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## ARTICLE

# Plasma lipidomic analysis to investigate putative biomarkers of P-glycoprotein activity in healthy volunteers

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## Abstract

P-glycoprotein (P-gp) is an efflux transporter involved in the bioavailability of many drugs currently on the market. P-gp is responsible for several drug–drug interactions encountered in clinical practice leading to iatrogenic hospital admissions, especially in polypharmacy situations. *ABCB1* genotyping only reflects an indirect estimate of P-gp activity. Therefore, it would be useful to identify endogenous biomarkers to determine the P-gp phenotype to predict in vivo activity prior to the initiation of treatment and to assess the effects of drugs on P-gp activity. The objective of this study was to assess changes in plasma lipidome composition among healthy volunteers selected on the basis of their *ABCB1* genotype and who received clarithromycin, a known inhibitor of P-gp. Untargeted lipidomic analysis based on liquid chromatography–tandem mass spectrometry was performed before and after clarithromycin administration. Our results revealed changes in plasma levels of some ceramides (Cers) {Cer(d18:1/22:0), Cer(d18:1/22:1), and Cer(d18:1/20:0) by ~38% ( $p < 0.0001$ ), 13% ( $p < 0.0001$ ), and 13% ( $p < 0.0001$ ), respectively} and phosphatidylcholines (PCs) {PC(17:0/14:1), PC(16:0/18:3), and PC(14:0/18:3) by ~24% ( $p < 0.001$ ), 10% ( $p < 0.001$ ), and 23.6% ( $p < 0.001$ )} associated with both *ABCB1* genotype and clarithromycin intake. Through the examination of plasma lipids, our results highlight the relevance of untargeted lipidomics for studying in vivo P-gp activity and, more generally, to safely phenotyping transporters.

## Study Highlights

### WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

P-glycoprotein (P-gp) is an efflux transporter involved in the interindividual pharmacokinetic variability of drugs that can influence therapeutic and toxic effects. Genetic polymorphisms in the *ABCB1* gene can explain some of the variability

Marie-Anne Lorient and Olivier Laprévôte are co-last authors.

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in P-gp activity but do not predict P-gp activity and its variations over time. The identification of endogenous probes to develop noninvasive and safe in vivo phenotyping of P-gp is challenging.

#### WHAT QUESTION DID THIS STUDY ADDRESS?

Are there lipidome changes related to *ABCB1* genotype and P-gp inhibition by clarithromycin highlighting putative endogenous biomarkers of P-gp?

#### WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

This study shows plasma variations in several lipid species, including ceramides and phospholipids, according to *ABCB1* genotype and P-gp activity and validates a strategy based on an untargeted lipidomic approach to study P-gp activity.

#### HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

Metabolomics could be an attractive methodology to discover endogenous substrates of P-gp to allow its phenotyping and thus predict drug response and anticipate potential drug–drug interactions.

## INTRODUCTION

In routine practice, drugs are prescribed by the physician at the doses recommended by the “Summary of Product Characteristic” or by expert societies. However, response and tolerance to medication are highly dependent on interindividual pharmacokinetic variability resulting in adverse drug reactions or lack of drug response. Age, gender, genetics, ethnicity, diseases, and drug–drug interactions (DDIs) are the main parameters explaining this variability through the enzymes and transporters involved in drug metabolism.<sup>1–4</sup> Thus, increasing efforts have been made over the past decades in pharmacogenetics and pharmacogenomics to provide personalized prescriptions without fully accounting for interindividual variability.<sup>5,6</sup> Pharmacometabolomics aims to determine metabolic signatures after drug administration that may reflect drug response and tolerance.<sup>7</sup> This field includes mapping drug effects on metabolism to identify pathways that contribute to drug response phenotypes.<sup>8</sup>

P-glycoprotein (P-gp), a member of the family of ATP-binding cassette (ABC) transporters, is a key transporter in the efflux of xenobiotic compounds across the plasma membrane.<sup>9</sup> An estimated 50% of currently available drugs are P-gp substrates.<sup>10,11</sup> Moreover, many drugs are inhibitors or inducers of this transporter, which is involved in DDI. In drug metabolism and pharmacokinetics, P-gp appears to be as important as cytochrome P450 (CYP450). Indeed, in preclinical and clinical trials, it is now recommended to assess the P-gp efflux of drug candidates.<sup>1</sup>

P-gp protein expression is dependent on *ABCB1* gene polymorphisms that likely explain some of the interindividual variability in drug response and tolerance.<sup>12</sup>

More than 50 single nucleotide polymorphisms (SNPs) have been reported in the *ABCB1* gene, most of which result in changes in P-gp expression and activity.<sup>10,12,13</sup>

This is particularly the case for SNPs located in exons 21 (G2677[A,T], A893[T,S]), and 26 (C3435T, Ile1145Ile), which are the most frequently encountered SNPs.<sup>14</sup> Indeed, these two SNPs are in strong linkage disequilibrium and mediate decreased P-gp efflux in several in vitro studies.<sup>14–16</sup> In *MDR1* knockout mice, brain accumulation of many P-gp substrates<sup>17</sup> and pharmacokinetic changes of digoxin and cyclosporine A, P-gp, substrate probes have been reported.<sup>18,19</sup> In humans, digoxin has been extensively studied to assess *ABCB1* polymorphism, demonstrating an increase in digoxin plasma concentration in healthy volunteers homozygous for the 3435TT variant.<sup>20</sup>

Despite the relevance of *ABCB1* genotyping to predict P-gp activity, it provides only partial information regarding the actual P-gp efflux capacities of individuals. Indeed, P-gp activity is known to depend on other individual factors, such as age, pathology or DDIs.<sup>1,2,21–23</sup> P-gp phenotyping by the so-called “cocktail approach” allows a more accurate assessment of the activity of this transporter in humans. This approach is based on the simultaneous measurement of P-gp and CYP450 isoform activities by administration of low doses of substrate probes. For example, the “Cologne Cocktail” and the “Geneva Cocktail” use the metabolic ratios of administered digoxin or fexofenadine, respectively, to determine P-gp activity.<sup>24,25</sup> However, the cocktail approach remains difficult to apply in clinical practice because it introduces additional iatrogenic risk, particularly for polypharmacy and high-risk DDI populations.<sup>25</sup>

