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RESEARCH TOPICS

FUNCTIONAL POLYMORPHISMS OF XENOBIOTICS METABOLIZING ENZYMES (XME)

Topic Editors
José A. Agúndez and Kathrin Klein

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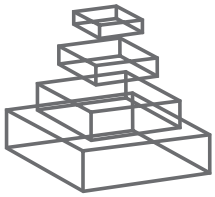

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FUNCTIONAL POLYMORPHISMS OF XENOBIOTICS METABOLIZING ENZYMES (XME)

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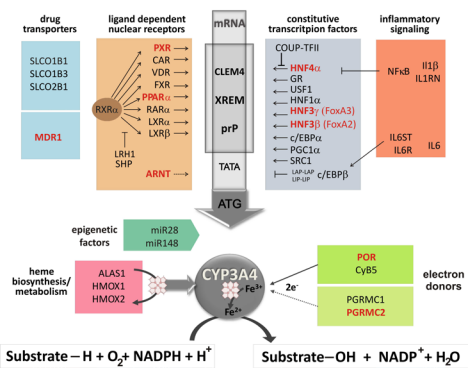

Regulation network of CYP3A4 phenotype expression. Figure taken from Klein K and Zanger UM (2013) Pharmacogenomics of cytochrome P450 3A4: recent progress toward the "missing heritability" problem. *Front. Genet.* 4:12. doi: 10.3389/fgene.2013.00012

The human genome harbours an impressive number of genes encoding enzymes that primarily metabolize drugs or other xenobiotics. Genetic and functional variation in these genes is tremendous and has complex consequences, depending, for example, on whether enzyme structure or expression is affected, or whether the produced metabolite is pharmacologically or toxicologically active or not. Despite numerous impressive examples of the impact of genetic variation on pharmacokinetics and drug response, today's knowledge is incomplete regarding most XME genes and fragmentary even for many well-investigated XMEs. This is one of the reasons why clinical pharmacogenetic studies are often controversial and clinical application in personalized medicine is presently limited. Advanced technology and ongoing large-scale projects are rapidly uncovering the existing genetic variation in all populations on earth, ultimately enabling the personal genome in the very near future. A wealth of mostly rare novel variants is awaiting functional characterization either by high-throughput expression/phenotyping techniques or by prediction using improved algorithms to estimate functional relevance.

With this Research Topic we would like to give an up-to-date overview about the current knowledge in this field by covering both, known hard facts as well as cutting-edge advancement in novel genetic and genomic variation of XMEs and their functional consequences. The scope of this Research Topic therefore includes all related areas of

experimental research, methodology, modeling and simulation, opinion and strategy, as well as historical or topical review. We encourage the submission of manuscripts. For illustration, some examples of potential Research Topics are given here, but the following list is not comprehensive:

- Novel prediction algorithms for functional effect of SNPs
- Novel structure comparison of amino acid variants with functional implication
- High-throughput method for recombinant expression and functional analysis
- Novel SNPs in XME gene and their functional relevance
- Interethnic and intraethnic variability of XME gene variants
- In vitro / in vivo comparison of phenotypic SNP effects
- Tissue-based genotype-phenotype or genotype/expression correlation analysis
- Substrate-dependent effects of genetic variants
- Identification of causal mutation or functional comparison of haplotypes
- Phenotypic effects of SNPs in non-coding (intronic, silent, 5'- 3'-) areas
- Relevance of microRNA polymorphisms for expression
- Toxicological relevance of XME gene variants
- Metabonomic relevance of XME gene variants
- XME Polymorphisms in animals
- Strategies on exploitation of 1000-genomes project data
- Protocol for XME gene-enrichment for next-generation-sequencing
- RNAseq approaches to dissect splice variants
- Review articles on history and current knowledge of particular gene or group of genes

This Research Topic will be of great interest to pharmacologists, toxicologists and geneticists around the world in order to improve their evidence-based pharmacogenetic strategies, extend the panel of functional and causal variants, and thus improve the benefit of complex and expensive clinical studies. Frontiers Research Topics are a fantastic opportunity to bring your research area to critical mass and to intensify collaborations. All Research Topic articles are freely available on the Internet and submitted to PubMed Central and other archiving facilities.

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EDITORIAL
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Functional polymorphisms of xenobiotics metabolizing enzymes—a research topic

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The human genome harbors an impressive number of genes encoding enzymes that primarily metabolize or transport drugs or other xenobiotics (XMEs). Genetic and functional variation in these genes is tremendous and has complex consequences, depending, for example, on whether enzyme structure or expression is affected, or whether the produced metabolite is pharmacologically or toxicologically active or not. Despite numerous impressive examples of the impact of genetic variation on pharmacokinetics and drug response, today's knowledge is incomplete regarding most XME genes and fragmentary even for many well-investigated XMEs. This is one of the reasons why clinical pharmacogenetic studies are often controversial and clinical application in personalized medicine is presently limited. Advanced technology and ongoing large-scale projects are rapidly uncovering the existing genetic variation in all populations on earth, ultimately enabling the personal genome in the very near future. A wealth of mostly rare novel variants is awaiting functional characterization either by high-throughput expression/phenotyping techniques or by prediction using improved algorithms to estimate functional relevance.

With this Research Topic we would like to give an up-to-date overview about the current knowledge in this field by covering both, known hard facts as well as cutting-edge advancement in novel genetic and genomic variation of XMEs and their functional consequences. Five major subtopics which include 20 research or review papers are included in this E-book. These are the following:

History and current knowledge of XMEs

- Clinical application of CYP2C19 pharmacogenetics toward more personalized medicine (Lee, 2013, review).
- Pharmacogenetics of cytochrome P450 2B6 (CYP2B6): advances on polymorphisms, mechanisms, and clinical relevance (Zanger and Klein, 2013, review).
- Pharmacogenetics of human ABC transporter ABCC11: new insights into apocrine gland growth and metabolite secretion (Ishikawa et al., 2013, review).
- Pharmacogenomics of cytochrome P450 3A4: recent progress toward the "missing heritability" problem (Klein and Zanger, 2013, review).

Clinical implications of XME gene variants

- *ABCB1* 4036A>G and 1236C>T polymorphisms affect plasma efavirenz levels in South African HIV/AIDS patients (Swart et al., 2012, research article).
- Genetic variations in drug-induced liver injury (DILI): resolving the puzzle (Stephens et al., 2012, opinion).
- MDMA, methamphetamine, and CYP2D6 pharmacogenetics: what is clinically relevant? (de la Torre et al., 2012, review).
- Molecular interactions between NAFLD and xenobiotic metabolism (Naik et al., 2013, review).
- Toward a clinical practice guide in pharmacogenomics testing for functional polymorphisms of drug-metabolizing enzymes. Gene/drug pairs and barriers perceived in Spain (Agúndez et al., 2012, perspective).

Inter/intraethnic variability of XME gene variants

- Characterization of the genetic variation present in *CYP3A4* in three South African populations (Drögemöller et al., 2013, research article).
- Frequencies of 23 functionally significant variant alleles related with metabolism of antineoplastic drugs in the Chilean population: comparison with Caucasian and Asian populations (Roco et al., 2012, research article).
- Pharmacogenomic diversity among Brazilians: influence of ancestry, self-reported color, and geographical origin (Suarez-Kurtz et al., 2012, review).

Regulation of XME gene expression

- Impact of the interaction between 3′-UTR SNPs and microRNA on the expression of human xenobiotic metabolism enzyme and transporter genes (Wei et al., 2012, research article).
- Molecular mechanisms of genetic variation and transcriptional regulation of *CYP2C19* (Helsby and Burns, 2012, review).

Pharmacogenetics in cancer therapy

- Impact of genetic polymorphisms on chemotherapy toxicity in childhood acute lymphoblastic leukemia (Gervasini and Vagace, 2012, review).
- Multilocus genotypes of relevance for drug metabolizing enzymes and therapy with thiopurines in patients with acute lymphoblastic leukemia (Stocco et al., 2013, review).
- Functional polymorphisms in xenobiotic metabolizing enzymes and their impact on the therapy of breast cancer (Vianna-Jorge et al., 2013, review).

- High-resolution melting analysis of the common c.1905+1G>A mutation causing dihydropyrimidine dehydrogenase deficiency and lethal 5-fluorouracil toxicity (Borràs et al., 2013, research article).
- Polymorphisms of phase I and phase II enzymes and breast cancer risk (Justenhoven, 2012, review).
- Analysis of the functional polymorphism in the cytochrome P450 *CYP2C8* gene rs11572080 with regard to colorectal cancer risk (Ladero et al., 2012, research article).

ACKNOWLEDGMENTS

We would like to thank all contributors for their valuable work helping us to present wide-ranged aspects in the field of pharmacogenetics and pharmacogenomics in this research topic. This E-book is of interest to pharmacologists, toxicologists and geneticists in order to improve their evidence-based pharmacogenetic strategies, extend the panel of functional and causal variants, and thus improve the benefit of complex and expensive clinical studies.

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REVIEW ARTICLE
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Clinical application of CYP2C19 pharmacogenetics toward more personalized medicine

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More than 30 years of genetic research on the *CYP2C19* gene alone has identified approximately 2,000 reference single nucleotide polymorphisms (rsSNPs) containing 28 registered alleles in the P450 Allele Nomenclature Committee and the number continues to increase. However, knowledge of *CYP2C19* SNPs remains limited with respect to biological functions. Functional information on the variant is essential for justifying its clinical use. Only common variants (minor allele frequency >5%) that represent *CYP2C19*2*, **3*, **17*, and others have been mostly studied. Discovery of new genetic variants is outstripping the generation of knowledge on the biological meanings of existing variants. Alternative strategies may be needed to fill this gap. The present study summarizes up-to-date knowledge on functional CYP2C19 variants discovered in phenotyped humans studied at the molecular level *in vitro*. Understanding the functional meanings of CYP2C19 variants is an essential step toward shifting the current medical paradigm to highly personalized therapeutic regimens.

Keywords: CYP2C19, functional genetics, personalized medicine, SNP, drug

INTRODUCTION

Pharmacogenetics incorporates genetic information into clinical decision making to avoid adverse drug effects and improve drug efficacy. Drug responses can be affected by three major factors: pharmacokinetics, pharmacodynamics, and the underlying molecular mechanisms of disease. Because the DNA sequence of *CYP2C19* is highly polymorphic, this may account for much of the variability in the pharmacokinetics of drugs metabolized by CYP2C19. CYP2C19 metabolizes a number of drugs, including the antiulcer drug omeprazole (Andersson et al., 1992), the antiplatelet drug clopidogrel (Mills et al., 1992), the anticonvulsant mephenytoin (Andersson et al., 1992; Bertilsson, 1995), the antimalarial drug proguanil (Helsby et al., 1991; Ward et al., 1991), the anxiolytic drug diazepam (Bertilsson, 1995; Wan et al., 1996; Qin et al., 1999), and certain antidepressants such as citalopram (Sindrup et al., 1993), imipramine (Skjelbo et al., 1991), amitriptyline (Bouman et al., 2011), and clomipramine (Nielsen et al., 1994). The phenotype of CYP2C19 metabolic capacity can be categorized based on genotypes and includes extensive metabolizers (EM, two wild-type functional alleles), intermediate metabolizers (IM, two reduced functional alleles or one null allele and a functional allele), and poor metabolizers (PM, two non-functional alleles) of drugs. Undesirable side effects such as prolonged sedation and unconsciousness have been observed after administration of diazepam in CYP2C19 PMs (Bertilsson, 1995). In addition, a diminished response to the antiplatelet drug clopidogrel has been found in CYP2C19 PMs (Hulot et al., 2006; Brandt et al., 2007; Mega et al., 2009). However, proton pump inhibitor drugs, including omeprazole and lansoprazole, exhibit a greater cure rate for gastric ulcers with *Helicobacter pylori* infections in PMs than in EMs due to higher plasma concentrations of the parent drugs in PMs (Sohn et al., 1997; Furuta et al., 1998). In any case, clinical decision strategies following *CYP2C19* genotyping suggest two regimens: an adjustment of the drug dose according to the genotype or an alternative drug choice. However, the greatest uncertainty is in the IM group, in which interindividual variation is clearly observed. The underlying mechanism for this variation remains unclear. Integration of other factors, such as clinical factors, environmental factors, and drug response-modulating factors may be needed to understand this variation. A successful launch of personalized medicine in relation to CYP2C19 drugs would be impossible without resolving the variation in the IM group. The majority of *CYP2C19* pharmacogenetic studies have been conducted using *CYP2C19*2*, **3*, and **17* variants. This article presents a collection of functional *CYP2C19* variants evidenced in human studies and discusses their utilities and limitations for clinical use.

DIVERSE CYP2C19 FUNCTIONAL VARIANTS

Four *CYP2C* genes have been identified in humans: *CYP2C8*, *CYP2C9*, *CYP2C18*, and *CYP2C19*. Among them, *CYP2C19* is the most polymorphic enzyme, and it metabolizes many important clinical drugs. Its activity can be inhibited by fluoxetine (Jeppesen et al., 1996), fluvoxamine (Jeppesen et al., 1996; Yao et al., 2003), lansoprazole, pantoprazole (Li et al., 2004), and ticlopidine (Tateishi et al., 1999), and can be induced by phenobarbital and rifampin (Madan et al., 2003). A number of *CYP2C19* polymorphisms have been identified. However, defining the quantitative value of altered functionality of the identified variant, such as the unequivocal percent value of wild-type activity, is difficult except for null alleles. One reason for this difficulty is from the different functional assay systems that include various molecular techniques with lab-to-lab variations. For example, to study protein-coding variants, enzyme expression systems have included yeast (Ferguson et al., 1998), baculovirus (Mankowski, 1999), and *Escherichia*

coli (Lee et al., 2009), and have depended on the preference or experimental conditions of the laboratory. All systems are useful biochemical tools for determining relative activity compared to wild-type activity *in vitro*. However, results of comparative parameters are not identical among the assay systems. In addition, not all *in vitro* assay systems reflect the same phenomena of those conditions *in vivo*. Therefore, a quantitative comparison of *CYP2C19* variants with wild-type *CYP2C19* has been difficult and should be interpreted with great caution.

New *CYP2C19* variants have been discovered by direct DNA sequencing using two kinds of human DNA samples, normal healthy subjects or phenotyped individuals. *CYP2C19* variants identified by DNA sequencing in individuals that have been characterized by clinicians as PMs or outliers include *CYP2C19*2*, **3*, **4*, **5*, **6*, **7*, **8*, **16*, **17*, and **26*, all based on comparisons to wild-type CYP2C19 activity *in vivo* or in *in vitro* functional studies, with the exception of *CYP2C19*16* (Wilkinson et al., 1989; Balian et al., 1995). Briefly, *CYP2C19*2* is the most common variant, which is a single base pair mutation in exon 5 (G > A), resulting an aberrant splice site (de Morais et al., 1994b). Creation of this splice site alters the reading frame of the mRNA beginning at amino acid 215 and produces a premature stop codon of 20 amino acids downstream (de Morais et al., 1994b); in that study, 7 of 10 Caucasians and 10 of 17 Japanese PMs for mephenytoin were homozygous for this mutation. *CYP2C19*3* has been found in Japanese PMs that were not homozygous for *CYP2C19*2* (de Morais et al., 1994a). *CYP2C19*3*, the result of the 636G > A mutation in exon 4, creates a premature stop codon. *CYP2C19*2* and **3* are responsible for the majority of PM phenotypes in the metabolism of CYP2C19 substrate drugs (Goldstein, 2001; Xie et al., 2001). *CYP2C19*4*, an A > G mutation in the initiation codon, was identified in Caucasian PMs of mephenytoin (Ferguson et al., 1998); in that study, *CYP2C19*4* cDNA was not expressed into the protein in a yeast expression system or an *in vitro* translation assay, whereas the *CYP2C19*1* cDNA was expressed and translated in both systems, suggesting a new PM allele. *CYP2C19*5*, a 1297C > T mutation in the heme-binding region, results in a Arg433Trp substitution and has been identified in a single Chinese PM of *S*-mephenytoin (Xiao et al., 1997) and 1 in 37 white PMs of *S*-mephenytoin (Ibeanu et al., 1998a). A recombinant enzyme activity study indicated that this allele abolishes the activity toward *S*-mephenytoin and tolbutamide, suggesting a PM allele (Ibeanu et al., 1998a). *CYP2C19*6*, a 395G > A mutation in exon 3, leads to an Arg132Gln substitution and has been found in a white PM outlier of mephenytoin (Ibeanu et al., 1998b). In this study, recombinant protein of *CYP2C19*6* prepared in an *E. coli* expression system exhibited negligible activity toward *S*-mephenytoin compared to that of wild-type, indicating that the *CYP2C19*6* allele contributes to the PM phenotype in whites. *CYP2C19*7* is a T > A mutation at the 5′ donor splice site of intron 5 and results in a PM allele. This allele has been found in a mephenytoin PM outlier of a Danish individual (Ibeanu et al., 1999). *CYP2C19*8*, a T358C change in exon 3 that results in a Trp120Arg substitution, was first identified in a French subject enrolled in a cancer risk study with mephenytoin activity (Benhamou et al., 1997). In a recombinant study, the *CYP2C19*8* protein exhibited a 90% decrease in activity for *S*-mephenytoin and a 70% reduction in tolbutamide activity compared to wild-type (Ibeanu et al., 1999). *CYP2C19*16* is a 1324C > T change in exon 9 located close to the heme-binding region that results in a Arg442Cys substitution, and was first identified in a Japanese individual with a low capacity to metabolize mephobarbital (Morita et al., 2004). Because this amino acid change is located close to the heme-binding site, it is proposed to have decreased activity for CYP2C19 substrate drugs. *CYP2C19*17* is an allele carrying −806C > T and −3042C > T found in the 5′ regulatory region (Sim et al., 2006). Individuals with *CYP2C19*17/*17* exhibit 35–40% lower omeprazole area under the plasma concentration-time curve values than the individuals having *CYP2C19*1/*1*, suggesting that this allele leads to an increased metabolizer phenotype. In Sim et al. (2006), a reporter assay showed increased transcriptional activity of *CYP2C19*17* and electrophoretic mobility shift assays showed specific binding of human hepatic nuclear proteins to an element carrying −806T but not −806C. *CYP2C19*26*, a 766G > A change in exon 5 resulting in a D256N substitution, was first identified in an omeprazole PM outlier of a Vietnamese individual; a recombinant enzyme activity assay prepared in an *E. coli* expression system showed a significant decrease in V_{max} for omeprazole (2.6-fold) and mephenytoin metabolism (twofold; Lee et al., 2009).

The majority of other alleles have been discovered in various human projects by using DNAs obtained from normal populations. *CYP2C19* variants that were discovered without phenotyping but which were characterized by *in vitro* systems include **9*, **10*, **11*, **12*, **13*, **14*, **15*, and **27*. A positive correlation between *in vitro* data and *in vivo* biological evidence would strengthen the incorporation of genetic data into clinical practice. Although more than 2,000 variants have been identified in the last several decades, alleles characterized via either *in vivo* or *in vitro* studies are limited to less than 20 variants, indicating a huge gap between the number of discovered single nucleotide polymorphisms (SNPs) and the number of functionally known SNPs. A summary of *CYP2C19* variants that have been discovered and characterized in phenotyped human subjects is presented in **Figure 1**. Clinical application of variant alleles with functional information is critical in the clinical setting for individualized drug therapy. Profiling of rare variants reflecting interindividual variability in drug responses could be difficult, but is needed for incorporating *CYP2C19* pharmacogenetics into the clinical decision making process.

Because *CYP2C19*2* and *CYP2C19*3* are the most common and defective variants (Goldstein et al., 1997; Xie et al., 2001), a number of reports have described the clinical significance of these variants. The promoter variant *CYP2C19*17* has been shown to be associated with increased CYP2C19 activity due to increased transcription of *CYP2C19*. Therefore, homozygous carriers of *CYP2C19*17* exhibit rapid clearance of CYP2C19 substrate drugs compared to wild-type (Sim et al., 2006; Ingelman-Sundberg et al., 2007; Rudberg et al., 2008). As a consequence, individuals with *CYP2C19*17* are likely to exhibit a lack of response to certain PPIs and antidepressants compared to EMs (*CYP2C19*1/*1*); this is due to rapid clearance of the drugs. Although most variation in the drug response can be explained by these three variants (*CYP2C19*2*, **3*, and **17*), high interindividual variation in subjects homozygous for *CYP2C19*1* has also been observed (Sim

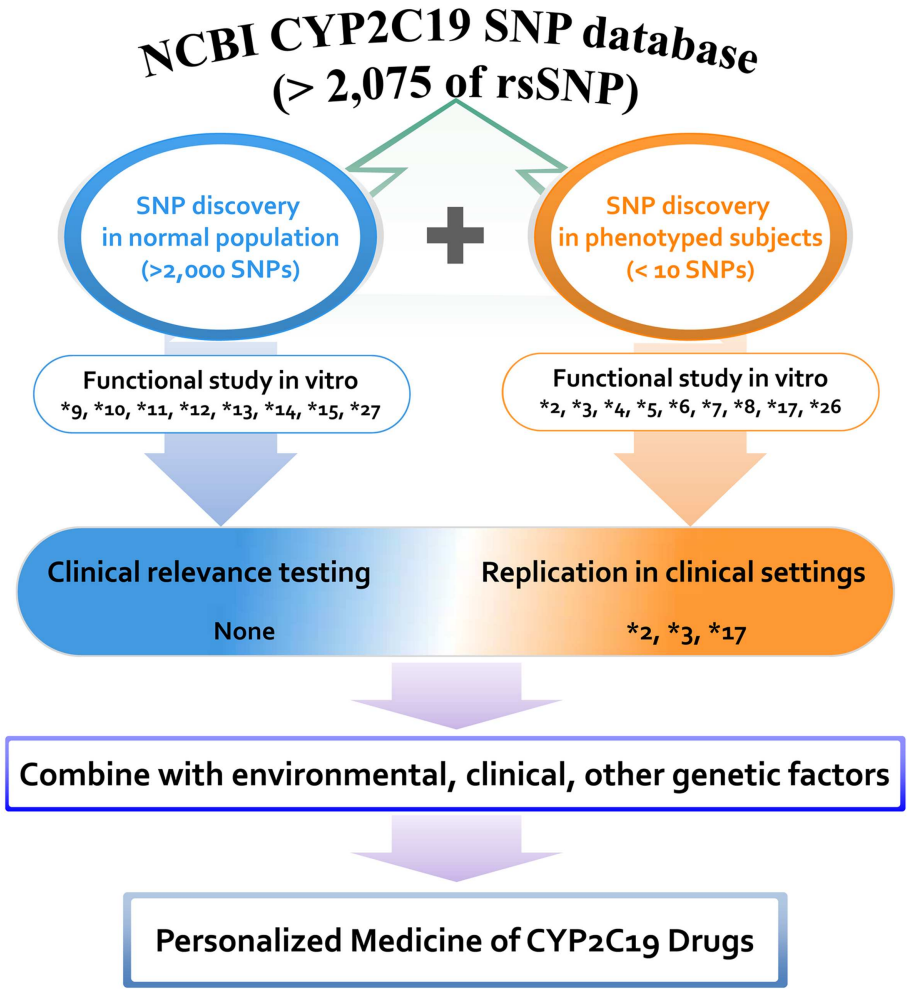

FIGURE 1 | Distribution of *CYP2C19* variants discovered and characterized in phenotyped human subjects and normal populations. A total of 2,075 rsSNPs have been reported in NCBI CYP2C19 SNP database at the present stage. Among them the number of SNPs that are functionally studied *in vitro* and validated *in vivo* is limited. Proposed scheme is for the personalized medicine of CYP2C19 substrate drugs.

et al., 2006). It is well-known that CYP2C19 activity is influenced by clinical factors, various inducers and inhibitors, drug–drug interactions, and other genetic polymorphisms in drug response-related genes. Because most studies have translated drug response data after genotyping for common alleles only, variants that have not been detected may be missing. More candidate SNPs may improve the translational process for drug responses. The limited amount of genotyping in current research may be due to the lack of data on the functional significance (*in vitro* or *in vivo*) of the allele and the low or undetermined allele frequency in the study population. Therefore, information on the functional significance of the variant allele would help researchers to include more candidate SNPs for genotyping in their clinical studies. This would ultimately improve translational quality in clinical settings and would be a beginning step for launching personalized medicine because physicians could easily communicate with patients using biological evidence for the drug response prediction instead of explaining the statistical risk without biological significance.

CURRENT CLINICAL UTILITIES OF *CYP2C19* POLYMORPHISMS

Many clinical drugs are metabolized by CYP2C19, and hence, the pharmacokinetics of CYP2C19 substrate drugs is influenced by *CYP2C19* polymorphisms. Altered CYP2C19 activity can be, at least in part, predicted by the *CYP2C19* genotype. The most extensively used polymorphic variants for genotype and phenotype association studies have been *CYP2C19*2*, **3*, and **17* (Goldstein, 2001; Xie et al., 2001; Desta et al., 2002). Ethnic differences in the frequency of *CYP2C19*2*, **3*, and **17* alleles are summarized in **Table 1**. Identification of PM alleles or the *CYP2C19*17* variant in patients and the modification of therapy regimens does not represent personalized drug therapy but is useful for the prevention of side effects. Because the human genome is very difficult to understand and drug responses are affected by several modulating genes with environmental factors, the development of personalized medicine for CYP2C19 substrate drugs using only *CYP2C19* and other modulating gene's genotypes is nearly impossible. However,

Table 1 | Frequencies of *CYP2C19* *2, *3, and *17 alleles in different ethnic populations.

Ethnic group	Allele no.	**2* (681G > A)	**3* (636G > A)	**17* (−806C > T)	Reference
		Splicing defect	W212X	Increased transcription	
WHITE					
Faroese	622	0.187	N.D	0.154	Pedersen et al. (2010)
Danish	552	0.150	N.D	0.201	Pedersen et al. (2010)
French	48	0.208	N.D	0.188	Berge et al. (2011)
Italian	720	0.094	0.008	N.D	Scordo et al. (2001)
Polish	250	0.116	N.D	0.272	Kurzawski et al. (2006)
Norwegian	664	0.181	0.006	0.220	Rudberg et al. (2008)
Sweden	370	0.160	N.D	0.200	Ramsjö et al. (2010)
Russians	580	0.114	0.003	N.D	Gaikovitch et al. (2003)
Caucasian	284	0.136	0.000	0.201	Myrand et al. (2008)
BLACK					
African-American	216	0.250	0.000	N.D	Bravo-Villalta et al. (2005)
African-American	472	0.182	0.008	N.D	Luo et al. (2006)
African-American	228	N.D	N.D	0.210	Kearns et al. (2010)
Ethiopian	380	N.D	N.D	0.179	Sim et al. (2006)
Nigerian	86	0.151	0.000	N.D	Babalola et al. (2010)
Egyptians	494	0.110	0.002	N.D	Hamdy et al. (2002)
ASIAN					
Korean	542	0.284	0.101	0.015	Kim et al. (2010)
Korean	100	0.290	0.050	0.020	Lee et al. (2009)
Chinese	136	N.D	N.D	0.044	Sim et al. (2006)
Chinese	800	0.247	0.033	0.012	Chen et al. (2008)
Japanese	530	0.279	0.128	0.013	Sugimoto et al. (2008)
Japanese	200	0.345	0.090	0.005	Myrand et al. (2008)
India	40	0.375	0.000	N.D.	Lamba et al. (2000)
India	906	0.350	0.010	N.D.	Jose et al. (2005)
Vietnamese	330	0.264	0.049	N.D.	Lee et al. (2007)
Thai	1548	0.290	0.030	N.D.	Tassaneeyakul et al. (2006)
Burmese	254	0.300	0.040	N.D.	Tassaneeyakul et al. (2006)
Karen	262	0.280	0.010	N.D.	Tassaneeyakul et al. (2006)
Jordanian	156	0.160	0.000	N.D.	Zalloum et al. (2012)
Iranians	206	0.120	0.100	N.D.	Hashemi-Soteh et al. (2012)

N.D., not determined.

several FDA-approved drugs are strongly affected by *CYP2C19* genotypes (see **Table 2**), and their labels include pharmacogenomic information (http://www.fda.gov/Drugs/ScienceResearch/ResearchAreas/Pharmacogenetics). Briefly, diazepam is demethylated by CYP2C19 (Jung et al., 1997), and hence its pharmacokinetics are affected by *CYP2C19* genetic polymorphisms. The plasma half-life of diazepam is approximately fourfold longer in individuals with PM genotypes than in individuals homozygous for wild-type *CYP2C19*1* (Wan et al., 1996; Qin et al., 1999). PMs may be at risk for toxic doses of diazepam and therefore more care is required to determine the diazepam dose for such subjects, especially for certain ethnicities because the frequency of PM differs between ethnic groups, being 3–6% in whites and blacks, 13–23% in Asians, and 38–79% in Polynesians and Micronesians (Kaneko et al., 1999; Xie et al., 2001). The *CYP2C19* genotype affects the metabolism of PPIs, resulting in altered cure rates for *H. pylori* infection in peptic ulcer patients. In a previous study, the cure rate of peptic or duodenal ulcers for Japanese patients that received dual therapy with omeprazole (20 mg/day for 2 weeks) and amoxicillin (2,000 mg/day for 2 weeks) was 100% in *CYP2C19* PMs, 60% for those who were heterozygous for one mutant allele, and 29% in individuals homozygous for the *CYP2C19*1* allele (Furuta et al., 1998). These differences are attributable to the *CYP2C19* PM genotype's impaired metabolism of PPIs, which leads to higher PPI plasma concentrations in PM individuals (Furuta et al., 2004). The contribution of *CYP2C19* to the metabolism of various PPIs differs. For example, in terms of plasma drug concentration-time curves, the ratio of the area under the curve of PMs versus EMs decreases in the following order: omeprazole, pantoprazole, lansoprazole, and rabeprazole (Funck-Brentano et al., 1997; Furuta et al., 2004). This is in agreement with the fact that the cure rate of rabeprazole is less dependent on the *CYP2C19* genotype compared to other PPIs. Patients with *CYP2C19*17* exhibit an enhanced response to clopidogrel with an increased risk of bleeding due to

Table 2 | Genetic polymorphisms in *CYP2C19* and their clinical consequences.

Drug	Therapeutic area	Clinical consequences
Diazepam	Psychiatry	Increased risk of sedation time and unconsciousness in PM genotype due to the prolonged half-life of diazepam
Omeprazole, lansoprazole	Gastroenterology	Increased cure rates due to increased half-life of the parent drugs in PM genotypes Decreased cure rates in the EM genotype
Clopidogrel	Cardiovascular	Decreased response to clopidogrel in the PM genotype due to low transformation into active metabolite and increased risk of recurrent MI, stroke, and stent thrombosis Increased risk of bleeding disorder in individuals homozygous for the *CYP2C19*17* allele due to increased inhibition of platelet function

PM, poor metabolizer; EM, extensive metabolizer; MI, myocardial infarction.

the higher rate of biotransformation into the active metabolite (Sibbing et al., 2010). In addition, an improved protective effect of clopidogrel after myocardial infarction has been observed in patients carrying the *CYP2C19*17* allele (Tiroch et al., 2010). The major enzymes involved in the production of the active metabolite of clopidogrel have been identified as CYP2C19, CYP3A4, and paraoxonase-1 (Clarke and Waskell, 2003; Bouman et al., 2011), although clopidogrel itself is a potent inhibitor of CYP2C19 and CYP3A4 (Richter et al., 2004). In general, patients that carry one or two CYP2C19 loss-of-function alleles exhibit diminished platelet inhibition after clopidogrel treatment compared to those with the EM genotype (Hulot et al., 2006; Brandt et al., 2007; Mega et al., 2009; Kubica et al., 2011). CYP2C19 PMs may not benefit from clopidogrel and thus alternative drugs, such as prasugrel, should be considered. No definitive recommendations have been established regarding dose adjustment of clopidogrel based on *CYP2C19* genotype testing. This may be due to the fact that other downstream genes, such as GPIIb/IIIa receptor genes, and other modulating factors can render a patient more sensitive or more resistant to clopidogrel despite having the same genotype. Larger-scale investigations using genotype-guided clopidogrel therapy compared to other therapy options would reveal the effectiveness of *CYP2C19* genotype testing for clopidogrel therapy.

FUTURE DIRECTIONS FOR CLINICAL APPLICATION

To date, the pharmacogenetic data on CYP2C19 clearly support that genetic variants alter the drug responses of its substrate drug. However, clinical application of CYP2C19 pharmacogenetics is limited to certain genotypes, mostly null alleles, and functionally known variants with a minor allele frequency of >5%. Clinical research using common variants only in case and control groups can enhance the statistical power of the evidence for that variant. However, the number of study populations that would benefit from pharmacogenomics research would be greatly reduced if such studies focused on common variants for strong statistical evidence. One goal of pharmacogenomics is to provide personalized medicine to each individual patient and to provide him or her an appropriate dose of the most appropriate drug. Therefore, more diversified investigations or strategies including low-frequency variants are needed to develop a standard practice for human applications. This may be ineffective or unrealistic for personalized medicine if clinical trials wait for the enrollment of a certain number of participants carrying the target genotype to satisfy the statistical power when assessing the role of that particular variant. The biological function of most rare variants remains unknown or has not been investigated *in vitro*, which has made physicians reluctant to test these individuals in clinical trials. Therefore, more data on functional genomics are needed particularly for rare variants. Although CYP2C19 is a major enzyme for the metabolism of certain drugs and influences their pharmacokinetics, there are many other genes that can modulate or mask the genetic effect of CYP2C19 polymorphisms, including genes involved in pharmacodynamics. Fortunately, recent whole genome sequencing and exome sequencing data have identified numerous variants, and these findings have led to genome-wide association studies using dense genomic markers on chips, resulting in the discovery of new determinants of drug responses. In conclusion, the translation of CYP2C19 pharmacogenetics into clinical practice is currently limited to a small number of functional variants, although more than 2,000 variants have already been discovered. More comprehensive and diverse research covering a large number of CYP2C19 variants will lay the foundation for improved personalized medicine in the future.

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Pharmacogenetics of cytochrome P450 2B6 (CYP2B6): advances on polymorphisms, mechanisms, and clinical relevance

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Cytochrome P450 2B6 (CYP2B6) belongs to the minor drug metabolizing P450s in human liver. Expression is highly variable both between individuals and within individuals, owing to non-genetic factors, genetic polymorphisms, inducibility, and irreversible inhibition by many compounds. Drugs metabolized mainly by CYP2B6 include artemisinin, bupropion, cyclophosphamide, efavirenz, ketamine, and methadone. *CYP2B6* is one of the most polymorphic CYP genes in humans and variants have been shown to affect transcriptional regulation, splicing, mRNA and protein expression, and catalytic activity. Some variants appear to affect several functional levels simultaneously, thus, combined in haplotypes, leading to complex interactions between substrate-dependent and -independent mechanisms. The most common functionally deficient allele is *CYP2B6*6* [Q172H, K262R], which occurs at frequencies of 15 to over 60% in different populations. The allele leads to lower expression in liver due to erroneous splicing. Recent investigations suggest that the amino acid changes contribute complex substrate-dependent effects at the activity level, although data from recombinant systems used by different researchers are not well in agreement with each other. Another important variant, *CYP2B6*18* [I328T], occurs predominantly in Africans (4–12%) and does not express functional protein. A large number of uncharacterized variants are currently emerging from different ethnicities in the course of the 1000 Genomes Project. The *CYP2B6* polymorphism is clinically relevant for HIV-infected patients treated with the reverse transcriptase inhibitor efavirenz, but it is increasingly being recognized for other drug substrates. This review summarizes recent advances on the functional and clinical significance of CYP2B6 and its genetic polymorphism, with particular emphasis on the comparison of kinetic data obtained with different substrates for variants expressed in different recombinant expression systems.

Keywords: bupropion, cyclophosphamide, cytochrome P450, drug metabolism, drug–drug interaction, efavirenz, pharmacogenetics, pharmacogenomics

INTRODUCTION

The cytochrome P450 (CYP) enzyme CYP2B6 is one of about a dozen human CYPs that are primarily involved in the biotransformation of drugs and other xenobiotics. The *CYP2B6* gene and its closely related pseudogene, *CYP2B7*, are located in a tandem head-to-tail arrangement within a large *CYP2* gene cluster on the long arm of chromosome 19 (Hoffman et al., 2001; **Figure 1**). The orthologous genes in dog, mouse, and rat are termed *CYP2B11*, *Cyp2b10*, and *CYP2B1*, respectively, but in contrast to other mammalian species, CYP2B6 is the only functional isozyme of its subfamily in humans (Nelson et al., 2004). Owing to the existence of extensive genetic polymorphism as well as strong inhibitors and inducers, its activity is highly variable in the population. For some clinically used drugs including the antiretroviral agents efavirenz and nevirapine, *CYP2B6* single nucleotide polymorphisms have been shown to be useful predictors of pharmacokinetics and drug response (reviewed in Zanger et al., 2007; Telenti and Zanger, 2008; Rakhmanina and van den Anker, 2010). However, recent data indicate that pharmacogenetic mechanisms are complex, appear on several levels of gene expression from the initial mRNA transcript to splice variants (pre-mRNA splicing and mRNA expression) to altered proteins, and affect function in various ways including substrate-dependent and substrate-independent effects. Several previous reviews are available that cover the biochemical pharmacology, molecular genetics, and pharmacogenetics of this enzyme at various degrees of detail (Ekins and Wrighton, 1999; Turpeinen et al., 2006; Hodgson and Rose, 2007; Zanger et al., 2007; Wang and Tompkins, 2008; Mo et al., 2009; Turpeinen and Zanger, 2012). The purpose of this review is to summarize recent advances in areas that have an impact on variable expression of CYP2B6 and the mechanisms and impact of *CYP2B6* polymorphism, as observed by various *in vitro* approaches as well as in *in vivo* studies, and to discuss their functional and clinical implications.

VARIABILITY OF EXPRESSION AND TRANSCRIPTIONAL REGULATION

Cytochrome P450 2B6 is primarily expressed in the liver where its contribution to the total microsomal P450 pool has been estimated

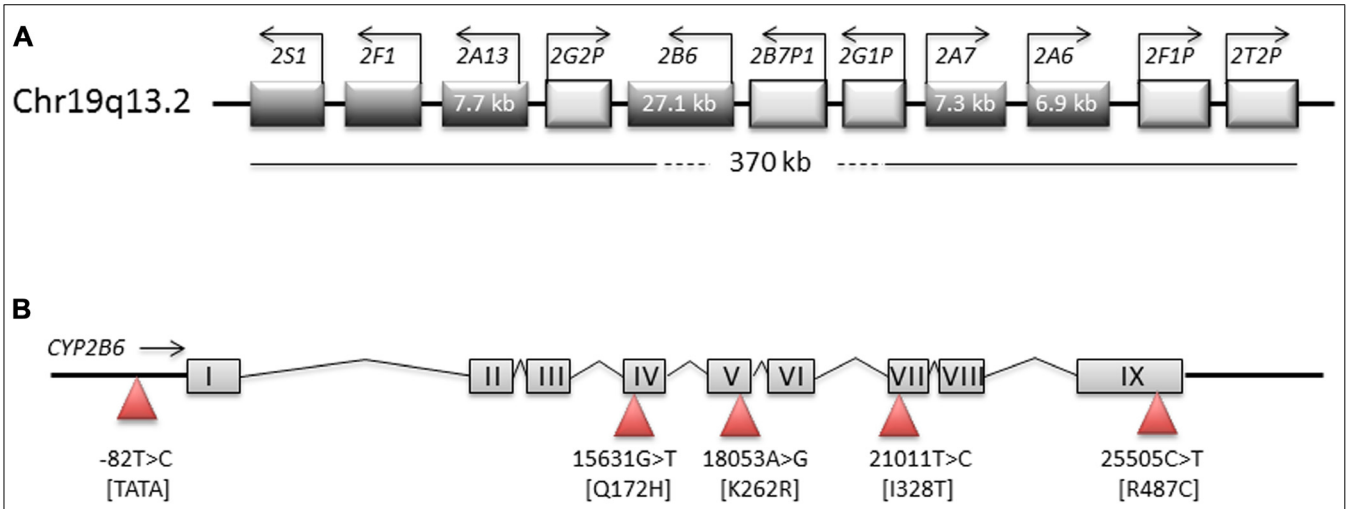

FIGURE 1 | Structure of genomic regions on chromosome 19. Schematic representation of the *CYP2ABFGST*-cluster on chromosome 19 **(A)** and of the *CYP2B6* gene **(B)**. Boxes in the **(A)** represent genes, with direction of transcription indicated by arrows. The **(B)** represents CYP2B6 exons and introns and shows the most important SNPs as triangles.

to be within a range of about 1–10%, with a large inter-individual variability at protein level of roughly 100-fold (see Zanger et al., 2007 for review and references therein). Although some earlier studies reported expression in only a fraction of human livers, newer studies with better antibodies found CYP2B6 to be present in all investigated human adult liver samples (Hofmann et al., 2008) while up to one-third of pediatric samples contained no detectable protein (Croom et al., 2009). In the latter study, ontogenic differences were studied in liver microsomes from 217 pediatric liver donors. Hepatic median CYP2B6 protein levels were about twofold higher in the period between birth and 30 days postnatal compared to fetal samples, and protein levels varied already over 25-fold in both of these age groups (Croom et al., 2009). Maturation effects may further depend on genotype, as suggested in a study on HIV-infected children treated with efavirenz (Sueyoshi et al., 1999; Wang et al., 2003; Faucette et al., 2004, 2007).

One of the most important factors contributing to intra- as well as inter-individual variability is enzyme induction, i.e., *de novo* protein synthesis following exposure to certain chemicals. Regulation of *CYP2B* gene expression represents the archetypal example of enzyme induction (Remmer et al., 1973). Human CYP2B6 is strongly inducible by several drugs including "classical" inducers such as rifampicin, phenytoin, and phenobarbital involving a so-called phenobarbital-responsive enhancer module (PBREM) at −1.7 kb of the *CYP2B6* gene promoter, and a distal xenobiotics-responsive enhancer module (XREM, −8.5 kb), to which pregnane X receptor (PXR, *NR1I2*) and/or constitutive androstane receptor (CAR, *NR1I3*) bind to mediate increased transcription (Sueyoshi et al., 1999; Wang et al., 2003; Faucette et al., 2004, 2007). Since other CYPs are regulated by overlapping sets of nuclear receptors, CYP2B6 is often co-induced with CYP2C enzymes and CYP3A4. CYP2B6 inducers identified to date include cyclophosphamide (Gervot et al., 1999), hyperforin (Goodwin et al., 2001), artemisinin antimalarials (Simonsson et al., 2003; Burk et al., 2005), carbamazepine (Oscarson et al., 2006; Desta et al., 2007), metamizole (Saussele et al., 2007; Qin et al., 2012), ritonavir (Kharasch et al., 2008), the insect repellent *N,N*-diethyl-*m*-toluamide (DEET; Das et al., 2008), statins (Feidt et al., 2010), efavirenz (Ngaimisi et al., 2010; Habtewold et al., 2011). Interestingly, in the latter study, gender influenced the inducibility of efavirenz 8-hydroxylation, which was higher in women than in the men (Ngaimisi et al., 2010). In addition to therapeutic drugs, pesticides were found to be powerful inducers of CYP2B6 and other CYPs through interaction with both PXR and CAR (Das et al., 2008). Induction of CYP2B6 and other cytochromes P450 and its clinical consequences has been reviewed by others (Pelkonen et al., 2008; Mo et al., 2009).

Sex differences in liver expression have been observed in a number of studies. Females liver donors had higher amounts of CYP2B6 mRNA (3.9-fold), protein (1.7-fold), and enzyme activity (1.6-fold) compared to male subjects in a study of 80 ethnically mixed samples (Lamba et al., 2003). In a study with 235 Caucasian liver donors, female samples had 1.6-fold higher expression level of CYP2B6 mRNA, however, this difference did not translate into higher protein and activity levels and no sex difference was found when only liver donors without presurgical drug exposure were considered (Hofmann et al., 2008). Discrepant effects of sex on pharmacokinetics of CYP2B6 substrates, which may be due to other confounders such as age or smoking status, were also found *in vivo*. Higher bupropion hydroxylation rates were found in adolescent females compared to males (Stewart et al., 2001) but not in adults (Hsyu et al., 1997). For efavirenz, several studies reported elevated plasma concentrations in female compared to male patients, which is in contrast to the above-mentioned *in vitro* findings and may be explained by other factors such as differences in body fat content and distribution (Burger et al., 2006; Nyakutira et al., 2008; Mukonzo et al., 2009). The influence of age on CYP2B6 expression may also depend on sex, as only males showed a significant increase of liver CYP2B6 at higher age (Yang et al., 2010).

Besides liver, CYP2B6 is also consistently expressed in different parts of respiratory and gastrointestinal tracts, including lung and nasal mucosa, and also in skin and the kidneys (Choudhary et al., 2003; Dutheil et al., 2008; Thelen and Dressman, 2009; Leclerc et al., 2010). The significance of CYP2B6 in these extrahepatic tissues is currently unknown, but it should be remembered that the enzyme is probably the most important one for many environmental toxins such as pesticides, and its presence in tissues with barrier function may thus contribute substantially to protection against these chemicals. In addition, the presence of CYP2B6 in brain has been demonstrated in human and primate brain tissue samples and smoking, alcohol consumption, and genetic polymorphism have been suggested to contribute to its variability in this organ (Miksys et al., 2003). In general, CYP levels in extrahepatic tissues are far below those of liver, but the localization to specific regions in the brain may contribute to the activation or inactivation of centrally acting drugs and to neurological side effects of certain medications or abused drugs, e.g., "ecstasy" [1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), see below]. This may also explain why efficacy for some centrally acting drugs is not well correlated to their plasma levels. The potential role of brain-expressed CYPs including CYP2B6 in the biotransformation of centrally acting drugs has been reviewed by others (Meyer et al., 2007; Ferguson and Tyndale, 2011).

THE CHEMICAL INTERACTION PROFILE OF CYP2B6

Recent studies have revealed crystal structures of the CYP2B6 wild-type and K262R variant in complex with various inhibitors at providing first views into its active site and its plasticity to adopt different conformations when binding different ligands (Gay et al., 2010; Shah et al., 2011; Wilderman and Halpert, 2012). Substrates of CYP2B6 are usually fairly lipophilic, neutral or weakly basic non-planar molecules with one or two hydrogen bond acceptors (Lewis et al., 1999, 2004). The CYP2B6 substrate selectivity comprises many diverse chemicals, including not only clinically used drugs but also many environmental chemicals such as pesticides (Turpeinen et al., 2006; Hodgson and Rose, 2007; Turpeinen and Zanger, 2012). Therapeutically important drugs metabolized primarily by CYP2B6 include the prodrug cyclophosphamide, which is converted to the direct precursor of the cytotoxic metabolites, phosphoramide mustard and acrolein, by 4-hydroxylation (Huang et al., 2000; Roy et al., 2005), the non-nucleoside reverse transcriptase inhibitor (NNRTI), efavirenz, which is 8-hydroxylated to become pharmacologically inactive (Ward et al., 2003; Desta et al., 2007), the atypical antidepressant and smoking cessation agent bupropion, which is converted to pharmacologically active hydroxybupropion (Faucette et al., 2000; Hesse et al., 2000; Turpeinen et al., 2005b), the anesthetics propofol (Court et al., 2001; Oda et al., 2001) and ketamine (Desta et al., 2012), the analgesic pethidine (meperidine; Ramírez et al., 2004); the μ-opioid receptor agonist, methadone (Totah et al., 2008), the antimalarial artemisinin (Svensson and Ashton, 1999; Asimus and Ashton, 2009), among numerous additional metabolic pathways of other drugs, to which CYP2B6 contributes in part, such as the antiretroviral, nevirapine (Erickson et al., 1999), and many others (Turpeinen and Zanger, 2012). Metabolic pathways suitable as probe for CYP2B6 activity include S-mephenytoin *N*-demethylation (Ko et al., 1998), bupropion hydroxylation (Faucette et al., 2000; Fuhr et al., 2007) and efavirenz, based on *in vitro* investigations (Ward et al., 2003; Desta et al., 2007).

Endogenous substances metabolized by the enzyme include arachidonic acid, lauric acid, 17beta-estradiol, estrone, ethinylestradiol, and testosterone 16α- and 16β-hydroxylation (Ekins et al., 1998).

Cytochrome P450 2B6 furthermore participates in the biotransformation of the abused drug "ecstasy" (*N*-methyl-3,4-methylenedioxymethamphetamine, MDMA), which is *N*-demethylated leading to potentially neurotoxic metabolites (Kreth et al., 2000). It also plays a minor role in nicotine metabolism (Yamazaki et al., 1999; Yamanaka et al., 2005). CYP2B6 has furthermore been found to be of importance in the metabolism of pesticides and other environmental chemicals and pollutants (Hodgson and Rose, 2007). In particular the bioactivating oxidation of the organophosphorus insecticides chlorpyrifos (Crane et al., 2012) and methyl parathion (Ellison et al., 2012a) to their more toxic oxon metabolites is mainly catalyzed by CYP2B6, a public health concern due to their worldwide use and documented human exposures (Ellison et al., 2012b). Further environmental substrates are the insecticide and endocrine disruptor methoxychlor, the extensively used insect repellent *N*,*N*-diethyl-*m*-toluamide (Das et al., 2008), profenofos and other pesticides (Abass and Pelkonen, 2012), as well as the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK; Smith et al., 2003), aflatoxin B1 (Code et al., 1997), and others (Hodgson and Rose, 2007; Abass and Pelkonen, 2012).

Several structurally unrelated drugs have been shown to inhibit CYP2B6 and many of them do that in a mechanism-based, irreversible manner (Turpeinen et al., 2006; Turpeinen and Zanger, 2012). The thienopyridine derivatives clopidogrel and ticlopidine are prodrugs that selectively inhibit platelet aggregation and have been in clinical use for the prevention of atherothrombotic events for several years. Both of them are potent mechanism-based inhibitors of CYP2B6 (Richter et al., 2004; Zhang et al., 2011a). The established anticancer agent, thioTEPA (*N*,*N*′,*N*′′-triethylenethiophosphoramide) was also found to be a highly selective and mechanism-based CYP2B6 inhibitor (Rae et al., 2002; Harleton et al., 2004; Richter et al., 2005). A comparison of several selective inhibitors revealed that 2-phenyl-2-(1-piperidinyl)propane is probably the most selective CYP2B6 inhibitor *in vitro* (Walsky and Obach, 2007). Recent *in vitro* observations identified the progesterone receptor antagonist, mifepristone (RU486; Lin et al., 2009); the anti-Parkinsonian agent selegiline (the R-enantiomer of deprenyl; Sridar et al., 2012), methadone (Amunugama et al., 2012), and tamoxifen (Sridar et al., 2012) as potent mechanism-based inhibitors. *In vivo* drug–drug interactions have been reported, for example, between thioTEPA and cyclophosphamide (Huitema et al., 2000), clopidogrel and bupropion (Turpeinen et al., 2005a), voriconazole and efavirenz (Liu et al., 2008; Jeong et al., 2009), clopidogrel and efavirenz (Jiang et al., 2012), and between ticlopidine and ketamine (Peltoniemi et al., 2011). Furthermore, certain non-pharmaceutical compounds like particular benzylpyridine derivatives have been characterized as very potent inhibitors of CYP2B6 (Korhonen

et al., 2007) and have been utilized for structural modeling experiments (Gay et al., 2010).

PHARMACOGENETICS OF *CYP2B6*

The *CYPalleles* website[1] currently lists 37 distinct star-alleles, i.e., gene haplotypes with a distinct variant amino acid sequence or with demonstrated functional effect (last accessed: February 21st, 2013). More than 30 amino acid-changing single-nucleotide polymorphisms (SNPs) occur in different combinations and together with additional non-coding variants and many more SNPs not yet assigned to particular haplotypes. The worldwide variations in SNP frequencies have been reviewed recently (Li et al., 2012). **Table 1** lists the most important variants in terms of frequency and functional impact and summarizes updated structural, functional, and frequency information for different ethnicities. In addition to the *CYPallele* website, further valuable information about *CYP2B6* SNPs and pharmacogenetics are available on the websites of *The Pharmacogenomics Knowledgebase*[2], the *NCBI portal for short genetic variations*, dbSNP[3], the *1000 Genomes Catalog of Human Genetic Variation*[4], as well as the NHLBI exome sequencing project[5].

[1]http://www.cypalleles.ki.se/cyp2b6.htm
[2]http://www.pharmgkb.org/
[3]http://www.ncbi.nlm.nih.gov/projects/SNP/
[4]http://www.1000genomes.org/
[5]http://EVS.gs.washington.edu

*CYP2B6*6* AND EFAVIRENZ: *IN VIVO, EX VIVO, IN VITRO*

The most common variant allele in all populations studied to date harbors two amino acid changes, Q172H and K262R, and is termed *CYP2B6*6*. This haplotype occurs in about 15 to over 60% of individuals, depending on ethnicity (**Table 1**). Although additional variants occur in the promoter and in introns, their functional impact appears to be of limited relevance and will not be further discussed here (Lamba et al., 2003; Hesse et al., 2004; Hofmann et al., 2008).

Since the discovery that CYP2B6 is the major enzyme for efavirenz 8-hydroxylation (Ward et al., 2003), pharmacogenetic studies have linked the Q172H variant to elevated plasma concentrations of efavirenz, indicating decreased enzyme function *in vivo*. This finding has been reproduced manifold in different ethnicities throughout the world (summarized by Telenti and Zanger, 2008; Rakhmanina and van den Anker, 2010). Three CYP2B6 polymorphisms, 15631G>T, 21011T>C, and an intron 3 SNP rs4803419, were also shown to be associated with efavirenz pharmacokinetics at genome wide significance (Holzinger et al., 2012).

The potent first-generation NNRTI of HIV-1 is recommended as initial therapy with two NRTIs in highly active antiretroviral therapy (HAART) regimes, but patients with subtherapeutic plasma concentrations can develop resistance and treatment failure, whereas those with too high plasma levels are at increased risk of central nervous system (CNS) side effects, which can lead to treatment discontinuation in a fraction of patients (King and Aberg, 2008). Q172H variant was furthermore associated

Table 1 | Summary data on selected genetic polymorphisms of *CYP2B6*.

CYP allele designation[a]	Key mutation(s)[b] rs number	Location, protein effect	Allele frequencies[c]	Functional effect
*CYP2B6*4*	g.18053(c.516) A>G rs2279343	K262R (isolated)	0.00 AA, Af 0.04 Ca 0.05–0.12 As	↑ Expression, moderate substrate-dependent effects
*CYP2B6*5*	g.25505(c.1459) C>T rs3211371	R487C	0.01–0.04AA, Af 0.09–0.12 Ca 0.05–0.12 Hs 0.01–0.04 As	↓ Expression, in part compensated by ↑ specific activity
*CYP2B6*6*	g.15631(c.516) G>T rs3745274 and g.18053(c.785)A>G rs2279343	Q172HK262R	0.33–0.5 AA, Af 0.10–0.21 As 0.14–0.27 Ca 0.62 PNG	↓ Expression; ↓activity with efavirenz *in vivo*; some other substrates show ↑ activity
*CYP2B6*18*	g.21011(c.983)T>C rs28399499	I328T	0.04–0.08 AA 0.05–0.12, Af 0.01 HS 0.00 As, Ca, PNG	↓ Expression and activity
*CYP2B6*22*	g.-82T>C rs34223104	promoter (TATA-box)	0.00–0.025 AA, Af, As 0.024 Ca, Hs	↑ Expression and activity ↑ Inducibility *in vitro*

[a] *According to CYPallele nomenclature homepage http://www.cypalleles.ki.se.*
[b] *Genomic (g.) and cDNA (c.) positions are given in bp.*
[c] *Selected frequencies of individual ethnicities (AA, African American; Af African; As Asian; Ca Caucasian; Hs, Hispanic; PNG, Papua New Guineans) compiled from dbSNP http://www.ncbi.nlm.nih.gov/SNP and from the literature cited in the text.*

with increased neurotoxicity and other CNS side effects (Haas et al., 2004; King and Aberg, 2008; Lubomirov et al., 2010; Ribaudo et al., 2010; Maimbo et al., 2011) with HAART-induced liver injury (Yimer et al., 2011), and with efavirenz treatment discontinuation and the associated risk of developing drug resistance (Ribaudo et al., 2006; Lubomirov et al., 2011; Wyen et al., 2011). Importantly, compound heterozygotes of 516T and another low activity allele (e.g., **11*, **18*, **27*, **28*) also predict high efavirenz plasma levels (Rotger et al., 2007; Ribaudo et al., 2010). In prospective, genotype-based dose adjustment studies the therapeutic dose of efavirenz could be successfully reduced and CNS-related side effects decreased (Gatanaga et al., 2007; Gatanaga and Oka, 2009). Using pharmacokinetic modeling and simulation it was suggested that *a priori* dose reduction in homozygous *CYP2B6*6* patients would maintain drug exposure within the therapeutic range in this group of patients (Nyakutira et al., 2008).

The *in vitro* data that have accumulated over the years on the *CYP2B6*6* allele draw a more complex picture with functional consequences on various levels including pre-mRNA splicing, protein expression, as well as substrate-dependent changes in enzyme activity and different sensitivity toward irreversible inhibition. While early studies using recombinantly expressed enzyme variants found higher 7-ethoxycoumarin O-deethylase activity for the Q172H variant (Ariyoshi et al., 2001; Jinno et al., 2003), in genotyped human livers (*ex vivo*), the **6* allele has been associated with approximately 50–75% decreased protein levels (Lang et al., 2001; Desta et al., 2007; Hofmann et al., 2008). An explanation for decreased protein expression was provided based on the observation that the c.516G>T SNP coding for Q172H in exon 4 (rs3745274, **Table 1**) was correlated to increased amounts of a hepatic splice variant that lacked exons 4–6, and concurrently to decreased amounts of the normal functional transcript. Recombinant expression of minigene constructs in mammalian cells proved that the c.516G>T variant was causally involved in erroneous splicing and lower expression of functional mRNA and protein (Hofmann et al., 2008). It has been hypothesized that binding of splice factors to an exonic splicing enhancer(s) located in exon 4 could be affected by the variant (Zanger and Hofmann, 2008; Sadee et al., 2011). Although reduced expression in liver appears to satisfactorily explain increased efavirenz plasma concentrations in individuals with **6/*6* genotype (Desta et al., 2007), recent *in vitro* data of expressed variants seem to indicate that the amino acid substitutions contribute to changes in catalytic activity of the enzyme. Structurally this is not easy to comprehend, because the Q172H and Lys262Arg amino acid changes occur in regions of the protein that are not directly located at the active site or that have been identified as substrate recognition sites (**Figure 2**).

Concerning efavirenz and also other substrates, the available *in vitro* data are however, not well in agreement with each other. **Table 2** summarizes kinetic parameters for bupropion and efavirenz for CYP2B6 enzyme variants obtained from different recombinant expression systems. Using *Escherichia coli* expression system, Zhang et al. (2011b) purified six *N*-terminally truncated expressed variants to homogeneity and reconstituted them with NADPH:cytochrome 450 reductase (POR) at a molar ratio of 1:2

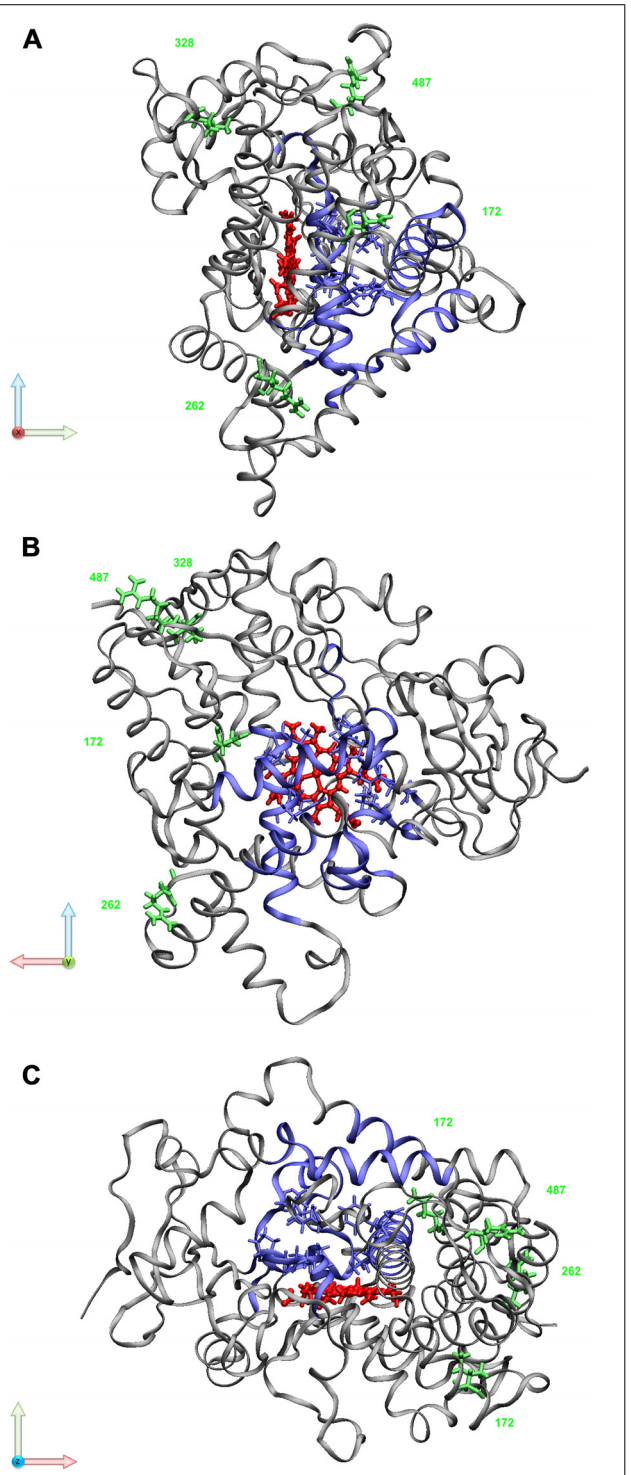

FIGURE 2 | CYP2B6 structural model. Selected variant residues are marked in green (Q172H, K262R, I328T, R487C); substrate recognition sites (SRS; Nguyen et al., 2008) and active site residues (ASR; Niu et al., 2011) are highlighted in blue; the prosthetic heme molecule is shown in red. PDB file 3IBD was visualized using VMD visual molecular dynamics viewer http://www.ks.uiuc.edu/Research/vmd/ (Humphrey et al., 1996). The protein sequence used for determining the crystal structure contains R at position 262 instead of the reference residue K. **(A)** view along *x*-axis (light red); **(B)** view along *y*-axis (light green); **(C)** view along *z*-axis (light blue)

Table 2 | Kinetic properties of recombinantly expressed CYP2B6 protein variants with bupropion and efavirenz.

Variant	System	Bupropion hydroxylation			Efavirenz 8-hydroxylation			Reference
		K_m (μM)	V_{max} (%)	CL_{int} (%)	K_m (μM)	V_{max} (%)	CL_{int} (%)	
2B6.1	COS-1	87	100	100	2.07	100	100	Radloff et al. (2013)
	E. coli	95	100	100	7.3	100	100	Zhang et al. (2011b)
	Sf9				7.7	100	100	Ariyoshi et al. (2011)
	Sf9	64	100	100	3.2	100	100	Xu et al. (2012)
2B6.6	COS-1	72	81	98	1.21	107	183	Radloff et al. (2013)
	E. coli	380	175	43	198	563	20	Zhang et al. (2011b)
	Sf9				12.4	81	50	Ariyoshi et al. (2011)
	Sf9	63	139	143	8.8	133	49	Xu et al. (2012)
2B6.4	*E. coli*	162	60	35	5.5	73	96	Zhang et al. (2011b)
	Sf9				9.16	169	142	Ariyoshi et al. (2011)
2B6.5	COS-1	65	44	59	1.15	46	83	Radloff et al. (2013)
	E. coli	134	66	47	53	1005	138	Zhang et al. (2011b)

Only studies which determined kinetic parameters (K_m, V_{max}, or K_{cat}) were included.

and measured efavirenz and bupropion kinetics. Using Sf9 insect cell cotransfection, CYP2B6.1, 2B6.4 and 2B6.6 were expressed in the presence of 10-fold excess of POR, i.e., under saturating conditions, to measure efavirenz kinetics (Ariyoshi et al., 2011). Another study determined both bupropion and efavirenz kinetics in protein preparations also derived from insect cells in the presence or absence of cytochrome b5 (CYB5) but at somewhat more variable ratios in regard to POR (Xu et al., 2012). Radloff et al. (2013) used the COS-1 expression system, where P450 monooxygenase activity is supported by endogenously expressed POR, to determine bupropion and efavirenz kinetics for several novel CYP2B6 variants in comparison to the known variants 2B6.1, 2B6.5, and 2B6.6.

The compilation of data in **Table 2** shows that differences between the variants were masked by differences between the expression systems. For example, efavirenz K_m was moderately decreased (58%) for COS-1 cell-expressed 2B6.6 compared to 2B6.1 but moderately larger for both insect cell-expressed proteins. The *E. coli*-expressed variant showed, however, 27-fold increased K_m. While the COS-1 proteins had almost identical V_{max}, one of the insect cellproteins had decreased V_{max} (81%), while the other had increased activity (133%). Again, the *E. coli*-expressed variant showed the biggest difference of almost sixfold higher activity for the variant. Similar discrepancies, albeit less dramatic, were found with bupropion as substrate (**Table 2**).

This data-comparison illustrates the problems that still exist with recombinant P450 expression systems, and particularly for CYP2B6, which appears to be an enzyme that sensibly reacts with activity changes to expression conditions. It is difficult to pin down the reasons for these differences exactly. Reconstitution of recombinant or even purified P450 with POR and CYB5 is a non-trivial problem especially if different protein variants shall be compared for quantitative kinetic parameters. Reconstitution under saturating conditions with respect to electron donators, e.g., at a POR:P450 ratio of 10, is a straightforward practical way, but in hepatocytes, POR is stoichiometrically underrepresented (ratio about 1:10) and may be limiting for monooxygenase activity (Gomes et al., 2009). Enzyme variants may interact differently with the electron donors and catalytic differences could thus depend on reconstitution conditions. In addition, *N*-terminal modifications required to achieve high expression in *E. coli* may interact with the DNA-polymorphisms to be analyzed. In the COS-cell system, on the other hand, the POR:P450 ratio can neither be controlled nor quantified because expression of P450 is too low for spectral quantitation.

Taken together, the data from expression systems indicated that catalytic differences may exist between CYP2B6.6 and CYP2B6.1. However, except for the *E. coli* study, the differences were rather modest and at present it cannot be concluded with certainty whether the CYP2B6.6 variant is catalytically more or less active compared to the wild-type, atleast for bupropion and efavirenz. Taken all evidence together, the decrease in hepatic expression due to erroneous splicing caused by the c.516G>T SNP (Desta et al., 2007; Hofmann et al., 2008) most plausibly explains most of the phenotypic *in vivo* activity differences observed with efavirenz and bupropion.

*CYP2B6*6* SNP-related functional differences were also observed with inhibitors. In contrast to the wild-type enzyme the recombinantly expressed K262R variant was not inactivated by efavirenz, but both enzymes were irreversibly inhibited by 8-hydroxyefavirenz (Bumpus et al., 2006; Bumpus and Hollenberg, 2008). Lower susceptibility to inhibition of the K262R variant and the CYP2B6.6 double variant compared to CYP2B6.1 was also found with respect to sertraline and clopidogrel, as well as several other potent drug inhibitors of CYP2B6 (Talakad et al., 2009). These data indicate a role of genetic polymorphisms in drug–drug interaction sensitivity of CYP2B6, a finding that warrants further investigation *in vivo*.

OTHER *CYP2B6* VARIANTS AND OTHER SUBSTRATES – *IN VITRO* STUDIES

The two amino acid changes that together constitute the **6* allele also occur in isolation, although at much lower frequencies (Klein et al., 2005; Zanger et al., 2007; **Table 1**). In most pharmacogenetic studies they are not being determined and functional data is therefore rare, especially concerning **9* (Klein et al., 2005). Data from recombinant systems as well as liver data suggest that the K262R variant possesses catalytic activities similar to the wild-type enzyme, although with different substrates moderately increased or decreased activity was observed (**Tables 2** and **3**; Desta et al., 2007; Hofmann et al., 2008). The allele with amino acid change [R487C] in exon 9 (**5* variant; **Table 1**) expresses very low levels of protein which does not translate into similarly reduced activities as measured with bupropion as well as efavirenz (Lang et al., 2001; Desta et al., 2007; Hofmann et al., 2008). The variant was shown to have higher specific activity compared to wild-type (Radloff et al., 2013). In human liver, this leads to partial compensation of low expression, finally resulting in a phenotype with moderately decreased activity with bupropion and efavirenz *in vivo*. This explains why *CYP2B6*5* was not associated with efavirenz pharmacokinetics in HIV patients (Burger et al., 2006).

The second most important functionally deficient allele is *CYP2B6*18* (c.983C>T [I328T]), which occurs predominantly in African subjects with allele frequencies of 4–11% (Mehlotra et al., 2007; Li et al., 2012). The I328T variant expressed no detectable protein or activity toward bupropion, 7-ethoxy-4-trifluoromethylcoumarin (7-EFC), selegiline and artemether in COS-1 cells whereas a partially defective protein was expressed in insect cells (Klein et al., 2005; Watanabe et al., 2010; Honda et al., 2011). This demonstrates another example for expression system-dependent differences. Most likely the 2B6.18 variant is temperature-sensitive and thus able to be expressed at the lower temperature (27°C) of insect cell culture but not at 37°C. Interestingly, the I328T+Q172H double variant expressed partially functional protein in HEK293 cells and in yeast (Wang et al., 2006), indicating that Q172H can stabilize the I328T variant. The **18* allele is thus phenotypically a null allele, at least *in vitro* with some substrates. This is supported by many *in vivo* studies (see below).

At least 12 additional null or low-activity alleles have been described and analyzed with various substrates (Lang et al., 2004; Klein et al., 2005; Rotger et al., 2007; Watanabe et al., 2010; Honda et al., 2011). Although they are rather rare in all investigated populations they may have profound effects on drug metabolism if present in compound heterozygous genotypes, e.g., in combination with **6* or **18* (Rotger et al., 2007). The *CYP2B6*22* allele is a gain-of-function variant associated with increased transcription *in vitro* (Zukunft et al., 2005) and with increased activity *in vivo* (Rotger et al., 2007). It was shown that a -82T>C exchange alters the TATA-box into a functional CCAAT/enhancer-binding protein binding site that causes increased transcription from an alternative downstream initiation site (Zukunft et al., 2005). Interestingly, the -82T>C polymorphism also confers synergistically enhanced *CYP2B6* inducibility by the PXR ligand rifampicin in human primary hepatocytes (Li et al., 2010).

New variants are discovered preferentially in previously uncharacterized ethnic groups. Restrepo et al. (2011) described two novel combinations of known amino acid variants in a Colombian population. Structural variants including a novel CYP2B6/2B7P1 duplicated fusion allele (*CYP2B6*30*) were found when individuals from various ethnicities were screened for copy number variations (Martis et al., 2012). Furthermore, three novel and five previously uncharacterized amino acid variants in different combinations (*CYP2B6*33* to **37*) were identified by resequencing the *CYP2B6* gene in a Rwandese cohort of HIV-1-infected patients (Radloff et al., 2013). The variants were then functionally studied by COS-1 cell expression and by *in silico* prediction tools. At least four of the variants were shown to result in complete or almost complete loss of function with bupropion and efavirenz as substrates. The detailed comparison of *in vitro* functionality of the variants with *in silico* prediction tools including a thorough substrate docking simulation analysis points at the challenge to deal with the hundreds of new variants that exist in all populations as currently uncovered by next generation sequencing approaches and large scale population projects (see links above).

Table 3 | Properties of recombinantly expressed CYP2B6 protein variants with other clinical substrates.

	Artemether[1] COS-7 cells (Honda et al., 2011)		Selegiline[2] COS-7 cells (Watanabe et al., 2010)		Chlorpyrifos[3] COS-1 cells (Crane et al., 2012)		Cyclophosphamide[4] Sf9 cells (Ariyoshi et al., 2011)		Cyclophosphamide[4] *E. coli* (Raccor et al., 2012)	
Variant	K_m (μM)	V_{max}(%)	K_m (μM)	V_{max}(%)	K_m (μM)	V_{max}(%)	K_m (mM)	V_{max}(%)	K_m (mM)	V_{max}(%)
2B6.1	3.1	100	48.2	100	1.84	100	2.68	100	3.6	100
2B6.6	6.72	416	56.6	169	1.97	254	1.62	99	4.0	155.2
2B6.4	2.73	196	45.8	147	1.09	1094	2.75	74	3.5	67.1
2B6.5	6.87	55	70.1	85	0.80	441			5.1	72.4

[1] *O-Demethylation.*
[2] *N-Demethylation (mean values were calculated for several expressions of CYP2B6.1).*
[3] *Desulfation.*
[4] *4-Hydroxylation.*

CLINICAL STUDIES WITH DIFFERENT DRUGS

The widely used anticancer and immunosuppressant prodrug cyclophosphamide depends on bioactivation to 4-hydroxycyclophosphamide for cytotoxic activity. Bioactivation is highly variable in cancer patients and has been attributed mainly to CYP2B6 *in vitro* and *in vivo* with contributions from CYP2C19 and CYP3A4 (Chang et al., 1993; Raccor et al., 2012). The case of cyclophosphamide 4-hydroxylation deserves particular attention, as it exemplifies substrate-dependent effects of CYP2B6 pharmacogenetics. Cyclophosphamide 4-hydroxylation was initially reported to be enhanced in livers genotyped *CYP2B6*6/*6* (Xie et al., 2003), which was confirmed in several later *in vivo* studies (Xie et al., 2006; Nakajima et al., 2007; Torimoto and Kohgo, 2008). However, other *in vivo* studies analyzing pharmacokinetics or clinical outcome also presented contradictory or negative results (Singh et al., 2007; Ekhart et al., 2008; Melanson et al., 2010; Yao et al., 2010; Raccor et al., 2012). *In vitro,* insect cell-expressed recombinant CYP2B6.4 [K262R] had lower activity for cyclophosphamide 4-hydroxylation (Ariyoshi et al., 2011; Raccor et al., 2012). The CYP2B6.4 and CYP2B6.6 variants thus display mirror-inverted catalytic activities toward efavirenz and cyclophosphamide, in that the former variant is the catalytically more active one with efavirenz, whereas the opposite is true for the latter variant (**Table 3**). A direct comparison of catalytic properties of the two variants with the reference enzyme expressed in insect cells supports this inverse behavior of the two variants toward these two substrates (Ariyoshi et al., 2011). Interestingly, several studies associated other variants including *CYP2B6*4, *5, *8*, and **9* with lower 4-OH cyclophosphamide formation *in vivo* or with worse outcome (Takada et al., 2004; Bray et al., 2010; Helsby et al., 2010; Joy et al., 2012). Taken together, the data concerning cyclophosphamide from both *in vivo* and *in vitro* indicate that *CYP2B6* polymorphism plays a role, although the studies are so far not yet conclusive. This may be explained by different study size design as well as lack of consistency in allele definition and genotype information among studies (Helsby and Tingle, 2011).

In addition to efavirenz, *CYP2B6* genotype also affects plasma levels of the antiretroviral drug nevirapine (Penzak et al., 2007; Mahungu et al., 2009). The impact of the *CYP2B6* 516G>T polymorphism on nevirapine exposure was confirmed and quantified in a pharmacometric analysis of nevirapine plasma concentrations from 271 patients genotyped for 198 SNPs in 45 ADME (absorption, distribution, metabolism, and excretion) genes and covariates (Lehr et al., 2011). Moreover, nevirapine-related cutaneous adverse events, which are most likely major histocompatibility complex (MHC) class I-mediated, were significantly influenced by *CYP2B6* polymorphism while hepatic side effects, most likely MHC class II-mediated, were unaffected by *CYP2B6* (Yuan et al., 2011).

CYP2B6 allele variants were also investigated in the context of the synthetic μ-opioid receptor agonist, methadone, which is metabolized by CYPs 3A4/5, 2B6, and 2D6, and used as a maintenance treatment for opioid addiction. In **6/*6* carriers (*S*)-methadone plasma levels were increased leading to potentially higher risk of severe cardiac arrhythmias and methadone associated deaths (Crettol et al., 2005; Eap et al., 2007; Bunten et al., 2011). Methadone dose requirement for effective treatment of opioid addiction was shown to be significantly reduced in carriers of this genotype (Levran et al., 2011).

CONCLUSION

The polymorphism of the *CYP2B6* gene has initially been studied by reverse genetics approach, i.e., starting from the identification of genetic variants in DNA and liver samples, followed by *in vitro* characterization of genotyped livers and expressed variant proteins. Clinical studies have then contributed to identify the variants that are important *in vivo*, and *in vitro* studies are again needed to identify and mechanistically explain causal variants. Nevertheless, C*YP2B6* pharmacogenetics has yet to be fully explored, especially with respect to combined effects of the involved variants on both expression and catalytic properties, the latter of which additionally depend on the substrate. While the relevance for HIV-1 therapy with efavirenz is well established and translational approaches have already been clinically tested, an increasing number of studies suggest clinical relevance for additional drug substrates.

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Pharmacogenetics of human ABC transporter ABCC11: new insights into apocrine gland growth and metabolite secretion

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Cell secretion is an important physiological process that ensures smooth metabolic activities and tissue repair as well as growth and immunological functions in the body. Apocrine secretion occurs when the secretory process is accomplished with a partial loss of cell cytoplasm. The secretory materials are contained within secretory vesicles and are released during secretion as cytoplasmic fragments into the glandular lumen or interstitial space. The recent finding that the non-synonymous single nucleotide polymorphisms (SNP) 538G > A (rs17822931; Gly180Arg) in the *ABCC11* gene determines the type of earwax in humans has shed light on the novel function of this ABC (ATP-binding cassette) transporter in apocrine glands. The wild-type (Gly180) of ABCC11 is associated with wet-type earwax, axillary osmidrosis, and colostrum secretion from the mammary gland as well as the potential risk of mastopathy. Furthermore, the SNP (538G > A) in the *ABCC11* gene is suggested to be a clinical biomarker for the prediction of chemotherapeutic efficacy. The aim of this review article is to provide an overview on the discovery and characterization of genetic polymorphisms in the human *ABCC11* gene and to explain the impact of *ABCC11* 538G > A on the apocrine phenotype as well as the anthropological aspect of this SNP in the *ABCC11* gene and patients' response to nucleoside-based chemotherapy.

Keywords: apocrine gland, earwax, axillary osmidrosis, breast cancer, mastopathy, 5-fluorouracil, tamoxifen

INTRODUCTION

ATP-binding cassette (ABC) proteins form one of the largest protein families encoded in the human genome (Dean et al., 2001; Holland et al., 2003). Hitherto more than 48 human ABC protein genes have been identified and sequenced (Klein et al., 1999). It has been reported that mutations of ABC protein genes are causative of several genetic disorders in humans (Dean et al., 2001). Many of the human ABC proteins are involved in membrane transport of drugs, xenobiotics, endogenous substances, or ions, thereby exhibiting a wide spectrum of biological functions (Schinkel and Jonker, 2003). Based on the arrangement of molecular structure components, i.e., nucleotide binding domains and topologies of transmembrane domains, the hitherto reported human ABC proteins have been classified into seven different sub-families (A to G; Klein et al., 1999; Borst and Elferink, 2002; Ishikawa, 2003).

In 2001, three research groups independently cloned two novel ABC transporters named ABCC11 and ABCC12 from the cDNA library of human adult liver (Bera et al., 2001; Tammur et al., 2001; Yabuuchi et al., 2001). These two genes have been found to be located on human chromosome 16q12.1 in a tail-to-head orientation with a separation distance of about 20 kb (**Figure 1**). The predicted amino acid sequences of both gene products show a high similarity to those of ABCC4 and ABCC5, which suggests that they have the typical structure of "full" ABC transporters with 12 transmembrane helixes and two ABCs. Interestingly, there is no putative mouse or rat orthologous gene corresponding to human *ABCC11* (Shimizu et al., 2003), which indicates that *ABCC11* is not an orthologous gene but rather a paralogous gene generated by gene duplication in the human genome. In contrast, *ABCC12* and its orthologous genes are found in several different species including humans, primates, and rodents (Shimizu et al., 2003; Ono et al., 2007). Transcript analyses suggest that human ABCC11 mRNA is ubiquitously expressed in human adult and fetal tissues (Tammur et al., 2001; Yabuuchi et al., 2001). High levels of ABCC11 mRNA were observed in breast cancer tissues (Bera et al., 2001; Yabuuchi et al., 2001). **Table 1** summarizes major findings in the research of the *ABCC11* gene.

When transfected exogenously, the ABCC11 wild-type (WT) protein was localized in the apical membrane of Madin–Darby canine kidney cells strain II (MDCK II cells; Bortfeld et al., 2006). The substrate specificity of ABCC11 WT was characterized in more detail by an *in vitro* transport assay with plasma membrane vesicles prepared from pig LLC-PK1 cells transfected with an ABCC11 WT expression vector (Chen et al., 2005). The results of this assay demonstrated that ABCC11 WT is able to transport a variety of lipophilic anions including cyclic nucleotides, glutathione conjugates such as leukotriene C_4 (LTC_4) and S-(2,4-dinitrophenyl)-glutathione (DNP-SG), steroid sulfates such

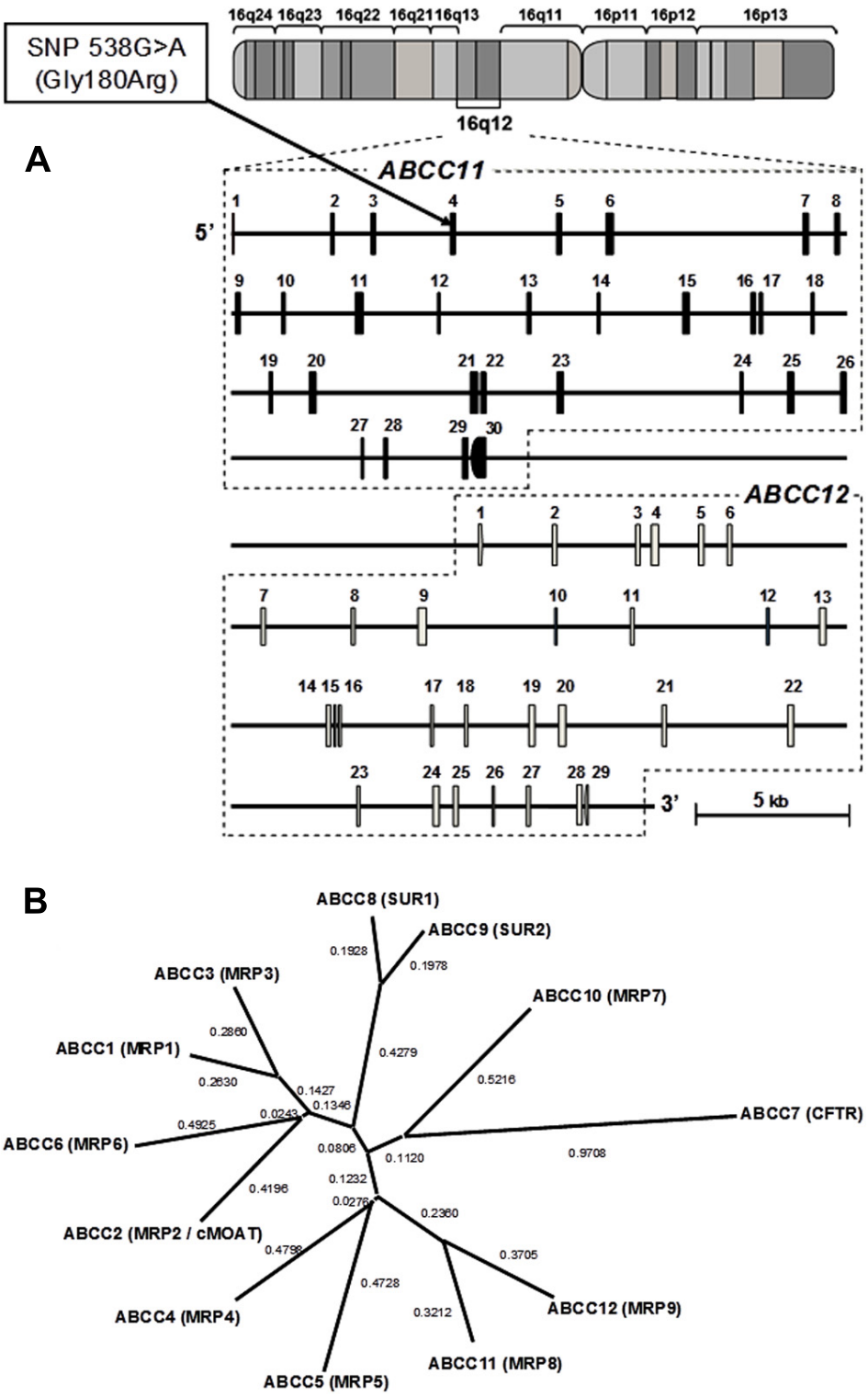

FIGURE 1 | Schematic illustration of the genomic structures of *ABCC11* and *ABCC12* genes on human chromosome 16q12.1. (A) The cytogenetic location of the *ABCC11* gene as well as the structures of exons and introns were analyzed by BLAST searches on the human genome. A non-synonymous SNP: 538G > A (Gly180Arg), an earwax determinant, is located in exon 4. **(B)** Phylogenetic tree of the ABCC subfamily including CFTR, SUR1, SUR2, and MRPs. The phylogenetic tree was modified from Toyoda et al. (2008). The phylogenetic relationships among members of the "C" sub-family of human ABC transporters were calculated by using the distance-based neighbor-joining methods (Saitou and Nei, 1987).

