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ORIGINAL ARTICLE

Cytochrome P450 phenotyping using the Geneva cocktail improves metabolic capacity prediction in a hospitalized patient population

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Abstract

Aims: Liver cytochromes (CYPs) play an important role in drug metabolism but display a large interindividual variability resulting both from genetic and environmental factors. Most drug dose adjustment guidelines are based on genetics performed in healthy volunteers. However, hospitalized patients are not only more likely to be the target of new prescriptions and drug treatment modifications than healthy volunteers, but will also be more subject to polypharmacy, drug–drug interactions, or to suffer from disease or inflammation affecting CYP activities.

Methods: We compared predicted phenotype based on genetic data and measured phenotype using the Geneva cocktail to determine the extent of drug metabolizing enzyme variability in a large population of hospitalized patients (>500) and healthy young volunteers (>300). We aimed to assess the correlation between predicted and measured phenotype in the two populations.

Results: We found that, even in cases where the genetically predicted metabolizer group correlates well with measured CYP activity at group level, this prediction lacks accuracy for the determination of individual metabolizer capacities. Drugs can have a profound impact on CYP activity, but even after combining genetic and drug treatment information, the activity of a significant proportion of extreme metabolizers could not be explained.

Conclusions: Our results support the use of measured metabolic ratios in addition to genotyping for accurate determination of individual metabolic capacities to guide personalized drug prescription.

KEYWORDS

cytochrome P450, drug metabolism, genotyping, phenotyping

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1 | INTRODUCTION

Among the enzymes involved in liver drug processing, cytochromes from the P450 family (CYPs) stand out both in terms of the number of drugs they process and the extent of their interindividual variability. **CYP2D6**, **2C9**, **2C19**, **1A2**, **2B6** and **3A** are the six major players in terms of drug metabolism.[1,2] They metabolize about 70–80% of all currently commercialized drugs. However, differences in expression and protein levels can reach over 100-fold with subsequent effect on enzymatic activities.[1,3] The resulting differences in metabolizing capacities arise from a combination of intrinsic and external environmental factors, such as genetic polymorphisms, drug–drug interactions, liver diseases, dietary habits, smoking or inflammation, respectively.[1,4,5]

Study of polymorphisms affecting CYP activities has led to the establishment of prediction algorithms enabling the computation of activity scores and classification of individuals in distinct metabolizer groups according to their genetic profile. This, in turn, has allowed the development of a number of genetic-based dose-adjustment guidelines to aid clinicians to adapt drug prescriptions, taking into consideration the variability in drug metabolizing enzymatic activities.[6,7] Today, thanks to these guidelines, genotyping has gained popularity for specific CYP-related drug dose adjustments in clinical practice.[8–14] Multiple tools predicting relevant drug–drug interactions and their effect on metabolism are available, allowing initiated physicians to take into account the contribution of environmental factors as well.[15–19]

Phenotyping is a more direct assessment of enzymatic activities than genotyping. It is performed by measuring the blood concentration of an enzyme-specific probe drug and its metabolite at a given time after probe drug administration. The readout of the assay is the ratio of the metabolite over the parent drug concentrations. Phenotyping has the advantage of taking into consideration not only the intrinsic metabolic capacity but also the combined contribution of environmental and medical factors influencing this capacity at any given time point.[7,20,21] Thus, while a single genotypic analysis provides life-long information, phenotyping should be considered as a snapshot of an individual's metabolizing capacity at the time of testing. Phenotyping needs to be repeated over time as the effects of external and even intrinsic factors might evolve.

What is known about this subject

- Interindividual variability in cytochrome P450 activity influence drug treatment response.
- International guidelines recommend determination of individual patient metabolizer status through inference from genetic information.
- Phenotyping is an alternative method for cytochrome activity assessment taking into account both genetic and environmental factors at a given time point.

What this study adds

- Large-scale phenotyping-genotyping comparison in heavily medicated hospitalized patients reveals important discrepancies between both measures.
- The quality of genotyping-phenotyping correlation in healthy individuals is highly enzyme dependent with: CYP2D6 and CYP2C19 > CYP2C9 and CYP1A2 > CYP2B6 and CYP3A.
- Significant numbers of individuals with extreme phenotype that are at high risk of adverse drug reactions are not detected through genotyping and concomitant medication intake.

Pharmacological studies are mostly conducted in healthy volunteers, reducing clinical risks and interindividual variability.[22] However, hospitalized patients are more likely to be exposed to new drug prescriptions or drug treatment modifications. Polypharmacy, disease-related complications and increased vulnerability, all increase the risk of adverse drug reactions. It is therefore important to determine whether guidelines, based mostly on data from healthy volunteers, are reliable models for hospitalized patients. We therefore compared genotyping and phenotyping data from more than 300 healthy volunteers and 500 hospitalized patients receiving antithrombotic drugs for cardiovascular problems. We calculated tolerance intervals based on our healthy volunteer cohort, identified individuals with extreme

metabolic activities and investigated the possible causes of their uncommon metabolic capacities. Our patient cohort represents a relatively global hospital population compared to previous studies focusing on sensitive (i.e., paediatric or psychiatric) or specific patients preselected on the basis of their unusual drug response.[23–25]

2 | METHODS

2.1 | Study population

The healthy volunteer population was recruited within the frame of a study on the evolution of absorption, distribution, metabolism and excretion (ADME) genes diversity and their corresponding phenotypic profiles in four distinct human populations located along the latitudinal transect from Addis Ababa (ADD, Ethiopia), to Muscat (MUS, Oman) and Alexandroupolis (ALE, Greece) and Prague (PRA, Czech Republic). In brief, healthy volunteers were recruited among students and staff belonging to the participating universities, as described in Mouterde et al.,[26] with samples collected from approximately 100 participants per country between November 2015 and March 2017. Information obtained for the healthy cohort included age, gender and body mass index (BMI), as well as usage habits of tobacco, alcohol, khat and contraceptive pill. Further information on the healthy cohort study is reported in Mouterde et al.[26] and Rollason et al.[27]

The patient population was recruited during two clinical studies on patients suffering from cardiovascular diseases hospitalized in the University Hospital of Geneva between June 2017 and July 2021. These two studies were known under the acronyms Daphne (Direct Anticoagulant PHarmacogeNEtic) and OptimAT (Antithrombotic Therapeutic Optimization in Hospitalized Patients Using Physiologically and Population-based Pharmacokinetic Modeling).[28,29] Available patient information includes BMI, age, gender, comedication, presence of active cancer or cirrhosis as well as liver and renal function and prescribed drug treatment.