Because of these limitations, an approach based on endogenous biomarkers of P-gp activity rather than drug cocktails would be very useful to phenotype individuals. Indeed, in addition to drugs, several endogenous

metabolites are substrates of P-gp, most of which are hydrophobic compounds.<sup>26,27</sup> Among them, lipids could be considered good biomarker candidates of P-gp activity. In support of this hypothesis, previous studies have described interactions between P-gp and some lipid species, including membrane phospholipids and sphingolipids (especially glucosylceramides).<sup>28–30</sup>

The objective of this study was to determine whether certain lipids could reflect P-gp activity and be used as endogenous probes. For this purpose, we investigated changes in plasma lipid profiles in healthy volunteers with known *ABCB1* genetic status (wild-type [WT], heterozygous variant [HetV], and homozygous variant [HomV] for haplotype “2677–3435”). In addition, to assess the correlation between the lipid profile changes and the level of P-gp transport activity, healthy volunteers received clarithromycin, an inhibitor of P-gp. The study was based on a comprehensive lipidomic analysis performed by liquid chromatography (LC) coupled with high-resolution mass spectrometry and tandem mass spectrometry (MS/MS).

## MATERIALS AND METHODS

### Chemical and reagents

Chloroform (Carlo Erba Reactifs SDS), acetonitrile, methanol, and isopropanol of LC–MS grade (J.T. Baker), 3,5-di-tert-4-butylhydroxytoluene (Sigma Aldrich), and methyl-tert-butyl-ether (Acros Organics Thermo Fisher Scientific) were used to prepare cell lipid extracts and mobile phases for reversed-phase liquid chromatography. LC–MS grade water (Sigma Aldrich) was used for sample preparation and analysis. All standard lipids were purchased from Avanti Polar Lipids.

### Plasma samples

#### Healthy volunteers

Plasma samples were obtained from a cohort of healthy volunteers established in a previous study.<sup>31</sup> Volunteers

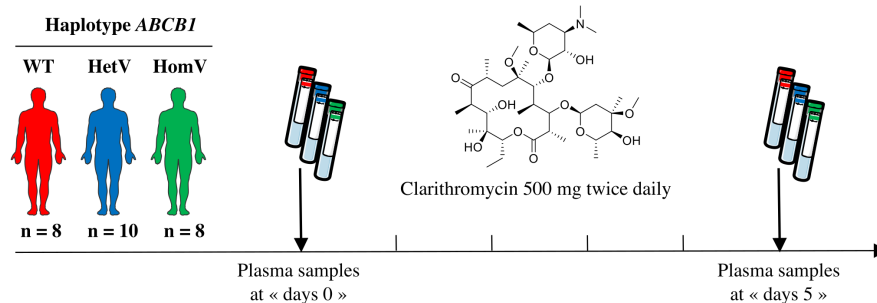
recruited in two Parisian clinical investigation centers located at the European Georges Pompidou Hospital and the Pitié-Salpêtrière Hospital between January 2011 and June 2014 were men, White race, aged between 18 and 45 years, and weighing between 55 and 85 kg. Volunteers had no history of allergy, gastric ulcer, chronic disease, recent acute episode, alcohol or tobacco intoxication, or psychotropic medication. The study was approved by the regional Ethics Committee (No. P100507-DRIVING, CPP Ile de France 10) and registered on [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (NCT01627665). All participants gave their written informed consent to participate.

### Study design

A total of 26 healthy volunteers were included in the present study and divided into three groups according to their *ABCB1* genotype: eight WT, 10 HetV, and eight HomV. Each volunteer received clarithromycin for 5 days twice daily at a dose of 500 mg. Clarithromycin is known to be an inhibitor of P-gp and CYP3A4, as reported in various *in vivo* studies.<sup>32,33</sup> Clarithromycin was provided by the Agence Générale des Equipements et Produits de Santé (AGEPS, AP-HP). Blood samples were taken from the volunteers before and after clarithromycin intake. The first one was obtained from a morning fasting sample at “day 0” before the first administration of clarithromycin. The second blood sample was taken during morning fasting after a 5-day course of clarithromycin (“day 5”; **Figure 1**). During the same period, the healthy volunteers did not receive any other drug to exclude potential drug interference on *in vivo* P-gp activity and to minimize sources of variability that could induce misinterpretation of the observed results.

### *ABCB1* genotyping

Genotyping for *ABCB1* was performed by allelic discrimination using the TaqMan method in the Department of Clinical Chemistry of the European Georges-Pompidou Hospital (Paris, France). The two SNPs analyzed are



**FIGURE 1** Study design. HetV, heterozygous variant; HomV, homozygous variant; WT, wild-type.

located on exon 21 (rs2032582; c.2677G>A/T and p. Ala893Thr/ser) and on exon 26 (rs1045642; c.3435C>T; and p.Ile1145Ile), forming a haplotype named “2677–3435.” The references of the reagents used were C\_11711720D\_40 and C\_11711720C\_30 for exon 21, and C\_7586657\_20 for exon 26, respectively.

## Lipidomic analysis

### Plasma samples preparation and lipid extraction

Lipidomic analysis was performed as previously described.<sup>34,35</sup> After thawing, the 52 plasma samples were diluted in ultrapure water (0.5 mL) spiked with a mixture of lipid internal standards at a final concentration of 1  $\mu$ M and sonicated for 5 min. Lipids were extracted using a chloroform/methanol/water (5:5:2, v/v/v) mixture containing 3,5-di-tert-4-butylhydroxytoluene 0.01% (w/v) as an antioxidant agent. Samples were subsequently centrifuged at 3000 rpm for 10 min, supernatants were collected, and solvents evaporated under reduced pressure at 45°C. Dry residues were resuspended in an acetonitrile/isopropanol/chloroform/water mixture (35:35:20:10 v/v/v/v) before injection into the ultraperformance (UP)LC–MS system.

