ($P = 0.975$, Mann-Whitney test). Median time from stent thrombosis to the response test was 585 d (i.q.r., 516–640; range, 274–835). None of the predefined conditions necessitating exclusion of a patient occurred between the end of follow-up and the clopidogrel response test.

A prospective assessment of pharmacokinetic and pharmacodynamic responses to clopidogrel was not feasible due to the rare incidence of definite stent thrombosis (<1% per year).

ADP-stimulated platelet responsiveness within an individual was reported to be genetically conserved and thus highly consistent over time[55]. However, platelet reactivity was demonstrated to be generally increased after coronary stent implantation for at least several days[56,57], and patients suffering from subacute stent thrombosis showed elevated post-event platelet reactivity over more than 6 months[58]. To prevent that procedural variables or acute ischemic or inflammatory conditions may confound the assessment of the genetic factors influencing platelet aggregation, we performed the clopidogrel response test at the earliest 9 months after the stent thrombosis event and 18 months after the index PCI.

Clinical laboratory methods

We performed all standard clinical laboratory measurements at a central laboratory. To prevent ascertainment bias all laboratory analyses, genotyping and data management were done by staff that was blinded to patients' status and outcome.

Blood collection and sample preparation

We drew samples of peripheral venous blood after a 12-h overnight fast with a loose tourniquet through a catheter inserted into the median antecubital vein of the left forearm.

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For the assessment of clopidogrel pharmacokinetics, blood samples were aspirated into 4.9 ml potassium EDTA tubes (final concentration of 1.2–2 mg EDTA per ml blood) (Sarstedt S-Monovette) immediately before and at 20, 30, 40, 60, 90, 120, 180, 240, and 360 min after oral administration of a single 600-mg clopidogrel dose (8 Iscover tablets, Bristol-Myers Squibb) with 200 ml water. Plasma was obtained by immediate centrifugation at 1,500 *g* for 10 min at 4 °C, aliquoted into 1.5 ml propylene tubes with patented stabilizer coating (patent no. DE 10 2004 046 159.7) and stored at –80 °C until analysis. Frozen plasma samples were thawed at 4 °C, spiked with internal standard, protein precipitated with acetonitrile (2:1, v v^{-1}) and centrifugated at 16,100 *g* for 30 min at 4 °C.

For optical aggregometry, after the first 2 ml were discarded blood samples were collected in 2.9 ml sodium citrate tubes (final concentration of 31.3 mg citrate per ml blood) (Sarstedt S-Monovette) immediately before and 360 min after administration of the clopidogrel and processed within 1–2 h.

Pharmacokinetic analysis

We analyzed the individual plasma concentration versus time data of clopidogrel, 2-oxo-clopidogrel, the thiol metabolite and the carboxylic acid metabolite by a compartmental model using WinNonlin version 4.1 software (Pharsight Corporation). To determine which compartmental model best fitted the data, we used the log likelihood function with Akaike's information criterion. The parameters characterized were: absorption rate constant (k_a), elimination rate constant (k_{el}), apparent clearance (CL / F), apparent volume of distribution (V / F, where F is the fraction of drug absorbed), lag time to appearance of the first non-zero concentration (t_{lag}), and area under the concentration-time curve extrapolated to infinity ($AUC_{0\rightarrow\infty}$).

A one-compartment model with lag time, first-order absorption and first-order elimination was selected as the best fitting pharmacokinetic model for all analytes ($r \geq 0.95$). Values of the maximum plasma concentration (c_{max}), the time to maximum plasma concentration (t_{max}) and of $AUC_{0\rightarrow\infty}$ were extracted from individual curve fits. Arithmetic means and standard deviations were presented. For analysis of pharmacokinetic-pharmacodynamic relationships we used c_{max} values, because these showed the strongest correlation with platelet response in our validation studies (see below) and in agreement with our previous studies[27,59].

Aggregometry

We measured platelet aggregation by light transmission aggregometry (LTA) in native platelet-rich plasma (PRP) after stimulation with 20 μM of adenosine diphosphate (ADP) using a 4-channel APACT aggregometer (LABiTec) as described[27,60]. We prepared PRP from citrated blood by centrifugation at 150 *g* for 15 min and platelet-poor plasma (PPP) as reference standard by centrifugation at 1,500 *g* for 20 min at room temperature. Platelet counts were not adjusted with autologous PPP to avoid a decrease in platelet aggregation[61]. Mean platelet count of PRP was 378 x 10^9 l^{-1} (range, 168–572). Aggregation was indicated as maximal percent change in light transmission with reference to unstimulated PRP (0% transmission) and PPP (100% transmission) after recording for 10 min at 37 °C under constant stirring. Inhibition of aggregation by clopidogrel was expressed as the difference (Δ aggregation, %) of predose aggregation minus postdose aggregation. We preferred initiation of aggregation with 20 μM ADP over lower ADP concentrations, because our previous studies revealed a lower intra-assay variability (CV, %), a higher sensitivity and a closer correlation of Δ aggregation values with clopidogrel pharmacokinetics, when we compared 20 μM ADP with 5 μM ADP[27,60]. Previous studies have shown that a plateau of maximal platelet inhibition after a single 600-mg clopidogrel

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dose was achieved within 3–4 h in about 90% of subjects and remained constant over approximately 24 h[62].