Table 1 | Historical overview on identification of the *ABCC11* gene and its function.

Year	Scientific progress	Reference
2001	Discovery of human *ABCC11 (MRP8)* gene	Bera et al. (2001), Tammur et al. (2001), Yabuuchi et al. (2001)
2003	Characterization of ABCC11 as a cyclic nucleotide efflux pump	Guo et al. (2003)
2005	*In vitro* characterization of substrate selectivity of ABCC11	Chen et al. (2005)
2006	Characterization as an apical efflux pump for steroid sulfates in CNS	Bortfeld et al. (2006)
2006	Identification of *ABCC11* SNP c.538G > A as the determinant of human earwax type	Yoshiura et al. (2006)
2007	Association between the degrees of apocrine colostrum secretion and *ABCC11* genotype	Miura et al. (2007)
2008	Involvement of ABCC11 in 5-fluorouracil resistance in lung cancer cell line	Oguri et al. (2007)
2008	Regulation of ABCC11 expression by estrogen in MCF7 cells	Honorat et al. (2008)
2009	Discovery of ubiquitination and proteasomal degradation of SNP c.538G > A variant	Toyoda et al. (2009)
2009	Association between axillary osmidrosis and ABCC11 wild-type	Nakano et al. (2009), Toyoda et al. (2009), Inoue et al. (2010)
2009	Japanese map of the earwax gene frequency: a nationwide collaborative study	Super Science High School Consortium (2009)
2010	Association between breast cancer risk and *ABCC11* wild-type in Japanese women	Ota et al. (2010), Toyoda and Ishikawa (2010)
2010	Involvement of ABCC11 in pemetrexed resistance in lung cancer	Uemura et al. (2010)
2011	No association between breast cancer risk and *ABCC11* wild-type in European women	Beesley et al. (2011), Lang et al. (2011)
2011	Down-regulation of ABCC11 protein in human breast cancer	Sosonkina et al. (2011)

as estrone 3-sulfate ($E_1$3S) and dehydroepiandrosterone 3-sulfate (DHEAS), glucuronides such as estradiol 17-β-D-glucuronide ($E_2$17βG), the monoanionic bile acids glycocholate and taurocholate, as well as folic acid and its analog methotrexate (MTX; Chen et al., 2005; Bortfeld et al., 2006). Chemical structures of these compounds are presented in **Figure A1** in Appendix. While ABCC11 transports a variety of organic anions, endogenous natural substrates for this transporter have not yet been identified.

GENETIC POLYMORPHISMS AND PHYSIOLOGICAL FUNCTION OF ABCC11

To date, more than 10 non-synonymous single-nucleotide polymorphisms (SNPs) have been reported in the human *ABCC11* gene (**Figure 2**). Among those SNPs, one SNP (rs17822931; 538G > A, Gly180Arg) determines the human earwax type (Yoshiura et al., 2006). Interestingly, this SNP (538G > A) exhibits wide ethnic differences in allelic frequency (**Table 2**). In Mongoloid populations in Asia, the frequency of the 538A allele is predominantly high, whereas the frequency of this allele is low among Caucasians and Africans (Yoshiura et al., 2006; Toyoda et al., 2008; **Figure 3A**). The frequency of the 538A allele exhibits a north-south and east-west downward geographical gradient with the highest peak in Korea. It is suggested that the 538A allele arose in northeast Asia and thereafter spread throughout the world (Yoshiura et al., 2006), apparently reflecting the inter-continental migration of *Homo sapiens* (**Figure 3B**). A similar geographical gradient was also observed in the frequency of the 2677G (Ala893) allele of the *ABCB1* (*P-glycoprotein*/*MDR1*) gene (Sakurai et al., 2007). In this regard, anthropological aspects of SNP 538G > A in the *ABCC11* gene are described in the following section.

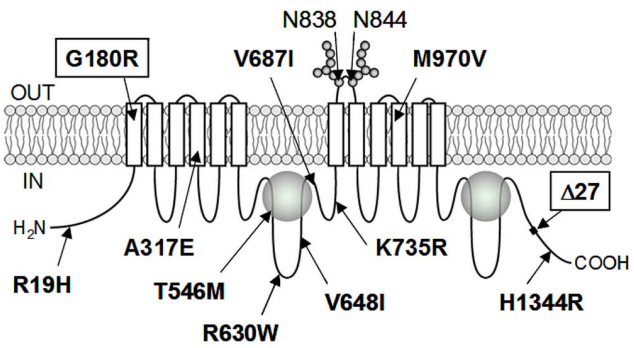

FIGURE 2 | Schematic illustration of ABCC11 protein structure and hitherto known non-synonymous SNPs. ABCC11 has a total of 12 transmembrane (TM) regions and two intracellular ATP-binding cassettes. Asn838 and Asn844 residing in an extracellular loop between transmembrane helices TM7 and TM8 are *N*-linked glycosylation sites in the ABCC11 WT protein. Locations of hitherto reported non-synonymous SNPs and Δ27 (a rare deletion mutation) are indicated in the putative structure of ABCC11. G180R and Δ27 are related to the formation of dry-type earwax.

Earwax (cerumen) is a secretory product of the ceruminous apocrine glands, which can be classified into two phenotypes in humans, wet (sticky) and dry. The dry-type is most commonly found within the Asian population, especially among Koreans, Japanese, and Chinese; whereas the wet-type is the dominant phenotype for many Africans and Caucasians. The 538A/A genotype gives the dry phenotype, whereas both the 538G/A and G/G genotypes give the wet phenotype. This relationship is consistent with observations that earwax type is a Mendelian trait and that the wet phenotype is dominant to the dry one.

Table 2 | Frequencies of *ABCC11* genotypes and allele c.538A among different ethnic groups.

Ethnic groups	Tribes or inhabitants	No. of individuals with genotypes			Number of individuals genotyped	Frequency of allele "A"
		AA (frequency)	GA	GG		
Korean[§]	Daegu city inhabitants	100 (1.000)	0	0	100	1.000
Chinese[§]	Northern and southern Han Chinese	42 (0.808)	10	0	52	0.904
Mongolian	Khalkha tribe[§]	126 (0.759)	36	4	166	0.867
Japanese	Nagasaki people (West-end prefecture of Japan mainland)[§]	87 (0.690)	35*	4	126	0.829
	Okinawa people (Southwest-end prefecture of Japan)	30 (0.517)	25	3	58	0.733
	Yonaguni islander (West-end island of Japan)[§]	13 (0.433)	15	2	30	0.683
	Ainu in Biratori-Nibutani village in Hokkaido[§]	31 (0.534)	23	2	58	0.733
Vietnamese	People from multiple regions	82 (0.536)	60	11	153	0.732
Thai	Northern Thai [Lahu, Shan, Lisu, Hmong, Akha, Mlaburi, and Karen (Mae-sot Thai) tribes combined]	215 (0.505)	163	48	426	0.696
	Central Thai in Bangkok	31 (0.633)	10	8	49	0.735
	Southern Thai (Orang Laut and Sakai tribes combined)	2 (0.026)	23	52	77	0.175
Indonesian	Dayak tribe in Kalimantan	12 (0.293)	23	9	41	0.573
	Toraja and Bugis tribes in Sulawesi	27 (0.270)	49	24	100	0.515
	Flores	18 (0.300)	25	17	60	0.508
	Sumba	9 (0.180)	16	25	50	0.340
	WA tribe in Irian Jaya	0 (0.000)	2	31	33	0.030
Malaysian	Sabah in North Borneo[§]	24 (0.393)	27	10	61	0.566
	Bentong tribe	8 (0.113)	40	23	71	0.394
Taiwanese	Taiwan Aborigine (Yami and Ami combined)	34 (0.330)	48	21	103	0.563
Native American[§]		6[†] (0.300)	8[†]	6	20	0.500
Filipino	Palawan	11 (0.229)	23	14	48	0.469
Easter Islander[§]		4 (0.148)	17	6	27	0.463
Pacific islander[§]		1 (0.143)	1	5	7	0.429
Bolivian[§¶]	Aymara	5 (0.167)	14	11	30	0.400
Kazakh		6 (0.200)	11	13	30	0.383
Native Paraguayan	Ayoreos	2 (0.040)	34	14	50	0.380
	Sanapana	0 (0.000)	14	61	75	0.093
Russian[§]		5 (0.045)	45	62	112	0.246
Solomon Islander[§]		2 (0.323)	25	35	62	0.234
French[§]	From the CEPH families	1 (0.083)	3	8	12	0.208
Andes people[§]		1 (0.100)	2†	6	10	0.200
Hungarian[§]		0 (0.000)	4	6	10	0.200
Jew[§]	Ashkenazi	0 (0.000)	4	6	10	0.200
Ukrainian[§]		0 (0.000)	15	27	42	0.179
Papuan	Papua New Guinea	1 (0.026)	11	26	38	0.171
American of European ancestry[§]	From CEPH families without the French and Venezuelans	1 (0.012)	16	65	82	0.110
Venezuelan[§]	Ye'Kuana village	0 (0.000)	3	11	14	0.107
	Sanuma village	0 (0.000)	0	19	19	0.000
Vanuatu islander	Aneityum and Santo islanders combined	1 (0.011)	17	74	92	0.103
Iberian[§]		0 (0.000)	2	8	10	0.100
Columbian		0 (0.000)	2	17	19	0.053
African	From various sub-Saharan nations	0 (0.000)	1	11	12	0.042
American of African ancestry[§]		0 (0.000)	0	10	10	0.000

*Data are from Yoshiura et al. (2006). [§] Examined for a 27-bp deletion (A27) in ABCC11; *One exceptional case of dry cerumen who has the deletion; [†] One each case of the deletion; [¶] Nine cases of the deletion.*

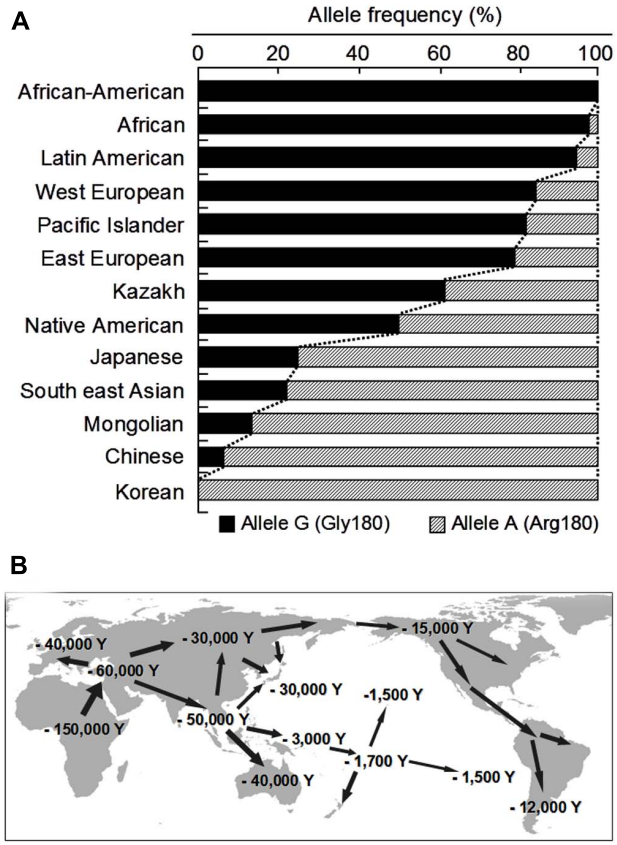

FIGURE 3 | The allele frequencies of the wild-type (WT; Gly180) and 538G > A (Arg180) variant of human *ABCC11* among different ethnic populations (A) and inter-continental migration of *Homo sapiens* (B). Data are from Yoshiura et al. (2006) and Toyoda et al. (2008).

Immunohistochemical studies with cerumen gland-containing tissue specimens revealed that the ABCC11 WT protein with Gly180 was expressed in the cerumen gland (Toyoda et al., 2009). The cerumen gland is one of the apocrine glands. In addition to their presence in the external auditory canal, apocrine glands can be found in the axillary region and breast, whose physical characteristics also are concerned with apocrine glands. In fact, there is a positive association among the wet earwax type, axillary osmidrosis (Yoo et al., 2006), and colostrum secretion from the breast (Miura et al., 2007).

ANTHROPOLOGICAL ASPECTS OF ALLELES 538G AND 538A

It is generally accepted that, since they migrated out-of-Africa, humans spread all over the world with great diversity (Cavalli-Sforza, 2005). Whilst the routes of migration followed by the ancient Mongoloid people remain obscure, two different routes have been proposed (**Figure 3B**). After branching from a main stream common to the ancient Caucasoids 150,000–60,000 years ago (Nei, 1982), the ancient Mongoloids migrated to Southern Asia. It is assumed that one branch remained there or further migrated more south-eastwardly through the so-called Sundaland and Sahuland, and finally reached the Australian continent 50,000–46,000 years ago (Bowler et al., 2003). The last wave of migration of people to the Southern Pacific islands took place more recently, 3,000–1,500 years ago. On the other hand, another branch migrated northward and reached an area around Lake Bikal of Siberia along the Altai mountains. Alternatively, a branch of the ancient Mongoloids might have directly migrated to Siberia from the common stream or migrated from South East Asia toward North East Asia (The HUGO Pan-Asian SNP Consortium, 2009). As an expansion of the last glaciation occurred 30,000–15,000 years ago, small tribes of the ancient Northern Mongoloids may have long been isolated by the glaciers.

As described above, wet/dry types of earwax are determined by the SNP c.538G > A in the *ABCC11* gene; G/A and G/G genotypes give the wet-type and A/A the dry-type. There is a hypothesis that a c.538G (wet-type) → c.538A (dry-type) mutation may have occurred some 40,000 years ago in a tribe of the ancient Northern Mongoloids (Ohashi et al., 2011). Subsequent spreading of the dry-type among the Mongoloids may be explained by a certain selective advantage of the mutation (Matsunaga, 1962), or dry cerumen might have been evolutionally neutral, which would have led to its spread as an attribute of genetic drift. Based on the geographical gradient distribution of the 538A allele with a peak in East Asia, especially in Northern China and Korea, one might assume that the c.538G → c.538A mutation arose somewhere in Siberia. The north-south downward gradient of the 538G allele from Northern China toward Japan and Southern Asia might reflect the Ancient Northern Mongoloid migrations. Similarly, the east-west gradient from Siberia toward Europe (Spitsyn and Afanasèva, 1989) is partly the consequence of Mongolian migration, especially as their invasions pushed westward from about A.D. 500 until A.D. 1500. The situation is much like the frequency of type B for the ABO blood group, which is high in Asia (>25%) and low (<10%) in Europe (Roychoudhury and Nei, 1988). Similar geographic distribution and ethnic differences are known for the *ALDH2* gene, one of the major determinants for alcohol sensitivity (Goedde et al., 1992; Luo et al., 2009).

The relatively high frequency of the 538A allele among present-day Native Americans suggests that their ancestors may have undertaken long journeys from Siberia through the Bering land-bridge (Beringia) to the American continent during the past 15,000 years (Horai et al., 1993; Bonatto and Salzano, 1997; Tokunaga et al., 2001; Dillehay, 2003). Based on recent craniometric studies of skeletons from archeological remains in the Baja California peninsula, however, it has also been postulated that an earlier migration wave from that via the northern route might have occurred from islands of south-eastern Asia by an ancestor common to both Palaeoamericans and Australians around 40,000–12,000 years ago (Gonzáles-José et al., 2003). Furthermore, the allelic data for South Americans revealing the 538A allele frequency of 0.400 in Bolivia, 0.200 in the Andes region, 0.093–0.380 in Paraguay, 0.053 in Colombia, and 0.000 in Venezuela rather favors a hypothesis of an ancient migration through a "pacific coast road" along the Andes mountain range.

ROUTES OF THE JAPANESE POPULATION

The Japanese population is considered to have a dual structure comprising descendants of mixtures between the ancient "Jomon" and "Yayoi" populations. The term "Jomon" is derived

from characteristic twisted cord striations or marks on earthenware used during a prehistoric time (13,000–3,000 years ago) in Japan. As it has been reported that the Jomon had occupied various areas of Japan prior to the Yayoi's appearance, they apparently were either assimilated or rather moved away from the Yayoi (Iizuka and Nakahashi, 2002; Temple et al., 2008; Temple, 2010). It is hypothesized that the dry earwax type was introduced by the Yayoi people to the Jomon population, where the wet-type had been predominant.

Since the admixture of the two Jomon and Yayoi populations is still not complete in Japan even now, the 538G allele frequency is higher in the rather remote areas where the Jomon moved away from the Yayoi's peopling route within the Japanese islands. The Ainu-Japanese people living in a Japanese northern island "Hokkaido" are aboriginal inhabitants of Japan. Based on morphological and mitochondrial DNA polymorphism studies, it has been hypothesized that both the Ainu- and the Okinawa-Japanese living in a Japanese southern island "Okinawa" are descendants of the ancient native Japanese, "Jomon" people (Horai et al., 1991). Molecular studies demonstrated that Ainu-Japanese still retain a certain degree of their own genetic uniqueness among surrounding populations, and exhibit considerable genetic distance from other East Asian populations (Tokunaga et al., 2001; Tajima et al., 2004). As far as wet-type of earwax and the 538G allele frequency are concerned, the Ainu- and Okinawa-Japanese people are not the direct descendants "Yayoi" from the Ancient Northern Mongoloids of Siberian origin.

To analyze the nationwide allele frequency, the Super Science High School (SSH) consortium collected a total of 1963 fingernail samples of pupils/students from at least one high school/university in every prefecture in Japan (Super Science High School Consortium, 2009). Although the 538G allele frequency varied among the 47 prefectures, the Gifu/Kyoto and Okinawa prefectures showed the lowest and highest values for the 538G allele, respectively. Other areas with low frequencies of the 538G allele included Northeastern Kyushu, Northern Shikoku, and Kinki districts, showing a belt-like zone, whereas those with high frequencies of the 538G allele next to Okinawa were the Southwestern Kyushu, Hiroshima prefecture, and Tohoku districts. Those observations strongly suggest that the admixture of "Jomon" and "Yayoi" populations is still not complete in Japan.

STRONG ASSOCIATION BETWEEN AXILLARY OSMIDROSIS AND THE GENOTYPE OF *ABCC11* 538G > A

Today in Japan, axillary osmidrosis is recognized as a disease that is covered by the national health insurance system. Axillary osmidrosis, which is exemplified by unpleasant odors, sweating and staining of clothes, is often perceived, especially by young women, as a distressing and troublesome problem (Wu et al., 1994). Axillary osmidrosis is a chronic skin condition characterized by an excessive, axillary malodor resulting from apocrine gland dysfunction (Hess et al., 2008). Certain people display an excessive fear, aversion or psychological hypersensitivity to unpleasant smells or odors. They tend to opt for aggressive surgical treatments and are sometimes categorized as having osmophobia. Interestingly, an association between wet-type earwax and axillary osmidrosis had already been recognized more than half a century ago (Matsunaga, 1962). Hence, the wet-type of earwax has frequently been used as one of diagnostic criteria and characteristics in the clinic. This relationship, however, had only been based on the observations of those two respective phenotypes. Therefore, there has been a need for objective evidence for diagnosis of axillary osmidrosis to prevent unnecessary treatments for such patients.

Table 3 | Association of *ABCC11* genotype with earwax type and axillary osmidrosis in Japanese subjects.

Earwax type		Genotype at *ABCC11* 538G > A			Axillary osmidrosis patients
		G/G	G/A	A/A	
Dry	262	0	0	262	0
Wet	300	23	277	0	182

Data are calculated from Inoue et al. (2010) and Nakano et al. (2009).

Recently, it has been reported that the ABCC11 WT allele is intimately associated with axillary osmidrosis as well as the wet-type of earwax (**Table 3**). Several studies have already concluded that the genotypes at *ABCC11* 538G > A would be useful biomarkers for the diagnosis of axillary osmidrosis (Nakano et al., 2009; Toyoda et al., 2009; Inoue et al., 2010; Martin et al., 2010). Therefore, it is suggested that genotyping of the *ABCC11* gene would provide an accurate and practical criterion for guidance of appropriate treatment and psychological management of patients (Toyoda et al., 2009; Inoue et al., 2010; Ishikawa and Hayashizaki, 2012). Rapid genotyping of the *ABCC11* gene is briefly described in Appendix.

Sweat produced by the axillary apocrine glands is odorless. Secretions from the apocrine glands, however, can be converted to odoriferous compounds by bacteria (*Corynebacteria*), which results in the formation of the unique "human axillary odor" (Shehadeh and Kligman, 1963). Axillary osmidrosis patients (538G/G homozygote or G/A heterozygote) were observed to have significantly more numerous and larger-sized axillary apocrine glands as compared with those in subjects carrying the A/A homozygote. Indeed, the 538G allele in the *ABCC11* gene is associated with axillary osmidrosis (Nakano et al., 2009; Toyoda et al., 2009; Inoue et al., 2010; Martin et al., 2010), and ABCC11 WT (Gly180) would be responsible for the secretion of preodoriferous compounds from the axillary apocrine gland. In primates, axillary odors may play a role in olfactory communication, although no documented behavioral or endocrine changes resulting from volatiles produced in the axillae have been reported to occur in humans. Previous studies have described the presence of androgen steroids in the axillary area. Androsterone sulfate (AS) and DHEAS were detected in an extract of axillary hairs in addition to high levels of cholesterol (Julesz, 1968). It was also demonstrated, following injection of radioactive pregnenolone or progesterone, that steroid secretion was concentrated in the axillary area (Brooksbank, 1970). In those studies, however, the axillary sweat collected from the skin surface contained a mixture of materials from apocrine, eccrine, and sebaceous glands, in addition to desquamating epidermal cells. In this respect, Labows et al. (1979) demonstrated that pure apocrine secretions contained at least two androgen steroids, AS and DHEAS, in addition to cholesterol. It is strongly suggested that

one of the physiological functions of ABCC11 WT is the active transport of steroid metabolites, such as AS and DHEAS, into the lumen of apocrine glands.

ENDOPLASMIC RETICULUM-ASSOCIATED DEGRADATION OF THE SNP VARIANT OF ABCC11

Why does one SNP (c.538G > A) in the human *ABCC11* gene affect the function of apocrine glands? To address this question, we have recently provided evidence that proteasomal degradation of the SNP variant (Arg180) of ABCC11 is the underlying molecular mechanism (Toyoda et al., 2009). ABCC11 WT with Gly180 is an *N*-linked glycosylated protein, which is localized within intracellular granules and large vacuoles as well as at the luminal membrane of secretory cells in the cerumen apocrine gland (Toyoda et al., 2009). *N*-linked glycosylation occurs at both Asn838 and Asn844 in the extracellular loop between transmembrane domains 7 (TM7) and 8 (TM8) of the ABCC11 WT protein. In contrast, the SNP variant (Arg180) lacks *N*-linked glycosylation and readily undergoes proteasomal degradation, most probably via ubiquitination. As a consequence, no granular or vacuolar localization was detected in the cerumen apocrine glands of people homozygous for the SNP variant.

Morphological differences were previously reported between the secretory cells of wet and dry types of human ceruminous glands (Shugyo et al., 1988). In the wet-type glands, the Golgi apparatus was reportedly well developed, whereas it was generally small in the corresponding cells of the dry-type. Furthermore, numerous intracellular granules were observed in the wet-type gland in close relationship to their well-developed Golgi apparatus, whereas intracellular granules were rare in the dry-type gland.

The endoplasmic reticulum (ER) and Golgi apparatus are the synthesis and maturation sites of proteins destined for the plasma membrane, the secretory and endocytic organelles, and secretion (Ellgaard et al., 1999; Helenius and Aebi, 2004). Efficient quality control systems have evolved to prevent incompletely folded proteins from moving along the secretory pathway. Accumulation of misfolded proteins in the ER would detrimentally affect cellular functions. Therefore, misfolded proteins may be removed from the ER by retrotranslocation to the cytosol compartment where they are degraded by the ubiquitin-proteasome system. This process is known as ER-associated degradation (ERAD; Mori, 2000; Ellgaard and Helenius, 2001; Hampton, 2002; Kleizen and Braakman, 2004). It is likely that the product of the SNP variant (Arg180) is recognized as a misfolded protein in the ER and readily undergoes proteasomal degradation. An electrostatic charge (either positive or negative) at amino acid 180 in the transmembrane domain 1 (TM1) might interfere with correct folding of the *de novo* synthesized ABCC11 protein in the ER (Toyoda et al., 2009). This ERAD processing of the SNP variant (Arg180) of ABCC11 may greatly influence the activity of ceruminous apocrine glands and determine the type of human earwax. Similar ERAD processing is considered to take place for the SNP variant (Arg180) of ABCC11 in axillary and mammary apocrine glands. **Figure 4** schematically illustrates the impact of this SNP on the cellular localization and function of ABCC11 in secretory cells of the apocrine gland. Asn838 and Asn844 are glycosylation target sites in the human ABCC11. The *N*-linked glycans are thought to be subjected to extensive modification as glycoproteins mature and move through the ER via the Golgi apparatus to their final destinations as, for example, intracellular granules and large vacuoles of secretory cells in the apocrine gland.

ABCC11 WILD-TYPE ALLELE AND BREAST CANCER RISK

In 1971, Petrakis (1971) first reported that international mortality and frequency rates for breast cancer seemed to be associated with the frequency of the allele for wet-type earwax. Caucasians and African–Americans in the USA as well as Germans exhibited approximately fourfold higher rates of breast cancer mortality as compared with Japanese and Taiwanese women (Petrakis, 1971). Nevertheless, the phenotypic association of the wet-type of earwax with breast cancer remained controversial (Petrakis, 1971; Ing et al., 1973).

At the present time, it is not well understood whether ABCC11 WT really contributes to breast cancer risk. Therefore, we have most recently carried out a genotyping study of the SNP 538G > A (Gly180Arg) for a total of 543 Japanese women to examine the association between the frequency rate of breast cancer and the allelic frequency of the G allele (WT). We obtained blood samples from patients with invasive breast cancer ($n = 270$) and control volunteers ($n = 273$) and genotyped the SNP c.538G > A in the *ABCC11* gene. The frequency of the 538G allele in the breast cancer patients was higher than that in the control volunteers. The odds ratio for the women with genotypes (G/G + G/A) to develop breast cancer was estimated as 1.63 (p-value = 0.026), suggesting that the 538G allele in the *ABCC11* gene is moderately associated with the risk of breast cancer (Ota et al., 2010). The relative ratio of breast cancer patients carrying the homozygous 538G/G allele was 1.77-fold greater than that of the corresponding healthy volunteers (Ota et al., 2010). This relative ratio was even greater than that (1.41-fold) for breast cancer patients carrying heterozygous alleles 538G/A. The G allele appears to be positively related to breast cancer frequency in the groups of Japanese women studied. In contrast, no significant association with breast cancer risk was observed in Europeans (Beesley et al., 2011; Lang et al., 2011).

We initially thought that some genetically determined variation(s) in the apocrine system might influence susceptibility to breast cancer, although the genetic determinant (538G > A SNP in *ABCC11*) was not known at that time. It is hypothesized that the function of ABCC11 *per se*, or metabolites transported by ABCC11, may stimulate the proliferation of apocrine gland cells to enhance the risk of mastopathy (**Figure 5**). This hypothesis is supported by evidence that apocrine glands are large in individuals carrying the WT allele of the *ABCC11* gene. So far as the cell cycle machinery is operating normally, proliferation of apocrine gland cells should be controlled to a certain extent. When a somatic mutation has occurred in *BRCA1*, *BRCA2*, *p53*, or *p21*, however, it can lead to deleterious and unregulated proliferation of those cells (**Figure 5**).

REGULATION OF *ABCC11* GENE EXPRESSION

In 2004, Bieche et al. (2004) reported that ABCC11 was up-regulated in estrogen receptor α-positive breast tumors, as compared with normal breast tissue. In contrast, Sosonkina et al. (2011) reported down-regulation of ABCC11 protein in human

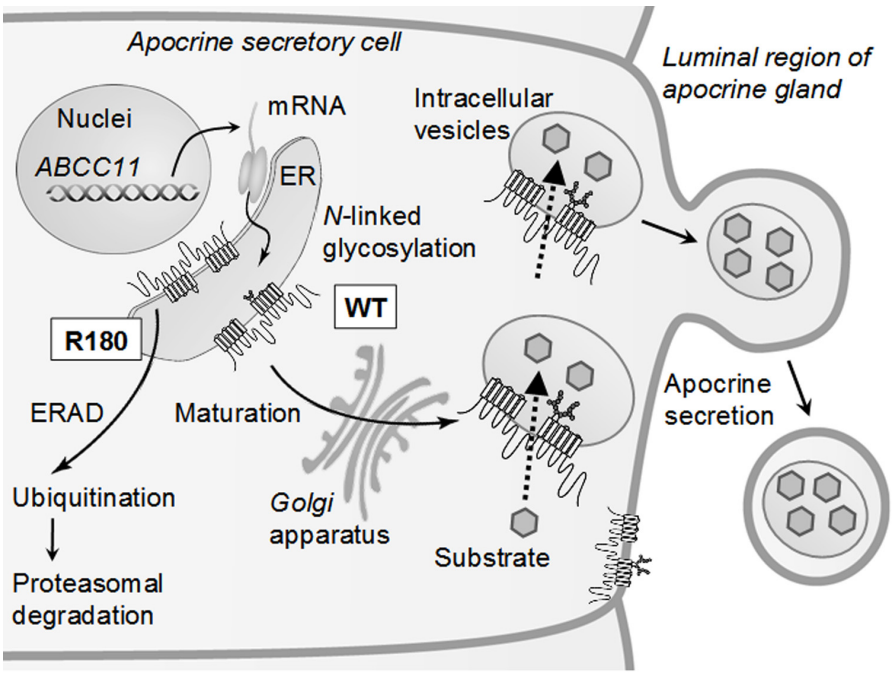

FIGURE 4 | Schematic illustration of intracellular sorting of ABCC11 WT and proteasomal degradation of the R180 (Arg180) variant in secretory cells of the apocrine gland. *De novo* synthesized ABCC11 WT is *N*-linked glycosylated at Asn838 and Asn844 in the ER, further processed in the Golgi apparatus, and destined for the membranes of intracellular granules and vacuoles. Ceruminous components are thought to be transported by ABCC11 WT and sequestered in intracellular granules and vacuoles. SNP variant R180 lacking *N*-linked glycosylation is recognized as a misfolded protein in the ER and readily undergoes ubiquitination and proteasomal degradation (ERAD pathway). ER, endoplasmic reticulum; ERAD, ER-associated degradation. This scheme is modified from Toyoda et al. (2009).

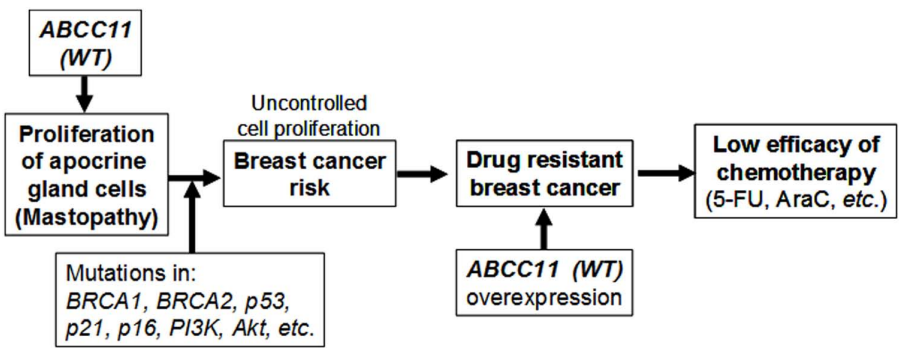

FIGURE 5 | The potential impact of *ABCC11 WT* (538G) on the apocrine phenotype, patients' response to nucleoside-based chemotherapy, and the potential risk of mastopathy and breast cancer. BRCA-1, breast cancer-1; BRCA-2, breast cancer-2; PI3K, phosphatidylinositol 3-kinase; ERα (+), estrogen receptor α-positive; 5-FU, 5-fluorouracil; AraC, cytarabine. This scheme is modified form Toyoda and Ishikawa (2010).

breast cancer. Park et al. (2006) investigated the mRNA levels of ABC transporter genes in breast cancer patients who underwent sequential weekly paclitaxel/FEC (5-fluorouracil, epirubicin, and cyclophosphamide) neoadjuvant chemotherapy. Their analysis showed that the expression of ABCC11 was increased (fold ratio = 2.71) in those patients with residual disease as compared with the patients having no pathologic evidence of any residual invasive cancer cells in the breast.

Honorat et al. (2008) have demonstrated that endogenous ABCC11 mRNA levels in breast cell lines are correlated with their estrogen receptor α-status. Interestingly, they found that ABCC11 expression was reduced *in vitro* by E_2 treatments. Furthermore, this E_2-dependent down-regulation of ABCC11 expression was blocked by co-treatment with tamoxifen, an E_2 antagonist. These findings suggest that ABCC11 expression is regulated directly or indirectly by estrogen receptor α and that the prolonged exposure of breast cancer cells to tamoxifen can lead to up-regulation of ABCC11.

Hauswald et al. (2009) have shown that some of the histone deacetylase inhibitors induced the expression of several ABC

transporters, including the *ABCC11* gene, to render acute myeloid leukemia cells resistant to a broad-spectrum of drugs. Molecular mechanisms underlying the induction remain to be elucidated. Since histone deacetylase inhibitors can be utilized in combination with conventional anti-cancer drugs in clinical trials, such induction of the ABCC11 WT may affect the efficacy of nucleoside-based chemotherapy.

RELEVANCE OF ABCC11 WT TO DRUG RESISTANCE IN CANCER CHEMOTHERAPY

The potential involvement of ABCC11 in drug resistance of breast cancer has recently been reported. For example, ABCC11 mRNA is found to be highly expressed in breast tumors (Bera et al., 2001; Yabuuchi et al., 2001; Bieche et al., 2004), and particularly in invasive ductal adenocarcinomas (available at: https://www.oncomine.org/resource/logn.html, accessed October 01, 2012). This expression is reportedly regulated by estrogen receptor-β (Honorat et al., 2008) and induced by 5-fluorouracil (5-FU; Oguri et al., 2007). Furthermore, it has been shown that ABCC11 is directly involved in 5-FU resistance by means of the efflux transport of the active metabolite 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP; Guo et al., 2003; Kruh et al., 2007; Oguri et al., 2007). It remains to be elucidated whether the expression of ABCC11 WT (538G) is related to drug resistance of breast cancer and high rates of mortality. Further clinical studies, including protein expression studies in tumors, will be needed to clarify the potential contribution of ABCC11 to breast cancer risk and prognosis, including drug resistance and chemosensitivity.

Because of their structural similarities, it could be anticipated that substrate specificity of ABCC11 would be related to those of ABCC4 and ABCC5. This indeed has been the case. Ectopic expression of ABCC11 in mammalian cells enhances the cellular efflux of cyclic nucleotides and confers resistance to certain anticancer and antiviral nucleotide analogs (Guo et al., 2003). In fact, it has been reported that ABCC11 WT has an ability to efflux cyclic nucleotides (e.g., cGMP and cAMP) and confers resistance to several antiviral and anticancer nucleotide analogs, such as 5′-FdUMP and 9′-(2′-phosohonylmethoxynyl)adenine (PMEA; Guo et al., 2003; Kruh et al., 2007; Oguri et al., 2007).

Therapy with nucleoside-derived drugs is characterized by inter-individual variability (Heinemann et al., 1988; Abbruzzese et al., 1991). Genetic variants that affect protein products involved in all steps leading to a drug's action may be major contributors to this heterogeneity of responses to nucleoside-based treatments. In particular, variants of drug metabolizing enzymes and transporters might affect the amount of drug needed for an efficient therapeutic response (Errasti-Murugarren and Pastor-Anglada, 2010).

Successful treatment of cancer remains a therapeutic challenge, with a high percentage of patients suffering from drug resistance or relapsed disease. One of such examples involves anti-leukemia treatment with nucleoside analogs, such as cytarabine (AraC). Guo et al. (2009) have recently presented evidence that expression of ABCC11 WT is an important factor affecting acute myeloid leukemia patient survival. It is very likely that the cause of treatment failure in those patients with high expression of ABCC11 WT is an increased extrusion of AraC from blast cells mediated by the transporter.

Uemura et al. (2010) have recently found that both gene and protein expression of ABCC11 were higher in pemetrexed (MTA)-resistant cells than in the parental cells. The MTA-resistant cells showed cross-resistance to MTX, which is a substrate for ABCC11, and intracellular MTX accumulation in MTA-resistant cells was lower than that in the parental cells. They then tested the effect of decreasing the expression of ABCC11 by siRNA and found that decreased expression of ABCC11 enhanced MTA cytotoxicity and increased intracellular MTX accumulation in MTA-resistant cells. These findings suggest that ABCC11 confers resistance to MTA by enhancing the efflux of the intracellular anti-cancer drug.

They further analyzed the relationship between the *ABCC11* gene expression and MTA sensitivity of 13 adenocarcinoma cell lines. In contrast to their expectation, there was no correlation. Instead, the 13 lung adenocarcinoma cell lines could be classified into three groups based on the genotypes of the ABCC11 SNP (538G > A); G/G, G/A, and A/A. The A/A group showed a significant reduction in the IC_{50} value of MTA compared with those values for the combined G/G and G/A groups, indicating that *ABCC11* 538G > A is an important determinant of MTA sensitivity. These results suggest that *ABCC11* 538G > A may be one of the biomarkers for selection of MTA treatment in adenocarcinomas. This finding, however, should be carefully evaluated by clinical studies to determine whether *ABCC11* 538G > A is truly a clinically important biomarker for the prediction of chemotherapeutic efficacy.

CONCLUSION

Apocrine secretion occurs when the secretory process is accomplished with a partial loss of cell cytoplasm. The secretory materials may be contained within the secretory vesicles or dissolved in the cytoplasm and then released during excretion as cytoplasmic fragments into the glandular lumen or interstitial space (Gesase and Satoh, 2003). Hitherto, apocrine secretory mechanisms have not been well characterized (Gesase and Satoh, 2003). Although the biochemical and physiological pathways that regulate the apocrine secretory process are not clearly known, our recent findings (Yoshiura et al., 2006; Toyoda et al., 2009; Inoue et al., 2010) that the SNP (538G > A, Gly180Arg) in the *ABCC11* gene determines both earwax type and axillary osmidrosis have shed light on the novel function of this ABC transporter in apocrine glands. Further studies are needed to explore the clinical significance of ABCC11 so as to elucidate whether there are any other diseases that involve apocrine secretion.

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APPENDIX

CLINICAL GENOTYPING OF SNP 538G > A (Gly180Arg) IN THE *ABCC11* GENE

The rapid growth of personalized medicine is being supported by emerging new technologies together with accumulating knowledge of pharmacogenomics. We tried to create a clinical method, the SmartAmp, to genotype the SNP 538G > A in the human *ABCC11* gene. The SmartAmp-based method enables us to detect genetic polymorphisms or mutations in about 30 min under isothermal conditions without requiring DNA isolation and PCR processes for sample preparation (Ishikawa and Hayashizaki, 2012). **Figure A2A** schematically illustrates the strategy of SNP detection by this clinical method. To determine the SNP 538G > A (Gly180Arg) in the *ABCC11* gene, we prepared one set of primers designated TP, FP, BP, OP, and CP (**Figure A2A**). The TPS discriminate the polymorphism 538G or 538A in the *ABCC11* gene, and the CPS inhibit the background amplification from mismatch sequence pairs (Toyoda et al., 2009; Ishikawa and Hayashizaki, 2012). These primers selectively recognized the SNP 538G > A of the *ABCC11* gene to discriminate homozygous 538G/G, heterozygous 538G/A, and homozygous 538A/A (**Figure A2A**). Thus, this genotyping method would provide a practical tool to support clinical diagnosis (Ishikawa and Hayashizaki, 2012).

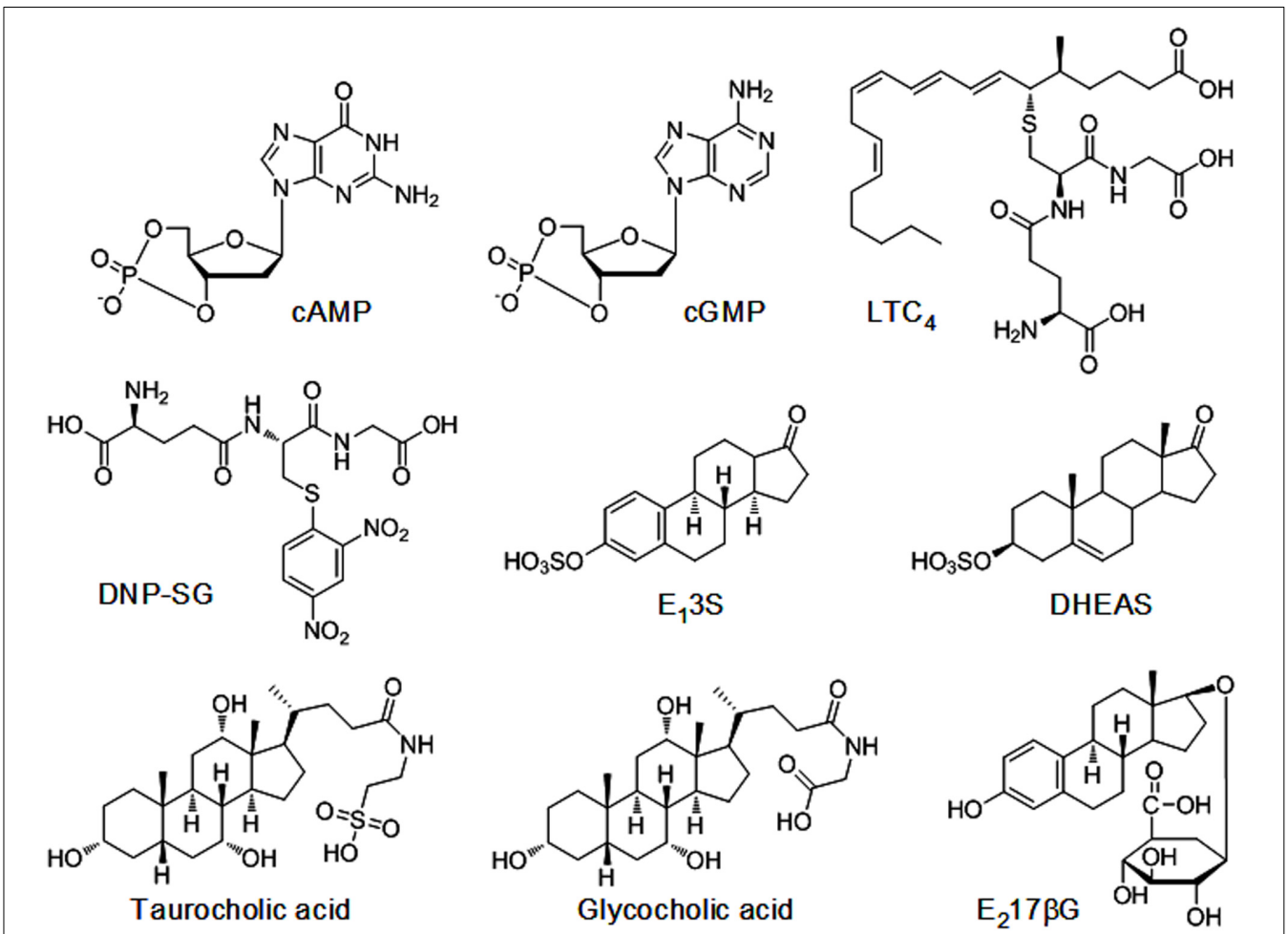

FIGURE A1 | Chemical structures of ABCC11 substrates. Cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), leukotriene C_4 (LTC_4), S-(2,4-dinitrophenyl)-glutathione (DNP-SG), estrone 3-sulfate ($E_1$3S), dehydroepiandrosterone 3-sulfate (DHEAS), and $E_2$17βG (estradiol 17-β-D-glucuronide).

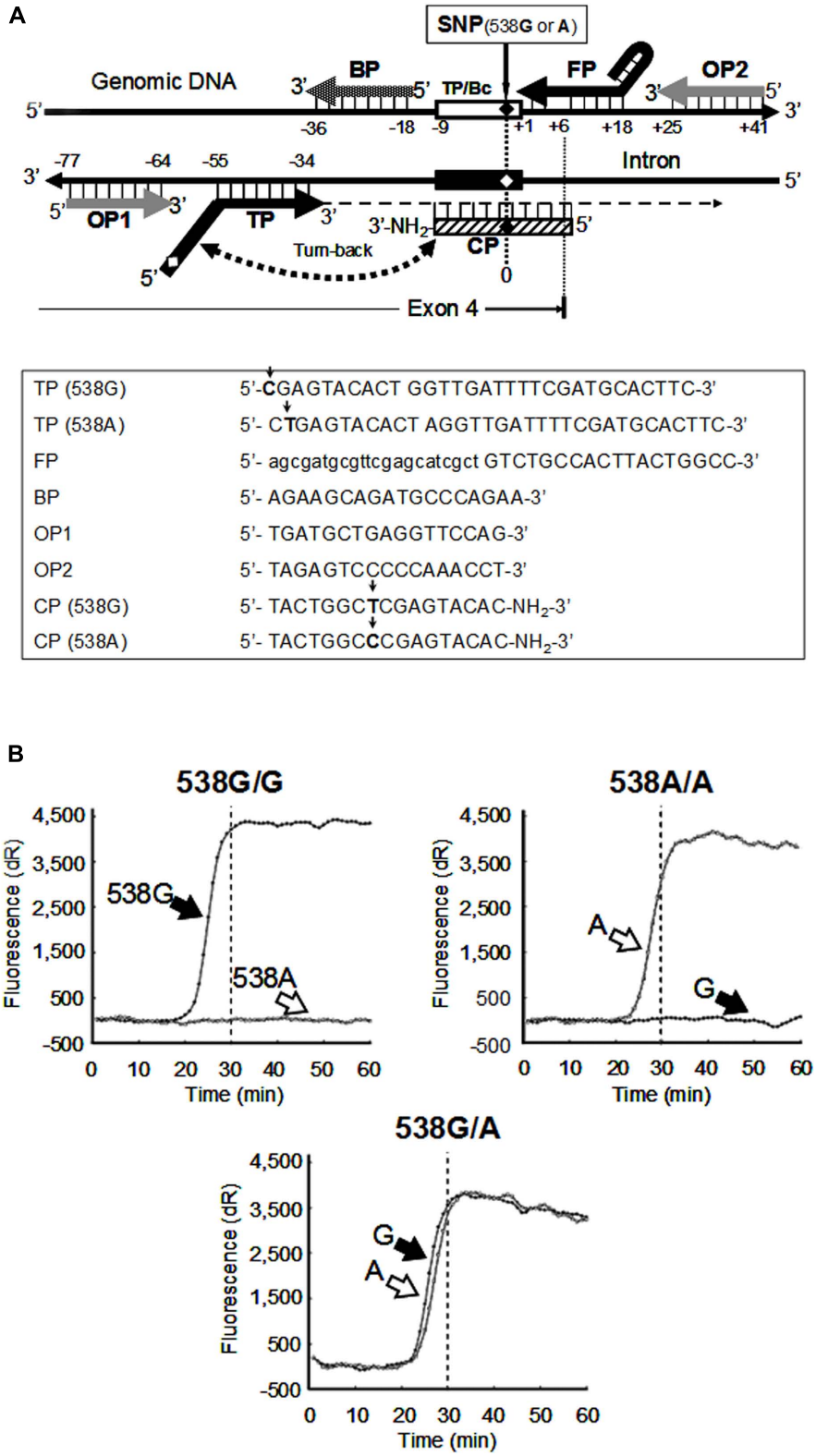

FIGURE A2 | Detection of SNP 538G > A in *ABCC11* gene. (A) Strategy and primers for the SmartAmp-based detection of SNP 538G > A residing in exon 4 of the *ABCC11* gene on chromosome 16q12 (upper panel) and the sequences of ABCC11 allele–specific primers needed for the SNP detection (lower panel). Arrows indicated the sequence difference between the WT and SNP alleles. **(B)** Time-courses of the SNP-detection reaction with ABCC11 allele-specific primers. Data are from Toyoda et al. (2009).

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Pharmacogenomics of cytochrome P450 3A4: recent progress toward the "missing heritability" problem

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CYP3A4 is the most important drug metabolizing enzyme in adult humans because of its prominent expression in liver and gut and because of its broad substrate specificity, which includes drugs from most therapeutic categories and many endogenous substances. Expression and function of CYP3A4 vary extensively both intra- and interindividually thus contributing to unpredictable drug response and toxicity. A multitude of environmental, genetic, and physiological factors are known to influence CYP3A4 expression and activity. Among the best predictable sources of variation are drug–drug interactions, which are either caused by pregnane X-receptor (PXR), constitutive androstane receptor (CAR) mediated gene induction, or by inhibition through coadministered drugs or other chemicals, including also plant and food ingredients. Among physiological and pathophysiological factors are hormonal status, age, and gender, the latter of which was shown to result in higher levels in females compared to males, as well as inflammatory processes that downregulate CYP3A4 transcription. Despite the influence of these non-genetic factors, the genetic influence on CYP3A4 activity was estimated in previous twin studies and using information on repeated drug administration to account for 66% up to 88% of the interindividual variation. Although many single nucleotide polymorphisms (SNPs) within the *CYP3A* locus have been identified, genetic association studies have so far failed to explain a major part of the phenotypic variability. The term "missing heritability" has been used to denominate the gap between expected and known genetic contribution, e.g., for complex diseases, and is also used here in analogy. In this review we summarize *CYP3A4* pharmacogenetics/genomics from the early inheritance estimations up to the most recent genetic and clinical studies, including new findings about SNPs in *CYP3A4* (**22*) and other genes (P450 oxidoreductase (POR), peroxisome proliferator-activated receptor alpha (PPARA)) with possible contribution to CYP3A4 variable expression.

Keywords: cytochrome P450, CYP3A4, pharmacogenomics, pharmacogenetics, drug metabolism, heritability

CYP3A4 DRUG SUBSTRATES AND PHENOTYPING

CYP3A4 is in the majority of individuals abundantly expressed in liver but population variability is extremely high (>100-fold). Average microsomal content has been estimated between ∼60 pmol/mg of microsomal protein (Ohtsuki et al., 2012), 110 pmol/mg (Klein et al., 2012), and ∼150 pmol/mg (Westlind-Johnsson et al., 2003). Hepatic expression of the other CYP3A enzymes CYP3A5, CYP3A7, and CYP3A43 is much lower in adult Caucasians, although CYP3A5 may contribute up to about 50% of the CYP3A pool in individuals with at least one *CYP3A5*1* allele and with low CYP3A4 expression (Hustert et al., 2001a; Kuehl et al., 2001; Koch et al., 2002; King et al., 2003; Klees et al., 2005; McCune et al., 2005). The role of the minor CYP3A forms has been reviewed by others (Burk and Wojnowski, 2004; Daly, 2006). CYP3A4 is thus one of the most abundantly expressed cytochromes in human liver contributing on average about 15–30% to the microsomal P450 pool. Therefore and owing to their extraordinarily broad substrate selectivity CYP3A enzymes play a major role in the metabolism of ∼30–40% of all clinically used drugs (Evans and Relling, 1999; Zanger et al., 2008). These include preferentially lipophilic and bulky compounds from almost all therapeutic categories, including tacrolimus, cyclosporin A, erythromycin, ifosfamide, tamoxifen, benzodiazepines, several statins, antidepressants, opioids, and many more, as summarized in several previous reviews (Bu, 2006; Liu et al., 2007; Zanger et al., 2008). As CYP3A4 is also an efficient steroid hydroxylase, it has in addition an important role in the catabolism of several endogenous steroids like testosterone, progesterone, cortisol, and bile acids (Patki et al., 2003; Bodin et al., 2005).

The high sequence similarity of >85% between the CYP3A isozymes CYP3A4 and CYP3A5 leads to highly similar substrate selectivity between these isoforms (Williams et al., 2002). CYP3A4 and CYP3A5 activities are therefore not specifically discriminable although some probe drugs showed higher selectivity for CYP3A4-including, for example, erythromycin *N*-demethylation (Wrighton et al., 1990) and atorvastatin ortho-hydroxylation (Feidt et al., 2010). Several *in vivo* test substrates are available and have been compared to each other, e.g., midazolam, erythromycin, quinine, and nifedipine (Liu et al., 2007). Endogenous marker activities have also been proposed as functional

CYP3A-markers, including the 6β-hydroxylation of cortisol and the 4β-hydroxylation of cholesterol, but some limitations due to considerable intraindividual variability exist (Chen et al., 2006; Fuhr et al., 2007). Remarkably, however, data obtained with different CYP3A substrates or endogenous markers are not generally well correlated to each other and have therefore to be selected carefully with respect to the expected change in activity and design of the study (Galteau and Shamsa, 2003; Chen et al., 2006; Fuhr et al., 2007). Functional CYP3A phenotyping therefore continues to be a non-trivial problem.

DEGREE OF HERITABILITY

CYP3A4 drug oxidation phenotypes are highly variable but unimodally distributed. Nevertheless there is indication of substantial heritability. For example, antipyrine 4-hydroxylation rate, which is mainly catalyzed by CYP3A4 (Engel et al., 1996), was reported to be largely inherited (85%) as shown in early twin studies (Penno et al., 1981). Moreover, a high degree of heritability for CYP3A4 drug oxidation capacity toward several of its substrates (erythromycin: 89% and midazolam: 96%) was estimated by a repeated drug administration approach (Ozdemir et al., 2000). A recent study used a classical twin model approach in combination with a St. John's Wort induction regimen and quinine sulfate metabolism as CYP3A4 activity measure to elucidate genetic versus non-genetic contribution to variable CYP3A4 induction. Although the uninduced levels were not recorded, the induced CYP3A4 activity heritability was estimated to be at least 66% and environmental factors like BMI, alcohol use, and smoking habit/quantity contributed at least 20% to the variability (Rahmioglu et al., 2011).

In contrast to the genetically polymorphic *CYP2D6*, which is mostly determined genetically with only minor contribution by environmental factors, most of the previously studied *CYP3A4* polymorphisms are either rare or lack phenotypic effect and are thus unable to explain a sizeable fraction of heritable variation. The first documented *CYP3A4* polymorphism is the frequently studied proximal promoter variant *CYP3A4*1B* (−392A > G, rs2740574) which occurs in white populations at ∼2–9% but at higher frequencies in Africans. This single nucleotide polymorphism (SNP) was initially found to be associated with higher tumor grade and stage in prostate cancer and showed higher nifedipine oxidase activity in human livers (Rebbeck et al., 1998). Association of *CYP3A4*1B* with markers of advanced disease was confirmed by some but not all further studies (Keshava et al., 2004; Perera et al., 2009). Although one *in vitro* study on the promoter variant found higher transcriptional activity in cell culture experiments using luciferase constructs, as well as changed protein binding by electrophoretic mobility shift assay (Amirimani et al., 2003), the functional effect of this variant remains questionable given the controversial data reported in several other *in vitro* and human liver studies (Wandel et al., 2000; Spurdle et al., 2002; Rodríguez-Antona et al., 2005; Klein et al., 2012). Also, a study with healthy volunteers phenotyped using the dextromethorphan/methoxymorphinan ratio failed to find an association of *CYP3A4*1B* or **2* with CYP3A4 activity (García-Martín et al., 2002). Recently, resequencing and haplotype tagging studies have been carried out at the *CYP3A* locus in ethnically diverse populations (Thompson et al., 2006; Schirmer et al., 2007; Perera et al., 2009; Perera, 2010). These studies addressed the problem of "missing heritability" of CYP3A4 phenotype variability by considering haplotype structure at the *CYP3A* locus, which was found to be of some relevance. A markedly increased occurrence of rare variants and the presence of a homogeneous group of long-range haplotypes at high frequency were observed in non-African populations (Thompson et al., 2004). Because of their involvement in the metabolism not only of naturally occurring foreign compounds, such as flavonoids, diterpenoids, and other herbal constituents (Zhou et al., 2007) but also of endogenous chemicals such as uroporphyrin (Franklin et al., 2000) it has been suggested that molecular adaptation to the changing environment may have occurred for genes at the *CYP3A* locus. Thus, it has been postulated that a region from about 40 kb upstream of the *CYP3A4* gene promoter to intron 6 was under positive selection in humans (Thompson et al., 2004; Chen et al., 2009). In addition, despite the controversy about the *CYP3A4*1B* allele, a "high haplotype homozygosity" in European Caucasians in contrast to African Americans was observed, which may indicate a negative selection pressure that eliminated the **1B* allele in non-African populations (Schirmer et al., 2006). It should also be mentioned that interaction between SNP/haplotype and gender have been reported to impact on gene expression, further complicating the situation (Schirmer et al., 2007).

An intronic variant rs4646450, located in the *CYP3A5* gene, has recently been associated with reduced tacrolimus dosage requirement in Japanese patients (Onizuka et al., 2011), with reduced endogenous serum dehydroepiandrosterone sulfate concentrations in over 14,000 individuals (Zhai et al., 2011) and was also associated with decreased protein expression and activity of CYP3A4 in human liver, explaining about 3–5% of hepatic variability (Klein et al., 2012). These data underline the importance of haplotype structure throughout the *CYP3A* locus, and they raise questions about causality of the various intronic and regulatory variants. It has been suggested generally that the most penetrant risk alleles may be of negative selective pressure and therefore be rare (Sadee, 2012). This may have led to their oversight in small sized screening studies or due to gene-environment interactions which can substantially mask genetic effects (Rahmioglu et al., 2011; Klein et al., 2012; Sadee, 2012).

Recently, however, an intron 6 variant rs35599367C > T (*CYP3A4*22*) was identified by a systematic screen for SNPs showing allelic mRNA expression imbalance in human liver (Wang et al., 2011). The effect of the variant was not confounded by sex or other variables and it accounted for 7% of the mRNA expression variability in a cohort of 93 liver samples. Expression in cultured cells transfected with minigenes containing exon 6, intron 6, and exon 7 reproduced the functional effect of the T-allele to decrease mRNA *in vitro*, suggesting that it could be the causal variant. As no associated splice variants or other hints to the exact mechanism were found, the variant was supposed to affect nascent RNA elongation rate (Wang et al., 2011; Sadee, 2012). In another recent study using 150 liver samples this variant was associated with decreased protein levels, but not when corrections were made for multiple testing (Klein et al., 2012).

Furthermore, the potential impact of CYP3A4 protein variants on drug–drug interaction was emphasized in a recent *in vitro* study

using recombinantly expressed variant CYP3A4 proteins 3A4.2, 3A4.7, 3A4.16, and 3A4.18, where inhibitory profile differences of itraconazole and cimetidine were described in relation to the CYP3A4 variants (Akiyoshi et al., 2011). Thus, although most of the protein variants are too rare to make an impact on a population scale, individual carriers of such variants may have risk not only for impaired drug metabolism but also for a different drug–drug interaction profile.

CYP3A4 POLYMORPHISMS – CLINICAL IMPACT

Most previous clinical studies that implemented *CYP3A* genotyping are not considered here because they used mainly the *CYP3A5*3* polymorphism as marker. The reader is referred to other reviews on this topic (Burk and Wojnowski, 2004; Daly, 2006). **Table 1** shows a selection of *CYP3A4*-including pharmacogenetic *in vivo* studies. Although the effects of the novel intron 6 variant were not very pronounced *in vitro*, its impact became more apparent by *in vivo* pharmacokinetic and pharmacodynamic studies. Significant association of *CYP3A4*22* with decreased $AUC_{0-\infty}$ ratio in atorvastatin-treated volunteers or 1.7- to 5-fold reduced statin dose of T-allele carriers compared to non-T carriers to achieve optimal lipid control was shown in volunteer and clinical studies (Wang et al., 2011; Klein et al., 2012). The association of *CYP3A4*22* with simvastatin lipid-lowering response was shown in another clinical study (Elens et al., 2011a). Furthermore, renal transplant recipients who were carriers of the low-expressor T-allele had a 33% reduced mean daily-dose requirement to reach the same tacrolimus blood concentration compared to homozygotes for the wild type allele (Elens et al., 2011b) and 1.6- to 2.0-fold higher dose-adjusted trough blood levels of tacrolimus and cyclosporine A in stable renal transplant patients (Elens et al., 2012), both indicating lower CYP3A4 activity. Despite these consistent reports, the rather low frequency of the intron 6 SNP [global minor allele frequency (MAF) 2.1%, Caucasians 3–8%] limits broader contribution to overall CYP3A4 variability.

CYP3A4 POLYMORPHISMS – END OF THE ROPE?

Databases are very helpful tools to get an overview on genetic variability. However, there are major collections which serve as basis for other online browsers (**Table 2**). The most important collection of data concerning cytochrome P450 gene haplotypes and *in vitro* as well as *in vivo* functional impact are summarized in the *CYPallele* homepage (updated last August 31, 2011)[1]. To date besides the reference *CYP3A4*1* another 21 alleles are included, comprising at least 21 amino acid changes and 2 frame shift variants. Although this collection is a useful resource for functional data related to the alleles, it only provides limited information on genetic variability. Further valuable information on *CYP3A4* SNPs and clinical pharmacogenetics is available on the homepage of *The Pharmacogenomics Knowledgebase PharmGKB*[2]. More comprehensive genetic information can be obtained from other databases, which collect SNP data of diverse projects, but in contrast to the *CYPallele* database or *PharmGKB* they lack cross reference to documented functional impact (**Table 2**). The dbSNP database[3] (build 137) currently provides information on 550 SNPs for the human *CYP3A4* gene of which 21% are located within the coding region, including 61 non-synonymous amino acid changes, 18 synonymous, and 4 frameshift mutations. Remarkably, for most of the coding SNPs no MAF or reliable population related distribution data is yet available. This may soon be overcome by new data releases of the international "*1000 Genomes Project*." This large international collaborative project aims to generate a "Deep Catalog of Human Genetic Variation" including the entire spectrum of all types of DNA changes from SNPs and small indels (insertions/deletions) to large structural variations like copy number variants and large deletions and insertions, as well as frequency information and haplotype context (The 1000 Genomes Project Consortium, 2010)[4]. Next generation sequencing technologies are used to sequence the complete diploid genomes of 2500 unidentified individuals from about 25 different populations. With the release of the Integrated Phase 1 Variant set in May 2012 phased genotype calls on 1092 samples for SNPs, short indels, and large deletions from low-coverage sequencing became available. However, no functional or medical information is being collected. The potential of the *1000 Genomes Project* for pharmacogenomics has been emphasized before (Gamazon et al., 2009). To illustrate how *1000 Genomes* data may be used in pharmacogenetics for fine analysis, e.g., in search for causal variants, **Figure 1** shows exemplarily the contribution of new SNP information to the genomic region of *CYP3A* flanking the *CYP3A5*3* SNP rs776746 (±250 kb). While in the *HapMap* data (**Figure 1A**) only eight SNPs were in strong linkage to rs776746, 49 SNPs from current *1000 Genomes* dataset were identified to be in strong LD, and interestingly also some moderately linked SNPs are located within *CYP3A4* (**Figure 1B**). Unfortunately, linkage information of SNPs with potential functional impact within the *CYP3A4* gene, like **1B* or **22*, is not available from both datasets, probably due to missing frequency data.

For the *CYP3A4* gene the current *1000 Genomes* database release (Integrated Phase 1 Variant Set; May 2012) contains in total 471 SNPs assigned to the major transcript (ENST00000336411), including NCBI dbSNP database content (build 134). The *1000 Genomes Project* to date (accessed September 2012) contributed 129 new SNPs to this collection. Besides 115 intronic variant positions 7 non-synonymous amino acid changes were previously unknown, of which four variants were reported on the *CYPallele* homepage (**4*, **8*, **11*, **21*) and only one was predicted to be deleterious by phenotype prediction tools SIFT and PolyPhen (**Table 3**). However, for most of the SNPs, availability of global MAF and population frequency data is still limited. Although most of the novel variants are rare, it should be noted that these can collectively make a marked contribution to the functional population variability. For individual patients, the combination of a rare deleterious variant with more frequent alleles of low function (compound heterozygosity) can be of predictive value. This was for example demonstrated for the polymorphic *CYP2B6* in a large clinical study with HIV patients under efavirenz

[1] http://www.cypalleles.ki.se/
[2] http://www.pharmgkb.org/
[3] http://www.ncbi.nlm.nih.gov/projects/SNP
[4] http://www.1000genomes.org

Table 1 | Selected pharmacogenetic *in vivo* studies with CYP3A4-relevant data.

	Substrate/metabolite	Study design	Subjects	Genotypes	Remarks/findings
Penno et al. (1981)	Antipyrine (orally)/4-OH-antipyrine	Twin study	Adult male unmedicated twins (10 unrelated, 10 monozygotic twins, 10 dizygotic twins)	No	Heritability 0.88
Ball et al. (1999)	Erythromycin, nifedipine	Five racial groups Hispanics, African American, Asian	802 Healthy volunteers	−292 (5′UTR) in *CYP3A4*	No effect on 3A4 dependent demethylation of erythromycin or nifedipine metabolism
Ozdemir et al. (2000)	10 Substrates	Repeated drug administration method	161 (Meta-analysis from 16 studies, literature search)	No	Genetic contribution to hepatic CYP3A4 activity 0.96–0.66
García-Martín et al. (2002)	Dextromethorphan/3′-methoxymorphinan ratio	Single oral dose with 24 h urine collection	76 Healthy volunteers (white subjects)	*CYP3A4*1B, *2, *4, *5, *6, *8, *11, *12, *13*	No association of **1B* with activity phenotype
Floyd et al. (2003)	Midazolam oral/systemic clearance; erythromycin breath test	Constitutive and induced with rifampicin	57 Healthy subjects (European, African American)	*CYP3A4*1B, CYP3A5*3, CYP3A5*6,* and *CYP3A5*7; MDR1* ex21/26	Fold increase after rifampicin is related to *CYP3A4*1B*
Hesselink et al. (2003)	Variability in cyclosporine and tacrolimus PK	Pharmacokinetic	110 + 64 Kidney transplant recipients	*CYP3A4*1B, CYP3A5*3, MDR1*_3435C > T	*CYP3A4*1B*: lower dose-adjusted trough tacrolimus levels
Hesselink et al. (2004)	Population PK of cyclosporine		151 Kidney and heart transplant recipients	*CYP3A4*1B* and **3, CYP3A5*3* and **6, MDR1*_3435C > T	*CYP3A4*1B*: significantly higher oral cyclosporine clearance
He et al. (2005)	Midazolam oral clearance		26 Healthy volunteers, mixed ethnicity	Novel variants/haplotypes	*CYP3A4*VI – CYP3A5*3A* with only limited impact on CYP3A metabolism
Wang et al. (2005)	Lipid-lowering by simvastatin; 6β-OH-cortisol/cortisol		211 Hyperlipidemic Chinese patients	*CYP3A4*4, *5, *6*	*CYP3A4*4* was related to a decrease of CYP3A4 activity, and seemed to increase the lipid-lowering effects of simvastatin
Diczfalusy et al. (2009)	4β-OH cholesterol/cholesterol	Rifampicin induction	24 Unrelated healthy volunteers	No	Plasma 4β-hydroxycholesterol has half-life of 17 days, low intraindividual variability

(Continued)

Table 1 | Continued

	Substrate/metabolite	Study design	Subjects	Genotypes	Remarks/findings
Diczfalusy et al. (2011)	4β-OH cholesterol/cholesterol, midazolam, quinine	Rifampicin induction; ritonavir inhibition	135 Tanzanian, 136 Swedes, 146 Korean	*CYP3A5*1*	4β-OH cholesterol/cholesterol is a good measure for long-term studies, midazolam/quinine rather for short term studies
Wang et al. (2011)	Required doses for optimal lipid control		235 Patients with stable doses of atorvastatin, simvastatin, or lovastatin	13 SNPs including *CYP3A4*1B* and **22*	T-allele required significant lower statin doses for optimal lipid control
Chen et al. (2011)	Methadone and metabolite plasma concentrations and withdrawal scores		366 Han Chinese patients with heroin addiction under methadone maintenance treatment	*CYP3A4* rs4646440/rs2242480 and others	*CYP3A4* rs4646440, rs2242480 significantly associated with severity of withdrawal symptoms and side effects
Suhre et al. (2011)	Urine metabolic traits	GWAS KORA S4 population study	862 Independent male (Germany)	GWAS	*CYP3A4* rs17277546-A associated to metabolic trait androsterone sulfate
Rahmioglu et al. (2011)	Variability of induced CYP3A4	Classical twin study, induction with St. John's wort	367 Twins; (99DZ, 63MZ, 43 singletons)	No	Basal activities not measured
Elens et al. (2011a)	Simvastatin-mediated cholesterol reduction	Rotterdam study subset	80 Incident simvastatin users	*CYP3A4*22*	*CYP3A4* rs35599367 reduced CYP3A4 activity; stronger simvastatin lipid-lowering response
Elens et al. (2011c)	Tac/CsA dose adjustment		99 Renal transplant recipients	*CYP3A4*22; CYP3A5*3*	Associated with altered tac/csA metabolism
Elens et al. (2011b)	Overall mean daily-dose requirement to reach the same predose tacrolimus blood concentration	International randomized controlled clinical trial (fixed dose, concentration controlled study)	185 Renal transplant recipients		*CYP3A4* rs35599367C > T associated with a significantly altered TAC metabolism and increased risk of supratherapeutic TAC concentrations early after transplantation
Zhai et al. (2011)	DHEAS levels	GWAS meta-analysis	14,846 Individuals	GWAS	*TRIM4*_rs17277546 in linkage to *3A4/5* SNPs
Elens et al. (2012)	CsA levels	Fixed dose concentration controlled study	172 *De novo* kidney transplant patients	*CYP3A4*22; CYP3A5*3 CYP3A4*1B ABCB1*_3435C > T	*3A4*22* carriers under CsA associated with worse renal function and delayed graft function due to reduced 3A4 activity

(Continued)

Table 1 | Continued

	Substrate/metabolite	Study design	Subjects	Genotypes	Remarks/findings
Klein et al. (2012)	Atorvastatin AUC	Single-dose PK study	56 Healthy volunteers	*CYP3A4*22* PPARa_rs4253728; ARNT_rs2134688; GR_rs258747; PGRMC2_rs3733260	*CYP3A4*22* and PPARa_rs4253728 associated with higher $AUC_{0-\infty}$ ratio
Rahmioglu et al. (2012)	Variability of induced CYP3A4; measure: urinary MR quinine/3-hydroxyquinine	GWAS classical twin study, induction with St. John's wort	310 Healthy female twins (from previous study Rahmioglu et al., 2011)	GWAS	No significant genome-wide associations to induced CYP3A4 activity; several genomic regions were highlighted that may play minor roles
Zochowska et al. (2012)	Cyclosporine A, sirolimus		100 Renal transplant recipients	*CYP3A4*1B*, *CYP3A5*3*	*CYP3A5*1* and/or *CYP3A4*1B* carriers require significantly higher doses of cyclosporine A to reach target levels

treatment. It was shown that several individuals with elevated efavirenz plasma concentrations could be predicted when rare variants were considered together with the common low-expressor **6*-allele (Rotger et al., 2007). Taking this into account, the knowledge on genetic variability of ADME (*A*bsorption *D*istribution *M*etabolism *E*xcretion) genes including *CYP3A4* in diverse populations will profit enormously from *1000 Genomes* and new candidate SNPs influencing *CYP3A4* expression and activity will certainly be discovered, although prediction or experimental testing of functional impact of the many novel variants pose a challenging task.

GENES INFLUENCING CYP3A4 PHENOTYPE OUTSIDE THE *CYP3A* LOCUS

Regulation of CYP3A4 phenotype expression is enormously complex including influences from networks of nuclear receptors and other transcription factors (Pascussi et al., 2008), hormonal and inflammatory pathways (Aitken et al., 2006), heme and protein synthesis, and degradation pathways, as well as components of monooxygenase complexes and their interaction partners (see in **Figure 2**). Potentially influential polymorphisms in these genes on *CYP3A4* expression have been studied only in recent years by single gene or pathway-directed approaches.

NADPH:cytochrome P450 oxidoreductase (POR) is a microsomal flavoprotein and an obligatory electron donator in the microsomal P450 monooxygenase reaction. In contrast to the multiplicity of CYPs, mammals have only a single *POR* gene. In humans the gene is located on chromosome 7q11.2 and spans about 72 kb, coding for a 680 amino acid protein. Complete deletion of the *por* gene in mouse is embryonically lethal most likely due to deficient adrenocortical steroidogenesis (Shen et al., 2002; Otto et al., 2003). In contrast, liver-specific *por* knockout leads to phenotypically and reproductively normal mice that accumulate hepatic lipids and have a drastically diminished capacity for hepatic drug metabolism (Gu et al., 2003; Finn et al., 2007).

The amount of POR in human liver is stoichiometrically ∼5- to 10-fold lower compared to the microsomal CYP pool, and hepatic POR levels are correlated to several P450 monooxygenase activities, suggesting that it represents a limiting factor (Huang et al., 2008a; Gomes et al., 2009). Functional polymorphisms in *POR* should thus be expected to influence CYP activity. In recent years rare POR missense mutations in humans were discovered that impair POR function and cause disordered steroidogenesis, ambiguous genitalia, and Antley–Bixler syndrome (Flück et al., 2004; Flück and Pandey, 2011). The *CYPalleles* website currently lists 41 distinct star-alleles, most of which represent very rare mutations, but common polymorphisms also exist (Huang et al., 2005, 2008a).

In particular the A503V variation (*POR*28*) is common with frequencies ranging from 19 to 37% in all major ethnicities. In recombinant systems the variant retained >50% of the wild type activity toward several CYPs (Huang et al., 2008b; Sandee et al., 2010) and CYP3A4 activity was influenced in a substrate-dependent way (Agrawal et al., 2010). In an *in vivo* study it was found that *POR*28* TT genotype was associated with a 1.6-fold increase in CYP3A midazolam 1′-hydroxylase activity compared with *POR*28* C carriers, a finding that could be replicated in an

Table 2 | Overview of databases providing valuable SNP/mutation data.

Name	link	Info[a]
Human CYPallele	http://www.cypalleles.ki.se/	Overview on functional SNPs in CYP
PharmGKB	http://www.pharmgkb.org/	Summarizing gene-drug-disease relationship, clinical PGx, PGx research, and many more; referring to dbSNP_build137
dbSNP	http://www.ncbi.nlm.nih.gov/projects/SNP/	NCBI database
1000 Genomes Project	http://browser.1000genomes.org/index.html	NGS project
SNPedia	http://www.snpedia.com/index.php/SNPedia	Provides information on the platforms including specific polymorphisms
MutDB (Mooney Lab)	http://www.mutdb.org/cgi-bin/mutdb.pl	Data from dbSNP (NCBI) and Swiss-Prot, includes SIFT prediction for amino acid variants
Database of genomic variation (DGV)	http://projects.tcag.ca/variation/	All SNPs from dbSNP, overview on structural genomic variations (CNV, segmental duplications/deletions, InDels)
GeneCards	http://www.genecards.org/index.shtml	dbSNP information in a compact overview with graphically illustrated frequencies
SNAP	http://www.broadinstitute.org/mpg/snap/	Displaying linkage graphically (R^2 measure)

[a] *PGx, pharmacogenomics; Swiss-Prot, manually annotated and reviewed protein knowledge base at the Swiss Institute of Bioinformatics Resource portal ExPASY; NGS, next generation sequencing; SIFT, "sorting intolerant from tolerant" protein variant prediction tool.*

independent cohort (Oneda et al., 2009). Similarly, in a cohort of allograft recipients under tacrolimus therapy, *POR*28* T-allele carriers had significantly higher tacrolimus dose requirements compared to non-carriers but only if they were genotypic CYP3A5 expressors (i.e., presence of at least one *CYP3A5*1* allele; De Jonge et al., 2011). Thus, the effect of this common *POR* variant appears to depend on the CYP isozyme as well as the substrate.

Numerous additional genetic *POR* variants have been identified (Hart et al., 2008; Huang et al., 2008a). With regard to CYP3A4, several variants were described to affect its function in liver, *in vitro* or *in vivo*. A multivariate analysis in human liver microsomes identified three intronic *POR* variants that affected several CYP activities and that accounted for 60% of the microsomal atorvastatin hydroxylase variability, in concert with the donor's sex (Gomes et al., 2009). Chen et al. analyzed six full length protein variants for their impact on CYP3A4-mediated testosterone hydroxylation and on CYP2B6-mediated bupropion hydroxylation in a recombinant system. Several *POR* variants showed markedly differential effects on both activities (Chen et al., 2012). *In vivo*, reduction in hepatic drug metabolizing CYP3A4 activities caused by *POR* mutations was also described in patients with disordered steroid metabolism (Flück et al., 2010). Taken together these recent advances indicate that *POR* variants are a complex but potentially relevant source of genetic variation for CYP3A-related drug oxidation phenotypes (Miller et al., 2011).

The xenosensors pregnane X-receptor (PXR, *NR1I2*) and constitutive androstane receptor (CAR, *NR1I3*) are important transcription factors in the regulatory network of *CYP3A4* constitutive and inducible expression. Both genes are polymorphic and genetic variants have been studied for impact on *CYP3A4* transcription (Lamba et al., 2005). Despite existence of numerous alternative transcripts, naturally occurring functional polymorphisms of *CAR* that contribute to CYP3A4 phenotypic expression are not well documented. In contrast, the functional impact of genetic *PXR* variants has been studied more thoroughly. For example, the protein variants V140M, D163G, and A370T, discovered in an exon sequence screening of *PXR* gene in DNA samples of several ethnicities, were shown to exhibit altered basal or induced transactivation activity of *CYP3A4* promoter using recombinant variant expression and promoter-reporter constructs (Hustert et al., 2001b). Resequencing analysis of 46 liver tissue samples of Caucasian origin and hepatocytes identified SNPs in the *PXR* promoter and intron 1 associated with CYP3A4 mRNA expression, mainly due to destruction of putative transcription factor binding sites within the regulatory region of *PXR* by polymorphisms in strong linkage (Lamba et al., 2008). Moreover, the *PXR*1B* haplotype, which is tagged by the SNPs 2654T > C and IVS6-17C > T, was related to doxorubicin clearance in Asian breast cancer patients (Sandanaraj et al., 2008). Further studies analyzing patient cohorts under fosamprenavir/lopinavir or artemisinin treatment gave additional hints to some *PXR* variants that may affect inducibility of CYP3A4 phenotype expression (Svärd et al., 2010; Piedade et al., 2012). The occurrence of two SNPs in the 3′UTR region of *PXR*, rs3732359 and rs3732360, has been shown to be associated with altered midazolam 1′-hydroxylation in a liver bank and with more pronounced effect in midazolam clearance of healthy volunteers of African American descent (Oleson et al., 2010). Two recent studies included *PXR* polymorphisms in their pathway targeted pharmacogenetic approach on variable CYP3A4 expression in liver samples (Lamba et al., 2010; Klein et al., 2012). Although in the former study *PXR* rs1523130 was significantly associated with *CYP3A4* mRNA expression by univariate analysis

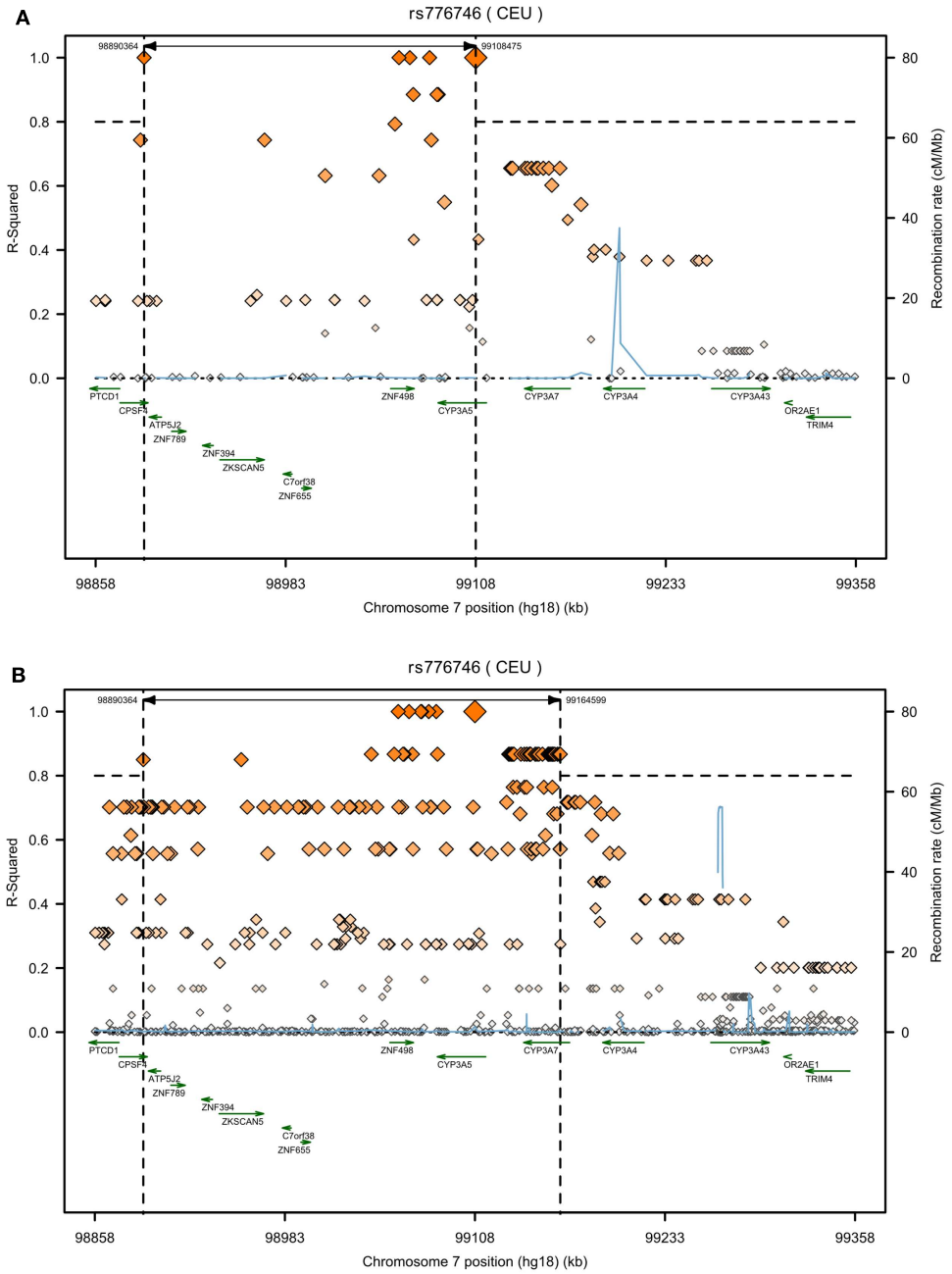

FIGURE 1 | Increase of SNP data information by the *1000 Genomes Project* within the CYP3A locus. LD-plots are generated for rs776746 using SNAP (http://www.broadinstitute.org/mpg/snap/ldplot.php); 250 kb region up- and downstream of the target SNP; genes are marked by green arrows, SNPs are shown with their R^2-square values calculated versus the target SNP rs776746 (red squares) and recombination rates are given in cM/Mb (blue line); dashed lines mark the threshold for strong linkage $R^2 = 0.8$ (horizontal) and the corresponding region of linked SNPs (vertical); **(A)** HapMap3 Release 2, CEU **(B)** *1000 Genomes* Pilot 1, CEU.

and in the multivariate model, the latter study did not document a significant contribution of *PXR* SNPs in univariate as well as in multivariate statistical models on mRNA, protein or activity. In summary, contribution of *PXR* variants to CYP3A4 expression remains controversial and may be limited and dependent on the population studied.