The healthy volunteer study was approved by the Ethics Committee of Geneva (CCER 2015-169), as well as by the Institutional Review Board of Charles University, Faculty of Sciences, Prague (Czech Republic); the National Research Ethics Review Committee and the Food, Medicine and Healthcare Administration and Control Authority of Ethiopia, Addis Ababa (Ethiopia); the Medical Research and Ethics Committee of Sultan Qaboos University, Muscat (Sultanate of Oman); and the Research Ethics Committee of Democritus, University of Thrace (Greece) for the Czech, Ethiopian, Omani and Greek healthy volunteers, respectively.[26,27] Both patient studies were approved by the Ethics Committee of Geneva (CCER 2016-01490 and CCER 2017-00225).

All studies respected the principles of the Declaration of Helsinki and were conducted according to national laws directing research on human subjects. All participants gave their written informed consent, and all studies have been registered on Clinicaltrial.gov under accession numbers NCT02789527, NCT03112525 and NCT03477331, respectively.

2.2 | Cytochrome P450 genotyping and CYP2D6 copy number determination

We collected a total of 323 saliva samples from healthy controls and 532 blood samples from patients for genotyping. DNA extraction was performed using standard commercial extraction kits and quantified either with a nanodrop (healthy volunteers) or the Qubit dsDNA BR assay (Thermo Fisher Scientific) (patient samples). All DNA samples were genotyped using an OpenArray® PGx Express panel operated on a QuantStudio 12K (Thermo Fisher Scientific). The *CYP2D6* copy number assay was performed in triplicate using TaqMan® probes from Thermo Fisher Scientific (Hs00010001_cn) located in exon 9 relative to the RNase P reference gene. Raw genetic data were processed using the Genotyper™ and CopyCaller™ software provided by Thermo Fisher Scientific. All genotyping techniques were performed with commercially available kits used according to manufacturer's instructions. OnlySNPs and samples with >95% completeness were retained, providing 838 (98%) successfully genotyped participants for the main analysis. Final SNP and sample mean genotyping call rates exceeded 99.7% both for the healthy participant and the patient cohort.

Detailed genotype assignment procedures are described in Supplementary Material S1. Default star allele nomenclature were assigned from the respective genotype of every study participant according to the Clinical Pharmacogenetics Implementation Consortium (CPIC) nomenclature guidelines, when available, or otherwise from the Pharmacogene Variation (PharmVar) Consortium database.[6,30,31] Activity scores and metabolizer status were derived from the star allele nomenclature according to the consensus guidelines available on the PharmGKB website (https://www.pharmgkb.org/page/pgxGeneRef).[32,33] CYP1A2 predicted activities were adjusted for smoking as previously described.[15]

2.3 | Cytochrome P450 phenotyping with the Geneva cocktail

All patients and healthy volunteers were phenotyped after overnight fasting using the Geneva cocktail approach.[34] Smoking and drinking habits as well as usual medication intake were recorded although abstention in the last 12 h before phenotyping was required for healthy volunteers. For patients receiving omeprazole as medication, its administration was suspended 24 h before phenotyping. The Geneva cocktail is composed of 50 mg **caffeine** (CYP1A2), 20 mg **bupropion** (CYP2B6), 10 mg **flurbiprofen** (CYP2C9), 10 mg **omeprazole** (CYP2C19), 10 mg **dextromethorphan** (CYP2D6), 1 mg **midazolam** (CYP3A4 and CYP3A5) and 25 mg fexofenadine (for P-glycoprotein transporter activity).[35] Despite the availability of fexofenadine transport kinetics from the phenotyping experiment, the current analysis was restricted to cytochrome activities as the PGx express array does not contain polymorphisms related to *ABCB1* gene. Due to a supply issue, no omeprazole was administrated to Greek healthy volunteers, which thus lack CYP2C19 phenotyping.[26]

Capillary blood samples were collected into a microfluidic system (HemaXis DB10, HemaXis, Switzerland) from a small finger prick (BD Microtainer, Contact-Activated Lancet, Plymouth, UK) 2 hours after cocktail intake.[34,35] The blood drop was applied at the entrance of the microfluidic channel. Once filled, the cover containing filter paper card (Perkin Elmer 226 Bioanalysis Card) was folded, transferring the exact blood volume onto the card. Blood spots were then kept at room temperature for 30 minutes to dry and sent to the clinical pharmacology and toxicology laboratory. Samples were stored at −20°C until analysis. The same team supervised the sample collection at all different sites.

The cocktail substrates and their CYP-specific metabolites were quantified in dried blood spots using the original high-performance liquid chromatography–tandem mass spectrometry method developed by Bosilkovska et al.[34] Samples from all the clinical trials were analyzed in the same laboratory at Geneva University Hospitals. The method was fully validated according to Food and Drug Administration (FDA) guidelines. Accuracy was included in the interval 92.2–111.1% for all drugs and their respective metabolites, whereas both intra-day and inter-day precision were below 11%.

2.4 | Tolerance interval calculation and extreme metabolizer status classification

To define patients with extreme metabolizer phenotypes, we calculated tolerance intervals based on normal metabolizers from the healthy volunteer cohort. Tolerance intervals mark the limits within which the values of a parameter (in our case the measured metabolic ratio [MR]) belonging to a defined proportion of a population will fall, based on a given confidence interval (CI). Compared to confidence intervals that describe sampling errors, tolerance intervals are larger and take into consideration not only sampling errors but also the true variance within a population. For each of the cytochromes except CYP3A5, we selected healthy volunteers carrying the reference **1/*1* genotype for the analysis. As 92% of the European population carries the *CYP3A5 *3* reduced activity allele, the *CYP3A5 *3/*3* combined with the *CYP3A4 *1/*1* genotype was chosen as the reference group for CYP3A activity, although it corresponds to a phenotypically intermediate metabolizer status. For CYP1A2 activity, we retained only non-smokers for the calculations considering the well-known impact of smoking on CYP1A2 induction.[36]

To avoid population structure bias resulting from the recruitment of the healthy control cohort in four different geographical areas in the establishment of the tolerance intervals, we tested the homogeneity of the groups. We probed the homogeneity of the population using the Kruskal-Wallis one-way ANOVA test followed by pairwise Wilcoxon rank sum test to determine if any of the populations differed significantly from the others.

Extreme metabolizers were defined as those with values outside the tolerance interval.

2.5 | Drug–enzyme interaction approach

For each patient we determined for each cytochrome whether the treatment received contained CYP inducers or inhibitors according to the information in Supplementary Material S6. Following identification of the extreme metabolizers we determined the proportion of them that could be attributed to either genetic and/or concomitant drug intake.

2.6 | Statistical analysis

All statistical calculations were performed using R (v3.5.1) and R studio (v1.1.463), PLINK (v1.07) and Microsoft® Excel (v 16.16.3).