We conducted a validation study in 25 healthy, clopidogrel-naïve volunteers to determine the optimal postdose time point for measurement of platelet aggregation by LTA. After a 12-h overnight fast subjects received a singe 600-mg clopidogrel dose and maximum plasma concentration (c_{max}) and area under the concentration-time curve ($AUC_{0\rightarrow\infty}$) of the active metabolite were tested for linear correlation with ADP (20 μM)-induced Δ aggregation values at 2, 4, 6, 8, 12, and 24 h postdose. The mean Δ aggregation values were 18.8, 26.1, 31.0, 29.7, 28.8, and 25.4%, respectively. The squared Pearson correlation coefficients r^2 between c_{max} and Δ aggregation were 0.573, 0.676, 0.778, 0.677, 0.575, and 0.475, respectively ($P < 0.001$ each) (**Supplementary Figure 9a**). The r^2 values for the correlation between $AUC_{0\rightarrow\infty}$ and Δ aggregation were 0.224, 0.306, 0.429, 0.385, 0.317, and 0.255, respectively ($P \leq 0.012$) (**Supplementary Figure 9b**). The maximum percentage of platelet inhibition developed at 6 h postdose, and this value also showed the highest correlation with both c_{max} and $AUC_{0\rightarrow\infty}$ values. Compared with earlier postdose time points, measurement of aggregation at 6 h accounted for interindividual differences in the rate of onset of platelet inhibition, and ensured that peak plasma levels of the active metabolite were passed through in all subjects (range, 28–114 min) and that the majority of free active metabolite was cleared from plasma. Until 6 h postdose the offset of platelet inhibition by recovery of platelets is negligible, but becomes apparent at later time points; the percentage of inhibited platelets begins to decrease[62], and consequently the correlation with c_{max} and $AUC_{0\rightarrow\infty}$ values became weaker. The pharmacodynamic platelet response involves irreversible binding of the active metabolite to the P2Y12 receptors on the platelet surface[63,64]. Therefore, the velocity of onset and the magnitude of platelet inhibition depend on the rate and extent of appearance of the active metabolite in the systemic circulation. C_{max} is a metric of the rate and the extent of active drug appearance, whereas $AUC_{0\rightarrow\infty}$ reflects merely the extent of the total systemic exposure to the active metabolite. Hence, the c_{max} values were not highly correlated with $AUC_{0\rightarrow\infty}$ values ($r^2 = 0.405$), and platelet inhibition at all postdose time points was stronger linked to c_{max} than to $AUC_{0\rightarrow\infty}$ values. Therefore, to yield highest consistency between platelet response and clopidogrel pharmacokinetics, for our mechanistic pharmacokinetic-pharmacodynamic investigations we compared c_{max} values of active metabolite with the relative maximum of platelet inhibition (at 6 h after a 600-mg clopidogrel dose).

We conducted a subsequent validation study to evaluate whether administration of multiple prior doses of clopidogrel influence the correlation between platelet inhibition and active metabolite concentrations in a clopidogrel loading test. After a wash-out phase of 14 d the 25 healthy volunteers received a maintenance dose of 75 mg clopidogrel every 24 h for 10 d. Twenty-four hours after the last maintenance dose and following a 12-h overnight fast the subjects were administered a 600-mg loading dose. The pharmacokinetic parameters were not different from the preceding 600-mg loading test in the clopidogrel-naïve state: mean (s.d.) c_{max} and $AUC_{0\rightarrow\infty}$ values of the active metabolite in pretreated versus non-pretreated subjects were 16.5 (6.33) versus 17.9 (10.9) ng ml^{-1} ($P = 0.747$) and 1,947 (625) versus 2,069 (816) ng min ml^{-1} ($P = 0.411$), respectively. There were also strong intercorrelations among the c_{max} values ($r^2 = 0.966$; $P < 0.001$) and among the $AUC_{0\rightarrow\infty}$ values ($r^2 = 0.816$; $P < 0.001$) of both tests. The mean 6-h postload platelet inhibition versus baseline aggregation (i.e. before the first maintenance dose) was not different from the assessment in non-pretreated subjects (34.2% versus 31.0%, $P = 0.407$), but the intercorrelation among both measurements was considerably weaker ($r^2 = 0.183$; $P = 0.033$). The mean 6-h postload platelet inhibition versus preload aggregation (i.e. at 24 h after the last maintenance dose) was only 12.3% and not intercorrelated with the corresponding platelet inhibition in the non-pretreated participants ($r^2 = 0.014$; $P =$