Based on the observation that CYP3A4 and the multi-drug resistance protein P-glycoprotein [P-gp, product of the *MDR1 (ABCB1)* gene] display largely overlapping substrate selectivity (Wacher et al., 1995; Patel and Mitra, 2001; Pal and Mitra, 2006), it has been hypothesized that changes in P-gp expression may influence the intracellular concentration of endogenous or

Table 3 | CYP3A4 coding SNPs provided by the *1000 Genomes Project* (selected are the SNPs for the major transcript ENST00000336411 from http://browser.1000genomes.org/Homo_sapiens/Search/Results?site=ensembl&q=cyp3a4) accessedSeptember 2012.

ID	Chr7: bp	Alleles	Class	Source	CYPallele (activity)[a]	Type	Amino acid	AA co-ordinate[b]	SIFT	PolyPhen
rs12721634	99381661	A/G	SNP	dbSNP	*14	nsc	L/P	15 (2)	Deleterious	Probably D
rs146568511	99377652	G/A	SNP	dbSNP		nsc	P/L	43 (2)	Deleterious	Probably D
rs56324128	99375702	C/T	SNP	dbSNP	*7	nsc, ss	G/D	56 (2)	Deleterious	Probably D
rs59418896	99375666	T/C	SNP	dbSNP		nsc	Y/C	68 (2)	Deleterious	Probably D
rs3091339	99370245	T/C	SNP	dbSNP		nsc	K/E	96 (1)	Deleterious	Probably D
rs142296281	99370218	G/A	SNP	dbSNP		nsc	R/W	105 (1)	Deleterious	Probably D
rs72552799	99367788	C/T	SNP	dbSNP, 1000G	*8 (decr)	nsc	R/Q	130 (2)	Deleterious	Probably D
rs4987161	99366081	A/G	SNP	dbSNP	*17 (decr)	nsc	F/S	189 (2)	Deleterious	Probably D
rs139541290	99366075	A/G	SNP	dbSNP		nsc	V/A	191 (2)	Deleterious	Probably D
rs55901263	99365994	G/C	SNP	dbSNP	*5	nsc	P/R	218 (2)	Deleterious	Probably D
rs75726589	99364854	A/G	SNP	dbSNP		nsc	L/P	233 (2)	Deleterious	Probably D
rs190354371	99361563	A/G	SNP	1000G		nsc	L/P	314 (2)	Deleterious	Probably D
1000GENOMES_7_99361548	99361548	T/C	SNP	1000G	*21	nsc	Y/C	319 (2)	Deleterious	Probably D
rs71581998	99359841	A/T	SNP	dbSNP		nsc	V/E	359 (2)	Deleterious	Probably D
rs67784355	99359829	G/A	SNP	dbSNP, 1000G	*11 (decr)	nsc	T/M	363 (2)	Deleterious	Probably D
rs113716682	99359715	A/G	SNP	dbSNP		nsc	L/P	401 (2)	Deleterious	Probably D
rs143966082	99359710	G/A	SNP	dbSNP		nsc	R/C	403 (1)	Deleterious	Probably D
rs72552797	99359685	G/A	SNP	dbSNP		nsc	P/L	411 (2)	Deleterious	Probably D
rs4986909	99359670	G/A	SNP	dbSNP	*13 (decr)	nsc	P/L	416 (2)	Deleterious	Probably D
rs4986910	99358524	A/G	SNP	dbSNP	*3	nsc	M/T	445 (2)	Deleterious	Probably D
rs72552796	99358521	C/T	SNP	dbSNP		nsc	R/K	446 (2)	Deleterious	Probably D
rs71583803	99358470	A/C	SNP	dbSNP		nsc	F/C	463 (2)	Deleterious	Probably D
rs78764657	99377692	G/C	SNP	dbSNP		nsc	H/D	30 (1)	Deleterious	Possibly D
rs140422742	99375669	T/C	SNP	dbSNP		nsc	K/R	67 (2)	Deleterious	Possibly D
rs57409622	99367428	G/A	SNP	dbSNP		nsc	R/W	162 (1)	Deleterious	Possibly D
rs71581996	99361591	C/A	SNP	dbSNP		nsc	A/S	305 (1)	Deleterious	Possibly D
rs188389063	99381698	G/C	SNP	1000G		nsc	L/V	3 (1)	Deleterious	Benign
1000GENOMES_7_99381694	99381694	A/G	SNP	1000G		nsc	I/T	4 (2)	Deleterious	Benign
COSM42988	99381647	C/T	Somatic_SNV	COSMIC		nsc	V/M	20 (1)	Deleterious	Benign
rs145582851	99364062	C/T	SNP	dbSNP		nsc	R/Q	268 (2)	Deleterious	Benign
rs148633152	99359730	A/G	SNP	dbSNP		nsc	I/T	396 (2)	Deleterious	Benign
rs149870259	99358596	T/C	SNP	dbSNP		nsc	K/R	421 (2)	Deleterious	Benign
rs28371760	99358498	A/-	del	dbSNP		nsc, fs	L/I	454 (1)	Deleterious	Benign
rs150559030	99358488	A/T	SNP	dbSNP		nsc	I/N	457 (2)	Deleterious	Benign
rs12721627	99366093	G/C	SNP	dbSNP	*16 (decr)	nsc	T/S	185 (2)	Tolerated	Possibly D
rs55785340	99365983	A/G	SNP	dbSNP	*2	nsc	S/P	222 (1)	Tolerated	Possibly D
COSM35658	99381689	C/T	Somatic_SNV	COSMIC		nsc	D/N	6 (1)	Tolerated	Benign
rs140355261	99381687	G/C	SNP	dbSNP		nsc	D/E	6 (3)	Tolerated	Benign
COSM42989	99381680	T/C	Somatic_SNV	COSMIC		nsc	M/V	9 (1)	Tolerated	Benign
rs55951658	99367825	T/C	SNP	dbSNP, 1000G	*4	nsc	I/V	118 (1)	Tolerated	Benign
rs147752776	99367818	A/G	SNP	dbSNP		nsc	I/T	120 (2)	Tolerated	Benign
rs4986907	99367427	C/T	SNP	dbSNP	*15	nsc	R/Q	162 (2)	Tolerated	Benign
rs72552798	99367404	C/T	SNP	dbSNP	*9	nsc	V/I	170 (1)	Tolerated	Benign
rs3208361	99366070	T/C	SNP	dbSNP		nsc	I/V	193 (1)	Tolerated	Benign
rs113667357	99366047	T/A/C	SNP	dbSNP		nsc	Q/H	200 (3)	Tolerated	Benign
rs181612501	99365992	A/G	SNP	1000G		nsc	F/L	219 (1)	Tolerated	Benign
rs3208363	99364798	A/C	SNP	dbSNP		nsc	S/A	252 (1)	Tolerated	Benign

(Continued)

Table 3 | Continued

ID	Chr7: bp	Alleles	Class	Source	CYPallele (activity)[a]	Type	Amino acid	AA co-ordinate[b]	SIFT	PolyPhen
1000GENOMES_7_99364768	99364768	C/T	SNP	1000G		nsc	E/K	262 (1)	Tolerated	Benign
rs28371759	99361626	A/G	SNP	dbSNP	*18	nsc	L/P	293 (2)	Tolerated	Benign
rs138675831	99361618	C/T	SNP	dbSNP		nsc	V/M	296 (1)	Tolerated	Benign
1000GENOMES_7_99361606	99361606	T/C	SNP	1000G		nsc	I/V	300 (1)	Tolerated	Benign
rs10250778	99359871	G/T	SNP	dbSNP		nsc	T/N	349 (2)	Tolerated	Benign
rs145669559	99359812	T/C	SNP	dbSNP		nsc	I/V	369 (1)	Tolerated	Benign
rs12721629	99359800	G/A	SNP	dbSNP	*12 (decr?)	nsc	L/F	373 (1)	Tolerated	Benign
rs142425279	99359734	T/C	SNP	dbSNP		nsc	M/V	395 (1)	Tolerated	Benign
rs139109027	99358581	T/C	SNP	dbSNP		nsc	N/S	426 (2)	Tolerated	Benign
rs1041988	99358566	A/G	SNP	dbSNP		nsc	I/T	431 (2)	Tolerated	Benign
rs4986913	99358459	G/A	SNP	dbSNP	*19	nsc	P/S	467 (1)	Tolerated	Benign
rs181210913	99358450	C/T	SNP	1000G		nsc	E/K	470 (1)	Tolerated	Benign
rs138105638	99364063	G/A	SNP	dbSNP		Stop	R/*	268 (1)	–	–
rs34784390	99364036–99364035	–/T	ins	dbSNP		fs		277 (2)	–	–
rs4646438	99364035–99364034	–/T	ins	dbSNP	*6	fs		277 (3)	–	–
rs72552795	99358466–99358465	–/G	ins	dbSNP		fs		465 (1)	–	–
rs67666821	99355807–99355806	–/T	ins	dbSNP	*20 (no)	fs		488 (1)	–	–

[a] *Derived from http://www.cypalleles.ki.se/; Information not provided by 1000 Genomes.*

[b] *Number corresponds to the codon, variant nucleotide of the triplet is given brackets; nsc, non-synonymous coding; fs, frameshift; D, damaging.*

exogenous substances that potentially lead to *CYP3A4* induction, thus contributing to CYP3A4 expression differences (Lamba et al., 2006). Although data supporting this hypothesis have been reported in one human liver association study (Lamba et al., 2010), they were not reproduced in another study (Klein et al., 2012).

In search for additional influential genes, Lamba et al. (2010) phenotyped 128 livers by quantitative real-time PCR for expression of *CYP3A* genes and identified a functional CCT-repeat polymorphism in the *FoxA2* (*HNF3α*) gene to be associated with higher expression of FoxA2 mRNA and its target genes *PXR* and *CYP3A4*. Polymorphisms in *FoxA2*, *HNF4α*, *FoxA3* (*HNF3γ*), *PXR*, *MDR1*, and the *CYP3A4* promoter together with sex explained 24.6% of the variation in hepatic CYP3A4 mRNA expression. However the study lacked information on the relevance of these variations for CYP3A4 protein and activity.

More recently, we carried out extensive candidate gene approaches on 150 Caucasian liver samples phenotyped for CYP1A2 and CYP3A4 mRNA and protein levels, as well as enzyme activity (Klein et al., 2010, 2012). With respect to CYP3A4, we identified SNPs in the Ah-receptor nuclear translocator (*ARNT*), glucocorticoid receptor (*GR*), progesterone receptor membrane component 2 (*PGRMC2*), and peroxisome proliferator-activated receptor alpha (*PPARA*) to be consistently associated with CYP3A4 phenotype in human liver in the multivariate analysis. Validation in an atorvastatin-treated volunteer cohort confirmed decreased atorvastatin-2-hydroxylation in carriers of *PPARA* SNP rs4253728. Moreover, homozygous carriers of the variant had reduced PPARα protein expression in liver, and shRNA-mediated *PPARA* gene knock-down in primary human hepatocytes decreased mRNA expression levels of *CYP3A4* by more than 50%. Multivariate analysis revealed that two linked *PPARA* SNPs alone explained ∼8–9% of the atorvastatin hydroxylase activity variation, whereas all genetic and non-genetic factors together accounted for ∼33% of atorvastatin 2-hydroxylase variation in this liver cohort (Klein et al., 2012). This result was somewhat unexpected, because PPARα had so far not been considered as a major regulator of *CYP3A4*, although inducing effects by fibrates had been noted before in human, but not mouse primary hepatocytes (Prueksaritanont et al., 2005; Rakhshandehroo et al., 2009). The interesting question of course is, whether *CYP3A4* is a direct target of PPARα or whether indirect regulation, e.g., via nuclear receptor crosstalk resulting in downregulation of PXR (Aouabdi et al., 2006; Takagi et al., 2008) is the causative mechanism. Recent data from our group suggest, however, that PPARα indeed binds to several non-consensus PPARα-response elements (PPREs) in the *CYP3A4* promoter to activate transcription in a ligand-dependent manner (Thomas et al., 2013). Because PPARα is a master regulator of lipid homeostasis and energy balance, these results indicate a novel connection between endogenous and xenobiotic metabolism.

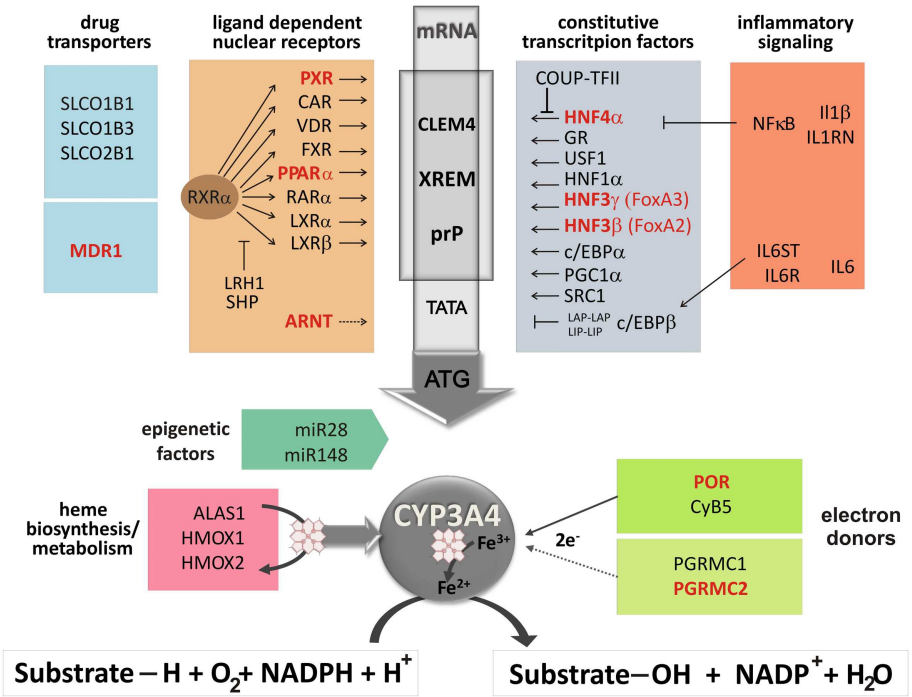

FIGURE 2 | Regulation network of CYP3A4 phenotype expression adapted from Klein et al. (2012). The scheme displays the promoter region of *CYP3A4* covering constitutive liver enhancer module CLEM4, xenobiotic responsive enhancer module XREM, and the proximal promoter prP. Genes of the constitutive and inducible pathways (Jover et al., 2002; Burk and Wojnowski, 2004) drug transporters, inflammatory signaling (Martínez-Jiménez et al., 2005; Aitken et al., 2006), possible miRNA interference (Pan et al., 2009), and of the heme biosynthesis/metabolism pathway as well as potential electron donors are listed. Genes with documented influential polymorphisms on CYP3A4 expression are depicted in red. Abbreviations: ALAS1, δ-aminolevulinate-synthase; ARNT, aryl hydrocarbon receptor nuclear translocator; CAR, constitutive androstane receptor; c/EBP, CCAAT/enhancer binding protein-α; Coup-TFII, COUP transcription factor II; CyB5, cytochrome B5; CYP3A4, cytochrome P450 3A4; FXR, farnesoid X-receptor; GR, glucocorticoid receptor; HMOX, heme-oxygenase; HNF1α, hepatocyte nuclear factor 1 homeobox A; FoxA2 (HNF3β), forkhead box A2; FoxA3 (HNF3γ), forkhead box A3; HNF4α, hepatocyte nuclear factor 4 α; IL1β, interleukin-1 β; IL1RN, interleukin-1 receptor antagonist; IL6, interleukin-6; IL6R, interleukin-6 receptor; IL6ST, interleukin-6 signal transducer; LRH1, liver receptor homolog 1; LXRα/β, liver X-receptor; MDR, multi-drug resistance protein; NF-κB, nuclear factor-κB subunits; PGRMC, progesterone receptor membrane component; POR, P450 oxidoreductase; PPARα, peroxisome proliferator-activated receptor α; RARα, retinoic acid receptor; RXRα, retinoid X-receptor; PXR, pregnane X-receptor; SHP, short heterodimer partner; SLCO, solute carrier organic anion transporters; USF-1, upstream stimulatory factor 1; VDR, vitamin D receptor.

NEW ASPECTS/OUTLOOK

Several genome-wide association studies with a total number of over 1000 human liver samples identified a large number of novel expression quantitative trait loci (eQTL) for numerous liver-expressed genes (Schadt et al., 2008; Yang et al., 2010; Innocenti et al., 2011; Schröder et al., 2013). Although the advantage of this approach is the unbiased way of investigation, results were rather disappointing with respect to CYPs, probably due to lack of statistical power. Thus, mainly polymorphisms in *CYP2D6* and *CYP3A5* were reproduced in these studies (Schadt et al., 2008; Schröder et al., 2013). Building up on one eQTL study, Yang et al. used the genome-wide SNP data of 466 livers to search for associations with enzyme activities determined for 9 CYPs (i.e., activity- or aQTLs). A total of 54 SNPs influencing 8 CYP activities were identified, of which 30 influenced CYP2D6 in *cis* (i.e., SNPs were located within ±1 Mb of the *CYP2D6* gene), whereas all remaining 24 SNPs were described as *trans*-acting elements, and only one of these (rs12041966 located on chromosome 1) influenced testosterone hydroxylation (Yang et al., 2010). Unfortunately, functional annotation of the *trans*-acting SNPs was not possible in this study, and these results thus await further experimental confirmation.

A further level of potential importance, which has not yet been explored in terms of genetic variability concerns the influence of epigenetic processes on pharmacologically relevant genes and drug response (Gomez and Ingelman-Sundberg, 2009). SNPs in DNA methylation regions or miRNAs and miRNA binding sites, as well as miRNA copy number variations may influence target gene expression (Schmeier et al., 2011). Thus, CYP3A4 expression was shown to be directly regulated by miRNAs but can also be influenced indirectly by miRNA regulation of transcriptional regulators such as PXR and VDR (Pan et al., 2009). However, polymorphic variation within this regulatory pathway has not yet been analyzed. Furthermore, additionally to regulatory polymorphisms (rSNPs) affecting transcription, structural RNA polymorphisms (termed srSNPs) are suggested to influence RNA function (splicing, turnover, translation) and to display promising biomarkers (Sadee et al., 2011; Lee et al., 2012).

The *ENCODE* project (*ENC*yclopedia *O*f *D*NA *E*lements)[5] systematically analyzes functional DNA elements in the human genome (e.g., binding data for more than 100 transcription factors, DNAse sensitive sites, methylation, chromatin interaction, and genotyping from multiple cell types) and may provide new hypotheses on functional consequences of SNPs located especially in non-coding DNA-regions (Bernstein et al., 2012; Gerstein et al., 2012; Yip et al., 2012).

In conclusion, the predictive power of currently known genetic polymorphisms with relevance for CYP3A4 *in vivo* phenotype is still far away from the expected 60–80% of genetic determination. The use of next generation sequencing approaches for the identification of causal variants in *CYP3A4* as well as the numerous genes of its many influential pathways may lead to the identification of many more rare and common DNA variants that together account for a sizeable fraction of this variability. The examples of *POR* and *PPARA* demonstrate that CYP3A4 variability is at least in part determined by polymorphisms in genes outside the *CYP3A4* locus. However, investigating and validating *trans*-acting factors is much more difficult due to the more indirect nature of interaction which is more likely to be masked by covariates, thus necessitating larger studies, *in vitro* or *in vivo*. In addition, mathematical algorithms are needed to combine many genetic variants, some of which contribute only small fractions to the total variability, into practically useful signatures for application on clinical studies and individualized medicine.

[5]http://genome.ucsc.edu/ENCODE/

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ABCB1 4036A>G and *1236C>T* polymorphisms affect plasma efavirenz levels in South African HIV/AIDS patients

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The *ABCB1* gene encodes P-glycoprotein, an ATP-dependent drug efflux pump, which is responsible for drug transport across extra- and intra-cellular membranes. The variability in the expression of *ABCB1* may contribute to variable plasma efavirenz concentration which results in variability in the levels of suppression of the human immunodeficiency syndrome virus (HIV). The aim of the study was to evaluate the role of polymorphisms in *ABCB1* gene on plasma efavirenz levels and treatment response in the form of change in viral load and CD-4 cell count in HIV/AIDS patients receiving efavirenz-containing highly active antiretroviral treatment regimens. Two hundred and eighty-two HIV-infected patients were recruited from Themba Lethu Clinic in Johannesburg and plasma efavirenz drug concentration levels were measured using LC-MS/MS. SNaPshot was used to genotype five known *ABCB1* single nucleotide polymorphisms (SNPs). Genotype-phenotype correlations were computed. The *ABCB1 4036A/G* and *4036G/G* genotypes were significantly associated with low plasma efavirenz concentrations ($P = 0.0236$), while the *ABCB1 1236C/T* and *1236T/T* genotypes were associated with high efavirenz concentrations ($P = 0.0282$). A haplotype *ABCB1 T-G-T-A* is reported that is associated with significantly increased plasma efavirenz levels. This is the first report on *61A>G*, *2677G>T/A*, and *4036A>G* SNPs in the South African population. *ABCB1* plays a role in determining the plasma concentrations of efavirenz and should be taken into account in future design of assays for genotype-based dosing of efavirenz-containing regimens.

Keywords: *ABCB1*, efavirenz, HIV/AIDS, South Africa, pharmacogenetics

INTRODUCTION

Efavirenz provides the backbone to first-line highly active antiretroviral treatment (HAART) in South Africa. HAART effectively suppresses human immunodeficiency syndrome virus (HIV) replication in the majority of patients (Mocroft et al., 2003). Thus, many HIV-infected patients are now living longer compared to the pre-HAART period. However, long term antiretroviral (ARV) treatment has its own challenges such as drug–drug interactions and the development of adverse drug reactions (ADRs). Drug–drug interactions are a major problem in HIV/AIDS patients due to co-morbidities such as TB and malaria.

FDA-approved ARV drugs, including efavirenz, indinavir, nelfinavir, and ritonavir are affected by the activities of the multidrug transporter P-glycoprotein (P-gp), coded by the *ABCB1* gene. *ABCB1* forms part of the ATP-binding cassette gene family with about 50 members and is located on chromosome 7q21.12, spanning 209.6 kb, and containing 29 exons (Bodor et al., 2005). Genetic variation in the *ABCB1* gene is known to alter mRNA stability or splicing activity (Fung and Gottesman, 2009). The three most common single nucleotide polymorphisms (SNPs) in the protein coding region of *ABCB1* are *1236C>T* (*rs1128503*), *3435C>T* (*rs1045642*), and *2677G>T/A* (*rs2032582*), where multiple alleles have been reported. The *1236C>T* SNP occurring in exon 13, does not result in an amino acid change, but may affect *ABCB1* expression through codon usage (Gu et al., 2004). The allele frequency of *1236T* variant ranges from 10% among South Africans (Dandara et al., 2011) to 90% among Asians (Ambudkar et al., 1999). The *2677G>T/A* SNP results in a change from serine to alanine or threonine at residue 893, but the effect of the changes on protein function is uncertain. The *3435T* allele has been associated with reduced expression of P-gp, although it is synonymous (Meissner et al., 2004). Large inter-ethnic variability has been reported for the *3435C>T* SNP with the *ABCB1 3435C* variant being the most frequent at 83, 58, 57, and 11% among Africans (Kenyans and Ghanaians), Asians (Chinese), Caucasians, and Yoruba individuals, respectively (Ameyaw et al., 2001). The *ABCB1 3435T* variant has been linked with good immune recovery in HIV/AIDS individuals, while the presence of the *ABCB1 2677T* variant has been strongly associated with virological failure (Motsinger et al., 2006). A few studies have suggested associations between *ABCB1* gene polymorphisms and variability in plasma efavirenz concentrations (Fellay et al., 2002; Mukonzo et al., 2009), but all the studies lack adequate sample size. There are conflicting reports on the effects of these SNPs on efavirenz treatment response (Cascorbi et al., 2001; Fellay et al., 2002; Cascorbi, 2006). Replication studies are thus necessary to understand the contribution of *ABCB1* gene variants to plasma efavirenz levels. Dandara et al. (2011) showed that genetic variants in *ABCB1* are frequent in the South African population, and this study is a continuation further evaluating the clinical significance of these SNPs. Therefore,

the aim of this study was to investigate the role of genetic polymorphisms in *ABCB1* on plasma efavirenz levels in HIV/AIDS patients in the South African population.

RESULTS

The mean age of the HIV/AIDS patients was 41.3 years, and more than 75% ($n = 227$) were female. Of the patients, 7 and 10% smoked and consumed alcohol. The clinical characteristic of the patients included viral load and CD-4 cell count (**Table 1**).

COMPARISON OF ALLELE FREQUENCIES AMONG WORLD POPULATIONS

The genotypes for all the SNPs were observed in the HIV/AIDS patients, except the *ABCB1 61G/G* (*rs9282564*) genotype. All *ABCB1* SNPs were in Hardy–Weinberg Equilibrium (HWE). The *ABCB1 61A/G* (*rs9282564*), *3435T/T* (*rs1045642*), *4036G/G* (*rs3842*), *1236T/T* (*rs1128503*), *2677T/A*, and *2677G/A* (*rs2032582*) genotypes were present at frequencies of 0.006, 0.024, 0.036, 0.015, 0.004, and 0.004, respectively, among the South Africans. No individuals with an *ABCB1 3435A* allele was observed in the South African cohort (**Table 2**), and this is similar to what was reported by Dandara et al. (2011). The allele frequencies of SNPs in the South Africans were compared to the allele frequencies reported previously in other populations (**Table 2**), available from previous studies or the HapMap project (http://hapmap.ncbi.nlm.nih.gov/).

CORRELATION OF GENETIC VARIATION WITH PLASMA EFAVIRENZ CONCENTRATION

The *ABCB1 4036A/G* and *4036G/G* genotypes were associated with significantly decreased efavirenz levels ($P = 0.0236$), compared to the *4036A/A* genotype (**Figure 1A**). Fewer individuals with the *ABCB1 4036G/G* genotype changed treatment compared to the individuals with the *4036A/A* genotype (**Table 3**). The *ABCB1 1236C/T* and *1236T/T* genotypes were associated with significantly higher plasma efavirenz concentrations, compared to the *1236C/C* genotype ($P = 0.0282$; **Figure 1C**). Compared to the *ABCB1 1236C/C* genotype, more individuals with the *1236T/T* genotype changed antiretroviral regimens 1 year post treatment initiation (**Table 3**). No difference was observed when comparing individuals with efavirenz concentration above 4 μg/mL to those with concentrations below 4 μg/mL, with respect to change in treatment regimens ($P = 0.571$). No significant differences were observed in efavirenz concentrations between the *ABCB1 2677G>T/A* and *ABCB1 3435C>T* genotypes (**Figures 1B,D**).

HAPLOTYPE ANALYSIS

Haplotype and efavirenz plasma levels for each patient are presented in supplementary **Table A1**. The haplotypes with respect to *1236C>T*, *2677G>T/A*, *3435C>T*, and *4036A>G* SNPs *C-G-C-A*, *C-G-C-G*, *C-G-T-G*, *T-G-C-A*, *T-G-T-A*, *T-G-T-G*, *T-T-T-A*, and *T-T-T-G* had the following frequencies in the HIV/AIDS patients; 0.67, 0.17, 0.04, 0.03, 0.06, 0.01, 0.001, and 0.01, respectively. The *ABCB1 T-G-T-A* haplotype had the highest mean plasma $\log_{10}$ efavirenz concentrations (0.90 μg/mL) compared to 0.49 and 0.65 μg/mL among patients with the *T-G-C-A* or *T-G-T-G* haplotypes, respectively (**Figure 2**). The efavirenz concentrations differed significantly between individuals with the *ABCB1 C-G-C-G* and *T-G-T-A* haplotypes ($P = 0.007$) and were still significant after Bonferroni's correction for multiple testing (cut-off significant $P < 0.01$).

Table 1 | Clinical characteristics of the South African HIV/AIDS patients.

Clinical characteristics	HIV/AIDS patients ($n = 301$)
Median HIV-RNA at baseline, copies/mL ± SD (range)	26917.71 ± 27133.50 (25–98400)
Median HIV-RNA at 6 months post-initiation of HAART, copies/mL ± SD (range)	1518.52 ± 9004.10 (0–75000)
Average CD-4 cell count at baseline, cells/μL ± SD (range)	136.09 ± 113.24 (2–605)
Average CD-4 cell count at 6 months post-initiation of HAART, cells/μL ± SD (range)	261.76 ± 137.68 (28–775)
ARV regimens	
3TC_TDF_EFV	9
AZT_3TV_EFV	11
d4T_3TC_EFV	222
d4T_3TC_LPVr	18
d4T_3TC_NVP	22
Average plasma efavirenz concentration, μg/mL (range)	4.64 (0.6–22)

UNIVARIATE AND MULTIVARIATE REGRESSION ANALYSIS OF EFAVIRENZ CONCENTRATION

Univariate regression analysis was performed to determine the effect of age, gender, tobacco smoking, alcohol use, BMI at baseline, CD-4 cell count, $\log_{10}$ HIV-RNA levels, *ABCB1* haplotypes, *ABCB1 1236C>T, 4036A>G, 3435C>T*, and *2677G>T/A* genotypes on plasma efavirenz concentrations (**Table 4**). The genotypes of the four SNPs *1236C>T*, *2677G>T/A*, *3435C>T*, and *4036A>G* significantly predicted efavirenz concentration individually and were, thus, included in the multivariate analysis together with age, gender, tobacco smoking, alcohol use and BMI at baseline. Stepwise backward regression analysis was then performed to identify the minimum set of independent variables that are predictive of plasma efavirenz levels and to determine the relative contribution of each variable to efavirenz concentration variability. Only three independent variables remained in the final model with $P < 0.05$, including *ABCB1 1236C>T* (standardized regression coefficient = 0.24; $P = 0.004$), *ABCB1 4036A>G* (standardized regression coefficient = 0.17; $P = 0.009$), and *ABCB1 2677G>T/A* (standardized regression coefficient = 0.36; $P = 0.047$). The adjusted coefficient of determination (R^2) for the regression was 0.16, indicating that 16% of the total variance in efavirenz concentrations was explained by the model. *ABCB1 1236C>T, 4036A>G*, and *2677G>T/A* genotypes accounted for 29, 23, and 17% (respectively) of the total variance in plasma efavirenz concentrations. When repeating the multivariate analysis among female patients only, the adjusted coefficient of determination (R^2) for the regression was 0.18, indicating that 18% of the total variance in efavirenz concentrations was explained by the model

Table 2 | Allele frequencies in the South Africans compared to other populations.

Population	Reference	*N*	61G	1236T	3435T	2677T	2677A	4036G
Black South Africans[#]	(Dandara et al., 2011)/This study	979	0.003	0.090	0.120	0.040	0.004	0.202
Sotho/Tswana South Africans	This study	127	0.000	0.106	0.110	0.012	0.004	0.165
Xhosa South Africans	This study	107	0.014	0.178*	0.210*	0.098*	0.005	0.224
Zulu South Africans	This study	139	0.000	0.119	0.140	0.022	0.000	0.209
Malawi	Brown et al. (2011)	30	N/A	N/A	0.210	0.000	0.000	N/A
Yoruba	Hapmap	226	0.000	0.124	0.111	0.000*	0.000	0.142
Luhya	Ikediobi et al. (2011)	89	N/A	0.110	N/A	N/A	N/A	N/A
Maasai	Ikediobi et al. (2011)	143	N/A	0.140	0.840*	N/A	N/A	N/A
African-American	Hapmap	46	0.000	0.136	0.071	0.077	0.000	0.000*
Caucasian	Hapmap	226	0.100*	0.451*	0.571*	0.340*	0.042*	0.142
Gujarati Indian	Hapmap	176	0.017	0.597*	0.597*	0.653*	0.000	0.163
Mexican	Hapmap	96	0.052*	0.460*	0.460*	0.430*	0.000	0.230
Toscan	Hapmap	176	0.062*	0.426*	0.466*	0.438*	0.000	0.138
Chinese	Ikediobi et al. (2011)	45	0.000	0.680*	0.580*	N/A	N/A	N/A
Japanese	Hapmap	90	0.000	0.587*	0.459*	0.552*	0.000	0.320*

*N/A, not available, *statistically significant difference from the frequencies among the Black South African group[#].*

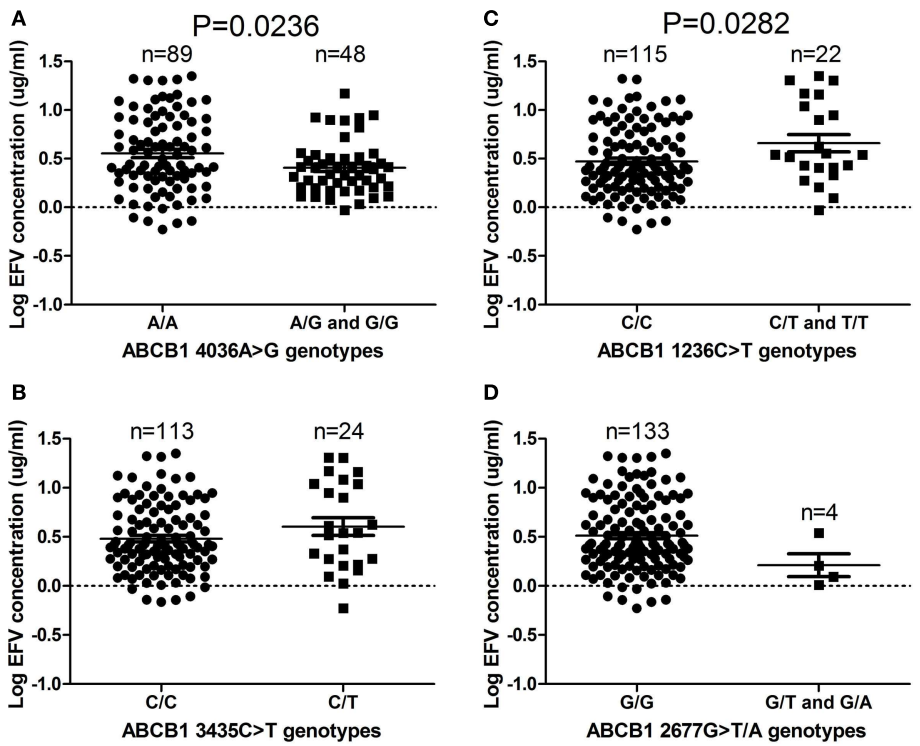

FIGURE 1 | (A–D) *ABCB1 4036A/G* and G/G genotypes ($P = 0.0236$) are associated with reduced efavirenz levels, while *1236C/T* and T/T genotypes ($P = 0.0282$) are associated with increased efavirenz levels in South African HIV/AIDS patients. Only significant *P*-values (without Bonferroni correction), using the dominant genetic model, are shown.

compared to the 16% explained when males and females were combined. However, the majority of HIV/AIDS patients in our study were female. Further statistical analysis comparing only the genetic- and non-genetic factors in the multivariate analysis, showed that the genetic factors alone explained 11% of variance in efavirenz levels, while the non-genetic factors only explained 3% compared to the 16% explained by the combined multivariate analysis.

Table 3 | Frequency of HIV/AIDS patients changing ART regimens within 3 months, 6 months and 1 year post-initiation of treatment*.

Genotype	Treatment initiation (*n*)	3 months	*P*-value	6 months	*P*-value	1 year	*P*-value
EFV-CONTAINING ARV REGIMEN							
3TC_TDF_EFV	9	0.11		0.00		0.11	
AZT_3TC_EFV	11	0.00	0.182	0.09	0.993	0.18	0.898
d4T_3TC_EFV	222	0.03		0.05		0.14	
1236C/C	192	0.04		0.05		0.12	
1236C/T	44	0.02	0.399	0.04	0.636	0.18	0.089
1236T/T	3	0.00		0.00		0.50	
3435C/C	192	0.04		0.05		0.13	
3435C/T	44	0.00	0.387	0.05	0.962	0.16	0.653
3435T/T	3	0.33		0.00		0.00	
2677G/G	229	0.03		0.06		0.13	
2677G/T	7	0.00	0.563	0.11	0.666	0.00	0.706
2677T/A and G/A	2	0.00		0.00		0.50	
4036A/A	153	0.03		0.05		0.14	
4036A/G	78	0.03	0.987	0.06	0.834	0.14	0.852
4036G/G	8	0.00		0.00		0.11	

**Only 282 patients had information on treatment regimens. ABCB1 61A>G was excluded based on being monomorphic.*

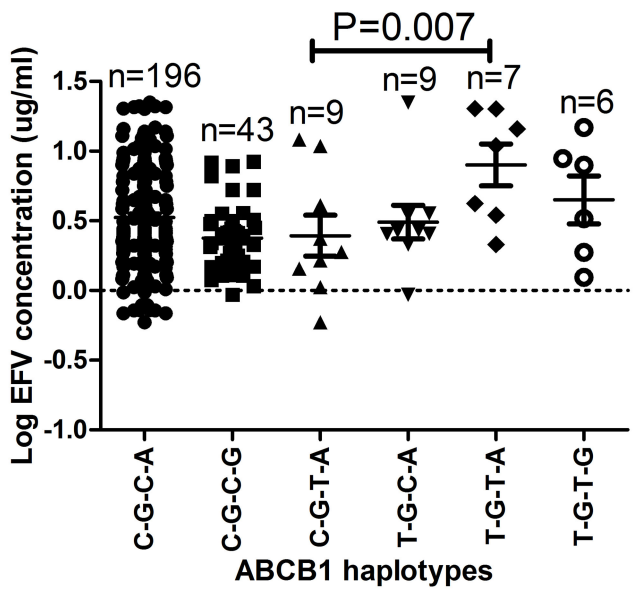

FIGURE 2 | The effect of *ABCB1* haplotypes on plasma efavirenz concentrations. Only significant *P*-values after Bonferroni's correction for multiple testing at significance $P < 0.01$ are shown. The *ABCB1 C-A-C-A*, *C-T-C-A*, and *T-T-T-G* haplotypes were only observed once and thus excluded from the analysis.

CORRELATION OF GENETIC VARIATION WITH CLINICAL PARAMETERS

The average CD-4 cell count and viral load of individuals with the different genotypes for *ABCB1 1236C>T* and *4036A>G* were compared at baseline and also 6 months post-initiation of ARV therapy. The *ABCB1 1236C/T* and *4036G/G* genotypes were associated with the biggest decreases in viral load at 6 months (as shown in **Figures 3A,B**). None of the individuals with the *ABCB1 1236T/T* genotype had information on viral load at baseline or 6 months post-initiation of treatment. The *ABCB1 1236T* allele is associated with decreased expression of P-gp (Gow et al., 2008) and the *ABCB1 4036G* allele is associated with increased expression of P-gp. However, there were no major genotype associated differences in the recovery of the CD-4 cells with all genotypes showing a positive response (**Figures 3C,D**).

DISCUSSION

HETEROGENEITY OF AFRICAN POPULATIONS IN TERMS OF GENETICS

To our knowledge, the present study is the first to report on the allele and genotype frequencies of the *ABCB1 61A>G*, *2677G>T/A*, and *4036A>G* polymorphisms in the South African population. However, the other SNPs have been previously reported by Dandara et al. (2011). This data contributes to the accumulation of information on genetic variants of pharmacogenetics relevance among Africans. The allele frequencies of the genetic polymorphisms in South Africans were also compared to the frequencies among other African, African-American, Asian, and Caucasian populations (**Table 2**). As expected there are significant differences in the allele frequencies between African populations and Caucasians, for example, the allele frequency of the *ABCB1 3435T* allele in African populations ranges from 0.07 to 0.12, but is present at a frequency of almost 0.6 in Caucasian individuals. Differences in allele frequencies between the South African population compared with other African populations were also observed. The allele frequencies of the *ABCB1 4036G*, *2677T*, and *2677A* alleles were different to the frequencies reported in the Yoruba individuals ($P < 0.0001$). The differences in allele frequencies between the African and Caucasian individuals show that therapeutic drugs, including efavirenz, may not have

Table 4 | Univariate and multivariate regression analysis of efavirenz concentration.

Independent variable	Log_{10} efavirenz, % (95%CI)	*P*	Contribution in model (%)
UNIVARIATE			
Age	0.01 (−0.06 to 0.08)	0.738	1.36
Gender	−7.34 (−21.5 to 6.81)	0.307	0.12
Tobacco smoking	−0.75 (−27.2 to 25.7)	0.956	2.60
Alcohol use	−12.4 (−34.3 to 9.45)	0.263	11.9
BMI at baseline	−0.70 (−2.16 to 0.76)	0.344	15.2
CD-4 cell count at baseline	0.01 (−0.06 to 0.07)	0.793	–
CD-4 cell count at 6 months	−0.04 (−0.09 to 0.02)	0.186	–
Log_{10} HIV-RNA at baseline	−11.9 (−21.3 to −2.44)	0.015	–
Log_{10} HIV-RNA at 6 months	10.4 (−7.26 to 28.0)	0.245	–
Genotype (dominant model)			
WT/WT	Ref		
ABCB1 1236C>T	18.6 (2.02 to 35.2)	0.028	28.9
ABCB1 4036A>G	−14.8 (−27.5 to −2.01)	0.024	23.2
ABCB1 3435C>T	12.3 (−3.90 to 28.4)	0.136	0.12
ABCB1 2677G>T/A	−30.0 (−66.4 to 6.47)	0.106	16.6
ABCB1 haplotypes	0.19 (−1.98 to 2.37)	0.862	–
MULTIVARIATE#			
ABCB1 1236C>T	24.2 (7.81 to 40.6)	0.004	–
ABCB1 4036A>G	−16.6 (−29.1 to −4.15)	0.009	–
ABCB1 2677G>T/A	−35.9 (−71.3 to −0.43)	0.047	–

#Only significant covariates in the multivariate regression analysis are shown.

similar effectiveness in different populations when given at standard dosages. Similarly, fine scale genetic structure exists within the African population which, therefore, should not be treated as one population.

IMPLICATIONS FOR DISEASE OR DRUG TREATMENT AND POSSIBLE DEVELOPMENT OF DIAGNOSTIC TOOLS

We observed lower plasma efavirenz concentrations among individuals with the *ABCB1 4036A/G* and *4036G/G* genotypes and this could perhaps be as a result of the disruption of a miRNA binding site in the 3′UTR of *ABCB1*. Five poorly conserved miRNAs namely; miR-129, miR-491, miR-4795, miR-561, and miR-4717 have been predicted to target the 3′UTR region surrounding the *ABCB1 4036A>G* SNP using the TargetScanHuman 6.1 miRNA target prediction software. Disruption of these sites could potentially cause reduced transport of efavirenz by *ABCB1* resulting in lower plasma efavirenz levels. In a different study among Ugandans, the *ABCB1 4036A/G* and *4036G/G* genotypes were associated with higher efavirenz bioavailability (Mukonzo et al., 2009). In the current study, *ABCB1 1236C/T* and *1236T/T* genotypes were associated with high plasma efavirenz concentrations, but there are conflicting reports on the effect of *ABCB1 1236C>T* genotypes in tacrolimus, cyclosporine, and sirolimus drug responses (Kuzuya et al., 2003; Anglicheau et al., 2004; Haufroid et al., 2004), making it difficult to draw conclusions. There are conflicting reports as well for the role or effects of *ABCB1 2677G>T/A* and *3435C>T* polymorphisms (Schwab et al., 2003; Leschziner et al., 2007). Haas et al. (2005) reported an association between the *ABCB1 3435T/T* genotype and a decreased likelihood of virologic failure and decreased resistance to efavirenz, but not with plasma efavirenz exposure.

Clinical parameters such as CD-4 cell count, viral load, disease stage, hemoglobin, AST, and ALT levels were used as indicators of ARV treatment efficacy, underlying liver disease and disease progression in the HIV/AIDS patients. As expected, efavirenz-containing HAART led to a general (48%) increase in CD-4 cell count (cells/μL) and a 94% decrease in viral load (copies/mL) when baseline levels where compared to levels at 6 months post-initiation of treatment. Failure of reduction in viral load and emergence of opportunistic infections after 6 months led to ARV switching. Sustained viral load after 6 months and the presence of opportunistic infections are indications of possible treatment failure or non-adherence. On the other hand, other studies have shown that high plasma efavirenz concentrations are associated with development of adverse drug events leading to drug discontinuation (Marzolini et al., 2001; Lubomirov et al., 2011; Wyen et al., 2011).

CONCLUSION

The current study showed that the drug transporter *ABCB1* contributes in predicting response to efavirenz treatment in the South African HIV/AIDS population. The *ABCB1 4036A>G* and *1236C>T* polymorphisms were significantly correlated with low and high plasma efavirenz concentration levels, respectively. However, this data should be taken together with the variation in *CYP2B6* which has a profound effect on efavirenz metabolism. The *CYP2B6 516G>T* SNP is known to be associated with high plasma efavirenz levels and the combined effect of *CYP2B6* together with *ABCB1* SNPs will be more informative in predicting response to efavirenz treatment. This strongly supports the development of a pharmacogenetic suite of gene variants to assist in deciding the HAART regimen for HIV/AIDS treatment in a clinical setting as well as the starting ARV dosage.

MATERIALS AND METHODS

RESEARCH PARTICIPANTS

All participants provided written informed consent and study approval was obtained from the University of Cape Town Health Science Faculty Research Ethics Committee, Cape Town, South Africa and the University of Witwatersrand Human Research Ethics Committee, Gauteng, South Africa. The research was performed in accordance with the guidelines of the Helsinki Declaration of 2008. Two hundred and eighty-two ($n = 282$) South African HIV/AIDS patients receiving efavirenz-based treatment for at least 6 months, were recruited to participate in this study. All subjects were of Bantu origin and comprised of Sotho/Tswana from Gauteng and Xhosa subjects from the Western Cape Province, South Africa. All subjects gave information on their ethnicity, age, health status (including self-reported adherence to treatment or pill counts), dietary, and smoking habits.

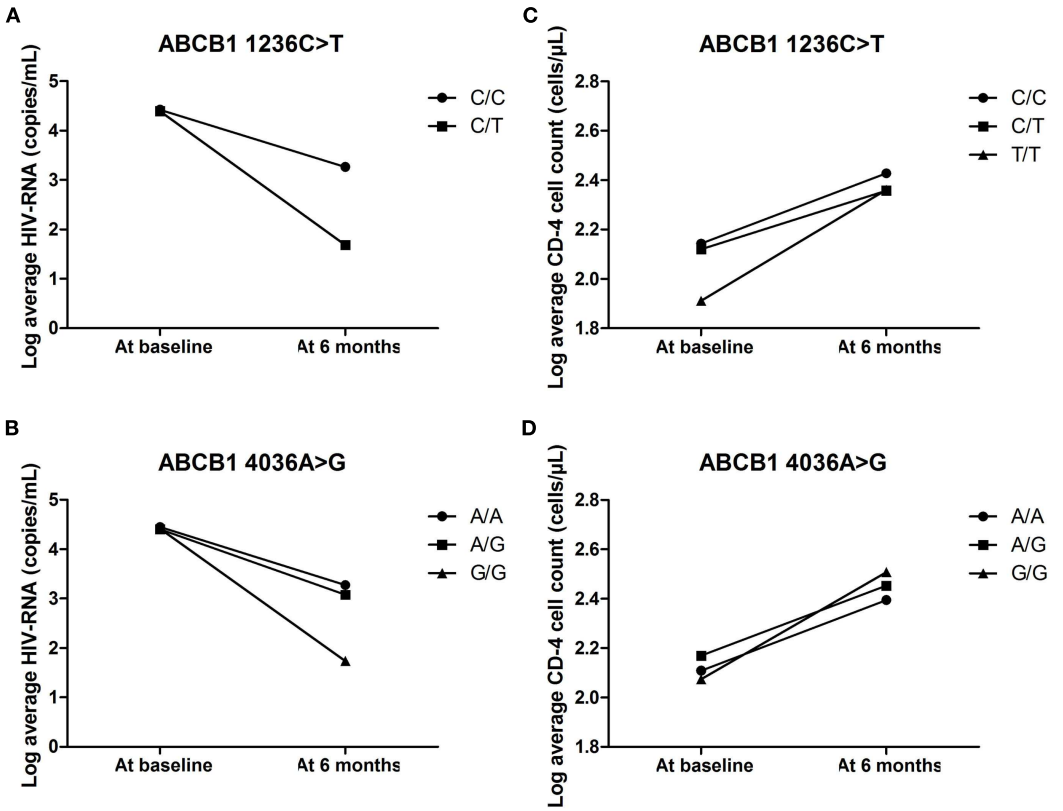

FIGURE 3 | (A–D) The effect of *ABCB1 4036A>G* and *1236C>T* on average HIV-RNA and CD-4 cell count at baseline and 6 months post-initiation of treatment.

A 5 mL whole blood sample was obtained from each subject, and used for plasma sample collection as well as DNA extraction. DNA was isolated using a salting-out method adapted from Gustafson et al. (1987) or the GenElute™ Blood Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA). Steady-state plasma samples were available for 137 HIV/AIDS patients 12–16 h post dose with efavirenz. Plasma efavirenz concentrations were determined by LC/MS/MS (API 4000 triple quadrupole MS/MS Applied Biosystems, South Africa) according to the method by Chi et al. (2002).

SELECTION OF SNPs AND GENOTYPING METHODS USED

Five previously reported SNPs in *ABCB1* [GenBank accession: NM_000927.4], namely *61A>G, 1236C>T, 2677G>T/A, 3435C>T*, and *4036A>G* were selected for investigation based on having minor allele frequencies above 10% in the African-Americans, South African, or other African populations and were genotyped using SNaPshot mini-sequencing (**Table 5**).

Each PCR reaction contained, 50 ng of genomic DNA, 1X Green GoTaq Flexi Reaction Buffer (Promega Corporation, Madison, WI, USA), 0.2 mM of each of the deoxynucleotide triphosphates (dNTPs; Bioline, London, UK); 1.5 mM $MgCl_2$ (Promega Corporation, Madison, WI, USA); 40 pmol of the forward and reverse primers (Integrated DNA Technologies, Inc., Coralville, IA, USA); 1 U of GoTaq Flexi DNA Polymerase (Promega Corporation, Madison, WI, USA). The PCR reactions were carried out using a "MyCycler Thermal cycler" from Bio-Rad. PCR conditions were as follows: 3 min at 94°C; 40 cycles of 94°C for 30 s, the annealing temperature specific to each SNP for 30 s, 72°C for 50 s; and 10 min at 72°C for final extension.

Five microliters of each PCR product was pooled and 10 μL of the combined PCR products were cleaned using 1.5 U shrimp alkaline phosphatase (Fermentas Life Sciences, Burlington, Canada) and 2 U *ExoI* (Fermentas Life Sciences, Burlington, Canada) in a total reaction volume of 20 μL. The shrimp alkaline phosphatase and *ExoI* reaction was incubated at 37°C for 1 h and the enzymes were inactivated at 75°C for 15 min. SNaPshot single base extension of the genetic polymorphisms was performed on the "GeneAmp® PCR System 9700 version 3.08″ (Applied Biosystems, Carlsbad, CA, USA) using the SNaPshot cycling programme as 96°C for 10 s, and then repeated for 25 cycles at 50°C for 5 s and 60°C for 30 s. The SNaPshot reaction (10 μL) contained 1 μL ABI Prism® SNaPshot™ Multiplex Kit (Applied Biosystems, CA, USA) and the pooled internal SNaPshot primers (Integrated DNA Technologies, Inc., Coralville, IA, USA). Following the SNaPshot reaction, the clean-up reaction was repeated using 1 U shrimp alkaline phosphatase using cycling conditions as mentioned before, and capillary electrophoresis was performed using a ABI 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). SNaPshot results were analyzed on the GeneMapper © Software version 4.1 (Applied Biosystems, Carlsbad, CA, USA).

Table 5 | PCR and SNaPshot amplification conditions for *ABCB1* SNPs (GenBank accession: NM_000927.4).

SNP	Primer sequence (5′-3′)	Ta (°C)	PCR product	References
4036A>G	F: CCTCAGTCAAGTTCAGAGTCTTCA R: TCACAGGCAGTTGGACAAG SNaPshot primer: TCTTGGCAGAAACTGCAAAAGGAGATTGAT	54°C	297	Designed
3435C>T	F: ACTCTTGTTTTCAGCTGCTTG R: AGAGACTTACATTAGGCAGTGACTC SNaPshot primer: ACTCGTCCTGGTAGATCTTGAAGGG	54°C	230	Rhodes et al. (2007)
2677G>T/A	F: ATGGTTGGCAACTAACACTGTTA R: AGCAGTAGGGAGTAACAAAATAACA SNaPshot primer: CTTCGACCTAAGTGGAGAATGAGTTATTCTAAGGA	54°C	206	Rhodes et al. (2007)
1236C>T	F: TGTGTCTGTGAATTGCCTTGAAG R: CCTCTGCATCAGCTGGACTGT SNaPshot primer: TTAATTAATCAATCATATTTAGTTTGACTCACCTTCCCAG	51°C	228	Rhodes et al. (2007)
61A>G	F: CTGCGGTTTCTCTTCAGGTC R: GATTCCAAAGGCTAGCTTGC SNaPshot primer: CTCCTTTGCTGCCCTCAC	51°C	149	Designed

STATISTICAL ANALYSIS

Statistical analysis was performed using the Graphpad Prism (Version 5, GraphPad Software Inc., San Diego, CA) and STATA (Version 11, StatSoft, USA) statistical programs. *ABCB1* haplotypes were inferred using Phase v2.1 (Stephens et al., 2001; Stephens and Donnelly, 2003; Stephens and Scheet, 2005). Pearson's χ^2-test and Fisher's exact test were used to compare the allele frequencies to results previously published in populations of different ethnicity. Fisher's exact test was also used to compare change in treatment regimen between the *ABCB1* genotypes. One-way analysis of variance, followed by Bonferroni's multiple comparison tests, was used to determine the effect of *ABCB1* haplotypes on plasma $\log_{10}$ efavirenz levels. Genotypes were dichotomized according to the dominant genetic model (wild-type = 0 and heterozygote/homozygote variants = 1). Univariate regression analysis was applied to $\log_{10}$ efavirenz concentrations as dependant variable and the percentage change in efavirenz levels, with the 95% CI, was calculated as 100 × regression coefficient. Multivariate regression analysis was performed by including covariates from the univariate analysis, followed by stepwise backward removal. TargetScanHuman 6.1 miRNA target prediction software were used to predict miRNA binding to the *ABCB1 3′UTR*. The following equation was used to calculate the sample size required to achieve a 99% confidence-interval: $N = [\text{DEFF} * \text{Np}(1-p)]/[(\text{d2/Z21}-\alpha/2 * (N-1) + p * (1-p)]$. DEFF is defined as the design effect, Z is the value for 99% confidence, d is an α-value = 0.05, while p is the frequency of the variant allele. A DEFF-value of 1 was used for random sampling, a Z-value of 1.96 and allele frequency of 0.1 was used to calculate the sample size of $N = 239$ samples. All statistical tests were performed two tailed, and statistical significance was defined as $P < 0.05$.

AUTHORS' CONTRIBUTIONS

Marelize Swart carried out all of the molecular genetic studies and drafted the manuscript. Yuan Ren and Peter Smith both carried out the LC/MS/MS analysis of plasma efavirenz concentration. Collet Dandara conceived of the study, designed, coordinated the study, collected all the samples, assisted with statistical data analysis, helped to draft the manuscript and approved the final version. All authors read and approved the final 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.

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APPENDIX

Table A1 | *ABCB1* haplotype composition for each HIV/AIDS patient where steady-state plasma efavirenz levels were available.

HIV/AIDS patients	ABCB1 haplotype combination*	Log_{10} efavirenz levels (μg/mL)
1	C-G-C-A/C-G-C-A	0.111
2	C-G-C-A/C-G-C-A	0.838
3	C-G-C-A/C-G-T-A	0.023
4	C-G-C-A/C-G-C-A	−0.013
5	C-G-C-A/C-G-C-A	0.676
6	C-G-C-A/C-G-C-G	0.507
7	C-G-C-A/C-G-T-A	0.275
8	C-G-C-A/C-G-C-G	0.199
9	C-G-C-A/C-G-C-G	0.428
10	C-G-C-A/C-G-C-G	0.327
11	C-G-C-A/C-G-T-A	−0.229
12	C-G-C-A/C-G-C-G	0.917
13	C-G-C-A/C-G-C-G	0.208
14	C-G-C-A/C-G-C-G	0.415
15	C-G-C-A/C-G-C-G	0.478
16	C-G-C-A/C-G-C-A	0.321
17	C-G-C-A/C-G-C-G	0.276
18	C-G-C-A/T-G-T-A	0.624
19	C-G-C-A/C-G-T-A	1.083
20	C-G-C-A/C-G-C-G	0.170
21	C-G-C-A/C-G-C-G	0.445
22	C-G-C-A/C-G-T-A	0.611
23	C-G-C-A/C-G-C-G	0.419
24	C-G-C-A/C-G-C-G	0.558
25	C-G-C-A/C-G-C-A	0.622
26	C-G-C-A/C-G-C-A	0.267
27	C-G-C-G/C-G-C-G	0.107
28	C-G-C-A/T-G-T-G	1.170
29	C-G-C-A/C-G-C-A	0.083
30	C-G-C-A/C-G-C-A	0.390
31	C-G-C-A/C-G-C-A	0.408
32	C-G-C-A/C-G-C-A	0.720
33	C-G-C-A/C-G-C-A	0.378
34	C-G-C-A/C-G-T-A	0.157
35	C-G-C-A/C-G-C-G	0.172
36	C-G-C-A/C-G-C-A	0.029
37	C-G-C-A/C-G-C-A	0.902
38	C-G-C-A/C-G-C-A	0.380
39	C-G-C-A/C-G-C-A	1.016
40	C-G-C-A/C-G-C-A	0.880
41	C-G-C-A/T-G-C-A	0.407
42	C-G-C-A/C-G-C-G	0.819
43	C-G-C-A/C-G-C-A	0.780
44	C-G-C-A/C-G-C-A	1.316
45	C-G-C-A/C-G-C-G	0.112
46	C-G-C-G/T-G-C-A	0.554
47	C-G-C-A/C-G-C-G	0.419
48	C-G-C-A/C-G-C-A	0.352
49	C-G-C-A/C-G-C-A	1.141
50	C-G-C-A/T-T-T-G	0.539
51	C-G-C-A/T-G-T-A	1.303
52	C-G-C-A/C-G-C-A	0.927
53	C-G-C-A/C-G-C-G	0.298
54	C-G-C-A/C-G-C-G	0.892
55	C-G-C-A/C-G-C-A	0.422
56	C-G-C-A/T-G-T-G	0.899
57	C-G-C-A/T-G-C-A	0.428
58	C-G-C-G/C-G-C-G	0.723
59	C-G-C-A/T-T-T-G	0.205
60	C-G-C-A/C-G-C-A	0.353
61	C-G-C-A/C-G-C-G	0.252
62	C-G-C-G/C-G-C-G	0.499
63	C-G-C-A/C-G-C-A	0.442
64	C-G-C-A/T-G-T-G	0.095
65	C-G-C-A/C-G-C-A	0.649
66	C-G-C-G/T-G-C-A	0.405
67	C-G-C-A/C-G-C-A	0.495
68	C-G-C-A/C-G-C-G	0.413
69	C-G-C-A/C-G-C-A	0.619
70	C-G-C-A/C-G-C-A	0.196
71	C-G-C-A/T-G-T-A	1.306
72	C-G-C-A/C-G-C-G	0.328
73	C-G-C-A/C-G-C-A	0.932
74	C-G-C-A/C-G-C-A	0.315
75	C-G-C-A/C-G-C-A	1.322
76	C-G-C-A/C-G-C-A	0.585
77	C-G-C-A/C-G-C-A	0.623
78	C-G-C-G/C-G-T-A	0.215
79	C-G-C-A/C-G-C-A	1.125
80	C-G-C-A/C-G-C-A	0.294
81	C-G-C-A/T-G-T-G	0.947
82	C-G-C-A/T-G-T-A	1.160
83	C-G-C-A/C-G-C-A	0.989
84	C-G-C-A/C-G-C-A	0.072
85	C-G-C-A/C-G-C-A	0.415
86	C-G-C-A/C-G-C-A	0.214
87	C-G-C-A/C-G-C-A	0.874
88	C-G-C-A/C-G-C-G	0.450
89	C-G-C-A/C-G-C-A	−0.140
90	C-G-C-A/C-G-T-A	1.037
91	C-G-C-A/C-G-C-A	0.381
92	C-G-C-A/C-G-C-A	−0.143
93	C-G-C-A/C-G-C-G	0.076
94	C-G-C-A/C-G-C-G	0.924
95	C-G-C-A/C-G-C-G	0.033
96	C-G-C-A/C-G-C-A	0.946
97	C-G-C-A/C-G-C-A	−0.164
98	C-G-C-G/T-G-C-A	−0.029
99	C-G-C-A/C-G-C-A	−0.105
100	C-G-C-A/C-G-C-A	0.645

(Continued)

(Continued)

Table A1 | Continued

HIV/AIDS patients	ABCB1 haplotype combination*	Log_{10} efavirenz levels (μg/mL)
101	C-G-C-A/C-G-C-A	0.911
102	C-G-C-A/C-G-C-G	0.394
103	C-G-C-A/T-G-T-G	0.274
104	C-G-C-A/C-G-C-G	0.313
105	C-G-C-A/C-G-C-G	0.164
106	C-G-C-A/C-G-T-A	0.371
107	C-G-C-A/C-G-C-A	0.264
108	C-G-C-A/C-T-C-A	0.010
109	C-G-C-A/C-G-C-G	0.111
110	C-G-C-A/C-G-C-A	1.109
111	C-G-C-A/C-G-C-A	0.671
112	C-G-C-A/C-G-C-A	0.324
113	C-G-C-A/C-G-C-A	0.431
114	C-G-C-A/T-G-C-A	0.431
115	C-G-C-A/C-G-C-A	0.566
116	C-G-C-A/C-G-C-A	0.780
117	C-G-C-A/T-G-T-G	0.516
118	C-G-C-A/C-G-C-G	0.276
119	T-G-C-A/T-G-T-A	0.541
120	C-G-C-A/C-G-C-A	0.821
121	C-G-C-A/T-G-T-A	1.038
122	C-G-C-A/C-G-C-A	0.364
123	C-G-C-A/C-G-C-A	1.106
124	C-G-C-A/C-G-C-A	0.584
125	C-G-C-A/C-G-C-A	1.093
126	C-G-C-A/C-G-C-A	0.692
127	C-G-C-A/C-G-C-G	0.340
128	C-G-C-A/C-G-C-A	0.751
129	C-G-C-A/C-G-C-A	0.192
130	C-A-C-A/C-G-C-A	0.092
131	C-G-C-A/C-G-C-A	0.192
132	C-G-C-A/C-G-C-A	0.106
133	C-G-C-A/C-G-C-A	0.941
134	C-G-C-A/C-G-C-A	0.204
135	C-G-C-A/T-G-C-A	1.348
136	T-G-C-A/T-G-T-A	0.329
137	C-G-C-A/C-G-C-G	0.271

**ABCB1 haplotypes with respect to 1236C>T-2677G>T/A-3435C>T-4036A>G, 61A>G was excluded from the haplotype based on being monomorphic.*

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OPINION ARTICLE
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Genetic variations in drug-induced liver injury (DILI): resolving the puzzle

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Despite stringent requirements for drug development imposed by regulatory agencies, drug-induced liver injury (DILI) is an increasing health problem and a significant cause for failure to approve drugs, market withdrawal of commercialized medications, and adoption of regulatory measures. The pathogenesis is yet undefined, though the rare occurrence of idiosyncratic DILI (1/100,000–1/10,000) and the fact that hepatotoxicity often recurs after re-exposure to the culprit drug under different environmental conditions strongly points toward a major role for genetic variations in the underlying mechanism and susceptibility. Pharmacogenetic studies in DILI have to a large extent focused on genes involved in drug metabolism, as polymorphisms in these genes may generate increased plasma drug concentrations as well as lower clearance rates when treated with standard medication doses. A range of studies have identified a number of genetic variants in drug metabolism Phase I, II, and III genes, including *cytochrome P450* (CYP) *2E1*, *N-acetyltransferase 2*, *UDP-glucuronosyltransferase 2B7*, *glutathione S-transferase M1*/*T1*, *ABCB11*, and *ABCC2*, that enhance DILI susceptibility (Andrade et al., 2009; Agundez et al., 2011). Several metabolic gene variants, such as *CYP2E1*c1 and *NAT2* slow, have been associated with DILI induced by specific drugs based on individual drug metabolism information. Others, such as *GSTM1* and *T1* null alleles have been associated with enhanced risk of DILI development induced by a large range of drugs. Hence, these variants appear to have a more general role in DILI susceptibility due to their role in reducing the cell's antioxidative capacity (Lucena et al., 2008). Mitochondrial superoxide dismutase (SOD2) and glutathione peroxidase 1 (GPX1) are two additional enzymes involved in combating oxidative stress, with specific genetic variants shown to enhance the risk of developing DILI (Lucena et al., 2010).

Nevertheless, there are discrepancies in the findings and many studies are based on small cohorts and subsequently insufficient to confirm a prominent association between genetic variability in drug metabolism and the risk of DILI. It is important to note that interethnic differences in allele frequency are present and should be considered as a source of heterogeneity when analysing diverse ethnicities. In addition, populations with low variant allele frequencies require high sample sizes to achieve adequate statistical power. Although significant associations between specific drug metabolism gene variants and DILI susceptibility have been found, the low relative risk associated with these variants prevents clinical translation, such as the development of predictive biomarkers (Agundez et al., 2012). Screening for specific risk genotypes identified to date prior to prescriptions would not adequately reduce the number of DILI incidents and could also prevent treatment for many risk allele carriers that would not develop DILI if taking the specific drug.

The introduction of genome-wide association (GWA) studies brought great expectations for the revelation of genetic components responsible for DILI susceptibility. Unfortunately, the outcome has yet not reached the anticipated results. Despite the wide coverage of variants across the entire genome with this technique, none of the previously identified drug metabolizing gene variants has been confirmed. In fact, only specific human leukocyte antigen (HLA) alleles have been significantly associated with idiosyncratic DILI in the GWA studies performed to date (Daly, 2012). The HLA genes are located in the major histocompatibility complex (MHC) region on chromosome 6 and play a prominent role in the human immune system. The class I and II HLA genes encodes for cell surface antigen presenting proteins while the class III derived proteins have variable functions, such as comprising the complement system. The class I and II genes display a large degree of polymorphisms. This variability stems from the need to successfully display a wide range of processed foreign peptides to T cell antigen receptors.

The lack of drug metabolizing gene associations could be due to limitations of the GWA technique as several factors restricting the GWA utility have been identified: (1) very rare genetic variants with very weak effects are not detectable by stringent statistical association analyses, (2) a high number of variants may be present for the same gene with different effects on protein function and the combination of variants may be even more complex in multifactorial diseases, such as DILI, (3) phenotypic diversity in DILI

cohorts may "dilute" the presence of specific genetic variations, (4) incomplete coverage of the genome due to technical performance or to variations in linkage disequilibrium strength between single nucleotide polymorphisms (SNPs) in the tested population and the population used to design the array, (5) systemic errors due to differences in variant frequencies between different ethnicities, (6) geographic diversity within the same ethnicity may lead to population-specific effects that, if not accounted for, may prevent identification of disease variants (Karlsen et al., 2010). The last point is particularly important in terms of DILI. Due to the relative rarity of this condition, a single center is unlikely to obtain sufficient cases for successful studies. Collaborative efforts are therefore needed to reach a high number of cases, which can come at the expense of population differences. In addition to array based GWA studies, whole-genome sequencing is fast approaching as a means of searching for genetic variations that contribute to medical conditions. The introduction of "next generation sequencing technologies" has led to a dramatic fall in sequencing cost and together with the increase in genetic information obtained, compared to more conventional array based techniques, whole-genome sequencing offers an enormous potential. Nevertheless, whole-genome sequencing is not without bioinformatic and analytical challenges and its usefulness in personalized medicine will require concerted efforts between multiple groups in a wide range of disciplines (Cordero and Ashley, 2012).

Genetic heterogeneity is common in complex diseases, which points toward a likelihood of multiple genes and pathways being implicated in DILI development. Changes in any of these genes or pathways may lead to the same phenotype, which complicates the identification of specific genetic risk factors. Moreover, the genetic redundancy existing in the human genome may very well compensate for the disruption of a single gene and consequently lead to a neutral effect. A combination of genetic variants affecting several genes simultaneously may therefore be required to produce a specific phenotype or cellular environment in which the presence of an interfering drug compound could lead to DILI. Hence, the importance here is not the individual variants *per se* but the resulting cellular phenotype, which could be reached through various polymorphic combinations. Future studies focusing on cellular/metabolic pathways and variant combinations in well-characterized cohorts are therefore needed. GWA studies have a clear advantage here due to their ability to screen a large number of variants simultaneously. However, the statistical side of this technique is somewhat lagging behind as single polymorphisms are generally considered individually, controlling the overall type I error rate by correcting for multiple testing. Considering biological pathways and variant interactions in GWA study analyses could potentially lead to more meaningful results and is currently being explored (Wang et al., 2010). This could lead us toward the ultimate goal in DILI studies, the identification of genetic risk factors to provide a better insight into the underlying pathogenesis and enable the development of new diagnostic tools along with new safer treatment strategies.

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MDMA, methamphetamine, and CYP2D6 pharmacogenetics: what is clinically relevant?

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In vitro human studies show that the metabolism of most amphetamine-like psychostimulants is regulated by the polymorphic cytochrome P450 isozyme CYP2D6. Two compounds, methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA), were selected as archetypes to discuss the translation and clinical significance of *in vitro* to *in vivo* findings. Both compounds were chosen based on their differential interaction with CYP2D6 and their high abuse prevalence in society. Methamphetamine behaves as both a weak substrate and competitive inhibitor of CYP2D6, while MDMA acts as a high affinity substrate and potent mechanism-based inhibitor (MBI) of the enzyme. The MBI behavior of MDMA on CYP2D6 implies that subjects, irrespective of their genotype/phenotype, are phenocopied to the poor metabolizer (PM) phenotype. The fraction of metabolic clearance regulated by CYP2D6 for both drugs is substantially lower than expected from *in vitro* studies. Other isoenzymes of cytochrome P450 and a relevant contribution of renal excretion play a part in their clearance. These facts tune down the potential contribution of CYP2D6 polymorphism in the clinical outcomes of both substances. Globally, the clinical relevance of CYP2D6 polymorphism is lower than that predicted by *in vitro* studies.

Keywords: MDMA, CYP2D6, methamphetamine, pharmacogenetics, ecstasy

INTRODUCTION

Amphetamine-type stimulants (ATS) make up a group of substances comprised of synthetic stimulants including amphetamine, methamphetamine, methcathinone, and ecstasy-group substances [e.g., 3,4-methylenedioxymethamphetamine (MDMA) and its analogues]. According to the latest report published by UNODC, ATS such as "ecstasy" and methamphetamine now rank as the world's second most widely abused drug type after cannabis (UNODC, 2011).

In vitro human studies show that the metabolism of most psychostimulants belonging to this class of compounds is regulated by the polymorphic cytochrome P450 isozyme CYP2D6. In addition, some of them behave both as substrates and inhibitors of CYPD6 and several other CYP isozymes (Wu et al., 1997; see Table 1). The gene (CYP2D6) environment (drug use, gender, ethnicity…) interaction has been evaluated in drug users in order to evaluate: (1) intensity of drug effects, (2) susceptibility to acute toxicity episodes and fatalities, (3) susceptibility to drug dependence, (4) contribution to drug induced neurotoxicity, and (5) drug-drug pharmacological interactions.

The fact that this polymorphic enzyme partially regulates metabolic disposition leads us to postulate that acute toxicity, drug abuse and dependence as well as, in some cases, long-term neurotoxicity could be influenced by CYP2D6 genetics (Sellers and Tyndale, 2000; de la Torre and Farré, 2004; Perfetti et al., 2009). Specifically, it was postulated that:

1. Subjects carrying genotypes which lead to enzymatic functional phenotypes should display an increased risk of drug abuse proportionate to their genotype (homozygous vs. heterozygous) and absolute level of enzyme activity.
2. Subjects carrying genotypes which lead to enzymatic functional phenotypes should display an increased risk of neurotoxicity proportionate to their genotype and absolute level of enzyme activity if the underlying mechanism is unrelated to a metabolic bioactivation.
3. Subjects carrying genotypes which lead to non-functional enzyme should experience greater risk of toxicity to a drug which is not metabolically inactivated, and might be less likely to acquire drug-taking behavior.

The present review will examine available clinical data to determine to what extent these postulates have been confirmed. Due to the fact that ATS are a broad class of compounds encompassing a number of substances, and that scant *in vivo* data from human studies are available for most of them (Wu et al., 1997), the review will focus mainly on the following two: MDMA (ecstasy) and methamphetamine. They will serve as archetypes in order to discuss the translation and clinical significance of *in vitro* to *in vivo* findings. Both compounds were chosen based on their differential interaction with CYP2D6 and their abuse prevalence in society.

CYP2D6, MDMA, AND METHAMPHETAMINE: BACKGROUND CONSIDERATIONS

CYP2D6 accounts for only a small percentage of total hepatic cytochrome P450 (1–2%), yet it is responsible for the metabolism of approximately 20–30% of marketed pharmaceuticals, including tricyclic antidepressants, selective serotonin reuptake inhibitor antidepressants, opioids, and antipsychotic, antiemetic, antiarrhythmic, and amphetamine-like drugs (Ingelman-Sundberg, 2005). *CYP2D6* exhibits a marked genetic polymorphism—over 70 alleles (www.cypalleles.ki.se/cyp2d6.htm) have been described whose combination leads to four phenotypes: poor, intermediate, extensive, and ultrarapid metabolizers (PM, IM, EM, and UM, respectively). Subjects with a PM phenotype lack two functional alleles; those with an IM have one reduced-activity allele and one non-functional allele or two reduced-activity alleles; whereas EM individuals have one or two functional alleles; and the UM phenotype is associated with gene duplications of functional alleles, with an increased protein expression (Zanger et al., 2004; Bogni et al., 2005). About 5–10% of Caucasians are PM, presenting a metabolic deficiency in CYP2D6 activity (Sachse et al., 1997).

CYP2D6 regulates MDMA O-demethylenation leading to the formation of 3,4-dihydroxymethamphetamine (HHMA) and the 4-hydroxylation of methamphetamine (pholedrine) (see **Figures 1** and **2**). Both compounds are, therefore, substrates of the same enzyme although the rates by which they are oxidized differ markedly. MDMA oxidation takes place at almost 100 times the rate of methamphetamine oxidation (see **Table 1**) (Lin et al., 1997). While methamphetamine is both a substrate and competitive inhibitor of CYP2D6, MDMA acts as a substrate and potent mechanism-based inhibitor (MBI) of the enzyme (Delaforge et al., 1999).

The east and south–east regions of Asia home to about one-third of the global population, have one of the most strongly established markets for ATS in the world, primarily for methamphetamine (UNODC, 2011). This is of relevance since the *CYP2D6*10* allelic variant, which encodes a hypofunctional enzyme, is carried by approximately 75% of the Asian population. An *in vitro* study comparing catalytically the *CYP2D6*10* allelic variant vs. *CYP2D6*1* (wild type), which is more prevalent in Caucasians, showed that the ratios of intrinsic clearance (Vmax/Km) of **1 to *10* for MDMA O-demethylenation was 123, and for methamphetamine 67, for the p-hydroxylation 157 of the N-demethylation (Ramamoorthy et al., 2001). This almost 100-fold difference in intrinsic clearance for both drugs, depending on the allelic variant considered, is of significance in the interpretation of clinical data. Findings observed in Caucasian populations with respect to the impact of *CYP2D6* polymorphism on drug effects are not always reproduced in Asian ones. This lack of reproducibility is due to the distribution of extreme phenotypes (UM and PM) in these populations. Globally, however, the rate of metabolic disposition of CYP2D6 substrates is slower in Asians.

Another aspect to be considered is the common belief that amongst drug abuser populations genotypes are distributed according to the same patterns as the general population. In the case of CYP2D6, assuming that PM subjects have a higher tendency to accumulate drug in the body, either these subjects discontinue drug consumption or autoregulate it by taking lower doses. Due to the relatively small size of the populations evaluated in clinical studies (in terms of guaranteeing a fair representation of all genotypes), the number of subjects carrying extreme genotypes is usually quite small. In fact, the authors of the present review believe that PM subjects are underrepresented in MDMA and other amphetamine-like compound user

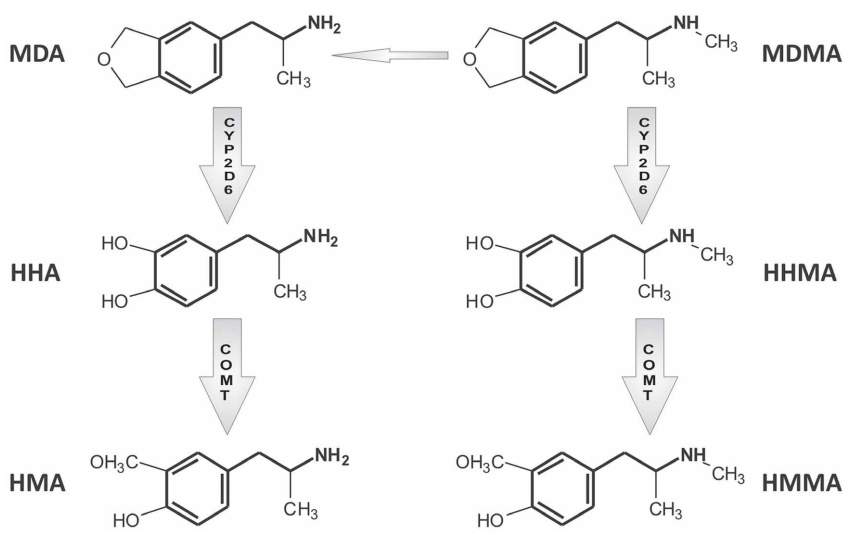

FIGURE 1 | Simplified scheme of MDMA main metabolic pathways. For a more detailed description, readers are referred to de la Torre et al. (2004). MDMA (3,4-methylendioxymethampetamine), MDA (3,4-methylendioxyampetamine), HHMA (3,4-dihydroxy methamphetamine), HMMA (3-methoxy-4-hydroxymethamphetamine), HHA (3,4-dihydroxyamphetamine), and HMA (3-methoxy-4-hydroxyamphetamine).

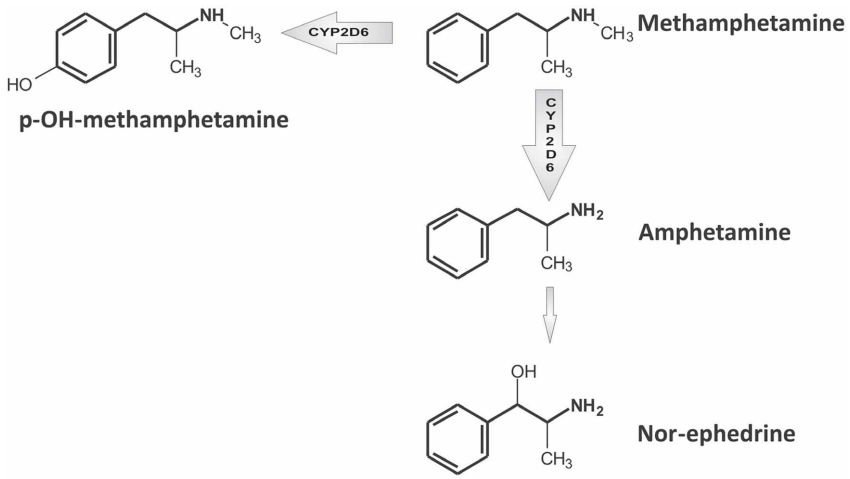

FIGURE 2 | Simplified scheme of methamphetamine main metabolic pathways. For a more detailed description, readers are referred to Shima et al. (2006).

populations probably because of the acute effects experienced. This bias has to be taken into consideration when interpreting clinical data.

MDMA PHARMACOLOGY AND METABOLIC DISPOSITION

MDMA is a psychostimulant drug that displays effects related to amphetamine-type drugs plus a number of distinctive ones (closeness to others, facilitation to interpersonal relationship, and empathy) that have been named by some authors as entactogen properties. MDMA is a potent releaser and/or reuptake inhibitor of presynaptic serotonin (5-HT), dopamine (DA), and norepinephrine (NE). These actions result from the interaction of MDMA with the membrane transporters involved in neurotransmitter reuptake and vesicular storage systems. MDMA is a mild inhibitor of monoamine oxidase (MAO) and also has direct action on several types of receptors including the 5-HT2 receptor, the M1 muscarinic receptor, the α2-adrenergic receptor, and the histamine H1 receptor.