We used Hardy Weinberg equilibrium (HWE) to assess genetic data integrity with a *P*-value cut-off set at .05/50 = 1E-03 following Bonferroni correction for multiple testing. Genetic consistency was determined by comparing obtained minor allele frequencies (MAFs) with databases reference for respective populations[6,37] as well as in-between the different cohorts integrated in the study. We used Spearman's rank correlations to assess predicted activity score versus measured MRs. Pearson's chi-square test was used to compare groups defined by categorical variables while the Welch two sample *t*-test was used for continuous variables. Tolerance intervals were calculated using the R Tolerance interval package for 90% of the population with a 95% confidence. Kruskal-Wallis ANOVA was used to test group consistencies and pairwise Wilcoxon rank sum tests for between-group comparison. Correlation between categorized clinical or demographic data with extreme metabolizer phenotypes was tested using Chi-square test, while for continuous variables such as age or BMI, we used the Welch *t*-test. For continuous laboratory variables (ASAT, ALAT, etc.), clinically relevant increase was defined as gender specific 3× the upper limit of normal (ULN) except for creatinine clearance where the cut-off for significant impairment was set at <60 mL/min/1.73m^2. Statistical test results are reported using APA guidelines.

The threshold for statistical significance was set at a *P*-value of <.05. Bonferroni multiple test corrections were applied where relevant.

2.7 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to Pharmacology, and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/24.[38]

3 | RESULTS

3.1 | Study population description and genetic data integrity

All patients were recruited at Geneva University Hospitals and the cohort is considered as being mainly of European (EUR) origin. The healthy volunteer cohort is composed of Ethiopian, Omani, Greek and Czech participants.

Demographic data are presented in Table 1. Considerable lifestyle differences are apparent in the four groups composing the healthy volunteer population, with much higher frequencies of tobacco and alcohol consumption in Europe than in Arabia or East Africa. Compared to healthy volunteers, the patient population is mostly of European origin (93.9%), significantly older and heavier, and has similar smoking and drinking habits as other European participants. The majority of the patients received anticoagulants or antithrombotic medication for either atrial fibrillation ($n = 233$), coronaropathy ($n = 199$) or venous thromboembolism ($n = 63$). Although generally very diverse, the prominent causes of hospitalization were cardiac or respiratory complications and infections. Of the 526 patients in the study, 20 (3.8%) had active cancers, seven (1.3%) suffered from cirrhosis and three (0.6%) patients had both. On average, each patient was taking five drugs. Clinical parameters describing the patient population are reported in Supplementary Material S4. There is a notable gender imbalance between the patients and the Ethiopian (ADD) volunteers versus the other groups.

HWE is conserved for all SNPs both in the patient and the control population, with the exception of *CYP2D6*-associated rs16947 ($P = 7E-04$) in the healthy volunteer group, possibly reflecting a bias resulting from the combination of four different populations. Indeed, the MAF for this common SNP varies considerably between world populations and the HWE is conserved in each single population when the four healthy volunteer groups are analyzed separately.

The choice of populations in the healthy volunteer study[26] limits the use of global cohort MAFs to control the consistency of the genotyping data, especially for polymorphisms with high interpopulation variability. The pooled MAFs for the two European populations (ALE, PRA) correlate well with expected values from the reference EUR population from the 1000 Genomes Repository reported in the Ensembl browser (Pearson correlation: $r(48) = .99$, $P < 2.2E-16$). No specific reference data for Ethiopian or Omani populations are available from the Ensembl browser, and MAF correlations between our study data and the closest related populations (AFR, SAS respectively) are relatively poor (Supplementary Material S2A). However, comparison between the star allele frequencies in our study populations and the ones from respective CPIC populations (Europe, Sub-Saharan Africa and the Near East) confirms the good correlation between our two European populations and reference values, with $r(20) = 1.00$, $P < 2.2E-16$. The population recruited in Oman shows also a very good correlation with reference values for the Near Eastern group, with $r(18) = 0.99$, $P < 2.2E-16$. The Sub-Saharan group remains a relatively poor proxy for our Ethiopian population ($r(20) = 0.77$, $P = 2.3E-05$), probably due to the very large diversity of the included populations. However, restricting the comparison to the two studies reporting only Ethiopian populations increases the correlation to $r(17) = 0.96$, $P = 2.8E-11$ (Supplementary Material S2B).

TABLE 1 Population characteristics.

	Healthy controls							Patients *vs.* all controls[b]
	All	ADD	MUS	ALE	PRA	EUR[a]	Patients	*P*-value
No. of participants	312	93	53	93	73	166	526	-
Gender								
Women/men	172/140	29/64	37/16	57/36	49/24	106/60	165/361	1.97e-11
% women	55.1	31.2	69.8	61.3	67.1	63.9	31.4	
Age [min.-max.][b]	23.3 ± 5.2 [18-45]	21.0 ± 2.0 [18-32]	27.5 ± 6.7 [19-45]	21.9 ± 4.3 [18-44]	25.2 ± 5.3 [20-44]	23.3 ± 5.0 [18-44]	70.9 ± 12.8 [24-97]	< 2.2e-16
BMI [min.-max.]	22.36 ± 3.62 [12.6 – 38.8]	21.00 ± 2.49 [16.9 – 28.6]	23.46 ± 4.35 [12.6 – 32.4]	22.93 ± 3.59 [16.2 – 38.8]	22.56 ± 3.89 [16.8 – 38.8]	22.77 ± 3.72 [16.2 – 38.8]	27.02 ± 5.38 [15.0 – 50.9]	< 2.2e-16
Smoking								
Yes/no	35/277	1/92	0/53	16/77	18/55	34/132	122/404	2.63e-05
% smokers	11.2	1.1	0.0	17.2	24.7	20.5	23.2	
Alcohol								
Yes/no	173/139	27/66	0/53	75/18	71/2	146/20	295/231	n.s.
% consumers	55.4	29.0	0.0	80.6	97.3	87.9	56.1	

Abbreviations: ADD = Addis Abeeba (Ethiopia), MUS = Muscat (Oman), ALE = Alexandroupolis (Greece), PRA = Prague (Czech Republic), EUR = European.

[a]EUR = PRA + ALE.

[b]*t*-test or Chi-square test for difference between patient and healthy controls (all). Multiple testing adjusted *P*-value cut-off < 0.05/6 = 8.3E-03.

For the patient population, the MAFs correlate very well with expected values for the European reference population, $r(48) = 1.00$, $P < 2.2E-16$, as well as with healthy controls of European origin from the current study, $r(48) = 0.98$, $P < 2.2E-16$ (Supplementary Material S2C).