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0.567). The r^2 values for the correlation of the 6-h postload platelet inhibition versus baseline with the c_{max} values of the active metabolite were 0.172 (P = 0.039) and with the $AUC_{0\rightarrow\infty}$ values 0.155 (P = 0.051) (**Supplementary Figure 9c**). The corresponding r^2 values for the correlation of the 6-h postload platelet inhibition versus preload were 0.001 (P = 0.877) and 0.012 (P = 0.607) (**Supplementary Figure 9d**). These findings indicate that multiple dosing does not alter the pharmacokinetics of clopidogrel. In particular, it is unlikely that the enzymes involved in clopidogrel metabolism could be saturated by administration of a daily maintenance dose (or by a single high dose of clopidogrel). The ranges of the maximum plasma concentrations of clopidogrel and its intermediate metabolite 2-oxo-clopidogrel were at least one order of magnitude below the K_m values of the metabolizing enzymes (CYP isozymes and PON1). Moreover, clopidogrel, 2-oxo-clopidogrel and the active metabolite were rapidly cleared from the systemic circulation with a mean half-life of <1 h. Hence, no cumulation of plasma concentrations occurs at usual clopidogrel dosing intervals of 24 h. By contrast, the pharmacodynamic response to clopidogrel underlies a ceiling effect. Administration of a clopidogrel loading dose in subjects already on maintenance treatment yields no substantial further increase of platelet inhibition. The potential individual increment of platelet inhibition caused by an additional clopidogrel dose depends on the residual platelet aggregation. Low peak levels of the active metabolite, a slow onset or a rapid offset of platelet inhibition are principally associated with higher levels of residual aggregation compared with the contrary conditions of high peak levels of the active metabolite, a rapid onset or a slow offset of platelet inhibition. Consequently, the c_{max} and $AUC_{0\rightarrow\infty}$ values of the active metabolite were insufficiently linked to platelet inhibition measures obtained after multiple maintenance doses of clopidogrel or after administration of an additional loading dose in individuals on clopidogrel maintenance treatment (**Supplementary Figure 9c,d**). Hence, we performed tests of pharmacokinetic-pharmacodynamic response to clopidogrel in individuals who were free of clopidogrel for a sufficient long period to allow for complete recovery of platelet function (and offset of any other potential residual effect of clopidogrel). This was ensured in the case-cohort patients who underwent the clopidogrel response test at the earliest 6 months after cessation of clopidogrel therapy.