The most frequent effects after MDMA administration are euphoria, well-being, happiness, stimulation, increased energy, extroversion, feeling close to others, increased empathy, increased sociability, enhanced mood, and mild perceptual disturbances. In addition, cardiovascular related somatic symptoms, autonomic effects (dry mouth, sweating, tremor, mydriasis tremor, jaw clenching, and restlessness), and moderate derealization have been observed (de la Torre et al., 2004).

MDMA induced acute toxic effects are related to its pharmacologic actions. Hyponatremia is an uncommon complication associated with inappropriate antidiuretic hormone (SIADH) secretion and excessive water intake. Fulminant hepatitis and hepatic necrosis have been described (Henry et al., 1992; de la Torre et al., 2004). Chronic use of MDMA is linked to a progressive neurodegeneration of the serotonergic neurotransmission system (Green et al., 2003).

Two main pathways are involved in MDMA metabolic clearance: (1) O-demethylenation partially regulated by CYP2D6 followed by catechol-O-methyltransferase (COMT)-catalyzed methylation (HMMA) and/or glucuronide/sulfate conjugation; and (2) N-dealkylation leading to 3,4-methylenedioxyamphetanmine (MDA), further subject to similar metabolic reactions as MDMA (O-demethylenation and O-methylation) (see **Figure 1**). MDMA metabolic clearance accounts for about 75% of plasma clearance and 30% of its metabolism is regulated by CYP2D6 (de la Torre et al., 2000; Segura et al., 2005).

MDMA CYP2D6 MECHANISM-BASED INHIBITION AND THE PHENOCOPYING PHENOMENON

As previously stated, whilst MDMA is metabolized by CYP2D6 it is also a potent MBI of the enzyme (de la Torre et al., 2000; Farré et al., 2004; Heydari et al., 2004; Yang et al., 2006). MBI occurs shortly after a single recreational dose, inactivating most hepatic CYP2D6 within 2 h, and returning to a basal level of CYP2D6 activity after at least 10 days (O'Mathúna et al., 2008; Yubero-Lahoz et al., 2011). This phenomenon is associated with a decrease in the amount of effective enzyme so that recovery of activity depends on its *de novo* synthesis (Liston et al., 2002). In MBI there is a rapid phenocopying to apparent PM status after a single dose of MDMA, which signifies that within 2 h subjects display the PM phenotype after drug intake, irrespective of their original genotype. Previous clinical trials have reported that the phenocopying phenomenon was observed in 67% of male subjects (O'Mathúna et al., 2008) and in 100% of the females (Yubero-Lahoz et al., 2011). Therefore, recreational MDMA users (including those who take repeated doses in the same session) are exposed to a higher probability of relative overdose and an increased risk of suffering adverse effects from CYP2D6 substrates (Farré et al., 2004).

MDMA AND CYP2D6 PHARMACOGENETICS

Most research evaluating the potential impact of CYP2D6 pharmacogenetics in MDMA pharmacology has been focused on acute effects. Preliminary *in vitro* studies showing that MDMA was a CYP2D6 substrate raised the possibility that subject carriers

Table 1 | Interaction of amphetamine-like and related psychostimulants with CYP2D6: affinity and inhibitory capacity.

Amphetamine derivatives	Ki (μM)[a]	Km (μM)
±3,4-Methylenedioxymethamphetamine (MDMA)	0.6 ± 0.6	2.2 ± 1.6[b]
±3,4-Methylenedioxyamphetamine (MDA)	1.8 ± 1.0	11.6 ± 5.4[b]
±-3,4-methylenedioxyethylamphetamine (MDE)		2.6 ± 1.4[b]
+Amphetamine	26.5 ± 1.5	
+Methamphetamine	25.0	39.6 ± 4.7[c]
±-2-Methoxyamphetamine	11.5 ± 0.5	
±-3-Methoxyamphetamine	17.5 ± 2.5	
±-4-Methoxyamphetamine (PMA)	24 ± 6	29.3 ± 4.4[d]
±-4-Hydroxyamphetamine	195 ± 45	
±-4-Hydroxymethamphetamine	60 ± 10	
p-Methoxymethamphetamine (PMMA)		4.6 ± 1.0[e]
4-methoxy-Nethylamphetamine (M-NEA)		19.5 ± 1.4[d]
N-butylamphetamine (NBA)		3.7 ± 0.3[d]
4-methoxy-N-butylamphetamine (M-NBA)		11.9 ± 1.8[d]
±-2,4,6-Trimethoxyamphetamine (2,4,6-TMA)	33 ± 12	
±-3,4,5-Trimethoxyampthetamine (3,4,5-TMA)	128 ± 3	
benzodioxolyl-butanamine (BDB)		0.8 ± 0.1[f]
N-methyl-benzodioxolyl-butanamine (MBDB)		1.0 ± 0.02[g]
4′-Methyl-α-pyrrolidinopropiophenone (MPPP)		9.8 ± 2.5[h]
4′-methoxy-α -pyrrolidinopropiophenone (MOPPP)		9.9 ± 2.5[i]
3′,4′-methylenedioxy-α-pyrrolidinopropiophenone (MDPPP)		13.5 ± 1.5[j]

[a] *(Wu et al., 1997).*
[b] *(Kreth et al., 2000).*
[c] *(Lin et al., 1997).*
[d] *(Bach et al., 1999).*
[e] *(Staack et al., 2004). Calculated as pmol/min/pmol P450.*
[f] *(Meyer et al., 2009b). Calculated as pooled human liver microsomes (pHLM 20 mg microsomal protein/mL, 400 pmol total P450/mg protein).*
[g] *(Meyer et al., 2009a). Calculated as pooled human liver microsomes (pHLM 20 mg microsomal protein/mL, 400 pmol total P450/mg protein).*
[h] *(Springer et al., 2003a). Calculated as pmol/min/pmol P450.*
[i] *(Springer et al., 2003b). Calculated as pmol/min/pmol P450.*
[j] *(Springer et al., 2003c). Calculated as pmol/min/pmol P450.*

of allelic variants leading to the PM phenotype for CYP2D6 might be at increased risk of acute toxicity episodes and higher abuse liability (Tucker et al., 1994; Henry and Hill, 1998; de la Torre et al., 1999). Conversely, since long-term neurotoxicity is believed to be mediated by metabolites formed after methylenedioxyphenyl ring-opening by CYP2D6 (de la Torre and Farré, 2004; Jones et al., 2005), PM might be protected against chronic toxicity (Perfetti et al., 2009). However, toxicological data do not seem to fully support these expectations since in a series of acute intoxications, high plasma MDMA concentrations have been reported although an overrepresentation of genotypes (homozygous for the **3* and **4* allelic variants examined) leading to the PM phenotype was not found (O'Donohoe et al., 1998; Schwab et al., 1999; Gilhooly and Daly, 2002). Unfortunately, few data are available concerning the clinical pharmacology of MDMA in PM individuals. A previous study reported that PM (homozygous **4/*4*) subjects display increased plasma concentrations, and an increased risk of hyperthermia, after a single dose of MDMA. A similar observation has been reported for another methylenedioxyamphetamine derivative,3,4-methylenedioxyethylamphetamine (MDE) (Kreth et al., 2000). Concerning MDMA, due to CYP2D6 autoinhibition and, therefore, the phenomenon of phenocopying towards the PM phenotype, effects experienced by EM subjects (*1/*1, $n = 6$, and *1/*4 $n = 3$) after two consecutive doses of the drug (Farré et al., 2004; de la Torre et al., 2005) are similar to those of PM ones (*4/*4). Other studies have reported that drug side effects are related individuals with low-activity of CYP2D6 (EM/IM category, comprising the following genotypes: **2/*9, *1/*10, *1/*41, *2/*41, *2/*35, *35/*35, and *35/*41*) displaying an increased induction of plasma hypo-osmolality, hyponatremia, and increased plasma antidiuretic hormone (vasopressin) after MDMA consumption (Aitchison et al., 2012). Moreover, the PM/IM (**4/*29, *5/*41, and *6/*41*) or the IM/IM (*41/*41) genotypes were related to a greater degree of increase in plasma cortisol concentration than the other CYP2D6 after MDMA intake (Wolff et al., 2012).

The formation of tioether adducts with quinones resulting from the auto-oxidation of the MDMA catechol type metabolites HHMA and HHA (3,4-dihydroxyamphetamine) is one of the hypotheses for MDMA induced neurotoxicity. These compounds can easily enter into redox cycling, generating radical oxygen species, which are the underlying mechanism of MDMA neurotoxicity. Genetic polymorphisms in CYP2D6 and catechol-O-methyltransferase, the combination of which are major determinants of steady-state levels of HHMA and HMMA, probably explain the interindividual variability seen in the recovery of N-acetyl-cysteinyl adducts from urine (N-Ac-5-Cys-HHMA and N-Ac-5-Cys-HHA). The recovery was marginally related to the CYP2D6 genotype among EM subjects (one vs. two functional alleles) ($p < 0.1$) and to the COMT *val158met* genotype ($p < 0.1$) of subjects. The recovery of N-Ac-5-Cys-HHMA was 2-fold higher among *met/met* subjects compared with the value for the *val/val* subjects (Perfetti et al., 2009).

MDMA DRUG–DRUG INTERACTIONS

MDMA, once taken, is not selective to CYP2D6 and interacts with several isozymes of P450. In fact, the contribution of CYP2D6 to MDMA metabolism has been reported to be less than 30% (Segura et al., 2005). Thus, several other P450 isoenzymes such as CYP1A2 and, to a lesser extent, CYP2B6 and CYP3A4 have the capacity to contribute to the microsomal oxidative metabolism of MDMA and MDA A recent study showed that while CYP2D6 was inhibited by MDMA, CYP1A2 increased its activity (Yubero-Lahoz et al., 2012). Another clinical trial showed there was a conversion from MDMA to HHMA *in vivo*, despite the CYP2D6 inhibition by paroxetine, suggesting alternative metabolic pathways (Segura et al., 2005). Other enzymes may, therefore, become

more predominant once CYP2D6 is inhibited which could further contribute to MDMA metabolic disposition.

The administration of inhibitors of CYP2D6 activity can influence the metabolism of MDMA, and in turn MDMA can inhibit drugs metabolized by CYP2D6. Previous administration of antidepressants with CYP2D6 inhibitory actions, such as paroxetine, reboxetine, or duloxetine, produce 15–30% of MDMA concentrations, but decrease concentrations of its metabolite HMMA by 40–50% (Segura et al., 2005; Farré et al., 2007; Hysek et al., 2011, 2012). Moreover, the pharmacological effects of MDMA are decreased, probably due to competition for the uptake transporter decreasing MDMA entry in neurons.

CYP2D6 is also the source of a number of drug–amphetamine interactions because it regulates the biotransformation of many therapeutic drugs. Antiretroviral drugs (ritonavir, a known CYP2D6 inhibitor) and MAO inhibitors have been reported to be the main cause of life threatening interactions with MDMA (Henry and Hill, 1998; de la Torre et al., 1999; Papaseit et al., 2012).

METHAMPHETAMINE PHARMACOLOGY AND METABOLIC DISPOSITION

Methamphetamine is an indirect sympathomimetic agent, similar in structure to amphetamine. Nevertheless, an added N-methyl group confers increased lipid solubility, allowing for more rapid diffusion into the central nervous system. Methamphetamine effects derive from their interaction with a number of neurotransmitter systems; primarily with the dopaminergic but also with serotonergic, noradrenergic, and glutamatergic systems. Acute adverse effects including cardiovascular and psychoactive ones are related to an excess of neurotransmitters. As previously explained, long-term methamphetamine induced effects are the result of a neurodegeneration of the dopaminergic system (Schep et al., 2010).

There are three main biotransformation pathways (see **Figure 2**) involved in methamphetamine metabolic clearance: (1) N-demethylation to produce amphetamine, (2) aromatic hydroxylation producing 4-hydroxymethamphetamine (pholedrine) with both reactions partially regulated by CYP2D6 (Lin et al., 1997), and (3) beta-hydroxylation to produce norephedrine. Metabolic clearance represents more than 50% of total plasma clearance (Cook et al., 1993).

METHAMPHETAMINE AND CYP2D6 PHARMACOGENETICS

A review of the current literature for genetic-association studies of methamphetamine use disorders, including 38 studies and 39 genes, showed that 18 genes were found to have a significant genotypic, allelic, and/or haplotypic association. Among these genes was CYP2D6 which was associated with methamphetamine dependence (Bousman et al., 2009). Of particular relevance was a report in which a total of 202 patients with methamphetamine dependence and 337 controls in a Japanese population were genotyped for *CYP2D6*1, *4, *5, *10*, and **14*. A significant association of the CYP2D6 genotype with methamphetamine dependence ($p = 0.03$) was reported. There were fewer patients carrying the hypofunctional alleles **10* and **14* alleles than in the control population, and in this population there were no PMs. IMs of CYP2D6 were significantly fewer among methamphetamine-dependent subjects than in controls ($p = 0.02$), with an odds ratio of 0.62 (95% confidence interval: 0.51–0.76). A potential conclusion of this study is that a lower CYP2D6 activity seems to confer some degree of protection against methamphetamine dependence (Otani et al., 2008).

In a study performed in Caucasians it was observed that EM ($n = 8$) and PM ($n = 3$) subjects administered with methamphetamine by the oral route (10 mg) displayed a similar area under the curve for plasma methamphetamine concentrations, despite the fact that p-hydroxymethamphetamine was only observed in PM subjects. PM subjects appeared to be more sensitive to the slope of plasma methamphetamine concentrations in several measurements of subjective effects. The data suggest that brain methamphetamine concentrations (in the absence of differences in plasma concentrations) are higher in PM subjects or that they have a steeper concentration-response relationship (Sellers and Tyndale, 2000).

Methamphetamine use may induce the following physical effects: anorexia, hyperactivity, dilated pupils, flushing, restlessness, dry mouth, bruxism, headache, cardiovascular alterations in heart rate, and blood pressure. It may cause rhabdomyolysis which has been associated with mortality. In a series of 18 autopsies genetic susceptibility to rhabdomyolysis was examined. Mutations of the following genes were studied: ryanodine receptor 1 (RYR 1), carnitine palmitoyltransferase II (CPT II), very long-chain acyl-CoA dehydrogenase (VLCAD), and CYP2D6. The conclusion was that there was no obvious relationship between the genetic mutations observed in this study and rhabdomyolysis (Matsusue et al., 2011).

Neuropsychological alterations seen in many methamphetamine users are often unrelated to its lifetime consumption or length of abstinence. In a series of 52 methamphetamine users the contribution of CYP2D6 polymorphism to variability observed in long-term effects was studied. EM subjects showed worse overall neuropsychological performance and were three times as likely to be cognitively impaired as IMs/PMs. Apparently, a more efficient metabolic disposition of methamphetamine is associated with a poorer cognitive performance. It has also been suggested that metabolism may generate metabolic species involved in the underlying mechanism of neurotoxicity (Cherner et al., 2010).

METHAMPHETAMINE DRUG—DRUG INTERACTION

The administration of inhibitors of CYP2D6 activity can influence the metabolism of methamphetamine, and methamphetamine can inhibit the metabolism of CYP2D6 substrates. The number of published drug-interaction studies with this class of substances is very scarce. Previous administration of bupropion, a known CYP2D6 inhibitor, produces a large increase of methamphetamine concentrations, and a reduction in amphetamine ones. The pharmacological effects of methamphetamine (cardiovascular and euphoria-like ones) were decreased by bupropion (Newton et al., 2005, 2006).

Antiretroviral drugs (ritonavir, a known CYP2D6 inhibitor) have been reported to be the main cause of life-threatening interactions with methamphetamine (Hales et al., 2000).

CONCLUDING REMARKS

The involvement of CYP2D6 polymorphism in the metabolic clearance of both MDMA and methamphetamine leads to the speculation that it should have an impact on acute and long-term drug toxicity and drug taking behavior.

Concerning acute effects, those subject carriers of alleles with a reduced functionality are at higher risk, for both MDMA and methamphetamine, of experiencing heightened pharmacological effects. Moreover, in combination with some environmental factors this may lead to acute toxicity episodes including death. Irrespective of the initial dose, the following one results in the MBI of MDMA and phenocopying to the PM phenotype thus diluting the variability incorporated by the genetic polymorphism and, consequently, putting all subjects at risk of acute effects. With respect to methamphetamine, because a large portion of drug users are of Asian origin, and carriers of the lower functionality allele **10*, most of these subjects should also experience increased effects.

Concerning the relevance of CYP2D6 polymorphism on drug abuse, preliminary data from methamphetamine suggest that an increased CYP2D6 functionality may lead to an increased abuse of the substance. An observation that is more relevant for methamphetamine, with a higher abuse liability, than MDMA.

Regarding neurotoxicity, only in the case of MDMA may a metabolic bioactivation be involved in long-term neurotoxic effects. Theoretically those subject carriers of CYP2D6 functional alleles (including those carriers of duplications) and with a low COMT activity should be the most efficient in generating metabolic neurotoxic species and, consequently, the most vulnerable to neurotoxicity. Again the MBI of MDMA should be taken into consideration after repeated doses.

Metabolic clearance of both methamphetamine and MDMA ranges from 50% to 75%, there is, therefore, a relevant contribution of renal excretion in plasma clearance. The fraction of metabolic clearance regulated by CYP2D6 is lower than 50% for both drugs and other isozymes of cytochrome P450 contribute to their clearance. Both factors combine with catalytic activities, and MBI behavior in case of MDMA, to tune down the potential contribution of CYP2D6 polymorphism in clinical outcomes of both substances. Although MDMA and methamphetamine are the most consumed ATS many substances of this group are substrates of CYP2D6. Thus, in possible future reports on other substances it would not be surprising to find that the CYP2D6 polymorphism has a strong role in the clinical outcome of drug users. The difficulty in performing controlled clinical studies with drug users stratified as a function of drug metabolizing polymorphisms, limits the evaluation of their clinical impact.

In summary, the genetic polymorphism of CYP2D6 and co-administration of CYP2D6 inhibitors may have less impact on the risk of acute toxicity than previously thought, whereas the role of metabolism by other cytochrome P450 enzymes and renal excretion assumes greater importance with regard to systemic exposure to unchanged drug. Globally, the clinical relevance of CYP2D6 polymorphism is lower than that predicted by *in vitro* studies.

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Molecular interactions between NAFLD and xenobiotic metabolism

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Non-alcoholic fatty liver disease (NAFLD), the hepatic manifestation of the metabolic syndrome, is a complex multifactorial disease characterized by metabolic deregulations that include accumulation of lipids in the liver, lipotoxicity, and insulin resistance. The progression of NAFLD to non-alcoholic steatohepatitis and cirrhosis, and ultimately to carcinomas, is governed by interplay of pro-inflammatory pathways, oxidative stress, as well as fibrogenic and apoptotic cues. As the liver is the major organ of biotransformation, deregulations in hepatic signaling pathways have effects on both, xenobiotic and endobiotic metabolism. Several major nuclear receptors involved in the transcription and regulation of phase I and II drug metabolizing enzymes and transporters also have endobiotic ligands including several lipids. Hence, hepatic lipid accumulation in steatosis and NAFLD, which leads to deregulated activation patterns of nuclear receptors, may result in altered drug metabolism capacity in NAFLD patients. On the other hand, genetic and association studies have indicated that a malfunction in drug metabolism can affect the prevalence and severity of NAFLD. This review focuses on the complex interplay between NAFLD pathogenesis and drug metabolism. A better understanding of these relationships is a prerequisite for developing improved drug dosing algorithms for the pharmacotherapy of patients with different stages of NAFLD.

Keywords: NAFLD, xenobiotic metabolism, nuclear receptors, phase I and II enzymes, transporters

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in Western countries with a wide disease spectrum. It ranges from the hepatic accumulation of lipids known as steatosis, non-alcoholic steatohepatitis (NASH) wherein steatosis is accompanied by inflammation and can further progress to cirrhosis and hepatocellular carcinoma. NAFLD is the hepatic manifestation of the metabolic syndrome as it is frequently associated with obesity, insulin resistance, hyperglycemia, hypertension, and dyslipidemia (Anderson and Borlak, 2008; Lewis and Mohanty, 2010; Cohen et al., 2011).

The accumulation of hepatic triglycerides due to an imbalance between free fatty acids uptake, *de novo* lipogenesis, oxidation, esterification, and secretion, is a hallmark of hepatic steatosis (Donnelly et al., 2005). Initially the progression of NAFLD to NASH was described as a two-hit event, with the accumulation of hepatic triglycerides being the first hit, which in turn triggers the second hit – inflammation and oxidative stress (Crespo et al., 2001; Browning and Horton, 2004; Sunny et al., 2011; Wree et al., 2011). An extended hypothesis proposed a reduced capacity of hepatic regeneration and the detrimental effects of free fatty acid lipotoxicity as the third hit (Feldstein et al., 2004; Day, 2006).

Several other factors contribute to the induction of NAFLD and/or to its progression. These include compromised adipose tissue storage and function (Calvert et al., 2007), upregulation of inflammatory factors, and reactive oxygen species (ROS; Crespo et al., 2001; Browning and Horton, 2004; Wree et al., 2011), the interaction of the intestinal microbial populations with the host through the inflammasome (Henao-Mejia et al., 2012), downregulation of the endoplasmic reticulum stress and protein ubiquitination pathways (*HSPA5*, *USP25*), and gene expression changes in cell development, morphology, movement, death, and antigen presentation pathways (Gawrieh et al., 2010). Insulin resistance also plays a detrimental role in the pathogenesis of NAFLD. Polymorphisms that may potentially explain this effect were identified in the insulin receptor-substrate 1 (*IRS-1*; Gly172Arg; Dongiovanni et al., 2010).

As a multifactorial disorder, NAFLD is characterized by interactions between genetic and environmental factors, thus proving it difficult to understand its manifestations (Fon Tacer and Rozman, 2011; Lorbek and Rozman, 2012). Moreover, the scarcity of robust non-invasive diagnostic methods represents an obstacle in accurately determining the prevalence of NAFLD (Ratziu et al., 2011). By current estimations, NAFLD has a prevalence rate of 6–35% with a median of 20%, depending on the population studied and the method of assessment (Vernon et al., 2011; Chalasani et al., 2012). Ethnic differences in the prevalence of NAFLD also exist, with a lower frequency in African–Americans compared to

Hispanic-Americans (Wagenknecht et al., 2009; Stepanova et al., 2010). It occurs in children (Roberts, 2007) and adults of all age groups, however conflicting observations have been made regarding the gender-specific risk of NAFLD (Bedogni et al., 2005; Chen et al., 2008b). The prevalence of NASH is much lower, affecting 2–5% of the population (Neuschwander-Tetri and Caldwell, 2003; Vernon et al., 2011), however, its frequency escalates with increasing age, body mass index (BMI), more severe forms of insulin resistance, hypertriglyceridemia, and poor liver function. Increased progression to NASH is observed in Hispanics, possibly due to the large-scale adaptation of western lifestyles (Browning et al., 2004). Obese individuals (BMI $> 30\,kg/m^2$) are at a higher risk of developing NAFLD, with a prevalence of 85–98% for NAFLD and >25% for NASH (Machado et al., 2006). Furthermore, diabetes mellitus (Type 2 Diabetes, T2D) is a major determinant of NAFLD with a 70% prevalence of NAFLD in some diabetic populations (Targher et al., 2006; Leite et al., 2009). However, NAFLD also occurs in approximately 18% of normal weight non-diabetic populations (de Alwis and Day, 2008).

Due to the lack of targeted drugs, NAFLD patients are usually treated by cholesterol-lowering statins, fibrates, or antidiabetics such as thiozolidinediones, sulfonylureas, etc. (Rozman and Monostory, 2010). The controversy regarding the NAFLD patients' benefits versus the potential harm due to liver toxicity is however, a matter of investigations and vivid debates.

NAFLD AND DRUG DISPOSITION

Liver is the major organ of endogenous and xenobiotic metabolism. In healthy livers, the metabolic processes are in homeostasis. A long-term disturbance of one or more metabolic pathways can provoke liver diseases. The intracellular accumulation of xeno- and endo-biotics is potentially toxic and is regulated at several levels including uptake, biotransformation, and elimination by drug metabolizing enzymes (DMEs). DMEs are classified as phase I, mainly cytochrome P450s (CYPs) that catalyze hydroxylation reactions, or phase II enzymes that are involved in conjugation reactions. Human phase I DME subfamilies CYP3A and CYP2C account for 50% of all hepatic CYPs and metabolize a large number of diverse drugs, e.g., lovastatin, tamoxifen, and R-warfarin. Phase II DMEs include UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), *N*-acetyltransferases, and several other transferases that transform compounds into more excretable forms. Transporters such as organic anion transporting polypeptide (OATP) and ATP-binding cassette (ABC)-transporters are responsible for the hepatocellular uptake and excretion of xenobiotics. The expression of phase I and II DMEs and transporters is regulated by a battery of nuclear receptors in a coordinated manner (Aleksunes and Klaassen, 2012).

Non-alcoholic fatty liver disease patients show differences in drug metabolism and its regulatory components, as summarized in **Tables 1–3**. Compared to normal subjects, a pediatric NAFLD population exhibited altered glucuronidation of acetaminophen, a commonly used analgesic and antipyretic agent known to cause acute hepatic failure (Barshop et al., 2011). Although the pharmacokinetic profile of acetaminophen in both the normal and NAFLD subjects was unaltered, this study highlighted changes in the biotransformation of the drug and the possibility of compensation by other excretory pathways in the presence of NAFLD. Similarly, the metabolism of ezetimibe, an intestinal cholesterol-uptake blocker, is altered in NASH patients due to differential localization of ABCC2 and ABCB1 efflux transporters, hence, resulting in plasma retention of the active glucuronide metabolite of ezetimibe (Hardwick et al., 2012b). Studies have also indicated a reduced efficacy of certain treatments in NAFLD condition, such as the novel oral hypoglycemic sitagliptin, a dipeptidyl peptidase-4 (DPP-4) inhibitor. T2D patients with NAFLD have increased serum DPP-4 activity, an enzyme that inhibits incretins such as glucagon-like peptide 1 (GLP-1), and thus, reduced efficacy of sitagliptin (Firneisz et al., 2010; Iwasaki et al., 2012).

Several drug metabolizing CYPs are downregulated in genetically modified (e.g., leptin deficient ob/ob, dysfunctional leptin receptor db/db mice) and diet-induced [e.g., high-fat diet, methionine- and choline-deficient (MCD) diet] NAFLD animal models and in patients with characteristics of the metabolic syndrome (Buechler and Weiss, 2011; Ghose et al., 2011). Genome wide association and candidate gene studies have identified single nucleotide polymorphisms (SNPs) in DMEs that associate with NAFLD prevalence, progression, or severity, highlighting the role of altered drug metabolism in NAFLD pathogenesis (Anstee et al., 2011; Lake et al., 2011). Thus, there is ample evidence for altered xenobiotic metabolism and efficacy in NAFLD patients.

A function of CYPs in hepatic lipid homeostasis is indicated by their broad role in microsomal oxidation, cholesterol biosynthesis, and their activation by lipids. Further proof of this role was observed from studies in the liver conditional CYPs reductase microsomal flavoprotein NADPH: CYP oxidoreductase (*Por*) null mice. POR is an essential redox partner of the microsomal CYPs. The liver conditional *Por* knockout mice display hepatomegaly, hepatic steatosis, and a reduced capacity of drug metabolism (Gu et al., 2003). One of the CYPs, CYP51, is an essential enzyme of cholesterol synthesis (Keber et al., 2011). Cholesterol and its metabolites are also known to contribute to lipotoxicity and inflammation-mediated progression of NAFLD to NASH (Caballero et al., 2009). The cholesterol synthesis pathway responds to feedback regulation by cholesterol, TNF-α stimulation (Tacer et al., 2007), and xenobiotics, such as statins (Rezen et al., 2008, 2009; Rozman and Monostory, 2010), thus emphasizing the hepatic crosstalk between endobiotic and xenobiotic metabolism and inflammation. Endobiotics and xenobiotics activate various nuclear receptors and thus influence the expression of genes involved in the various hepatic metabolic pathways (Gao and Xie, 2010). The interplay between metabolism of endobiotics and xenobiotics is a frequent cause of drug side effects that can now be explained at the molecular level (Hafner et al., 2011; Rezen, 2011).

NUCLEAR RECEPTORS REGULATING DMEs

Pregnane X receptor

Pregnane X receptor (PXR, NR1I2) is a ligand-activated nuclear receptor that upon activation forms a heterodimer with retinoid X receptor (RXR) and regulates the expression of a wide range of DMEs (Tolson and Wang, 2010). Apart from being activated by xenobiotics, it also responds to endobiotics including bile acids (Xie et al., 2001) and steroid hormones (di Masi et al., 2009). In mice, PXR activation results in hepatic steatosis due to

Table 1 | Nuclear receptors and transcription factors in interaction between NAFLD and drug metabolism.

Nuclear receptor/ transcription factor	Targets	Association with NAFLD
Pregnane X receptor (PXR)	*CYP2C9* *CYP3A4* *CYP2B6* *UGT1A1* *MDRP1 P-glycoprotein* *CD36*	Activation in mice causes hepatic steatosis due to enhanced lipogenesis, decreased β-oxidation, and increased uptake of fatty acids via CD36 activation (Zhou et al., 2006b) A NAFLD population of European descent displayed strong association between rs2461823/A and rs7643645/G-containing haplotypes and disease severity irrespective of BMI and HOMA index (Sookoian et al., 2010)
Constitutive androstane receptor (CAR)	*CYC2C9* *CYP2B6* *CYP3A4* *UGT1A1*	*Car*+/+ mice fed MCD diet develop increased liver fibrosis (Yamazaki et al., 2007) Activation results in the induction of aberrant hepatic DNL and insulin resistance via the expression of THRSP (Anderson et al., 2009; Rezen et al., 2009; Breuker et al., 2010) Lowers plasma concentration of HDL (Masson et al., 2008)
Farnesoid X receptor (FXR)	*CYP7A1* *BSEP*	Deficiency in a mouse model of hypercholesterolemia fed on a HFD results in features of NASH (Kong et al., 2009) *FXR*1B* (-1T) is associated with decreased FXR expression and functionality (Marzolini et al., 2007)
Liver X receptor (LXR)	*SREBP-1c* *PPARγ* *chREBP* *CD36* *ABC1, ABCG1, ABCG5, ABCG8*	Involved in lipid biosynthesis, cholesterol and bile acid homeostasis, and fatty acid uptake (Handschin and Meyer, 2005; Rezen et al., 2011) Plays a crucial function in glucose tolerance, insulin secretion, and adipocyte size (Efanov et al., 2004; Gerin et al., 2005) rs17373080[G] polymorphism in *LXR*β associated with 20–30% lower risk of T2D prevalence but a higher risk of obesity (Dahlman et al., 2009; Solaas et al., 2010)
Peroxisome proliferator-activated receptor (PPAR)	*ACS* *CPT-I* *SULT 1C1, 1C2, 1E1, 2A1, 2A2, 3A1* *UGT1A1, UGT1A3, UGT1A6, UGT2B4*	Fibrates are utilized to treat patients with elevated plasma triglycerides PPARα activates fatty acid oxidation and hepatic lipid hydrolysis and downregulates hepatic triglyceride secretion (Kersten et al., 1999; Pyper et al., 2010; Rakhshandehroo et al., 2010) *Ppar*α-deficient mice develop hepatic steatosis on a high-fat diet (HFD; Abdelmegeed et al., 2011) Potential protective role for the *Val227Ala* variant of *PPARα* against obesity compared to wild-type variant (Chen et al., 2008a)
Nuclear factor erythroid 2-related factor 2 (Nrf2)	*GST* *HO-1, Nqo1, GCLC* *Mrp2/ABCC2*	Null mice on MCD diet exhibit increased hepatic steatosis, inflammation, and oxidative stress (Chowdhry et al., 2010) Rats fed with an MCD diet display Nrf2-dependent upregulation of oxidative stress response (Lickteig et al., 2007)

enhanced sterol regulatory element-binding protein-1c (SREBP-1c)-independent lipogenesis, decreased β-oxidation, and increased uptake of fatty acids via fatty acid translocase (FAT/CD36) activation (Zhou et al., 2006b). The activation of CD36 by PXR in mice occurs directly or *via* the activation of peroxisome proliferator-activated receptor gamma (PPARγ; Tontonoz et al., 1998; Zhou et al., 2006b). PXR also plays a role in glucose metabolism (Gao and Xie, 2012). It inhibits gluconeogenesis by inactivating hepatocyte nuclear factor-4 (HNF-4) and forkhead box protein O1 (FOXO1), both of which are positive regulators of gluconeogenic genes (Bhalla et al., 2004; Kodama et al., 2004). Genetic association studies in a European NAFLD population indicated an association of the *PXR* rs2461823[A] and rs7643645[G]-containing haplotypes and disease severity, irrespective of BMI and homeostatic model assessment (HOMA) index (Sookoian et al., 2010). Although PXR activation increases steatosis, PXR-dependent counteraction of inflammation by inhibition of nuclear factor kappa-B (NFκB) has also been documented in human and mouse samples (Zhou et al., 2006a). In support of the previous statement, rats treated with a PXR activator pregnenolone-16α-carbonitrile (PCN) displayed reduced hepatic fibrosis and necrosis in response to a fibrogenesis-inducing agent carbon tetrachloride (CCl_4; Marek et al., 2005). It is thus plausible that *PXR* polymorphisms associated with NAFLD may affect disease severity by lowering PXR activity, thus resulting in increased susceptibility to NASH. Another link between PXR and NAFLD is through the modulation of DMEs. PXR induces the expression of CYP2C9 (Gerbal-Chaloin et al., 2002), which metabolizes the anti-diabetic drug rosiglitazone known to reduce liver fat. Since rosiglitazone is used in NAFLD patients with hyperglycemia and IR (Ratziu et al., 2008), patients with *PXR*

Table 2 | Phase I drug metabolizing enzymes implicated in the pathogenesis of NAFLD.

Phase 1 DME	Drugs metabolized/transported	Association with NAFLD
CYC2C9	Roziglitazone (anti-diabetic) Sulphanylureas (anti-diabetic) Warfarin (anti-coagulant) Tamoxifen (selective estrogen receptor modulator)	Loss-of-function variants associated with increased response to sulfonylurea drugs, a NAFLD treatment, and an increased glycemic response in the treatment of T2D patients (Zhou et al., 2010) mRNA and enzyme activity increases with NAFLD progression (Fisher et al., 2009b)
CYP3A4	Atorvastatin (statin) Simvastatin (statin) Lovastatin (statin) Fibrates (anti-dyslipidemia) Nateglinide (anti-diabetic) Docetaxel (anti-cancer)	Expression and activity affected by SNPs in the *PXR* coding, non-coding, and promoter regions, in *PPARα* and *POR* (Zhang et al., 2008; Gomes et al., 2009; Klein et al., 2012) Intron 6 SNP rs35599367[T] in *CYP3A4* results in decreased expression and activity of CYP3A4 and carriers of the T allele require significantly lower doses of statins to treat dyslipidemia (Elens et al., 2011; Wang et al., 2011) CYP3A activity shows a negative correlation with the severity of steatosis (Kolwankar et al., 2007) Displays sexual dimorphism with elevated expression in premenopausal women with a more favorable lipid profile, compared to men (Wolbold et al., 2003)
CYP2E1	Propranolol (beta-blocker) Paracetamol (analgesic)	Catalyzes fatty acid oxidation in hepatic microsomal compartments and is implicated in NASH development (Williams, 2004) NAFLD and NASH patients and animal models display enhanced expression of CYP2E1 and lipid peroxidation (Robertson et al., 2001), with increased localization to areas in the liver with oxidative stress injuries, leptinemia, reduced adiponectin levels and insulin resistance in NAFLD (Weltman et al., 1996, 1998) Contrasting observations indicated decreased *CYP2E1* mRNA and protein levels and no changes in its activity at progressive stages of NAFLD (Aubert et al., 2011) *Cyp2e1*-null mice that still displayed lipid peroxidation had increased expression of *Cyp4a10* and *Cyp4a14* genes (Fisher et al., 2009b; Mitsuyoshi et al., 2009)
CYP4A	Fatty acid derivatives	Enhanced activity results in increased production of ROS, thus contributing to steatohepatitis In contrast, *Pparα*-null mice on an MCD diet are more prone to developing NASH in the absence of *Cyp4a* induction (Leclercq et al., 2000; Hardwick et al., 2009)

gene variants may theoretically suffer from aberrant rosiglitazone metabolism. *PXR* is thus a potential pharmacogenetic marker for thiazolidinedione treatments. Furthermore, PXR is a strong modulator of CYP3A4, the major phase I DME in humans. Several SNPs that affect the expression of CYP3A4 reside within the PXR coding, non-coding, and promoter regions (Zhang et al., 2008). Since many NAFLD patients are treated with drugs metabolized by CYP3A4, further pharmacogenetic evaluation of patients with these *PXR* variants is required (**Table 1**).

Constitutive androstane receptor

Constitutive androstane receptor (CAR, NR1I3) is also a key regulator of xenobiotic and endobiotic metabolism (Moore et al., 2000; Handschin and Meyer, 2005). Upon ligand activation, CAR is translocated to the nucleus where it binds to DNA elements of DME genes such as *CYP2B6*, *CYP3A4 CYP2Cs*, and others (Sueyoshi and Negishi, 2001; Gerbal-Chaloin et al., 2002; Faucette et al., 2006; Chen and Goldstein, 2009), as well as phase II enzymes involved in glucuronidation (Sugatani et al., 2005), sulfation, and drug transport (Tolson and Wang, 2010). Several studies also indicate a role of CAR in energy homeostasis (Wada et al., 2009). Hence, the activation of CAR for regulation of energy homeostasis may affect drug metabolism (Hafner et al., 2011). SREBP-1c, which is upregulated in hepatic steatosis, inhibits CAR and thus, may further contribute to aberrant xenobiotic and endobiotic metabolism (Roth et al., 2008). *Car*+/+ mice fed with MCD diet, known to induce NASH (Rinella et al., 2008), developed enhanced liver fibrosis due to lipid peroxidation, inducible nitric oxide synthase (iNOS), and increased CYP induction compared to *Car*−/− mice (Yamazaki et al., 2007). However, no difference in hepatic lipid accumulation was observed between *Car*+/+ and *Car*−/− mice, indicating that CAR may be involved in later stages of NAFLD progression and hepatocarcinogenesis (Takizawa et al., 2011). Furthermore, observations in *Car*−/− mice and human hepatocytes highlight the role of CAR activation in the induction of aberrant hepatic *de novo* lipogenesis and insulin resistance by enhancing the expression of thyroid hormone-responsive spot 14 protein (*THRSP*; Anderson et al., 2009; Breuker et al., 2010). Activation of CAR in mice with 1,4-Bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) increased serum triglycerides and hepatic fatty acid synthesis and repressed adaptation to hyperlipidemia, which is expected to favor the development of NAFLD (Rezen et al., 2009). Contradictory observations arise from other mouse models, where CAR has been linked to improved fatty liver due to decreased lipogenesis, increased β-oxidation, improved glucose tolerance, and insulin sensitivity (Dong et al., 2009; Gao et al., 2009). In mice, CAR also regulates cholesterol and bile acid metabolism by lowering plasma high-density lipoprotein (HDL)

Table 3 | Phase II drug metabolizing enzymes and transporters implicated in the pathogenesis of NAFLD.

Phase II DME/transporter	DRUGS metabolized/transported	Association with NAFLD
Glutathione-*S*-transferases (GSTs)	Chlorambucil (anti-cancer) Busulfan (anti-cancer) Cyclophosphamide (anti-cancer)	GSTM2, GSTM4, and GSTM5 mRNA levels decreased in patients with steatosis and NASH (Ip et al., 2003) Overall GST activity decreased with disease progression, accompanied by a reduced pool of glutathione, highlighting the depleted ability to combat oxidative stress in NAFLD patients (Younossi et al., 2005) Lower expression in Caucasians compared to African–Americans (Hardwick et al., 2010) *GSTM1*-null genotype present at a higher frequency in NAFLD subjects (Stepanova et al., 2010)
Sulfotransferases (SULTs)	Acetaminophen (analgesic) Albuterol (β_2-adrenergic agonist) Terbutaline (β_2-adrenergic agonist) Hormonal contraceptives	SULT2B1b has anti-lipogenic properties by suppressing the LXR-SREBP1c interaction, resulting in decreased hepatic and serum level of lipids in *Ldlr*-null mice on a HFD (Hori et al., 2007, 2009) *SULT1A2* expression is downregulated in NASH patients compared to control obese individuals (Bai et al., 2012) SULT1C4 and SULT4A1 have increased mRNA and protein levels in human NASH samples compared to control and steatosis samples (Younossi et al., 2005)
UDP glucuronosyltransferases	Non-steroidal anti-inflammatory drugs Opioids Anti-depressants Anti-psychotics	Mice with severe hepatic steatosis induced by a high-fat and high-sucrose diet, display increased expression of *Ugt1a1* and *Ugt1a6* via interaction with CAR and PXR (Hardwick et al., 2012a) The *UGT1A1*6* allele has a protective effect against NAFLD in a population of obese Taiwanese children (Osabe et al., 2008)
ABCC2	Pravastatin (statin) Vinblastine (anti-cancer) Ceftriaxone (antibiotic)	Decreased in rodent models of obesity, NAFLD and NASH and normalized on roziglitazone treatment (Lin et al., 2009) rs17222723 and rs8187710 variants in the *ABCC2* are significantly associated with NAFLD patients and clinical and histological parameters (Geier et al., 2005; Fisher et al., 2009a; Martin et al., 2010)
Uptake transporters (NTCP, OATP1a1, 1a4, 1b2, 2b1, OAT2, and OAT3)	Atorvastatin (statin) Pravastatin (statin) Rosuvastatin (statin) Non-steroidal anti-inflammatory drugs Captopril (anti-hypertension)	Downregulation of uptake transporters in the transition from steatosis to NASH rather than between control and steatotic samples (Sookoian et al., 2009)

and reverse cholesterol transport, possibly *via* downregulation of apolipoprotein A1 (ApoA1; Masson et al., 2008). *CAR* polymorphisms have not yet been linked to metabolic diseases; however, its role in glucose and lipid metabolism and its functional redundancy with PXR highlights that the *CAR* gene is an important candidate for NAFLD association studies (Rezen et al., 2009).

FXR and LXR

Farnesoid X receptor (FXR) and Liver X receptor (LXR) are not major regulators of xenobiotic metabolism, but they play an important role in the metabolism of cholesterol and bile acids (Rezen et al., 2011). FXR/NR1H4 is the predominant regulator of bile acid synthesis and secretion, thereby lowering hepatic cholesterol levels. The first and rate-limiting step of bile acid synthesis from cholesterol is catalyzed by cholesterol 7α-hydroxylase (CYP7A1). The activation of FXR in primary human and mouse hepatocytes results in decreased transcription of *CYP7A1* due to an indirect negative feedback mechanism (Goodwin et al., 2000; Holt et al., 2003). Furthermore, activated FXR upregulates the expression of CYP3A4, which hydroxylates some common bile acids into more soluble forms (Gnerre et al., 2004), as well as the bile salt export pump (BSEP; Ananthanarayanan et al., 2001; Plass et al., 2002; Song et al., 2008). Deficiency of FXR in an *Ldlr*−/− mouse model of hypercholesterolemia on high-fat diet results in features of NASH, such as macrosteatosis, hepatocyte ballooning, and inflammation (Kong et al., 2009). A common SNP, *FXR* 1B (-1T)*, has been identified in the sequence flanking the start codon of *FXR* in European, African, Chinese, and Hispanic-American populations. It associates with decreased FXR expression and functionality, which may contribute to the pathogenesis of metabolic disorders (Marzolini et al., 2007). However, association of this *FXR* polymorphism with NAFLD in humans has not yet been identified.

Liver X receptor plays important roles in lipid biosynthesis as well as cholesterol and bile acid homeostasis (Handschin and Meyer, 2005; Rezen et al., 2011). Typical activators of LXR are oxysterols such as 22(R)-hydroxycholesterol (22(R)-HC), 24(S),

25-epoxycholesterol, and 25-hydroxycholesterol (Jakobsson et al., 2012). LXR activation not only increases cholesterol catabolism but also hepatic lipogenesis through activation of SREBP-1c, PPARγ, or carbohydrate response element-binding protein (chREBP; Lee et al., 2008). LXR and PXR share many target genes (Boergesen et al., 2012) and both regulate the uptake of fatty acids into hepatocytes *via* expression of FAT *CD36* (Zhou et al., 2006b). Although LXR activates *Cyp7a1* expression during bile acid synthesis in rodents, it does not have an effect on human *CYP7A1* expression (Goodwin et al., 2003). In humans, the LXRα isoform is mainly expressed in tissues involved in lipid metabolism, where it regulates the expression of cholesterol transporter genes, such as the ABC transporters *ABC1* (Schwartz et al., 2000), *ABCG1* (Sabol et al., 2005), *ABCG5* (Repa et al., 2002), and *ABCG8* (Repa et al., 2002). LXRβ, the ubiquitously expressed isoform and the only isoform present in pancreatic β-cells, does not play a role in cholesterol homeostasis (Alberti et al., 2001) but has a crucial role in glucose tolerance, insulin secretion, and adipocyte size (Efanov et al., 2004; Gerin et al., 2005). The rs17373080[G] polymorphism in *LXR*β associates with a 20–30% lower risk of T2D but with a higher risk of obesity, as observed in two independent studies (Dahlman et al., 2009; Solaas et al., 2010). This is in accordance with data on *Lxr*β-null mice that display a lean phenotype with glucose intolerance (Gerin et al., 2005). According to the best of our knowledge, *LXR* polymorphisms have not yet been linked to NAFLD.

Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors (PPARs) are transcription factors that are activated by endogenous ligands, such as fatty acids, and synthetic ligands, such as the hypolipidaemic fibrates and the insulin-sensitizing thiozolidinediones. Like PXR and CAR, they form heterodimers with RXR and transactivate numerous target genes with vital roles in metabolism by binding to PPAR response elements (PPRE; Nielsen et al., 2008; van der Meer et al., 2010). The identified subtypes *PPAR*α, *PPAR*γ, and *PPAR*β/δ have different tissue-specificities and functions (Kallwitz et al., 2008). Whilst *PPAR*γ is highly expressed in adipose tissue and functions in adipocyte differentiation, *PPAR*α functions as a major regulator of lipid and glucose metabolism in the liver. *PPAR*β/δ is ubiquitously expressed with a wide array of functions. *PPAR*α-agonists, the fibrates, are utilized to treat patients with elevated plasma triglycerides (Sirtori and Franceschini, 1988) due to the ability of PPARα to activate fatty acid oxidation and hepatic lipid hydrolysis by regulating acyl CoA synthetase (*Acs*), carnitine palmitoyl transferase I (*Cpt-1*), mitochondrial β-oxidation enzymes, and hepatic lipases in addition to downregulating *apoC-III* and decreasing hepatic triglyceride secretion (Kersten et al., 1999; Pyper et al., 2010; Rakhshandehroo et al., 2010). Accordingly, *Ppar*α-deficient mice develop hepatic steatosis on a high-fat diet (Abdelmegeed et al., 2011).

A case-control study of NAFLD patients highlighted a potentially protective role for the *Val227Ala* variant of *PPAR*α against obesity compared to subjects with the wild-type receptor (Chen et al., 2008a). The adipocyte differentiation regulator, PPARγ, plays an important role in lipid homeostasis and insulin sensitivity by enhancing fatty acid and insulin-dependent glucose uptake in adipose tissue (Kallwitz et al., 2008). Moreover, novel *PPAR*γ agonists acting mainly on adipose *Ppar*γ prevent formation of steatotic livers in mice by improving insulin resistance, upregulating adiponectin, and downregulating leptin expression and secretion (Zheng et al., 2011). *Ppar*γ is expressed at low levels in the liver but is upregulated in rodent fatty livers, contributing to hepatic triglyceride accumulation with a protective effect to dyslipidemia and insulin resistance in other tissues (Gavrilova et al., 2003). Upregulation of hepatic *PPAR*γ is also observed in obese NAFLD patients (Pettinelli and Videla, 2011).

PPARα affects the expression of several phase II enzymes such as SULTs and UGT (Runge-Morris and Kocarek, 2009) as well as of *CYP3A4* and several other CYPs in humans (Rakhshandehroo et al., 2009; Klein et al., 2012). Contrasting observations have been made regarding the regulation of DMEs by PPARα in human versus mouse. While treatment of human primary hepatocytes with the selective PPARα agonist WY14 643 resulted in the activation of several drug metabolizing CYPs including *CYP3A4*, *CYP2B6*, *CYP2C8*, and *CYP1A2*, none of the mouse gene orthologs were regulated (Rakhshandehroo et al., 2009). Downregulation of *CYP3A4* in the presence of *PPAR*α variants that result in decreased hepatic PPARα protein levels was also found by genetic association analysis and confirmed in a human atorvastatin volunteer study (Klein et al., 2012). Moreover, activation of PPARα by agonists downregulates the expression of representatives genes of the *Sult 1*, 2, 3, and *5* families, specifically in female rats (Alnouti and Klaassen, 2008). However, in human hepatocytes activation of PPARα resulted in the upregulation of *SULT2A1* via a functional PPRE, further emphasizing species and gender differences in the functionality of PPARα (Fang et al., 2005). Protein and mRNA levels of UGT1A1 increased upon PPARα activation in rat and human hepatocytes (Jemnitz et al., 2000; Richert et al., 2003). Additionally, *UGT1A3*, *UGT1A4*, and *UGT1A6* are upregulated in human hepatocytes and transgenic mice carrying the human *UGT1* locus (Senekeo-Effenberger et al., 2007). *UGT2B4* is also enhanced after treatment of human hepatocytes with PPARα agonists (Barbier et al., 2003). Functional PPREs have been identified in the 5′-flanking regions of *UGT1A1*, *UGT1A3*, *UGT1A6*, and *UGT2B4* genes, thus providing evidence that these genes are direct targets of PPAR (Barbier et al., 2003; Senekeo-Effenberger et al., 2007). The widespread use of drugs metabolized by SULTs and UGTs such as hormonal contraceptives, acetaminophen, β_2-adrenergic agonists, anti-depressants, and non-steroidal anti-inflammatory drugs highlights the implications of altered PPARα activation on xenobiotic metabolism in NAFLD patients.

Nuclear factor erythroid 2-related factor 2

Nuclear Factor Erythroid 2-related factor 2 (NRF2) is a transcription factor that responds to oxidative/electrophilic stimuli by releasing from its repressor Kelch-like ECH associating protein 1 (Keap1) in the cytosol, translocating to the nucleus, binding to antioxidant response elements (AREs) upstream of numerous phase II DME genes, and genes involved in redox balance and oxidative stress response [e.g., heme oxygenase-1 (*HO-1*), NAD(P)H:quinone oxidoreductase-1 (*NQO1*)] and activating their transcription (Wu et al., 2012). NRF2 also regulates the

glutathione synthesis enzyme, glutamate cysteine ligase catalytic (*GCLC*). The expression of a canalicular biliary efflux transporter, multidrug resistance protein 2 (*MRP2/ABCC2*) and sinusoidal transporters, *MRP3* and *MRP4* is also regulated by NRF2 in mouse liver and HepG2 cells in response to oxidative stress and xenobiotics, thus providing further evidence that phase II enzymes and efflux transporters are regulated simultaneously (Vollrath et al., 2006; Aleksunes et al., 2008). MRP2 is involved in the excretion of reduced and oxidized glutathione and hence plays an important role in detoxification and against oxidative stress. *Nrf2*-null mice on MCD diet exhibit increased hepatic steatosis accompanied by inflammation and oxidative stress (Chowdhry et al., 2010). Similarly, in rats on MCD diet, the NRF2-dependent genes involved in the oxidative stress response were upregulated (Lickteig et al., 2007). Thus, NRF2 appears to have a crucial role in the pathogenesis of NAFLD.

Thus, it is evident that as many of the lipids that accumulate in obesity and steatosis, such as fatty acids, cholesterol, or bile acids, are endogenous ligands of nuclear receptors, their deregulation may not only exacerbate the deregulated metabolic processes in NAFLD patients but also result in deregulated xenobiotic metabolism.

PHASE I DMEs

CYP3A

The CYP3A sub-family of DMEs plays a predominant role in the metabolism of statins. Statins, in monotherapy and in combination with other lipid-lowering drugs or antioxidants, are beneficial in NAFLD patients by improving dyslipidemia (Athyros et al., 2011; Fon Tacer and Rozman, 2011). The inter-individual variability in the response to statins varies in NAFLD patients based on their risk for cardiovascular diseases (Maroni et al., 2011). Moreover, the CYP3A4 drug metabolizing activity is also a factor influencing inter-individual variability and hence, is relevant to NAFLD patients undergoing statin therapy. The level of CYP3A protein correlates negatively with the severity of steatosis in humans (Kolwankar et al., 2007). No changes were found in the CYP3A4 mRNA level in human fatty liver samples at various stages of NAFLD progression, however a trend of decreasing activity and protein levels was observed (Fisher et al., 2009b). In another study, CYP3A4 activity significantly decreased in macrosteatotic fatty livers and cultured human hepatocytes treated with fatty acids (Donato et al., 2006, 2007). *CYP3A4* also displays sexual dimorphism with approximately twofold elevated expression in premenopausal women (Wolbold et al., 2003), who display a more favorable lipid profile compared to men (Williams, 2004). An intron 6 polymorphism in *CYP3A4* (rs35599367[T]) results in decreased expression and activity of CYP3A4, with carriers of the T allele requiring significantly lower doses of statins (Elens et al., 2011; Wang et al., 2011). In accordance with the reduced CYP3A4 expression in NAFLD, studies to determine the association of the rs35599367 *CYP3A4* polymorphism in NAFLD cohorts will enable the elucidation of statin dose selection in these patients. Moreover, genetic variants in other factors implicated in NAFLD, endobiotic, and xenobiotic metabolism such as *PXR*, *PPARα*, and *POR* have also been associated with altered CYP3A4 expression and activity (Zhang et al., 2008; Gomes et al., 2009; Klein et al., 2012). These studies emphasize the high level of variability in responses to statin treatments and may provide a basis for dose selection in NAFLD patients based on CYP3A4 status (**Table 2**).

CYP2C9

CYP2C9 is the most abundant CYP of the CYP2C sub-family in human liver microsomes, accounting for the metabolism of a large number of clinically important drugs, especially some with a narrow therapeutic index, such as warfarin. The expression of CYP2C9 is coordinated by nuclear receptors such as CAR and PXR in association with nuclear factors and coactivators such as hepatocyte nuclear factor-4 alpha (HNF-4α) and PPARγ coactivator-1 alpha (PGC-1α), which is also involved in energy homeostasis (Chen and Goldstein, 2009). CYP2C9 has been closely associated with adverse drug reactions. Its mRNA and enzyme activity increase with NAFLD progression, hypoxia, and at later stages of NASH in humans (Fisher et al., 2009b). Previous observations linking CYP2C9 with arachidonic acid metabolism and vasocontraction in hypoxic conditions (Pokreisz et al., 2006) may possibly provide an explanation for elevated CYP2C9 in progressive NAFLD. Approximately 50 variants have been identified in the *CYP2C9* gene to date, with the *CYP2C9*2* and *CYP2C9*3* loss-of-function alleles as the most important. Heterozygotes and homozygotes for these polymorphisms are common in Caucasians, with frequencies of approximately 10–17 (*CYP2C9*2)* and 7% (*CYP2C9*3*). Both polymorphic alleles were associated with increased response to anti-diabetic sulfonylurea drugs and an increased glycemic response in T2D patients (Zhou et al., 2010). As NAFLD patients are treated with sulfonylureas, genotyping is clinically relevant. Further studies are needed to identify the association of the *CYP2C9*2* and *CYP2C9*3* variants with adverse drug reactions such as hypoglycemia and weight gain resulting from sulfonylurea treatment.

CYP2E1

CYP2E1, a fatty acid (Ω-1)-hydroxylase, catalyzes the oxidation of many low molecular weight molecules, including ethanol and acetone, a product of fatty acid oxidation. An important catalytic feature of CYP2E1 is the generation of ROS such as superoxide anion radical and hydrogen peroxide as a result of uncoupling of oxygen consumption with NADPH oxidation and as a by-product of lipid peroxidation (Robertson et al., 2001; Caro and Cederbaum, 2004). It is also involved in the biotransformation of xenobiotics such as acetaminophen, resulting in the generation of toxic reactive metabolites (Aubert et al., 2011). NAFLD and NASH patients and the MCD diet-fed rat model of NASH display enhanced expression of CYP2E1, which is in contrast to all other drug metabolizing CYPs, and elevated lipid peroxidation (Weltman et al., 1996, 1998; Videla et al., 2004) with increased localization to hepatic areas with oxidative stress injuries. Obese females with steatosis and NASH display elevated CYP2E1 protein levels and a positive correlation between the c2 allele of *Rsa1/Pst1* polymorphisms in *CYP2E1* and liver injury (Varela et al., 2008). Mice with silenced diacylglycerol acyltransferase 2 (*Dgat2*) on MCD diet display elevated *Cyp2e1* expression that correlates with increased lipid peroxidation and oxidative damage, thus highlighting the

role of CYP2E1 in the progression to NASH in response to increased hepatic free fatty acids (Yamaguchi et al., 2007). An upregulation in *CYP2E1* has also been associated with leptinemia, reduced adiponectin levels, and insulin resistance in NAFLD (Aubert et al., 2011). This phenomenon is reversed in patients who have undergone bariatric surgery with resulting decreases in weight and hepatic steatosis (Bell et al., 2010). With the robust cellular protection mechanisms intact, increases in pro-oxidant molecules and CYP2E1 are counteracted by increased levels of glutathione (GSH). However, most NASH rodent models display lower GSH, indicating defects in the oxidative stress response pathways in progressive NAFLD. Nitrosylation of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) is crucial in a *Cyp2e1*-overexpressing mouse model of NAFLD (Kathirvel et al., 2010) because increased levels of iNOS generates reactive nitrogen species (RNS), which nitrosylate antioxidant enzymes and decrease their activity. Thus, *CYP2E1* polymorphisms that associate with the progression of NAFLD to NASH may possibly trigger the combined detrimental effects of both ROS and RNS, which in combination with toxic metabolites from xenobiotic biotransformation may result in further aggravated liver injury in NAFLD patients.

Observations in a NAFLD pediatric population have indicated a direct correlation between lipid peroxidation and disease severity irrespective of CYP2E1 levels; however, the small sample size of this study and the possibility of alternative mechanisms of lipid peroxidation in early onset hepatic steatosis cannot be excluded (Bell et al., 2011). Other groups have also previously indicated decreased CYP2E1 mRNA and protein levels and no changes in CYP2E1 activity at progressive stages of NAFLD (Fisher et al., 2009b; Mitsuyoshi et al., 2009). Thus, no conclusive role of CYP2E1 in NAFLD can be described. Interestingly, *CYP4A* genes seem to compensate for microsomal lipid oxidation in the absence of CYP2E1 as observed in *Cyp2e1*-null mice that display lipid peroxidation and increased expression of *Cyp4a10* and *Cyp4a14* (Leclercq et al., 2000; Hardwick et al., 2009). This observation may possibly explain the absence of changes in CYP2E1 activity in some NAFLD populations.

CYP4A

CYP4A enzymes ω-hydroxylate fatty acids into dicarboxylic acids that are preferentially oxidized by peroxisomes. Genes of the *CYP4A* sub-family are induced by PPARα-agonists and in conditions of fasting. The enhanced activity of CYP4A results in increased production of ROS, thus contributing to steatohepatitis. In contrast, PPARα agonists prevent NASH by increasing β-oxidation. Moreover, *Ppar*α-null mice on MCD diet are more prone to developing NASH in the absence of *Cyp4a* induction (Ip et al., 2003). These observations suggest that the anti-steatotic effects of PPARα may be more potent than its activation of *CYP4A* genes, hence overriding the ROS-generating effects of CYP4A. In the absence of PPARα alternative oxidative stress mechanisms may act as causal factors.

PHASE II DMEs

Phase II DMEs are conjugative, detoxification enzymes that transform substrates into more excretable inactive forms or on the other hand may also be involved in bioactivation. Glutathione-*S*-transferases (GSTs) are present as different isoforms Alpha, Mu, and Pi and conjugate electrophilic compounds with reduced GSH (Hayes et al., 2005). While a GST A and P are upregulated with disease progression in the livers of NAFLD patients, GST M is significantly downregulated, thus highlighting the differential regulation of GST isoforms in NAFLD progression; however, the overall GST activity was decreased in these samples (Hardwick et al., 2010). GSTM2, GSTM4, and GSTM5 mRNA levels are expressed at lower levels in patients with steatosis and NASH (Younossi et al., 2005). GSTs play a significant role in controlling oxidative stress by conjugating harmful by-products of oxidative stress with GSH (Hayes et al., 2005). Decreased GST activity in progressive NAFLD samples was accompanied by a reduced pool of GSH, highlighting the depleted ability to combat oxidative stress, a causal factor for NASH (Hardwick et al., 2010). The antioxidant, *S*-adenosyl-L-methionine (SAM) provides the cysteine moiety for the generation of GSH. Several rodent studies have indicated a decrease in SAM on a high-fat diet (Kwon do et al., 2009; Buechler and Weiss, 2011). Furthermore, GSTs have a lower expression in Caucasians compared to African–Americans, who have a lower prevalence of NAFLD (Stepanova et al., 2010). The *GSTM1*-null genotype, shown to confer a higher risk of T2D, is also present at a higher frequency in NAFLD subjects compared to control (Hori et al., 2007, 2009). Thus, decreased activity of GSTs play a plausible role in NAFLD progression as a result of increased damage by oxidative stress (**Table 3**).

Sulfotransferases are involved in sulfation of several endogenous steroids and xenobiotics. The sulfation of oxysterols by SULT2B1b has anti-lipogenic properties by suppressing the LXR-SREBP-1c interaction, resulting in significantly lower hepatic and serum lipids as observed in low-density lipoprotein receptor (*Ldlr*)-null mice on a high-fat diet (Bai et al., 2012). Moreover, *SULT1A2* gene expression is downregulated in NASH patients compared to control obese individuals (Younossi et al., 2005). SULT2A1 is upregulated by PPARα agonists in primary human hepatocytes, but not in rat hepatocytes, due to the presence of a PPRE in the 5′ region of the gene (Fang et al., 2005; Runge-Morris and Kocarek, 2009). Thus, downregulation of PPARα observed in NAFLD may have implications in the altered expression of SULT2A1. However, only two SULT isoforms, SULT1C4 and SULT4A1, whose regulation and function are largely unknown, have increased mRNA and protein levels in human NASH samples compared to control and steatosis samples (Hardwick et al., 2012a). A previous association of SULT4A1 in deregulated metabolic homeostasis makes it a good candidate for further studies in the context of NAFLD (Kiba et al., 2009).

UDP glucuronosyltransferases are involved in the glucuronidation of 40–70% of all clinical drugs in humans (Wells et al., 2004). *UGT1A1*, *1A3*, *1A4*, *1A6*, and *2B4* are induced by PPARα-agonists in primary human hepatocytes and PPREs have been identified in these genes (Runge-Morris and Kocarek, 2009). Mice on high-fat and high-sucrose diet, which develop severe hepatic steatosis, display elevated expression of *Ugt1a1* and *Ugt1a6* mediated by CAR and PXR (Osabe et al., 2008). A study in a pediatric NAFLD population identified *UGT1A1* as a risk factor for NAFLD. The

*UGT1A1*6* allele in the coding region has a protective effect against NAFLD in obese Taiwanese children (Lin et al., 2009). UGT1A1 is involved in the glucuronidation of heme after breakdown to bilirubin. The ability of bilirubin to oxidize ROS may provide protection against the progression of NAFLD. Additionally, the high prevalence of unconjugated hyperbilirubinemia was detected in NAFLD patients (25.4%) that were diagnosed with less severe forms of NAFLD (Kumar et al., 2012). However, the absence of changes in glucuronidation activity in human steatosis and NASH liver samples warrants the need for further studies to investigate the role of UGTs in NAFLD (Hardwick et al., 2012a).

TRANSPORTERS

Solute carrier transporters are uptake transporters that transport molecules from the blood into the hepatocyte. Studies in rat and human samples have indicated a coordinated downregulation of uptake transporter genes in NASH, such as the sodium/bile acid transporter (*NTCP*), organic anion transporting polypeptide *1a1* (*OATP1a1*), *1a4*, *1b2*, *2b1*, *OAT2*, and *OAT3*. The expression of these transporters is significantly altered in the transition from steatosis to NASH rather than between control and steatotic samples (Fisher et al., 2009a; Lake et al., 2011). These changes appear to be hepatoprotective to prevent the accumulation of toxic intermediates and xenobiotics in the diseased liver. However, they have major implications in therapeutic regimens in NAFLD patients in terms of dose selection and side effects of drugs due to excessive accumulation (**Table 3**).

Transporters on the hepatocyte canalicular membranes are involved in the secretion of several endobiotics and xenobiotics *via* the bile. ABC-transporters are the most extensively studied and are altered in steatotic and NASH livers (Buechler and Weiss, 2011). Of particular interest is Mrp2/Abcc2, which is decreased in several rodent models of obesity, NAFLD and NASH and is normalized upon rosiglitazone treatment (Geier et al., 2005; Fisher et al., 2009a; Martin et al., 2010). Furthermore, the *rs17222723* and *rs8187710* variants in *ABCC2* significantly associate with NAFLD and clinical and histological parameters (Sookoian et al., 2009). Decreased levels of ABCC2 protein may result in hampered secretion of bile, leading to the accumulation of cholesterol and drug-related toxicities. As mentioned previously, this may result from impaired NRF2 function.

CONCLUSION AND FUTURE DIRECTIONS

The high prevalence of NAFLD is concerning in terms of general population health and also drug treatment regimens. A recent study in mice has identified that the feed-forward cycle of continuous exposure to high-fat diet over two generations leads to a significantly higher degree of obesity, NAFLD, insulin and leptin resistance, and epigenetic modifications resulting in increased lipogenesis and ER stress in future generations (Li et al., 2011). If these observations are also true for humans, the rising epidemics of obesity and NAFLD will expand exponentially in the absence of serious efforts to tackle these conditions.

With the widespread prevalence of NAFLD, the proportion of patients with steatotic livers undergoing drug therapies for various disorders has also increased. The variability of drug treatment responses in these patients highlights the need for personalized therapeutic regimens. As detailed in this review, several components of the drug metabolism pathway are significantly affected in the presence of NAFLD. Similarly, genetic variations in DMEs and nuclear receptors associate with NAFLD with either positive or negative prognosis. Hence, inter-dependent interactions and common confounding factors exist between the pathogenesis of NAFLD and altered drug metabolism. As a majority of the DMEs are also involved in the metabolism of steroids and other lipids, polymorphisms in DMEs resulting in non-functional proteins may further aggravate the prognosis of NAFLD. The utility of identified genetic associations to determine NAFLD disease susceptibility, improve drug sensitivity or prevent adverse drug reactions holds great potential. Further efforts to characterize DMEs and identify risk factors for adverse drug reactions or treatment efficacies in NAFLD populations may lead to the utilization of innovative interdisciplinary strategies to provide a better insight into the pharmacokinetic profile of drugs and their efficacy. Although the implementation of these findings in the clinic is still a long-term goal with hurdles to pass, novel technologies and increasing interest in this field continues to increase our understanding of NAFLD and its interactions with drug metabolism.

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Toward a clinical practice guide in pharmacogenomics testing for functional polymorphisms of drug-metabolizing enzymes. Gene/drug pairs and barriers perceived in Spain

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The development of clinical practice recommendations or guidelines for the clinical use of biomarkers is an issue of great importance with regard to adverse drug reactions. The potential of pharmacogenomic biomarkers has been extensively investigated in recent years. However, several barriers to implementing the use of pharmacogenomics testing exist. We conducted a survey among members of the Spanish Societies of Pharmacology and Clinical Pharmacology to obtain information about the perception of such barriers and to compare the perceptions of participants about the relative importance of major gene/drug pairs. Of 11 potential barriers, the highest importance was attributed to lack of institutional support for pharmacogenomics testing, and to the issues related to the lack of guidelines. Of the proposed gene/drug pairs the highest importance was assigned to HLA-B/abacavir, UGT1A1/irinotecan, and CYP2D6/tamoxifen. In this perspective article, we compare the relative importance of 29 gene/drug pairs in the Spanish study with that of the same pairs in the American Society for Clinical Pharmacology and Therapeutics study, and we provide suggestions and areas of focus to develop a guide for clinical practice in pharmacogenomics testing.

Keywords: biomarkers, adverse drug reactions, pharmacogenomics, clinical recommendations, clinical relevance

Functional polymorphisms of drug-metabolizing enzymes are a major factor involved in adverse drug reactions. The development of pharmacogenomic biomarkers has evolved in recent years, mainly in a frame where these biomarkers are intended to be used as outcome biomarkers, that is, to substitute for a clinical outcome or predict an outcome of a disease or toxicity following treatment. While the most conservative pharmacogenomics views aim to stratify patient populations (patient selection biomarkers) into those who should or should not receive a given drug (Green and Guyer, 2011), other guidelines are intended to adjust drug dose based on pharmacogenomics tests (see for instance Swen et al., 2011). The limitations of pharmacogenomics-based dose adjustment are analyzed elsewhere (Agúndez et al., 2012) and will not be discussed here, but it should emphasized that part of the disenchantment experienced with pharmacogenomics in recent years is related to the overoptimistic expectation of making a safe and reliable personalized dose adjustment based on pharmacogenomics tests. Today we know that interindividual variability in drug metabolism and response exists, even within individuals with identical pharmacogenomics genotypes, and so

pharmacogenomics is simply another factor to be considered in dose adjustment.

But in even the most conservative approaches to pharmacogenomics (whether individuals would respond to a determined drug, or what patient's odds are to experience adverse effects with a determined drug), many factors hamper rapid development of the clinical use of pharmacogenomics tests. These factors have been analyzed in detail elsewhere (Deverka et al., 2007; Haga and Burke, 2008; Agúndez, 2009; Agúndez et al., 2009, 2012; Relling et al., 2010; Matheis et al., 2011; Relling and Klein, 2011), but the relative importance of these factors has received little attention. In the frame of the Carlos III Institute of Health (ISCIII) Spanish Research Network of Adverse Reactions to Allergens and Drugs, we conducted a survey in 2012 among members of the Spanish Societies of Pharmacology and Clinical Pharmacology, together with other Spanish clinicians and geneticists closely related to pharmacogenomics, on the most important challenges to clinical implementation of pharmacogenomic tests. The profile of the participants was as follows: 59% were practicing physicians, 21% had an academic research profile, 16% were clinical laboratory professionals, and 5% worked in the pharmaceutical industry. The survey in Spain was designed to include the same gene/drug pairs and the same evaluation criteria as in a US survey (Relling and Klein, 2011) to obtain results which could be directly compared. In addition, we included a survey regarding potential barriers to implementing the use of pharmacogenomics testing (Agúndez et al., 2012). The responses were closed, ranking from 1 to 10 for every item. The ranks obtained for gene/drug pairs were re-scaled in a scale from 1 to 5 to make results comparable to those from the US survey (Relling and Klein, 2011).

Figure 1 summarizes the results, which indicated three major groups of barriers. The most relevant group was related to low institutional promotion, and the second corresponded to lack of clinical guidelines, protocols, and other factors related to validity of pharmacogenetics tests. The barriers in this second group are closely related to one another and, in our opinion, are a major cause of lack of promotion and support for healthcare systems. A third group of barriers with less perceived importance in the survey was related to economical and institutional issues, knowledge of the pharmacogenomic background of the Spanish population and ethical, legal, or social implications.

Because most of the main barriers were related to lack of clinical guidelines and protocols, we included in our survey the same 29 gene/drug pairings listed in a previous study carried out among members of the Clinical Pharmacogenetics Implementation Consortium (CPIC; see http://www.pharmgkb.org/page/cpic) and members of the American Society for Clinical Pharmacology and Therapeutics (Relling and Klein, 2011). The highest ranked pairs (based on the perceived importance of the data linking the drug to the gene variation) were HLA-B/abacavir, UGT1A1/irinotecan, and CYP2D6/tamoxifen (**Figure 2**). While most gene/drugs pairs received a similar rank in both studies, the comparison revealed that in the Spanish study the gene pairs CYP2C9/celecoxib and CYP2D6/nortriptyline had higher ranks than in the US study, whereas the pairs CYP2C19/clopidogrel, GP6D/chloroquine, and both genes related to warfarin were ranked lower. Nevertheless, looking at the whole picture, the ranks are similar for most gene/drug pairs, thus indicating that guidelines for gene/drug pairs such as those which are currently being elaborated by CPIC members seem to be well suited for Spain, and probably for other European countries with a similar genetic background. The few discrepancies observed in **Figure 2** may be related to the differences in the pattern of drug use in the US and in Spain. CPIC guidelines on some of the gene/drug pairs mentioned have already been published (see for instance (Johnson et al., 2011; Crews et al., 2012; Wilke et al., 2012) and many others are currently underway (see http://www.pharmgkb.org/page/cpicGeneDrugPairs). Hopefully

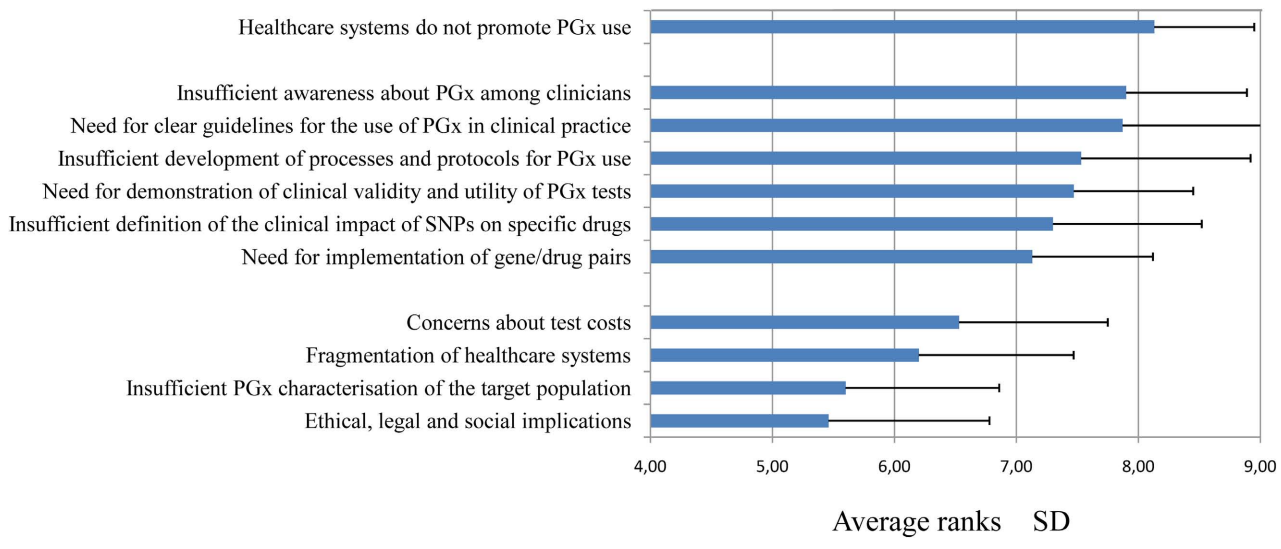

FIGURE 1 | Highest-ranking barriers to implementing the use of pharmacogenomics testing, based on a survey of Spanish Societies for Pharmacology and Clinical Pharmacology members in 2012. Data related to average importance (on a scale of 1–10) are plotted along the *x*-axis.

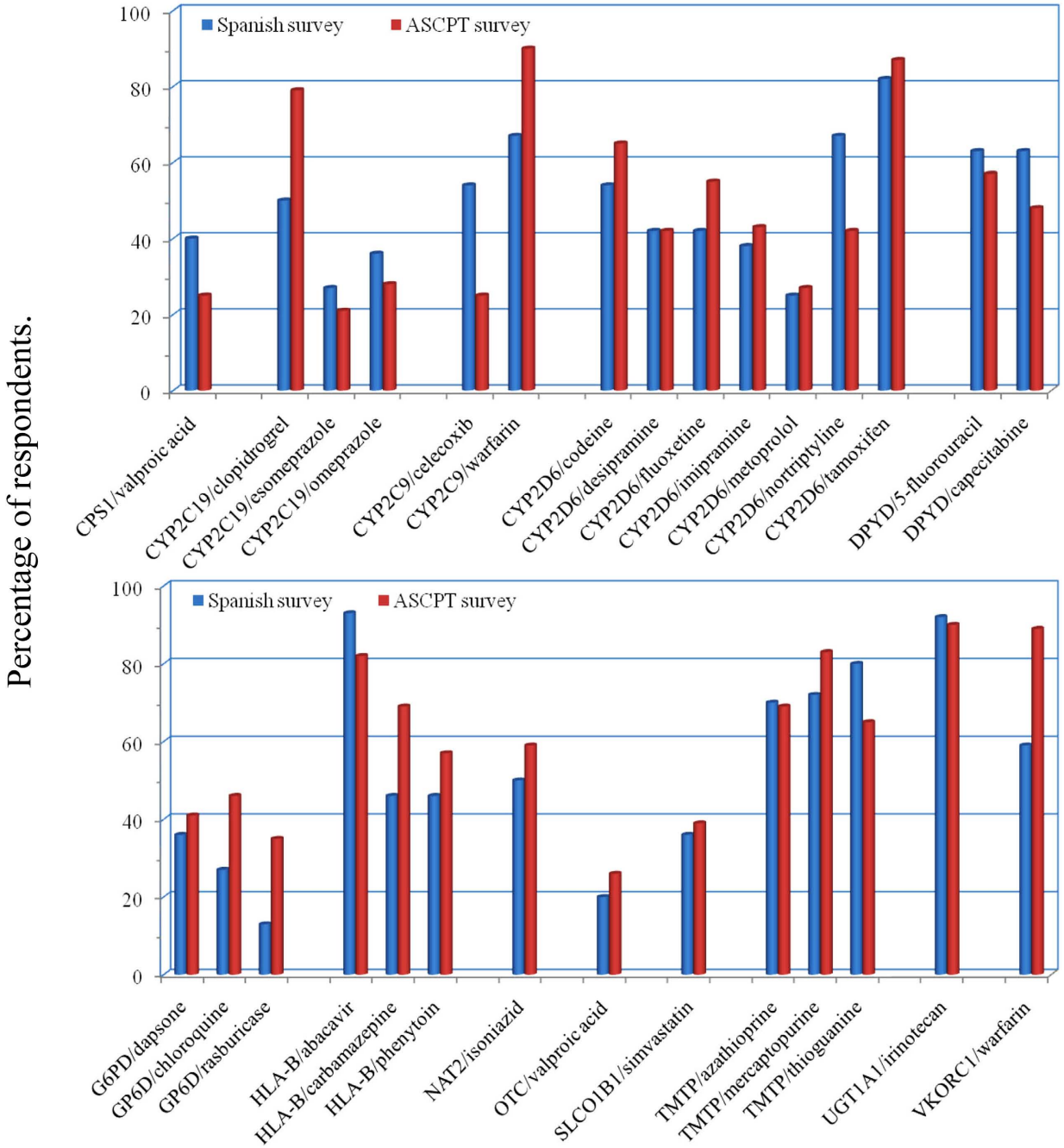

FIGURE 2 | Highest-ranking gene/drug pairs, based on a survey of Spanish Societies for Pharmacology and Clinical Pharmacology members in 2012 (blue bars), compared to a published survey of American Society for Clinical Pharmacology and Therapeutics (ASCPT) members conducted by CPIC (red bars). Data related to the percentages of respondents who ranked the gene/drug pairs as 1 or 2 (on a scale of 1–5) are plotted along the *y*-axis.

we will soon have guidelines available for all relevant gene/drug pairs.

Nevertheless, some remaining questions related to interethnic and intraethnic genetic variability (Garcia-Martin et al., 2006; Garcia-Martin, 2008; Borobia et al., 2009; Restrepo et al., 2011), refinement in phenotype inference (Agúndez et al., 2008; Ruiz et al., 2012), and detailed studies on the contribution of additional factors which may modify the strength of the association of gene/drugs pairs need to be dealt with. These studies should include gene expression and regulation, patient's metabolic profile with a given drug, and relevant lifestyle and environmental factors that influence drug metabolism or drug response. Once guidelines and protocols are ready, the major groups of barriers shown in **Figure 1** will be weakened. And, in consequence, this

will hopefully lessen the impact of the first and most determinant barrier, since we believe that institutional support and promotion for the use of pharmacogenomics biomarkers will greatly improve as the influence of the other barriers decline.

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Characterization of the genetic variation present in *CYP3A4* in three South African populations

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The CYP3A4 enzyme is the most abundant human cytochrome P450 (CYP) and is regarded as the most important enzyme involved in drug metabolism. Inter-individual and inter-population variability in gene expression and enzyme activity are thought to be influenced, in part, by genetic variation. Although Southern African individuals have been shown to exhibit the highest levels of genetic diversity, they have been under-represented in pharmacogenetic research to date. Therefore, the aim of this study was to identify genetic variation within *CYP3A4* in three South African population groups comprising of 29 Khoisan, 65 Xhosa and 65 Mixed Ancestry (MA) individuals. To identify known and novel *CYP3A4* variants, 15 individuals were randomly selected from each of the population groups for bi-directional Sanger sequencing of ~600 bp of the 5′-upstream region and all thirteen exons including flanking intronic regions. Genetic variants detected were genotyped in the rest of the cohort. In total, 24 SNPs were detected, including *CYP3A4*12*, *CYP3A4*15*, and the reportedly functional *CYP3A4*1B* promoter polymorphism, as well as two novel non-synonymous variants. These putatively functional variants, p.R162W and p.Q200H, were present in two of the three populations and all three populations, respectively, and *in silico* analysis predicted that the former would damage the protein product. Furthermore, the three populations were shown to exhibit distinct genetic profiles. These results confirm that South African populations show unique patterns of variation in the genes encoding xenobiotic metabolizing enzymes. This research suggests that population-specific genetic profiles for *CYP3A4* and other drug metabolizing genes would be essential to make full use of pharmacogenetics in Southern Africa. Further investigation is needed to determine if the identified genetic variants influence CYP3A4 metabolism phenotype in these populations.

Keywords: *CYP3A4*, pharmacogenetics, South African populations, Xhosa, mixed ancestry, Khoisan

INTRODUCTION

The human CYP3A enzymes are regarded as the most prominent Cytochrome P450 (CYP) subfamily in facilitating the elimination of drugs, other xenobiotic compounds and endogenous molecules from the body (Lamba et al., 2002). The pharmacogenetically relevant CYP3A4 is responsible for metabolizing 50–60% of all clinically prescribed drugs (Guengerich, 1999) and is listed among The Pharmacogenetics and Pharmacogenomics Knowledge Base's (PharmGKB's) "very important pharmacogenes" (http://www.pharmgkb.org/gene/PA130?tabType=tabVip). CYP3A4 can be inhibited by drugs (e.g., ketoconazole and ritonavir) and is often involved in unfavorable drug-drug interactions, due to its ability to accommodate two or more similar or dissimilar molecules in its active site (Sevrioukova and Poulos, 2013). The enzyme is predominantly expressed in the liver and small intestine (Shimada and Guengerich, 1989). Expression has as much as 40-fold variation between individual human livers and a 10-fold variation in the metabolism of CYP3A4 substrates *in vivo* (Shimada and Guengerich, 1989; Lown et al., 1995; Guengerich, 1999). While complex regulatory pathways and environmental factors are important, it is suspected that a portion of this inter-individual variation can be attributed to genetic variants located within the coding gene regions as well as its core regulatory regions, which affect either the expression level or the functional protein of the gene (Steimer and Potter, 2002; Lamba et al., 2002).