### UPLC–HRMS–ESI+/- analysis

The LC–MS analysis of lipid extracts was performed under both positive and negative electrospray ionization conditions with a UPLC system (Waters) combined with a SynaptG2 (Q–TOF) mass spectrometer (Waters) as previously described.<sup>34–36</sup> Chromatographic separation was achieved on an Acquity CSH C18 column (100 mm  $\times$  2.1 mm; 1.7  $\mu$ m; Waters). Elution was performed using a binary gradient system consisting of 10 mM ammonium acetate in an acetonitrile/water mixture (40:60, v/v) as solvent A and 10 mM ammonium acetate in an acetonitrile/isopropanol mixture (10:90, v/v) as solvent B. The eluent increased from 40% B to 100% B in 10 min and was held at 100% B for 2 min before returning to 40% B. The flow rate was kept at 0.4 mL/min, the column oven was set at 50°C, and the injection volume was 5  $\mu$ L. The source parameters were as follows: capillary voltage, 2400 V; cone voltage, 45 V; source temperature, 120°C; desolvation temperature, 550°C; cone gas flow, 20 L/h; and desolvation gas flow, 1000 L/h. Leucine enkephalin (2 ng/mL) was used as an external reference compound (Lock–Spray; Manchester, UK) for mass correction. In data-dependent acquisition mode, MS full scans were followed by five MS/MS scans performed on

the five most intense ions above an absolute threshold of 1000 counts at the detector. Selected parent ions were fragmented at collision energies ranging from 20 to 40 eV. The scan duration for both MS and MS/MS was 0.2 s. In full-scan mode, the data were acquired between  $m/z$  50 and 1200 using a resolution of 20,000 FWHM at  $m/z$  500. Data acquisition was managed using Waters MassLynx software (version 4.1; Waters MS Technologies). The order of sample injections into the UPLC/MS/MS system was orthogonalized to avoid any bias. A mixture of 65 standard lipids belonging to nine of the main lipid classes: fatty acid (FA), phosphatidic acid, phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylglycerol, ceramide (Cer), sphingomyelin (SM), hexosylceramide, monoacylglycerol, diacylglycerol (DG), and triacylglycerol (TG) at a final individual concentration of 1  $\mu$ M was also periodically injected throughout the analytical batch.

### Data preprocessing and lipid identification

For both ion modes, raw data files were converted into universal open source mzXML files with MSConvert 3.0 and processed using MZmine 2.5 software. MS and MS/MS spectra were extracted with a mass detection noise level set at 1E2 and 0E0, respectively. Chromatograms were built with the ADAP algorithms (minimum group size of five scans, a group intensity threshold of 1000, and an  $m/z$  tolerance of 10 ppm).<sup>37</sup> The ADAP wavelet chromatogram deconvolution algorithm was used with the following settings: signal-to-noise ratio=10, coefficient/area ratio=60, peak duration range=0.05–0.5 min, and retention time wavelet range=0.0–0.2. Chromatograms were deisotoped using the isotopic peaks grouper algorithm using an  $m/z$  and a retention time ( $t_R$ ) tolerance of 10 ppm and 0.1 min, respectively. Peak alignment was performed through the join aligner method using the following parameters:  $m/z$  and  $t_R$  tolerance of 10 ppm and 0.15 min, respectively. Each MS/MS scan was associated with the corresponding MS scan using an  $m/z$  and  $t_R$  tolerance of 10 ppm and 0.15 min, respectively. The peak list was finally gap-filled using the so-called module “same RT and  $m/z$  range gap filler” with  $m/z$  tolerance of 5 ppm. Lipids were annotated based on exact mass and retention time measurements with the “custom database” module using an in-house database. These preprocessing steps led to two matrices (from ESI+ and ESI– analyses), listing for the annotated lipids  $m/z$ ,  $t_R$  and peak area values. The structure assignment of lipid species was based on MS and MS/MS data using a mass tolerance window of 5 and 15 ppm, respectively. Identification was also supported by  $t_R$  values through comparison of experimental data to



expected values calculated using  $t_R$  prediction models.<sup>38</sup> MS/MS spectra were used to fully determine phospholipid fatty acid composition. The fatty acyl  $sn_1$  and  $sn_2$  positions of the major regioisomers were determined in the negative ion mode using the relative intensity of carboxylate fragment anions as previously described.<sup>35,39</sup> The lipid amounts, expressed as  $\mu\text{M}$ , were estimated using the internal standard lipid mixture spiked in plasma samples before extraction.<sup>40</sup> Lipid species intensities were individually normalized to those of the corresponding internal standards chosen in the same subclass. The standard lipid mixture includes one lipid molecule representative of each investigated subclass. Lipid species used as internal standards contain a fatty acid side chain with a total odd carbon number or are deuterated derivatives and thus cannot overlap with the endogenous lipids present in the plasma samples. The resulting matrices were then normalized and filtered as previously described according to a correlation coefficient.<sup>38</sup> Only those variables whose intensity is sufficiently correlated among the 1/1, 1/3, and 1/6 quality controls (QCs) are retained, with a threshold set at 0.7. A second filtration is performed according to the coefficient of variation (CV) calculated for each value from the intensities of the 1/1 QCs. Only the variables with a CV lower than 30% are kept.

## Statistical analysis

Multivariate statistical analyses (MSAs) were performed to search for possible correlations between identified lipids and biological data (clarithromycin exposure conditions, *ABCB1* genotype). Data were analyzed using SIMCA-P+ software (Umetrics).<sup>41</sup> First, we performed an unsupervised MSA (i.e., principal component analysis [PCA]), with no a priori indication of whether each sample belongs to a group, to highlight the distribution of variables according to the greatest variance. Next, we used two types of supervised MSAs: partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA).<sup>42</sup>

Statistical analysis was performed using GraphPad Prism 9.0.0 software (GraphPad, GraphPad Software). The verification for normality of the distribution of the variables was performed by an Agostino-Pearson test. For each annotated lipid, following log transformation, a paired  $t$ -test was performed to compare the means of individuals following clarithromycin exposure according to *ABCB1* with a risk set at 0.05. The false discovery rate (FDR) was applied post hoc with an alpha risk set at 0.1.<sup>43</sup> The significance thresholds were as follows: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$ .