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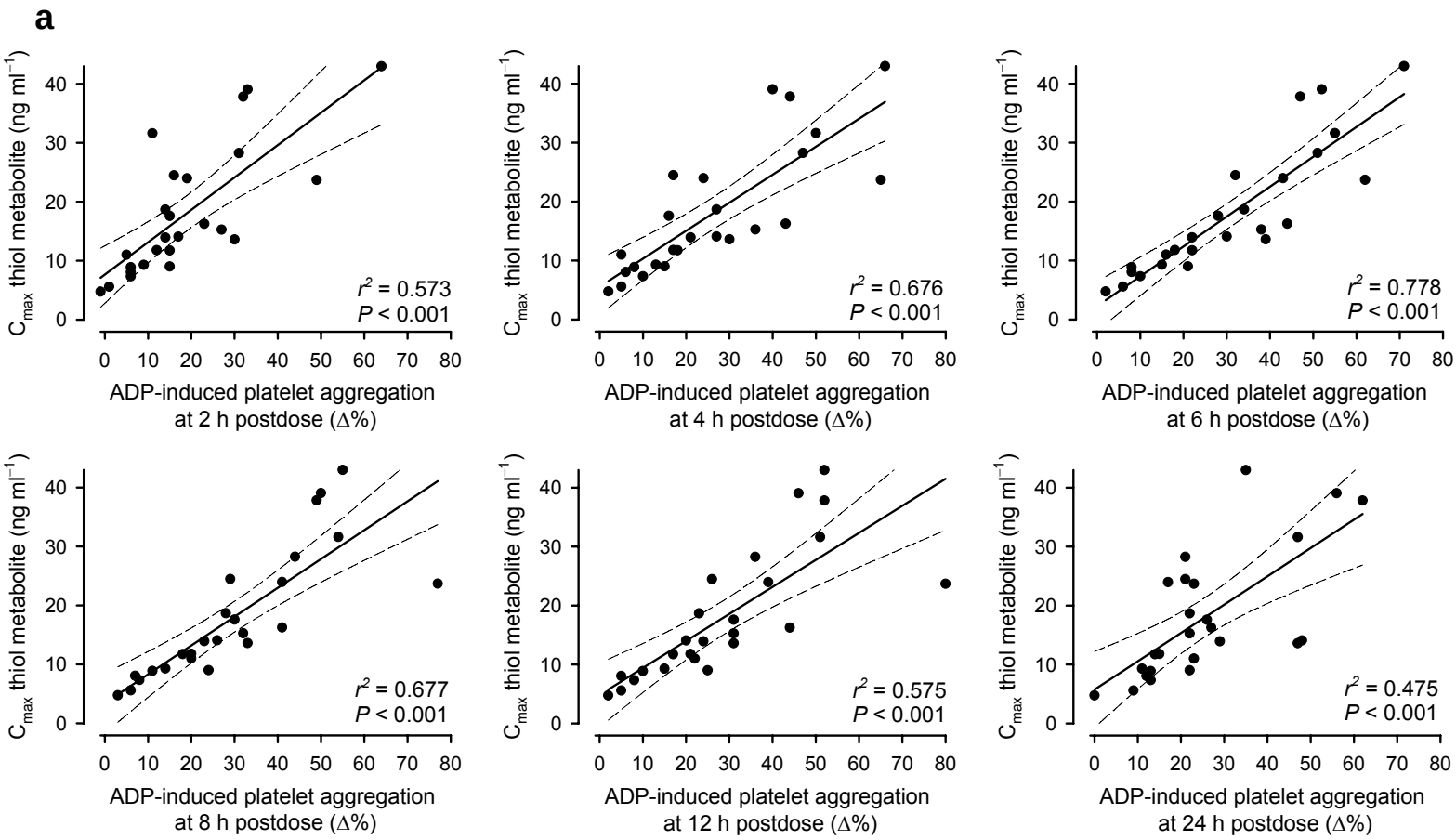
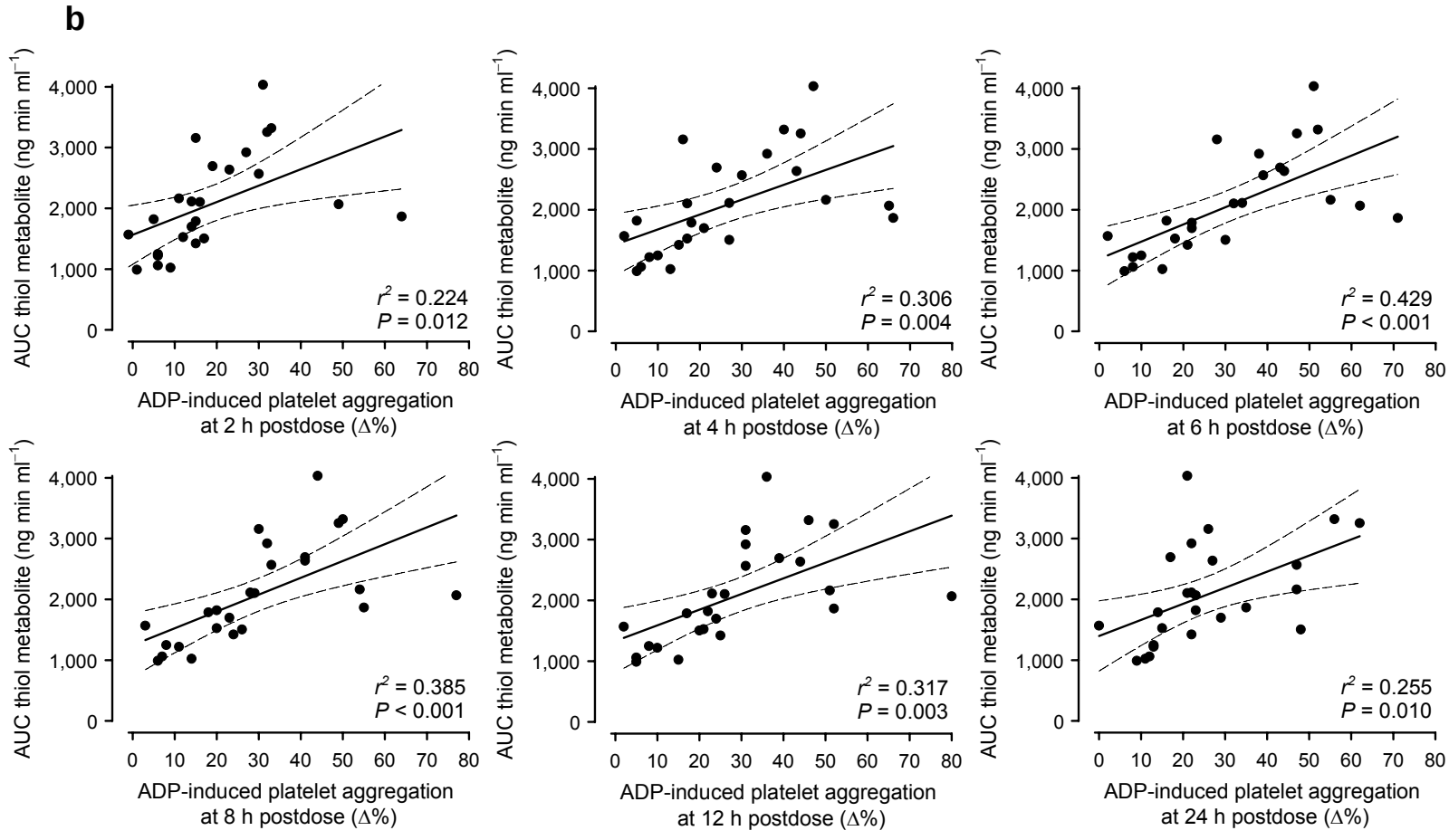
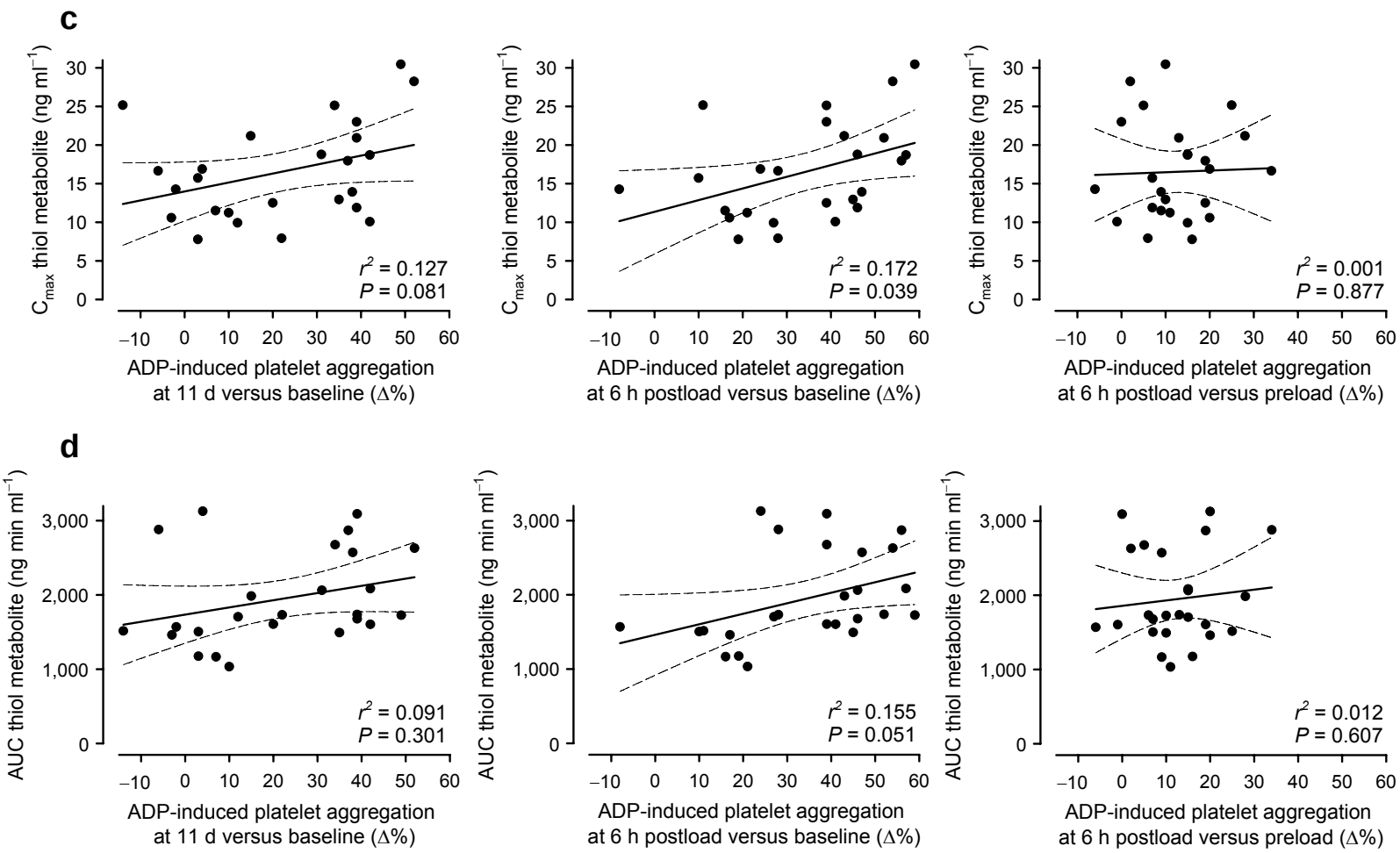