3.2 | CYP activity prediction potential in healthy controls

Having established that the genotypes of the healthy volunteer cohort accurately reflect respective population-specific profiles, we looked at the correlation between the measured MRs and the activity score computed for each CYP enzyme. Spearman's rank correlations for all healthy volunteers clearly highlight the differences in predicting the enzymatic activity of the different CYPs (Table 2). While the correlations obtained for CYP2D6 and CYP2C19 are highly significant, and those for CYP2C9 and CYP1A2 above the *P*-value threshold level, those for CYP2B6 and CYP3A activities are not significant. Restricting the correlation analysis to healthy volunteers from Europe, in order to avoid possible bias resulting from inclusion of populations with different genetic backgrounds, did not drastically change the observations, indicating that the current genotype-derived prediction potential accuracy is relatively limited (Table 2).

After classifying each volunteer to their respective metabolizer groups (UM for ultra-fast, NM for normal, IM for intermediate or PM for poor metabolizers) according to their genotype, we compared the measured enzymatic activity distribution across the different metabolizer groups for each CYP (Figure 1). As expected, we observed an increasing median MR trend along PM < IM < NM < UM metabolizer categories. However, although Wilcoxon rank sum tests showed statistically significant differences between some of the neighboring groups, the plots demonstrated considerable MR overlap between categories, hampering with MR cut-off determinations for metabolizer categories. Similar results were observed when restricting the analysis of MR distribution to healthy volunteers of European origin (Supplementary Material S3).

In summary, in the healthy individual cohort, CYP2D6 and CYP2C19 display the best correlations and metabolizer group discriminations, followed by CYP2C9 and CYP1A2, while no phenotype–genotype correlation could be observed for CYP2B6 nor CYP3A. Taken together, the results show a substantial variability in inferred CYP activity, limiting our capacity to predict actual enzymatic activity from genotype information only. Moreover, even for those CYPs associated with the highest correlation coefficients and statistically significant group differences, the observed overlaps between categories emphasize the difficulty in predicting MRs at the individual level.

3.3 | Variability in CYP metabolic ratios in patients

To assess the relative impact of different factors on MR variability associated with the different predicted groups of metabolizers, we reproduced the correlation analysis of predicted *vs.* measured enzymatic activity in the patient population (Table 2). Interestingly, while the correlation coefficients for CYP2C9 and CYP2D6 have relatively similar values between the patient and healthy cohorts, the ones between genotype-predicted activity score and measured MR for CYP1A2 and especially CYP2C19 appear lower in the patient group.

The boxplots depicting the MR distribution for each metabolizer group in the patient cohort are shown in Figure 2. Comparing the MR distributions in patients (Figure 2) versus healthy controls (Figure 1) reveals a global trend of increase in intragroup variability in patients, coupled with a considerable increase in the maximal MR values in the case of CYP2D6 and CYP2C9. This increase in variability and upper-level values is highlighted in Table 3, which reports the patient over healthy volunteer ratios for the calculated coefficients of variations (CVs) obtained for each metabolizer status, as well as the maximal MR value measured for each CYP. As shown in Table 3, not all CYPs and metabolizer groups are equally affected. The largest increase in variability is seen for CYP2C19 PMs, with CV reaching up to 9.3-fold higher values for patients than controls.

Intergroup differences for each CYP were re-assessed using pairwise Wilcoxon rank sum tests comparing MRs belonging to different metabolizer categories. In contrast to the healthy volunteer cohort, in the patient cohort the distinction between CYP2C9 metabolizer groups allows significant separation of the PM and IM categories (Figure 2). On the other hand, for CYP2C19, only a slight increase in the intragroup variability was observed, which was not coupled with

TABLE 2 Spearman's rank correlations between activity scores and measured metabolic ratios.

	Healthy controls (all)			Healthy controls (EUR)			Patient population		
	n	*r*	*P*-value[b]	*n*	*r*	*P*-value[b]	*n*	*r*	*P*-value[b]
CYP2D6	288	0.60	< 2.2E-16	155	0.71	< 2.2E-16	487	0.55	< 2.2E-16
CYP2C19	217	0.48	6.02E-14	72	0.57	1.56E-07	521	0.17	1.43E-04
CYP2C9	309	0.29	1.80E-07	163	0.28	2.04E-04	522	0.35	< 2.2E-16
CYP1A2[a]	226	0.29	1.02E-05	132	0.22	ns	436	0.14	3.02E-03
CYP2B6	312	0.01	ns	166	0.08	ns	523	0.16	1.58E-04
CYP3A	300	0.10	ns	158	0.05	ns	504	0.14	1.94E-03

[a]Including adjustment for tobacco smoking.
[b]Multiple testing adjusted *P*-value cut-off < 0.05/6 = 8.3E-03.