## RESULTS

### Clarithromycin contents of plasma samples

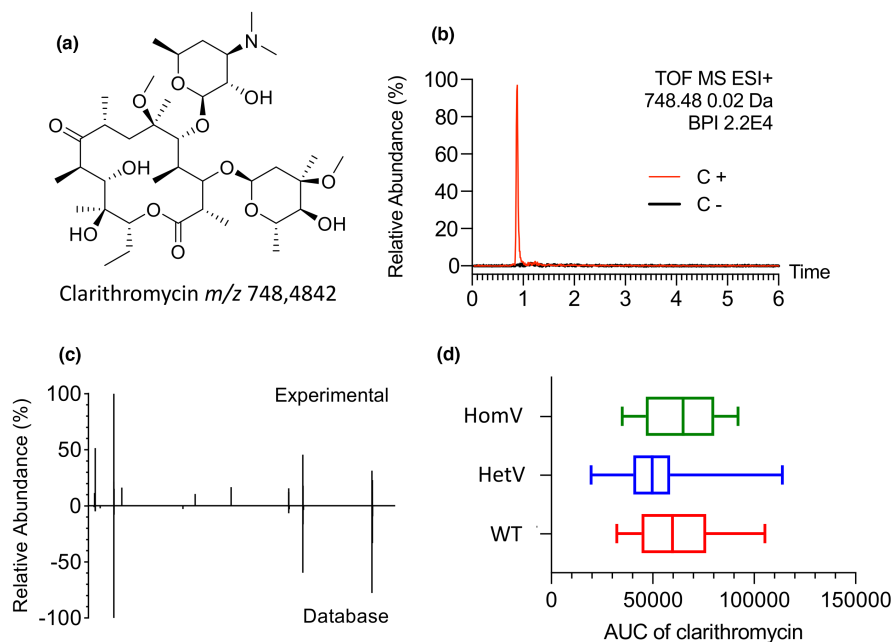
Because of its hydrophobic character ( $\log P = 3.16$ ), clarithromycin was efficiently extracted together with lipids (Figure 2a) and an estimation of its plasma concentration was performed (Figure 2b,c). No significant differences in clarithromycin plasma content were found according to the *ABCB1* genotype of healthy volunteers ( $p = 0.5456$ ; Figure 2d).

### Validation of the lipidomic analysis

To ensure the validity of LC-MS data acquisition, QCs were prepared from an equivolumetric mixture of each sample (QC 1/1), which was diluted to the third (QC 1/3) and the sixth (QC 1/6). QC samples were injected throughout the whole analytical sequence. The PCA score plot corresponding to QC samples exhibited clustering of the injection replicates corresponding to QC 1/1, QC 1/3, and QC 1/6 (Figure S1).

### Lipids identification and semiquantification

Lipidomic analysis by UPLC-HRMS-ESI+/- performed on the 52 plasma samples allowed the formal identification of 343 lipids included in five classes and 10 subclasses: FAs, TG, DG, PC, PE, PS, phosphatidylinositol (PI), SM, Cer, and cholesteryl ester (CE; Table S1). Score plots of PCA, PLS-DA, and OPLS-DA showed no separation between sample groups based on *ABCB1* genotype or clarithromycin intake (data not shown). Furthermore, the FDR test performed on all the above lipids ( $p < 0.05$ ) indicated that no lipid exhibited a change in its plasma level following administration of clarithromycin regardless of *ABCB1* genotype ( $n = 26$ ; Figure 3a). In contrast, in *ABCB1* WT healthy volunteers ( $n = 8$ ), administration of clarithromycin resulted in decreased plasma concentrations of 3 ceramides: Cer(d18:1/22:0), Cer(d18:1/22:1), and Cer(d18:1/20:0; Figure 3b). In *ABCB1* HetVs ( $n = 10$ ), no changes in plasma lipids were observed (Figure 3c). Finally, in *ABCB1* HomVs ( $n = 8$ ), clarithromycin induced an increase in plasma concentrations of 10 PC, 3 PI, and 28 TG (Figure 3d). In addition, when taken together WT and HetV ( $n = 18$ ), clarithromycin intake decreased the plasma concentrations of eight ceramides, 7 PE, 1 PI, and 21 TG (Figure 3e). However, when HetV and HomV



**FIGURE 2** Clarithromycin identification and semi-quantification. (a) Chemical structure of clarithromycin. (b) Chromatogram reconstructed at  $m/z$  748.48 corresponding to the  $[M+H]^+$  ion of clarithromycin in a healthy volunteer prior (C $-$ ) and following (C $+$ ) clarithromycin intake. (c) Comparison of the MS/MS spectrum of clarithromycin from experimental data and the GNPS database. (d) Clarithromycin AUC determined in plasma samples of healthy volunteers by HPLC-HRMS depending on the *ABCB1* status. AUC, area under the curve; HetV, heterozygous variant; HomV, homozygous variant; HPLC, high-performance liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; WT, wild-type.

subjects were considered together ( $n=18$ ), no lipids showed changes in their plasma levels (Figure 3f).

### Ceramides plasma levels

In the WT group, clarithromycin induced a mean decrease in the plasma levels of Cer(d18:1/22:0), Cer(d18:1/22:1), and Cer(d18:1/20:0) by  $\sim 38\%$  ( $p < 0.0001$ ),  $13\%$  ( $p < 0.0001$ ), and  $13\%$  ( $p < 0.0001$ ), respectively. This decrease was observed in 100% of the WT samples. In the HetV group, the mean decreases were  $\sim 17\%$  ( $p < 0.05$ ) and  $6\%$  ( $p < 0.05$ ) for Cer(d18:1/22:0) and Cer(d18:1/22:1), respectively. No change in Cer(d18:1/20:0) was observed. This decrease was observed in 70% of HetV samples (Figure 4a–c). Finally, in the HomV group, no change in plasma ceramide levels was found. It should be noted that no correlation between clarithromycin and ceramide concentrations was established regardless of the *ABCB1* genotype group considered (Table S2).