Few pharmacogenetically-relevant polymorphisms have been identified in the *CYP3A4* gene; however, some polymorphisms have been associated with, amongst others, immunosuppressant dose requirements (Elens et al., 2011), clopidogrel response variability (Angiolillo et al., 2006), and withdrawal symptoms and adverse reactions in patients receiving methadone treatment

(Chen et al., 2011). Furthermore, a rare haplotype, *CYP3A4*20*, results in a complete loss of function (Westlind-Johnsson et al., 2006), while *CYP3A4*1B* is suspected to alter the expression levels of CYP3A4 (Westlind et al., 1999), although conflicting results have been reported (Wang et al., 2011). Although genetic variants in the *CYP3A4* gene have been extensively studied in populations such as Caucasians, Asians, and African-Americans, little research has been conducted in present-day African populations, including those indigenous to South Africa (Warnich et al., 2011). Not only are these research disparities observed in candidate gene studies, but they also extend to recent large scale re-sequencing projects such as the 1000 Genomes Project, which although comprehensively examining the genomic variation present in many individuals, provides no information for South African populations (1000 Genomes Consortium, 2010).

We have therefore tried to aid in addressing the disparity of pharmacogenetic data that exists for South African populations by analyzing three of the diverse population groups, which are representative of: (1) the most ancient population group: the Khoisan, (2) the most globally-admixed population group: the South African Mixed Ancestry (MA) population, and (3) the largest language family in South Africa: the Bantu-speaking population group, represented by the Xhosa population. The ancient Khoisan population used in this study consisted of individuals from the !Kung and Khwe linguistic groups (Chen et al., 2000). These individuals are descendant from people of the latter Stone Age and are believed to be some of the first lineages of *Homo sapiens* (Kaessmann and Pääbo, 2002). The MA population, with Xhosa, Khoisan, European, and Asian ancestral contributions, has been shown to exhibit the highest levels of admixture across the globe (Tishkoff et al., 2009) and is therefore of interest for pharmacogenetic applications as genetic variants present in many different populations may affect these individuals as has been reported for other admixed populations such as those from Brazil (Suarez-Kurtz, 2005, 2010; Suarez-Kurtz et al., 2012). Lastly, 9 of the 11 official South African languages are classified as Bantu languages (Warnich et al., 2011), spoken by ∼75% of the total South African population, and therefore it is imperative that representatives of this group are included in pharmacogenetic research. In this study, we utilized the Xhosa population, which are representative of the Nguni-speaking tribes (Warnich et al., 2011) and are the biggest Bantu-speaking population in the Western Cape of South Africa, where this research was conducted.

In our experience, it is important that pharmacogenes, such as the *CYP* genes are comprehensively characterized in South African populations, as we have discovered both novel alleles and unique variation profiles for the *CYP2C19* and *CYP2D6* genes (Drögemöller et al., 2010; Wright et al., 2010). It is hoped that the comprehensive characterization of *CYP3A4* in these populations will aid future *CYP3A4* genotype-phenotype studies in African populations to determine whether functionally relevant *CYP3A4* polymorphisms exist that have an impact on drug metabolism phenotype. We therefore screened the 5′-flanking region and thirteen exonic regions of the *CYP3A4* gene in the three South African populations described above in order to determine which common allelic variants, novel or previously characterized, are present.

MATERIALS AND METHODS

CLINICAL SAMPLES

Ethical approval was obtained for this study from the Human Research Ethics Committee of Stellenbosch University (S12/07/190) and informed consent was acquired from all participants. Genomic DNA (gDNA) was available for 29 Khoisan, 65 Xhosa, and 65 MA healthy individuals. The Khoisan samples used in this study were collected from !Kung and Khwe speaking individuals from the Schmidtsdrift region of the Kalahari desert in the Northern Cape Province of South Africa (Chen et al., 2000), while samples from the Xhosa and MA populations were collected from the Western Cape Province of South Africa.

POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION

Primers were designed to amplify ∼600 bp of the 5′-upstream region and all 13 exons with flanking intronic regions of *CYP3A4* (GenBank: AF280107.1; Ensembl ID: ENSG00000160868) (refer to **Table 1** for primer sequences). PCR amplifications were carried out in a total reaction volume of 25 μl, with each reaction containing 20–30 ng of gDNA, 10 pmol of each primer, 320 μM dNTPs, 4 mM $MgCl_2$, 0.5 U BIOTAQ™ DNA polymerase and 1X Reaction Buffer. All reagents were supplied by Bioline, London, UK. The reaction cycle conditions consisted of an initial denaturation step at 94°C for 3 min, followed by 30 cycles of 15 s denaturation at 94°C, 15 s annealing at varying temperatures (refer to **Table 1** for specific annealing temperatures), and 30 s extension at 72°C, with a final extension at 72°C for 5 min.

DNA SEQUENCING

To identify common *CYP3A4* genetic variation occurring in each of the three populations, 15 individuals were randomly selected from every population groups for bi-directional sequence analysis, allowing for detection of alleles with a frequency of more than 10%, with 95% confidence. The PCR products from each of the 13 amplicons were purified with SureClean (Bioline) and bi-directionally sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA), after which capillary electrophoresis was performed by the Central Analytical Facility of Stellenbosch University on a 3130*xl* Genetic Analyser (Applied Biosystems). The obtained sequences were subsequently compared to the reference sequence (GenBank: AF280107.1; Ensembl ID: ENSG00000160868) to detect the presence of variants. The generated sequence data also served to ensure that the reaction conditions used did not amplify any of the associated *CYP3A4* isoforms or pseudogenes. The effect of the detected variants was determined using the software programs Sorting Intolerant From Tolerant (SIFT), Polymorphism Phenotyping (PolyPhen) and the Alternative Splice Site Predictor (ASSP) (http://www.es.embnet.org/~mwang/assp.html; Ramensky et al., 2002; Ng and Henikoff, 2003).

GENOTYPING OF THE DETECTED VARIANTS

To determine the frequencies of the genetic variants detected in the 5′-flanking region and the coding regions of the *CYP3A4* gene through sequence analysis, an additional 14 individuals from

Table 1 | Sequencing Primers and PCR conditions.

Region	Primer name	Sequence (5′-3′)	T_m (°C)	Product size (bp)
5′-upstream	5′_F 5′_R	CAG AAG GGA TGA CAT GCA GA GGC TAT GTG CAT GGA GCT TT	60	767
Exon 1	E1_F E1_R	GAT TCT TTG CCA ACT TCC AAG GAT TAG CAC CCC AAG TCC AA	60	363
Exon 2	E2_F E2_R	GCA GGA AAG GAC CTG ATG AA AAG CTG CTC TTG GCA ATC AT	60	323
Exon 3	E3_F E3_R	TGA CGT CTC CAA ATA AGC TTC C AGG TTG ACA AGA GCT TCA TCC	60	301
Exon 4	E4_F E4_R	AGG ATC AAA GTC TGG CTT CC GGA TGA AGT GGA CGT GGA AC	60	305
Exon 5	E5_F E5_R	TCT AGC ATA GGG CCC ATC AC CA GTG GAC TAC CCC TTG GAA	60	352
Exon 6	E6_F E6_R	CCA AGG GGT AGT CCA CTG AA GGA ATA ACC CAA CAG CAG GA	55	362
Exon 7	E7_F E7_R	TGG AGT GTG ATA GAA GGT GA TTG TGA CAG GGG GCT GAT AG	55	516
Exon 8	E8_F E8_R	TGC TCC AGG TAA ATT TTG CAC CAA ACC CCA CTT TCT GCA TT	60	369
Exon 9	E9_F E9_R	CAT CCT GCT TTC CAA GGA CCT GCA TGC CTC TAG AAA GTG	60	418
Exon 10	E10_F E10_R	TGA TGC CCT ACA TTG ATC TGA CTG CCA GTA GCA ACC ATT TG	60	391
Exon 11	E11_F E11_R	CCC GAA TGC TTC CCA CCT GGC AGA ATA TGC TTG AAC CAG	60	506
Exon 12	E12_F E12_R	GAC TGA AAG CTC CTA TAG TGT C CCA TGC TAA TCT ACA TGG GCT	60	598
Exon 13	E13_F E13_R	GCC ATC ATA CCT AAT AAT CTG G AT GTG CAG GAA AGC ATC TGA	60 Xhosa 55 Others	988

5′, 5′-upstream; E, exon; F, forward; R, reverse.

the Khoisan population and 50 individuals each from the Xhosa and MA populations were genotyped using a combination of restriction fragment length polymorphism (RFLP) analysis and additional sequence analyses. In cases where no restriction sites were created or destroyed by the SNPs of interest, mutagenic primers were designed to introduce artificial restriction enzyme recognition sequences (refer to **Table 2** for the primer sequences used for RFLP genotyping). Amplification using the mutagenic primers was performed by means of a nested PCR, using 1 in 200 dilutions of PCR product as template, to avoid co-amplification of isoforms and pseudogenes. The nested PCR conditions were identical to those used during original PCR amplification, except that the cycle number and $MgCl_2$ concentration were changed to 25 cycles and 2 mM, respectively (refer to **Table 2** for annealing temperatures). To ensure that the RFLP assays were successful, samples with known genotypes were selected as controls for each of the individual restriction enzyme analyses. Due to the large number of variants detected in the exon 7 amplicon, all the individuals from all three of the population groups were sequenced for this region, rather than utilizing individual RFLP genotyping assays. Additionally, due to the fact that the RFLP genotyping assay for rs57409622 in exon 6 would detect both the presence of this SNP and the adjacent rs4986907 (allele defining SNP of *CYP3A4*15*), any individuals testing positive for this assay underwent bi-directional sequencing to determine which one, or both, of the SNPs were in fact present. For genotyping specifications, including a list of the specific restriction enzymes used, refer to **Table 2**.

Table 2 | PCR_RFLP primers, PCR conditions, and genotyping assays.

Position in gene	Allele	rs number	Genotyping assay	Primer name	Sequence (5′-3′)	T_m (°C)	Product size (bp)
−392 A>G	*CYP3A4* 1B*	rs2740574	*Mbo*II	5′_F_m1 5′_R	GGA CAG CCA TAG AGA CAA GGG **g**A GGC TAT GTG CAT GGA GCT TT	55	350
−292 T>G		Novel	*Rsa*I	5′_F 5′_R_m	CAG AAG GGA TGA CAT GCA GA CCT CCT TTG AGT TCA TAT TCT ATG AGG TAT C**g**T	55	575
−215 T>A		rs144721069	*Hpy188*III	5′_F_m2 5′_R	TGT GTG TGT GAT TCT TTG CCA ACT TC**t c**AG G GGC TAT GTG CAT GGA GCT TT	55	181
3847 A>G		Novel	*Nla*III	E2_F_m E2_R	GCA GGA AAG GAC CTG ATG AAC A**c**A T AAG CTG CTC TTG GCA ATC AT	55	323
5916 T>C		rs12721625	*Bse*NI	E3_F E3_R	TGA CGT CTC CAA ATA AGC TTC C AGG TTG ACA AGA GCT TCA TCC	60	301
13969 G>A		Novel	*Apo*I	E5_F E5_R	TCT AGC ATA GGG CCC ATC AC CA GTG GAC TAC CCC TTG GAA	60	352
14268 C>T	*CYP3A4* 23*	rs57409622	*Aci*I/Sequencing	E6_F_m/E6_F E6_R	GAT GTG TTG GTG AGA AAT CTG AG**a** C GGA ATA ACC CAA CAG CAG GA	55	362/154
14269 G>A	*CYP3A4* 15*	rs4986907	*Aci*I/Sequencing	E6_F_m/E6_F E6_R	GAT GTG TTG GTG AGA AAT CTG AG**a** C GGA ATA ACC CAA CAG CAG GA	55	362/154
15619 A>G		rs111768354	Sequencing				
15628 C>T		rs4987159	Sequencing				
15649 A>T	*CYP3A4* 24*	rs113667357	Sequencing				
15753 T>G		rs2687116	Sequencing				
15783 T>C		rs4987160	Sequencing				
15804 T>G		rs28988584	Sequencing				
15837 T>A		rs12721622	Sequencing				
17829 T>C		Novel	*Psi*I	E9_F E9_R	CAT CCT GCT TTC CAA GGA CCT GCA TGC CTC TAG AAA GTG	60	418
20230 G>A	*CYP3A4* 1G*	rs2242480	*Rsa*I	E10_F E10_R	TGA TGC CCT ACA TTG ATC TGA CTG CCA GTA GCA ACC ATT TG	60	391

(Continued)

Table 2 | Continued

Position in gene	Allele	rs number	Genotyping assay	Primer name	Sequence (5′-3′)	T_m (°C)	Product size (bp)
20309 G>C		rs4986911	*Hind*III	E10_F E10_R_m	TGA TGC CCT ACA TTG ATC TGA CAG TGA AAG AAT CAG TGA TTA **a**G	55	327
20327 T>C		rs34738177	*Mbo*II	E10_F E10_R	TGA TGC CCT ACA TTG ATC TGA CTG CCA GTA GCA ACC ATT TG	60	391
21896 C>T	*CYP3A4* 12*	rs12721629	*Pst*I	E11_F E11_R_m	CCC GAA TGC TTC CCA CCT CAT CTT TTT TGC AGA CCC TCT **gc**A	55	334
23081 C>T		rs12721620	*HpyCH4*IV	E12_F E12_R	GAC TGA AAG CTC CTA TAG TGT C CCA TGC TAA TCT ACA TGG GCT	60	598
25721 A>G		rs3735451	*Bsa*JI	E13_F E13_R	GCC ATC ATA CCT AAT AAT CTG G AT GTG CAG GAA AGC ATC TGA	60 Xhosa 55 Others	988

5′, 5′-upstream; E, exon; F, forward; R, reverse; m, mutagenic primer; lowercase and bold letters, mutagenic bases for the introduction of restriction enzyme recognition sites.

STATISTICAL ANALYSIS

Allele frequencies of the *CYP3A4* genetic variants detected in the three population groups were compared using MedCalc Version 12.3.0 (http://www.medcalc.org/calc/odds_ratio.php). Furthermore, we compared the frequencies of the allele defining SNPs detected in the three South African populations to the frequencies reported by the 1000 Genomes Browser (http://browser.1000genomes.org/) and HapMap Phase I + II Project data (http://hapmap.ncbi.nlm.nih.gov/). The 1000 Genomes Browser contains allele frequency information for the African (AFR), American Admixed (AMR), East Asian (ASN) and European (EUR) populations; while the HapMap project contains frequency data for European (CEU), Chinese (CHB), Japanese (JPT) and Nigerian (YRI) populations. Deviations from Hardy-Weinberg equilibrium (HWE) were determined using either a Pearson chi-squared analysis or an analogue to Fisher's exact test, depending on observed genotype distribution, in Tools for Population Genetic Analysis (TFPGA) Software v1.3 (http://www.marksgeneticsoftware.net/tfpga.htm). *P*-values of <0.05 were considered statistically significant.

RESULTS

VARIANT DETECTION

This study detected 24 SNPs in 45 individuals from three South African populations using *CYP3A4* DNA sequencing. Three of the intronic SNPs and one SNP in the 5′-flanking region are novel. Genotyping of rs12721624 in intron 8 and rs147972695 in intron 12 could not be performed in the entire cohort, due to technical difficulties. Genotyping of the remaining 22 SNPs was successful, and all SNPs were in HWE (refer to **Table 3** for the positions and frequencies of the detected SNPs). The previously described alleles *CYP3A4*1B* and **1G* were present in all three populations, while *CYP3A4*12* and *CYP3A4*15* were only present in the Xhosa population. Furthermore, two novel alleles, designated *CYP3A4*23* and *CYP3A4*24*, which are characterized by the two non-synonymous SNPs, resulting in p.R162W and p.Q200H, were detected. *CYP3A4*24* was present in all three population groups, while *CYP3A4*23* was present in the Xhosa and Khoisan populations. Of particular interest, the amino acid change caused by R162W (*CYP3A4*23*) was predicted by both the SIFT and PolyPhen algorithms to affect the function of the protein product. None of the variants were predicted to alter any splice-sites.

POPULATION VARIANT FREQUENCY COMPARISONS

When examining the successfully genotyped variants in the three South African populations, we noticed that the allele frequencies for several SNPs differed significantly between the population groups ($P < 0.05$) (refer to **Table 4**). The smallest difference was seen when the allele frequencies of the Khoisan and Xhosa populations were compared, with the allele frequencies of three SNPs differing significantly between these two population groups. With regards to the comparisons of (1) the Khoisan and MA populations, and (2) the Xhosa and MA populations, five and six SNPs showed significant allele frequency differences, respectively.

The novel *CYP3A4* alleles, although detected in the South African populations (refer to **Table 3**), were present at frequencies of less than 1% in the populations recorded on the 1000 Genomes

Table 3 | *CYP3A4* variants detected in the three South African populations.

Position in gene (ENSG00000160868)	Allele	rs number	Region	Amino acid substitution	Allele frequencies (%)		
					Khoisan ($n = 29$)	Xhosa ($n = 65$)	MA ($n = 65$)
−392 A>GG>A	*CYP3A4* 1B*	rs2740574	5′-flanking		76.79	73.02	45.90
−292 T>G		Novel	5′-flanking		7.14	0.00	0.81
−215 T>A		rs144721069	5′-flanking		0.00	0.81	0.85
3847 A>G		Novel	Intron 1		0.00	8.59	0.00
5916 T>C		rs12721625	Intron 2		0.00	1.56	1.56
13969 G>A		Novel	Intron 5		0.00	0.00	2.31
14268 C>T	*CYP3A4* 23*	rs57409622	Exon 6	R162W	3.57	0.77	0.00
14269 G>A	*CYP3A4* 15*	rs4986907	Exon 6	R162Q	0.00	2.38	0.00
15619 A>G		rs111768354	Exon 7	G190G	1.72	3.85	3.17
15628 C>T		rs4987159	Exon 7	I193I	0.00	4.62	3.17
15649 A>T	*CYP3A4* 24*	rs113667357	Exon 7	Q200H	10.34	3.08	3.17
15753 T>G		rs2687116	Intron 7		75.86	77.69	45.38
15783 T>C		rs4987160	Intron 7		10.34	3.85	3.85
15804 T>G		rs28988584	Intron 7		0.00	3.85	2.38
15837 T>A		rs12721622	Intron 7		10.34	10.00	3.85
17024 C>T*		rs12721624	Intron 8		0.00	0.00	3.33
17829 T>C		Novel	Intron 9		8.62	0.78	0.78
20230 G>A	*CYP3A4* 1G*	rs2242480	Intron 10		91.38	93.85	60.00
20309 G>C		rs4986911	Intron 10		15.52	9.68	6.35
20327 T>C		rs34738177	Intron 10		0.00	1.61	0.78
21896 C>T	*CYP3A4* 12*	rs12721629	Exon 11	L373F	0.00	2.34	0.00
23081 C>T		rs12721620	Intron 11		1.92	20.31	10.83
25721 A>G		rs3735451	Intron 12		76.92	87.70	50.00
25739 C>T*		rs147972695	Intron 12		0.00	3.33	0.00

Allele frequencies are given for the variant allele.

**Due to RFLP genotyping failure, these SNPs were only genotyped in the 45 sequenced individuals.*

Browser. When examining the previously described *CYP3A4* alleles, in the case of *CYP3A4*12* and *CYP3A4*15*, the data from the 1000 Genomes Browser was not available for all populations, therefore we utilized frequency data from the HapMap Project. Both of these variants were not detected in any of the HapMap populations, however, it should be noted that frequency data for the *CYP3A4*15* SNP was not available for the YRI population. These SNPs were also absent in the MA and Khoisan populations, but were present in the Xhosa population at a frequency of at 2.3% and 2.4%, respectively. With regards to the remaining potentially functional *CYP3A4* allele, namely *CYP3A4*1B*, the frequencies of this variant in the three South African populations as well as those reported by the 1000 Genomes Browser differed substantially, as demonstrated by **Figure 1**.

Table 4 | All allele defining variants and SNPs displaying significant differences in allele frequencies between the Khoisan ($n = 29$), Mixed Ancestry ($n = 65$), and Xhosa ($n = 65$).

Position in gene	Allele	rs number	Khoisan-mixed ancestry		Khoisan-xhosa		Mixed ancestry-xhosa	
GenBank: AF280107.1			***P*-Value**	**OR (95% CI)**	***P*-Value**	**OR (95% CI)**	***P*-Value**	**OR (95% CI)**
−392 A>GG>A	*CYP3A4* 1B*	rs2740574	0.000	0.257 (0.125–0.525)	0.592	0.818 (0.392–1.705)	0.000	3.189 (1.876–5.421)
−292 T>G		Novel	0.619	1.769 (0.187–16.713)	0.041	21.343 (1.129–403.534)	0.093	15.894 (0.628–402.140)
3847 A>G		Novel	0.713	2.094 (0.041–106.852)	0.094	0.087 (0.005–1.508)	0.029	0.042 (0.002–0.716)
14268 C>T	*CYP3A4* 23*	rs57409622	0.137	10.138 (0.478–214.853)	0.206	4.778 (0.424–53.807)	0.566	0.391 (0.016–9.687)
14269 G>A	*CYP3A4* 15*	rs4986907	0.713	2.094 (0.041–106.852)	0.430	0.302 (0.015–5.935)	0.202	0.144 (0.007–2.818)
15649 A>T	*CYP3A4* 24*	rs113667357	0.059	3.519 (0.953–12.993)	0.053	3.635 (0.985–13.414)	0.964	1.033 (0.253–4.2220)
15753 T>G		rs2687116	0.000	0.264 (0.132–0.529)	0.783	1.108 (0.534–2.298)	0.000	4.191 (2.447–7.180)
17829 T>C		Novel	0.025	11.981 (1.367–105.025)	0.025	11.981 (1.367–105.025)	1.000	1.000 (0.062–16.163)
20230 G>A	*CYP3A4* 1G*	rs2242480	0.000	0.142 (0.053–0.378)	0.540	1.439 (0.450–4.603)	0.000	10.167 (4.583–22.553)
21896 C>T	*CYP3A4* 12*	rs12721629	0.707	2.128 (0.042–108.589)	0.437	0.307 (0.016–6.030)	0.202	0.144 (0.008–2.817)
23081 C>T		rs12721620	0.083	0.161 (0.021–1.268)	0.013	0.077 (0.010–0.583)	0.043	0.477 (0.232–0.978)
25721 A>G		rs3735451	0.001	0.300 (0.143–0.629)	0.076	2.140 (0.922–4.965)	0.000	7.133 (3.718–13.685)

Variants showing significant differences between the South African populations are indicated in shaded areas ($P < 0.05$).

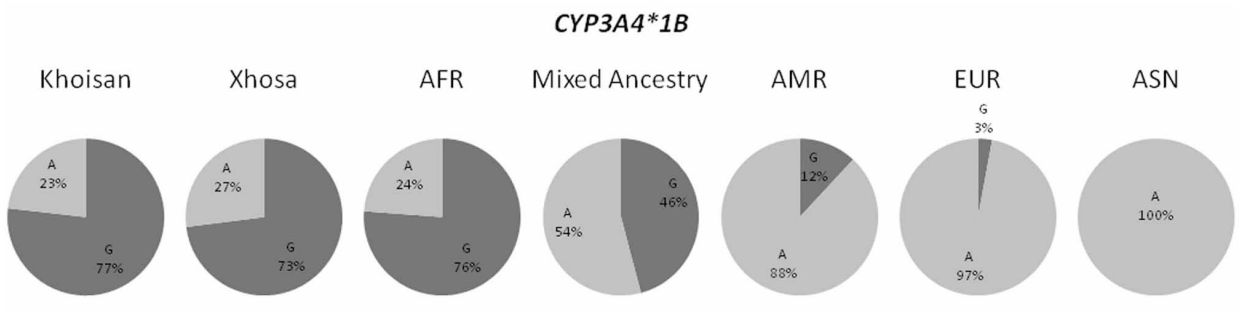

FIGURE 1 | Distribution of the *CYP3A4*1B* allele frequencies in all three South African populations and the four populations present in the 1000 Genomes Browser. (AFR, African; AMR, American Admixed; ASN, East Asian; EUR, European).

DISCUSSION

CYP3A4 GENETIC VARIATION IN THE THREE SOUTH AFRICAN POPULATIONS

To our knowledge, this was the first study in which the entire coding region of the *CYP3A4* gene was screened for common genetic variation in any Southern African population. This study identified a total of 24 variants in the three South African population groups, which included the discovery of four novel SNPs (i.e., ∼17% of the total genetic variation). Overall this study revealed the presence of the previously described *CYP3A4*1B*, *CYP3A4*1G*, *CYP3A4*12,* and *CYP3A4*15* alleles, in addition to two novel alleles, *CYP3A4*23* and *CYP3A4*24*. The number of novel alleles reported here is in accordance with the number of novel alleles that we have detected previously through the re-sequencing of other *CYP* genes in South African populations (Drögemöller et al., 2010; Wright et al., 2010). Prior to this study, these novel *CYP3A4* alleles had not been recorded on the *CYP* allele database and were present at very low frequencies in the populations described on the 1000 Genomes Browser. Both were present in the Xhosa and Khoisan populations examined in this study and *CYP3A4*24* was additionally detected in the MA population. The p.R162W amino acid change in exon 6, characterizing *CYP3A4*23*, may have functional consequences for the CYP3A4 protein, as arginine is a positively charged and hydrophilic amino acid; while tryptophan is a polar, aromatic and hydrophobic amino acid. The likely functional consequences of this variant are in agreement with the predictions made by both the SIFT and PolyPhen algorithms. Although the p.Q200H variant, characterizing *CYP3A4*24*, was not predicted to change the function of the protein product, the presence of this variant has also been reported in the genome of another Khoisan individual sequenced by Schuster et al. (2010), which correlates to the fact that the frequencies of both novel alleles were highest in the Khoisan population (Drögemöller et al., 2011). Additionally, the low frequency of these variants in the 1000 Genomes populations in comparison to the presence of these alleles at varying frequencies in the South African population groups, highlights the unique genomic composition of South African populations. Thus, results obtained from other population groups cannot be directly inferred onto the South African populations and comprehensive re-sequencing studies such as this one are required to characterize South African genomes.

The recent discovery of the *CYP3A4*22* allele confirmed that novel alleles may have functional relevance to the field of pharmacogenetics (Wang et al., 2011). This allele was initially found to influence RNA expression and statin dose requirement (Wang et al., 2011). These findings have subsequently been replicated with regards to statin therapy and the allele has additionally been shown to influence the dose requirements of the immunosuppressants, tacrolimus, and cyclosporine (Elens et al., 2011, 2012). *CYP3A4*22* is characterized by the intron 6 SNP rs35599367, which, however, was not genotyped in the current study as the aim of the study was to examine only coding regions, including the exon-intron boundaries, and the core promoter region of the gene. Furthermore, this variant does not occur in the 1000 genomes AFR or ASN populations and occurs at very low frequencies (2–5%) in the EUR and AMR populations and is thus unlikely to occur at pharmacogenetically relevant frequencies in the South African populations (http://browser.1000genomes.org/Homo_sapiens/Variation/Population?r=7:99365816993668 16;source=dbSNP;v=rs35599367;vdb=variation;vf=11936818). The reported functional significance of this intronic *CYP3A4*22* variant does, however, highlight the importance of non-coding regions. The significance of these areas, including regions that are not in close proximity to the gene has been further emphasized by the recent release of the ENCODE data (ENCODE Project Consortium et al., 2012). These data suggest that in the future additional analyses to examine the variation present in such areas, including the functional significance of the four novel non-coding SNPs identified by this study, are warranted.

The detection of novel variants in this study highlights the fact that although large re-sequencing studies such as the 1000 Genomes Project have played an integral role in characterizing the human variome (1000 Genomes Consortium, 2010), novel variation still exists, underlining the importance of re-sequencing studies such as this one. These re-sequencing studies are specifically required in African populations, as these populations have been under-represented in genomic studies to date (Rosenberg et al., 2010; Drögemöller et al., 2011). Furthermore, it may be important to compare results obtained by next generation sequencing studies to those obtained through the use of Sanger sequencing. Although the throughput of next generation sequencing studies is beyond comparison, it may be beneficial to evaluate the accuracy of next generation sequencing

for the complex and polymorphic *CYP* genes, whose sequences show high similarity to one another and to their corresponding pseudogenes (Drögemöller et al., 2011). This may be particularly important with regards to the genotyping of *CYP3A4*, which shows between 76 and 88% sequence similarity to the *CYP3A43*, *CYP3A5,* and *CYP3A7* genes (http://www.ensembl.org/Homo_sapiens/Gene/Compara_Paralog?g=ENSG00000160868;r=7:99354604-99381888) and is thus likely to be affected by the consequences of misalignment or co-amplification of other genes during the use of high-throughput technologies.

Of the previously identified alleles that were detected in this study, both *CYP3A4*1B* and *CYP3A4*12* have been reported to have functional relevance for pharmacogenetic applications. The high frequency *CYP3A4*1B* is characterized by a 5′-upstream c.−392A>G point mutation in a regulatory element, namely the putative nifedipine-specific element, which has been linked to altered gene expression *in vitro* (Amirimani et al., 2003; Georgitsi et al., 2011). Furthermore, this allele has been associated with various disease states such as prostate cancer and secondary leukemias (Lamba et al., 2002). Of relevance to pharmacogenetic applications, PharmGKB lists this SNP as affecting the metabolism of a number of therapeutic drugs, although the level of evidence for variant-drug associations is still low currently (http://www.pharmgkb.org/rsid/rs2740574; Whirl-Carrillo et al., 2012). The lack of pharmacogenetic evidence for this allele is further questioned by the results obtained by Wang et al. (2011) and the functional significance of this variant may require further examination. On the other hand, while *CYP3A4*1B* appears to affect the expression of *CYP3A4*, *CYP3A4*12* (p.L373F) affects the protein product. p.L373 has been identified as one of the key residues affecting substrate binding, cooperativity and regioselection of metabolism (Sevrioukova and Poulos, 2013) and therefore the amino acid change has been shown to result in a protein that amongst others, displays an altered testosterone metabolite profile and a four-fold increase in the *Km* value for 1′-OH midazolam formation (Eiselt et al., 2001). While *CYP3A4*1B* occurs at a relatively high frequency, both *CYP3A4*12* and *CYP3A4*15* occur at low frequencies, possibly limiting the relevance that these two variants may have for pharmacogenetic applications, especially when considering their absence from the HapMap populations. Similarly, the lack of applicability of the SNPs defining *CYP3A4*3, CYP3A4*13, CYP3A4*17,* and *CYP3A4*18* to pharmacogenetics in the South African setting is also likely as they were not detected in this study or a previous study (Ikediobi et al., 2011). These conclusions should however, be made with caution, as the relatively frequent occurrence of rare variants in African populations (Tishkoff et al., 2009) cannot be ignored and the effect of such variants should possibly also be taken into account when considering the implementation of pharmacogenetics in the African context.

VARIANT FREQUENCY DIFFERENCES BETWEEN THE THREE SOUTH AFRICAN POPULATIONS

When comparing the significant differences in allele frequencies between the three population groups, it was observed that the three groups differed significantly from one another for eight SNPs (refer to **Table 4**). These results reflect the unique genomic compositions of South African populations (Warnich et al., 2011) and indicate that the results of one South African population are not always representative of another South African population. When looking at the three populations independently, the Khoisan and Xhosa were shown to be the most similar to one another, while the differences observed between the Khoisan and MA and the Xhosa and MA were comparable. The fact that the MA population showed the greatest number of genetic differences may be explained by the large number of ancestry contributions, other than the Xhosa and Khoisan, that have been made to this population (Schlebusch et al., 2009; De Wit et al., 2010; Quintana-Murci et al., 2010; Warnich et al., 2011). The large degree of similarity observed between the Xhosa and Khoisan is to be expected and can be explained by the large ancestry contribution that the Khoisan have made to the Xhosa population (De Wit et al., 2010; Warnich et al., 2011).

The differences in allele frequencies observed for the *CYP3A4*1* sub-allele, *CYP3A4*1B*, between the different population groups (refer to **Figure 1**), serves as an excellent illustration of how pharmacogenetic applications may differ between population groups. It is important to remember that drugs designed to optimally treat one population group based on the presence of a certain allele, may be harmful to another population group for which the opposite allele is dominant. **Figure 1** shows how the *CYP3A4*1B* allele is more frequent in the African populations (Khoisan, Xhosa and AFR), while in the ASN and EUR population groups the opposite allele occurs more often. Interestingly, both the MA and AMR admixed populations show allele frequencies that are intermediate between the African and EUR/ASN populations. Furthermore, the MA is more similar to the African populations, while the AMR is more similar to the non-African populations. These results are in accordance with the ancestral history of these population groups. The MA have ancestry contributions from the Xhosa, Khoisan, European, and Asian populations (Schlebusch et al., 2009; Tishkoff et al., 2009; De Wit et al., 2010; Quintana-Murci et al., 2010), which explains why although the frequencies of the variants in this population are between the African and EUR/ASN populations, they are more similar to the African populations. On the other hand, the AMR population, which consists of the Mexicans, Puerto Ricans, Columbians, and Peruvians (http://www.1000genomes.org/faq/which-populations-are-part-your-study), is more similar to the EUR/ASN population groups due to the larger ancestry contribution that these populations have made to the AMR, when compared to the ancestry contribution of Africans (Galanter et al., 2012).

The allele frequency differences observed in admixed populations, as previously reported in admixed Brazilian populations (Suarez-Kurtz, 2005, 2010; Suarez-Kurtz et al., 2012), bring to light an important consideration for the implementation of pharmacogenetics. Individuals within admixed populations are likely to exhibit different levels of ancestry contributions, as has been shown with the use of STRUCTURE analyses for both the MA (Tishkoff et al., 2009) and Brazilian populations (Suarez-Kurtz, 2010). Population based pharmacogenetic testing is thus unlikely to detect all pharmacogenetically relevant variants and it may be necessary to implement pharmacogenetics on an

individualized level. In the context of South Africa with its diverse population groups, which exhibit both rare variants and variants from several different population sources (Warnich et al., 2011), this may be especially important. However, before this goal can be realized it will be necessary to consider whether individualized treatment will be feasible in the resource limited settings of the country.

CONCLUSIONS

Although this study identified both novel and known SNPs of functional significance in all three population groups, due to the current lack of validated evidence regarding the pharmacogenetic application of *CYP3A4*, the relevance of these SNPs in the clinical setting remains unknown. The SNP markers detected in the current study should therefore be included in genotyping panels in future pharmacogenetic association studies involving CYP3A4 substrate medications. Nonetheless, this study provides an excellent example of how re-sequencing studies are required in African populations in order to identify variation that remains novel. These differences in allele frequencies were not only seen when comparing the South African populations to other populations, but also when comparing them to each other. These results demonstrate that a one-size-fits-all approach is not ideal when implementing therapeutic treatment regimes, also within the South African context.

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Frequencies of 23 functionally significant variant alleles related with metabolism of antineoplastic drugs in the Chilean population: comparison with Caucasian and Asian populations

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Cancer is a leading cause of death worldwide. The cancer incidence rate in Chile is 133.7/100,000 inhabitants and it is the second cause of death, after cardiovascular diseases. Most of the antineoplastic drugs are metabolized to be detoxified, and some of them to be activated. Genetic polymorphisms of drug-metabolizing enzymes can induce deep changes in enzyme activity, leading to individual variability in drug efficacy and/or toxicity. The present research describes the presence of genetic polymorphisms in the Chilean population, which might be useful in public health programs for personalized treatment of cancer, and compares these frequencies with those reported for Asian and Caucasian populations, as a contribution to the evaluation of ethnic differences in the response to chemotherapy. We analyzed 23 polymorphisms in a group of 253 unrelated Chilean volunteers from the general population. The results showed that *CYP2A6*2*, *CYP2A6*3*, *CYP2D6*3*, *CYP2C19*3*, and *CYP3A4*17* variant alleles are virtually absent in Chileans. *CYP1A1*2A* allele frequency (0.37) is similar to that of Caucasians and higher than that reported for Japanese people. Allele frequencies for *CYP3A5*3*(0.76) and *CYP2C9*3*(0.04) are similar to those observed in Japanese people. *CYP1A1*2C*(0.32), *CYP1A2*1F*(0.77), *CYP3A4*1B*(0.06), *CYP2D6*2*(0.41), and *MTHFR*T(0.52) allele frequencies are higher than the observed either in Caucasian or in Japanese populations. Conversely, *CYP2C19*2* allelic frequency (0.12), and genotype frequencies for *GSTT1* null (0.11) and *GSTM1* null (0.36) are lower than those observed in both populations. Finally, allele frequencies for *CYP2A6*4*(0.04), *CYP2C8*3*(0.06), *CYP2C9*2*(0.06), *CYP2D6*4*(0.12), *CYP2E1*5B*(0.14), *CYP2E1*6*(0.19), and *UGT2B7*2*(0.40) are intermediate in relation to those described in Caucasian and in Japanese populations, as expected according to the ethnic origin of the Chilean population. In conclusion, our findings support the idea that ethnic variability must be considered in the pharmacogenomic assessment of cancer pharmacotherapy, especially in mixed populations and for drugs with a narrow safety range.

Keywords: polymorphisms, biomarkers, CYP450, MTHFR, antineoplastic, biotransformation, pharmacogenetics, pharmacogenomics

INTRODUCTION

Cancer is a leading cause of death worldwide and the total number of cases globally is increasing. The number of cancer deaths is projected to increase 45% from 2007 to 2030 (from 7.9 million to 11.5 million deaths), influenced in part by an increasing and aging global population. The estimated rise takes into account expected slight declines in death rates for some cancers in high resource countries. New cases of cancer in the same period are estimated to increase from 11.3 million in 2007 to 15.5 million in 2030 (WHO, 2011). In Chile cancer have a rate of 133.7 × 100,000 inhabitants, is the second cause of death after cardiovascular diseases with a sustained increase in the time both, in the rates and in proportion of deaths. Chile has a number of 30,000 new cases and 36,000 hospitalizations per year. The first cause of cancer

death in Chilean population is gastric cancer for both genders, with a rate of 19.8 × 100,000 inhabitants, followed by lung cancer (15 × 100,000 inhabitants), prostate (10.4 × 100,000 inhabitants), and gallbladder (8.1 × 100,000 inhabitants; DEIS, 2011).

Though the principal achievements in the fight against cancer has been those related to preventive measures, the success in the treatment of an established cancer depends on the cancer stage when detected, on the type of cancer, and on its location. It is quite infrequent to find patients considered as "therapeutical success" because there is always a possibility that a tumor process may appear back (Arrastoa, 1998).

Chemotherapy for cancers has progressed from its introduction to clinical practice and constitutes the mainstay modality of therapy in these pathologies. Nevertheless, its use is limited by the inability to predict the response. In most cases the therapy choice is empirical. Nowadays there are more than 100 antineoplastic drugs, which are used either alone or combined. A combined therapy allows that drugs with different mechanism of action work together to destroy the larger possible amount of tumor cells, in order to reduce the possibility of resistance to a particular antineoplastic drug. The therapy to select, the dose, the method of administration and the frequency, and duration of the treatment, will depend on the type of cancer, its location, the rate of growth, how it is affecting the normal functions of the body, as well as on the patient's general health condition. Usually therapies can be managed by means of cycles that alternate drug administration with washout periods that allow healthy cells to recover from the effect of the medication.

The biotransformation of drugs, including antineoplastic agents, is done basically in two phases: phase I, catalyzed mainly by the cytochrome P450 system and phase II, by transferases that catalyze reactions of conjugation of xenobiotics with diverse molecules of endogenous nature as glucuronic acid, sulfates, acetate, glutathione, or some amino acids. The final outcome of both phases is to increase the hydrophilicity of chemicals facilitating the excretion through urine or bile (Rooney et al., 2004).

There is limited information available in human beings regarding the metabolism and pharmacokinetics interaction of antineoplastic drugs. Nevertheless, it is well known that clinical significant interactions exist between drugs that can render them less efficient if used simultaneously, and in some cases, produce unexpected effects.

The cytochrome P450 (CYP) system is the most important metabolic system responsible for the oxidation of numerous chemotherapeutic agents, and it is responsible to a great extent for the variability observed in response to drugs. For example, some enzymes of the CYP3A family play an important role in the metabolism of epidophilotoxin, ifosfamide, tamoxifen, taxol, and vinca alkaloids. Cytochrome P450 enzymes are also of great importance in the study of chemotherapy resistance. (Kivisto et al., 1995; Yao et al., 2000; Lin and Yu, 2001; García-Martín et al., 2006a; Quiñones et al., 2008).

Genetic polymorphisms of CYP enzymes can produce deep changes in enzyme activity, thus determining the individual response to a certain drug leading to poor, intermediate, extensive, or ultrarapid metabolizer phenotypes (Ingelman-Sundber, 2005; Jin, 2005). On the other hand, the glucuronidation of drugs is carried out by the UDP Glucuronyl transferases (UGT), which are also polymorphic in human beings, adding to the diversity of this reaction. In this respect, UGT2B7 has unique specificity for 3,4-catechol estrogens and estriol, suggesting that it may play an important role in regulating the level and activity of these potent estrogen metabolites. It is also able to conjugate major classes of drugs such as analgesics (morphine), carboxylic non-steroidal anti-inflammatory drugs (ketoprofen), anticarcinogens (all-trans retinoic acid), and tamoxifen. 802C/T mutation leads to UGT2B7 variants UGT2B7*1 (Y268) and UGT2B7*2 (H268) which has been suggested to increase the activity of the enzyme (Ritter et al., 1990; Coffman et al., 1997; Barre et al., 2007; Lazarus et al., 2009). Similarly, the human glutathione-*S*-transferases (GSTs), are polymorphic isoenzymes which show a wide subcellular distribution. GST alpha (α), kappa (κ), mu (μ), pi (π), omega (ω), sigma (σ), theta (θ), and zeta (Z are being studied as possible genetic biomarkers of cancer and its chemotherapeutic treatment (Guengerich et al., 1992; Quiñones et al., 1999; Bredschneider et al., 2002). These enzymes are fundamental in the oxidative processes and detoxification of a wide variety of xenobiotics, including many chemotherapeutic drugs (Massad-Massade et al., 1997; Sargent et al., 1999; Clapper, 2000; Bredschneider et al., 2002; Petros et al., 2005). These polymorphisms have been postulated also as biomarkers for susceptibility to diverse types of cancer (IARC, 1999; Clapper, 2000; Au, 2001; Quiñones et al., 2001; Acevedo et al., 2003; Keshava et al., 2004; Lee et al., 2006; Cordero et al., 2010; Singh et al., 2011) showing marked interethnic differences (Stephens et al., 1994; Muñoz et al., 1998; Garte et al., 2001; Yasuda et al., 2008).

Another relevant enzyme is methylene tetrahydrofolate reductase (MTHFR) which converts 5,10-methylene tetrahydrofolate to 5-methyl tetrahydrofolate. This reaction is required for the multistep process that converts the amino acid homocysteine to another amino acid, methionine. Polymorphisms of this enzyme predispose to serious bone marrow toxicity during treatment with agents that inhibit folate synthesis (e.g., methotrexate; Chiuslo et al., 2002; Yang et al., 2012).

Because of enzymes CYP1A1, CYP1A2, CYP2A6, CYP3A4/5, CYP2C8, CYP2C9, CYP2C19, CYP2E1, CYP2D6, GSTM1, GSTT1, UGT2B7, and MTHFR take part in the metabolism of oncological drugs (**Table 1**), the main goal of this study was to determine the allele frequencies of variants of these enzymes in a group representative of the Chilean population in order to describe genetic polymorphisms that might be useful in public health programs, and to compare these frequencies with other populations, as the first approximation to the evaluation of ethnic differences in the response to chemotherapies.

MATERIALS AND METHODS

STUDY POPULATION

Blood samples were obtained from 253 unrelated volunteers living in Santiago of Chile (both sexes, 27–55 years old). The study group has a 37% Amerindian-Caucasian admixture, as determined by ABO blood group distribution (Valenzuela, 1988). The research was authorized by the Ethics Committee of the Faculty of Medicine of the University of Chile. All subjects signed an authorized

informed consent. **Table 1** shows general characteristics of the studied population.

DNA EXTRACTION

Extraction of genomic DNA was done by a standard procedure from whole blood using a commercial kit (High Pure PCR Template Preparation Kit, Roche Diagnostics®) and DNA samples were stored at -20°C until further analysis.

GENOTYPING

Genomic DNAs from peripheral blood were amplified by PCR using specific primers for detection of the specific allelic variants in study. For detection of polymorphisms *CYP1A1*2A*, *CYP1A1*2C*, *CYP1A2*1F*, *CYP2E1*5B*, *CYP2E1*6*, *CYP2A6*2*, *CYP2A6*3*, *CYP2A6*4*, *CYP2D6*2*, *CYP2C9*2*, *CYP2C19*2*, *CYP3A4*1B*, *CYP3A4*17*, *CYP3A5*3*, and *UGT2B7*2*, amplicons were surrendered to digestion with the appropriate restriction enzyme to be analyzed through electrophoresis, in agarose gel (2%) or polyacrylamide gel (16%) according to methods previously reported (Hayashi et al., 1999; Kitagawa et al., 1999; Quiñones et al., 1999; Cavalli et al., 2001; LEE et al., 2005; Lin et al., 2005). *CYP2D6*3* and *CYP2D6*4* were analyzed by means of allele-specific PCR (Heim and Meyer, 1990; Amrithraj et al., 2006). *CYP2C9*3*, *CYP2C19*3*, and *CYP2C8*3* polymorphisms were analyzed with *Taqman* probes in an ABI 7500 Real Time PCR system, using specific probes previously described (Agúndez et al., 2009). Deletions of *GSTM1* and *GSTT1* were analyzed through PCR using β-globin gene as control (Quiñones et al., 1999; Rebbeck et al., 1999). Heterozygous and homozygous non-null individuals could not be differentiated, therefore double null genotypes (−/−) are the null genotypes reported. Finally, to detect *MTHFR* C677T polymorphism we use a commercial Real Time kit (Roche Diagnostics®).

RESULTS AND DISCUSSION

Pharmacogenetic research is directed to identify genes or gene products associated with diseases and, especially, allelic variants in enzymes of biotransformation that alter the individual response to drugs. These variants can modify the magnitude of the pharmacological effect, the threshold of toxicity, the efficacy of the drug, side effects, and drug–drug interactions. In this respect, it is particularly important at the time, to define pharmacogenetic profiles of patients with cancer to determine suitable dosages, to improve efficacy, to avoid adverse reactions of the traditionally used drugs, and to develop new drugs according to the genetic – metabolic profile of the patients (Wilkinson, 2000). Moreover, ethnicity plays an important role in pharmacokinetics and pharmacodynamics of drugs (Ling and Lee, 2011; Kurose et al., 2012) giving rise a more complex situation in "mestizo" populations as South American people. In this sense, Chilean population is a genetic admixture originated primarily between Caucasian (mainly Spaniards) and native-American (mainly Mapuches) from a first single migration of Asians from Siberia 15,000 years ago through Beringia (Reich et al., 2012) and secondly from immigration, mostly from Germany, Croatia, France and Italy. This is a restriction to extrapolate the dosage of drugs with clinical studies performed in other ethnic groups. Another restriction is the poor information about the biotransformation enzyme polymorphisms in Chilean population. According to this, we have studied genetic polymorphisms of several enzymes, in a sub-group of the Chilean population, which metabolize mainly antineoplastic drugs used for chemotherapy in health institutions of Chile (**Table 1**). General characteristics of the analyzed population are shown in **Table 2**. The mean age identifies a young adult population which has, in average, a normal mean weight, height, and body mass index and are a representative sample of the middle class Chilean population, which is supported by the Amerindian-Caucasian admixture (37%).

The allele and genotype frequencies for metabolic enzymes included in this research are shown in the **Table 3**. Due to DNA shortage, not all DNA samples were analyzed for all polymorphisms. In **Table 4** we compare allele frequencies found in this study in relation to Caucasian and Asian populations.

Our results show the absence of variant alleles *CYP2A6*3*, *CYP2C19*3*, and *CYP3A4*17* such as it was also observed for Caucasians. In Japanese people *CYP2A6*3* and *CYP3A4*17* are also absent, but the *CYP2C19*3* allele has a frequency of 0.11. *CYP1A1*2A* and *CYP2A6*2* allele frequencies are similar to Caucasians but higher than the reported for Japanese people. *CYP3A5*3* and *CYP2C9*3* frequencies are similar to those in Japanese people, but different to the Caucasian people. *CYP1A1*2C*, *CYP1A2*1F*, *CYP3A4*1B*, and *CYP2D6*2* allelic frequencies are higher than those observed either in Caucasian or Japanese populations. *MTHFR*T allele frequency is higher than the observed in Caucasian and Japanese population, but also than the frequency reported previously by Nitsche et al. (2003) in other group of the Chilean population. We suggest that this difference could be explained by different genetic composition of the previously studied group, which could be more similar to Caucasians. Unfortunately, Nitsche et al. no reported the Amerindian-Caucasian admixture percentage to evaluate this point.

On the other hand, *CYP2C19*2*, *GSTT1* null and *GSTM1* null frequencies are lower than those reported in Caucasian or Japanese population. Finally for *CYP2C8*3*, *CYP2C9*2* and *3, *CYP2D6*3*, *CYP2D6*4*, *CYP2E1*5B* and *6, as well as for *UGT2B7*2* the frequencies described for Chileans are intermediate in relation to those described for Caucasian and Japanese population (Sullivan-Klose et al., 1996; Nasu et al., 1997; Chen et al., 1998, 1999; Chida et al., 1999; Paris et al., 1999; Sachse et al., 1999; Bhasker et al., 2000; Matsuo et al., 2001; Murata et al., 2001; Roy et al., 2005; Skarke et al., 2005; Nakajima et al., 2006; Krishnakumar

Table 1 | General characteristics of the studied population.

Sex	Women	Men	Total
Number	155	98	253
Age (years)*	33.6 ± 13.6	28.7 ± 10.8	31.7 ± 12.8
Weight (kg)*	61.8 ± 9.1	74.9 ± 10.1	66.8 ± 11.4
Height (m)*	1.60 ± 0.06	1.73 ± 0.07	1.65 ± 0.09
BMI (Kgm^{-2})*	24.1 ± 3.3	25.0 ± 2.9	24.5 ± 3.2
A_{A-C}: 37%**			

Information is expressed as averages ± SD; BMI: body mass index.

***Amerindian-Caucasian Admixture (%).*

Table 2 | Some antineoplastic drugs, substrates of polymorphic enzymes analyzed in this research (http://www.pharmacologyweekly.com/content/pages/cytochrome-cyp-p450-enzyme-medication-herbs-substrates; Quiñones et al., 2008).

Drugs	Enzyme	Cancer
Cisplatin	GSTM, GSTT	Breast
Cyclophosphamide	CYP2B6, CYP2C19, CYP3A4	Leukemia, lymphoma
Dacarbazine	CYP1A1, CYP1A2, CYP2E1	Melanoma, sarcoma, lymphoma
Docetaxel	CYP2C8, CYP3A, CYP1B1	Breast, lung, stomach
Doxorubicin	CYP3A4	Sarcoma
Ellipticine	CYP3A4, CYP1A	Leukemia, myeloma, lymphosarcoma
Etoposide	CYP3A4, CYP2E1, CYP1A2	Testicle, lung, breast, leukemia, lymphoma
Ifosfamide	CYP2B6, CYP3A4	Sarcoma, testicle
Imatinib	CYP3A4, CYP3A5	Leukemia
Irinotecan	CYP3A4/5 UGT1A1	Colon and rectum
Methotrexate	MTHFR	Leukemia
Mitoxantrone	CYP3A4, CYP1B1	Leukemia, lymphoma
Paclitaxel	CYP2C8, CYP3A4	Breast, lung, ovary
Phortress (2-(4-aminophenyl)-benzotiazol)	CYP1A1, CYP1B1	Ovary, breast
Procarbazine	CYP1A1, CYP2B6	Lymphoma, brain, lung, melanoma, testicle
Tamoxifen	CYP3A4, CYP2D6, CYP2C9, CYP2C19, CYP1B1, UGT2B7	Breast
Tegafur	CYP2A6, CYP2C8, CYP1A2	Colon, rectum, stomach
Teniposide	CYP2C19, CYP3A4/5	Leukemia, lung, brain, bladder, myeloma
Thiotepa	CYP2B6, CYP3A4	Bladder
Topotecan	CYP3A4/5	Ovary, lung
Vinblastine	CYP3A4/5	Lymphoma, osteosarcoma
Vincristine	CYP3A4/5	Lymphoma
Vindesine	CYP3A4/5	Lung
Vinrelbine	CYP3A4/5	Lung

et al., 2012; Kurose et al., 2012), which is expected because Chilean population is considered a mixed ethnicity between both races.

Some limitations in this study should be pointed out. Only some polymorphism of the many (>80) CYP2D6 known were genotyped. We select only CYP2D6 polymorphisms that have shown better reported relationship with plasma levels of antineoplastic drugs and those that have higher allele frequencies, based on a previous pilot study in Chilean subjects (Dr. Monica Acuña, Personal Communication). Similarly, same criteria were used for choosing the other studied polymorphisms, based on both literature and our own previous research. Nevertheless, some potentially relevant CYP variants are currently under investigation in our laboratory (CYP2D6*2xN, CYP2D6*5, CYP2D6*10, CYP2C8*3, and CYP2C8*4), to complement the results shown in this paper.

Additionally, we have no data for other relevant polymorphic enzymes, such as for example, CYP1B1 and CYP2B6, responsible for the metabolism of several antineoplastic drugs (Quiñones et al., 2008) and UDP Glucuronyl transferase 1A1, involved in metabolism of irinotecan (Dias et al., 2012). We did not analyze thiopurine *S*-methyltransferase (TPMT), a cytosolic phase II enzyme involved in the metabolism of azathioprine, thiopurine, and thioguanine (Zhou, 2006). However the frequency of four allelic variants of this gene (*2, *3A, *3B, and *3C) were analyzed previously in Chilean population by Alvarez et al. (2009) showing that the presence of *3A allele is the most prevalent, which is similar to Caucasians, giving a first approach to the use of this polymorphism in clinical practice in Chilean patients.

Another limitation of the present research is the use of Japanese population as proxy of the ancestral Asians of Chilean people. We use this population as reference due to two main facts: (a) there is very good and complete information about these polymorphisms in Japanese people and (b) recently, have been reported no great differences among Japanese and other Asian populations, particularly with respect to Chinese population (Kurose et al., 2012).

On the other hand, drug–drug pharmacological interactions, some epigenetic and environmental factors, and alternative metabolic routes should not be excluded to describe response to antineoplastic agents, which is *per se* a multifactorial event. Thus, the research in this area must identify these factors and potential gene – environment interactions that modulate response to these drugs.

Some polymorphisms have been studied in other South American countries (Isaza et al., 2000; Gaspar et al., 2002; Fernández et al., 2004; Gattas et al., 2004; Vianna-Jorge et al., 2004; Almeida et al., 2005; Lizcano Fernández, 2005; Rossini et al., 2006; Schlawicke et al., 2007; Canalle et al., 2008; Rodríguez et al., 2008; Castaño-Molina et al., 2009; Dorado et al., 2011), but the comparison with Chilean Mestizo population is very difficult because of the divergent origin of these populations. Chilean population is different from other South American countries, from Brazilian people for example, which have African and Portuguese contribution with great pharmacogenomic diversity, or from Argentina and Uruguay populations, which are multiethnic countries, with Amerindian-European admixture, but mostly with Italians (Lizcano Fernández, 2005). In this respect, in South America, the region one of the most diverse genetic background in the world, four main components have contributed to the present-day population: Amerindians (pre-Columbian inhabitants); Iberians (conquerors) who dominated the continent until the nineteenth century, Africans (imported as slaves by the colonizers); and post-independence immigrants from overseas (mostly Italy and Germany but also from France, South Asia, and Japan). Therefore, we suggest that studies of these pharmacogenes in Chileans should be used to develop pharmacogenetic tools for this specific population, rather than extrapolating results obtained to other populations.

Table 3 | Genotype and allele frequencies of CYP1A1, CYP1A2, CYP2A6, CYP3A4/5, CYP2C8, CYP2C9, CYP2C19, CYP2E1, CYP2D6, UGT2B7, GSTM1, GSTT1 y MTHFR polymorphisms in a Chilean mestizo population.

CYP1A1*2A	***n***	**%**	**CYP1A1*2C**	***n***	**%**	**CYP1A2*1F**	***n***	**%**	**CYP2A6*2**	***n***	**%**	**CYP2A6*3**	***n***	**%**	**CYP2A6*4**	***n***	**%**
*1/*1	112	44.27	*1/*1	79	43.89	*1A/*1A	18	7.11	*1A/*1A	244	100.00	*1A/*1A	253	100.00	*1A/*1A	244	100.00
*1/*2A	95	37.55	*1/*2C	86	47.78	*1A/*1F	81	32.02	*1A/*2	9	0.00	*1A/*3	0	0.00	*1A/*4	0	0.00
*2A/*2A	46	18.18	*2C/*2C	15	8.33	*1F/*1F	154	60.87	*2/*2	0	0.00	*3/*3	0	0.00	*4/*4	9	0.00
TOTAL	253	100.0	TOTAL	180	100.00	TOTAL	253	100.00	TOTAL	253	100.00	TOTAL	253	100.00	TOTAL	253	100.00
fwt	0.63		*f**1	0.68		*f**1A	0.23		*f**1A	0.98		*f**1A	1.00		*f**1A	0.96	
*f**2A	0.37		*f**2C	0.32		*f**1F	0.77		*f**2	0.02		*f**3	0.00		*f**4	0.04	
CYP2C8*3	***n***	**%**	**CYP2C9*2**	***n***	**%**	**CYP2C9*3**	***n***	**%**	**CYP2C19*2**	***n***	**%**	**CYP2C19*3**	***n***	**%**	**CYP2D6*2**	***n***	%
*1A/*1A	157	87.22	*1A/*1A	225	88.93	*1A/*1A	167	92.78	*1A/*1A	201	79.45	*1A/*1A	253	100.00	*1A/*1A	88	34.78
*1A/*3	23	12.78	*1A/*2	26	10.28	*1A/*3	13	7.22	*1A/*2	44	17.39	*1A/*3	0	0.00	*1A/*2	121	47.83
*3/*3	0	0.00	*2/*2	2	0.79	*3/*3	0	0.00	*2/*2	8	3.16	*3/*3	0	0.00	*2/*2	44	17.39
TOTAL	180	100.00	TOTAL	253	100.00	TOTAL	180	100.00	TOTAL	253	100.00	TOTAL	253	100.00	TOTAL	253	100.00
*f**1A	0.94		*f**1A	0.94		*f**1A	0.96		*f**1A	0.88		*f**1A	1.00		*f**1A	0.59	
*f**3	0.06		*f**2	0.06		*f**3	0.04		*f**2	0.12		*f**3	0.00		*f**2	0.41	
CYP2D6*3	***n***	**%**	**CYP2D6*4**	***n***	**%**	**CYP2E1*5B**	***n***	**%**	**CYP2E1*6**	***n***	**%**	**CYP3A4*1B**	***n***	**%**	**CYP3A4*17**	***n***	**%**
*1A/*1A	248	98.02	*1A/*1A	198	78.26	*1A/*1A	135	75.00	*1A/*1A	123	68.33	*1A/*1A	225	88.93	*1A/*1A	253	100.00
1A/*3	5	1.98	1A/*4	50	19.76	*1A/*5B	39	21.67	*1A/*6	45	25.00	*1A/*1B	28	11.07	*1A/*17	0	0.00
*3/*3	0	0.00	*4/*4	5	1.98	*5B/*5B	6	3.33	*6/*6	12	6.67	*1B/*1B	0	0.00	*17/*17	0	0.00
TOTAL	253	100.00	TOTAL	253	100.00	TOTAL	180	100.00	TOTAL	180	100.00	TOTAL	253	100.00	TOTAL	253	100.00
*f**1A	0.99		*f**1A	0.88		*f**1A	0.86		*f**1A	0.81		*f**1A	0.95		*f**1A	1.00	
*f**3	0.01		*f**4	0.12		*f**5B	0.14		*f**6	0.19		*f* *1B	0.06		*f**17	0.00	
CYP3A5*3	***n***	**%**	**MTHFR (C677T)**	***n***	**%**	**UGT2B7*2**	***n***	**%**	**GSTM1**	***n***	**%**	**GSTT1**	***n***	**%**			
*1A/*1A	17	6.72	CC	36	20.00	*1/*1	68	26.88	null	161	63.64	null	160	88.88			
*1A/*3	86	33.99	CT	100	55.56	*1/*2	170	67.19	null	92	36.36	null	20	11.11			
*3/*3	150	59,29	TT	44	20.00	*2/*2	15	5.93	TOTAL	253	100.00	TOTAL	180	100.00			
TOTAL	253	100.00	TOTAL	180	24.44	TOTAL	253	100.00									
*f**1A	0.24		fc	0.48		*f**1	0.60										
*f**3	0.76		ft	0.52		*f**2	0.40										

Table 4 | Comparison of allelic frequencies of CYP1A1, CYP1A2, CYP2A6, CYP3A4, CYP3A5, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, GSTM1, GSTT1, UGT2B7, and MTHFR in Caucasian, Japanese, and Chilean populations.

					Alle Frequencies			
Enzyme	**Allele**	**rs**	**Gene change (protein change)**	**Enzyme activity**	**Chilean**	**Caucasian**	**Japanese**	**Reference**
CYP1A1	*2A	rs4646903	T3801C	Increased	0.37	0.36	0.09	Murata et al. (2001)
	*2C	rs1048943	A2454G (I462V)	Decreased	0.32	0.22	0.05	Murata et al. (2001)
CYP1A2	*1F	rs762551	C-163A	Higher inducibility	0.77	0.68	0.61	Sachse et al. (1999), Skarke et al. (2005), Chida et al. (1999)
CYP2A6	*2	rs1801272	T479A (L160H)	None	0.02	0.02	0.00	Nakajima et al. (2006), Chen et al. (1999)
	*3	rs56256500	CYP2A6/CYP2A7 hybrid	Decreased?	0.00	0.00	0.00	
	*4	rs3892097	Deletion	None	0.04	0.00	0.19	
CYP3A4	*1B	rs2740574	A-392G	Decreased	0.06	0.04	0.00	Paris et al. (1999)
	*17	rs4987161	T15615C	Decreased	0.00	0.00	0.00	
CYP3A5	*3	rs776746	A6986G Splicing defect	Decreased	0.76	0.70	0.75	Kurose et al. (2012), Roy et al. (2005)
CYP2C8	*3	rs10509681	G2130A, A30411G (R139K, K399R)	Decreased	0.06	0.16	0.00	Agúndez et al. (2009)
CYP2C9	*2	rs1799853	C3608T (R144C)	Decreased	0.06	0.08	0.00	Agúndez et al. (2009), Sullivan-Klose et al. (1996), Nasu et al. (1997)
	*3	rs1057910	A42614C (I359L)	Decreased	0.04	0.06	0.03	
CYP2C19	*2	rs28399504	G19154A Splicing defect	None	0.12	0.14	0.23	Kurose et al. (2012)
	*3	rs4986893	G17948A W212X	None	0.00	0.00	0.11	
CYP2D6	*2	rs16947	C2850T (R296C)	Normal	0.41	0.32	0.13	Kurose et al. (2012)
	*3	rs35742686	del2549A(259 Frame shift)	None	0.01	0.02	0.00	
	*4	rs3892097	G1846A Splicing defect	None	0.12	0.21	0.00	
CYP2E1	*5B	rs2031920/ rs3813867	G-1293C/C-1053T	Decreased	0.15	0.04	0.20	Krishnakumar et al. (2012)
	*6	rs6413432	T7632A	Decreased	0.22	0.08	0.29	Krishnakumar et al. (2012)
GSTM1	null	SNP500Cancer ID – GSTM1-02	Null deletion	None	0.20	0.45	0.55	Kurose et al. (2012)
GSTT1	null	SNP500Cancer ID – GSTT1-02	Null Deletion	None	0.11	0.52	0.20	Kurose et al. (2012)
UGT2B7	*2	rs7439366	C802T (H268Y)	Decreased	0.40	0.49	0.27	Bhasker et al. (2000)
MTHFR	T	rs1801133	C677T (A222V)	Thermolabile enzyme	0.52	0.41	0.32	Matsuo et al. (2001), Chen et al. (1998)

The frequencies observed in metabolic polymorphism studied in Chilean population were distinct from paternal races. These results contribute to better understanding of the basis of ethnic variation in drug metabolism and response (Agúndez, 2004; García-Martín et al., 2006b; Yasuda et al., 2008), and suggest a complex genetic profile of this "mestizo" population, which should be considered in pharmacotherapy, especially for drugs with a narrow safety range, particularly in cancer chemotherapy (García-Martín, 2008; Quiñones et al., 2008). These established genotype frequencies may be used for studying the phenotype variation in further studies. Thus, it may be a good contribution for further studies on the clinical application of pharmacogenomics in Asian-Caucasian mixed races.

CONCLUSION

Profound variation in polymorphisms of metabolizing enzymes have been described in diverse populations, including enzymes that take part in the metabolism of chemotherapeutics drugs (50). In this sense, our results agree with these observations when we compare the analyzed sub-group with Asian and Caucasian populations (**Table 4**).

As Chilean population represents a mixed ethnicity mainly between native-Americans and Caucasians (mostly Spaniards) the data obtained might help to understand inter ethnic differences not only for single polymorphisms, but also the function of simultaneous polymorphisms in metabolic genes in each subject, to explain differences in response to chemotherapy.

This investigation contributes to have a first pattern of several relevant polymorphisms in metabolizing enzymes (*CYP1A1*, *CYP1A2*, *CYP2A6*, *CYP2C8*, *CYP2C9*, *CYP2C19*, *CYP2D6*, *CYP2E1*, *CYP3A4/5*, *GSTM1*, *GSTT1*, *UGT2B7*, *and MTHFR*) in Chilean people, which can give course for a genetic – population investigation of these polymorphisms helping to the understanding of susceptibility to drugs and pathologies in this population, which already has been suggested by our group of research for some specific genes (Quiñones et al., 1999, 2001, 2008; Acevedo et al., 2003; Lee et al., 2006; Cordero et al., 2010).

The knowledge of genetic variants involved in the metabolism of the antineoplastic drugs in the Chilean population will help to the prediction of their clinical efficacy and/or toxicity, and therefore, will help us to the design personalized cancer treatments to improve therapeutic response and diminish the adverse effects improving cost-efficacy of treatments.

Finally, based on scientific literature and our experience, we believe that, at least in Chilean population, MTHFR/methotrexate, GST/cisplatin, and CYP2D6/tamoxifen are the potential more relevant gene/drug pairs which are closer for monitoring use in clinical practice.

AUTHORS' CONTRIBUTIONS

Angela Roco: AB, ES; Luis Quiñones: AB, ES, FG; José AG. Agúndez: FG; Elena García-Martín: FG; Valentina Squicciarini: AB; Carla Miranda: AB; Joselyn Garay: AB; Nancy Farfán: AB; Dante Cáceres: ES; Carol Ibarra: AB; Nelson Varela: AB; Iván Saavedra: FG.

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Pharmacogenomic diversity among Brazilians: influence of ancestry, self-reported color, and geographical origin

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By virtue of being the product of the genetic admixture of three ancestral roots: Europeans, Africans, and Amerindians, the present-day Brazilian population displays very high levels of genomic diversity, which have important pharmacogenetic/-genomic (PGx) implications. Recognition of this fact has prompted the creation of the Brazilian Pharmacogenomics Network (Refargen), a nationwide consortium of research groups, with the mission to provide leadership in PGx research and education in Brazil, with a population heath impact. Here, we present original data and review published results from a Refargen comprehensive study of the distribution of PGx polymorphisms in a representative cohort of the Brazilian people, comprising 1,034 healthy, unrelated adults, self-identified as white, brown, or black, according to the Color categories adopted by the Brazilian Census. Multinomial log-linear regression analysis was applied to infer the statistical association between allele, genotype, and haplotype distributions among Brazilians (response variables) and self-reported Color, geographical region, and biogeographical ancestry (explanatory variables), whereas Wright's F_{ST} statistics was used to assess the extent of PGx divergence among different strata of the Brazilian population. Major PGx implications of these findings are: first, extrapolation of data from relatively well-defined ethnic groups is clearly not applicable to the majority of Brazilians; second, the frequency distribution of polymorphisms in several pharmacogenes of clinical relevance (e.g., *ABCB1*, *CYP3A5*, *CYP2C9*, *VKORC*) varies continuously among Brazilians and is not captured by race/Color self-identification; third, the intrinsic heterogeneity of the Brazilian population must be acknowledged in the design and interpretation of PGx studies in order to avoid spurious conclusions based on improper matching of study cohorts.

Keywords: biogeographical ancestry, Brazilian pharmacogenomic network, F_{ST} statistics, pharmacogenomic diversity, population admixture, refargen

INTRODUCTION

The present-day Brazilian population, in excess of 190 million people, is highly heterogeneous and admixed, as result of five centuries of mating between native Amerindians, Europeans, and sub-Saharan Africans. This fact renders inappropriate extrapolation of pharmacogenetic/-genomic (PGx) data derived from well-defined ethnic groups to the majority of Brazilians. Recognition of this fact has prompted the creation of the Brazilian Pharmacogenomics Network or Refargen (Suarez-Kurtz, 2004), a nationwide consortium of research groups, mostly from academia[1]. In consonance with its mission to provide leadership in PGx research and education in Brazil, with impact on population heath (Suarez-Kurtz, 2009), Refargen has recently concluded a comprehensive study of the distribution of PGx polymorphisms among Brazilians. In this article, we will present original data and review previously published results (Suarez-Kurtz et al., 2010, 2012a,b,c; Pena et al., 2011; Sortica et al., 2012) from the Refargen study and discuss the PGx implications of the findings for Brazilians and possibly other admixed populations of the Americas.

The study cohort consisted of 1,034 healthy, unrelated adults recruited in the North, Northeast, Southeast, and South regions of Brazil (**Figure 1**). Each individual signed a written informed consent and was asked to self-identify according to the classification scheme adopted by the Brazilian Census[2], which relies on self-perception of skin color. Accordingly, the subjects were distributed into three groups: *branco* (White, $n = 342$), *pardo* (Brown, $n = 350$), and *preto* (Black, $n = 342$). The term Color is capitalized throughout the text, to call attention to its special meaning in the context of the Brazilian Census classification. This cohort is considered representative of the present-day Brazilian population since 99% of Brazilians self-identify in one of the three Color categories, and 93% live in one of the four regions, included in the study[3]. Individuals from the Center-West region (7% of the

[1] http://www.refargen.org.br

[2] http://www.ibge.gov.br

[3] http://www.sidra.ibge.gov.br/bda/tabela/listabl.asp?z=t&c=262

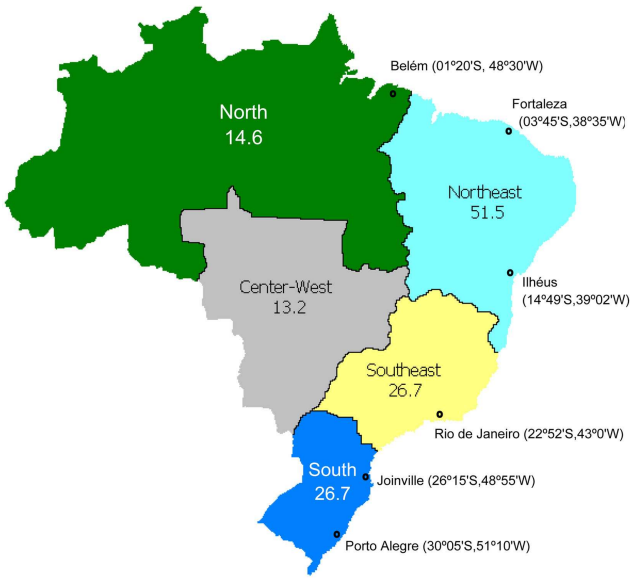

FIGURE 1 | Map of Brazil, showing its five geographical regions, their populations (in millions), and cities where individuals were recruited for the Refargen study, reviewed in this article.

Brazilian population) and those classified as "Yellow" (meaning Asian descendants, 0.7%) or Amerindian (0.3%) were not included in the study. We genotyped 44 loci in 12 pharmacogenes (**Table 1**) which modulate drug metabolism (*CYP2B6*, *CYP2C8*, *CYP2C9*, *CYP2C19*, *CYP2D6*, *CYP3A5*, *COMT*, and *TPMT*), transport (*ABCB1*, *SLCO1B1*, and *SLCO1B3*) and effect (*VKORC1*). Pharmacogenomics Knowledge Base (PharmGKB[4]) lists all these genes, except *SCLO1B3*, as "Important PGx genes (VIP)" and two thirds of the 44 polymorphisms investigated as "Important Variants."

We will initially present data for the overall cohort and for each Color group within this cohort. **Figure 2** shows frequency histograms of the total number of minor alleles identified in each individual. No statistically significant difference (Kruskal–Wallis test $p = 0.92$) was detected across the three Color groups, the median (interquartile range) number of polymorphisms being 17 (14–20), 16 (13–18), and 16 (13–19) in White, Brown, and Black individuals. This adds to 18.9% of the total number of alleles genotyped at the 44 loci in the overall cohort. However, the allele frequency at 11 of these loci differed significantly (chi square $p < 0.05$) across the Color groups. The pharmacogenes affected were *ABCB1* (2 SNPs), *CYP2C8* (1) *CYP3A5* (3), *NAT2* (3), *SLCO1B1* (1), *SCLO1B3* (2 SNPs, which are in complete LD) and *VKORC1* (2). We applied the Wright's F_{ST} statistics (Wright, 1951) to estimate the extent of PGx divergence among the three Color strata, and observed mean F_{ST} values of 0.005 (SD 0.006), 0.013 (0.017), and 0.004 (0.005) for pair-wise comparisons of White vs. Brown, White vs. Black, and Brown vs. Black, respectively (Suarez-Kurtz et al., 2012b). According to Wright's qualitative guidelines (Wright, 1978), F_{ST} values lower than 0.05 denote low genetic diversity, whereas values between 0.05 and 0.15 indicate moderate diversity. As shown in **Table 1**, only three SNPs, namely *CYP3A5*3* and the linked *SLCO1B3* 334T > C and 699G > A transitions exceeded, and two other SNPs (*ABCB1* 2677G > nonG and *CYP3A5*6*) approached, the F_{ST} threshold for moderate genetic divergence in White vs. Black Brazilians in the entire cohort. Not surprisingly, these were the SNPs with the smallest p values for the Kruskal–Wallis analyses of frequency distribution in the overall cohort (<0.0001–0.0006, **Table 1**). Taken together, the F_{ST} analyses in the overall cohort suggest low PGx divergence at all loci interrogated in self-identified Brown vs. White or Black individuals, whereas moderate divergence was observed at three, and possibly five loci (out of the 44 investigated) in pair-wise comparisons of White vs. Black Brazilians.

DISTRIBUTION OF PHARMACOGENETIC POLYMORPHISMS AMONG BRAZILIANS ACCORDING TO COLOR CATEGORIES AND GEOGRAPHICAL REGIONS

With an area of 8,511,960 Km^2, Brazil is a country of continental size (the fifth largest in the world) and different regions have diverse population histories. For instance, the North had a large influence of the Amerindian root, the Northeast had a history of strong African presence due to slavery and the South was mostly settled by European immigrants (Pena et al., 2011). We have applied multinomial log-linear regression analyses (Suarez-Kurtz et al., 2010, 2012c; Sortica et al., 2012) to infer the statistical association between allele, genotype, and haplotype distributions among Brazilians (response variables) and self-reported Color and geographical region (explanatory variables). This procedure obviates the need for correction for multiple comparisons, because the main effects and interaction terms are tested simultaneously within each regression context. **Table 2** illustrates results from this exercise, applied to selected genes affecting drug metabolism (*CYP2C8*, *CYP2C9*, and *CYP2C19*), transport (*ABCB1* and *SLCO1B1*) and response (*VKORC1*). Color *per se* associates significantly with the frequency distribution of *CYP2C8* and *CYP2C9* variant alleles, *ABCB1* and *SLCO1B1* haplotypes, and *VKOC1 3673G > A* alleles and genotypes; no association is observed with respect to the *CYP2C19* polymorphisms. Color in combination with geographical region is significantly associated with distribution of *CYP2C8* and *CYP2C9* alleles, *ABCB1* and *SLCO1B1* haplotypes, whereas geographical region *per se* associates with *CYP2C8* and *CYP2C9* allele frequency.

We explored further the PGx heterogeneity among Brazilians by the F_{ST} statistics. First, we performed pair-wise comparisons between Color groups within each geographical region, and detected significant differences in the distribution of F_{ST} values for White vs. Brown ($P < 0.0001$, ANOVA) and White vs. Black ($P < 0.0001$), but not Brown vs. Black individuals, across regions (Suarez-Kurtz et al., 2012b). This implies that the extent of pharmacogenetic divergence between Whites and Non-Whites (i.e., Black and Brown individuals) varies significantly among regions. The data presented in **Figure 3** supports this interpretation: we show that 10 selected polymorphisms in *ABCB1*, *CYP2D6*, *CYP3A5*, *SLCO1B1*, *SCLO1B3*, and *VKORC1* display moderate divergence between Whites and Blacks in the South, compared

[4]http://www.pharmgkb.org/search/browseVip.action?browseKey=annotatedGenes

Table 1 | Distribution of pharmacogenetic polymorphisms among Brazilians.

Gene	Polymorphism	Id #	Effect	Minor allele frequency				Chi square	F_{ST}		
				Overall	White	Brown	Black	*P* value	White vs. Black	White vs. Brown	Brown vs. Black
ABCB1	1267C > T	rs1128503	G412G	0.380	0.412	0.362	0.312	0.18	0.011	0.003	0.003
	2677G > T/A	rs2032582	S193A/T	0.370	0.417	0.343	0.221	**0.0006**	0.044	0.006	0.019
	3435C > T	rs1045642	I1145I	0.427	0.458	0.411	0.317	**0.037**	0.021	0.002	0.009
COMT	472G > A	rs4680	V158M	0.408	0.437	0.374	0.411	0.52	0.001	0.004	0.001
CYP2B6	64C > T	rs8192709	R22C	0.081	0.067	0.099	0.078	0.60	0.001	0.003	0.001
	785A > G	rs2279343	K262R	0.303	0.295	0.300	0.374	0.22	0.007	0.000	0.006
	1459C > T	rs3211371	R487C	0.172	0.213	0.135	0.127	0.068	0.013	0.011	0.000
	516G > T	rs3745274	Q172H	0.369	0.393	0.325	0.458	0.055	0.004	0.005	0.019
CYP2C8	805A > T	rs11572103	I269F	0.047	0.026	0.060	0.106	**0.012**	0.026	0.007	0.007
	416G > A	rs11572080	R139K	0.098	0.125	0.075	0.058	0.08	0.014	0.007	0.001
	792C > G	rs1058930	I264M	0.038	0.040	0.039	0.020	0.54	0.004	0.000	0.003
CYP2C9	430C > T	rs1799853	R144C	0.108	0.137	0.082	0.066	0.08	0.004	0.001	0.007
	1075A > C	rs1057910	I359L	0.052	0.049	0.059	0.026	0.26	0.014	0.008	0.001
	1080C > G	rs28371686	D360E	0.005	0.004	0.005	0.009	1.0	0.001	0.000	0.000
	1003C > T	rs28371685	R335W	0.008	0.010	0.005	0.009	0.78	0.000	0.001	0.000
CYP2C19	681G > A	rs4244285	Splicing defect	0.115	0.106	0.120	0.144	0.58	0.003	0.001	0.001
	636G > A	rs4986893	W212X	0.000	0.000	0.000	0.003	1.0	0.000	0.000	0.000
	−806C > T	rs12248560	Increased transcription	0.169	0.168	0.167	0.175	0.98	0.000	0.000	0.000
CYP2D6	−1584C > G	rs1080985	Promoter region	0.218	0.235	0.207	0.178	0.64	0.003	0.003	0.000
	31G > A	rs769258	V11M	0.035	0.043	0.027	0.023	0.60	0.002	0.002	0.000
	100C > T	rs1065852	P34S	0.136	0.150	0.124	0.115	0.79	0.003	0.002	0.000
	1023C > T	rs28371706	T107I	0.044	0.021	0.057	0.111	0.08	0.025	0.011	0.003
	1661G > C	rs1058164	V136V	0.456	0.461	0.445	0.482	0.89	0.000	0.000	0.001
	1846G > A	rs3892097	Splicing defect	0.117	0.134	0.105	0.080	0.61	0.003	0.004	0.000
	2549A > del	rs35742686	Frame shift	0.009	0.011	0.007	0.006	0.60	0.000	0.000	0.000
	2615-2617delAAG	rs5030656	K281del	0.013	0.014	0.011	0.013	1.0	0.001	0.002	0.001
	2850C > T	rs16947	R296C	0.390	0.352	0.417	0.480	0.23	0.016	0.003	0.005
	3183G > A	rs59421388	V287M	0.026	0.011	0.038	0.056	0.25	0.021	0.011	0.002
	4180G > C	rs1135840	S486T	0.531	0.513	0.541	0.582	0.74	0.004	0.000	0.002
CYP3A5	6986A > G	rs776746	Frame shift	0.698	0.785	0.627	0.541	<**0.0001**	**0.067**	0.030	0.007
	14690G > A	rs10264272	Splicing defect	0.027	0.004	0.039	0.105	**0.0001**	0.049	0.014	0.017
	23132insT	rs413003343	Frame shift	0.024	0.013	0.027	0.079	**0.003**	0.025	0.003	0.013
SLCO1B1	388A > G	rs2306283	N130D	0.553	0.498	0.601	0.635	**0.036**	0.019	0.011	0.001
	463C > A	rs11045819	P155T	0.118	0.118	0.122	0.097	0.77	0.001	0.000	0.002
	521T > C	rs4149056	V174A	0.135	0.135	0.143	0.089	0.24	0.005	0.000	0.007
SLCO1B3	334T > G	rs4149117	S112A	0.741	0.799	0.702	0.592	**0.0003**	**0.051**	0.013	0.013
	699G > A	rs7311358	M233I	0.741	0.799	0.702	0.592	**0.0003**	**0.051**	0.013	0.013
TMPT	238G > C	rs1800462	A80P	0.008	0.001	0.014	0.014	0.36	0.006	0.006	0.000
	460G > A	rs1800460	A154T	0.010	0.009	0.011	0.010	0.82	0.000	0.000	0.000
	719A > G	rs1142345	Y240C	0.026	0.017	0.037	0.017	0.46	0.000	0.004	0.004
VKORC1	3673G > A	rs9923231	Reduced transcription	0.333	0.371	0.306	0.238	**0.038**	0.021	0.005	0.006
	5808C > T	rs2884737	Intronic	0.193	0.255	0.135	0.128	**0.003**	0.026	0.023	0.000
	6853G > C	rs8050894	Intronic	0.392	0.404	0.386	0.357	0.65	0.002	0.000	0.001
	9104G > A	rs7294	3-UTP	0.375	0.376	0.372	0.379	0.65	0.000	0.000	0.000

Bold numbers in the column "chi square" indicate statistically significant P values; bold numbers in the "White vs. Black" column indicate moderate pharmacogenetic divergence.