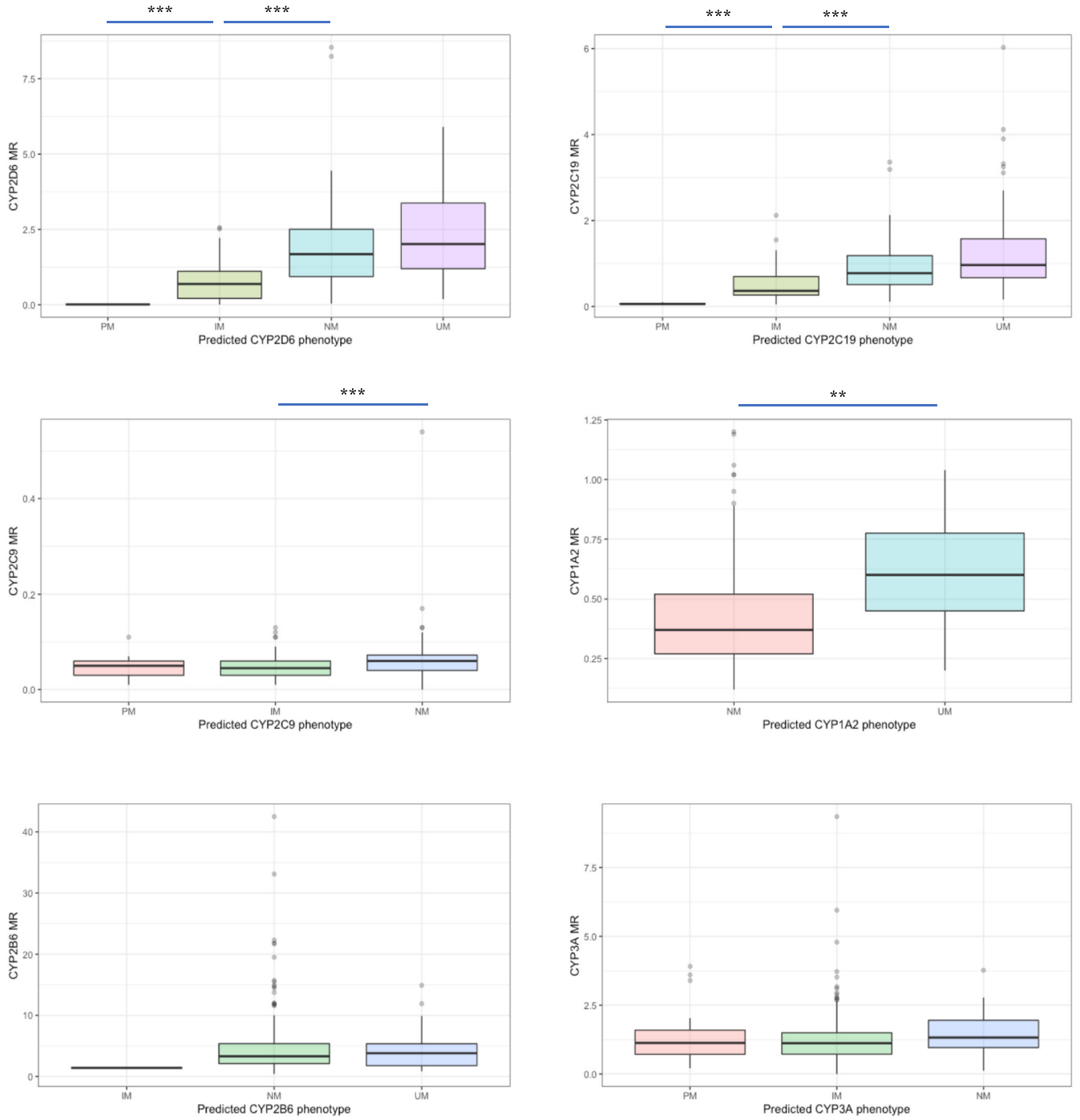

FIGURE 1 Metabolic ratio distribution according to metabolizer group in healthy volunteers. For each CYP enzyme tested, the metabolizer group per healthy volunteer was determined from its genotype and the MR distribution was plotted accordingly. "BH" multiple pairwise test adjusted *P*-value statistics corresponding to Wilcoxon rank sum test. ***$P < .0001$, **$P < .001$, *$P < .01$.

an increase in maximal value, resulting in an attenuation of metabolizer intergroup differences. Except for PMs, intragroup variability for the CYP2D6 metabolizer categories remained similar and, while the MR values are higher in patients than in healthy volunteers, the effect is less important than for CYP2C9. The distinction between metabolizer groups for CYP2D6 is similar in patients versus healthy volunteers, while for CYP1A2 and CYP2B6 the intergroup differences are more pronounced (Figures 1 and 2).

3.4 | Determination of MR boundaries for extreme metabolizer phenotypes

Considering the geographic heterogeneity of our healthy volunteer cohort, we found that the inter-population differences for the carriers of the reference genotype for CYP2D6, CYP2C19, CYP3A and CYP1A2 activity were not statistically significant. In contrast, the MUS (Oman) populations differed significantly from the