### Phospholipids plasma levels

Clarithromycin decreased the plasma levels of some PE species, especially PE (16:0/20:4; 23.7%,  $p < 0.01$ ) and PE (18:0/20:4; 15.6%,  $p < 0.05$ ), in WT samples. Decreased concentrations of these two PE species were observed in

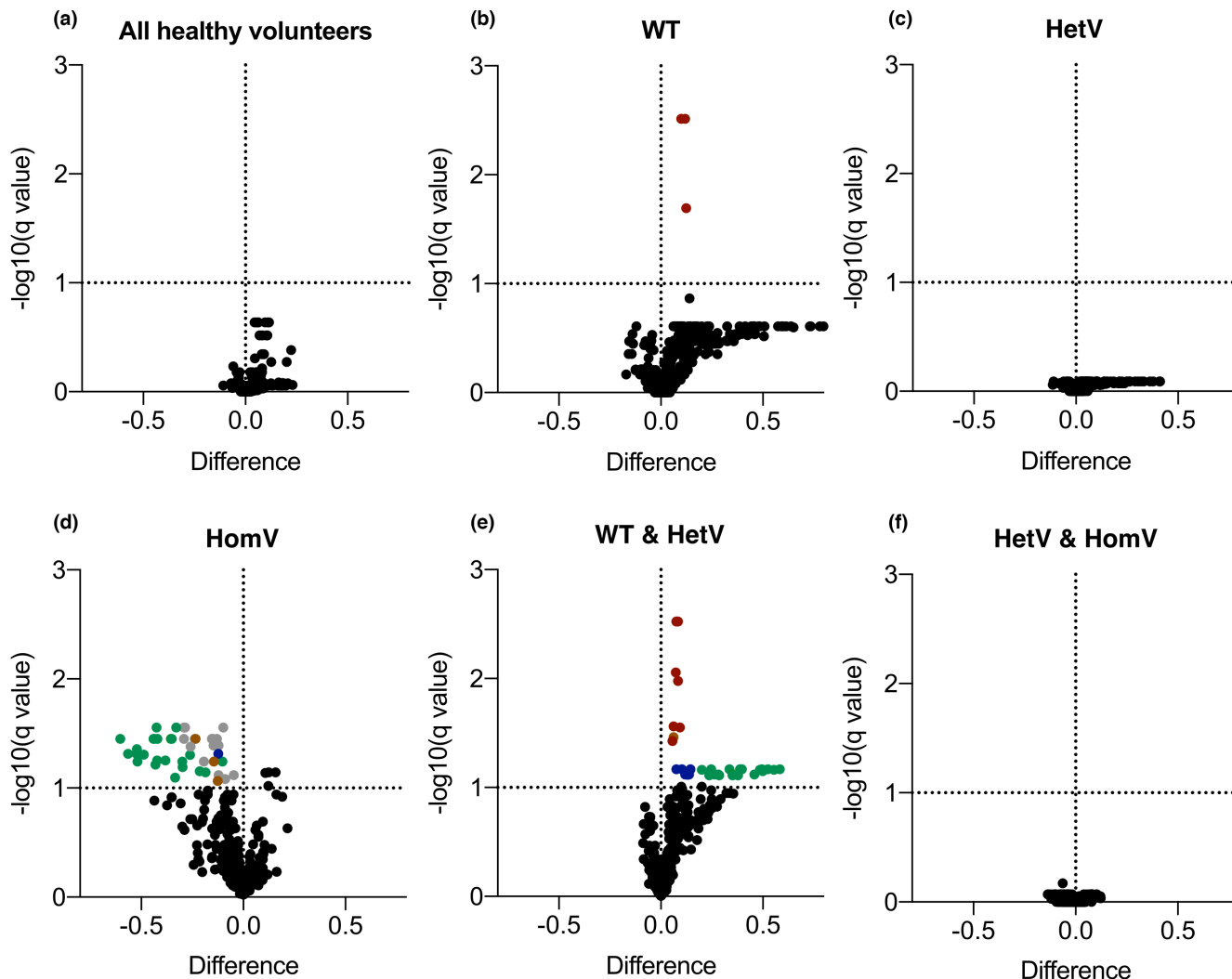
100% of WT. In contrast, an increase of 34.6% ( $p < 0.01$ ) in PE 16:0/20:4 was observed in HomV (Figure 5a,b) and was found in 80% of corresponding samples.

In addition, clarithromycin decreased the plasma levels of some PI in WT and HetV subjects, for instance, PI (18:0/20:4) in WT (6.6%,  $p < 0.05$ ) and HetV (3.8%,  $p < 0.01$ ). The decrease in PI (18:0/20:4) involved 80% of WT and HetV subjects (Figure 5c).

Several PCs showed higher plasma levels after clarithromycin administration in HomV but not in WT and HetV. Indeed, PC(17:0/14:1), PC(16:0/18:3), and PC(14:0/18:3) exhibited mean concentration increases of  $\sim 24\%$  ( $p < 0.001$ ),  $10\%$  ( $p < 0.001$ ), and  $23.6\%$  ( $p < 0.001$ ), respectively. This increase was observed in 100% of HomV samples (Figure 6a–c).

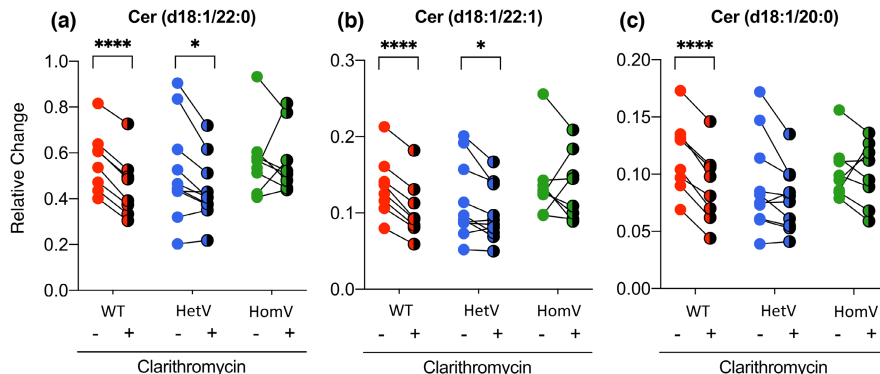
### Ratios Cer/PC

Plasma concentrations of Cer(d18:1/22:0), Cer(d18:1/22:1), Cer(d18:1/20:0), and PC(17:0/14:1), PC(16:0/18:3), and PC(14:0/18:3) determined in the three *ABCB1* genotype groups were used to calculate ratios Cer/PC prior to clarithromycin intake (Figures S2 and S3). Ratios Cer/PC(14:0/18:3) were maximal and were used to compare the three groups. The ratio of Cer(d18:1/22:0)/PC(14:0/18:3) was higher in HomV than in WT ( $p < 0.05$ ) and HetV ( $p < 0.01$ ). Cer(d18:1/22:1)/PC(14:0/18:3) increased in HomV compared to WT ( $p < 0.05$ )