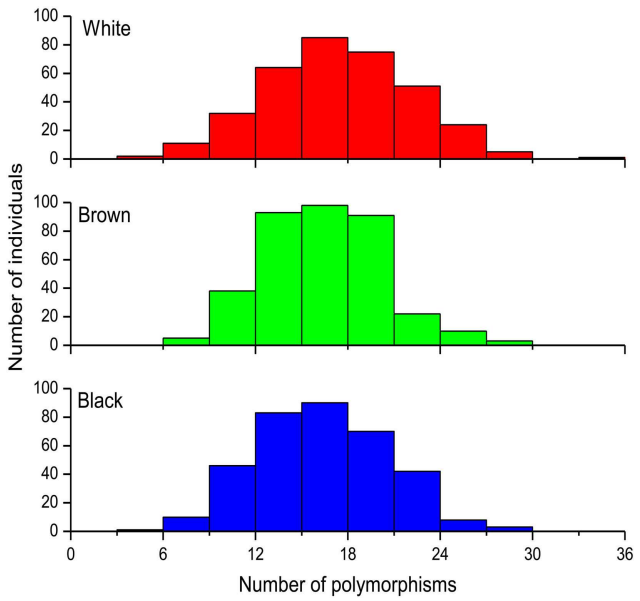

FIGURE 2 | Frequency histograms of the distribution of 44 PGx polymorphisms among self-identified (White $n = 342$), Brown ($n = 352$), and Black Brazilians ($n = 342$). The data represent the total number of minor alleles at the 44 loci, in each individual.

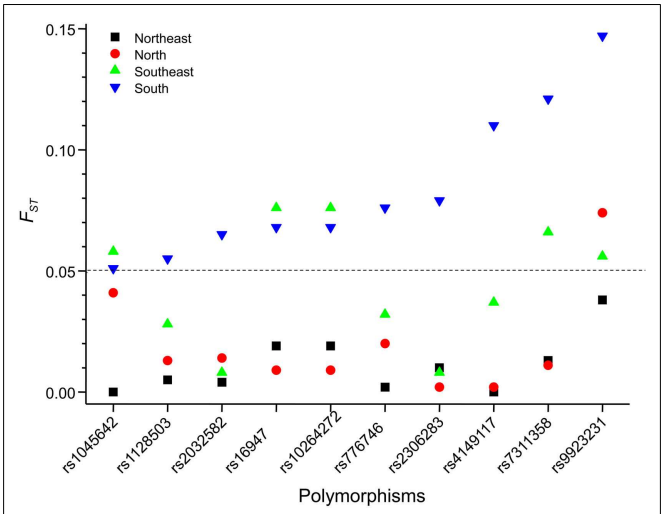

FIGURE 3 | Allele-specific F_{ST} values for 10 PGx polymorphisms (x-axis) in White vs. Black Brazilians recruited at the North, Northeast, Southeast, and South regions. The dashed line shows the threshold F_{ST} values (0.05) for moderate genetic divergence. The genes and loci for each polymorphism are presented in **Table 1**.

Table 2 | Multinominal log-linear analyses of the distribution of pharmacogenetic polymorphisms alleles among Brazilians according to self-reported color and geographical region.

Gene	Alleles	Explanatory variables[a]		
		Color	Geographical region	Color: geographical region
CYP2C8	*2, *3, *4	<0.0001	0.71	0.04
CYP2C9	*2, *3, *5, *11	<0.0001	0.23	0.01
CYP2C19	*2, *3, *17	0.60	0.11	0.33
ABCB1	haplotypes[b]	<0.001	0.001	0.013
SLCO1B1	haplotypes[c]	<0.001	0.001	0.003
VKORC1	3673A allele	0.0004	0.07	0.11
	3673A genotype	0.002	0.18	0.19

[a] *p values associated to the "main effects" (Color and geographical region) and their "interaction."*

[b] *Haplotypes comprising the 1236C > T, 2677G > nonG, and 3435C > T loci.*

[c] *Haplotypes comprising the 388A > G, 463C > A, and 521T > C loci.*

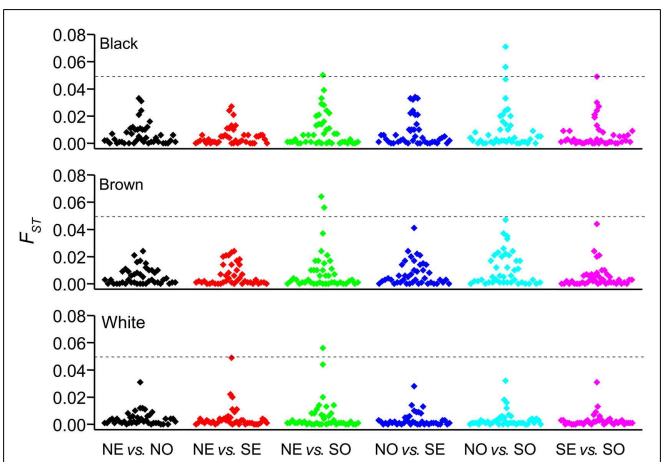

FIGURE 4 | Allele-specific F_{ST} values (y-axis) for pair-wise comparisons between geographical regions (x-axis). Data are presented separately for self-identified Black (top panel), Brown (middle panel), and White (bottom panel) individuals. Each symbol correspond to one of the 44 polymorphisms listed in **Table 1**. The dashed line shows the threshold F_{ST} values (0.05) for moderate genetic divergence. NO, North; NE, Northeast; SE, Southeast; SO, South.

to five, one, and zero in the Southeast, North, and Northeast, respectively. In a second exercise, we compared F_{ST} values for each Color between regions and present the results in **Figure 4**. Of the 792 (44 polymorphisms × six pair-wise regions × three Color groups) comparisons, only three SNPs among Black, one among Brown, and one among White individuals exceeded the threshold ($F_{ST} = 0.05$) for moderate PGx divergence. Taken together, these F_{ST} results extend the conclusions of the multinomial analyses described above, that the distribution of PGx polymorphisms among Brazilians is influenced by self-reported Color, geographical region, and the interaction of these two variables. Collectively, these data reflect the notorious heterogeneity of the Brazilian population and highlight the inappropriateness of ascribing PGx polymorphisms' frequencies for "Brazilians" based on data from one or more Color strata recruited at a given region (or city).

IMPACT OF BIOGEOGRAPHICAL ANCESTRY ON THE DISTRIBUTION OF PHARMACOGENETIC POLYMORPHISMS AMONG BRAZILIANS

These analyses were based on the individual proportions of European, African, and Amerindian ancestry, estimated using a panel of short insertion/deletion polymorphisms, validated as ancestry-informative markers (Bastos-Rodrigues et al., 2006), and the STRUCTURE clustering software (Pritchard et al., 2000). These data, available for 965 subjects confirmed that the vast majority of Brazilians, irrespective of self-reported Color, share European and African ancestries in variable proportions, and a sizable number of individuals display also distinct Amerindian ancestry (Suarez-Kurtz and Pena, 2006, 2007; Suarez-Kurtz et al., 2010; Pena et al., 2011). The average proportions of European ancestry decrease progressively from self-reported White (mean 0.80, SD 0.21, $n = 325$), to Brown (0.62, 0.29, 322) and then to Black individuals (0.46, 0.20, 318), and the opposite trend is observed with respect to African ancestry, which averaged 0.10 (SD 0.14) in White, 0.25 (0.26) in Brown, and 0.42 (0.29) in Black persons. However, the individual proportions of European and African ancestry varies widely, and most importantly, as a continuum within each of these three Color categories, whereas the individual proportion of Amerindian ancestry remains relatively constant across the three groups, ranging from 0.10 to 0.13. To describe the association between PGx polymorphisms and the estimated individual biogeographical ancestry we fitted non-linear logistic regression modeling using maximum likelihood estimation. A consistent finding in these analyses (Suarez-Kurtz et al., 2007a,b, 2010, 2012c; Estrela et al., 2008; Vargens et al., 2008) is that the frequency distribution of PGx polymorphisms among Brazilians is best fit by continuous functions of the individual proportions of African and European ancestry. This is illustrated in **Figures 5** and **6**. In **Figure 5** we show that the probability of having the wild-type (C/G/C) and the T/G/C *ABCB1* haplotypes increases continuously with the increase in African ancestry, whereas the opposite trend is observed for the T/nonG/T haplotype. **Figure 6** shows that the odds of having the heterozygous, and to a lesser extent, the homozygous variant genotype at the *VKORC1* 3673G > A locus increase progressively as the individual proportion of European ancestry increases. For comparison, we also display in **Figure 6** the distribution of *VKORC1* 3673G > A genotypes among Portuguese, by far the most important source of European migrants from Brazil, and individuals from Angola and Mozambique, two former Portuguese colonies in Africa, and origin of enslaved Africans brought to Brazil.

Considering that the European and African components together account for 89% of the diversity in individual genetic ancestry in the Refargen cohort (Pena et al., 2011), it might be anticipated that: (a) the greater the difference in frequency of a given polymorphism between Europeans and sub-Saharan Africans, the wider the range of frequency variation among Brazilians; (b) the range of variation among Brazilians will be smaller than the difference in frequency between Europeans and Africans, because of the admixture of these ancestral roots in Brazilians. We have previously verified both these predictions for polymorphisms in *VKORC1* (Suarez-Kurtz et al., 2010) and within the *CYP2C* cluster (Suarez-Kurtz et al., 2012c). We applied the F_{ST} statistics

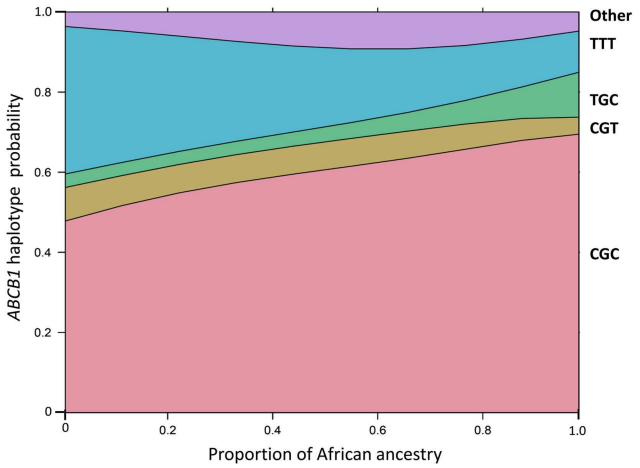

FIGURE 5 | Effect display for the distribution of *ABCB1* haplotypes in the logit model fit to the data for African ancestry in 965 Brazilians. The haplotypes comprising the 1236C > T, 2677G > nonG, and 3435C > T SNPs are shown at the right of the plot. The individual proportion of African ancestry is shown in the *x*-axis. The *y*-axis is labeled on the probability scale. The plot was generated as described by Venables and Ripley (2002) and implemented as function "multinom" available in the R package "nnet." Data from Sortica et al. (2012).

to examine these predictions in 38 polymorphisms which were genotyped in the Refargen cohort and also in the HapMap project. In **Figure 7** we shown the pair-wise F_{ST} values for each polymorphism in HapMap CEU vs. YRI cohorts – taken as proxies of the European and sub-Saharan African ancestral roots of Brazilians, respectively – and Brazilians with >90% European ancestry vs. Brazilians with >80% African ancestry. The attenuation of pharmacogenetic divergence between the Brazilian groups compared to the HapMap populations is evident.

CONCLUDING REMARKS AND PERSPECTIVES

The kaleidoscopic diversity of the admixed Brazilian population, with tri-hybrid biogeographical ancestry in Europe, Africa, and America adds complexity to, but also creates advantages for PGx research. Advantages include the opportunity to explore PGx associations in individuals with heterogeneous genetic ancestry under similar environmental and socio-economical conditions, and to gather information on peoples that are excluded or underrepresented in clinical drug trials, such as sub-Saharan Africans and Native Americans. A major challenge to PGx studies in Brazil is population stratification, which if not controlled for, will confound the outcomes of PGx association studies. Our studies describe ways to control for this caveat, by combining ancestry-informative markers and appropriate statistical approaches. A distinct message that emerges from these studies is that race/color categorization does not capture the distribution of PGx polymorphisms among Brazilians, which is best modeled by continuous functions of the individual proportions of European and African ancestry, irrespective of self-identified Color (Suarez-Kurtz, 2010). Recognition of this fact is important in the design and interpretation of PGx clinical trials in Brazilians but does not imply that PGx-informed drug

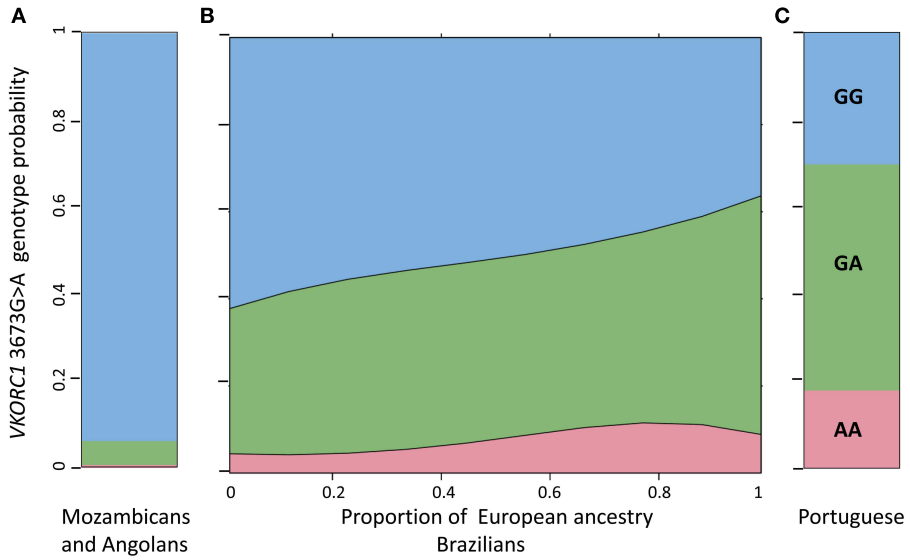

FIGURE 6 | Effect display for the distribution of *VKORC1* 3673G > A genotypes in the logit model fit to the data for African ancestry in 965 Brazilians (B). For comparison the frequency of each genotype in a cohort of Angolans and Mozambicans [$n = 216$, **(A)**] and in a Portuguese cohort [$n = 89$, **(C)**] are also shown. The individual proportion of African ancestry in Brazilians is shown in the *x*-axis. The *y*-axis represents the genotype probability for Brazilians and the observed genotype frequency for the African and Portuguese cohorts. Data from Suarez-Kurtz et al. (2010). The plot for Brazilians was generated as described in **Figure 5**.

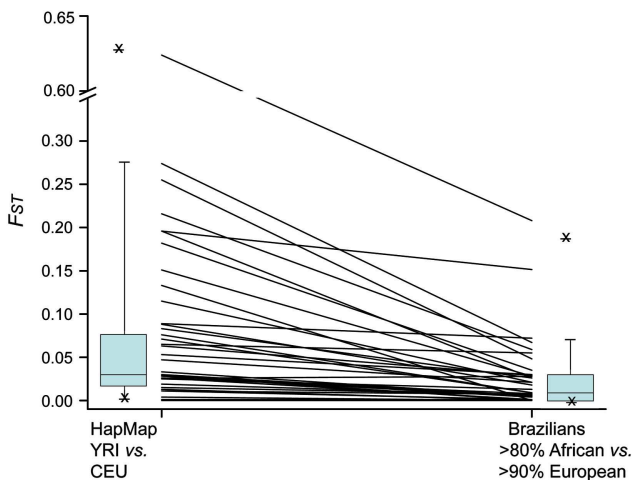

FIGURE 7 | Allele-specific F_{ST} values for 38 PGx polymorphisms in HapMap CEU vs. YRI and in Brazilians with >90% European ancestry vs. Brazilians with >80% African ancestry. The lines connect the F_{ST} values for each polymorphism in the two data sets and the box plots at the left and right summarize the ensemble of the data for each set.

prescription requires investigation of individual ancestry. Rather, individual genotyping should be directed to PGx polymorphisms of proven clinical utility for the specific medical condition being treated, irrespective of biogeographical ancestry.

Drug assessment and regulatory processes in Brazil are carried out by the National Health Surveillance Agency, ANVISA, an independently administered, financially autonomous agency, managed by a Collegiate Board of Directors[5]. ANVISA has the mandate to grant, and withdraw, product registration permits within its areas of activity, which comprise medicines for human use. Registration of new medicines do not require, that clinical trials be carried out in the Brazilian population, and evaluation of the medicine's efficacy and toxicity is based mainly, if not exclusively, on foreign data. Despite the increasing enrolment of non–Caucasian subjects in global drug development programs, most data submitted to ANVISA derive from white Europeans and North Americans. We have recently shown that there is little pharmacogenetic divergence between the HapMap CEU cohort of European extraction and White Brazilians, such that only *CYP3A5∗3* among 44 polymorphisms exceeded the F_{ST} threshold for moderate divergence. By contrast, F_{ST} analyses revealed very large divergence between CEU and Black Brazilians for *CYP3A5∗3* and moderate divergence for eight other polymorphisms, including another *CYP3A5* SNP (*CYP3A5∗6*) and SNPs in the *ABCB1*, *SLCO1B3*, and *SLCO1B1* genes. These findings represent a caveat against extrapolation of PGx data from European-derived ("Caucasian") cohorts to the ensemble of Brazilians.

Admixture is common in all developing nations in the American continent, although the relative contributions of the three major ancestral roots – native American, European, and sub-Saharan African – vary among these nations, as well as among ethnic groups and geographical regions within a given country. Hence, extrapolation of conclusions drawn from PGx studies in Brazilians to other admixed Latin American populations

[5]http://www.anvisa.gov.br/eng/index.htm

must take into account the specific patterns of population structure and diversity across the Americas. Therapeutic drugs are usually developed and investigated for their safety and efficacy in geographical and ethnical populations that do not encompass the diversity of Latin American peoples. Drivers and barriers to the adoption of PGx in developing countries, and specific ways in which these countries could benefit from PGx-based drug therapy deserve greater attention from academic and industrial scientists, prescribers, and legislators in developing nations across the Americas. This goal is not likely to be achieved simply by mandates to include subjects from ethnic minorities in clinical drug trials, especially when these groups are labeled by phenotypes which do not accurately reflect genetic ancestry (Suarez-Kurtz, 2005, 2010; Suarez-Kurtz and Pena, 2006, 2007).

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Impact of the interaction between 3′-UTR SNPs and microRNA on the expression of human xenobiotic metabolism enzyme and transporter genes

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Genetic variation in the expression of human xenobiotic metabolism enzymes and transporters (XMETs) leads to inter-individual variability in metabolism of therapeutic agents as well as differed susceptibility to various diseases. Recent expression quantitative traits loci (eQTL) mapping in a few human cells/tissues have identified a number of single nucleotide polymorphisms (SNPs) significantly associated with mRNA expression of many XMET genes. These eQTLs are therefore important candidate markers for pharmacogenetic studies. However, questions remain about whether these SNPs are causative and in what mechanism these SNPs may function. Given the important role of microRNAs (miRs) in gene transcription regulation, we hypothesize that those eQTLs or their proxies in strong linkage disequilibrium (LD) altering miR targeting are likely causative SNPs affecting gene expression. The aim of this study is to identify eQTLs potentially regulating major XMETs via interference with miR targeting. To this end, we performed a genome-wide screening for eQTLs for 409 genes encoding major drug metabolism enzymes, transporters and transcription factors, in publically available eQTL datasets generated from the HapMap lymphoblastoid cell lines and human liver and brain tissue. As a result, 308 eQTLs significantly ($p < 10^{-5}$) associated with mRNA expression of 101 genes were identified. We further identified 7,869 SNPs in strong LD ($r^2 \geq 0.8$) with these eQTLs using the 1,000 Genome SNP data. Among these 8,177 SNPs, 27 are located in the 3′-UTR of 14 genes. Using two algorithms predicting miR-SNP interaction, we found that almost all these SNPs (26 out of 27) were predicted to create, abolish, or change the target site for miRs in both algorithms. Many of these miRs were also expressed in the same tissue that the eQTL were identified. Our study provides a strong rationale for continued investigation for the functions of these eQTLs in pharmacogenetic settings.

Keywords: eQTL, xenobiotic metabolism enzyme and transporter, microRNA, pharmacogenetics, 3′-UTR

INTRODUCTION

Xenobiotic metabolizing enzymes and transporters (XMETs) are involved in biotransformation and detoxification of carcinogens, environmental toxins, and therapeutic drugs (Carlsten et al., 2008; Korkina et al., 2009). In humans, the process of biotransformation and detoxification of xenobiotics by XMETs can be divided into three phases: modification (phase I) primarily by enzymes of the cytochromes P450 superfamily; conjugation (phase II), e.g., glucuronidation by UDP-glucuronosyl transferase; and excretion (phase III) mainly by membrane transporters. XMETs are expressed in almost all tissue types, centrally and locally protecting the entire body against the damages caused by various natural and synthetic compounds. XMETs are highly expressed in digestive tract and especially in the liver, the most important organ for central metabolism (Conde-Vancells et al., 2010). Variations in the expression and activity of these XMETs lead to significant inter-individual difference in the disposition of exogenous chemicals including absorption, distribution, metabolism, and excretion (ADME) of pharmaceutical drugs. On the other hand, many XMETs are also found to be very abundant in non-digestive tract tissues/cells, e.g., brain, lung, bladder, and blood (Pavek and Dvorak, 2008). These XMETs could affect the local response to certain drugs at the site of action. Meanwhile, due to the crucial role of XMETs in detoxification of carcinogens and toxins, genetic variation in XMETs function in specific tissues/organs is also an important mechanism underlying genetic susceptibility to certain diseases, e.g., those XMETs expressed in lung and bladder may modify cancer risk. Recent genome-wide association studies have identified polymorphisms at the *UGT1A* locus strongly associated with urinary bladder

cancer risk (Selinski et al., 2012). XMETs are sensitively regulated by various nuclear receptors (NRs) and transcription factors (TFs). These *trans*-acting regulators play a pivotal role in mediating cellular response to exposure to xenobiotics by modulating the transcription of XMETs, thus significantly contributing to the variability in the function of XMETs (Bourgine et al., 2012).

Identifying the DNA polymorphisms leading to the variations in XMET function is a major area of interest in pharmacogenetic and genomic research. To date, numerous studies focused on individual XMET genes have discovered a large number of sequence variations, many of which alter protein coding sequence and consequently affecting the activity of XMETs (Adjei et al., 2003; Hildebrandt et al., 2004; Ji et al., 2005; Moyer et al., 2007; Mrozikiewicz et al., 2011). Meanwhile, even more variants were suggested to quantitatively modulate gene transcription (Pavek and Dvorak, 2008). Recently, genome-wide mapping for gene expression quantitative trait loci (eQTLs) in a few human tissues/cells offered unprecedented opportunities to identify the most influential single nucleotide polymorphisms (SNPs) determining gene expression level of XMETs (Gamazon et al., 2010). However, unlike the variants located in the protein coding sequences for which the causality for altered enzyme activity can be more easily understood, how eQTLs affect gene transcription is largely unknown. Understanding the underlying mechanisms will lead to identification of novel causative DNA variants for XMET function as well as reliable pharmacogenetic markers.

MicroRNAs (miRs) are single stranded, about 22-nucleotides (nt) long, evolutionarily conserved, and function as important posttranscriptional regulators of mRNA expression by binding to the 3′-UTR of target mRNAs (Ambros, 2004; Bartel, 2004). MiRs are involved in various developmental and physiological processes by negatively regulating gene expression (Zhang et al., 2007). Over 30% of all protein-coding genes were estimated to be regulated by miRs (Brennecke et al., 2005; Krek et al., 2005; Lewis et al., 2005; Lim et al., 2005). Due to the conservation of the miR target site, SNPs located in 3′-UTR sequences may abolish or create a miR target, thus significantly affecting the mRNA expression (Saunders et al., 2007). Previous studies have suggested that many XMETs are regulated by miRs (Tsuchiya et al., 2006; Takagi et al., 2010; Patron et al., 2012). Several studies also demonstrated that SNPs in XMET gene 3′-UTRs led to different levels of enzyme activity (Saunders et al., 2007; Chin et al., 2008). Hence, we hypothesized that it may be an important mechanism that common SNPs or their linkage disequilibrium (LD) proxies located in the XMET gene 3′-UTR sequences alter mRNA expression via interference with miR targeting. In order to identify these candidate SNPs that may significantly modulate XMET expression, in this study we used multiple published human eQTL datasets to perform an *in silico* screening for SNPs that highly correlated with mRNA level of 409 major XMET genes. The significant SNPs and/or their LD proxies located in the gene 3′-UTRs were selected to predict a potential interference with miRs. We found that 27 SNPs located in the 3′-UTR of 14 XMET genes are likely associated with gene expression via altering miR binding.

MATERIALS AND METHODS

SELECTION OF eQTLs

The general strategy for the data analysis was presented in **Figure 1**. We used the published eQTLs datasets generated from the HapMap lymphoblastoid cell lines (LCLs; Montgomery et al., 2010), human liver (Schadt et al., 2008), and human brain (Gibbs et al., 2010). Although additional eQTL datasets in human LCLs are also available, we chose to use the one by Montgomery et al. (2010) which utilized high-throughput sequencing for the quantification of gene expression, as this technology has been suggested to produce more accurate gene expression data. To our knowledge, all datasets were collected from tissue/cells derived from individuals of Caucasian in origin. We used the online tool[1] to search statistically significant eQTLs. As our study was focused on *cis*-acting eQTLs, we used a cut-off of $p = 10^{-5}$ for significance, considering the window for genomic region (500 kb) of each gene and the potential number of SNPs (1 in every 100–1,000 bp).

SEARCH FOR SNPs IN LD WITH eQTLs

To search SNPs in LD with significant eQTLs, we used the SNAP[2] program to screen the 1,000 Genome SNP data within 500 kb range of the eQTLs of interest in the CEU population with a LD level cut-off of $R^2 = 0.8$. Annotation for the location of eQTLs and their proxies relative to the gene structure was also collected with

[1] http://www.ncbi.nlm.nih.gov/gtex/GTEX2/gtex.cgi
[2] http://www.broadinstitute.org/mpg/snap/ldsearch.php

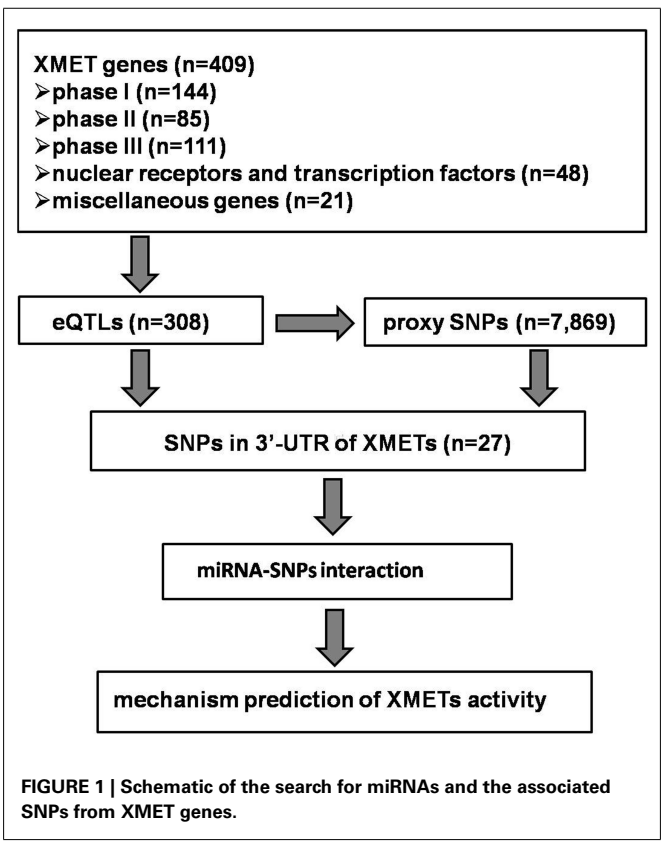

FIGURE 1 | Schematic of the search for miRNAs and the associated SNPs from XMET genes.

the program. Only SNPs and/or their proxies located within the 3′-UTR of the studied genes of interest were retained for further analyses.

PREDICTION OF SNP-miR INTERACTION

In order to predict the potential SNP-miR interaction, two programs, MicroSNiPer[3] and PolymiRTS[4] were used. The major difference between the two programs is the algorithm used to predict the target site of miRs. The PolymiRTS program used the TargetScan[5]; Lewis et al., 2005; Friedman et al., 2009) algorithm (Bao et al., 2007). In contrast, the MicroSNiPer program used the FASTA (Pearson and Lipman, 1988) alignment program to determine if a change in a nucleotide in 3′-UTR sequence would change the miR binding capability, based on the requirement of perfect Watson–Crick match to the seed 2–7 nt of miRs (Lewis et al., 2005). To be conservative, we used 7-mers match as the cut-off value for a positive prediction.

RESULTS

GENOME-WIDE eQTL ANALYSIS OF XMETs

Expression quantitative traits loci were screened for all 409 major XMET genes, including 144 phase I, 85 phase II and 111 phase III genes, 48 NRs, and transcription factor genes as well as another 21 genes related to drug ADME (**Table A1** in Appendix). As a result, a total of 308 significant ($p < 10^{-5}$) eQTLs were identified from 101 XMET genes. These include nine in LCL, 83 in liver, and 221 in brain tissues. Five SNPs were found as eQTLs shared in two tissue types: rs1023252 in both LCL and brain tissues, rs11101992, rs156697, rs2071474, and rs241440 in both liver and brain tissues (**Figure 2**). Among the total of 308 eQTLs, 20 SNPs were found to be located in the 3′-UTR region; 3 SNPs were in the 5′-UTRs; 171 SNPs were intronic; 8 and 6 SNPs were synonymous and non-synonymous coding variants, respectively; and 12 and 15 SNPs were located in the upstream and downstream flanking region of the genes, respectively. The remaining 73 SNPs were located in intergenic regions.

[3] http://cbdb.nimh.nih.gov/microsniper
[4] http://compbio.uthsc.edu/miRSNP/
[5] http://www.TargetScan.org/

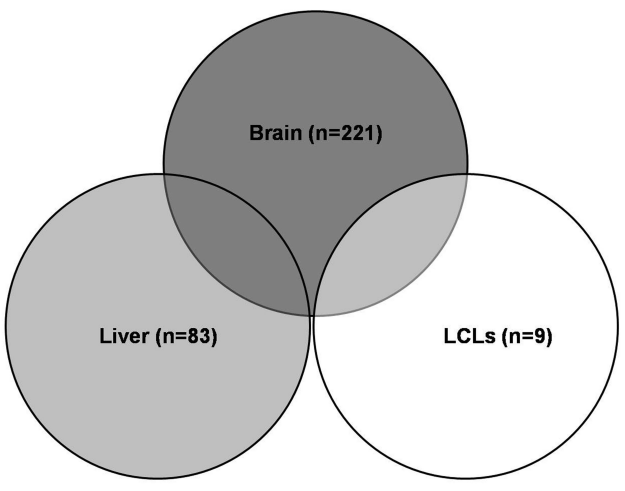

FIGURE 2 | Significant eQTLs in different tissues. A total of 308 significant eQTLs were identified, including 9 eQTLs in LCL, 83 in liver, and 221 in brain tissues. Five eQTLs were shared in two tissue types.

eQTLs AND THEIR LD PROXIES

We chose to screen the 1,000 Genome SNP dataset as this would produce the most comprehensive coverage for the SNPs that may be in LD with a given eQTL. A total of 7,869 SNPs with significant LD with 260 eQTLs were identified. Combined with the remaining 48 eQTLs which had no reliable proxies in the 1,000 Genome dataset, a total of 8,177 SNPs (308 eQTLs and 7,869 proxy SNPs) were included in the subsequent analyses.

PREDICTION OF miR-SNPs INTERACTION

Of the 112 eQTLs and proxies located in the 3′-UTR sequences, 27 SNPs were found in the 3′-UTR of 14 genes of interest. The remaining SNPs were located in nearby genes thus were excluded from the subsequent analysis. These SNPs were all common SNPs with their minor allele frequency (MAF) $\geq$0.067. Among the 27 SNPs, 12 were found in liver, and 15 were identified in brain tissue. More detailed information for these SNPs was listed in **Table A2** in Appendix.

We focused our study on the association between miRs and these 27 SNPs in the 14 genes. After screened with the two algorithms, MicroSNiPer (Barenboim et al., 2010) and PolymiRTs (Gong et al., 2012), all the 27 SNPs apart from rs11807 (which is not predicted to be in a target site in PolymiRTs database) were found to potentially create, abolish, or alter the target site for miRs in both algorithms. Notably, 34 miRs were predicted by both algorithms to interact with 19 of these SNPs (**Table A2** in Appendix). Of these 34 overlap miRs, except for rs2480256 of CYP2E1 which is not located in the seed sequence of hsa-miR-570-3p, all the remaining SNPs were found to be located in the seed sequence of miR targets.

To further validate the interaction between miRs and SNPs, we investigated whether the identified miRs were expressed in the same tissue as the identified eQTL. We used the GEO datasets (GSE21279 and GSE26545) to screen miR expression in liver and brain tissues, respectively (Hou et al., 2011; Hu et al., 2011). Since many predicted miRs were new and not probed by the published platforms, we thus only concentrate on the list of miRs probed in the platforms. Overall, over 74% (20 out of 27) of the identified miR-SNPs were found to have at least one predicted miR co-expressed with the gene of interest in the same tissue.

We further aimed to investigate whether these 27 SNPs are more likely to be targeted by miRs especially by the co-expressed miR in liver and brain tissues, compared to random-selected 3′-UTR SNPs with similar MAF. No statistical significance were found, possibly due to the limited power caused by the small number ($n = 27$) of SNPs involved (data not shown).

DISCUSSION

Although a large number of DNA variants affecting the function of XMETs have been identified, and many of them have been well linked with clinical response to pharmacotherapy or disease susceptibility (Motsinger-Reif et al., 2010), genetic variations in the

activity of most XMETs remain incompletely explained. Recent studies continue to discover novel functional variants in XMET genes (Ramsey et al., 2012). Meanwhile, genome-wide association studies have found a number of XMET SNPs without previously known function significantly associated with different phenotypes in humans (Teichert et al., 2009; Estrada et al., 2012). These studies consistently suggested that additional sequence variants with fundamental role in XMET function have not been identified. Recent eQTL mapping in human tissues provided an opportunity to discover functional XMET polymorphisms at the genome-wide level. However, questions remain whether the identified eQTLs are causal for the altered gene expression and via what mechanism. Our study provides a comprehensive evaluation for this question in major human XMET genes, and generated a list of candidate SNPs that may modulate XMET genes via interference with miR targeting in multiple human tissue types.

Single nucleotide polymorphisms located in the gene 3′-UTRs could have great impact on miR targeting. It has been demonstrated that the entire 3′-UTR sequence could play important roles in miR function in addition to miR target sites (Hu and Bruno, 2011). In particular, negative selection in humans is stronger on computationally predicted conserved miR binding sites than on other conserved sequence motifs in 3′-UTRs, and polymorphisms in predicted miR binding sites are highly likely to be deleterious (Chen and Rajewsky, 2006). Gong et al. (2012) mapped SNPs to the 3′-UTRs of all human protein coding genes. Their results showed that among the 225,759 SNPs identified in 3′-UTRs, over 25% of SNPs potentially abolished 90,784 original miR target sites, while another 25% created a similar number of putative miRNA target sites. Besides these *in silico* studies, a number of SNPs altering miR targeting have been experimentally demonstrated to be associated with multiple diseases as well as drug metabolism and environmental procarcinogen detoxification (Abelson et al., 2005; Tan et al., 2007; Yu et al., 2007; Yokoi and Nakajima, 2011). Although the seed sequences for miR binding are critical and highly conserved, recent studies have also suggested that 3′-UTR sequences outside of the seed sequences, e.g., flanking sequences may be equally important for miR targeting by controlling the accessibility of the miR or local RNA structure (Grimson et al., 2007). For example, a SNP (829C > T) located 14 bp downstream of a miR-24 binding site in the 3′-UTR of human dihydrofolate reductase gene (*DHFR*) was demonstrated to affect *DHFR* expression by interfering with miR-24 function, resulting in *DHFR* over expression and methotrexate resistance (Mishra et al., 2007). By using two algorithms predicting potential SNP-miR interaction, we suggested that 27 eQTLs or their proxies in high LD for 14XMET genes may function through interference with one or more miRs, with most of the SNPs located in the seed sequences. Meanwhile, the majority (20 out of 27) of the identified miR-SNPs were found to have predicted miR co-expressed with the gene of interest in the same tissue. Although no statistically significant enrichment of miR targeting for these SNPs, the strong trends observed here warrants further experimental validations.

Our findings may also provide useful information in addition to the previous observations on the function of these SNPs. Previous studies demonstrated that SNP rs2480256 in the *CYP2E1* gene was significantly associated with systemic lupus erythematosus (Liao et al., 2011). Another study showed that cyclosporine A concentration in serum was significantly correlated with the genotype of the *CYP3A5* rs15524 polymorphism (Onizuka et al., 2011). In addition, a *GSTM3* haplotype including rs1537236 was significantly associated with a decreased growth for maximum mid-expiratory flow rate (MMEF) in a large population-based lung function study (Breton et al., 2009). SNP rs11807 in the 3′ region of *GSTM5* was found to be associated with hypertension (Delles et al., 2008). Our results thus may help further elucidate the mechanism(s) by which the SNPs are involved in the susceptibility to these specific phenotypes.

In conclusion, our study summarized the potentially interacting SNP-miRs that may affect the expression of major XMET gene, which may ultimately facilitate to elucidate the mechanism how these genes are regulated as well as how they are involved in the genetic variations in drug metabolism and disease pathogenesis. Further investigations are necessary to corroborate the hypotheses generated in this study.

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APPENDIX

Table A1 | Major XMETs and related genes investigated in this study.

Phase I ($n = 144$)	Phase II ($n = 85$)	Phase III ($n = 111$)	Nuclear receptors and transcription factors ($n = 48$)	Miscellaneous genes ($n = 21$)
AADAC	AANAT	ABC1	AHR	CRABP1
ABP1	ACSL1	ABCA1	AHRR	CRABP2
ADH1A	ACSL3	ABCA2	AIP	CYB5A
ADH1B	ACSL4	ABCA3	ARNT	GZMA
ADH1C	ACSM1	ABCA7	ARNT2	GZMB
ADH4	ACSM2B	ABCA8	CREBBP	MT1A
ADH5	ACSM3	ABCB1	EP300	MT1B
ADH6	AGXT	ABCB10	ESR1	MT1F
ADH7	AS3MT	ABCB11	ESR2	MT1H
ADHFE1	ASMT	ABCB4	FOXA2	MT1M
AKR1A1	BAAT	ABCB5	FOXO1	MT1X
AKR1B1	CCBL1	ABCB6	HIF1A	MT2A
AKR1B10	CES5A	ABCB7	HIF3A	MT3
AKR1C1	COMT	ABCB8	HNF4A	MT4
AKR1C2	DDOST	ABCB9	HSP90AA1	MTHFR
AKR1C3	GAMT	ABCC1	KEAP1	POR
AKR1C4	GGT1	ABCC10	NCOA1	RBP1
AKR1CL1	GLYAT	ABCC11	NCOA2	RBP2
AKR1D1	GNMT	ABCC12	NCOA3	TP53
AKR1E2	GSTA1	ABCC12	NCOR1	TXN
AKR7A2	GSTA2	ABCC2	NCOR2	TXN2
AKR7A3	GSTA3	ABCC3	NFE2L2	
AKR7L	GSTA4	ABCC4	NR0B2	
ALDH16A1	GSTA5	ABCC5	NR1H2	
ALDH18A1	GSTK1	ABCC6	NR1H3	
ALDH1A1	GSTM1	ABCC8	NR1H4	
ALDH1A2	GSTM2	ABCC9	NR1I2	
ALDH1A3	GSTM3	ABCD4	NR1I3	
ALDH1B1	GSTM4	ABCG2	NR3C1	
ALDH1L1	GSTM5	ABCG8	NR3C2	
ALDH2	GSTO1	ALD	NR5A2	
ALDH3A1	GSTO2	AQP1	PPARA	
ALDH3A2	GSTP1	AQP7	PPARD	
ALDH3B1	GSTT1	AQP9	PPARG	
ALDH3B2	GSTT2	ATP6V0C	PPARGC1A	
ALDH4A1	GSTT2B	ATP7A	PPARGC1B	
ALDH5A1	GSTZ1	ATP7B	PPRC1	
ALDH6A1	HNMT	KCNK9	PTGES3	
ALDH7A1	INMT	MARCKSL1	RARA	
ALDH8A1	MGST1	MDR/TAP	RARB	
ALDH9A1	MGST2	MRP	RARG	
AOC2	MGST3	MVP	RXRA	
AOC3	MPST	OABP	RXRB	
AOX1	NAA20	OATP2	RXRG	
BCHE	NAT1	SLC10A1	THRA	
CBR1	NAT2	SLC10A2	THRB	
CBR3	NNMT	SLC15A1	TRIP11	
CBR4	PNMT	SLC15A2	VDR	
CEL	PTGES	SLC16A1		

(Continued)

Table A1 | Continued

Phase I ($n = 144$)	Phase II ($n = 85$)	Phase III ($n = 111$)	Nuclear receptors and transcription factors ($n = 48$)	Miscellaneous genes ($n = 21$)
CES1	SAT1	SLC18A2		
CES2	SULT1A1	SLC19A1		
CES3	SULT1A2	SLC19A2		
CES4	SULT1A3	SLC19A3		
CES7	SULT1A4	SLC1A1		
CYP11A1	SULT1B1	SLC1A2		
CYP11B1	SULT1C2	SLC1A3		
CYP11B2	SULT1C3	SLC1A6		
CYP17A1	SULT1C4	SLC1A7		
CYP19A1	SULT1E1	SLC21A5		
CYP1A1	SULT2A1	SLC22A1		
CYP1A2	SULT2B1	SLC22A11		
CYP1B1	SULT4A1	SLC22A12		
CYP20A1	SULT6B1	SLC22A16		
CYP21A2	TPMT	SLC22A2		
CYP24A1	TST	SLC22A3		
CYP26A1	UGT1A1	SLC22A4		
CYP26B1	UGT1A10	SLC22A5		
CYP26C1	UGT1A3	SLC22A6		
CYP27A1	UGT1A4	SLC22A7		
CYP27B1	UGT1A5	SLC22A8		
CYP27C1	UGT1A6	SLC22A9		
CYP2A13	UGT1A7	SLC25A13		
CYP2A6	UGT1A8	SLC28A1		
CYP2A7	UGT1A9	SLC28A2		
CYP2B6	UGT2A1	SLC28A3		
CYP2C18	UGT2A3	SLC29A1		
CYP2C19	UGT2B10	SLC29A2		
CYP2C8	UGT2B11	SLC29A3		
CYP2C9	UGT2B15	SLC29A4		
CYP2D6	UGT2B17	SLC2A1		
CYP2E1	UGT2B28	SLC31A1		
CYP2F1	UGT2B4	SLC38A1		
CYP2J2	UGT2B7	SLC38A2		
CYP2R1	UGT3A1	SLC38A5		
CYP2S1	UGT3A2	SLC3A1		
CYP2U1		SLC3A2		
CYP2W1		SLC47A1		
CYP39A1		SLC47A2		
CYP3A4		SLC5A4		
CYP3A43		SLC6A3		
CYP3A5		SLC6A4		
CYP3A7		SLC7A11		
CYP46A1		SLC7A5		
CYP4A11		SLC7A6		
CYP4A22		SLC7A7		
CYP4B1		SLC7A8		
CYP4F11		SLCO1A2		
CYP4F12		SLCO1B1		
CYP4F2		SLCO1B3		
CYP4F22		SLCO1C1		

(Continued)

Table A1 | Continued

Phase I ($n = 144$)	Phase II ($n = 85$)	Phase III ($n = 111$)	Nuclear receptors and transcription factors ($n = 48$)	Miscellaneous genes ($n = 21$)
CYP4F3		SLCO2A1		
CYP4F8		SLCO2B1		
CYP4V2		SLCO3A1		
CYP4X1		SLCO4A1		
CYP4Z1		SLCO4C1		
CYP51A1		SLCO5A1		
CYP7A1		SLCO6A1		
CYP7B1		TAP1		
CYP8B1		TAP2		
DHRS2		VDAC2		
DHRS4		VDAC3		
DHRS9				
DPYD				
EPHX1				
EPHX2				
ESD				
FMO1				
FMO2				
FMO3				
FMO4				
FMO5				
HSD17B10				
KCNAB1				
KCNAB2				
KCNAB3				
KDM1A				
KDM1B				
MAOA				
MAOB				
NQO1				
NQO2				
PAOX				
PON1				
PON2				
PON3				
PTGIS				
PTGS1				
PTGS2				
SPR				
SUOX				
TBXAS1				
UCHL1				
UCHL3				
XDH				

Table A2 | Putative miRNAs associated with SNPs in the 3′-UTR region.

Gene	Classification	SNP	Tissue	Putative miRNAs		
				microSNiPer	**PolymiRTs**	**Overlap**
ALDH16A1	Phase I	rs1055637	Liver	hsa-miR-4265 hsa-miR-1231 hsa-miR-3120-5p hsa-miR-4322 hsa-miR-4669 hsa-miR-4726-3p	hsa-miR-3151 hsa-miR-4447 hsa-miR-4472 **hsa-miR-491-5p** hsa-miR-132-5p hsa-miR-4669	hsa-miR-4669
CYP2E1	Phase I	rs2480256	Liver	**hsa-miR-570**	**hsa-miR-570-3p**	**hsa-miR-570-3p**
CYP2E1	Phase I	rs2480257	Liver	hsa-miR-4762-5p	hsa-miR-5582-3p **hsa-miR-570-3p**	
CYP2U1	Phase I	rs8727	Liver	**hsa-miR-549** hsa-miR-125b-2*	**hsa-miR-549**	**hsa-miR-549**
CYP3A5	Phase I	rs15524	Liver	hsa-miR-562 **hsa-miR-501-5p** hsa-miR-500b **hsa-miR-500a** hsa-miR-4668-3p hsa-miR-3973 **hsa-miR-362-5p**	**hsa-miR-500a-5p** hsa-miR-5680	**hsa-miR-500a-5p**
CYP3A7	Phase I	rs10211	Liver	N/A	**hsa-miR-125a-5p** **hsa-miR-125b-5p** hsa-miR-345-3p hsa-miR-3920 hsa-miR-4319 hsa-miR-4732-3p hsa-miR-670	
EPHX2	Phase I	rs1042032	Brain	hsa-miR-4476 hsa-miR-4533 hsa-miR-2392 **hsa-miR-432*** hsa-miR-761 hsa-miR-183 hsa-miR-3665 hsa-miR-32390	hsa-miR-183-5p hsa-miR-2392	hsa-miR-2392 hsa-miR-183-5p
EPHX2	Phase I	rs1042064	Brain	**hsa-miR-31** **hsa-miR-576-3p** **hsa-miR-22** hsa-miR-4696	hsa-miR-4696	hsa-miR-4696
GSTM3	Phase II	rs1109138	Brain	hsa-miR-4766-3p hsa-miR-2964a-3p **hsa-let-7i***	N/A	
GSTM3	Phase II	rs1537236	Brain	hsa-miR-4762-5p hsa-miR-4470	**hsa-miR-182-5p** hsa-miR-4470	hsa-miR-4470
GSTM3	Phase II	rs1537235	Brain	hsa-miR-4790-3p	**hsa-miR-409-5p**	
GSTM3	Phase II	rs3814309	Brain	hsa-miR-4421 hsa-miR-3182 hsa-miR-1237 **hsa-miR-486-5p** hsa-miR-4793-3p hsa-miR-3120-5p hsa-miR-4527 **hsa-miR-29b**	hsa-miR-3130-3p hsa-miR-4793-3p	hsa-miR-4793-3p

(Continued)

Table A2 | Continued

Gene	Classification	SNP	Tissue	Putative miRNAs		
				microSNiPer	**PolymiRTs**	**Overlap**
GSTM5	Phase II	rs11807	Liver	hsa-miR-1202	N/A	
				hsa-miR-1227		
				hsa-miR-1973		
MGST3	Phase II	rs8133	Liver	hsa-miR-875-3p	**hsa-miR-582-3p**	**hsa-miR-582-3p**
				hsa-miR-582-3p	hsa-miR-875-3p	hsa-miR-875-3p
				hsa-miR-4698	hsa-miR-224-3p	hsa-miR-3688-3p
				hsa-miR-4694-3p	hsa-miR-3688-3p	hsa-miR-4694-3p
				hsa-miR-4495	hsa-miR-4694-3p	
				hsa-miR-411*	hsa-miR-522-3p	
				hsa-miR-3688-3p		
ATP7B	Phase III	rs928169	Liver	hsa-miR-4734	hsa-miR-4447	hsa-miR-4472
				hsa-miR-4430	hsa-miR-4472	hsa-miR-4481
				hsa-miR-4481	hsa-miR-4481	hsa-miR-4745-5p
				hsa-miR-4472	hsa-miR-4745-5p	hsa-miR-4785
				hsa-miR-3652	hsa-miR-4785	
				hsa-miR-3135b	hsa-miR-4787-5p	
				hsa-miR-4745-5p		
				hsa-miR-3944-3p		
				hsa-miR-1275		
				hsa-miR-491-5p		
				hsa-miR-4446-3p		
				hsa-miR-4498		
				hsa-miR-194*		
				hsa-miR-122		
				hsa-miR-4734		
				hsa-miR-4430		
				hsa-miR-3652		
				hsa-miR-4309		
				hsa-miR-4785		
				hsa-miR-3198		
				hsa-miR-1298		
SLC31A1	Phase III	rs10759637	Liver	hsa-miR-4448	hsa-miR-3672	
				hsa-miR-3119	hsa-miR-4524a-3p	
				hsa-miR-4461		
TAP2	Phase III	rs13501	Brain	hsa-miR-3198	hsa-miR-1289	hsa-miR-1289
				hsa-miR-1289	hsa-miR-3198	hsa-miR-3198
				hsa-miR-4309	hsa-miR-4294	hsa-miR-4309
				hsa-miR-3127-5p	hsa-miR-4309	
					hsa-miR-5702	
TAP2	Phase III	rs17034	Brain	hsa-miR-4772-3p	**hsa-miR-1271-3p**	
					hsa-miR-4763-5p	
					hsa-miR-550a-3-5p	
					hsa-miR-550a-5p	
					hsa-miR-4327	
					hsa-miR-636	
TAP2	Phase III	rs241451	Brain	hsa-miR-1260	hsa-miR-4684-5p	hsa-miR-4684-5p
				hsa-miR-4758-3p		
				hsa-miR-4684-5p		
TAP2	Phase III	rs241452	Brain	hsa-miR-1206	hsa-miR-1206	hsa-miR-1206
				hsa-miR-1		
				hsa-miR-4789-5p		

(Continued)

Table A2 | Continued

Gene	Classification	SNP	Tissue	Putative miRNAs		
				microSNiPer	**PolymiRTs**	**Overlap**
TAP2	Phase III	rs241453	Brain	hsa-miR-4298 hsa-miR-1302	hsa-miR-1302 hsa-miR-4298	hsa-miR-1302 hsa-miR-4298
TAP2	Phase III	rs241454	Brain	hsa-miR-4476 hsa-miR-4779	hsa-miR-4476 hsa-miR-4533 hsa-miR-3173-3p hsa-miR-4779	hsa-miR-4476 hsa-miR-4779
TAP2	Phase III	rs241455	Brain	**hsa-miR-130a*** **hsa-miR-323-3p**	hsa-miR-2116-3p **hsa-miR-130a-5p** **hsa-miR-23a-3p** **hsa-miR-23b-3p** hsa-miR-23c hsa-miR-3680-5p hsa-miR-4798-3p	**hsa-miR-130a-5p**
TAP2	Phase III	rs241456	Brain	hsa-miR-3940-5p hsa-miR-4507 **hsa-miR-92a-1*** hsa-miR-4450	hsa-miR-2110 hsa-miR-3150a-3p hsa-miR-4450 **hsa-miR-450a-3p** **hsa-miR-1270** hsa-miR-3676-5p hsa-miR-4531 hsa-miR-4683 hsa-miR-620	hsa-miR-4450
TAP2	Phase III	rs2857101	Brain	hsa-miR-944 hsa-miR-4795-3p hsa-miR-183*	**hsa-miR-126-5p** hsa-miR-4795-3p hsa-miR-944	hsa-miR-944 hsa-miR-4795-3p
UGT2A1	Phase II	rs4148312	Liver	hsa-miR-548t hsa-miR-548ah hsa-miR-3662 hsa-miR-3646 hsa-miR-3609 **hsa-miR-340** **hsa-miR-1245** **hsa-miR-106a**	hsa-miR-3662 **hsa-miR-548c-3p** hsa-miR-3609 hsa-miR-548ah-5p **hsa-miR-548n** hsa-miR-548t-5p	hsa-miR-3662 hsa-miR-3609 hsa-miR-548ah-5p hsa-miR-548t-5p
ARNT	Nuclear receptors	rs11552229	Liver	hsa-miR-4716-5p	hsa-miR-4717-3p	

The miRs expressed in the tissue where the eQTL was identified are highlighted in bold.

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Molecular mechanisms of genetic variation and transcriptional regulation of *CYP2C19*

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Inherited variation in the function of the drug metabolizing enzyme CYP2C19 was first observed 40 years ago. The SNP variants which underpin loss of CYP2C19 function have been elucidated and extensively studied in healthy populations. However, there has been relatively meagre translation of this information into the clinic. The presence of genotype-phenotype discordance in certain patients suggests that changes in the regulation of this gene, as well as loss of function SNPs, could play a role in deficient activity of this enzyme. Knowledge of the molecular mechanisms which control transcription of this gene, reviewed in this article, may aid the challenge of delivering *CYP2C19* pharmacogenetics into clinical use.

Keywords: *CYP2C19*, pharmacogenetics, transcription factors, epigenetics, miRNA

INTRODUCTION

CYP2C19 is important in the metabolism of many clinically relevant drugs (Desta et al., 2002), particularly for several pro-drugs that require hepatic activation including clopidogrel (Bauer et al., 2011; Begg et al., 2012). The first reports of an autosomal recessive inherited trait that resulted in poor metabolism of the prototypical CYP2C19 substrate, mephenytoin, appeared many years ago (Kupfer et al., 1979). The correlation between the poor metabolizer phenotype and loss of function genotype has been comprehensively studied in healthy populations (Desta et al., 2002). Screening for these genetic variants is one approach to individualize therapy for drugs which are substrates for this enzyme. However to be useful genotype must be predictive of phenotype, not only in healthy populations but also in the clinical context (Helsby, 2008). The presence of genotype-phenotype discordance in certain morbidities (Williams et al., 2000; Frye et al., 2002; Helsby et al., 2008), suggests that an acquired deficiency in the activity of this enzyme also occurs. Factors which regulate *CYP2C19* transcription could play an additional role in the pharmacogenomic variation of this enzyme. The current knowledge of the molecular mechanisms which control expression of the *CYP2C19* gene, such as the coding and regulatory region *cis*-variants as well as *trans*-acting epigenomic factors, are reviewed in this article.

CYP2C19 GENE VARIANTS

The role of *cis*-acting variants of *CYP2C19* have been extensively characterized and the polymorphic expression of these genetic variants results in inter-individual variation in CYP2C19 activity. More than 28 variant alleles in *CYP2C19* have been identified (http://www.cypalleles.ki.se/, access date August 19, 2012) and are summarized in **Table 1**. Many of these variants have relatively low frequency however, the SNPs which lead to the *CYP2C19*2* and *CYP2C19*3* alleles are common and have been the most extensively studied. These SNPs, c.681G>A, and c.636G>A, cause aberrant splicing and a premature stop codon respectively and result in null function. Individuals who are homozygous variant for either of these alleles are poor metabolizers of certain drugs. The allele frequency of these null function variants varies with ethnicity (Xie et al., 2001; Sistonen et al., 2009). A particularly high prevalence of both *2 and *3 is observed in Vanuatu and Papua New Guinea, accounting for up to 70.8% (*2) and 13.3% (*3) of these alleles in the Vanuatu population, and up to 42.3% and 31.5%, respectively, in the Papua New Guinea population (Kaneko et al., 1997; Hsu et al., 2008). This exceptionally high expression in Melanesia may reflect an unidentified evolutionary pressure. For further information on these null function alleles and the drug substrates of CYP2C19 readers are directed to review articles that have focused on *CYP2C19* pharmacogenetics such as Desta et al. (2002). In addition to genetic variation in the coding region of the gene, promoter region variation may also influence transcriptional expression and ultimately activity. The *CYP2C19*17* allele (g.–3402C>T and g.–806C>T) has been the focus of studies to identify increased function variants of this gene. Ethnic variation in the prevalence of this allele is also observed with a relatively low allele frequency (<5%) in Japanese and Chinese populations, compared with a higher incidence in European and African populations (∼15–30%) (Li-Wan-Po et al., 2010).

REGULATORY POLYMORPHISMS

CYP2C19 17*

*CYP2C19*17* was first identified in 2006 (Sim et al., 2006). Electrophoretic mobility shift assays (EMSA) detected binding of human hepatic nuclear proteins at −806T but not −806C. A potential GATA binding site at this position was identified

Table 1 | *CYP2C19* gene variants.

Allele	Characteristic SNP[a]			Functional change	References
	cDNA	Gene	Effect		
*CYP2C19*1*	None[1]	None	None	Normal	Romkes et al., 1991
*CYP2C19*2*	681G>A[2]	19154G>A	Splicing defect	Non-functional	De Morais et al., 1994b; Ibeanu et al., 1998b; Fukushima-Uesaka et al., 2005; Lee et al., 2009; Satyanarayana et al., 2009a
*CYP2C19*3*	636G>A[3]	17948G>A	Premature stop codon (W212X)	Non-functional	De Morais et al., 1994a; Fukushima-Uesaka et al., 2005
*CYP2C19*4*	1A>G[4]	1A>G	GTG initiation codon	Non-functional	Ferguson et al., 1998; Scott et al., 2011
*CYP2C19*5*	1297C>T[5]	90033C>T	R433W	Non-functional	Xiao et al., 1997; Ibeanu et al., 1998a
*CYP2C19*6*	395G>A	12748G>A	R132Q	Non-functional	Ibeanu et al., 1998b
*CYP2C19*7*		19294T>A	Splicing defect	Non-functional	Ibeanu et al., 1999
*CYP2C19*8*	358T>C	12711T>C	W120R	Decreased *in vitro*	Ibeanu et al., 1999
*CYP2C19*9*	431G>A	12784G>A	R144H	Decreased *in vitro*	Blaisdell et al., 2002
*CYP2C19*10*	680C>T	19153C>T	P227L	Decreased *in vitro*	Blaisdell et al., 2002
*CYP2C19*11*	449G>A	12802G>A	R150H	Similar to wild type *in vitro*	Blaisdell et al., 2002
*CYP2C19*12*	1473A>C	90209A>C	X491C; 26 extra amino acids	Unstable *in vitro*	Blaisdell et al., 2002
*CYP2C19*13*	1228C>T	87290C>T	R410C	Similar to wild type *in vitro*	Blaisdell et al., 2002
*CYP2C19*14*	50T>C	50T>C	L17P	Not determined	Blaisdell et al., 2002
*CYP2C19*15*	55A>C	55A>C	I19L	Not determined	Blaisdell et al., 2002
*CYP2C19*16*	1324C>T[6]	90060C>T	R442C	Not determined	Morita et al., 2004
*CYP2C19*17*		3402C>T; −806C>T		Increased transcription *in vitro*; Should not be termed Ultrarapid (UM)	Sim et al., 2006
*CYP2C19*18*	986G>A	80156G>A; 87106T>C	R329H	Not determined	Fukushima-Uesaka et al., 2005
*CYP2C19*19*	151A>G	151A>G; 87106T>C	S51G	Not determined	Fukushima-Uesaka et al., 2005
*CYP2C19*20*[7]	636G>A	17948G>A	Premature stop codon (W212X) and D360N	Non-functional	Fukushima-Uesaka et al., 2005
*CYP2C19*21*[8]	681G>A	19154G>A; –98T>C	splicing defect and A161P	Non-functional	Fukushima-Uesaka et al., 2005; Satyanarayana et al., 2009a
*CYP2C19*22*	557G>C	17869G>C	R186P	Not determined	Matimba et al., 2009
*CYP2C19*23*	271G>C	12455G>C	G91R	Not determined	Zhou et al., 2009
*CYP2C19*24*	1004G>A; 1197A>G	80174G>A; 87259A>G	R335Q	Not determined	Zhou et al., 2009
*CYP2C19*25*	1344C>G	90080C>G	F448L	Not determined	Zhou et al., 2009
*CYP2C19*26*	766G>A	19239G>A	D256N	Decreased *in vitro*	Lee et al., 2009
*CYP2C19*27*		–1041G>A		Decreased *in vitro*	Drögemöller et al., 2010
*CYP2C19*28*	1120G>A	−2020C>A; −1439T>C; 80290G>A	V374I	No significant decrease *in vitro*	Drögemöller et al., 2010

[a] *Only major SNP or alteration(s) responsible for the phenotype of the corresponding allele are shown. Adapted from http://www.cypalleles.ki.se/*

[1] *The presence of additional SNP can further sub-classify individuals as *1B (99C>T; 991A>G) or *1C (991A>G). This results in an I331V change but does not alter activity.*

[2] *The presence of additional SNP can further sub-classify individuals as *2A, *2B, *2C, and *2D. Of these variants *2C and *2D harbor a SNP in the 5′ promoter region (−98T>C) that may have a functional effect.*

[3] *The presence of additional SNP can further sub-classify individuals as *3A (1251A>C) and *3B (1078G>A; 1251A>C).*

[4] *The presence of −3402C>T; −806C>T SNP in the promoter can further sub-classify individuals as *4B.*

[5] *The presence of 99C>T; 991A>G, can further sub-classify individuals as *5B.*

[6] *Existence of the CYP2C19*2 polymorphism 681G>A on the same allele cannot be excluded.*

[7] *Also known as CYP2C19*3B.*

[8] *Also known as CYP2C19*2C.*

in silico and it was hypothesized that the −806C>T variant could result in increased transcription of *CYP2C19*. However, to date GATA-dependent transactivation at the −806C>T site has not been directly demonstrated. Indeed it is of note that following co-transfection with GATA-4 or GATA-6, −806T>C variant reporter constructs did not have increased luciferase activity compared with wildtype constructs (Mwinyi et al., 2010b). Although GATA may not be involved, transfection of reporter constructs of the −0.9 Kb of the 5′ flanking region into mice lead to an increase in transcription in the −806T mutant compared with wildtype construct. However a range of overlapping individual luciferase activities were observed in wildtype and mutant constructs. To date no direct evidence of correlations between *CYP2C19*17* genotype status and increased transcription or protein expression in human liver biobanks has been reported. Despite the lack of direct evidence this genotype is often described as increasing the expression of the enzyme (protein) and many investigators have categorized individuals who carry this variant allele as ultra-rapid metabolizers (UM). When CYP2C19 activity is measured *in vivo* using drug to metabolite ratios of probe substrates such as omeprazole it is clear that the mean activity of CYP2C19 is higher in homozygous *17/*17 subjects than in individuals with the *1/*1 genotype (Baldwin et al., 2008). However, the activity in *17/*17 subjects overlaps the heterogenous activity observed in *1/*1 subjects (Baldwin et al., 2008). Similar effects have been observed with other drugs such as escitalopram, clopidogrel and voriconazole (Li-Wan-Po et al., 2010). The high activity in some *1/*1 subjects may be due to other currently unidentified increased activity variants. However currently *17/*17 subjects do not appear to be a separate population and fall within the normal distribution of wildtype CYP2C19 activity, therefore should not be classified as an ultra-rapid phenotype. Moreover, the ultra-rapid metabolizer phenotype observed with CYP2D6 substrates is typically due to gene duplication and associated copy number variation, and it is important to note that copy number variation for *CYP2C19*, appears to be absent (Drögemöller et al., 2010; Devendran et al., 2012). Further identification of additional SNP in the 5′-up-stream region of *CYP2C19* may clarify the wide heterogeneity of activity in *1/*1 individuals.

OTHER PROMOTER REGION VARIANTS

Publication of the promoter sequence of *CYP2C19* gene (Genbank accession #AF354181) led to the identification of eight SNP in the −1.833 Kb promoter region (Arefayene et al., 2003). Resequencing of genomic DNA from 92 individuals of varied ethnicity identified 13 SNP in the −1.46 Kb up-stream region of the gene (Blaisdell et al., 2002). Extensive characterization of the 5′-regulatory region of *CYP2C19* also identified a further seven SNP novel variants in the enhancer region and five SNP in the promoter region in Japanese subjects (Fukushima-Uesaka et al., 2005). Eight novel SNP were also detected in the −1.7 Kb promoter region in a South Indian population (Satyanarayana et al., 2009b). More recently resequencing of −2.095 Kb of the 5′-up-stream region of *CYP2C19* identified two additional novel SNP (g.−2030C>T and g.−2020C>A). These SNPs, in combination with a previously identified SNP in the 5′promoter (g.−1439T>C) and the g.80290 G>A SNP in exon 7, result in the *CYP2C19*28* genotype (**Table 1**) (Drögemöller et al., 2010). Extensive ethnic variation in the frequency of promoter/enhancer region SNP is evident from the above studies. Identification of the functional effects of these 35 novel SNP identified up-stream of the translational start site is important for our understanding of the variable expression and activity of this enzyme.

Regions of negative and positive regulatory control of *CYP2C19* were observed following transient expression of luciferase reporter deletion constructs in HepG2 cells (Arefayene et al., 2003). Transient expression into HepG2 cells of luciferase reporter deletion constructs between positions −153 bp and −17 bp significantly decreased luciferase activity, suggesting effects on transcription factor binding. In contrast deletion from −650 bp to −363 bp increased luciferase activity, indicating the presence of repressor regulation in this region. Using nine different constructs of the *CYP2C19* 5′ promoter region (−1.6 Kb) transfected into HepG2 cells, Satyanarayana et al. (2011) showed that the presence of either the −98T>C SNP in combination with −1498T>G or the combination of −98T>C, −779A>C, −1051T>C, and −1418C>T, significantly increased luciferase activity. The SNP −98T>C is within both a potential CCAAT displacement protein (CDP) binding site and a potential GATA-1 site (Satyanarayana et al., 2009b). *In silico* analysis indicated that interaction of the CDP repressor with its putative binding site was weaker in the presence of the −98C variant whereas GATA-1 had a high predicted binding activity in the presence of the normal −98T. Hence the functional consequence may be that in wildtype subjects (−98TT or CT) repression of GATA-1 binding will be greater than in homozygous −98CC subjects. Indeed, the presence of −98TT genotype appears to decrease the activity of the enzyme compared with the −98CT or −98CC genotype in subjects probed *in vivo* with proguanil (Satyanarayana et al., 2009a). This is suggestive of a functional effect of a transcriptional repressor at this region of the promoter, in agreement with the early data from Arefayene et al. (2003). It is of interest to note that −98T>C displays linkage with the c.681G>A (*2) SNP (Fukushima-Uesaka et al., 2005; Satyanarayana et al., 2009b) as *CYP2C19*21*, also known as *CYP2C19*2C* (**Table 1**).

Most recently the g.−1041G>A SNP in the *CYP2C19*27* allele (**Table 1**) has also been demonstrated to have functional consequences. Significantly decreased luciferase activity was observed in a construct transfected into HepG2 cells, whereas the promoter region SNP (−2030C>T;−1439T>C) in the *CYP2C19*28* allele did not significantly decrease activity (Drögemöller et al., 2010). Thus, to date only three of the 35 SNP identified in the proximal five region of *CYP2C19* appear to be associated with changes in gene transcription: −806C>T (*17), −1041G>A (*27) and −98T>C (*21).

TRANSCRIPTION FACTOR BINDING SITES IN *CYP2C19*

Many predicted or putative sites for transcription factor binding have been reported for *CYP2C19*. However, functional transcription factor binding sites have only been demonstrated for the ligand activated nuclear receptors ERα (NR3A1) (Mwinyi et al., 2010a), CAR (NR1I3), and GR (NR3C1) (Chen et al., 2003), and

the transcription factors HNF3γ (FOXA3) (Bort et al., 2004) and GATA-4 (Mwinyi et al., 2010b), (**Figure 1**).

The constitutive androstane receptor response element (CAR-RE) at −1891/−1876 in the promoter region of *CYP2C19* has been shown to functionally active. Binding of CAR protein occurs as a monomer or heterodimer with the retinoid X receptor (RXR) and pregnane X receptor (PXR). Deletion of this site completely abolishes binding (Chen et al., 2003). Expression of *CYP2C19* appears to be more sensitive to the effects of co-transfection with CAR than with PXR, nevertheless luciferase activity can be induced by the PXR activator rifampicin (Chen et al., 2003), a known inducer of CYP2C19 activity in patients (Feng et al., 1998). There is a significant correlation between *CAR* mRNA and *CYP2C19* transcription in human liver (Wortham et al., 2007). Deletion constructs have demonstrated the functional activity of a glucocorticoid responsive element (GRE) at −1750/−1736b, and dexamethasone can also induce the expression of a *CYP2C19* construct containing this GRE in transfected HepG2 and Caco2 cells (Chen et al., 2003).

A functional estrogen response element (ERE) half site has been identified in *CYP2C19* at position −151/−147 (Mwinyi et al., 2010a). This GGTCA motif binds ERα but not ERβ. Both 17-β estradiol and 17-α ethinylestradiol down-regulate reporter luciferase activity in Huh-7 transfected cells co-transfected with ERα. However, the partial agonists 4-hydroxytamoxifen and raloxifene had no effect on *CYP2C19* transcription (Mwinyi et al., 2010a). Interestingly mutation of this ERE half site decreases but does not abolish luciferase activity. Chromatin immunoprecipitation of Huh-7 cells combined with q-PCR demonstrated that ERα was associated with this ERE half site in the *CYP2C19* promoter. 17-α ethinylestradiol stimulated this interaction of ERα with the promoter whereas 4-hydroxytamoxifen abolished the interaction. Preliminary data suggested that treatment of primary hepatocytes with 17-β estradiol or 17-α ethnylestradiol decreased *CYP2C19* mRNA expression (Mwinyi et al., 2010a). This is in contrast to recent data which found that estradiol did not influence the expression of *CYP2C19* (Choi et al., 2012). The biotransformation of estrogens by CYP enzymes results in a half-life of estradiol in human hepatocytes of <34 min. Thus concentration dependent ligand binding may influence transactivation of *CYP2C19* by ERα in hepatocytes.

In addition to the three ligand-activated transcription factors described above, two additional transcription factor proteins have also been demonstrated to be important in *CYP2C19* transcription:- FOXA3 and GATA-4. Three hepatocyte nuclear factor 3 gamma (HNF3γ) sites, (−313/−298, −560/−545, −623/−608), have been identified in the *CYP2C19* promoter (Bort et al., 2004). Cotransfection with HNF3γ in luciferase reporter assays as well as overexpression of HNF3γ in HeLa and hepatoma cells significantly increased expression of *CYP2C19* (Bort et al., 2004). HNF3γ is member of the forkhead box/winged helix family of transcription factors and is also known as FOXA3. This transcription factor is important in the expression of liver-specific genes and the development of hepatic lineage. In contrast, co-transfection with HNF4α, did not increase luciferase expression despite the presence of HNF4α sites at −186/−174, −152/−140 (Kawashima et al., 2006). These sites do not appear to be functional as deletion constructs, indicating that HNF4α cannot increase transcription of *CYP2C19* (Bort et al., 2004) and also indicate that HNF4α protein does not bind to *CYP2C19* (Bort et al., 2004; Rana et al., 2010).

GATA-4 is a member of the zinc-finger transcription factor family. Two adjacent GATA binding motifs (TATC) have been detected in the *CYP2C19* promoter at −165/−162 and −159/−156 (Mwinyi et al., 2010b). Wildtype and deletion constructs containing destructive mutations in each of the GATA sites were transfected into HepG2 or Huh-7 cells. Significant up-regulation of luciferase activity of these constructs

FIGURE 1 | Functional transcription factor binding sites in the 2.0 kb 5′-promoter region of *CYP2C19*. The binding factor CAR (constitutive androstane receptor; NR113) acts at the CAR response element (−1891/−1876 bp). NR113 can also hetero-dimerise with retinoid X receptor (RXR) and pregnane X receptor (PXR) to transactivate *CYP2C19* (Chen et al., 2003). The glucocorticoid receptor (GR; NR3C1) acts at the glucocorticoid response element (GRE) at position −1750/−1736 bp (Chen et al., 2003). Hepatocyte nuclear factor 3 gamma (HNF3γ) is member of the forkhead box/winged helix family of transcription factors and is also known as FOXA3. Three functional FOXA3 sites, (−313/−298, −560/−545, −623/−608 bp) exist in the *CYP2C19* promoter (Bort et al., 2004). GATA-4 is a member of the zinc-finger transcription factor family. Two adjacent GATA binding motifs (TATC) are found between −165/−156 bp, and GATA-4 predominantly binds to site I (−165/−162 bp). The repressor protein, Friend of GATA (FOG-2), attenuates the effect of GATA-4 binding (Mwinyi et al., 2010b). Estrogen receptor-α (ERα) binds to the estrogen receptor element (ERE) half site at −151/−147 bp. Mutation of this site decreases but does not abolish transcription (Mwinyi et al., 2010a).

was observed when co-transfected with either GATA-4 or GATA-2. Deletion of this double GATA binding site completely abolished transcription. However, EMSA analysis demonstrated nuclear extracts predominantly bind to site I (−165/−162) and chromatin immunoprecipitation confirmed that GATA-4 was associated with the *CYP2C19* promoter. The GATA repressor protein friend of GATA 2 (FOG-2), also known as zinc finger protein multitype-2, attenuates the effect of GATA-4 and may also have a role to play in the regulation of *CYP2C19* transcription. GATA-4 is an important liver associated transcription factor (Molkentin, 2000).

More recently, it has been proposed that the following transcription factors and binding sites may also be important in the transcription of *CYP2C19*:- ATF-2 (−806 to −786), CEBP-β (−1505/−1491 and −1443/−1429), CDP repressor protein (−105/−87), GATA-1 (−103/−91) and an additional GRE (−828/−810), however further functional analysis is required to establish the relevance, if any, of these factors (Satyanarayana et al., 2011). The weak correlations between *PXR, ARNT,* and *HNF1α* genes with *CYP2C19* mRNA expression in human liver suggests that these transcriptional regulators do not extensively contribute to *CYP2C19* expression (Wang et al., 2011).

Trans ACTING FACTORS

In contrast to *cis* regulation due to genetic variants, the *trans*-acting factors which control *CYP2C19* expression have been largely ignored. Knowledge of epigenomic control of *CYP2C19*, via factors such as altered expression of transcription factor genes or the effects of noncoding RNA, is limited. A number of studies have identified that environmental (rather than inherited genetic) effects such as pregnancy, old age, cancer, and congestive heart failure (Williams et al., 2000; Frye et al., 2002; McGready et al., 2003; Ishizawa et al., 2005) can all lead to an acquired alteration in CYP2C19 activity. The observed change in activity can lead to genotype-phenotype discordance, such that a poor metabolizer status can be observed in individuals who are not homozygous variant for null function alleles. This may be due to *CYP2C19* gene down-regulation as has been observed following incubation of the inflammatory cytokines, IL-6 and TGF-β, with primary human hepatocytes (Aitken and Morgan, 2007). The mechanisms for this down-regulation of *CYP2C19* have not been elucidated to date but could include direct effects on gene transcription (e.g., CpG methylation), up-stream effects on the expression or activity of transcription factors or post-translational regulation of *CYP2C19*.

EPIGENETICS

Tissue specific regulation of genes can be the result of epigenetic regulation and it is notable that the expression of *CYP2C19* is restricted to the liver and intestine (Läpple et al., 2003; Hayashi et al., 2011; Bourgine et al., 2012). Quantification of gene transcripts (cDNA copy number) indicates the same range and median expression of *CYP2C19* mRNA in the intestine as in the liver. However, the authors could not detect an intra-individual correlation between *CYP2C19* expression in samples of liver and intestine from each patient expression. Hence there may be independent regulation of transcription of *CYP2C19* in these tissues (Läpple et al., 2003). One possible mechanism of tissue specific expression is epigenetic control via methylation of CpG islands in the gene or by histone modifications such as acetylation.

Remarkably little is known about epigenetic regulation of *CYP2C19* (Ingelman-Sundberg et al., 2007). However a small number of CpG islands can be detected in the gene (**Figure 2**). Methylation of CpG sites in the promoter region of a target gene can affect the physical binding of transcription factors to regulate gene expression. In the case of *CYP2C19* these CpG islands are not associated with the promoter region. However, DNA methylation can also act via an indirect mechanism on chromatin configuration. There is currently no data regarding the methylation status of the *CYP2C19* CpG islands.

DNA methylation can lead to a phenomenon known as allelic expression imbalance. The difference in expression levels between two alleles of *CYP2C19* typically occurs due to genetic polymorphisms (e.g., a heterozygous carrier of *3). However epigenetic silencing of an allele (e.g., methylation) can also result in the preferential expression of one of the two alleles. Allelic expression imbalance is assessed directly by the relative quantitation of an intragenic marker allele in cells or tissues. In the absence of any *cis*-acting control on transcription the allelic ratio should be 1

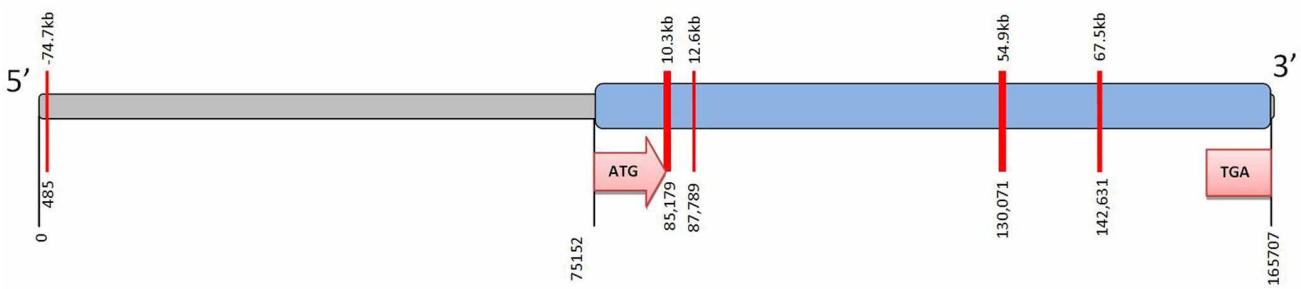

FIGURE 2 | CpG islands identified in *CYP2C19*. The complete *CYP2C19* sequence (Ensembl Gene ID ENSG00000165841; National Centre for Biotechnology Information (NCBI); Entrez core nucleotide sequence NM 000769) was analyzed using CpG Island finder, http://cpgislands.usc.edu/. Potential CpG island regions were determined using the following parameters %GC = 50–55%, ObsCpG/ExpCpG = 0.6, length = 100–500 bp, gap between adjacent islands = 100 bp. Up to five CpG islands were identified in *CYP2C19*. Notably most of these CpG islands are down-stream of the ATG initiation codon (i.e., within the coding region of the gene) and are not associated with the 5′proximal promoter. The CpG islands are shown as red bars at 485, 85179, 87789, 130071, and 142631 bp. The 5′ upstream region is shown in grey and the coding region of the gene in blue.

(i.e., equal amounts from each allele). In liver samples from heterozygous individuals, the *cis*-acting variant of *CYP2C19* (*2; rs4244285) accounts for the majority of the allelic expression imbalance observed. However, there was only a weak correlation ($p = 0.047$) between *CYP2C19* mRNA expression and this SNP in these 96 human livers. This suggests that variability in *CYP2C19* expression is not fully accounted for by known coding region polymorphisms. Interestingly, the non-coding marker SNP in intron 3, (rs 4388808), was associated with up to 47% of allelic expression imbalance for *CYP2C19* (Wang et al., 2011). This confirms that in addition to *cis*-acting polymorphic variants, there are factors which influence the regulatory control of *CYP2C19* RNA transcription or stability. Hence epigenetic factors may also affect the hepatic expression level of *CYP2C19*.

POST-TRANSLATIONAL REGULATION OF *CYP2C19*

In addition to transcriptional regulation, post-transcriptional regulation may influence the expression of *CYP2C19*. Noncoding RNA, such as microRNA (miR), can bind to recognition sites (MRE) in the 3′-untranslated region (3′UTR) or in the coding region of target genes and thereby repress gene translation. The role of miR regulation of *CYP2C19* was until recently not known, as *in silico* prediction of miR regulation of *CYP2C19* was not available due to the lack of information about the 3′-UTR of the gene (Ramamoorthy and Skaar, 2011). However, it has recently been reported that the 3′UTR of *CYP2C19* contains two putative MRE for miR-103/107 at 222–242 bp and 138–152 bp down-stream of the stop codon. These MRE contain one nucleotide mismatch, however, ectopic addition of precursors of miR-103/107 to human hepatocytes significantly down-regulated CYP2C19 immunoreactive protein (Zhang et al., 2012). This preliminary data suggests that post-transcriptional regulation of the constitutive expression of *CYP2C19* may be an additional contributing factor to inter-individual variation in the expression of this enzyme in subjects who do not express SNP variants.

CONTROL OF TRANSCRIPTION FACTOR ACTIVITY

Another mechanism which could account for variation in the regulation of *CYP2C19* expression is the effect of both genomic and environmental factors which influence transcription factor-binding to the promoter region. SNP variants present in the promoter region have been discussed above, however up-stream effects on the expression or activity of transcription factors may also play a role in *CYP2C19* transcription. It is important to appreciate that as well as altered expression of transcription factor genes the function of ligand-activated factors (ERα, CAR, GR) can be influenced by variation in the levels of endogenous ligands, such as estrogens and glucocorticoids. Hence environmental factors may influence the activity of transcription factors important for *CYP2C19* transcription. The ability of ERα, CAR, GR, FOXA3, and GATA-4 to interact with other transcription factors may add further complexity to the regulation of *CYP2C19*. For example, estrogen-dependent activation of ERα results in binding to the ERE. This appears to result in a down-regulation of *CYP2C19* transcription (Mwinyi et al., 2010a). Changes in CYP2C19 activity have been reported in women during pregnancy and whilst using oral contraceptives (McGready et al., 2003), suggesting a regulatory role for estrogens on CYP2C19 activity. However, ligand-independent activation of ERα also occurs. ERα can act via a non-classical pathway to alter the activities of other transcription factors (e.g., Sp1, AP-1, or NF-kappaB) at their cognate sites on DNA. The role of interactions of ER-α with other transcription factors that regulate *CYP2C19* cannot be discounted. This may account for why mutation constructs of the ERE site decrease but do not abolish *CYP2C19* transcription in the presence of ligand activated ER-α (Mwinyi et al., 2010a). In addition, GATA-4 co-operates with FOXA3 to stimulate albumin gene transcription in liver cells (Cirillo et al., 2002). Moreover, FOXA3 and GATA-4 can act as pioneer factors. Once bound these pioneer factors relax the adjacent chromatin to allow other factors to bind. Interestingly GATA-4 appears to be able to direct the association of ERα in certain contexts (Miranda-Carboni et al., 2011).