a
C_{max} thiol metabolite (ng ml^{-1})
r^2 = 0.573
P < 0.001
ADP-induced platelet aggregation at 2 h postdose (Δ%)
r^2 = 0.676
P < 0.001
ADP-induced platelet aggregation at 4 h postdose (Δ%)
r^2 = 0.778
P < 0.001
ADP-induced platelet aggregation at 6 h postdose (Δ%)
r^2 = 0.677
P < 0.001
ADP-induced platelet aggregation at 8 h postdose (Δ%)
r^2 = 0.575
P < 0.001
ADP-induced platelet aggregation at 12 h postdose (Δ%)
r^2 = 0.475
P < 0.001
ADP-induced platelet aggregation at 24 h postdose (Δ%)


b
AUC thiol metabolite (ng min ml^{-1})
r^2 = 0.224
P = 0.012
ADP-induced platelet aggregation at 2 h postdose (Δ%)
r^2 = 0.306
P = 0.004
ADP-induced platelet aggregation at 4 h postdose (Δ%)
r^2 = 0.429
P < 0.001
ADP-induced platelet aggregation at 6 h postdose (Δ%)
r^2 = 0.385
P < 0.001
ADP-induced platelet aggregation at 8 h postdose (Δ%)
r^2 = 0.317
P = 0.003
ADP-induced platelet aggregation at 12 h postdose (Δ%)
r^2 = 0.255
P = 0.010
ADP-induced platelet aggregation at 24 h postdose (Δ%)

Supplementary Figure 9 continued.