**FIGURE 3** Volcano-plot of the global lipidomic analysis. Volcano-plot of the 343 lipids identified in healthy volunteers ( $n=26$ ) prior and following clarithromycin intake (a), in WT healthy volunteers ( $n=8$ ) (b), in HetVs ( $n=10$ ) (c), in HomVs ( $n=8$ ) (d), in WT and HetVs ( $n=18$ ) (e) and in HetV and HomVs ( $n=18$ ) (f). The colored points represent the lipids displaying a change in plasma level following clarithromycin intake: ceramides (●), phosphatidylinositol (●), phosphatidylethanolamine (●), phosphatidylcholine (●) and triacylglycerol (●). ( $p < 0.05$  and FDR-adjusted  $p < 0.1$ ). FDR, false discovery rate; HetV, heterozygous variant; HomV, homozygous variant; WT, wild-type.

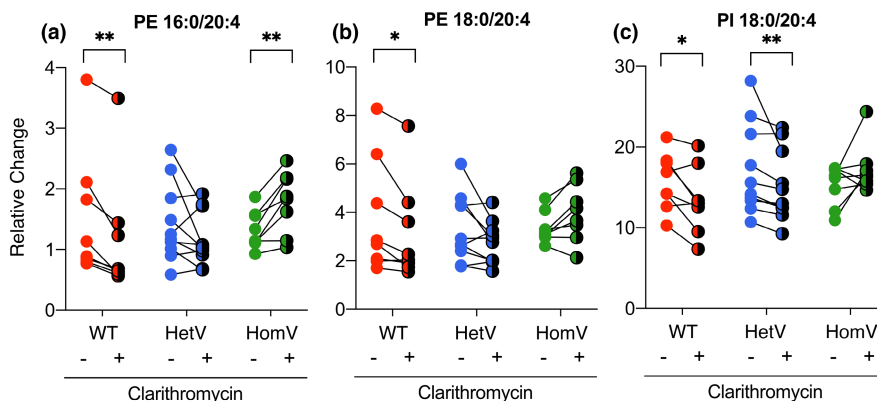
**FIGURE 4** Ceramides plasma levels. Variations in plasma levels of Cer(d18:1/22:0) (a), Cer(d18:1/22:1) (b), Cer(d18:1/20:0) (c) following clarithromycin intake ( $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ ). Cer, ceramide; HetV, heterozygous variant; HomV, homozygous variant; WT, wild-type.



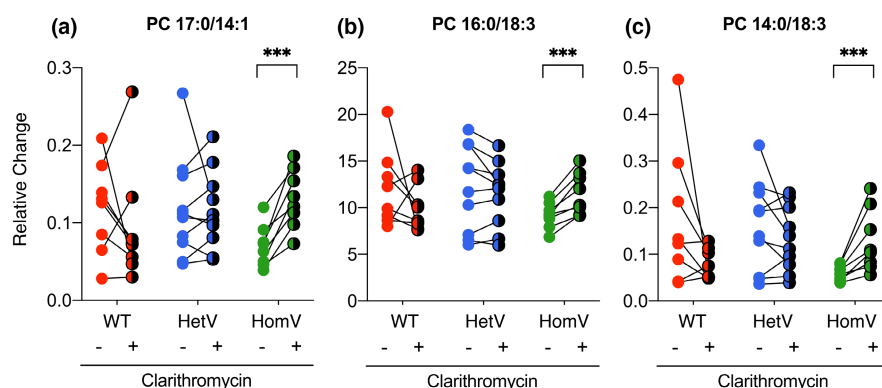
and HetV ( $p < 0.01$ ). Finally, the ratio of Cer(18:1/20:0)/PC(14:0/18:3) was higher in HomV than in HetV ( $p < 0.05$ ; Figure 7a–c). In addition, the Cer/PC ratios were increased

when HomV was compared to WT and HetV together (Cer(d18:1/22:0),  $p < 0.001$ , Cer(d18:1/22:1),  $p < 0.001$ , and Cer(d18:1/20:1),  $p < 0.01$ ; Figure 7d–f).





**FIGURE 5** Phosphatidylinositol and phosphatidylethanolamine plasma levels. Variations in plasma levels of PE 16:0/20:4 (a), PE 18:0/20:4 (b), and PI 18:0/20:4 (c) following clarithromycin intake ( $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ ). HetV, heterozygous variant; HomV, homozygous variant; PE, phosphatidylethanolamine; PI, phosphatidylinositol; WT, wild-type.



**FIGURE 6** Phosphatidylcholine plasma levels. Variations in plasma levels of PC 17:0/14:1 (a), PC 16:0/18:3 (b), PC 14:0/18:3 (c) following clarithromycin intake ( $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ ). HetV, heterozygous variant; HomV, homozygous variant; PC, phosphatidylcholines; WT, wild-type.