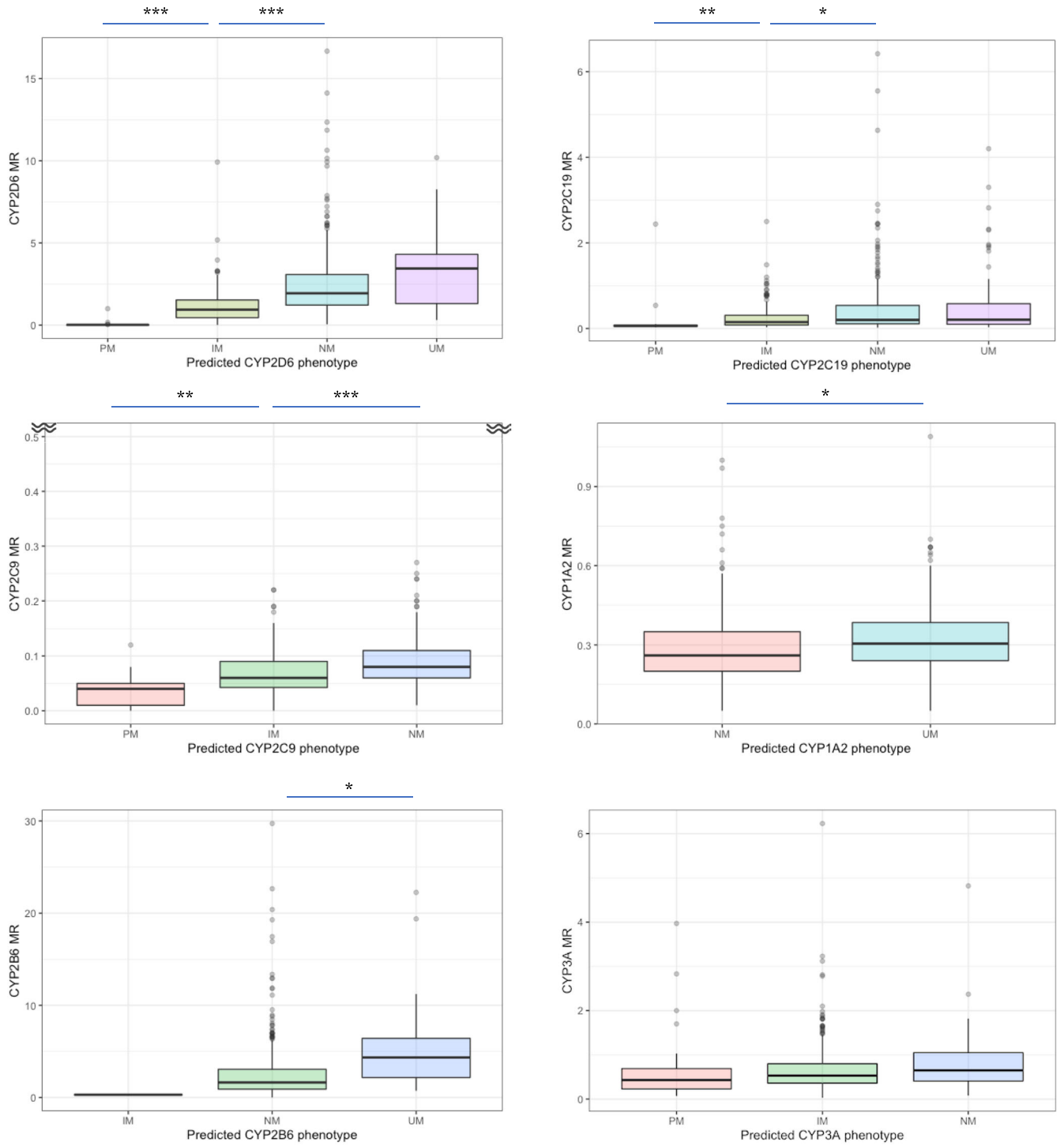

FIGURE 2 Metabolic ratio distribution according to metabolizer group in patients. For each CYP enzyme tested, the metabolizer group per patient was determined from its genotype and the MR distribution was plotted accordingly. For the sake of clarity, the extreme outliers (i.e. >5-fold increase relative to median value for rapid metabolizers) for CYP3A ($n = 13$) and CYP2C9 ($n = 6$) are not depicted on the corresponding graphs. "BH" multiple pairwise test adjusted *P*-value statistics corresponding to Wilcoxon rank sum test. ***$P < .0001$, **$P < .001$, *$P < .01$.

other populations for both CYP2C9 and CYP2B6 activities, while the ALE (Greece) differed from PRA (Czech Republic) in CYP2B6 only. The tolerance intervals were calculated excluding those populations. The details of the selection are presented in Supplementary Material S5. The 90% tolerance intervals were calculated on the chosen populations with a 95% CI. The results are displayed in Table 4, along with the number of participants used for these calculations and the corresponding descriptive statistics.

Application of tolerance intervals to the entire cohort of healthy volunteers reveals a number of outliers displaying either extremely

TABLE 3 Comparison between patient and healthy volunteer MR values.

			UM	NM	IM	PM	Min-max MR Max MR ratio
CYP2D6	Patient	*n*	16	285	164	26	$LLOD^2$–16.77
		MR	3.51 (±2.96)	2.48 (±2.25)	1.16 (±1.12)	0.06 (±0.19)	
	Healthy	*n*	30	155	94	9	$LLOD^2$–8.54
		MR	2.40 (±1.63)	1.84 (±1.30)	0.76 (±0.60)	0.01 (±0.01)	
	Ratio[a]	CV	1.2	1.3	1.2	**4.2**	Max MR: **2.0**
CYP2C19	Patient	*n*	158	217	128	18	0.02–6.42
		MR	0.44 (±0.61)	0.52 (±0.82)	0.28 (±0.35)	0.22 (±0.57)	
	Healthy	*n*	118	100	79	13	0.04–6.03
		MR	1.26 (±0.93)	0.93 (±0.61)	0.54 (±0.42)	0.06 (±0.02)	
	Ratio[a]	CV	1.9	**2.4**	1.6	**9.3**	Max MR: 1.1
CYP2C9	Patient	*n*	-	311	190	21	LLOD–1.93
		MR	-	0.10 (±0.11)	0.07 (±0.04)	0.04 (±0.03)	
	Healthy	*n*	-	208	94	7	LLOD–0.54
		MR	-	0.06 (±0.04)	0.05 (±0.02)	0.05 (±0.04)	
	Ratio[a]	CV	-	1.7	1.2	1.1	Max MR: **3.6**
CYP1A2	Patient	*n*	104	332	-	-	0.05–1.10
		MR	0.34 (±0.16)	0.29 (±0.13)	-	-	
	Healthy	*n*	23	202	-	-	0.09–1.20
		MR	0.62 (±0.24)	0.42 (±0.21)	-	-	
	Ratio[a]	CV	1.2	0.9	-	-	Max MR: 0.9
CYP2B6	Patient	*n*	17	507	1		0.4–42.5
		MR	6.17 (±6.17)	2.48 (±2.94)	0.3 (± -)	-	
	Healthy	*n*	30	281	1		LLOD–29.7
		MR	4.23 (±3.29)	4.58 (±4.58)	1.4 (± -)	-	
	Ratio[a]	CV	0.8	0.8	-	-	Max MR: 1.4
CYP3A	Patient	*n*	-	64	397	47	0.03–6.23
		MR	-	0.84 (±0.70)	0.66 (±0.53)	0.63 (±0.72)	
	Healthy	*n*	-	54	230	16	LLOD–9.35
		MR	-	1.47 (±0.70)	1.25 (±0.96)	1.46 (±1.17)	
	Ratio[a]	CV	-	1.7	1.1	1.4	Max MR: 0.7

Abbreviations: CV, coefficient of variation; LLOD, lower limit of detection; MR: metabolic ratio.

[a]Ratio of the coefficients of variability (CV) or maximal metabolic ratio values (MR) for patients over that for healthy controls.

TABLE 4 MR tolerance intervals obtained from selected genotype-predicted reference metabolizer groups of healthy volunteers.

CYP	Genotype	Populations Included	*n*	Mean	SD	CV	Min	Max	TI (90%)
CYP2D6	*1/*1	All	51	2.34	1.37	58.5	0.53	8.54	0.64-6.31
CYP2C9	*1/*1	ADD, ALE, PRA	170	0.059	0.043	72.9	0.010	0.540	0.025 – 0.113
CYP2C19	*1/*1	All	68	0.93	0.61	65.6	0.11	3.36	0.22 – 2.72
CYP1A2	*1/*1 Non-smokers	All	35	0.39	0.26	66.7	0.12	1.20	0.10 – 1.08
CYP2B6	*1/*1	ADD, PRA	117	4.14	4.38	105.8	0.59	42.5	0.87 – 11.62
CYP3A	*3A4: *1/*1* *3A5: *3/*3*	All	200	1.24	1.00	80.6	0.01	9.35	0.14 – 5.68

Abbreviations: CV = coefficient of variation (%); standard deviation; TI = 95% tolerance interval for 90% of the population.

high or extremely low enzymatic activities for each of the six CYPs analyzed (Table 5). While the number of outliers is particularly elevated for CYP2D6, more than half of these cases can be explained by the corresponding patient's genotype. Similarly, most of the CYP2C9 and CYP2C19 extreme metabolizer phenotypes could be attributed to the detected polymorphisms, indicating not only that a large proportion of interindividual variability for these enzymes is genetically determined, but also that the relevant SNPs have been characterized. It is noteworthy that half of the CYP2C9 slow metabolizers belong to the Greek (ALE) population where the frequency of CYP2C9 low activity alleles is higher compared to the other populations. None of the clinical parameters available (BMI, smoking, alcohol consumption, khat chewing or use of oral contraceptives) could explain the extreme metabolizer phenotypes observed for any of the CYPs. Interestingly, the majority (82.4%) of the unexplained CYP2D6 extreme slow metabolizers belong to non-European populations. For CYP1A2, the wide MR distributions of **1/*1* reference genotype, combined with the small sample size used to determine tolerance intervals, could explain the very small number of extreme metabolizers identified. Interestingly, our results indicate that there is a prevalence of CYP3A slow metabolizers in the Greek (ALE) population while fast CYP2B6 metabolizers are primarily from Oman (MUS).