Supplementary Figure 9. Correlation of clopidogrel active metabolite pharmacokinetics with platelet inhibition in a validation study of 25 healthy individuals.

(**a,b**) Plots of the maximum plasma concentration (c_{max}) (**a**) and of the area under the concentration-time curve extrapolated to infinity (*AUC*) (**b**) of the active metabolite against adenosine diphosphate (ADP, 20 μM)-induced platelet aggregation predose versus postdose at 2, 4, 6, 8, 12, and 24 h after administration of a single 600-mg clopidogrel loading dose to clopidogrel-naïve individuals.

(**c,d**) Plots of c_{max} values (**c**) and of *AUC* values (**d**) against ADP (20 μM)-induced platelet aggregation following administration of a 75-mg clopidogrel maintenance dose every 24 h over 10 d. Plots against three different Δ aggregation values are presented: (1) aggregation at 11 d (24 h after the last maintenance dose) versus aggregation before clopidogrel administration (baseline); (2) aggregation at 6 h after a 600-mg clopidogrel loading dose (that was administered at 24 h after the last maintenance dose) versus aggregation at baseline; (3) aggregation at 6 h after a 600-mg clopidogrel loading dose (that was administered at 24 h after the last maintenance dose) versus aggregation immediately before administration of the loading dose (preload).

Individual values and linear regression lines with 95% confidence intervals are displayed.

r^2 denotes squared Pearson correlation coefficient. $P < 0.05$ is considered a statistically significant correlation.

Determination of PON1 activity in plasma

We assessed PON1 activity independently by the hydrolysis rates of paraoxon and phenylacetate, respectively, in adoption of a previously described method[18]. The Q192 and R192 allozymes of PON1 were reported to hydrolyze phenylacetate at similar rates, whereas paraoxon was more rapidly hydrolyzed by the R variant[65].

For determination of arylesterase activity, EDTA plasma of 250-fold dilution was incubated in 500 µl reaction mixtures of 9 mM Tris-HCl pH 8.0, 0.9 mM $CaCl_2$ and 3.4 mM phenylacetate at 22 °C for 5 min, and formation of phenol was monitored every 30 s at 270 nm using a Beckman DU-600 spectrophotometer.

For determination of paraoxonase activity, EDTA plasma of 20-fold dilution was incubated in 200 µl reaction mixtures of 10 mM Tris-HCl pH 8.0, 2 mM $CaCl_2$, 1 M NaCl and 1.5 mM paraoxon at 22 °C for 10 min, and formation of *p*-nitrophenol was monitored every 60 s at 405 nm using a Molecular Devices EMax microplate spectrophotometer.

For each sample the enzyme-specific increase in absorbance (optical density (*OD*)) was calculated by linear regression as *OD* min^{-1} (ml plasma)$^{-1}$ and corrected for *OD* increase of spontaneous substrate hydrolysis in blank reaction mixtures. Specific *OD* increase was converted into rates of formation (nmol min^{-1} (ml plasma)$^{-1}$) of phenol and *p*-nitrophenol, respectively, by the use of calibration functions with authentic standards. Intra-assay precision of the analysis of five replicates of five individual samples at one day was 2.2% for phenylacetate and 1.5% for paraoxon. Inter-assay precision of the analysis of five replicates of five individual samples at five days was 4.8% and 3.7%, respectively. Since PON1 enzyme activity was shown to depend on the presence of free calcium[65], we compared the hydrolysis rates in EDTA plasma with heparin plasma and serum drawn in random order from the same individual. Assessment of the samples from 30 individuals revealed mean differences in hydrolysis activity to heparin plasma of –0.3% (phenylacetate) and 1.1% (paraoxon), respectively; mean differences to serum were –3.4% and –2.7%, respectively. This demonstrates that the PON1 activity was not significantly diminished by the residual EDTA concentration in the diluted incubation samples that were supplemented with an at least 10-fold molar excess of free calcium.