Understanding the genomic control of *CYP2C19* expression is important in order to increase our understanding of the observed phenotype-genotype discordance in morbidity. This acquired deficiency may influence the sensitivity and specificity of *CYP2C19* pharmacogenetic tests in clinical contexts. A correlation between *CYP2C19* mRNA and the expression of *CYP2C9* and *CYP3A4* has been observed (Wang et al., 2011) and Bayesian network analysis suggests that *CYP2C19* is the master regulator of *CYP2C9, CYP3A7, CYP3A4,* and *CYP3A43* (Yang et al., 2010). Hence further study of the mechanisms which regulate *CYP2C19* may also increase our understanding of the regulation of other important drug metabolizing enzymes.

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Impact of genetic polymorphisms on chemotherapy toxicity in childhood acute lymphoblastic leukemia

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The efficacy of chemotherapy in pediatric acute lymphoblastic leukemia (ALL) patients has significantly increased in the last 20 years; as a result, the focus of research is slowly shifting from trying to increase survival rates to reduce chemotherapy-related toxicity. At the present time, the cornerstone of therapy for ALL is still formed by a reduced number of drugs with a highly toxic profile. In recent years, a number of genetic polymorphisms have been identified that can play a significant role in modifying the pharmacokinetics and pharmacodynamics of these drugs. The best example is that of the *TPMT* gene, whose genotyping is being incorporated to clinical practice in order to individualize doses of mercaptopurine. However, there are additional genes that are relevant for the metabolism, activity, and/or transport of other chemotherapy drugs that are widely use in ALL, such as methotrexate, cyclophosphamide, vincristine, L-asparaginase, etoposide, cytarabine, or cytotoxic antibiotics. These genes can also be affected by genetic alterations that could therefore have clinical consequences. In this review we will discuss recent data on this field, with special focus on those polymorphisms that could be used in clinical practice to tailor chemotherapy for ALL in order to reduce the occurrence of serious adverse effects.

Keywords: acute lymphoblastic leukemia, pharmacogenetics, toxicity, chemotherapy, genetic polymorphisms

INTRODUCTION

In the last 20 years, the chemotherapy treatment of pediatric acute lymphoblastic leukemia (ALL), the most common malignancy in children, has reached success rates of up to 90%. This achievement has allowed limiting the administration of damaging cranial irradiation to rare cases with central nervous system (CNS) infiltration (Laningham et al., 2007). However, the clinical routine of chemotherapy treatments in these patients includes intrathecal and high intravenous doses of various drugs with a highly toxic profile. This is therefore a source of a wide variety of complications that add up to those caused by peripheral blood cell depression or the disease itself (Vagace and Gervasini, 2011). In fact, the toxicity of chemotherapy is a common cause of morbidity and mortality in children with ALL, as well as a frequent source of sequelae at mid-long term. These adverse effects are often the consequence of direct toxicity in healthy tissue, as a result of the low specificity displayed by these drugs and become more frequent as the treatment is intensified. In particular, central neurotoxicity is a major clinical concern in pediatric patients (Vagace et al., 2012).

In this scenario, it is obvious that any factor able to modify either the pharmacokinetics or pharmacodynamics of chemotherapy drugs holds the potential to be critical for the occurrence of serious adverse effects in ALL patients. One such factor is the presence of genetic polymorphisms in genes coding for drug-metabolizing enzymes, transporters, or drug targets. Genetic association studies in this field have traditionally been focused on efficacy parameters in general and survival in particular and therefore the body of work on the association with toxicity is still scarce.

In this review we will summarize what is currently known about how genetic variability can affect the toxicity induced by the main drugs used in the chemotherapy of ALL (see summary in **Table 1**). A special focus will be put on the discussion on those genetic analyses with the potential to tailor chemotherapy regimes in the ALL setting.

L-ASPARAGINASE

L-asparaginase is a standard component in the initial treatment of childhood ALL which induces the depletion of the essential amino acid L-asparagine in the tumor cells resulting in inhibition of protein synthesis.

This drug has been related to serious adverse effects in ALL patients, such as acute pancreatitis (Flores-Calderon et al., 2009) or cerebrovascular accidents (Gugliotta et al., 1992). In addition, hypersensitivity reactions occur in up to 25% of the patients and 10% of those may experience life-threatening anaphylactic reactions (Cortijo-Cascajares et al., 2012). Studies on the pharmacogenetics of asparaginase in ALL are in their early stages. A large study recently carried out at St Jude Children's Hospital by Chen et al. (2010) interrogated more than 500 000 single nucleotide polymorphisms (SNPs) in 485 children with ALL and found that five mutations (rs4958351, rs10070447, rs6890057, rs4958676, and rs6889909) in the *GRIA1* gene were associated with the occurrence of hypersensitivity to the drug. *GRIA1* encodes a subunit of the AMPA receptor, a tetrameric ligand-gated ion channel that transmits glutamatergic signals in the brain. Glutamate not only has a role as a neurotransmitter, but also as an immunomodulator (Pacheco et al., 2007) and the study by

Table 1 | Summary of genes and polymorphisms with a putative relevant role in chemotherapy-induced toxicity in acute lymphoblastic leukemia

Gene	Polymorphism	Drug affected; main effect	Reference
ABCB1	C3435T, G2677T/A, C1236T	IMT[a]; Less common toxicity-related dose reduction	Gurney et al. (2007)
CYP2B6	*2A, *4	CFD[b]; Hemorrhagic cystitis, oral mucositis	Rocha et al. (2009)
DCK	Several SNPs	Ara-C[c]; higher susceptibility to drug effects	Hartford et al. (2009)
DHFR	19-bp deletion	MTX[d], hepatotoxicity	Ongaro et al. (2009)
GRIA1	Several SNPs	ASP; higher risk of hypersensitivity	Chen et al. (2010)
ITPA	c.94C > A	6-MP; fever, hepatotoxicity	Stocco et al. (2009), Wan Rosalina et al. (2011)
ITPA	IVS + 21A > C	6-MP; higher risk of myelotoxicity	Hawwa et al. (2008)
MTHFR	C677T	MTX; neurotoxicity, hepatotoxicity, myelosuppression	Strunk et al. (2003), Mahadeo et al. (2010), D'Angelo et al. (2011), Yang et al. (2012)
MTHFR	A1298C	MTX[e], hematological toxicity	Kantar et al. (2009)
MTRR	A66G	MTX, oral mucositis	Huang et al. (2008)
RFC1	G-80A	MTX, overall toxicity	Shimasaki et al. (2006), Imanishi et al. (2007)
TPMT	*2, *3A, *3C	6-MP; Acute hematopoietic toxic effects	McLeod et al. (1999), Weinshilboum (2001), Pui et al. (2004)

[a] *Only data on chronic myeloid leukemia patients are available;* [b] *Several types of leukemia were included in the study;* [c] *Preliminary data on cell lines;* [d] *Adult patients;* [e] *Controversial association.*
6-MP, 6-mercaptopurine; MTX, methotrexate; ASP, L-asparaginase; CFD, cyclophosphamide; Ara-C, cytosine arabinoside; IMT, imatinib

Chen et al. (2011) provides the first link between *GRIA1* polymorphisms and an immune-related phenotype such as the occurrence of hypersensitivity to L-asparaginase. A later genome-wide study by the same group using the HapMap lymphoblastoid cell lines tested more than 2 million SNPs and identified the aspartate metabolic routes as the most likely candidate pathway for asparaginase sensitivity.

Finally, polymorphisms in other genes that mediate the antileukemic effect of asparaginase, such as the asparaginase synthetase gene (*ASNS*), the basic region leucine zipper activating transcription factor 5 (*ATF5*), or the argininosuccinate synthase 1 (*ASS1*), have been associated to lower event-free survival of ALL patients, albeit the authors reported no associations with adverse effects (Rousseau et al., 2011).

CYCLOPHOSPHAMIDE

Cyclophosphamide, an oxazophosphorine, bifunctional DNA alkylating agent, is crucial in the treatment of most pediatric and adult malignancies, including ALL. Cyclophosphamide is bioactivated in the liver by various enzymes of the hepatic P450 system including CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4, and CYP3A5 (Cox, 1979; Parekh and Sladek, 1993; Dirven et al., 1994; Hayes and Pulford, 1995; Ludeman, 1999; Huang et al., 2000), whilst detoxification of active metabolites is mainly mediated by aldehyde dehydrogenases (ALDH1A1 and ALDH3A1; Parekh and Sladek, 1993) and glutathione S-transferases (GSTA1, GSTM1, GSTP1, and GSTT1; Hayes and Pulford, 1995). These enzymes are known to have functional polymorphisms, some of which have been found to impact toxicity associated with cyclophosphamide-based therapies in several pathologies (Zhong et al., 2006; Cho et al., 2010).

A study conducted in patients with leukemia who underwent hematopoietic stem cell transplantation showed that carriers of *CYP2B6*2A* or *CYP2B6*4* variant alleles treated with cyclophosphamide were at higher risk of developing hemorrhagic cystitis and oral mucositis, respectively (Rocha et al., 2009). Interestingly, at least the *CYP2B6*4* variant has been related to increased enzyme activity (Lang et al., 2001; Kirchheiner et al., 2003), and presumably this could lead to increased bioactivation of the drug and therefore could explain the observed higher incidence of toxicity in the patients. Numerous other *CYP2B6* alleles that have been associated with changes in the enzyme activity/expression (www.cypalleles.ki.se/cyp2b6.htm) and therefore they could be relevant with regard to cyclophosphamide-induced side effects. It should also be remarked that the *CYP2B6* gene exhibits a large intraethnic variability. Indeed, novel allelic variants and different linkage disequilibrium values have been described in certain populations (Restrepo et al., 2011), which should be taking into account when implementing tailored genotyping protocols aimed to determine the potential of *CYP2B6* as a biomarker of drug response.

Allan et al. (2001) have reported that the 105Val allele of the *GSTP1* Ile105Val polymorphism was overrepresented in a group of patients with therapy-related acute myeloid leukemia. Interestingly, this association was only observed in therapies including GSTP1 substrates such as cyclophosphamide. The authors hypothesized that an overexposure to the drug due to reduced detoxification capabilities could have played a role in leukemogenesis. Moreover, the same variation of the *GSTP1* gene has been suggested to be one of the factors determining a higher neurotoxicity of ifosfamide, a closely related agent (Zielinska et al., 2005). A later report showed that the presence of the 677C-1298C

haplotype in the methylenetetrahydrofolate reductase (*MTHFR*) gene was also associated to secondary acute myeloid leukemia after cyclophosphamide treatment of hematologic malignancies (Guillem et al., 2007).

Other polymorphisms reportedly associated with increased cyclophosphamide-induced toxicity are *CYP2C19*2* (Takada et al., 2004; Ngamjanyaporn et al., 2011), *CYP3A4*1B* (Su et al., 2010), *GSTM1/T1* null (Cho et al., 2010), *ALDH3A1*2* and *ALDH1A1*2* (Ekhart et al., 2008), *ABCC4* rs9561778 (Low et al., 2009), or *ABCG2 Q141K* (rs2231142; Kim et al., 2008). It should be stated that some of these studies were conducted in patients with hematological malignancies but there are as yet no available data on ALL.

Cyclophosphamide is a chemotherapeutic agent used in a broad array of malignancies, which somewhat hampers the reproducibility of these findings. Therefore, larger studies and more consistent populations are needed in order to unequivocally establish the impact of the aforementioned SNPs and to identify other variants that could account for increased toxicity in cyclophosphamide-based therapies in ALL.

CYTOSINE ARABINOSIDE

Cytosine arabinoside (Cytarabine, Ara-C) is an antimetabolite widely used in acute leukemia, which is associated with several adverse side effects, including myelosuppression, infections, mucositis, neurotoxicity, and acute pulmonary syndrome (Hartford et al., 2009). Candidate gene approaches have been used to identify genetic variables that are important in susceptibility to Ara-C. These studies have mainly focused on genes in the pharmacokinetic pathway of the drug. For instance, a common polymorphism, A79C (rs2072671), in the cytidine deaminase (*CDA*) gene, which catalyzes the rapid deamination of Ara-C, results in lower enzyme activity and hence a decreased rate of Ara-C metabolism (Kirch et al., 1998). Interestingly, Bhatla et al. (2009) have shown that Ara-C-related mortality was significantly elevated in carriers of the 79CC genotype in children with acute leukemia. Moreover, Ciccolini et al. (2012) have related this SNP to life-threatening toxicities induced in a girl with lymphoma treated with Ara-C.

Hartford et al. (2009) utilized an unbiased whole-genome approach to find polymorphisms that might predict the susceptibility to the cytotoxic effects of Ara-C in cell lines derived from persons of European (CEU) and African (YRI) ancestry. The authors identified a unique pharmacogenetic signature consisting of four SNPs explaining 51% of the variability in sensitivity to ara-C among the CEU and five SNPs explaining 58% of the variation among the YRI. These unique genetic signatures comprised novel target genes, most importantly *GIT1*, *RAD51AP1*, and *SLC25A37*, which can be studied further in functional studies. Furthermore, the authors examined 64 SNPs in the deoxycytidine kinase gene (*DCK*), which catalyzes the most essential step in the Ara-C activation pathway. Their conclusions show that cells that carried the *DCK* A70G (Ile24Val) polymorphism, which affects protein function, had an increased sensitivity to Ara-C (Hartford et al., 2009). In this regard, a later study has suggested that another *DCK* SNP, C-360G, is associated with the occurrence of mucositis after low-dose Ara-C in pediatric ALL patients (Banklau et al., 2010).

Recently, a study by Xu et al. (2012) seems to confirm the importance of both *CDA* and *DCK* genes as important loci that should be further investigated regarding the outcome of Ara-C-based chemotherapy in leukemia patients.

CYTOTOXIC ANTIBIOTICS

Doxorubicin (adriamycin) and daunorubicin (daunomycin) are anthracycline antibiotics commonly used in the treatment of leukemias whose main concern is their well-known dose-related cardiotoxicity (Gilladoga et al., 1976).

The contribution of pharmacogenetic factors across the doxorubicin biochemical pathway is not well established, but the drug is characterized by inter-individual variation in pharmacokinetic and pharmacodynamic parameters, and genetic variation has been suggested to account for at least part of this variability (Jamieson and Boddy, 2010; Lal et al., 2010). For instance, SNPs in the *ABCB1* and *SLC22A16* transporter genes have been shown to increase exposure levels that could result in a higher incidence of adverse effects (Lal et al., 2007, 2008). No studies on the impact of these mutations on the incidence of adverse effects in ALL patients have as yet been conducted. In patients of breast cancer treated with doxorubicin, Bray et al. (2010) have shown that several SNPs in the *SLC22A16* influx transporter gene (A146G, T312C, and T755C) are related to lower incidence of dose delay, indicative of less toxicity; although it should be noted that the authors considered their own findings as preliminary.

In addition, *in vitro* studies have shown that the wild-type allele of the Val88Ile (262G > A, rs1143663) SNP in the carbonyl reductase 1 (*CBR1*) gene, involved in doxorubicin metabolism, exhibits a higher rate of synthesis of cardiotoxic metabolites (Gonzalez-Covarrubias et al., 2007). The conclusions of this work seem to indicate that the small percentage of individuals of African ancestry (to which this SNP is confined) who are homozygous for the low-activity Ile88 allele would therefore be at lower risk of cardiotoxicity. Paradoxically, this would imply that a majority of the African population is at-risk for doxorubicin-induced toxicity. Interestingly enough, a study by Hasan et al. (2004) evaluated 100 African American patients who underwent doxorubicin-based combination therapy and found that they appeared to suffer cardiotoxicity from doxorubicin three times more frequently than previously studied populations. Because of the low sample size analyzed and the lack of head-to-head comparison, larger studies in a multiracial setting seem necessary to clarify this finding.

With regard to daunorubicin, a recent work has shown a trend toward significant increase in the drug systemic exposure in patients carrying the C-allele of the *CBR1* G312C (Leu73Leu, rs25678) polymorphism, which could elevate the risk of drug-induced toxicity (Varatharajan et al., 2012). In any case, it should be stated that the genetic associations reported for these two anthracyclines are still relatively recent and have not been consistently observed yet.

Mitoxantrone is an anthracycline analog which is a known substrate for ABC efflux transporters such as those encoded by the *ABCB1* and *ABCG2* genes (Kodaira et al., 2010). To date, there are no studies in the ALL setting testing the plausible hypothesis that genetic polymorphisms in these genes could affect mitoxantrone disposition and hence modulate the response and side

effects to the drug. However, Cotte et al. (2009) have determined the frequencies of seven *ABCB1* and *ABCG2* SNPs in multiple sclerosis patients treated with mitoxantrone. Several associations were reported regarding the clinical response rate, but no relevant differences in genotype frequencies were observed in a subset of patients with severe hematological or cardiac side effects. However, it was intriguing that one patient presenting cardiomyopathy after a low dose of mitoxantrone was found to carry an uncommon genotype with homozygous variant alleles in two *ABCB1* (G2677T/A, rs2032582 and C3435T, rs1045642) and one *ABCG2* locus (Gln141Lys, rs2231142), in addition to a variant allele for the *ABCC2* C-24T (rs717620) SNP (Cotte et al., 2009).

ETOPOSIDE

Etoposide is a topoisomerase II inhibitor used in a variety of malignancies. This drug is a substrate for the P-glycoprotein transporter, CYP3A4 and CYP3A5 isoforms (Relling et al., 1994), whose expression is partly regulated by the vitamin D receptor (VDR; Drocourt et al., 2002), and a number of phase II metabolizing enzymes, including GSTs and UGT1A1 (Watanabe et al., 2003). Therefore, alterations in these genes hold the potential to be relevant for the drug concentrations and clinical effects. However, to our knowledge there are as yet no studies investigating the association of polymorphisms in the etoposide pathways with the occurrence of adverse effects, mainly dose-limiting myelosuppression, in patients treated with the drug. Currently, there are only indications based on the impact of these genetic variations on the level of exposure to the drug. For instance, a study in 109 children diagnosed with ALL showed that carriers of both the *CYP3A5*3/*3* and *GSTP1* Ile/Ile genotype displayed a lower drug clearance, although this association was only observed in African Americans one month after treatment (Kishi et al., 2004). Moreover, the *ABCB1* C3435T SNP was found to be an independent predictor of etoposide clearance disregarding ethnicity. In contrast, one year after treatment, the *UGT1A1* 6/6 (**1/*1*), *VDR* intron 8 GG and *VDR* Fok 1 CC genotypes predicted higher clearance in African Americans (Kishi et al., 2004). Despite that the drug pharmacokinetics were shown to correlate with the incidence of adverse effects, no analyses of the association between SNPs and toxicity were conducted by the authors (Kishi et al., 2004).

Two genome-wide studies by an American research group have identified both genomic regions and SNPs associated with cellular sensitivity to etoposide. Huang et al. (2007) identified 63 genetic variants that contributed to etoposide-induced cytotoxicity through the evaluation of cell growth inhibition in cell lines from multi-generational pedigrees. The variants were present in genes whose expression had previously been related to altered cell sensitivity to etoposide, such as *AGPAT2*, *IL1B*, and *WNT5B*, but also in other genes not yet known to be associated with sensitivity to this agent. A limitation of this study was that candidate genes known to contribute to the pharmacokinetics of etoposide, e.g., *CYP3A*, *UGT1A1*, and *ABCB1*, are not expressed or are expressed at very low levels in the lymphoblastoid cell lines utilized.

Using the same cell models, Bleibel et al. (2009) later identified 22 unique SNPs in four genes among three chromosomes significantly associated with cytotoxic phenotypes at one or more treatment conditions. Genes implicated were *UVRAG*, a DNA repair gene, *SEMA5A*, which encodes semaphoring-5A protein involved in axonal guidance during development, the *SLC7A6* transporter gene, which participates in nitric oxide synthesis that ultimately induces apoptosis and *PRMT7*, encoding the protein arginine methyltransferase that catalyzes an irreversible protein modification. All these processes would be altered in the presence of functional polymorphisms and could presumably lead to the observed increased cytotoxicity in etoposide-treated cells.

Unfortunately, and despite the interesting background provided by these two genome-wide reports, there are as yet no clinical studies that have investigated the role of any of the aforementioned genetic variants in patients treated with etoposide.

IMATINIB

Imatinib is a Bcr-Abl tyrosine kinase inhibitor that is specifically used in the treatment of Phy + leukemia. The drug is demethylated to *N*-desmethyl-imatinib by CYP3A4/5, with other CYP450 isoforms playing a less important role (Peng et al., 2005). Indeed, coadministration of inhibitors and inducers of CYP3A activity results in significant modifications of the drug's pharmacokinetics (Bolton et al., 2004), which show a wide interindividual variability (Judson et al., 2005). In order to explain this variability, pharmacogenetics studies have been carried out to find genetic determinants that could modify the pharmacological response to the drug. Most of these studies have aimed to find genetic markers of resistance. Thus, a number of polymorphisms in genes coding for transporters (*ABCB1*, *ABCG2*, *SLC22A1*), drug metabolizing enzymes (*CYP3A5*), proteins with involved in the nucleotide excision repair pathway (*EERC*) and proteins related to leukemogenesis (*SOCS1* and *PTPN22*) have been associated to the efficacy of the treatment (Dulucq and Krajinovic, 2010; Kong et al., 2012; Vivona et al., 2012). These studies have mainly been conducted in patients with chronic myeloid leukemia (CML) and gastrointestinal stromal tumors (GIST), probably because of the higher incidence of these two diseases.

On the other hand, pharmacogenetic studies on imatinib-related toxicity are scarce and therefore the clinical impact of genetic polymorphisms on the occurrence of adverse effects has to be inferred from their effect on the drug blood levels, as it has been reported that the severity of the side effects seems to correlate with the drug's pharmacokinetics (Judson et al., 2005). In this regard, two studies have shown a reduced oral clearance of the drug in patients carrying the *CYP2D6*4* (Gardner et al., 2006) or *ABCG2 421A* variant alleles (Petain et al., 2008), which could hypothetically result in increased adverse effects. Interestingly, Gurney et al. (2007) reported a higher drug clearance but also a less common toxicity-related dose reduction in CML patients on imatinib who were carriers of the TT genotype at each of three key positions in the *ABCB1* gene (1236, 2677, and 3435).

These data are not yet sufficiently conclusive to translate into individual drug dose adjustments and therefore further studies are still needed to analyze other genetic variants that can help individualize imatinib therapy.

MERCAPTOPURINE

6-Mercaptopurine (6-MP) is an antimetabolite that has been used for 40 years in the treatment of a variety of diseases. In

particular, 6-MP in orally daily regimen associated with weekly methotrexate (MTX) is the backbone of maintenance chemotherapy for ALL. The drug is metabolized toward active, and toxic, 6-thioguanine nucleotides (6-TGN) that are responsible for the elevated myelotoxicity of this chemotherapy agent.

The thiopurine methyltransferase (*TPMT*) gene, codes for a key enzyme in the metabolism of 6-MP (**Figure 1**) and other related thiopurine drugs such as 6-thioguanine and azathioprine. This enzyme is affected by functional polymorphisms that have been shown to produce a defective enzyme, amongst them, *TPMT*2* (G238C), *TMPT*3A* (G460A, A719G), and *TPMT*3C* (A719G) account for 90% of the enzymatic deficiency in most populations (Weinshilboum, 2001). Approximately one in 300 hundred individuals are homozygous for these variants and therefore lack TPMT activity, which results in high levels of 6-TGN and acute hematopoietic toxic effects, greater risk for radiation-induced brain tumors and a greater likelihood of chemotherapy-induced acute myeloid leukemia (Pui et al., 2004). Because of this, extreme caution should be exerted in those children with ALL who lack TPMT activity and are scheduled to receive 6-MP. In turn, these subjects are far less prone to experience a relapse (Lennard and Lilleyman, 1996). Dose reductions of up to 90% have been proved useful in these patients (McLeod et al., 2000). For heterozygous carriers the administration of 50% of the standard dose is recommended, although there has been a certain degree of controversy as to whether these patients with intermediate activity may benefit from lower doses (McLeod et al., 1999; Stanulla et al., 2005).

Having said that, the *TPMT* polymorphism does not explain all 6-MP-induced adverse effects, and some severe toxicities leading to life-threatening conditions remain unexplained (Palmieri et al., 2007). Additional SNPs in genes encoding enzymes involved in 6-MP metabolism and transport might contribute to the drug-induced toxicity. This is the case of the inosine triphosphate pyrophosphatase (ITPA) enzyme, which catalyzes one of the intermediate steps of 6-MP metabolism (**Figure 1**). Two SNPs in the *ITPA* gene with a frequency of roughly 10% in Caucasians (Adam de Beaumais and Jacqz-Aigrain, 2012), namely a non-synonymous C94A transition (rs1127354, Pro32Thr) and the intronic IVS2 + 21A > C mutation have been related with defective enzyme activity (Heller et al., 2004) leading to higher risk of myelotoxicity and hepatotoxicity in ALL pediatric patients (Hawwa et al., 2008; Stocco et al., 2009; Wan Rosalina et al., 2011).

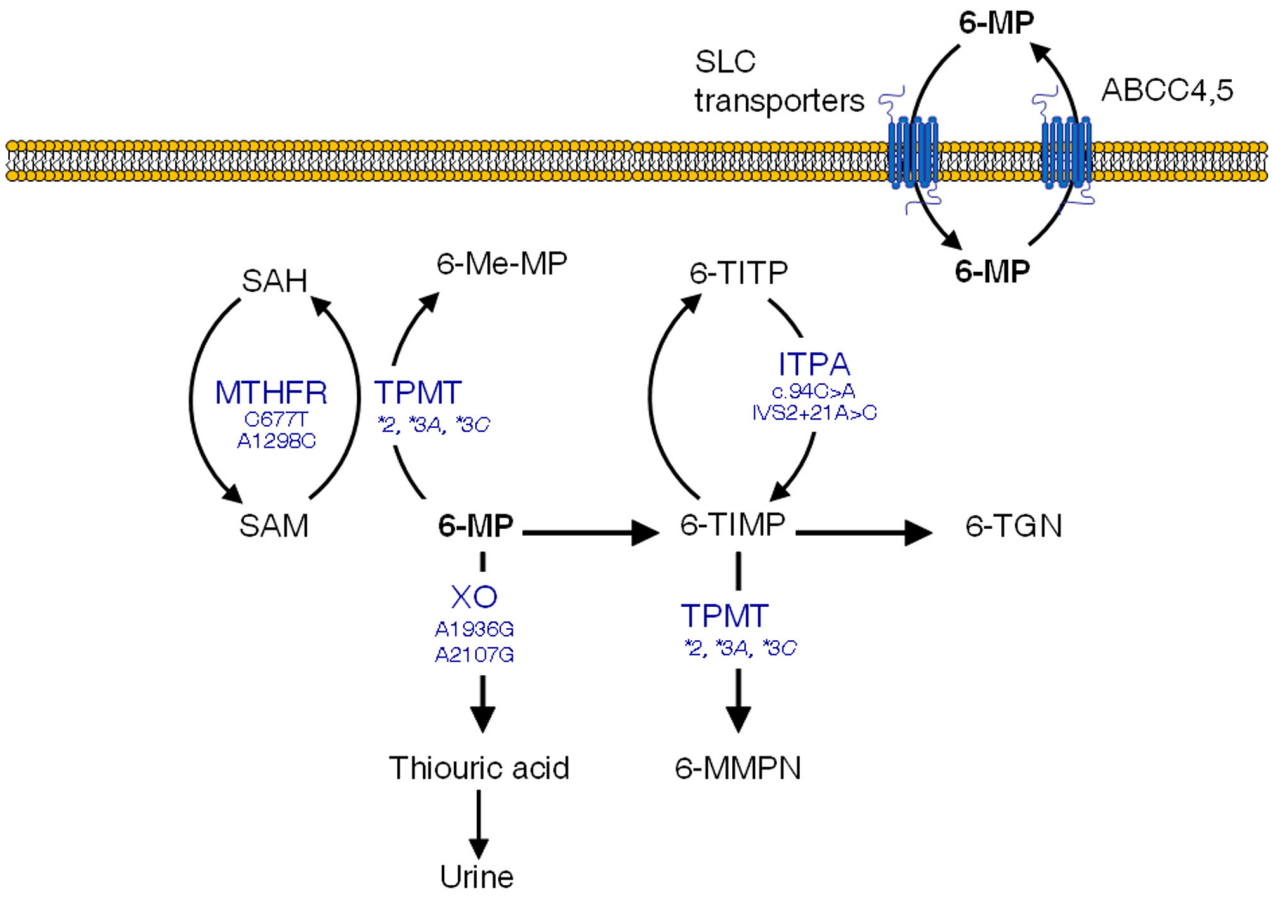

FIGURE 1 | 6-mercaptopurine (6-MP) intracellular pathways with polymorphisms suggested to increase the occurrence of adverse effects. SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; TPMT, thiopurine S-methyltransferase; XO, xanthine oxidase; 6-Me-MP, 6-methyl-mercaptopurine; 6-TIMP, 6-thioinosine monophosphate; 6-TITP, 6-thioinosine triphosphate; ITPA, inosine triphosphate pyrophosphatase; 6-MMPN, 6-methyl-mercaptopurine nucleotides; 6-TGN, 6-thioguanine nucleotides

In addition, *MTHFR* SNPs have been shown to be more common in subjects with low TPMT activity (Karas-Kuzelicki et al., 2009), probably because of their impact on S-adenosylmethionine, which functions as a cofactor for TPMT. Other polymorphisms in genes involved in 6-MP disposition such as xanthine oxidase or *ABCC4* have also been suggested to impact clinical outcomes, although there are as yet no available data in ALL patients (Hawwa et al., 2008; Ban et al., 2010).

The use of the TPMT genotyping for tailoring ALL therapy, and the putative inclusion of other polymorphisms in these genetic tests will be discussed in the final chapter of this review.

METHOTREXATE

Methotrexate (MTX) is a folate inhibitor widely employed in the chemotherapy of hematologic malignancies and various solid tumors. This drug is the cornerstone for therapy of ALL and has been the focus of a number of pharmacogenetic studies aimed to identify genetic determinants of its toxicity (reviewed elsewhere Gervasini, 2009). Its actions on folate metabolism follow a complex pattern that includes several transporters and metabolizing enzymes whose function and/or expression have been suggested to be altered by genetic polymorphisms.

Briefly, the reduced folate carrier 1 (*RFC1, SLC19A1*) is responsible for the entry of MTX in the cell (Moscow et al., 1995), whilst the drug is pumped out by a variety of ATP-binding cassette (ABC) efflux transporters (Strand et al., 1999). In the cell, MTX is metabolized to active polyglutamates, which are responsible for the disruption of the folate metabolic pathway by inhibiting enzymes that are essential for the DNA (Chabner et al., 1985). These include thymidylate synthase (TS; Szeto et al., 1979) and dihydrofolate reductase (DHFR; Galivan, 1980). Another key enzyme in the folate pathways is methylenetetrahydrofolate reductase (MTHFR), which produces 5-methyl-tetrahydrofolate (THF) from 5,10-methylene-THF, a major intermediary that is in turn synthesized by serine hydroxymethyltransferase (SHMT1). Finally, the production of 5-methyl-THF is pivotal for biotransformation of homocysteine to methionine, which involves two major enzymes, namely methionine synthase (MS) and methionine synthase reductase (MTRR). **Figure 2** depicts an overview of these intracellular pathways pointing out those SNPs that have been related with the occurrence of MTX-induced toxicity.

MTHFR has by far been the most extensively studied gene in association studies in ALL. Two SNPs, C677T (rs1801133) and A1298C (rs1801131), have been related to increased MTX toxicity (Kantar et al., 2009; D'Angelo et al., 2011). However, a recent meta-analysis (Yang et al., 2012), as well as the general perception in the literature (reviewed by Gervasini, 2009) lead to think that C677T is the only SNP that plays a clinically significant role, albeit contradictory results also exist (Chatzidakis et al., 2006; Kishi et al., 2007; Huang et al., 2008).

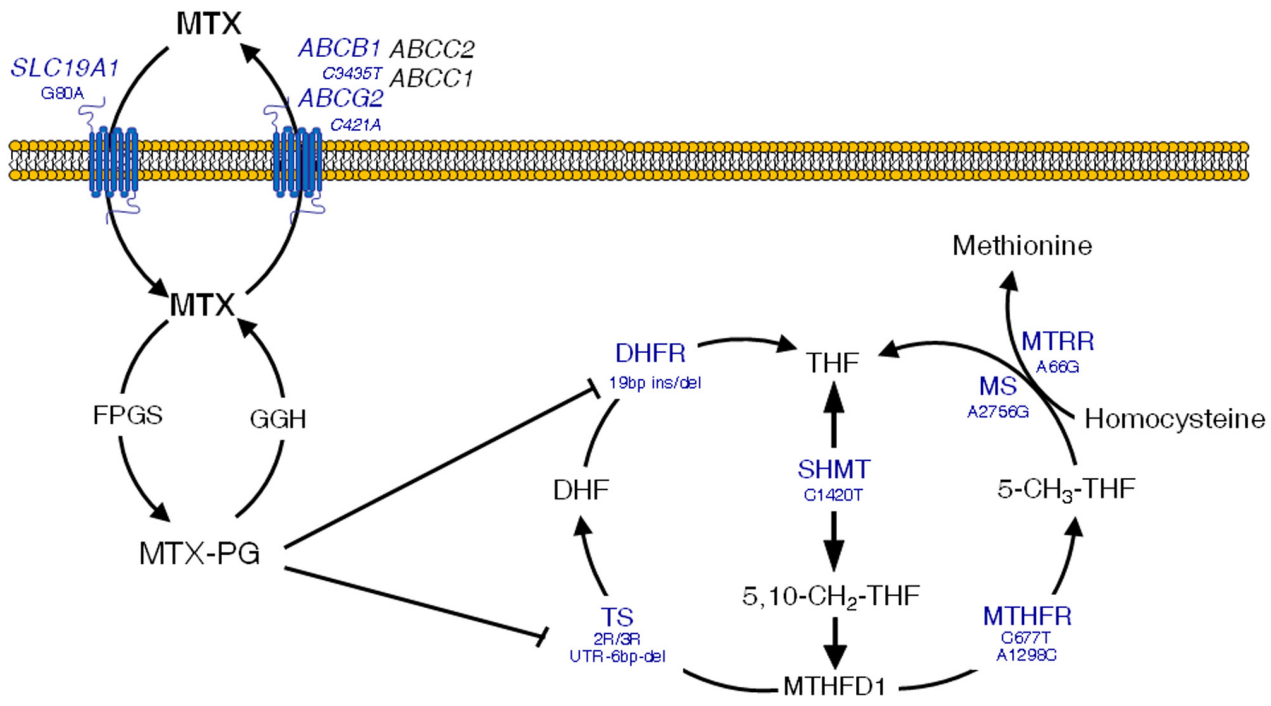

FIGURE 2 | Overview of methotrexate (MTX) mechanisms of action in the folate metabolic pathway. Clinically significant polymorphisms are shown for the main genes involved. MTX-PG, methotrexate polyglutamates; *SLC19A1*, solute carrier 19A1; *ABCC1* and *2*, ATP-binding cassette C1 and 2 transporters; *ABCG2*, ATP-binding cassette G2 transporter, breast cancer resistance protein; *ABCB1*, ATP-binding cassette B1 transporter, multidrug resistance; FPGS, folylpolyglutamyl synthase; GGH, γ-glutamyl hydrolase; *TS*, thymidylate synthase; UTR, untranslated region; *DHFR*, dihydrofolate reductase; *SHMT*, serine hydroxymethyltransferase; *MTHFD1*, methylenetetrahydrofolate dehydrogenase; *CCND1*, cyclin D1; *ATIC*, aminoimidazole 4-carboxamide ribonucleotide (AICAR) transformylase; *MS*, methionine synthase; *MTRR*, methionine synthase reductase. Adapted with permission from Gervasini (2009).

Most notably, given the severity of the syndrome, the 677T allele has been suggested to induce MTX-related neurotoxicity (mainly subacute leukoencephalopathy) in young ALL patients (Strunk et al., 2003; Mahadeo et al., 2010; Vagace et al., 2011, 2012). It should be noted that this association has only been observed in clinical case reports. In fact, a study in 53 children with ALL treated with high-dose MTX failed to confirm a relevant role of the 677T allele in the drug-induced neurotoxicity developed by nine of the patients (Kishi et al., 2003). In order to elucidate this controversy, it would be desirable to genotype large populations of pediatric ALL patients who underwent MTX-related neurotoxicity. However, the lack of homogeneity of treatment protocols, which makes multicenter studies hard to conduct, the low number of patients affected, and the more than likely influence of other SNPs, have so far been insurmountable obstacles.

A 19 bp ins/del polymorphism in the *DHFR* gene has also been associated with increased toxicity in adult leukemia patients treated with MTX (Ongaro et al., 2009). In the same manner, the *MTRR* A66G SNP has been related to increased risk of developing toxicity in children with ALL treated with high doses of MTX (Huang et al., 2008), although there seems to be no relation with CNS side effects (Krajinovic et al., 2005).

Other genes that could also be involved in the development of MTX-induced toxicity are the ABC efflux transporters. Their location in the blood-brain barrier makes tempting to speculate that polymorphisms in these genes could lead to drug accumulation in the brain and subsequent neurotoxicity. For instance, the combined presence of the C421A (rs2231142, Gln141Lys) SNP in the *ABCG2* gene (encoding the Breast Resistance Cancer Protein, BCRP transporter) and the C3435T transition in *ABCB1* (coding for P-glycoprotein) has been related to the occurrence of encephalopathy in children with ALL treated with MTX (Erdilyi et al., 2008). Finally, the G-80A polymorphism in the RFC1 influx transporter, which determines intracellular levels of MTX, has been associated with increased overall toxicity in ALL patients (Shimasaki et al., 2006; Imanishi et al., 2007; Kishi et al., 2007).

It should be noted that large studies on the association between MTX toxicity and genetics in childhood ALL are still scarce and mostly focus on *MTHFR* variants. Studies carried out in other pathologies have found evidences of increased toxicity in the presence of variant alleles in additional ABC transporters (Campalani et al., 2007; Ranganathan et al., 2008), or in the *SHMT1* (Weisman et al., 2006) and *TS* genes (Campalani et al., 2007). Therefore, we should not rule out the implication of these polymorphisms in MXT-related side effects experienced by ALL patients.

VINCRISTINE

The wide pharmacokinetic interindividual variability shown by vincristine, and the occurrence of its dose-limiting neurotoxicity remains largely unpredictable (Moore and Pinkerton, 2009). Renbarger et al. (2008) have showed different grades of neurotoxicity in Caucasians compared to African Americans, suggesting that genetics could influence vincristine-induced toxicity.

The main candidate gene for pharmacogenetic studies is *CYP3A5*, since it is known to contribute between 55 and 95% to total vincristine metabolism (Dennison et al., 2006). In addition, the drug is a substrate for P-glycoprotein (Song et al., 1999), which makes the encoding *ABCB1* gene another suitable candidate for pharmacogenetic studies. Two reports have evaluated the clinical effect of common SNPs (*CYP3A5*3* and *ABCB1* C3435T and G2677T) in these genes in pediatric ALL patients treated with vincristine. However, the authors failed to find a significant association with increased occurrence of side effects such as impaired motor activity or constipation (Plasschaert et al., 2004; Hartman et al., 2010).These two studies combined added up to only 86 genotyped patients, which is clearly not sufficient to rule out the involvement of these candidate genes in vincristine toxicity.

PERSPECTIVES AND CONCLUSION

The utility of pharmacogenetics in clinical routine has turned to be lower than anticipated, as numerous barriers to implementing individualized medicine have appeared over the years (Agundez et al., 2012). Cases in which a genetic test is sufficient to significantly affect a given therapy are discouragingly uncommon (Groenen, 2011) and the leukemia setting is not an exception. Indeed, only 1–2% of marketed drugs have pharmacogenomic-based recommendations (Agundez et al., 2012) and amongst the drugs commonly used in the chemotherapy of pediatric ALL, only 6-MP, thioguanine and azathioprine labels include genetic testing (*TPMT*) as a recommendation to help individualize therapy. However, steps are being taken in order to revert this situation. The increasing availability of low-cost, high-throughput genetic platforms that allow the simultaneous screening of hundreds of polymorphisms, the education of health practitioners or the implementation of multicenter networks aimed to improve the safety of new drugs, are some of the measures that hopefully will help improve the impact of pharmacogenetics on clinical routine. It should also be pointed out that genetic variability in several of the targets for which genetic testing is recommended by the FDA and other corporations has been shown to modify the risk of acute leukemia (Agundez, 2004), which certainly enhances the utility of these tests.

The body of work on the clinical impact of *TPMT* and *MTHFR* polymorphisms on ALL does not mirror the limited current knowledge on the pharmacogenetics of the other drugs that constitute the core of chemotherapy for this disease. Thus, significantly less information is available with regard to vincristine, asparaginase, cyclophosphamide etc. The *TPMT* gene polymorphism provides the best example of the value of applied pharmacogenetics in ALL and clinical oncology in general. It is now widely acknowledged that the initial dose of 6-MP treatment should be based on the *TPMT* genotype (Relling et al., 2011), thus allowing the clinician to identify patients at higher risk of toxicity. However, as it was described in the 6-MP chapter, there is still some interindividual variability in the response to this drug that cannot be explained only by the *TPMT* genotype (Palmieri et al., 2007). Therefore, the implementation of additional genetic analyses to identify polymorphisms in the *ITPA*, *MTHFR*, *XO* and other genes in the 6-MP intracellular pathway, as well as the study of their epistatic interactions seem to be reasonable steps to take in order to better adjust 6-MP doses in ALL patients. Furthermore, pharmacogenomics alone may not be sufficient to explain all the interindividual variability in 6-MP response and efforts should be

undertaken in the coming years to create more precise algorithms that can help predict drug response.

With regard to *MTHFR*, the reasons for the present controversy regarding the influence of its genetic variation on MTX-induced toxicity are diverse. For instance, the wide variety of diseases in which MTX has been proved useful has paradoxically hampered the reproducibility of the results of genetic association studies, because of the heterogeneity of the patients analyzed. Moreover, other unknown variants in the same gene, epistatic interactions with other genes and, most likely, the combination of these factors, may result in genetic backgrounds with different susceptibilities to MTX-induced toxicity. The challenge seems to be to identify which genetic factors and genetic combinations are those in specific populations.

Some ALL chemotherapy regimens contemplate MTX dose reductions for subjects homozygous for the 677T variant or for those carrying both 677CT and 1298AC heterozygous genotypes. One could consider at least premature to make this kind of dose adjustments based on data that are still controversial. In fact, MTX was not included in the list of drugs mentioned in the *Table of Pharmacogenomic Biomarkers in Drug Label* issued by the Food and Drug Administration (FDA) last year (FDA, 2011). It is probably naive to believe that the determination of SNPs in just one gene (*MTHFR*) belonging to a highly complex intracellular pathway, such as that of MTX, is enough to accurately anticipate the occurrence of adverse effects.

As a general rule, it would be logical to think that the identification of combinations of mutations in several genes along the pathway of a given drug must be more helpful in terms of identifying subjects at-risk of toxicity than a single-SNP approach (Vagace et al., 2011). This is crucial in the case of drugs with intricate intracellular routes as it is the case of MTX or 6-MP. Genome-wide approaches such as the ones reported by (Huang et al., 2007), Bleibel et al. (2009), and Chen et al. (2011) are able to evaluate whole pathways to identify key routes that can be later studied in detail. In addition, the inclusion of other clinical and demographic factors may increase the predictive value of pharmacogenetic models (Wessels et al., 2007).

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Multilocus genotypes of relevance for drug metabolizing enzymes and therapy with thiopurines in patients with acute lymphoblastic leukemia

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Multilocus genotypes have been shown to be of relevance for using pharmacogenomic principles to individualize drug therapy. As it relates to thiopurine therapy, genetic polymorphisms of *TPMT* are strongly associated with the pharmacokinetics and clinical effects of thiopurines (mercaptopurine and azathioprine), influencing their toxicity and efficacy. We have recently demonstrated that *TPMT* and *ITPA* genotypes constitute a multilocus genotype of pharmacogenetic relevance for children with acute lymphoblastic leukemia (ALL) receiving thiopurine therapy. The use of high-throughput genomic analysis allows identification of additional candidate genetic factors associated with pharmacogenetic phenotypes, such as TPMT enzymatic activity: *PACSIN2* polymorphisms have been identified by a genome-wide analysis, combining evaluation of polymorphisms and gene expression, as a significant determinant of TPMT activity in the HapMap CEU cell lines and the effects of *PACSIN2* on TPMT activity and mercaptopurine induced adverse effects were confirmed in children with ALL. Combination of genetic factors of relevance for thiopurine metabolizing enzyme activity, based on the growing understanding of their association with drug metabolism and efficacy, is particularly promising for patients with pediatric ALL. The knowledge basis and clinical applications for multilocus genotypes of importance for therapy with mercaptopurine in pediatric ALL is discussed in the present review.

Keywords: acute lymphoblastic leukemia, mercaptopurine, pharmacogenetics, multilocus genotypes, TPMT, ITPA, PACSIN2

INTRODUCTION

The principle of personalized therapy is the identification and application of features associated with treatment response, to select adequate medications and their doses, in order to offer to patients the most effective treatment, with the lower incidence of adverse events (Cheok and Evans, 2006). Among the several features that can be used to personalize therapy, demographic, clinical, and pharmacological ones have been considered. The application of therapy targeted according to these features, related to different treatment outcomes, has greatly improved the effectiveness and safety of therapy, in particular for patients with pediatric cancer, such as acute lymphoblastic leukemia (ALL).

PERSONALIZED THERAPY IMPROVES TREATMENT EFFECTS: THE PARADIGM OF CHILDHOOD ALL

Optimal use of existing antileukemic agents and improved supportive care in contemporary clinical trials have improved the 5-year survival rate of childhood ALL above 85% in developed countries, a disease that was universally fatal in the sixties; moreover, molecular characteristics of leukemia cells have been shown to influence treatment response (Pui and Evans, 2006; Pui et al., 2012).

Pharmacological therapy for childhood ALL consists in protocols in which specific treatment approaches may differ but consistently comprise three major treatment phases: remission induction therapy followed by consolidation/intensification therapy and then continuation/maintenance treatment to eliminate residual leukemic cells (Pui and Evans, 2006). Several medications are used in these treatment phases, comprising various lympholytic and cytotoxic drugs such glucocorticoids (i.e., prednisone, dexamethasone), asparaginase and vincristine, which are particular important for the induction of disease remission. The purine analog mercaptopurine is a key medication for the successful treatment of childhood ALL, in particular for the consolidation and continuation therapies and is used in combination with the folate analog methotrexate: for the success of ALL treatment, the 18–24 months of adequate maintenance therapy comprising mercaptopurine and methotrexate have a key role and are necessary to prolong and consolidate the remission obtained during the initial treatment phases (Pui and Evans, 2006; Paugh et al., 2010; Stocco et al., 2010).

GENETIC FEATURES MAY INFLUENCE RESPONSE TO THERAPY

Genetic polymorphisms for genes involved in drug metabolism, transport and molecular mechanism of action can alter the concentration of active metabolites and the molecular function of drugs' targets and therefore the efficacy and safety of pharmacological therapies (Paugh et al., 2011; Pinto et al., 2012). These genetic polymorphisms could therefore function as biomarkers for toxicity and efficacy, allowing the identification of patients with modified sensitivity, because of their genetic characteristics involving drug pharmacokinetics and pharmacodynamics. While many associations between single genetic polymorphisms and drug effects have been clearly demonstrated, showing that inherited genomic variation causes substantial interindividual differences in drug effects, the clinical implementation of these associations is still limited (Relling and Klein, 2011). This is due mainly to the lack of freely available, peer-reviewed, updatable, and detailed gene/drug clinical practice guidelines and even to the very high standards many clinicians and regulators hold for pharmacogenetic evidence (Relling et al., 2010). One of the main efforts to provide these guidelines, which could base the clinical implementation of pharmacogenomics, is that of the Clinical Pharmacogenetics Implementation Consortium (CPIC; Relling and Klein, 2011). CPIC was established in 2009 and consists of members of the Pharmacogenomics Research Network, the main US-based research network in this field, supported by the PharmGKB staff, one of the most important resources for curated pharmacogenomics knowledge (McDonagh et al., 2011), and other affiliated experts in pharmacogenetics, pharmacogenomics and laboratory medicine (Relling and Klein, 2011).

Currently, the CPIC has provided guidelines for pharmacogenetic implementation for 7 medications: abacavir, allopurinol, clopidogrel, codeine, simvastatin, thiopurines, and warfarin (https://www.pharmgkb.org/page/cpic). The process of guidelines definition and preparation is still ongoing and other potential guidelines may be of interest, such as inosine triphosphate pyrophosphatase (ITPA) genetic polymorphism and ribavirin (Fellay et al., 2010), for which a good amount of evidence and replication has been made (Ochi et al., 2010; Thompson et al., 2010; D'Avolio et al., 2012). Moreover, besides CPIC, other research groups have been putting together similar guidelines, such as the European Dutch and German translational pharmacogenomics research teams (Swen et al., 2008, 2011; Schwab and Brauch, 2012).

For the pharmacological therapy of pediatric ALL, several examples have been reported of genetic polymorphisms influencing drug response and toxicity, such as for prednisone polymorphisms of *SMARCB1* (Pottier et al., 2007, 2008) and *GST-M1* (Marino et al., 2009), for methotrexate solute carrier organic anion transporter family member 1B1 (SLCO1B1) (Trevino et al., 2009; Ramsey et al., 2012), for vincristine *ABCB1* and *CYP3A4*/*CYP3A5* (Paugh et al., 2010); however, the only drug – gene pair with a validated guideline published by CPIC that is fully relevant for therapy of pediatric ALL is that of mercaptopurine and thiopurine-*S*-methyltransferase (TPMT). Indeed, for mercaptopurine, genetic polymorphisms of *TPMT* have been demonstrated to influence drug metabolism and its effects, constituting one of the most studied and significant example of associations between drug clinical effects and a genetic polymorphism (Paugh et al., 2011). In lymphoid tissues, mercaptopurine is converted to its active metabolites, the thioguanine nucleotides (TGNs) and is inactivated primarily to methylmercaptopurine by TPMT (Stocco et al., 2010; Zaza et al., 2010). TPMT is encoded by a gene that has non-synonymous single-nucleotide polymorphisms, leading to reduced TPMT activity. In the majority of world populations studied to date, ∼1 in 180 to 1 in 3,700 individuals (depending on ethnicity) inherit two non-functional variants of the *TPMT* gene, 3–14% are heterozygous, and the rest are homozygous wild-type. With chronic conventional doses of mercaptopurine, patients who inherit two inactive *TPMT* alleles universally experience severe myelosuppression, because of accumulation of high levels of cellular TGNs; a high proportion (30–60%) of patients heterozygous for a *TPMT* variant allele does not tolerate full doses of mercaptopurine, again because of excessive TGNs. Three *TPMT* single-nucleotide polymorphisms account for more than 90% of inactivating alleles and therefore genotyping tests have a high likelihood of being informative. Characterization of TPMT deficiency by genotyping for the most common inactivating single-nucleotide polymorphisms can prospectively identify patients at higher risk of mercaptopurine hematopoietic toxicity; such genotyping is recommended in US Food and Drug Administration-approved labeling (Paugh et al., 2010, 2011).

The diagnosis of TPMT deficiency allows the rational reduction of mercaptopurine dosages while other concurrent cytotoxic agents remain at their usual unadjusted doses, thereby avoiding toxicity without compromising efficacy. For patients with ALL taking mercaptopurine, the CPIC guidelines indicate that these subjects with the homozygous variant should start with drastically reduced dose (i.e., reduce daily dose 10-fold and reduce frequency to thrice weekly instead of daily) and in case of myelosuppression, emphasis should be on reducing mercaptopurine over other agents; patients heterozygous for *TPMT* variant alleles (intermediate activity) should start at 30–70% of full dose and again, in case of myelosuppression, emphasis should be on reducing mercaptopurine over other agents. Patients with normal *TPMT* should begin therapy with normal starting dose and adjust doses of mercaptopurine (and of any other myelosuppressive therapy) without any special emphasis on mercaptopurine compared to other agents. For all genotypes, in case of dose adjustment, the guidelines specify to allow 2–6 weeks to reach steady state after each dose adjustment, with longer time needed for patients with inactive allele(s) as compared to patients with functional ones (Relling et al., 2011; **Table 1**). Indeed, it has been shown that, in an ALL protocol using mercaptopurine, prospective adjustment of mercaptopurine based on TPMT status allowed successful treatment of patients with variant *TPMT* at a reduced dose, with toxicity and efficacy comparable to those in patients with wild-type *TPMT* (Relling et al., 2006; Stocco et al., 2009).

MULTILOCUS GENOTYPES

Most of the associations evaluated in the literature and those ready for clinical implementation, i.e. with published curated guidelines, are considering single gene influencing response to a particular drug. However, one of the most important, in terms of relevance and diffusion of drug treatment and potential improvement of guideline application to influence drug use in the clinical setting

Table 1 | Recommended dosing of thiopurines by thiopurine methyltransferase phenotype.

TPMT status	Mercaptopurine		Thioguanine	
	Effects on mercaptopurine metabolism	**Dosing recommendations for mercaptopurine**	**Effects on thioguanine metabolism**	**Dosing recommendations for thioguanine**
Homozygous wild-type or normal, high activity	Lower concentrations of TGNs metabolites, higher methylTIMP, this is the "normal" pattern	Start with normal starting dose (e.g., 75 mg/m^2/day) and adjust doses of mercaptopurine (and of any other myelosuppressive therapy) without any special emphasis on mercaptopurine compared to other agents	Lower concentrations of TGNs metabolites, but note that TGNs after thioguanine are 5–10× higher than TGNs after mercaptopurine	Start with normal starting dose. Adjust doses of thioguanine and of other myelosuppressive therapy without any special emphasis on thioguanine.
Heterozygote or intermediate activity	Moderate to high concentrations of TGNs metabolites; low concentrations of methylTIMP	Start with reduced doses (start at 30–70% of full dose: e.g., at 50 mg/m^2/day or 0.75 mg/kg/day) and adjust doses of mercaptopurine based on degree of myelosuppression and disease-specific guidelines. In those who require a dosage reduction based on myelosuppression, the median dose may be ∼40% lower (44 mg/m^2/day) than that tolerated in wild-type patients (75 mg/m^2/day). In setting of myelosuppression, and depending on other therapy, emphasis should be on reducing mercaptopurine over other agents	Moderate to high concentrations of TGNs metabolites; but note that TGNs after thioguanine are 5–10× higher than TGNs after mercaptopurine	Start with reduced doses (reduce by 30–50%) and adjust doses of thioguanine based on degree of myelosuppression and disease-specific guidelines. In setting of myelosuppression, and depending on other therapy, emphasis should be on reducing thioguanine over other agents
Homozygous variant, mutant, low, or deficient activity	Extremely high concentrations of TGNs metabolites; fatal toxicity possible without dose decrease; no methylTIMP metabolites	Start with drastically reduced doses (reduce daily dose by 10-fold and reduce frequency to thrice weekly instead of daily, e.g., 10 mg/m^2/day given just 3 days/week) and adjust doses of mercaptopurine based on degree of myelosuppression and disease-specific guidelines. In setting of myelosuppression, emphasis should be on reducing mercaptopurine over other agents	Extremely high concentrations of TGNs metabolites; fatal toxicity possible without dose decrease	Start with drastically reduced doses (reduce daily dose by 10-fold and dose thrice weekly instead of daily) and adjust doses of thioguanine based on degree of myelosuppression and disease-specific guidelines. In setting of myelosuppression, emphasis should be on reducing thioguanine over other agents

TGNs, thioguanine nucleotide; TIMP, thioinosine monophosphate (secondary metabolite of mercaptopurine); TPMT, thiopurine-S-methyl transferase (Relling et al., 2011).

are the guidelines for warfarin, the most commonly used oral anticoagulant worldwide (Johnson et al., 2011); indeed, these guidelines consider genetic variability at two loci: one for the hepatic drug metabolizing enzyme CYP2C9 and one for the target enzyme of warfarin, that is vitamin K-epoxide reductase (VKORC1). *CYP2C9* and *VKORC1* genetic polymorphisms account for up to 18 and 30%, respectively, of the variance in stable warfarin dose among patients of European ancestry: these common polymorphisms in both genes affect warfarin pharmacokinetics (*CYP2C9*) and pharmacodynamics (*VKORC1*) and modulate the therapeutics dose necessary to maintain the optimal level of drug effect (i.e., anticoagulation), preventing the risk of adverse events due to low efficacy or excessive anticoagulation (i.e., respectively thrombosis or bleeding). Combination of *CYP2C9* and *VKORC1* genetic polymorphisms is important to select the most appropriate dose to start therapy with warfarin: patients are classified by the multilocus genotype in a 2×2 table, according to the combined effects of the most relevant polymorphisms in each gene (two SNPs for *CYP2C9* and one SNP for *VKORC1*) in three levels of warfarin starting dose; this table is currently inserted in the US Food and Drug's Administration approved warfarin label. More complex algorithms, comprising even relevant demographic and clinical patient's characteristics affecting warfarin efficacy, such as age, smoking status and interacting drugs have been developed; some of these algorithms consider even additional genetic information besides *CYP2C9/VKORC1* multilocus genotype, such as polymorphisms of the *CYP4F2* and *GGCX* genes. It has been shown that warfarin dosing criteria considering genetics outperform non-genetic clinical algorithms and are particularly beneficial for patients requiring relatively low or high doses of the medication (i.e., <21 mg/week or >49 mg/week), that however are $\sim$40% of all patients: thanks to genetic based dose selection, these patients reach their optimal dose level more quickly and therefore with a lower risk of developing adverse events. Moreover, these criteria are particularly important for patients starting warfarin therapy, while are less useful for already established treatments. The development of these important therapeutic guidelines, considering a multilocus genotype affecting warfarin dose requirements illustrates how genetic information in more than one gene can be of clinical relevance to guide therapy for a single medication. Other similar guidelines considering multiple loci are in development, such as *CYP2D6*/*CYP2C9* multilocus genotype for tricylic antidepressants (Consortium, 2012).

MULTILOCUS GENOTYPES OF RELEVANCE FOR THERAPY PERSONALIZATION OF PEDIATRIC ALL

TPMT AND ITPA AND MAINTENANCE THERAPY FOR PEDIATRIC ALL

In addition to *TPMT*, other genetic factors may alter the effects of mercaptopurine, although their clinical importance has not been as well characterized. It has been shown that once mercaptopurine treatment for childhood ALL is individualized for *TPMT*, the effect of genetic polymorphisms in inosine triphosphate pyrophosphatase (ITPA) emerges (Stocco et al., 2010). ITPA is an enzyme that catalyzes the hydrolysis of inosine triphosphate (ITP) to inosine monophosphate (IMP). IMP is a central intermediate in purine metabolism and can be converted to ITP and to ATP via AMP or to GTP via GMP. The putative role of ITPA is to protect cells from the accumulation of potentially harmful nucleotides, such as ITP or deoxy-ITP, which may be incorporated into nucleic acids; indeed, it has been demonstrated by knock-down experiments performed in HeLa cells, that ITPA has a significant role in preventing base analog induced apoptosis, DNA damage, and mutagenesis in human cells (Menezes et al., 2012). In humans, ITPA displays a genetically determined polymorphic activity (Marsh and Van Booven, 2009; Stocco et al., 2010). Characterization of *ITPA* haplotype structure has shown that the SNP rs1127354 is the most relevant polymorphism in determining ITPA low enzymatic activity (von Ahsen et al., 2008; Stocco et al., 2010). Our recent study assessed the influence of non-functional variant alleles of *TPMT* and *ITPA* on mercaptopurine metabolism and toxicity in patients with ALL whose mercaptopurine doses were adjusted based on *TPMT* genotype (Stocco et al., 2009). This study revealed that the cumulative incidence of severe adverse effects (grade 3–4 febrile neutropenia) in patients receiving maintenance therapy that includes mercaptopurine individualized for TPMT is significantly greater among patients who have inherited an *ITPA* variant allele; this association remained significant when the analysis was limited to only life threatening events (i.e., grade 4 fever and neutropenia). Our recent study has documented that inheritance of a non-functional variant allele for either *TPMT* or *ITPA* is associated with significant modification in the metabolism of mercaptopurine during treatment of ALL. Although the importance of the *TPMT* genetic polymorphism is very well known and characterized, this was the first report showing a significant effect of the *ITPA* genetic polymorphism in the context of mercaptopurine therapy that has been individualized based on *TPMT* genotype. We documented significantly higher concentrations of the methylated nucleotide metabolites of mercaptopurine in leukemia cells and erythrocytes of patients who have inherited a non-functional *ITPA* allele. In contrast, the inheritance of a variant *ITPA* allele was not associated with differences in TGN concentrations in either leukemia cells or erythrocytes. Although ITPA is known to be involved in mercaptopurine metabolism, the mechanism by which ITPA variant alleles influence the accumulation of methylated thionucleotides has not been fully elucidated (Stocco et al., 2009, 2010).

A recent study has replicated the observation of the effects of the combined *TPMT* and *ITPA* genotype on the mercaptopurine pharmacokinetics and in particular on the concentration of methylated-mercaptopurine-nucleotides: among 66 children with ALL, treated according to EORTC 58951 protocol, comprising mercaptopurine at a dose of 50 mg/m^2/day and methotrexate at a dose of 20 mg/m^2/week, methylated-mercaptopurine-nucleotides concentrations were low in patients with *TPMT* variant/*ITPA* wild-type multilocus genotype, intermediate in wild-type/wild-type patients and high in patients with wild-type *TPMT*/*ITPA* variant (Adam de Beaumais et al., 2010).

It is known that ethnic differences for genotype frequencies may influence treatment efficacy in ALL: for example, it has been reported that the component of genomic variation that co-segregated with Native-American ancestry was associated with risk of relapse, even after adjusting for known prognostic factors (Yang et al., 2011). The allele frequencies of *TPMT* and *ITPA* polymorphisms show significant inter-ethnic variability: in

particular for rs1127354 of *ITPA* allele frequency of the variant is known to be ∼20% in Asian populations, ∼6% in Caucasians, and ∼2% in Hispanics, while for *TPMT*, the most common variants (rs1142345, rs1800460 and rs1800462) have a frequency of ∼1% in Asians, ∼5% in Caucasians, and ∼10% in Hispanics. Therefore, it is interesting that for *TPMT* and *ITPA*, frequencies of the variant alleles associated with different metabolism of mercaptopurine, seem to be almost reversal in the two populations (Marsh and Van Booven, 2009) and *ITPA* variants seem to be predominant in the Asian population. Indeed several recent studies of patients of Asian ethnicity seem to underline significant effects of *ITPA* polymorphisms on thiopurines' efficacy and toxicity in patients with ALL, but even when these medications are used as immunosuppressants in other pathologies (Okada et al., 2009; Yamamoto et al., 2010). For children with ALL, a recent study in 90 Indian patients, on maintenance therapy according to the MCP-841 protocol (Advani et al., 1999) with mercaptopurine at a dose of 75 mg/m^2 for 12 weeks, showed an independent role for both *TPMT* and *ITPA* in terms of association with the incidence of hematological toxicity; moreover, the multilocus genotype *TPMT*/*ITPA* was associated with a gene-dosage effect: percentage of reduction in total leukocyte count (i.e., the average leukocyte count on days 43, 71, and 99 of maintenance therapy) resulted in ∼40% for a patient with a wild-type genotype at both the *TPMT* and *ITPA* loci and increased proportionally to the number of risk alleles (i.e., variant inactive alleles for *TPMT* or *ITPA*) up to almost 70% in patients with three or more risk alleles at the *TPMT* and *ITPA* loci (Dorababu et al., 2012a). Analysis of epistasis by multifactor dimensionality reduction (Hahn et al., 2003) confirmed synergistic interactions between *TPMT* and *ITPA* variant alleles, in terms of their association with hematological toxicity during ALL maintenance therapy for this cohort of Indian children. Another recent study considered 100 Korean patients with pediatric ALL and evaluated in these patients 18 loci in 16 candidate genes of pharmacogenetic interest, including *TPMT* and *ITPA*, and their association with survival rate. Even if this study did not seem to confirm a strong difference for TPMT and ITPA gene variants between a western population of reference and the Korean patients, there was a significant effect of *ITPA* genotype, but not of *TPMT*, on the event free survival rate, which was lower in *ITPA* variants. *TPMT* genotype was however associated with the tolerance of mercaptopurine and methotrexate, evaluated as the dose of the medications used during the last cycle of maintenance therapy: indeed, as expected, patients with variant *TPMT* were selected to be treated with lower doses of mercaptopurine; unfortunately, data about the effect of *ITPA* genotype on the doses of antimetabolites was not reported (Kim et al., 2012).

Tanaka et al. (2012) have measured the activity of ITPA in 65 Japanese children with pediatric ALL, showing that patients with lower activity of this enzyme tolerated lower doses of mercaptopurine during maintenance therapy and presented increased probability of hepatoxicity.

In Asian populations, therefore, polymorphisms of *ITPA* seem to be of particular relevance for the effects of mercaptopurine in children with ALL, given the low incidence of patients with variant *TPMT*, compared to patients of Caucasian ethnicity (Marsh and Van Booven, 2009). However, it is known that other genetic polymorphisms may be of particular importance for Asian patients, such as SNP rs3765534 in the transporter MRP4, that is polymorphic only in patients of Asian ethnicity and that has been shown to modulate thiopurines intracellular levels by regulating the efflux of the thionucleotides (Krishnamurthy et al., 2008; Stocco et al., 2010).

On these bases, to understand the pharmacogenetics and improve treatment with thiopurines in the Asian populations, larger prospective studies are needed, considering even multilocus genotypes at loci of known relevance, such as *TPMT*, *ITPA*, and *MRP4*.

MULTILOCUS GENOTYPE *TPMT* – *SLCO1B1* – *PACSIN2* AND EFFECTS ON SEVERE MUCOSITIS DURING CONSOLIDATION THERAPY FOR PEDIATRIC ALL

During consolidation therapy for pediatric ALL, patients are treated with weekly 24 h infusions of high dose methotrexate, up to 5 g/m^2 and daily oral mercaptopurine with doses that range from 25 to 50 mg/m^2. Therapy with this association of antimetabolites has a very important role in preventing the relapse of the disease, after remission induction; however consolidation therapy is associated with the development of adverse effects, in particular gastrointestinal toxicity, such as stomatitis and mucositis, which cause major discomfort for the patient and can be severe, preventing the children from normal food intake and requiring parenteral nutrition. To avoid adverse events related to consolidation therapy, one of the most common approaches used in therapeutic protocols for ALL worldwide is the administration of leucovorin, a source of folic acid, that contrasts the cytotoxic effects of methotrexate and its association with mercaptopurine. Most protocols for ALL worldwide measure the concentration of methotrexate in patients' blood at the end of each infusion and administer leucovorin if methotrexate is then still present at significant concentrations: for example, in the Italian AIEOP-BFM ALL 2000 protocol, leucovorin was administered every 6 h at a dose of 7.5 mg/m^2, if methotrexate concentration resulted higher than 0.5 μmol/l at 48 h from the beginning of the infusion and until methotrexate concentration dropped below 0.25 μmol/l (Conter et al., 2010; Schrappe et al., 2011). Consolidation therapy lasts from 2 weeks up to 3 months depending on the treatment protocol and therefore the length of the therapy is too short to implement therapeutic monitoring of mercaptopurine metabolites concentration, which are useful when the drug is taken for at least 2 months (Lennard and Lilleyman, 1989). Advanced protocols for treatment of ALL, developed at St. Jude Children's Research Hospital in Memphis, evaluate the clearance of methotrexate during the infusion and either adjust the speed of infusion of the drug to a target concentration in the subsequent course (Total XV protocol) or in the same course (Total XVI protocol). This procedure requires a quick and efficient turnaround of the samples for the measurement of methotrexate concentration, which need to be analyzed in a few hours timeframe, so that the clearance of methotrexate can be estimated during the infusion, and the medication's administration speed can be adapted to reach the desired concentration threshold (i.e., 33 μM for low risk patients, 65 μM for standard-high risk patients; Pui et al., 2009). Therapeutic monitoring of methotrexate during consolidation therapy has significantly improved patients'

tolerance to this association treatment with antimetabolites; however, about 5% of pediatric patients still develop severe stomatitis/mucositis, with consequences that can be life threatening. The study of pharmacogenetic determinants of severe gastrointestinal (GI) toxicity during consolidation therapy has lead to major breakthroughs in recent years, which hopefully will lead to even better treatment of patients with ALL, completely preventing the occurrence of this major adverse event. In particular, a recent genome-wide study analyzed 500,568 germline single-nucleotide polymorphisms to identify how inheritance affects methotrexate plasma disposition among 434 children with ALL who received 3,014 courses of methotrexate at 2–5 g/m^2 (Trevino et al., 2009). This study lead to the identification of polymorphisms in *SLCO1B1*, as the most significant associations (p-value $< 10^{-9}$) with methotrexate clearance, even after adjusting for age, race, sex, and methotrexate regimen. In particular, the most significant polymorphism was the intronic rs11045879, which is in linkage disequilibrium with the functional SNP rs4149056; these same polymorphisms were associated even with severe GI toxicity during consolidation therapy, mostly severe stomatitis and mucositis. This observation was confirmed by subsequent studies (Lopez-Lopez et al., 2011; Ramsey et al., 2012). Therefore *SLCO1B1* polymorphisms are significant determinants for the occurrence of severe GI toxicity and in particular stomatitis/mucositis, during consolidation therapy for pediatric ALL, by an effect on methotrexate disposition: indeed the gene product of *SLCO1B1* is a transporter which mediates the sodium-independent uptake of organic anions such as methotrexate and may play an important role in the clearance of bile acids and organic anions.

Another relevant study has considered the role of genetic determinants of mercaptopurine toxicity during consolidation therapy, together with *SLCO1B1*: this study on adverse effects considered 189 children with ALL and evaluated the association between genetic determinants of *TPMT* activity in patients and the incidence of severe GI toxicity. The frequency of GI toxicity (grade 3–4 mucositis) in this population was 8.5%; among these patients, deficiency in TPMT activity predisposed to an increased incidence of severe GI toxicity during consolidation therapy which included methotrexate (2 g/m^2/week) and mercaptopurine (75 mg/m^2/day, in all patients during consolidation therapy, regardless of TPMT genotype). Indeed, among nine patients with a variant TPMT allele, the frequency of GI toxicity was 33%, compared with 7.2% in patients with wild-type TPMT. As previously reported (Trevino et al., 2009), the *SLCO1B1* SNP rs11045879 was also associated with the incidence of GI toxicity: indeed none of the patients with the *SLCO1B1* CC or CT genotype had GI toxicity, whereas 11.8% of the patients with the wild-type *SLCO1B1* TT genotype had this side effect. Moreover, this study identified, through the HapMap model system, an additional determinant of TPMT activity, the *PACSIN2* gene which resulted as the highest correlated gene to TPMT activity, in an analysis combining polymorphisms and expression, all measured in the 30 HapMap CEU trios (Stocco et al., 2012). The most significant *PACSIN2* SNP in the HapMap analysis for TPMT activity, rs2413739, was also significantly associated with TPMT activity in patients with ALL, independently from *TPMT* genotype: the CC genotype for the rs2413739 SNP displayed a higher TPMT activity in comparison to the TT genotype. Moreover, *PACSIN2* SNP rs2413739 also had a significant association with GI toxicity during consolidation therapy: the frequency of toxicity was 2.1, 9.1, and 13.2%, respectively, for the CC, CT, and TT genotype (Stocco et al., 2012). The effects of *PACSIN2* polymorphism on the incidence of severe mucositis during consolidation therapy for pediatric ALL were confirmed in another cohort of patients, considering 67 cases developing the adverse event during therapy according to the protocol AIEOP-BFM ALL 2000, which involves four weekly infusion of methotrexate at the dose of 2 g/m^2 and concomitant daily treatment with mercaptopurine at the dose of 25 mg/kg. Analysis in the validation cohort was done by a case-control design and each case was matched to two controls from the same protocol based on sex, age, ALL lineage and ALL risk classification, confirming a significant effect of *PACSIN2* SNP rs2413739 on the incidence of severe mucositis during the consolidation therapy of pediatric ALL (Stocco et al., 2012). Interestingly, in the discovery cohort, the effects of *TPMT*, *SLCO1B1* and *PACSIN2* polymorphisms were independent from each other, both in a multivariate logistic regression model and in a classification and regression tree analysis and could be combined in a multilocus genotype of potential importance to predict the incidence of severe mucositis in children with ALL treated with consolidation therapy comprising the combination of methotrexate and mercaptopurine (**Figure 1**).

GENOME-WIDE ANALYSIS OF SNPs ASSOCIATED TO CLINICAL RESPONSE IN PEDIATRIC ALL: IMPLICATIONS FOR THE PHARMACOGENETICS OF MERCAPTOPURINE

Genome-wide analysis, if adequately powered, has great potential in elucidating and understanding the genomic component associated with interindividual differences in phenotypes, even of pharmacogenetic interest. This has been shown to be true in model systems like the HapMap cell lines, in which statistical power is obtained mainly by combining genomic information at the level of gene expression and genetic polymorphisms, with the advantage that the phenotypes can be characterized with great accuracy and consistency (Wheeler and Dolan, 2012). This has led for example to the identification of *PACSIN2* as a significant determinant of TPMT activity in the cell lines, with effects reproducible in patients with ALL mentioned above (Stocco et al., 2012). However, the greatest potential of the genome-wide approach resides really in the analysis of patients' samples: if the study is adequately designed and powered and the phenotypes are well collected, this approach can provide unpredictable insights on the phenotype of interest, potentially leading to major breakthroughs in the understanding of the genomic basis of inter-patient variability, even of pharmacogenetic traits. Several such examples exist in the literature: besides the already mentioned role of SLCO1B1 in the disposition of methotrexate in children with ALL (Trevino et al., 2009), this same transporter was shown to be involved in statins' induced myopathy (Link et al., 2008); another example of genome-wide studies is the discovery of a role for ITPA in anemia induced by the anti-viral agent ribavirin (Fellay et al., 2010).

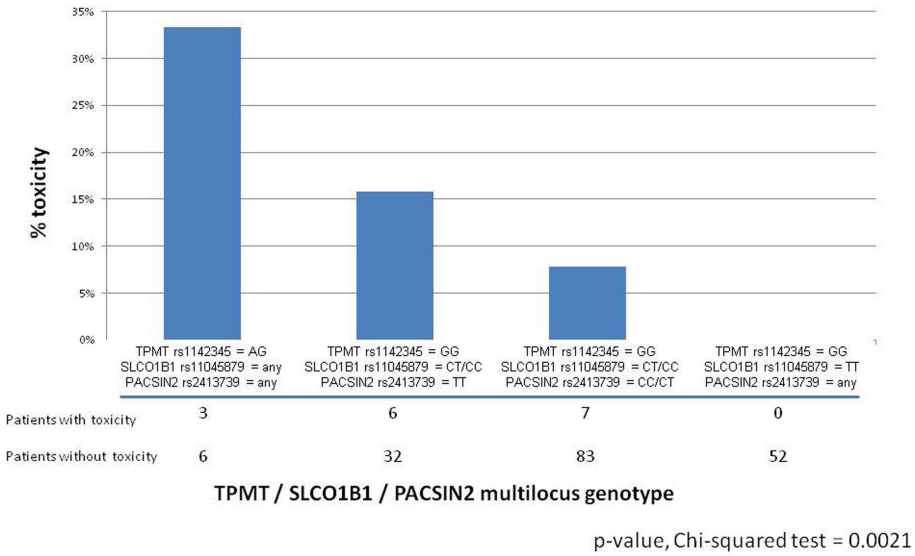

FIGURE 1 | Barplot reporting the percentage of patients developing severe (Grade 3-4) GI toxicity during consolidation therapy in patients with ALL treated according to the St Jude Total 13B protocol as a function of TPMT rs1142345 / SLCO1B1 rs11045879 / PACSIN2 rs2413739 multilocus genotype (Stocco et al., 2012).

While in pediatric ALL genome-wide interrogation is complicated by the relative small number of patients available, St. Jude Children's Hospital was able to publish some genome-wide studies on leukemia pharmacogenetics, thanks to access to uniformly treated and well characterized patients and phenotypes (Trevino et al., 2009; Yang et al., 2009, 2012; Kawedia et al., 2011). Among these genome-wide studies of pharmacogenetic interest, some have generated data that could be of particular interest for the identification of multilocus genotypes of relevance for the treatment of ALL with thiopurines. In particular, these studies considered genetic polymorphisms associated with outcome to therapy evaluated as minimal residual disease (MRD) (Yang et al., 2009) or disease relapse (Yang et al., 2012); while these very important clinical phenotypes of patients with ALL are not related directly only to mercaptopurine, the genetic features identified are related even to disposition of antileukemic drugs and may be of relevance for mercaptopurine effects too and should be therefore considered.

The study on MRD considered two independent cohorts of children with newly diagnosed ALL: 318 patients in St Jude Total Therapy protocols XIIIB and XV and 169 patients in Children's Oncology Group trial P9906. This study identified 102 SNPs associated with MRD in both cohorts, including five SNP in interleukin 15 (*IL15*). Twenty one of these SNPs were also associated with drug disposition (evaluated as methotrexate clearance, etoposide clearance, or methotrexate polyglutamates concentration), generally linking greater drug exposure with MRD eradication. While concentration of mercaptopurine metabolites was not evaluated in this study, the effects on the disposition of methotrexate, that is associated with mercaptopurine both during consolidation and maintenance therapy, suggest that these SNPs may be of interest to build multilocus genotypes useful for therapy personalization of pediatric ALL also with mercaptopurine.

FURTHER DEVELOPMENT OF MULTILOCUS GENOTYPES: EPISTASIS AND GENE-ENVIRONMENT INTERACTIONS

Phenotypes of pharmacogenetic interest are complex, particularly those describing patients' response to a medication, both in terms of efficacy and incidence of adverse events: it is likely that different genetic features, together with environmental factors, contribute to the interindividual variability of these phenotypes. Indeed, it is known that the effect of genetic polymorphisms is stronger when it refers to a pharmacokinetic phenotype and strength of the association reduces with the increasing complexity of the phenotype: for example, the effect of *TPMT* genotype is extremely strong on TPMT activity and the strength of the association is reduced, while still significant, considering more complex phenotypes such as the concentration of mercaptopurine metabolites and, even more, considering parameters of clinical response to the medication: the thinning of the association strength is due to the increasing complexity of the phenotype and the augmented potential role of environmental and additional genetic factors (Relling et al., 2011). Moreover, for complex phenotypes such as the response to a medication, the effects of a genetic factor may depend on other genetic variations and environmental factors, a phenomenon that is defined respectively as gene-gene interaction/epistastis or as gene-environment interaction (Moore and Williams, 2009). Methods have been developed to study consistently and efficiently the role of multiple genetic and environmental factors on complex phenotypes defined by discrete traits, such as those of pharmacogenetic interest (e.g., clinical response to a medication or occurrence of adverse events; Hahn et al., 2003; Gilbert-Diamond and Moore, 2011). This method is called multifactor dimensionality reduction (MDR) and allows collapsing multi-dimensional genetic information into a single dimension, thus permitting the detection of epistasis: MDR is a non-parametric method and

interactions are detected by a constructive induction approach, in particular by classifying multiple loci as high risk or low risk, depending on whether they are more common in affected or in unaffected subjects; this pooling allows reducing the dimensionality of the multilocus data to one dimension (Hahn et al., 2003). The new multilocus genotype variable is then evaluated for its ability to classify and predict the phenotype of interest (i.e., drug response): different approaches have been used to perform these computations; originally, however, it was done by cross-validation and permutation testing and recently extensions and variations of the method have been developed, which allow the calculation of odds ratios and application of Fisher's test to increase model robustness (Moore and Williams, 2009). Interestingly, it has been reported that MDR allows the identification of significant gene–gene interaction in the absence of a statistically significant main effect by a single genotype; moreover, it was mathematically proved that MDR is the best method to discriminate multilocus genotypes for clinical endpoints. MDR has been successfully applied to detecting gene–gene and gene–environment interactions for a wide variety of different complex phenotypes, such as incidence of human diseases and other clinical endpoints (Gilbert-Diamond and Moore, 2011). Recently this method has been applied even for studies of pharmacogenomics for thiopurines and methotrexate (Dervieux et al., 2012; Dorababu et al., 2012a,b; Kim et al., 2012), even if its application to this field is still limited and there is great potential for discovery, in particular to detect and elucidate multilocus genotypes associated with genome-wide studies of complex pharmacogenetic phenotypes.

CONCLUSION

Consideration of genetic biomarkers can improve therapy of pediatric ALL: the role of *TPMT* genetic polymorphism on mercaptopurine induced toxicity in children with ALL has been clearly defined and clinical guidelines have been developed to tailor treatment with this medication on the basis of TPMT status. Multilocus genotypes have been shown to be able to increase the amount of interindividual variability in a phenotype of clinical relevance explained: for example, the incidence of severe GI toxicity during consolidation therapy has been shown recently to be independently related to *TPMT*, *SLCO1B1*, and *PACSIN2* genetic polymorphisms. Identification, proper testing, and validation of multilocus genotypes hold great potential in further refining the clinical utility of pharmacogenetics to improve treatment of children with ALL by reducing treatment-related adverse events.

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Functional polymorphisms in xenobiotic metabolizing enzymes and their impact on the therapy of breast cancer

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Breast cancer is the top cancer among women, and its incidence is increasing worldwide. Although the mortality tends to decrease due to early detection and treatment, there is great variability in the rates of clinical response and survival, which makes breast cancer one of the most appealing targets for pharmacogenomic studies. The recognition that functional *CYP2D6* polymorphisms affect tamoxifen pharmacokinetics has motivated the attempts of using *CYP2D6* genotyping for predicting breast cancer outcomes. In addition to tamoxifen, the chemotherapy of breast cancer includes combinations of cytotoxic drugs, which are substrates for various xenobiotic metabolizing enzymes. Because of these drugs' narrow therapeutic window, it has been postulated that impaired biotransformation could lead to increased toxicity. In the present review, we performed a systematic search of all published data exploring associations between polymorphisms in xenobiotic metabolizing enzymes and clinical outcomes of breast cancer. We retrieved 43 original articles involving either tamoxifen or other chemotherapeutic protocols, and compiled all information regarding response or toxicity. The data indicate that, although *CYP2D6* polymorphisms can indeed modify tamoxifen pharmacokinetics, *CYP2D6* genotyping alone is not enough for predicting breast cancer outcomes. The studies involving other chemotherapeutic protocols explored a great diversity of pharmacogenetic targets, but the number of studies for each functional polymorphism is still very limited, with usually no confirmation of positive associations. In conclusion, the application of pharmacogenetics to predict breast cancer outcomes and to select one individual's chemotherapeutic protocol is still far from clinical routine. Although some very interesting results have been produced, no clear practical recommendations are recognized yet.

Keywords: breast cancer, gene polymorphisms, xenobiotic metabolizing enzymes, hormone therapy, chemotherapy, efficacy, toxicity

INTRODUCTION

Breast cancer is the most frequent type of cancer in women (Jemal et al., 2011), and the second leading cause of cancer-related death in women worldwide (DeSantis et al., 2011). The incidence rates of breast cancer are increasing both in developed and in developing countries (Ferlay et al., 2010), whereas the mortality rates have decreased in the last decade [World Health Organization (WHO), 2004; Ferlay et al., 2010; DeSantis et al., 2011; Jemal et al., 2011], probably because of the investments in early detection and in new pharmacological approaches (Berry et al., 2005). Although the recent decrease in the mortality rates proves the efficacy of the current therapeutic protocols, the optimization of available therapies is crucial. Most anticancer drugs are highly toxic, and many patients suffer with adverse reactions that might be persistent throughout the treatment, and sometimes even irreversible. In addition to their obvious impact in patients' quality of life, drug toxicities may also require dose delays, treatment modifications, or even treatment interruption, contributing to the great variability that is usually observed in breast cancer clinical outcomes. This scenario makes breast cancer one of the most appealing targets for evaluation of pharmacogenomic strategies toward personalized medicine.