3.5 | A high number of patients have unexplained extreme metabolizer phenotype

We then determined the number of patients presenting an extreme metabolizer phenotype by applying the calculated tolerance intervals to the patient cohort and identified the proportion of patients that could have been predicted on the basis of their genotype and/or the presence of CYP-affecting drugs in their treatment. The results are presented in Table 6. Drug classification as CYP inducer or inhibitor and an overview of the prevalence of those drugs in our patient cohort is available in Supplementary Material S6. In total, we listed more than 200 different drugs administered to the patient cohort. Of these, 58 are known inducers or inhibitors of one or more of the CYP enzymes. Although the strength of drug–cytochrome interaction depends on each pair and might be cumulative, this was not taken into consideration, and the presence or absence of a drug in the treatment regimen was recorded as a dichotomous yes/no value.

Compared to the results obtained with the healthy cohort, there were significantly more CYP2B6 ($X^2(1,838) = 56.7$, $P = 5.0E\text{-}14$) and CYP2C19 ($X^2(1,838) = 207.1$, $P < 2.2E\text{-}16$) slow metabolizers, as well as CYP2C9 fast metabolizers ($X^2(1,838) = 55.6$, $P = 8.7E\text{-}14$) in the patient cohort. The presence of comedication reducing CYP2C19 and CYP2B6 enzymatic activities seems to be responsible for a large fraction of these slow metabolizer patients. In contrast, there is no clear explanation for the high number of CYP2C9 fast metabolizers observed.

For CYP2D6, the results obtained in the patients and the healthy volunteer cohorts are very similar, including the proportion of cases that can be explained through genetics. The presence of inhibitors in patient drug treatments further explained some of the extreme CYP2D6 metabolizers. For CYP1A2 and CYP3A, the number of extreme metabolizers was slightly higher in the patients compared with the healthy volunteer cohort and drugs appeared to contribute to the reduction of CYP3A enzymatic activity. While neither genetics

TABLE 5 Description of the healthy volunteers with extreme phenotypes.

CYP	Group	*n*	% of the population	% explained by genotype	*n* Unexplained	Comments
CYP2D6	slow	86	27.6	60.5	34	82.4% of the genetically unexplained slow phenotypes belong to non-EUR
	fast	2	< 1	0.0	2	
	all	88	28.2	59.1	36	
CYP2C19	slow	18	8.2	83.3	3	
	fast	8	3.7	75.0	2	
	all	26	8.3	80.7	5	
CYP2C9	slow	15	4.8	73.3	4	55.3% of the slow metabolizers are from Greece (ALE)
	fast	2	< 1	0.0	2	
	all	17	5.4	64.7	6	
CYP1A2	slow	1	< 1	0.0	1	
	fast	2	< 1	0.0	2	
	all	3	< 1	0.0	3	
CYP2B6	slow	10	3.2	0.0	10	77.8% of the fast metabolizers are from Oman (MUS)
	fast	18	5.8	11.1	16	
	all	28	9.0	7.1	26	
CYP3A	slow	14	4.5	0.0	14	71.4% of the slow metabolizers are from Greece (ALE)
	fast	2	< 1	0.0	2	
	all	16	5.1	0.0	16	

TABLE 6 Description of the patients with extreme phenotype.

CYP	Group	*n*	% of the population	% explained by genotype	% explained by drug treatment	% explained total[a]
CYP2D6	slow	126	24.0	62.7	37.3	82.5
	fast	20	3.8	15.0	0.0	15.0
	all	146	27.8	56.2	32.2	73.3
CYP2C19	slow	293	55.7	33.5	70.0	79.5
	fast	8	1.5	37.5	0.0	37.5
	all	301	57.2	33.6	68.1	78.4
CYP2C9	slow	26	4.9	80.8	38.5	92.3
	fast	93	17.7	0.0	3.2	3.2
	all	119	22.6	17.6	10.9	22.7
CYP1A2	slow	14	2.7	0.0	35.7	35.7
	fast	2	< 1	50.0	0.0	50.0
	all	16	3.0	6.3	31.3	37.5
CYP2B6	slow	121	23.0	0.8	71.9	71.9
	fast	13	2.5	15.4	0.0	15.4
	all	134	25.5	2.2	64.9	66.4
CYP3A	slow	16	3.0	37.5	68.8	87.5
	fast	1	0.2	0.0	0.0	0.0
	all	31	5.9	29.0	41.9	61.3

[a]Percentage of cases that can be explained using either genotype or comedication, or both.

nor comedication seemed to have an important influence on CYP1A2 activity, it is interesting to note that the two extreme fast metabolizers are active smokers, a factor known to induce this enzyme. Moreover, three out of the 10 patients with cirrhosis have extremely slow CYP1A2 metabolizer capacity, which is a statistically significant result according to a Chi-square test ($X^2(1,540) = 14.6$, $P = 1.3E\text{-}04$).

Among the other clinical parameters evaluated, it is apparent that the majority of fast CYP3A and CYP2B6 metabolizers are women, although the Chi-square statistics only reaches marginal significance ($P < .05$). Conversely, the majority of CYP3A slow metabolizers are men (13/16), although the size of this group is too small to reach statistical significance. Finally, we observed no significant correlation between BMI, alcohol consumption, presence of an active cancer or inflammation (measured through plasma levels of c-reactive protein), hepatic function (assessed by aminotransferases ASAT, ALAT and alkaline phosphatase serum levels), or renal function (assessed by blood creatinine levels) with any extreme metabolizer group.

4 | DISCUSSION

In this study, we showed that the measured phenotypic variability and distribution for four out of the six main CYP enzymes studied is larger in a relatively homogeneous European hospitalized patient population than in a cohort of healthy volunteers recruited in four different geographic regions. We found that even CYPs with a good genotype–phenotype correlation cannot be reliably used to deduce metabolizer status at the individual level. Indeed, although CYP2C19 has the highest proportion of extreme metabolizers that could be explained through a combination of genetics and drug–drug interaction, many patients displaying MR values within the tolerance interval carry the same mutations or take similar drugs as the extreme metabolizers. Thus, while genetics and drug–CYP interaction data are highly valuable for group classification and useful to understand unusual reactions retrospectively, they seem to lack the sensitivity and specificity required to be used as a clinical guide.