## DISCUSSION

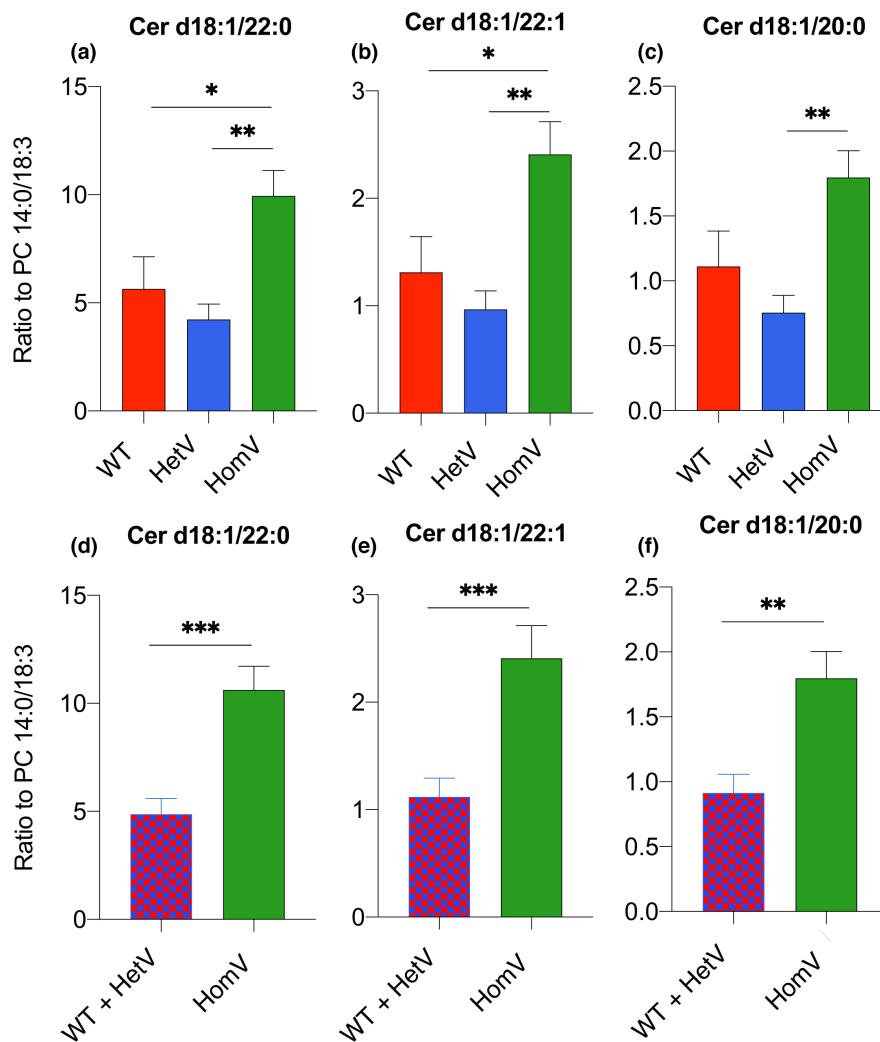
In addition to cytochrome P450 enzymes, it is now recognized that drug transporters and, more specifically, P-gp have a major impact on drug bioavailability. Due to its wide range of drug substrates, P-gp is potentially involved in many DDIs, leading to significant iatrogenic damage, especially in frail and polymedicated patients. Because *ABCB1* genotyping is insufficient to predict P-gp activity, identification of endogenous probes associated with the activity of this efflux drug transporter would be valuable in routine clinical practice to perform noninvasive phenotyping and thus better manage DDI. In this study, we characterized significant plasma lipidome changes among 26 healthy volunteers selected according to *ABCB1* genotype (WT, HetV, and HomV) who received the P-gp inhibitor clarithromycin for 5 days.<sup>31</sup> The results show different plasma changes in several ceramides and phospholipids depending on the *ABCB1* genotype.

The untargeted analysis performed in plasma samples obtained from healthy volunteers identified and semiquantified 343 lipids belonging to five classes and 10 subclasses. Depending on the *ABCB1* genotype, changes in plasma levels of several lipids were induced by clarithromycin.

Indeed, in WT subjects, clarithromycin intake induced a decrease in Cer (d18:1/22:0), Cer (d18:1/22:1), and Cer (18:1/20:0). In HomV, 10 PCs were increased, and three PIs were decreased. Considering WT and HetV together, clarithromycin intake was associated with a decrease in the concentration of eight ceramides, seven PE, and one PI. Hereafter, the aforementioned lipids will be referred to as “discriminant lipids.”

In the present study, clarithromycin intake induced a moderate decrease in plasma Cer (d18:1/22:0), Cer (d18:1/22:1), and Cer (d18:1/20:0) levels in WT and, to a lesser extent, a decrease in Cer (d18:1/22:0) and Cer (d18:1/22:1) in HetV. This change in plasma Cer homeostasis was observed in 100% of WT and 70% of HetV individuals but not in HomV individuals. This result could indicate a P-gp-mediated cellular efflux of this lipid species. This hypothesis is supported by the fact that in HetV subjects, a codominance pattern for SNPs 3435 and 2677 of *ABCB1* is known to induce a decrease in P-gp expression and efflux capacity, respectively.<sup>44,45</sup> P-gp was previously shown to be involved in the intracellular transport of sphingolipids between the cytoplasm and Golgi apparatus in cancer cells with a multidrug resistance phenotype.<sup>46</sup> Moreover, in transfected Madin-Darby canine kidney cell lines treated

**FIGURE 7** Ratio between three ceramides and the phosphatidylcholine PC(14:0/18:3). In these different graphs, three ceramides, namely cer(d18:1/22:0), cer(d18:1/22:1) and cer(d18:1/20:1), are presented as a ratio with PC(14:0/18:3) within the three different groups of healthy volunteers (a–c) or considering healthy volunteers WT and HetV within the same group (d–f). Significance is set for an alpha risk at 0.05 (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ). Cer, ceramide; HetV, heterozygous variant; HomV, homozygous variant; PC, phosphatidylcholines; WT, wild-type.



with cadmium, P-gp mediates ceramide efflux, preventing cell apoptosis.<sup>47</sup> With respect to PC, in 100% HomV, a moderate increase in PC(17:0/14:1), PC(16:0/18:3), and PC(14:0/18:3) was displayed, but no change was found in the WT and HetV samples. P-gp has been previously described as exhibiting flippase activity, and this efflux protein switches xenobiotics as well as short-chain phospholipids from the inner to the outer leaflet of the plasma membrane.<sup>48,49</sup> Finally, many ABC family transporters are known to exert flippase activity on endogenous membrane lipids.<sup>50,51</sup> The ubiquitous nature of P-gp and its expression both at the plasma membrane and at the membrane of certain cell organelles suggest that the direction of variation of discriminating lipids may change according to the nature of the lipids found in plasma, which could explain the results obtained concerning ceramides and phosphatidylcholines. We found no differences in plasma levels of discriminant lipids among our three groups of healthy volunteers prior to clarithromycin administration. We speculate that this may be due to adaptive mechanisms to maintain cellular homeostasis of lipid levels, depending on *ABCB1* genotype. However, the use of clarithromycin

allowed for us to demonstrate a decrease in some ceramides in WT subjects and, conversely, an increase in some PCs in HomM. Because of this “mirror” regulation, Cer/PC ratios were calculated from the concentrations of the discriminating lipids belonging to these two subclasses, allowing us to demonstrate an anti-correlation between two lipid classes.<sup>52</sup> It helps to erase as much interindividual variability as possible within human plasma samples. Thus, our analyses allowed us to characterize three Cer/PC ratios that could correlate with P-gp activity.