Genotyping

Genomic DNA was extracted from EDTA whole blood samples stored at –80 °C using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. The gene regions harboring the variant alleles *CYP2C9*2* (rs1799853), *CYP2C9*3* (rs1057910), *CYP2C19*2* (rs4244285), *CYP2C19*3* (rs4986893), *CYP2C19*4* (rs28399504), *CYP2C19*5* (rs56337013),*CYP2C19*6* (rs72552267), *CYP2C19*7* (rs72558186), *CYP2C19*8* (rs41291556), *CYP2C19*17* (rs12248560), *CYP3A4*1B* (rs2740574), *CYP3A4*1G* (rs2242480), *CYP3A4*2* (rs55785340), *CYP3A4*3* (rs4986910), *CYP3A5*3* (rs776746), *PON1* Q192R (rs662), and *ABCB1* C3435T (rs1045642) (accession numbers of the U.S. National Center for Biotechnology SNP database) were amplified by means of a polymerase chain reaction. The amplified DNA fragments were separated by agarose gel electrophoresis and purified from the excised gel slices using the Wizard SV Gel and PCR Clean-Up System (Promega). Sequencing of the purified PCR products was performed bidirectionally with the ABI PRISM BigDye Terminator v2.0 Cycle Sequencing Kit on an ABI PRISM 3100 Genetic Analyzer instrument (Applied Biosystems) using sequence specific forward and reverse oligonucleotide primers. Primer design was done using Primer3 software[66]. All sequences were analyzed independently by three investigators to prevent genotyping errors. A polymorphism was analyzed when the call rate was ≥95%. In the case of ambiguous readings genotyping was repeated using freshly extracted genomic DNA.

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Statistical analysis and sample size calculation

We compared demographic, clinical and genotype characteristics between cases and subcohort controls by univariate Cox regression (accounting for different timing of events) or binary logistic regression (assuming coincidence of events). To account for the case-cohort design, subcohort noncases were weighted with the inverse of the sampling fraction according to the method of Barlow *et al*.[1]. We used multivariate, stepwise forward Cox proportional hazards models to assess predictors of event-free time to stent thrombosis (or another clinical outcome). Potential explanatory variables that were identified by univariate regression ($P < 0.20$) or demographic, clinical or genetic variables that have previously been associated with response to clopidogrel were considered. The best fitting explanatory models were determined under the condition that every variable in the model was statistically significant at the 0.05 level. In addition, multivariate Cox regression models including all independent potentially explanatory variables were calculated to analyze the sensitivity of hazard estimates obtained in stepwise forward models to further adjustment. Moreover, hazard estimates obtained in univariate Cox models with the *PON1* genotype as explanatory variable were adjusted with clopidogrel activity variables to assess the magnitude of interaction between variables. The absence of high multicollinearity was assumed if no pair of covariates had a Pearson product-moment correlation coefficient $|\rho| \geq 0.80$ (corresponding to a variance inflation factor (*VIF*) ≥ 2.77). The proportional hazards assumption was satisfied if the log-minus-log plots (with log time scaling) for genotypes or tertiles of activity parameters did not cross.

We used multivariate, stepwise forward linear regression to evaluate associations of the *PON1* genotype with paraoxonase activity, clopidogrel pharmacokinetics and platelet inhibition. The coefficient of determination R^2 was calculated to express the variance explained between the *PON1* genotype and an activity variable and among the activity variables.

The association tests of unlinked genetic markers of different genes with stent thrombosis were considered as independent hypotheses tests, which did not require an adjustment of *P* values for multiple testing. Alternatively, to assess the probability that a statistically significant association ($P < 0.05$) between a tested genetic variant and stent thrombosis was actually a false-positive finding, we determined the false-positive reporting probability (FPRP) according to the method of Wacholder *et al*.[16]. Based on the mechanistic evidence that PON1 type Q192 and R192 allozymes determine clopidogrel bioactivity, a relative high prior probability of 0.1 and a prior odds ratio of 3.0 for the association with the risk *PON1* QQ192 genotype were assigned; a stringent FPRP level of noteworthiness of 0.2 was prespecified.

The discriminatory power of predictor variables for distinguishing between cases and noncases was evaluated by nonparametric receiver operating characteristic (ROC) curves using the area under the ROC curve as a measure of diagnostic effectiveness[23].

We evaluated deviation of observed genotype frequencies from the Hardy-Weinberg equilibrium by Fisher's exact test. Pairwise analysis of linkage disequilibrium between the *PON1* Q192R polymorphism and other selected genetic polymorphisms was performed by Haploview version 4.2 software (Broad Institute). Blocks of linkage disequilibrium were analyzed in the white populations of the HapMap database[46] and the Genome Variation Server[67] using the 'Solid Spine of LD' algorithm with a minimum D' value of 0.8 internal to Haploview 4.2. All other inferential statistics were done with PASW version 18.0.1 software (SPSS Inc.).

We used Fisher's exact test for a priori sample size calculation. The open cohort of all eligible PCI patients was augmented until at least 40 patients with non-fatal incident stent thrombosis could be enrolled in the clopidogrel response test. The frequency of *PON1* QQ192

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homozygous individuals among PCI patients without stent thrombosis was estimated as 36%, extrapolating available genotyping data of 1,023 PCI cohort patients (365 QQ, 493 QR, and 165 RR). Supposing that a clinically important genetic association with stent thrombosis would be indicated by an odds ratio of ≥3.0 for the QQ192 genotype compared with either QR192 or RR192, we calculated that a subcohort of 70 patients was required to achieve a power of 0.80. Estimating a drop-out rate of 50% due to nonadherence, unwillingness to consent or impossibility to contact, we took a random sample of 140 individuals.