We chose to work with tolerance intervals that are able to take into account the population variability based on a relatively wide dataset rather than using MR cut-off values generally defined using a small population of well-characterized volunteers sampled under very specific conditions.[34] Despite the interpopulation variability in our healthy volunteer cohort, the tolerance intervals calculated here agree well with the ones derived by Darnaud et al. based on literature data.[39] However, while our tolerance intervals have the advantage of highlighting the very extreme metabolizers in our cohorts, they are of limited clinical relevance and are not meant as cut-off values in routine practice. Ideally, any dose adaptation calculations ought to be based on robust models taking into account the magnitude of the PK parameter variations associated with each drug–drug and drug–gene interaction. Integration of measured MR values in drug PK models would therefore be a valuable contribution to refined precision medicine.

Beyond these considerations, a striking feature highlighted by our results is the distinct predictability pattern of each CYP enzyme and,

although we found patients with extreme metabolizer status in all groups, their numbers and causes were highly enzyme-specific. The wide distribution of CYP2D6 enzyme activity is apparent in both patient and healthy volunteer cohorts but is especially pronounced in the healthy volunteers in accordance with its known important interpopulation variability. However, the well-studied genotype–phenotype correlation for CYP2D6 enables a relatively confident prediction of its measured activity. Although a high number of drug treatments affect CYP2D6 activity, the proportion of extreme metabolizers did not differ significantly between healthy volunteers and the current patient population, in contrast to what has been observed earlier in a psychiatric patient population where CYP2D6-inhibiting drugs are even more prevalent.[24] Predicted CYP2C19 activity is significantly correlated with the measured MRs in the healthy volunteer cohort. However, with a large proportion of the patient population receiving proton pump inhibitors (PPI), MR values outside the tolerance interval are observed in more than half of the total patient population, some with extremely low CYP2C19 activity. *CYP2C9* has a moderate genotype–phenotype correlation, and intragroup variability profiles are similar in healthy volunteer and patient cohorts with maximal MR values corresponding to outliers with extremely high ratios. We identified a considerable number of unexplained CYP2C9 fast metabolizers in the patient cohort that cannot be attributed to any drug in our list. However, neither genetic nor environmental influences on cytochrome activities are fully described and new information is continuously published. For instance, both co-amoxicillin and chronic alcohol consumption have been associated with increased CYP2C9 activity and could together account for 70% of those unexplained cases.[40] For CYP2B6, CYP3A and CYP1A2, the lack of correlation between phenotype and genotype is problematic even in healthy participants as none of the SNPs present in the genotyping array had any significant effect on average MRs (Supplementary Material S8). This most likely resulted in overlarge tolerance interval estimation. Indeed, the small number of extreme CYP3A metabolizers were mostly affected by CYP3A activity altering drug intake. While for CYP2B6, the large number of individuals with extreme slow enzymatic activity could be attributed to inhibitors, such as clopidogrel and prasugrel, that might be overrepresented in cardiovascular patients, suggesting caution for extrapolation of this findings to other patient groups.

This study suffers a number of limitations, wiht one of the most important being the quality of the definition of normal metabolizers, as the characterization of individuals carrying the reference genotype is strongly biased whenever the genetic contribution to the phenotype is poorly defined. Indeed, the determination of metabolic status from genotypic data will always be limited by the selection of the SNPs included in the analysis and the on-going identification of new genetic variants are bound to continuously improve our prediction potential. For instance, a recently reported new CYP2C haplotype could contribute to the MRs of some of the unexplained fast metabolizer phenotype observed.[41]

Another important drawback is the difference in genetic ancestry and lifestyle habits between the healthy volunteer cohort and the patient population that prevented us from pooling the datasets. This is, for instance, reflected by the non-European origin of the majority of unexplained slow CYP2D6 metabolizers. For CYP1A2, we observed a clear difference in MR distribution between European and non-European populations carrying the **1A/*1A* genotype (Supplementary Material S5) even when considering only non-smoking participants (Supplementary Material S7). Our data for CYP1A2 is in line with the reported substantial environmental inducibility of this enzyme. Moreover, when considering that most pharmacogenetic studies have so far been conducted in the Europeans, the observed widespread MR distribution in this population may have confounded the SNP–activity relationship.

We did not address the complexity of drug–CYP interactions in the current study and considered intake of CYP-interacting drugs as a dichotomic value. However real-life clinical translation of any metabolizer characterization requires integration of parameters such as the strength and magnitude of CYP–drug interactions, and in case of polypharmacy, the impact of drug–drug interactions on CYP activity, considering that the effect of different drug treatments could be additive, competitive or synergic, or even compensatory if inducers and inhibitors of the same CYP are administered together. The development of mathematical models for the prediction of MR integrating genetics, comedication and patient parameters such as BMI, gender or age lies outside the scope of this paper but the use of mixed models combined with regularization techniques to minimize the confounding bias would be an interesting further step, especially for vulnerable populations where cocktail administration is problematic such a children or pregnant women.

In conclusion, our results suggest that, even if the CYP polymorphisms considered for the evaluation of enzymatic activities can, for some cytochromes and specific metabolizer groups, explain a substantial portion of the observed interindividual variability and do reflect metabolizer activity at group level, the accuracy of the overall genotype-derived prediction potential at the level of individuals needs to be further improved to enable reliable evidenced-based medical applications. For instance, based on our results, CYP2C19 substrates with narrow therapeutic indexes such as phenytoin or tricyclic antidepressants (TCAs),[9,12,42] should be administered with extra caution in hospital settings. Although taking into account the influence of drugs on enzymatic activity increases the prediction potential to a considerable extent for some of the CYPs, the proportion of unexplained extreme metabolizers remains important, justifying the use of phenotyping to assess metabolizer status in routine clinical practice. In any case, the combination of genotyping and phenotyping results remains the most powerful means to unravel the causes underlying extreme metabolizer phenotypes. Finally, in terms of cost-effectiveness of phenotyping, we have shown here that without phenotyping, a considerable number of patients are potentially subject to classification errors in terms of metabolic capacity with varying degrees of associated risk and consequent health costs. As the running costs of phenotyping are comparable to the costs of genotyping, implementation of routine phenotype testing appears to be a logical step towards the improvement of personalized medicine.

AUTHOR CONTRIBUTIONS

Y.G. and Y.D. conceived and designed the analysis; M.M., C.L., P.G. and J.T. collected the data; C.F.S., J.L.R., V.R., E.S.P., S.B., S.A.Y., G.Y. and V.C. contributed data or provided analysis tools; Y.G. performed the analysis and wrote the paper. All co-authors helped revising the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available on request from the corresponding author. The dataset is not publicly available due to privacy or ethical restrictions.

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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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