The analytical procedure used allowed us to detect and semiquantify clarithromycin among the healthy volunteers. These data were used to check drug compliance during the study protocol and to assess a putative correlation between clarithromycin and plasma levels of discriminant lipids. Although clarithromycin was detected in all plasma samples, indicating good adherence with the protocol of the volunteers studied, no significant change in drug plasma levels was observed between the three *ABCB1* genotype groups. Furthermore, no correlation between clarithromycin plasma concentrations and plasma levels of discriminant lipids was found. Thus, the

variations in lipid plasma levels are specifically related to the *ABCB1* genotype of the healthy volunteers and not to clarithromycin compliance. Furthermore, the clear clustering of the injection replicates corresponding to QC 1/1, QC 1/3, and QC 1/6 validated the analytical quality of the data obtained in ESI+ and ESI-, confirming that the differences between the biological samples are not related to analytical but biological variations.

The substrates of P-gp are mostly hydrophobic compounds, and several studies suggest that there are interactions between P-gp and some classes of lipids.<sup>28–30</sup> Indeed, using a pharmacometabolomic approach to phenotype P-gp, the lipidome-based strategy, including the most apolar fraction of the metabolome, appeared to be the most appropriate for discovering endogenous probes correlated with P-gp activity. The untargeted lipidomic analysis performed here has several advantages and some limitations. Since the advent of this discipline in 2003, numerous technical advances have allowed the comprehensive analysis of hundreds of lipids in a single analysis of biological samples.<sup>53</sup> Our method of lipid annotations published elsewhere facilitates and increases the reliability of lipid identification in lipidomic analysis.<sup>36</sup> This strategy allows for the identification of a large part of the lipidome despite the limited number of commercially available standard lipids. However, the method used does not identify certain classes of lipids that may be substrates of P-gp, such as sterols.<sup>54,55</sup> Because of the ubiquitous tissue distribution of P-gp, it is not possible to establish the source of identified lipids from plasma samples. In addition, there are methodological differences within the lipidomic community that can lead to significant inter- and intralaboratory variability in terms of lipid detection and quantification.<sup>56</sup> For this purpose, Bowden et al. attempted to define typical values for each lipid species in SRM-1950 (the commercial standard used for inter-laboratory studies) from 31 different laboratories.<sup>56</sup> It was satisfying to find that each lipid subclass identified in our study was on the same order of magnitude as in SRM-1950.

The choice to use clarithromycin as a P-gp inhibitor is also questionable. It is a validated P-gp inhibitor in *in vivo* studies and is relatively safe. However, clarithromycin is an antibiotic and can therefore lead to changes in the microbiome. Clarithromycin also interacts with the OATP1B1 and OATP1B3 transporters (uptake transporters), the MRP2 transporter (efflux transporter), and cytochrome P450 3A4, which may also be responsible for interindividual variability in our group of healthy volunteers.<sup>57</sup> Here, the lack of lipidome changes regardless of *ABCB1* genotype allows for us to assume that there is no link between the observed discriminant lipids and these other metabolic actors or microbiome modifications. Moreover, there were only few individuals with the *CYP3A5\*3* and *CYP3A4\*22* genotypes and were distributed across the *ABCB1* haplotype groups (two HetV in WT *ABCB1* and HetV *ABCB1*

group for *CYP3A5\*3*, one HetV in WT *ABCB1* and HetV *ABCB1* group and two HetV in HomV *ABCB1* group for *CYP3A4\*22*). We can therefore assume that this has very little influence on the results.

Another main limitation of our study is the small number of healthy volunteers enrolled. A validation cohort on a larger number of healthy volunteers would be necessary to reproduce the results. The homogeneous character of our cohort of healthy volunteers in terms of age, sex, and lifestyle represents a suitable exploratory mode for the search for P-gp biomarkers, as it enables variability outside the *ABCB1* genotype to be limited to a maximum. However, further studies involving a larger population will be needed to extrapolate the results to patients. In addition, the lack of established correlation between the different Cer/PC ratios identified and probe drugs of P-gp activity was a limitation. Further experimental approaches on cell lines or *in vivo* studies are needed to assess correlations between the Cer/PC ratios and the metabolic ratio obtained by using a validated probe drug (e.g., digoxin) for P-gp activity. This study also fails to confirm that the discriminant lipids are P-gp substrates, as the variations observed may be the result of co-regulatory mechanisms between *ABCB1* and genes encoding lipid metabolism enzymes.<sup>46</sup> Broader *in vitro* mechanistic studies are therefore required to substantiate these hypotheses.

## CONCLUSION

Based on a lipidomic analysis encompassing 10 lipid subclasses, our study reports, for the first time, changes in plasma lipid levels according to *ABCB1* genotype and P-gp activity among healthy volunteers. Indeed, significant variations in several ceramide and phospholipid species were observed according to *ABCB1* genotype and upon clarithromycin intake. Our results may reflect the involvement of P-gp flippase activity in ceramide and phospholipid species. In this context, different ceramides and phospholipids were characterized as endogenous and noninvasive biomarkers related to *ABCB1* genotype and P-gp activity in the presence of an inhibitor, not requiring administration of exogenous compound to assess P-gp activity *in vivo*. Using an untargeted lipidomic analysis, our study provides an efficient strategy for the identification of biomarker candidates of potential interest in the therapeutic management of P-gp drug substrates or interfering molecules.

## AUTHOR CONTRIBUTIONS

T.D., R.M., N.A., M.A.L., and O.L. wrote the manuscript. T.D., R.M., O.L., and M.A.L. designed the research. T.D., R.M., I.G.-T., X.D., P.M., J.-E.S., A.B., C.N., and M.P. performed the research. T.D., R.M., N.A., M.A.L., and O.L. analyzed the data.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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