Replication study

To evaluate the reliability and validity of the results obtained in the case-cohort study and to extend the potential clinical relevance of our findings, we conducted an independent replication study that assessed the association of the *PON1* Q192R genotype and the paraoxonase plasma activity with the risk of stent thrombosis and other predefined clinical end points in patients on clopidogrel therapy.

Replication study population

The replication study was conducted in 1,982 patients with a clinical presentation of acute coronary syndromes (ACS), with and without ST-segment elevation, who had undergone PCI with successful stent placement (according to the definition in the case-cohort study). Eligible were unrelated, between 18 and 80 years old male and female patients of self-reported European ancestry, if they were hospitalized within 24 h after onset of symptoms, received a loading dose of 600 mg clopidogrel at least 2 h before PCI and a maintenance dose of 75 mg clopidogrel daily for 12 months after PCI and 100 mg aspirin daily for at least 12 months. Exclusion criteria were previous stent thrombosis, active bleeding and bleeding diathesis, platelet count $<150 \times 10^9\ l^{-1}$, severe renal or hepatic disorder, hematologic disorder, chronic inflammatory or autoimmune disorder, active malignancy, alcohol or drug addiction, a body-mass index below 18.5 or above 40 kg m^{-2}, use of an oral anticoagulant, use of any other antiplatelet drug than clopidogrel or aspirin after enrollment or use of any other antiplatelet drug than aspirin within 5 days before enrollment, use of vitamin or mineral supplements, use of contraceptives or hormone replacement therapy, premature cessation of clopidogrel or aspirin or nonadherence to therapy or nonattendance to any of the scheduled control visits.

All eligible patients who were admitted between June 30, 2007 and July 1, 2009 to the coronary care units of our centers and who provided a whole blood sample for genetic testing and a plasma sample prior to clopidogrel administration for paraoxonase activity testing were included. All clinical assessments were done by investigators who were blind to the genetic information and the paraoxonase plasma activity of the patients. All laboratory analyses, genotyping and data management were done by staff that was blinded to patients' status and outcome.

Replication study design and sample size calculation

We conducted a prospective cohort study in 1,982 patients with ACS over follow-up period of 12 months after the index PCI. The study aimed to test the association of the *PON1* Q192R genotype and the paraoxonase plasma activity and other genetic markers with the occurrence of predefined clinical end points.

Follow-up visits were scheduled at hospital discharge and at 1, 3, 6, 9, and 12 months after the index PCI. Patients were monitored by standardized interview, physical examination and laboratory testing. Patients were considered adherent to therapy by the investigating physician if

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they had not discontinued the use of clopidogrel or aspirin for more than 5 days within the time after the last visit or before occurrence of a primary or secondary study end point.

2,164 patients met the eligibility criteria, 182 were excluded: 42 did not consent to genetic or paraoxonase activity testing, in 9 patients genotyping provided ambiguous readings, 71 had prematurely discontinued clopidogrel or aspirin or were nonadherent, and 60 were lost to follow-up for unknown reason. Finally, 1,982 patients were included in analyses.

End point definitions: Primary end point was incident fatal and non-fatal definite stent thrombosis during the 12-months of follow-up. Secondary end points were fatal and non-fatal myocardial infarction; fatal and non-fatal ischemic stroke; the composite of death from vascular causes, non-fatal myocardial infarction, or non-fatal ischemic stroke; death from nonvascular causes; and major bleeding. Definite stent thrombosis was evaluated according to the Academic Research Consortium criteria. Myocardial infarction was evaluated in accordance with the Joint ESC/ACCF/AHA/WHF Task Force universal definition[68]. Ischemic stroke was defined as a new focal loss of neurologic function of ischemic vascular origin (assessed by computed tomographic or magnetic resonance imaging, or autopsy), with residual symptoms lasting at least 24 h or leading to death. Death from vascular cause was defined as death from cardiovascular or cerebrovascular cause and any death without clearly documented nonvascular cause. Major bleeding was defined as fatal bleeding, symptomatic intracranial bleeding, intrapericardial bleeding requiring cardiac tamponade, bleeding leading to substantial hypotension requiring intravenous pressor drugs or surgical intervention, bleeding leading to clinically significant disability, intraocular bleeding leading to the loss of vision, or bleeding leading to a drop in the hemoglobin level of at least 3.0 g dl^{-1} or necessitating transfusion of at least 2 units of red blood cells or equivalent whole blood.

Fisher's exact test was used for a priori sample size calculation. Based on previous findings of clinical trials with clopidogrel in patients with ACS[69,70], we estimated a 12-months cumulative incidence of definite stent thrombosis of 2%. Supposing a frequency of the *PON1* QQ192 genotype of 36% and considering an odds ratio of 3 for the association of QQ192 with stent thrombosis, we calculated that a cohort of 1,850 individuals was required to achieve a power of 0.90.

The protocol of the study was approved by the institutional reviewing boards, and participants gave written informed consent.

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