The current treatment of breast cancer consists of combinations of surgery and adjuvant or neoadjuvant therapeutic approaches, including radiotherapy, cytotoxic chemotherapy, hormonal therapy, and targeted therapy. The treatment choice is routinely based on the estimated risk of recurrence, considering the clinical stage at diagnosis and molecular predictive factors (Soerjomataram et al., 2008). Nevertheless, breast cancer is a very heterogeneous disease, with a continuous grading in tumor histology (Hayes et al., 2001), different cellular origins (Anderson and Matsuno, 2006), and great molecular diversity (Danova et al., 2011), which make prognostic estimates a difficult task, especially in early-stage tumors. In an attempt to improve the classical pathology-driven classification of breast tumors, a series of efforts are currently in course, including the description of gene expression patterns (Perou et al., 2000; Sørlie et al., 2001), and of genomic signatures (Banerji et al., 2012; Nik-Zainal et al., 2012; Stephens et al., 2012). In addition to molecular variations in the tumor, the individual genetic diversity may also contribute for the great heterogeneity in treatment

outcomes. Thus, inherited sequence variations (polymorphisms) in genes involved in the pharmacokinetics and pharmacodynamics of anticancer drugs may affect both their efficacy and safety (O'Donnell and Ratain, 2012; Ruiz et al., 2012).

The chemotherapy of breast cancer includes different options of drug combinations. Anthracycline-based protocols have become the standard adjuvant and neoadjuvant chemotherapy for most patients in view of clinical evidences of improved efficacy in comparison to other previously used protocols (Hassan et al., 2010). More recently, taxanes, such as docetaxel or paclitaxel, were added to anthracycline-based protocols, further reducing the risk of recurrence (De Laurentiis et al., 2008; Martín et al., 2010; Jacquin et al., 2012). The most usual protocols for breast cancer chemotherapy nowadays are: docetaxel, doxorubicin, and cyclophosphamide (TAC); docetaxel, epirubicin, and cyclophosphamide (TEC); cyclophosphamide, doxorubicin, and 5-fluorouracil, followed by docetaxel (CAF-T); doxorubicin and cyclophosphamide (AC); and doxorubicin and cyclophosphamide, followed by paclitaxel or docetaxel (AC-P or AC-T). The older protocol cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) is also still used.

Cytotoxic antineoplastic drugs have narrow therapeutic window, and small variations in their plasma concentrations may lead to clinically significant toxicity. Taxanes, for example, may cause severe bone marrow dysfunction, and their toxic effects present great interpatient variability, which appear to be due to interindividual differences in pharmacokinetic parameters (Engels et al., 2011). Accordingly, the biotransformation of taxanes is mainly mediated by hepatic CYP450s, which may occur in different isoforms, with distinct functional activities, as a consequence of polymorphisms in their coding genes. Doxorubicin and epirubicin may cause severe cardiotoxicity (Doyle et al., 2005; Pinder et al., 2007; Gianni et al., 2008) and bone marrow dysfunction (Hershman et al., 2007; Patt et al., 2007). Their biotransformation includes reductions by carbonyl reductases (CBR1 and CBR3) and by aldoketoreductases (AKR1A1 and AKR1C3; Lal et al., 2010). Epirubicin also undergoes conjugation by uridine diphosphate-glucuronosyltransferase 2B7 (UGT2B7; Innocenti et al., 2001). Finally, glutathione (*S*)-transferases (GSTs) may also participate to detoxification. Like the CYP450s, all these xenobiotic metabolizing enzymes are coded by polymorphic genes, which make them potential targets for pharmacogenomic evaluations.

In addition to the cytotoxic antineoplastic drugs, tamoxifen is perhaps the most appealing target for breast cancer pharmacogenomics. It was approved by the FDA in 1977, and since then, it is still the drug with the most striking effect on patients' survival, reducing the annual risk of recurrence by 39% after a 5-year treatment [Early Breast Cancer Trialists Collaborative Group (EBCTCG), 2005]. The antitumor effects of tamoxifen are mediated by selective modulation of the estrogen receptor, which can be detected in more than two thirds of breast tumors, and to consequent inhibition of estrogen-dependent cell proliferation. However, in spite of the undisputable efficacy and long-term benefits of tamoxifen for breast cancer patients, there is great interindividual variability in the degree of response. Thus, approximately half of the estrogen receptor-positive tumors do not respond to tamoxifen therapy (Jaiyesimi et al., 1995; Osborne, 1998; Buzdar, 2001), and the 15-year recurrence probability in early breast cancer patients treated with tamoxifen for 5 years is approximately one third [Early Breast Cancer Trialists Collaborative Group (EBCTCG), 2005].

The great interindividual variability observed in the degree of response to tamoxifen can be ascribed to different causes, including failures in patient adherence, drug interactions, and genetic variations affecting tamoxifen pharmacokinetics (Hoskins et al., 2009). This variability is of special concern in premenopausal women, who cannot receive aromatase inhibitors, and therefore have less therapeutic options. Thus, it seems crucial to find strategies to ensure tamoxifen response, minimize, or predict individual variability, and improve disease outcomes. Besides the interpatient variability in the degree of response, another motivation for pharmacogenomic studies would be tamoxifen safety. The main concern refers to the risk of endometrial cancer and of thromboembolic events, although these are quite rare events (Fisher et al., 1998). The most common side effect of tamoxifen therapy is hot flushes, which are intrinsically correlated with tamoxifen's antiestrogenic activity. Although hot flushes do not represent a life threat, and might become more tolerable with therapy continuation, they can be so intense that patients stop tamoxifen use.

Tamoxifen is considered a prodrug, with very little affinity for the estrogen receptor in its original structure (Coezy et al., 1982). The pharmacological actions of tamoxifen are most likely due to its metabolites (Coezy et al., 1982; Robertson et al., 1982), which are generated in the liver by numerous phase I and II reactions (Mürdter et al., 2011). The major metabolite is *N*-desmethyl-tamoxifen, which is generated by CYP3A4/5 and accounts for approximately 90% of tamoxifen metabolites (Desta et al., 2004). However, *N*-desmethyl-tamoxifen shows little affinity for the estrogen receptor when compared to two other metabolites, 4-hydroxy-tamoxifen and 4-hydroxy-*N*-desmethyl-tamoxifen (endoxifen; Coezy et al., 1982; Jordan, 1982; Robertson et al., 1982). Because 4-hydroxy-tamoxifen and endoxifen have similar potencies in suppressing estrogen-dependent cell proliferation, but the latter appears to be generated in higher concentrations, endoxifen is believed to be the major active metabolite *in vivo* (Johnson et al., 2004; Lim et al., 2005). Endoxifen generation is mainly dependent on CYP2D6 activity (Desta et al., 2004), and initial observations suggested that genetic polymorphisms in its coding gene, *CYP2D6*, could affect the activity of CYP2D6, resulting in reduced plasma level of endoxifen (Jin et al., 2005). Corroborating this notion, *CYP2D6* polymorphisms responsible for reduced CYP2D6 activity were associated with worse breast cancer outcomes in postmenopausal estrogen receptor-positive patients treated with tamoxifen (Goetz et al., 2005). These results prompted the FDA to recommend, in 2006, an update in the tamoxifen package insert, alerting for the increased risk of breast cancer recurrence in patients who are CYP2D6 poor metabolizers.

The awareness of the potential impact of *CYP2D6* polymorphisms in tamoxifen pharmacokinetics and pharmacodynamics has motivated a series of pharmacogenomic studies, designed to explore the possibility of using *CYP2D6* genotyping for predicting clinical outcomes in breast cancer patients receiving tamoxifen. According to more recent data, it seems clear that genetic polymorphisms that modulate CYP2D6 activity can indeed modify

endoxifen plasma levels (Kiyotani et al., 2010; de Graan et al., 2011; Irvin et al., 2011; Lim et al., 2011; Mürdter et al., 2011). However, the impact of such pharmacokinetic changes on the individual degree of response to tamoxifen is less clear, and *CYP2D6* genotyping alone has been not enough for predicting breast cancer outcomes in clinical settings (Abraham et al., 2010; Rae, 2011; Regan et al., 2012). It appears, thus, that the pharmacological actions of tamoxifen may be more complex than initially thought, with its antiestrogenic activity being dependent not on a single metabolite, but on a composite action of them (Rae et al., 2011). As a consequence of this new assumption, other genetic polymorphisms affecting the pharmacokinetics of tamoxifen might have additional influences on breast cancer outcomes, and should also be considered in pharmacogenomic studies. Likewise, there might be combined influences of genetic polymorphisms on the pharmacokinetics/dynamics of both tamoxifen and other cytotoxic chemotherapeutic drugs, which would require much more complex study designs for evaluation of their impact in clinical settings.

In the present review, we aimed to compile all information available on pharmacogenomic studies involving polymorphisms in xenobiotic metabolizing enzymes and their consequences in clinical outcomes of breast cancer. The analyzed studies explore either tamoxifen or the antineoplastics used in chemotherapy. Instead of focusing on selected positive associations, we performed a systematic review of all published data, evaluating the reported effects on both response and toxicity, as well as all the recorded information of null associations.

MATERIALS AND METHODS

A systematic review of the literature data was conducted, which was performed via electronic search of the MEDLINE database (available at PUBMED), and included articles available until August 2012. The search terms were selected using the controlled vocabulary MeSH for the PubMed database. The main search was as follows: ["polymorphism, genetic" (MeSH Terms)] AND ["breast neoplasms" (MeSH Terms)] AND [enzyme (Text Word) OR "enzymes" (MeSH Terms)] AND [breast cancer (Title/Abstract)] AND (xenobiotic OR drug) NOT ["Review" (Publication Type)]. The following filters were used: Published in the last 10 years; Humans; English; Female.

All abstracts were retrieved, and were used for selection of articles to be used in the review. The pre-defined inclusion criteria were: original articles, including clinical trials, prospective, or retrospective observational studies, describing correlations between polymorphisms in xenobiotic metabolizing enzymes and any clinical response or outcome of breast cancer patients under therapeutic treatment. The exclusion criteria were: letters, commentaries, editorials, or case reports; studies involving only the susceptibility of developing cancer; studies involving only drug transporters or other enzymes not responsible for xenobiotic metabolism; studies involving only pharmacokinetic analyses or correlation with histopathological features, without evaluation of clinical outcomes.

Two reviewers performed the selection of articles to be included in the review according to previously defined inclusion and exclusion criteria, and extracted information for data compilation. A third reviewer examined the lists of selected and excluded articles in order to confirm the eligibility, and checked all the extracted information.

The compiled information was evaluated separately for tamoxifen and for other chemotherapeutic protocols, and focused on the associations between polymorphisms and drug efficacy or drug safety. All the null associations were also collected, compiled, and analyzed.

RESULTS

The literature search resulted in 158 abstracts among the bibliographic references from PUBMED. The abstract reading led to the selection of 30 original articles involving tamoxifen use, and 22 original articles involving any kind of breast cancer chemotherapy. The excluded items (106) consisted of non-original articles (4), articles not involving xenobiotic metabolizing enzymes (85), articles evaluating only the susceptibility to breast cancer (10), articles evaluating only the correlation with histopathological features (3), articles involving only pharmacokinetic analyses (2), 1 article evaluating clinical outcomes not related to breast cancer treatment, and 1 article not involving humans. Among the 49 selected abstracts, 2 articles could not be retrieved, and 7 articles did not fit the inclusion criteria. The excluded articles after full-text reading were: 2 articles not involving xenobiotic metabolizing enzymes, 1 article based on previously published data, 1 article analyzing breast cancer patients together with other cancer patients, 1 article involving only pharmacokinetic analyses, 1 article evaluating clinical outcomes not related to breast cancer treatment, and 1 article which was retracted. The selected articles consisted of 23 original studies involving tamoxifen use, and 20 original studies involving other chemotherapeutic protocols (**Figure 1**).

The selected studies were mostly based on prospective, observational designs, and focused on the evaluation of different breast cancer outcomes, defined either as primary or secondary endpoints. Some studies also included pharmacokinetic evaluations or other endpoints not directly related to breast cancer treatment. The extracted information was restricted to polymorphisms in xenobiotic metabolizing enzymes and to any clinical outcome directly correlated to breast cancer treatment. The results are distributed in five Tables: **Tables 1** and **2** present the compiled data involving tamoxifen treatment, and comprise results on both efficacy and safety. **Table 1** presents only statistically significant associations, whereas **Table 2** presents only null associations. **Tables 3–5** present the compiled data involving any adjuvant, neoadjuvant, or palliative chemotherapeutic protocol. **Table 3** presents the significant associations involving efficacy outcomes, **Table 4** presents any significant association involving toxicities, and **Table 5** presents all reported null associations, involving either efficacy or safety outcomes.

Among the 20 studies involving tamoxifen use, 16 explored the effects of *CYP2D6* polymorphisms, either considering polymorphisms individually or in their combined genotypes and expected phenotypes. The other four articles evaluated polymorphisms in other genes different from *CYP2D6*. Thus, Nowell et al. (2002) examined the relationship between the *SULT1A1*2* allele and survival in a cohort of 337 women (160 receiving tamoxifen); Tucker et al. (2005) evaluated the impact of *CYP3A5* polymorphisms on

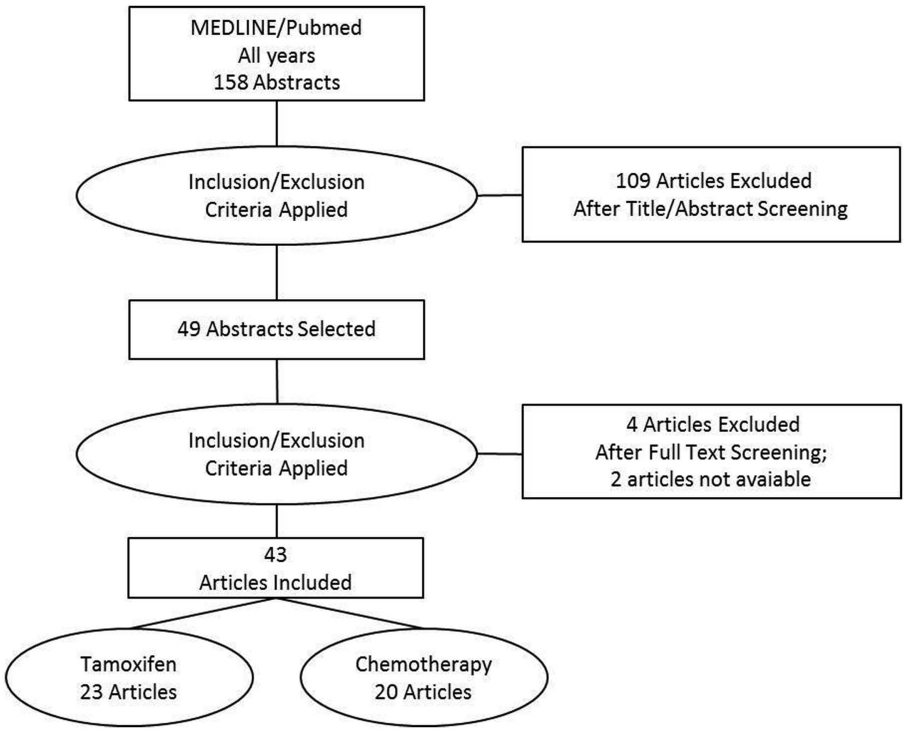

FIGURE 1 | Flow chart of the systematic review of literature data.

tamoxifen side effects in 98 postmenopausal women; Jernström et al. (2009) investigated the frequency of *CYP2C8* and *CYP2C9* polymorphisms in relation to disease-free survival in a prospective series of 652 breast cancer patients (297 receiving tamoxifen), and Ruiter et al. (2010) evaluated the impact of *CYP2C19*2* and *CYP2C19*3* on breast cancer mortality rate in 215 breast cancer patients (80 using tamoxifen). Among the 16 studies evaluating *CYP2D6* polymorphisms, 7 studies also analyzed other genes: Nowell et al. (2005) analyzed *UGT2B15* polymorphisms; Wegman et al. (2005) examined variants of *SULT1A1*; Wegman et al. (2007) evaluated *CYP3A5*, *SULT1A1*, and *UGT2B15*; Okishiro et al. (2009) analyzed *CYP2C19*; Kiyotani et al. (2010) did not evaluate polymorphisms involving xenobiotic metabolizing enzymes, but examined gene polymorphisms affecting drug transporters (ABCB1, ABCC2, ABCG2); and Moyer et al. (2011) examined *SULT1A1* and *CYP2C19* polymorphisms.

The compiled results in **Table 1** indicate that some functional polymorphisms affecting the enzymatic activities of CYP2D6, CYP3A5, CYP2C8, CYP2C19, SULT1A1, or UGT2B15 could significantly affect breast cancer outcomes. Thus, *CYP3A5*3* and *CYP2C19*2* seem to be beneficial with regards to the risk of recurrence or to breast cancer-specific survival, respectively. All the other polymorphisms with reported significant effects on breast cancer clinical outcomes, i.e., *CYP2C8*3*, *CYP2D6*4*, *CYP2D6*6*, *CYP2D6*10*, *SULT1A1*2*, and *UGT2B15*2*, were associated with an apparent worse response to tamoxifen, resulting in higher risk of tumor recurrence, shorter breast cancer-specific survival, or shorter overall survival.

With regards to *CYP2D6*4*, the work by Wegman et al. (2007) represents an exception, since it shows an apparent beneficial effect of the genotype *CYP2D6*1/*4* in relation to recurrence-free survival. This apparently protective effect, however, was not seen for patients homozygous for the allele **4*, who were similar to patients with the wild-type genotype in relation to recurrence-free survival. Although there are no other reports suggesting any beneficial effect of *CYP2D6* polymorphisms in relation to breast cancer outcomes, there is much inconsistency in the supposed detrimental effects of the variant alleles with regards to the risk of recurrence. Thus, only Bijl et al. (2009), with a very limited number of patients, reported an apparent trend between heterozygous and homozygous variants of the allele *4. Goetz et al. (2005) only found significant effects when comparing the homozygous variant genotype **4/*4* to the homozygous wild-type genotype **1/*1*. All the other published studies involving *CYP2D6*4* indicate no significant effect for the variant allele, either in heterozygosis or in homozygosis (**Table 2**). The lack of significant independent effect of the allele *4 cannot be attributed to low statistical power, since it was confirmed by the two largest studies (Abraham et al., 2010; Regan et al., 2012). The study by Abraham et al. (2010) also show no significant effects on breast cancer outcomes regarding the *CYP2D6* variant alleles *5, *9, *10, and *41. The lack of significant effects with the variant genotypes of *CYP2D6*10* had already been reported by Okishiro et al. (2009) and Toyama et al. (2009).

Other studies analyzed multiple *CYP2D6* polymorphisms (alleles **3*, **4*, **5*, **6*, **7*, **10*, **14*, **21*, **36–*36*, **41*) in different combinations, and, based on their expected functional impact on

Table 1 | Effects of polymorphisms in xenobiotic metabolizing enzymes on breast cancer outcomes after tamoxifen treatment.

Gene	SNP	Reference	Population	Design	*N*	Reference group[a]	Affected group[b]	Outcome	Main results	
									Calculation (95% CI)	*p*-Value
CYP2D6	*4	Goetz et al. (2005)	Adjuvant	Cohort	223	**1/*1*	**4/*4*	RFT	KM–LR[c]	0.03
						**1/*1*	**4/*4*	DFS	KM–LR[c]	0.02
		Wegman et al. (2007)	Adjuvant	Cohort	677	**4/*4*	**1/*4*	RFS	KM–LR[c]	0.04
		Bijl et al. (2009)	All incident users	Cohort	85	**1/*1*	**4/*4*	BCSS	HR; 4.1 (1.1–15.9)	0.041
						**1/*1*	**1/*4* or **4/*4*	BCSS	HR; 2.1 (1.1–4.2)	0.031
						**1*	**4*	BCSS	HR; 2.0 (1.1–3.4)	0.015
	*6	Abraham et al. (2010)	Adjuvant	Case-cohort	3155	**1/*1*	**1/*6*	BCSS	HR; 1.95 (1.1–3.4)	0.02
	*10	Lim et al. (2007)	Adjuvant, palliative	Cohort	202	**1/*1*	**10/*10*	TTP	KM–LR[c]	0.0032
		Xu et al. (2008)	Adjuvant	Cohort	152	**1/*1*	**10/*10*	5-Years DFS	HR; 4.7 (1.1–20.0)	0.04
COMBINED *CYP2D6* GENOTYPES										
		Newman et al. (2008)[d]	Adjuvant	Cohort	115	EM or Het-IM	IM	RFS	KM–LR[c]	0.031
						**1/*1*	PM	TTR	HR; 2.9 (0.9–9.4)	0.076
						**1/*1*	PM	OS	HR; 3.5 (0.8–15.4)	0.079
		Schroth et al. (2009)[e]	Adjuvant	Retrospective cohort	1325	EM	PM	TTR	HR; 1.90 (1.10–3.3)	0.02
		Schroth et al. (2010)[f]	Adjuvant	Cohort	492	EM	PM	RFS	HR; 2.77 (1.3–5.9)	0.011[g]
		Kiyotani et al. (2010)[h]	Adjuvant	Cohort	282	EM	Het-EM/IM	TTR	HR; 1.40 (1.0–1.9)	0.03
						**1/*1*	**1/V*	RFS	HR; 4.44 (1.3–15.0)	0.000036[g]
						**1/*1*	*V/V*	RFS	HR; 9.52 (2.8–32.4)	0.000036[g]
		Teh et al. (2012)[i]	Non-palliative	Cohort	95	EM	IM	RFS	OR; 13.14 (1.54–109.9)	0.004
CYP2C8	*3	Jernström et al. (2009)	Adjuvant	Cohort	297	**1/*1*	**1/*3* or **3/*3*	BCSS	HR; 9.10 (1.4–59.6)	0.021
						**1/*1*	**1/*3* or **3/*3*	DFS	HR; 8.56 (1.5–1.1)	0.015
CYP2C8–CYP2C9	**3–*2*	Jernström et al. (2009)	Adjuvant	Cohort	297	**1/*1*	**1/*3–*1/*2*	DFS	HR; 2.54 (1.5–5.79)	0.015
CYP2C19	*2	Ruiter et al. (2010)	All incident users	Cohort	80	**1/*1*	**2* Carriers	BCSS	HR; 0.26 (0.08–0.9)	0.03
		van Schaik et al. (2011)	Palliative	Cohort	499	**1/*1*	**2* Carriers	TTF	HR; 0.72 (0.57–0.90)	0.04
	*17	van Schaik et al. (2011)	Palliative	Cohort	499	**1/*1*	**17* Carriers	DFS	HR; 0.66 (0.46–0.95)	0.025

(Continued)

Table 1 | Continued

Gene	SNP	Reference	Population	Design	*N*	Reference group[a]	Affected group[b]	Outcome	Main results	
									Calculation (95% CI)	*p*-Value
CYP3A5	*3	Wegman et al. (2007)	Adjuvant	Cohort	677	*1/*1* or *1/*3	*3/*3	RFS	HR; 0.13 (0.02–0.9)	0.03
SULT1A1	*2	Nowell et al. (2002)	Adjuvant	Cohort	160	*1/*1* or *1/*2	*2/*2	OS	HR; 2.9 (1.1–7.6)	
		Wegman et al. (2007)	Adjuvant	Cohort	677	*1/*2* or *2/*2	*1/*1	RFS (2 years TAM)	HR; 0.33 (0.1–0.96)	0.04
COMBINED GENOTYPES										
SULT1A1 and *UGT2B15*		Nowell et al. (2005)	Adjuvant	Case-cohort	162	*SULT1A1* (*1/*1* or *1/*2), *UGT2B15*1/*1*	*SULT1A1*2/*2*, *UGT2B15* (*1/*2* or *2/*2)	OS	HR; 4.40 (1.2–16.5)	0.03[g]
						SULT1A1 (*1/*1* or *1/*2), *UGT2B15*1/*2*	*SULT1A1*2/*2*, *UGT2B15* (*1/*2* or *2/*2*	PFS	HR; 3.79 (1.2–12.1)	0.03[g]
CYP2D6 and *CYP2C19*		van Schaik et al. (2011)	Palliative	Cohort	499	*CYP2D6*1/*1*, *CYP2C19* *1/*1*	*CYP2D6* (*1/*4 or *4/*4), *CYP2C19* (*1/*17* or *17/*17)	TTF	KM–LR[c]	0.031

BCSS, breast cancer-specific survival; DFS, disease-free survival; TTF, time-to-treatment failure; EM, extensive metabolizer; Het, heterozygous; Homo, homozygous; HR, hazard ratio; IM, intermediate metabolizer; KM–LR, Kaplan–Meier curves and Log-Rank test; OR, odds ratio; OS, overall survival; PFS, progression-free survival; PM, poor metabolizer; RFS, recurrence-free survival; RFT, relapse-free time; RR, risk relative; TAM, tamoxifen; TTP, time to progression; TTR, time to recurrence; V, variant alleles. [a]*The Reference group includes alleles, genotypes, haplotypes, or phenotypes considered as reference for evaluation of outcome parameters. The designation *1 indicates the reference allele sequence for the analyzed SNP, but may include non-analyzed sequence variations.* [b]*The Affected group includes alleles, genotypes, haplotypes, or phenotypes with significantly different outcome parameters, when compared to the Reference group.* [c]*The Affected group had a worse outcome.* [d]*Newman et al. (2008) – PM: two copies of the CYP2D6*3, CYP2D6*4, or CYP2D6*5 alleles; a group with concomitant use of a potent CYP2D6 inhibitor in wild-type individuals or moderate inhibitor in heterozygous patients.* [e]*Schroth et al. (2009) – PM: presence of two alleles *3, *4, or *5; EM: absence of alleles *3, *4, *5, *10, and *41; Het-EM/IM: presence of two alleles *10 or *41, or presence of only one allele *3, *4, or *5;* [f]*Schroth et al. (2010) – EM: presence of alleles *1, *2, or *35; PM: presence of two alleles *3, *4, *5, *6, *7, or *8;* [g]*Ptrend values.* [h]*Kiyotani et al. (2010) – V: *4, *5, *10, *14, *21, *36, or *41;* [i]*Teh et al. (2012) – EM: absence of alleles *xN, *4, *5, *10, and *14; Het-IM: *1/*10; IM: *10/*10 or heterozygous null alleles (*1/*4, *1/*5, *1/*14).*

Table 2 | Null associations between polymorphisms in xenobiotic metabolizing enzymes and breast cancer outcomes after tamoxifen treatment.

Gene	SNP	Reference	Population	Design	*N*	Compared groups[a]			Outcome
CYP2D6	**4*	Goetz et al. (2005)	Adjuvant	Cohort	223	**1/*1* or **1/*4*	vs.	**4/*4*	RFS
						**1/*1* or **1/*4*	vs.	**4/*4*	DFS
						**1/*1* or **1/*4*	vs.	**4/*4*	Hot flushes
		Nowell et al. (2005)	Adjuvant	Case-cohort	162	**1/*1*	vs.	**4* Carrier	PFS
		Wegman et al. (2005)	Adjuvant	Cohort	112	**1/*1*	vs.	**1/*4* or **4/*4*	RFS
		Wegman et al. (2007)	Adjuvant	Cohort	677	**1/*1*	vs.	**4/*4*	RFS
						**1/*1*	vs.	**1/*4* or **4/*4*	RFS
		Abraham et al. (2010)	Adjuvant	Case-cohort	3155	**1/*1*	vs.	**4* Carrier	BCSS
		Regan et al. (2012)	Adjuvant	Cohort	4393	**1/*1*	vs.	**4/*4*	BCFI
						**1/*1*	vs.	**1/*4*	BCFI
		van Schaik et al. (2011)	Palliative	Cohort	499	**1/*1*	vs.	**4* Carrier	TTF
	**5*	Abraham et al. (2010)	Adjuvant	Case-cohort	3155	**1/*1*	vs.	**5* Carrier	BCSS
	**9*	Abraham et al. (2010)	Adjuvant	Case-cohort	3155	**1/*1*	vs.	**9* Carrier	BCSS
	**10*	Okishiro et al. (2009)	Adjuvant	Cohort	173	**1/*1* or **1/*10*	vs.	**10/*10*	RFS
						**1/*1* or **1/*10*	vs.	**10/*10*	Bone mineral density
						**1/*1* or **1/*10*	vs.	**10/*10*	Total cholesterol
						**1/*1* or **1/*10*	vs.	**10/*10*	Endometrial thickness (1 year TAM)
		Toyama et al. (2009)	Adjuvant	Cohort	156	**1/*1*	vs.	**1/*10* or **10/*10*	DFS
						**1/*1*	vs.	**1/*10* or **10/*10*	DFS
						**1/*1*	vs.	**1/*10* or **10/*10*	BCSS
		Abraham et al. (2010)	Adjuvant	Case-cohort	3155	**1/*1*	vs.	**10* Carrier	BCSS
		Xu et al. (2008)	Adjuvant	Cohort	152	**1/*1*	vs.	**10* Carrier	5-Year DSS
	**41*	Abraham et al. (2010)	Adjuvant	Case-cohort	3155	**1/*1*	vs.	**41* Carrier	BCSS
Combined *CYP2D6* genotypes		Abraham et al. (2010)[b]	Adjuvant	Case-cohort	3155	**1/*1*	vs.	PM or IM	BCSS
						**1/*1*	vs.	PM	BCSS
		Regan et al. (2012)[d]	Adjuvant	Cohort	4393	EM	vs.	PM	BCFI
						EM	vs.	IM	BCFI
						EM	vs.	PM or IM	BCFI
						EM	vs.	PM	Hot flushes
						EM	vs.	IM	Hot flushes
						EM	vs.	PM/IM	Hot flushes
		Teh et al. (2012)[c]	Non-palliative	Cohort	95	EM	vs.	Het-IM	RFS
		Newman et al. (2008)[e]	Adjuvant	Cohort	115	**1/*1*	vs.	PM	TTR
						**1/*1*	vs.	PM	OS
CYP2C8	*4	Jernström et al. (2009)	Adjuvant	Cohort	297	**1/*1*	vs.	**1/*4* or **4/*4*	BCSS
CYP2C9	*2	Jernström et al. (2009)	Adjuvant	Cohort	297	**1/*1*	vs.	**1/*2* or **2/*2*	BCSS
	*3					**1/*1*	vs.	**1/*3* or **3/*3*	BCSS
CYP2C19	**2* and **3*	Okishiro et al. (2009)	Adjuvant	Cohort	173	**1/*1*, **1/*2*, or **1/*3*	vs.	*2/*2, *2/*3, or *3/*3	RFS
						**1/*1*, **1/*2*, or **1/*3*	vs.	*2/*2, *2/*3, or *3/*3	Bone mineral density
						**1/*1*, **1/*2*, or **1/*3*	vs.	*2/*2, *2/*3, or *3/*3	Total cholesterol

(Continued)

Table 2 | Continued

Gene	SNP	Reference	Population	Design	*N*	Compared groups[a]			Outcome
						*1/*1, *1/*2, or *1/*3	vs.	*2/*2, *2/*3, or *3/*3	Endometrial thickness (1 year TAM)
	*2	van Schaik et al. (2011)	Palliative	Cohort	499	*1/*1	vs.	*2 Carrier	DFS
	*17	Moyer et al. (2011)	All incident users	Cohort	190	*1/*1	vs.	*1/*17 or *17/*17	DFS
		van Schaik et al. (2011)	Palliative	Cohort	499	*1/*1	vs.	*17 Carrier	TTF
CYP3A5	*3	Goetz et al. (2005)	Adjuvant	Cohort	223	*1/*1 or *1/*3	vs.	*3/*3	RFS
						*1/*1 or *1/*3	vs.	*3/*3	DFS
						*1/*1 or *1/*3	vs.	*3/*3	Hot flushes
		Tucker et al. (2005)	Adjuvant	Cohort	98	*1/*1	vs.	*1/*3 or *3/*3	Nausea
						*1/*1	vs.	*1/*3 or *3/*3	Migraines
						*1/*1	vs.	*1/*3 or *3/*3	Depression
						*1/*1	vs.	*1/*3 or *3/*3	Vaginal discharge
						*1/*1	vs.	*1/*3 or *3/*3	Vaginal dryness
						*1/*1	vs.	*1/*3 or *3/*3	Insomnia
						*1/*1	vs.	*1/*3 or *3/*3	Hot flushes
	*6	Tucker et al. (2005)	Adjuvant	Cohort	98	*1/*1	vs.	*1/*6 or *6/*6	Nausea
						*1/*1	vs.	*1/*6 or *6/*6	Migraines
						*1/*1	vs.	*1/*6 or *6/*6	Depression
						*1/*1	vs.	*1/*6 or *6/*6	Vaginal discharge
						*1/*1	vs.	*1/*6 or *6/*6	Vaginal dryness
						*1/*1	vs.	*1/*6 or *6/*6	Insomnia
						*1/*1	vs.	*1/*6 or *6/*6	Hot flushes
SULT1A1	*2	Wegman et al. (2005)	Adjuvant	Cohort	112	*1/*1	vs.	*1/*2 or *2/*2	RFS
		Wegman et al. (2007)	Adjuvant	Cohort	677	*1/*1	vs.	*1/*2 or *2/*2	RFS
	Copy number	Moyer et al. (2011)	All incident users	Cohort	190	≤ 2	vs.	>2	DFS
UGT1A8	*3	Ahern et al. (2011)	Non-palliative	Case-cohort	541	*1/*1	vs.	*1/*3 or *3/*3	RFS
UGT2B7	*2	Ahern et al. (2011)	Non-palliative	Case-cohort	541	*1/*1	vs.	*2/*2	RFS
UGT2B15	*2	Nowell et al. (2005)	Adjuvant	Case-cohort	162	*1/*1	vs.	*1/*2	RFS
						*1/*1	vs.	*2/*2	
		Wegman et al. (2007)	Adjuvant	Cohort	677	*1/*1	vs.	*1/*2, *2/*2	RFS
		Ahern et al. (2011)	Non-palliative	Case-cohort	541	*1/*1	vs.	*2/*2	RFS

Abbreviations: BCFI, breast cancer-free interval; BCSS, breast cancer-specific survival; DFS, disease-free survival; DSS, disease-specific survival; EM, extensive metabolizer; Het, heterozygous; Homo, homozygous; IM, intermediate metabolizer; PFS, progression-free survival; PM, poor metabolizer; RFS, recurrence-free survival; TTF, time-to-treatment failure; V, variant alleles. [a]*The designation *1 indicates the reference allele sequence for the analyzed SNP, but may include non-analyzed sequence variations.* [b]*Abraham et al. (2010) – PM: presence of at least one allele *4, *5, or *6; IM: presence of one or two alleles *9, *10, or *41;* [c]*Teh et al. (2012) – EM: absence of alleles *xN, *4, *5, *10, and *14; Het-IM: *1/*10; IM: *10/*10 or heterozygous null alleles (*1/*4, *1/*5, *1/*14);* [d]*Regan et al. (2012) – PM: presence of alleles *3, *4, *6, or *7; IM: *41/*41 or *41/PM;* [e]*Newman et al. (2008) – PM: two copies of the CYP2D6*3, CYP2D6*4, or CYP2D6*5 alleles; a group with concomitant use of a potent CYP2D6 inhibitor in wild-type individuals or moderate inhibitor in heterozygous patients.*

Table 3 | Effects of polymorphisms in xenobiotic metabolizing enzymes on breast cancer outcomes after chemotherapy.

Gene	SNP	Reference	Population/design	Protocol	*N*	Reference group[a]	Affected group[b]	Outcome	Main results	
									Calculation (95% CI)	*p*-Value
CYP1A1	*m2*	Chacko et al. (2005)	Adjuvant, neoadjuvant, metastatic	Not informed	79	*wt/wt*	*wt/m2* or *m2/m2*	BCSS	HR; 18.3 (2.4–140)	0.005
CYP1B1	**3*	Marsh et al. (2007)	Stages IIIA – IV	Paclitaxel-based	84	**1/*1 or *1/*3*	**3/*3*	PFS	KM–LR[c]	0.037
CYP2B6	**2*	Bray et al. (2010)	Adjuvant	AC	230	**1/*1*	**1/*2*	TTP	KM–LR[c]	0.002
	**4*	Bray et al. (2010)	Adjuvant	AC	230	**1/*1*	**4/*4*	OS	KM–LR[c]	0.05
	**9*	Bray et al. (2010)	Adjuvant	AC	230	**1/*1*	**9/*9*	OS	KM–LR[c]	0.003
CYP3A4	**1B*	Gor et al. (2010)	Adjuvant	CAF (+ CTX/thiotepa)	350	**1B/*1A*	**1A/*1A*	DFS	HR; 2.79 (1.5–5.1)	0.001
GSTA1	**B*	Sweeney et al. (2003)	Neoadjuvant	CTX-based		**A/*A or *A/*B*	**B/*B*	OS	HR; 0.5 (0.3–0.8)	0.01
GSTP1	*313A > G*	Huang et al. (2008)	Adjuvant	FEC	192	*A/A (Ile/Ile)*	*A/G (Ile/Val) or G/G (Val/Val)*	Early relapse[d]	χ^2 Test[c,e]	0.014
	313A > G	Zhang et al. (2011)	Neoadjuvant	EPI and CTX	120	*G/G (Val/Val)*	*A/A (Ile/Ile) or A/G (Ile/Val)*	Pathological response	OR; 0.4 (0.2–0.96)	0.024
GSTT1	Deletion (null)	Chacko et al. (2005)	Adjuvant, neoadjuvant, metastatic	Not informed	79	Non-null	Null	BCSS	HR; 0.2 (0.0–0.9)	0.039
UGT2B7	*His268Tyr*	Parmar et al. (2011)	Adjuvant	EPI	205	Two *268Tyr* alleles	At least one *268His* allele	DFS	HR; 2.64 (1.2–5.7)	0.014
COMBINED GENOTYPES										
GSTP1/ SOD2	*313A > G*	Bewick et al. (2008)	Metastatic/retrospective cohort	MITOX and CTX	95	*GSTP1 AA, and SOD2 CC or CT*	*GSTP1 GG or AG, and SOD2 TT*	BCSS	HR; 2.17 (1.1–4.2)	0.013
	313A > G/16C > T	Bewick et al. (2008)	Metastatic/retrospective cohort	MITOX and CTX	96	*GSTP1 AA, and SOD2 CC or CT*	*GSTP1 GG or AG, and SOD2 TT*	PFS	HR; 2.89 (1.4–5. 9)	0.002

*Abbreviations: AC, adriamycin and cyclophosphamide; BCSS, breast cancer-specific survival; CAF, cyclophosphamide, adriamycin, and 5-fluorouracil; CTX, cyclophosphamide; DFS, disease-free survival; EPI, epirubicine; FEC, 5-fluorouracil, epirubicine, and cyclophosphamide; HR, hazard ratio; KM–LR, Kaplan–Meier curves and Log-Rank test; MITOX, mitoxantrone; OR, odds ratio; OS, overall survival; PFS, progression-free survival; RR, relative risk; TEC, docetaxel, epirubicine, and cyclophosphamide; TTP, time to progression. WT, wild-type allele. [a]The Reference group includes alleles, genotypes, haplotypes, or phenotypes considered as reference for evaluation of outcome parameters. The designation *1 indicates the reference allele sequence for the analyzed SNP, but may include non-analyzed sequence variations. [b]The Affected group includes alleles, genotypes, haplotypes, or phenotypes with significantly different outcome parameters, when compared to the Reference group. [c]The Affected group had a worse outcome. [d]Early relapse was designated as the development of new recurrent or distant metastatic lesions within 2 years after receiving postoperative chemotherapy; [e]χ^2 test.*

Table 4 | Effects of polymorphisms in xenobiotic metabolizing enzymes on the toxicity to chemotherapy for breast cancer.

Gene	SNP	Reference	Population	*N*	Protocol	Outcome	Reference group	Affected group	Main results	
									Calculation (95% CI)	*p*-Value
CBR3	*G11A*	Fan et al. (2008)	Neoadjuvant	99	Doxorubicin and docetaxel	Leucopenia at nadir[a]	*GG*	*GA, AA*	Trend test	0.019
						Thrombocytopenia at nadir[a]	*GG*	*GA, AA*	Trend test	0.026
CYP1B1	**3*	Rizzo et al. (2010)	Adjuvant, neoadjuvant, or metastatic	95	Taxanes	Hypersensitivity[b]	*CG* or *GG*	*CC*	OR; 0.136 (0.05–0.37)	0.014
CYP2B6	**2*	Bray et al. (2010)	Adjuvant	230	AC	Toxicity and dose delay	*CT* or *TT*	*CC*	$\chi 2$ Test[c]	0.013
CYP3A5	**3*	Tsai et al. (2009)	Adjuvant or neoadjuvant	59	TEC	Febrile neutropenia	**3/*3*	**1/*3*	RR; 7.17 (1.10–3.55)	$p < 0.05$
		Tsai et al. (2009)	Adjuvant or neoadjuvant	59	TEC	Fever	**3/*3*	**1/*3*	RR; 3.29 (1.03–10.05)	$p < 0.05$
		Tsai et al. (2009)	Adjuvant or neoadjuvant	59	TEC	Neutropenia	**3/*3*	**1/*3*	RR; 3.29 (1.03–10.5)	$p < 0.05$
GSTP1	*Ile105Val*	Zárate et al. (2007)	Adjuvant	95	Anthracycline-based	Hematological toxicity (grades 3 or 4)	*A/A (Ile/Ile)* or *A/G (Ile/Val)*	*G/G (Val/Val)*	HR; 6.4 (1.05–39)	0.044
		Yao et al. (2010)	Adjuvant	874	CAF or CMF	Neutropenia (grades 3 or 4)	*A/A (Ile/Ile)*	*A/G (Ile/Val)* or *G/G (Val/Val)*	OR; 0.63 (0.41–0.97)	0.04
		Yao et al. (2010)	Adjuvant	874	CAF or CMF	Leucopenia	*A/A (Ile/Ile)*	*A/G (Ile/Val)* or *G/G (Val/Val)*	OR; 0.59 (0.39–0.89)	0.01
		Zhang et al. (2011)	Neoadjuvant	120	EPI and CTX	Severe toxicity (grades 3 or 4)	*G/G (Val/Val)*	*A/A (Ile/Ile) or A/G (Ile/Val)*	OR; 0.35 (0.13–0.78)	0.006

Abbreviations: AC, driamycin and cyclophosphamide; BCSS, breast cancer-specific survival; CAF, cyclophosphamide, adriamycin, and 5-fluorouracil; CMF, cyclophosphamide, metothrexate, and 5-fluorouracil; CTX, cyclophosphamide; DFS, disease-free survival; EPI, epirubicine; FEC, 5-fluorouracil, epirubicine, and cyclophosphamide; HR, hazard ratio; IM, intermediate metabolizer; KM–LR. Kaplan–Meier curves and Log-Rank test; MITOX, mitoxantrone; OR, odds ratio; OS, overall survival; PFS, progression-free survival; RR, relative risk; TEC, docetaxel, epirubicine, and cyclophosphamide; TTP, time to progression. [a]Nadir was defined as the lowest count documented within 21 days of doxorubicin administration; [b]The hypersensitivity reactions were characterized by acute dyspnea, flushing of the face, chest constraint, hypotension, and rash; [c]χ^2 test.

Table 5 | Null associations between polymorphisms in xenobiotic metabolizing enzymes and breast cancer outcomes after chemotherapy.

Gene	SNP	Reference	Population	Protocol	*N*	Outcome	Compared groups
CBR3	*730G > A*	Fan et al. (2008)	Neoadjuvant	Doxorubicin and docetaxel	99	Hematological toxicity or tumor reduction[a]	*GG*, *GA*, and AA
CYP2B6	**3*, **5*, **8*	Bray et al. (2010)	Adjuvant	AC	230	DFS and toxicity	*1/*1 vs. any variant
	**2*, **3*, **4*, **5*, **8*, and **9*	Gor et al. (2010)	Adjuvant	CAF (+ CTX/thiotepa)	350	DFS	*1/*1 vs. any variant
	**9*	Yao et al. (2010)	Adjuvant	CAF or CMF	449	Hematological toxicity	*GG* vs. *GT* or *TT*
CYP2C8	**1*, **2*, **3*, and **4*	Rizzo et al. (2010)	Adjuvant, neoadjuvant, metastatic	Taxanes	95	Hematological toxicity, neurotoxicity, and hypersensitivity[b]	*1/*1 vs. any variant
CYP2C9	**2* and **3*	Bray et al. (2010)	Adjuvant	AC	230	BCSS and toxicity	*1/*1 vs. any variant
		Gor et al. (2010)	Adjuvant	CAF (+ CTX/thiotepa)	350	DFS	*1/*1 vs. any variant
CYP2C19	**2*	Bray et al. (2010)	Adjuvant	AC	230	BCSS and toxicity	*1/*1 vs. any variant
CYP2D6	**10*	Gor et al. (2010)	Adjuvant	CAF (+ CTX/thiotepa)	350	DFS	*G/G* vs. *G/A* vs. *A/A*
CYP3A4	**4*	Tsai et al. (2009)	Adjuvant or neoadjuvant	TEC	59	Toxicity	**1/*1* vs. **1/*4*
	**5*	Tsai et al. (2009)	Adjuvant or neoadjuvant	TEC	59	Toxicity	**1/*1* vs. **1/*5*
	**18*	Tsai et al. (2009)	Adjuvant or neoadjuvant	TEC	59	Toxicity	**1/*1* vs. **1/*18*
	**1B*	Yao et al. (2010)	Adjuvant	CAF or CMF	456	Hematological toxicity	**1/*1* vs. **1/*1B* or **1B/*1B*
	**1G*	Zhang et al. (2011)	Neoadjuvant	EPI and CTX	120	Pathological response and toxicity	*CC* vs. *CT* vs. *TT*
CYP3A5	**3*	Bray et al. (2010)	Adjuvant	AC	230	BCSS and toxicity	*1/*1 vs. any variant
		Gor et al. (2010)	Adjuvant	CAF (+ CTX/thiotepa)	350	DFS	**1/*3*, **1/*1*, **3/*3*
		Zhang et al. (2011)	Neoadjuvant	EPI and CTX	120	Pathological response and toxicity	
	**6*	Gor et al. (2010)	Adjuvant	CAF (+ CTX/thiotepa)	350	DFS	**1/*1* vs. **1/*6*
CYP3A7	**2*	Zhang et al. (2011)	Neoadjuvant	EPI and CTX	120	Pathological response and toxicity	*AA*, *AT*, *TT*
GSTA1	rs3957356-69A > G	Yao et al. (2010)	Adjuvant	CAF or CMF	414	Hematological toxicity	*GG* vs. *GA* or *AA*
GSTM1	*Null*	Gor et al. (2010)	Adjuvant	CAF (+ CTX/thiotepa)	350	DFS	Null vs. non-null
		Oliveira et al. (2010)	Neoadjuvant	FEC	40	Pathological response	Null vs. non-null
		Mishra et al. (2011)	Neoadjuvant	CAF – docetaxel	45	Pathological response	Null vs. non-null
		Saadat et al. (2012)	Neoadjuvant	CAF or TAC	101	Pathological response	Null vs. non-null
GSTO2	rs156697 (Asn142Asp)	Saadat et al. (2012)	Neoadjuvant	CAF or TAC	101	Pathological response	*Asn/Asn*, *Asn/Asp*, *Asp/Asp*
GSTP1	*Ala114Val*	Bewick et al. (2008)	Metastatic	MITOX and CTX	95	PFS and BCSS	*AA*, *AG*, *GG*
	Ile105Val	Bewick et al. (2008)	Metastatic	MITOX and CTX	95	PFS and BCSS	*CC*, *CT*, *TT or GG*, *GA*, *AA*
		Gor et al. (2010)	Adjuvant	CAF (+ CTX/thiotepa)	350	DFS	*GG*, *GA*, *AA*
		Oliveira et al. (2010)	Neoadjuvant	FEC	40	Pathological response	*Ile/Val* vs. *Ile/Ile*
		Yao et al. (2010)	Adjuvant	CAF or CMF	874	DFS	*GG*, *GA*, *AA*

(Continued)

Table 5 | Continued

Gene	SNP	Reference	Population	Protocol	*N*	Outcome	Compared groups
GSTT1	*Null*	Gor et al. (2010)	Adjuvant	CAF (+ CTX/thiotepa)	350	DFS	Null vs. non-null
		Oliveira et al. (2010)	Neoadjuvant	FEC	40	Pathological response	Null vs. non-null
		Mishra et al. (2011)	Neoadjuvant	CAF – docetaxel	45	Pathological response	Null vs. non-null
		Saadat et al. (2012)	Neoadjuvant	CAF or TAC	101	Pathological response	Null vs. non-null
GSTZ1	rs7975 *(Glu32Lys)*	Saadat et al. (2012)	Neoadjuvant	CAF or TAC	101	Pathological response	*Glu/Glu*, *Glu/Lys*, *Lys/Lys*

Abbreviations: AC, adriamycin and cyclophosphamide; BCSS, breast cancer-specific survival; CAF, cyclophosphamide, adriamycin, and 5-fluorouracil; CMF, cyclophosphamide, metothrexate, and 5-fluorouracil; CTX, cyclophosphamide; DFS, disease-free survival; EPI, epirubicine; FEC, 5-fluorouracil, epirubicine, and cyclophosphamide; MITOX, mitoxantrone; OR, odds ratio; OS, overall survival; PFS, progression-free survival; TEC, docetaxel, epirubicine, and cyclophosphamide. [a]Tumor reduction was defined as ≥25% tumor reduction after the first cycle of doxorubicin; [b]The hypersensitivity reactions were characterized by acute dyspnea, flushing of the face, chest constraint, hypotension, and rash.

the activity of CYP2D6 enzyme, characterized individual metabolizing phenotypes (**Tables 1** and **2**). Although some studies have suggested that reduced CYP2D6 enzymatic activity might contribute for higher risk of breast cancer recurrence (**Table 1**), the recent results of the large studies by Abraham et al. (2010) and Regan et al. (2012) indicate no significant differences in relation to breast cancer-specific survival, either for poor or intermediate CYP2D6 metabolizers, which were characterized based on alleles **3*, **4*, **5*, **6*, **7*, **10*, and **41* (**Table 2**).

With regards to other polymorphisms that could affect tamoxifen metabolism, there are fewer studies available, and none of them are based on multicenter large populations. In addition, the published data are more diverse in relation to the polymorphisms and to the outcomes that were analyzed, with some polymorphisms being evaluated in only one study. Therefore, the available information does not support combined analyses, and any general conclusion is only tentative.

The only study involving *CYP2C8* (alleles **3* and **4*) or *CYP2C9* (alleles **2* and **3*) was the work by Jernström et al. (2009), who described an increased risk of early breast cancer-related deaths associated with the haplotype *CYP2C8*1/*3/CYP2C9*1/*2* (**Table 1**). According to the authors, the effect appears to be driven by *CYP2C8*3*, which presents strong, but not complete linkage disequilibrium with *CYP2C9*2*. The authors found no significant effects associated with *CYP2C8*4*, *CYP2C9*2*, or *CYP2C9*3*, when evaluated independently (**Table 2**). With regards to *CYP2C19*, Okishiro et al. (2009) analyzed the alleles **2* and **3*, Ruiter et al. (2010) analyzed only the allele **2*, and Moyer et al. (2011) analyzed the allele **17*. Ruiter et al. (2010) were the only ones to describe an apparent protective effect for the allele **2*, with longer breast cancer-specific survival among patients carrying any variant genotype (**Table 1**). *CYP3A5* was analyzed by Goetz et al. (2005), Tucker et al. (2005), and Wegman et al. (2007). Tucker et al. (2005) evaluated *CYP3A5*3* and *CYP3A5*6*, whereas the other two studies analyzed only *CYP3A5*3*. Wegman et al. (2007) reported a significantly improved recurrence-free survival among *CYP3A5*3*-homozygous patients (**Table 1**), which was not seen by Goetz et al., 2005; **Table 2**). Tucker et al. (2005) did not evaluate response outcomes, and reported no significant association between *CYP3A5* polymorphisms and adverse events during tamoxifen treatment (**Table 2**). *SULT1A1* was studied by Nowell et al. (2002), Wegman et al. (2005), Wegman et al. (2007), and Moyer et al. (2011). The first three studies evaluated the polymorphism *SULT1A1*2*, whereas Moyer et al. (2011) analyzed the number of gene copies in breast tumors. The study by Nowell et al. (2002) was the only one to report a significant effect of the homozygous **2/*2* on patients' overall survival (**Table 1**). Wegman et al. (2007) also reported significant detrimental effects of the variant allele **2* on recurrence-free survival after 2 years of tamoxifen treatment (**Table 1**), but this association was no longer existent after 5 years of tamoxifen treatment (**Table 2**). The polymorphisms *UGT1A8*3*, *UGT2B7*2*, and *UGT2B15*2* were analyzed by Ahern et al. (2011), who found no significant associations between their variant alleles and recurrence-free survival in breast cancer patients under adjuvant or neoadjuvant treatment with tamoxifen (**Table 2**). *UGT2B15*2* was also analyzed by Nowell et al. (2002) and Wegman et al. (2007). Nowell et al. (2002) were the only ones to report a significant higher risk of disease progression for patients carrying the variant allele **2* (either in heterozygosis or in homozygosis) in combination with the genotype *SULT1A1*2/*2* (**Table 1**). In conclusion, there is no consistency among the few and sparse results regarding polymorphisms in xenobiotic metabolizing enzymes different from CYP2D6 and their impact on tamoxifen efficacy or safety. Taken together, these results do not suggest clinically relevant implications of such pharmacogenetic targets for tamoxifen treatment.

The published data regarding functional polymorphisms in xenobiotic metabolizing enzymes and breast cancer chemotherapy present even greater diversity in design than observed for studies involving tamoxifen. As expected, these studies involve different patients' subgroups, and comprise distinct chemotherapeutic protocols. The study designs are also very diverse in the selection of polymorphisms and in analyzed outcomes, with some studies evaluating response outcomes, whereas others focus only in adverse effects. **Table 3** summarizes all the available data for significant associations with response outcomes. The compiled information indicates worse outcomes (higher recurrence risk or shorter breast cancer-specific survival) for the polymorphisms *CYP1A1m2*, *CYP1B1*3*, *CYP2B6*2*, **4*, and **9*, *CYP3A4*1B*, *GSTP1A313G*, and *UGT2B7His268Tyr*, whereas a

beneficial effect is observed for the variant genotype *GSTA1*B/*B* (better overall survival) and for the *GSTT1*Null genotype (longer breast cancer-specific survival). Bewick et al. (2008) found no significant effect for *GSTP1A313G* when evaluated independently, but reported a combined effect of *GSTP1A313G* variant genotypes and *SOD2C16CT* variant homozygous genotype resulting in shorter progression-free survival and breast cancer-specific survival for patients using mitoxantrone and cyclophosphamide.

Table 4 summarizes all the available data for significant associations with chemotherapy-related toxicity outcomes. The compiled results indicate higher risk of severe hematological reactions (neutropenia, leucopenia, or thrombocytopenia) for patients with *CBR3 G11A* or *GSTP1A313G* variant genotypes. The other analyzed polymorphisms (*CYP1B1*3*, *CYP2B6*2*, *CYP3A5*3)* showed beneficial effects in relation to the risk of hematological toxicities for patients using anthracycline-based protocols and/or taxanes.

Finally, **Table 5** summarizes all the null associations involving polymorphisms in xenobiotic metabolizing enzymes and breast cancer outcomes after chemotherapy. The compiled data comprise various studies evaluating many polymorphisms, but, in most cases, there is only one study for each polymorphism. Because most studies are based on a relatively small number of patients using specific protocols, the compiled information is very diffuse and does not allow definite conclusions or general assumptions. Therefore, the results must be analyzed independently, considering the particularities of each study.

The only polymorphisms that were analyzed by at least two independent studies with similar designs were *CYP2B6*2*, **3*, **5*, **8*, and **9*, *CYP2C9 *2* and **3*, and *CYP3A5*3*, which were studied by Bray et al. (2010) and Gor et al. (2010), evaluating survival in patients under adjuvant chemotherapy with anthracycline-based protocols. *GSTM1null* was also studied under similar conditions by Oliveira et al. (2010), Mishra et al. (2011), and Saadat et al. (2012), who evaluated the pathological response to neoadjuvant chemotherapy with anthracyclines and taxanes. In the case of *CYP2B6*2*, Bray et al. (2010) reported a significant association between the heterozygous variant genotype and shorter time to progression for patients under adjuvant chemotherapy with the protocol AC (**Table 3**), whereas Gor et al. (2010) found no significant effect on disease-free survival for patients under adjuvant chemotherapy with the protocol CAF (+ CTX/Thiotepa; **Table 5**). In the case of *CYP3A5*3*, Tsai et al. (2009) found that patients carrying the heterozygous **1/*3* genotype demonstrated more side effects of fever, pleural effusion, and febrile neutropenia than those with the homozygous **3/*3* genotype (**Table 4**). Bray et al. (2010), evaluating dose delays in adjuvant chemotherapy with AC, found no significant associations with *CYP3A5**3 variant alleles (**Table 5**), which the authors interpreted as no significant differences in clinically relevant toxicities. Zhang et al. (2011), evaluating severe toxicities (grade 3 or 4) to neoadjuvant chemotherapy with EPI and CTX, also found no significant associations with *CYP3A5**3 variant alleles (**Table 5**). It is not clear whether the discrepancies in the association results reported by different studies involving the same polymorphisms can be attributed to distinct chemotherapeutic protocols, or to other uncontrolled causes of variability.

DISCUSSION

The field of pharmacogenetics (or pharmacogenomics) has developed with the goal of identifying genetic causes of interindividual differences in pharmacological response, and of using such genetic information to predict one individual's profile of drug safety and efficacy. In this regard, pharmacogenetic studies are designed to evaluate the correlation between genotypes and phenotypes, and, therefore, provide scientific evidence for the implementation of individualized drug prescriptions, as part of a conduct of personalized medicine. Nevertheless, the characterization of phenotypes may not be easy to accomplish, especially in clinical settings, or when they require invasive procedures. In addition, the actual therapeutic goal is the final clinical outcome, which is, therefore, usually taken as the endpoint of pharmacogenetic studies. One limitation, however, is that clinical outcomes are often the result of complex and overlapping variables, which may have different genetic and non-genetic causes. As a consequence, the strict genotype-phenotype correlation may be compromised, and the results of pharmacogenetic studies may be difficult to interpret. This is exactly the scenario of breast cancer treatment: although there are theoretical bases and practical evidences that genetic influences may indeed affect the pharmacological response, there is great uncertainty about the usefulness of the genetic information to actually predict clinical outcomes and even more on the confidence of using such individual genetic information to modify one's therapeutic conduct.

The first therapeutic target to drive the attention of pharmacogenetic studies to breast cancer therapy was CYP2D6 in view of its apparent strong genotype-phenotype correlation. Thus, various literature reports indicated that genetic variations in *CYP2D6* affect the availability or the functional activity of the corresponding enzyme (Jin et al., 2005), and that such variations in the enzymatic activity ultimately lead to altered levels of tamoxifen metabolites (Lim et al., 2007, 2011; Kiyotani et al., 2010; de Graan et al., 2011; Irvin et al., 2011; Mürdter et al., 2011). In addition, parallel observations indicated that altered levels of the most active tamoxifen metabolite, endoxifen, result in reduced binding to the estrogen receptor and to lower signaling transduction (Coezy et al., 1982; Lim et al., 2006). Taken together, these results have reinforced the notion that *CYP2D6* polymorphisms could be useful to predict one individual's response to tamoxifen, and that *CYP2D6* genotyping might help the selection of the antiestrogenic drug or the definition of tamoxifen dosing. The promises of this rationale in relation to possible improvements in breast cancer outcomes has led to the expansion of pharmacogenomic studies to other therapeutic targets in breast cancer antineoplastic chemotherapy, as well as to other targets of tamoxifen pharmacokinetics.

The evaluation of the literature production involving the pharmacogenetics of breast cancer indicates a great number of studies. The current review, which was focused on pharmacokinetic targets, and more specifically, on functional polymorphisms of xenobiotic metabolizing enzymes, has initially retrieved 158 references on the subject. Although the criteria for the systematic search

included the mention to xenobiotic metabolizing enzymes in the article's title or abstract, most of the retrieved documents explored different targets as their main research subject. The most frequent targets besides xenobiotic metabolizing enzymes that were identified in the current search were drug transporters, which might contribute to antineoplastics' pharmacokinetics, affecting drug distribution and disposition, and aromatases, which modulate the availability of estrogens, and the estrogen receptor. The selection of documents exploring functional polymorphisms of xenobiotic metabolizing enzymes resulted in 43 original articles, 23 devoted to tamoxifen, and 20 dealing with various antineoplastic protocols. Among the articles involving tamoxifen, *CYP2D6* was the main pharmacogenetic target, whereas multiple xenobiotic metabolizing enzymes were explored with regards to different cytotoxic antineoplastics. This conjunct of original articles on the pharmacogenetics of breast cancer is still relatively limited in number and very diverse in design. Consequently, general conclusions are difficult to extract at this point, and no clear recommendations can be made for application in clinical practice. Nevertheless, the use of the same terms for bibliographic search without the restriction to original articles indicates that 24 reviews were published on the subject in the same period of time. Although the analysis of the reviews was not part of our systematic search, an overview of the publications indicates that *CYP2D6* predominates as the chosen subject, probably as a reflex of the great enthusiasm after the earliest studies. Some of the most recent reviews already include data on other xenobiotic metabolizing enzymes, but no systematic review had been presented before.

The studies on *CYP2D6* polymorphisms and their influences on tamoxifen efficacy show many discrepancies, which can be accounted to multiple factors, including variations in study design, in the definitions of breast cancer disease, and in the population characteristics. Thus, in relation to study design, different *CYP2D6* polymorphisms are analyzed, and some studies use tumor tissue for genotype assessment, which can compromise the accurate characterization of the number and types of *CYP2D6* alleles. There are also variations in the therapeutic regimen, including adjuvant, neoadjuvant and palliative treatment, and evaluation of different disease outcomes. The prescribed tamoxifen dose may also vary, and, in most studies, there is no control or documentation of drug adherence and of concomitant use of CYP2D6 inhibitors. The population characteristics also present great variability, including different ethnic and cultural backgrounds, which can interfere with risk estimates. Finally, the percentage of postmenopausal women may also be an interfering factor, if there is no control of the use of aromatase inhibitors after the completion of tamoxifen treatment.

A meta-analysis published by Seruga and Amir (2010) analyzed data from 10 studies assessing CYP2D6 genotype and clinical outcomes in breast cancer. The authors found significant heterogeneity in the definition of comparison groups between studies, but suggested that normal CYP2D6 function was associated with a trend toward improved disease-free survival (HR 2.07, 95% CI 0.96–4.49, $P = 0.06$). The most recent work on the subject, by Regan et al. (2012), enrolled a large number of postmenopausal patients treated with tamoxifen for 5 years, as part of the BIG 1–98 trial, and showed no association between CYP2D6 metabolizing phenotype and the risk of recurrence (characterized by the breast cancer-free interval), with or without previous chemotherapy. The patients who were identified as poor or intermediate metabolizers (based on alleles *2, *3, *4, *6, *10, *17, and *41) did not have worse disease outcomes than extensive metabolizers, or higher chance of presenting hot flushes as side effects. The authors argued that CYP2D6 metabolizing phenotype is not the correct surrogate for predicting symptoms or outcome of tamoxifen-treated postmenopausal women, and advocated that *CYP2D6* pharmacogenetic testing to determine whether adjuvant tamoxifen should be given to postmenopausal women with endocrine-responsive breast cancer is not justified (Regan et al., 2012). Although very large in the number of patients enrolled, and with a well-planned prospective design, one limitation of the work by Regan et al. (2012), as acknowledged by the authors, is the use of tumor samples for genotyping, which could lead to some misclassification of metabolizing phenotypes. In addition, the trial did not provide data on concomitant medications, and therefore, a possible interference of CYP2D6 inhibitors cannot be ruled out.

Two other large studies involving breast cancer survivors who used tamoxifen (Abraham et al., 2010; Madlensky et al., 2011) also suggested the lack of association between *CYP2D6* genotypes, or estimated CYP2D6 metabolizing phenotypes, and breast cancer outcomes. Thus, Abraham et al. (2010) found that only the allele *6 was associated with lower breast cancer-specific survival, whereas the other alleles (*4, *5, *9, *10, and *41) had no significant effects. Madlensky et al. (2011) found no significant associations between CYP2D6 metabolizing phenotypes and the risk of breast cancer recurrence or second breast cancer. The latter authors, however, reported that patients with endoxifen concentrations lower than 5.97 ng/mL were at higher risk of breast cancer recurrence, and that the proportion of decreased CYP2D6 metabolizing phenotype was higher in this group. In addition to CYP2D6 metabolizing phenotype, the authors identified other variables, such as excess weight and low tamoxifen levels (suggesting failures in adherence), which were associated with low endoxifen levels, but not independently associated with breast cancer outcomes. The authors suggested the existence of a non-linear dose-response effect for tamoxifen, and proposed that a threshold of endoxifen must be achieved for the therapeutic effect of tamoxifen. Finally, the authors argued that the metabolizing phenotype alone is not enough to determine whether tamoxifen is of potential benefit to any individual patient (Madlensky et al., 2011).

Another aspect with pharmacogenetic interest in relation to tamoxifen is the occurrence of side effects, especially hot flushes, which are intrinsically correlated with the suppression of estrogen signaling, and thus expected to be associated with endoxifen levels. Some authors have proposed that hot flushes could serve as surrogates of tamoxifen efficacy, and that their absence could indicate patients at higher risk of recurrence (Cuzick et al., 2008; Mortimer et al., 2008). As a logical consequence, it has been hypothesized that poor CYP2D6 metabolizers would be less likely to experience moderate to severe hot flushes (Goetz et al., 2005; Henry et al., 2009), whereas, in contrast, extensive metabolizers would be more prone to prematurely discontinue tamoxifen, possibly as a consequence of severe hot flushes (Rae et al., 2009). The assumed correlation between CYP2D6 metabolizing phenotypes and hot

flushes, however, was based on few and weak associations, with no (or only border-line) statistical significance, which were not validated in the recent large prospective study by Regan et al. (2012). Unfortunately, there have been no similar large observational studies evaluating CYP2D6 metabolizing phenotypes and hot flushes as possible causes of non-adherence or non-persistency to tamoxifen treatment. It would be also interesting to have studies evaluating the correlation between endoxifen levels and the occurrence of severe hot flushes.

The metabolism of tamoxifen involves other metabolizing enzymes than CYP2D6, such as CYP3A4/5, 2C8/9, SULT1A1, UGT1A8, UGT2B7, and UGT2B15, which might also have an impact on the availability of endoxifen and other metabolites, and therefore contribute for the heterogeneity in breast cancer outcomes. However, there are few studies evaluating genetic polymorphisms on such metabolic activities and their consequences on tamoxifen efficacy. In addition, the assumption that tamoxifen efficacy is mainly dependent on the availability of endoxifen must also be considered with a certain caution. Although endoxifen has greater affinity for the estrogen receptor than tamoxifen or *N*-desmethyl-tamoxifen (Coezy et al., 1982; Jordan, 1982; Robertson et al., 1982), and higher plasma concentrations than 4-hydroxy-tamoxifen (Johnson et al., 2004; Lim et al., 2005), it has been estimated that tamoxifen and its metabolites other than endoxifen are capable of nearly saturating estrogen receptors, with 99.94% occupancy (Dowsett and Haynes, 2003). Therefore, impaired tamoxifen metabolism may not represent a full limitation for tamoxifen efficacy, other cellular mechanisms must be considered when evaluating tamoxifen resistance. Thus, Kim et al. (2011) have shown that tumor cells with low mRNA expression of the estrogen receptor (*ESR1*) present increased tamoxifen resistance when compared to cells with high-level mRNA expression, regardless of endoxifen concentrations. Finally, recent studies have suggested that tamoxifen and its metabolites may have secondary pharmacological actions, such as blockade of voltage-dependent Ca^{2+} channels (Kuo et al., 2012), vasodilation (Montenegro et al., 2011), and aromatase inhibition (Lu et al., 2012). The possible impact of such additional pharmacological mechanisms on breast cancer is not known yet.

The evaluation of studies involving chemotherapeutic protocols for breast cancer therapy indicates that many xenobiotic metabolizing enzymes other than CYP2D6 are also being considered as possible pharmacogenetic targets. These studies indicate great diversity of antineoplastic protocols and a balanced interest in both efficacy and toxicity. The number of studies, however, is still very limited, with only one or very few studies for each pharmacogenetic target, and no confirmation of positive associations. The only exception appears to involve *GSTP1 Ile105Val*, since three different studies (Zárate et al., 2007; Yao et al., 2010; Zhang et al., 2011), exploring anthracycline-based protocols, suggest higher risk of severe hematological toxicity (neutropenia or leucopenia) for patients with variant genotypes. Although there are some inconsistencies regarding the heterozygous genotype, the combined results appear to indicate that the presence of valine instead of isoleucine, which results in decreased enzymatic activity (Watson et al., 1998), would favor higher plasma concentrations of the chemotherapeutic drugs, with consequent increased toxicity. Such increased toxicity, as an apparent consequence of increased plasma concentrations, does not seem to have a direct correlation with better response profile. Thus, although Zhang et al. (2011) have reported better pathological response for patients with the *G/G (Val/Val)* genotype after neoadjuvant therapy, Huang et al. (2008) found that patients with variant genotypes had higher rates of early relapse after adjuvant treatment, and other authors found no significant effects of the *GSTP1 Ile105Val* polymorphism on the therapeutic response after adjuvant (Gor et al., 2010; Yao et al., 2010) or neoadjuvant treatment (Oliveira et al., 2010).

The above results point an important aspect of pharmacogenetic studies in clinical oncology, which is the apparent higher variability in the results involving response outcomes than in those related to drugs' toxicities. This is not surprising considering that, in addition to the subjects' polymorphisms, tumors might also present mutations, as well as epigenetic variations that could affect cellular response to chemotherapy. Secondly, an individual clinical response also involves many non-genetic factors that are difficult to control, such as lifestyle, comorbidities, drug interactions, and treatment adherence. Finally, in the case of breast cancer, clinical outcomes have a long time frame, which make them more difficult to study in a controlled design. Although it is logical that prospective observational studies combining the evaluation of pharmacogenetic data and non-genetic patients' characteristics would be ideal for modeling breast cancer clinical outcomes, their practical implementation is certainly a challenge.

Another important issue raised by the example of the results involving *GSTP1 Ile105Val* involves the translation of pharmacogenetic data into the clinical practice of oncology. Let's assume that a genotype-phenotype is established, and that it involves a pharmacokinetic target, with an apparent plasma concentration-dependent relationship. In such scenario, should one individual's dose be adjusted based on the identified genotype? For example, should patients with the homozygous variant *GSTP1* genotype receive lower doses as an attempt to reduce their risk of severe neutropenia, and therefore avoid treatment delays or interruption that could compromise the final response outcomes? Although the genotype-phenotype relationship might be recognized when evaluating the risk estimates in a population, there is no absolute correspondence between genotypes and outcomes at the individual level. In addition, there is certainly great concern about reducing the dose, and consequently increasing the risk of lower response. Thus, it seems unlikely that such prophylactic dose adjustments based on an individual genotype would even be tested in a clinical trial, and ultimately incorporated for oncological treatments. Alternatively, should patients at estimated higher risk of toxicity receive some prophylactic or supportive treatment? For example, in the case of patients with the variant *GSTP1* genotype, should stimulating factors be considered as a strategy to minimize their risk of neutropenia? There is no doubt that the idea of being able to choose a drug guided by an individual genotype, as it was initially considered for tamoxifen vs. aromatase inhibitors, is more appealing as a practical pharmacogenetic conduct in oncology than the hard and risky task of defining one individual's antineoplastic

dose. Nevertheless, the current recommendations of the American Society of Clinical Oncology (ASCO) do not include routine *CYP2D6* testing to select the endocrine therapy (Burstein et al., 2010).

CONCLUSION AND PERSPECTIVES

The application of pharmacogenetics to predict breast cancer therapeutic outcomes and to select one individual's chemotherapeutic protocol is still far from clinical routine. Most studies used the candidate-gene approach, and evaluated single or few SNPs in metabolic pathways. In addition, because of the difficulties of conducting large trials, most studies explored the most common genetic variations. Although some very interesting results have been produced, no clear practical recommendations are recognized yet. The current challenge is to simultaneously evaluate multiple genes and pathways, including rarer variants, and to consider their combined effects on drug efficacy and toxicity. Such endeavor will require large, multicentric studies, and longer and well-controlled follow-ups, in order to produce reliable information, aiming at consequent practical applications for breast cancer therapy.

These above conclusions regarding the constraints for clinical applicability of pharmacogenomic data in breast cancer management meet the consensus view on the use of qualifying biomarkers in drug safety (Agúndez et al., 2012). It appears, thus, that the limitations of pharmacogenomic studies are not a particularity of Oncology, and that the use of genetic information as biomarkers requires medical and scientific consensus and the development of adequate guidelines for clinical practice. Research consortia appear to be good opportunities to explore these goals.

AUTHORS' CONTRIBUTIONS

Juliana Simões Festa-Vasconcellos performed the bibliographic searches, retrieved and selected abstracts, draw the Figure, helped reviewing the data from tables, and helped revising the manuscript. Sheyla Maria Torres Goulart-Citrangulo reviewed the articles involving tamoxifen, and compiled the data in **Tables 1** and **2**. Marcelo Sobral Leite reviewed the articles involving any kind of chemotherapy, and compiled the data in **Tables 3–5**. Rosane Vianna-Jorge conceived, designed, and coordinated the study, reviewed, and analyzed the data, wrote, and revised the manuscript. All authors read and approved the final manuscript.

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High-resolution melting analysis of the common c.1905+1G>A mutation causing dihydropyrimidine dehydrogenase deficiency and lethal 5-fluorouracil toxicity

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Dihydropyrimidine dehydrogenase (DPD) deficiency is a pharmacogenetic syndrome associated with life-threatening toxicity following exposure to the fluoropyrimidine drugs 5-fluorouracil (5-FU) and capecitabine (CAP), widely used for the treatment of colorectal cancer and other solid tumors. The most prominent loss-of-function allele of the *DPYD* gene is the splice-site mutation c.1905+1G>A. In this study we report the case of a 73-year old woman with metastatic colorectal cancer who died from drug-induced toxicity after the first cycle of 5-FU-containing chemotherapy. Her symptoms included severe neutropenia, thrombocytopenia, mucositis and diarrhea; she died 16 days later despite intensive care measures. Post-mortem genetic analysis revealed that the patient was homozygous for the c.1905+1G>A deleterious allele and several family members consented to being screened for this mutation. This is the first report in Spain of a case of 5-FU-induced lethal toxicity associated with a genetic defect that results in the complete loss of the DPD enzyme. Although the frequency of c.1905+1G>A carriers in the white population ranges between 1 and 2%, the few data available for the Spanish population and the severity of this case prompted us to design a genotyping procedure to prevent future toxic effects of 5-FU/CAP. Since our group had previously developed a high-resolution melting (HRM) assay for the simultaneous detection of *KRAS*, *BRAF*, and/or *EGFR* somatic mutations in colorectal and lung cancer patients considered for EGFR-targeted therapies, we included the *DPYD* c.1905+1G>A mutation in the screening test that we describe herein. HRM provides a rapid, sensitive, and inexpensive method that can be easily implemented in diagnostic settings for the routine pre-therapeutic testing of a gene mutation panel with implications in the pharmacologic treatment.

Keywords: dihydropyrimidine dehydrogenase, DPD, *DPYD*, 5-fluorouracil, 5-FU, capecitabine, toxicity, HRM

INTRODUCTION

Dihydropyrimidine dehydrogenase (DPD; EC 1.3.1.2) is the initial rate-limiting step in the catabolism of endogenous pyrimidines, as well as in fluoropyrimidine drugs such as 5-fluorouracil (5-FU) and its oral prodrug capecitabine (CAP), widely used in the treatment of colorectal cancer and other solid tumors. With a predominant expression in the liver, DPD rapidly catalyzes the reduction of more than 80% of the 5-FU administered (Heggie et al., 1987); thus, a reduced enzymatic activity increases the half-life of the drug, resulting in excess accumulation and toxicity (Ezzeldin and Diasio, 2004; Lee et al., 2004; van Kuilenburg et al., 2008). DPD activity is highly variable in the population, as it depends on many factors such as gender, circadian rhythms, drug interactions and genetic polymorphisms (Mercier and Ciccolini, 2006); with an estimated 3–5% of individuals experiencing low or deficient activity (Yen and McLeod, 2007).

DPD deficiency (OMIM 274270) is an autosomal recessive disorder described in pediatric patients presenting with thymine-uraciluria and major symptoms of convulsion and psychomotor retardation, although asymptomatic cases also exist (Webster et al., 2001). A common trait in these patients is the complete deficiency of DPD due to homozygosity or compound heterozygosity of inactivating mutations in the *DPYD* gene (van Kuilenburg et al., 1999). Likewise, the complete or partial loss of DPD function in cancer patients carrying *DPYD* mutant alleles is known to cause severe life-threatening hematologic and gastrointestinal toxicity after 5-FU administration (Amstutz et al., 2011). Accounting for 50–75% of severe 5-FU-related toxicities (Ciccolini et al.,

2010), DPD deficiency has been defined as a pharmacogenetic syndrome and is on the FDA's list of approved biomarkers (http://www.fda.gov/Drugs/ScienceResearch/ResearchAreas/Pharmacogenetics/ucm083378.htm). Lethal toxicities have been reported in DPD deficient patients treated with either 5-FU (Milano et al., 1999; Raida et al., 2001; van Kuilenburg et al., 2001, 2003; Ezzeldin et al., 2003; Magné et al., 2007; Morel et al., 2007; Saif et al., 2007a; Gross et al., 2008) or CAP (Ciccolini et al., 2006; Largillier et al., 2006; Saif et al., 2007b; Deenen et al., 2011). Indeed, combined pharmacogenetic syndromes with a fatal outcome have been associated with concomitant mutations in the *DPYD* and *UTG1A1* genes (Steiner et al., 2005; Mounier-Boutoille et al., 2010).

DPYD is a 843-kb, single copy gene located on chromosome 1p22 that comprises 23 exons and appears to be highly polymorphic, with more than 50 variants reported (Takai et al., 1994; Yokota et al., 1994; Wei et al., 1998). However, only three individual variants have been consistently associated with 5-FU toxicity in case-control studies (Amstutz et al., 2011): the two non-synonymous substitutions c.1679T>G (I560S) and c.2846A>T (D949V), which result in low enzyme activity but are very rare; and c.1905+1G>A (formerly IVS14+1G>A or *DPYD**2A), a point mutation in the splice donor site that results in a 165-bp deletion in the mRNA, due to skipping of exon 14, and lack of functional DPD expression (Meinsma et al., 1995; Vreken et al., 1996; Wei et al., 1996).

The c.1905+1G>A mutation has been the most frequently studied in the context of 5-FU toxicity as it proved to be the most prevalent among patients with complete DPD deficiency (52%) (van Kuilenburg et al., 1999) and was detected in 24% of cancer patients suffering grade 4 leucopenia, with the majority of them being heterozygous (Raida et al., 2001). Moreover, large general population screenings for the c.1905+1G>A mutation showed 1–2% of heterozygous carriers (Raida et al., 2001; van Kuilenburg et al., 2001), rendering this allele attractive for routine mutation screening. However, subsequent studies indicated a north-south gradient in Europe, so the proportion of 5-FU toxicity cases explained by the c.1905+1G>A variant varied greatly due to population frequency differences and sampling effects (Amstutz et al., 2011).

In Spain, the only studies addressing the prevalence of this mutation were conducted in colorectal cancer patients treated with 5-FU (Paré et al., 2010) and CAP (Salgado et al., 2007), showing heterozygote frequencies of 0% (0/234) and 1.7% (1/58), respectively. Some cases of 5-FU/CAP-induced severe toxicity in DPD-deficient patients have been reported in our country, mainly via communications at congresses or in pharmacy journals (Gironés Sarrió et al., 2005; López Sobella et al., 2008; Rubio Salvador et al., 2012). In the most recent report, and the only study to perform a genetic analysis, one toxic death was attributed to heterozygosity of an unspecified mutant *DPYD* allele (Rubio Salvador et al., 2012). It is also worth mentioning the case of a Spanish woman, reported in a French study, who died from 5-FU toxicity due to heterozygosity for the c.464T>A mutation (Morel et al., 2007).

The relatively high frequency of the c.1905+1G>A variant, with 1.3% of heterozygote carriers according to 1000 Genomes data (rs3918290 polymorphism in 1000GENOMES:EUR population at http://browser.1000genomes.org), together with the widespread use of 5-FU/CAP and the severity of the associated toxicities, prompted us to develop a fast and reliable method to identify high-risk individuals prior to undergoing pyrimidine-based chemotherapy. Since we had previously developed a high-resolution melting (HRM) assay to detect somatic *KRAS*, *BRAF*, and *EGFR* mutations in tumor samples from patients considered for EGFR-targeted therapies (Borràs et al., 2011), we included the detection of the *DPYD* c.1905+1G>A mutation in this screening test.

The HRM method is based on a PCR amplification using a saturating intercalating dye, followed by DNA strand separation in a temperature gradient, during which the fluorescence is registered with a high resolution. Thus, the melting curves obtained for homozygous and heterozygous samples differ significantly. Likewise, for somatic mutations, the presence of mutated alleles results in abnormal melting profiles.

Herein, we report the first case in Spain of a patient with 5-FU-induced lethal toxicity due to homozygosity for the c.1905+1G>A mutation, and describe a HRM assay for the routine testing of cancer patients prior to 5-FU/CAP therapy.

MATERIALS AND METHODS

DNA SAMPLES

The study was conducted in accordance with the Declaration of Helsinki and was approved by the internal Clinical Research Ethics Committee (CEIC) of the Hospital de Terrassa (Spain). Informed consent was obtained from all the participants and has been archived by the authors. Genomic DNA samples were obtained from a patient who died from 5-FU-induced toxicity and her close relatives. In the case of the index patient, the DNA was extracted from stored frozen blood samples and the consent for genotyping was provided by the relatives after the patient's death. DNA isolation from peripheral blood lymphocytes was performed automatically by the MagNaPure Compact Instrument (Roche Applied Science, Barcelona, Spain) according to the manufacturer's protocol.

PCR AMPLIFICATION AND SEQUENCING

A newly designed forward primer (5′-TATGGCCCTGGACAAAGCTC-3′) was combined with an existing reverse primer for *DPYD* exon 14 (5′-CAGCAAAGCAACTGGCAGATT-3′) (Kumar et al., 2007) to generate a 239-bp amplicon. Primer specificity and melting temperatures were analyzed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast). PCR amplification was conducted in a 50 µl final volume containing: 1x PCR buffer, 1.5 mM $MgCl_2$, 500 nM primers, 2 µl genomic DNA (32 ng to 1.7 µg), 200 µM dNTPs, 2.5 U of BioTaq polymerase (Bioline, Ecogen, Barcelona, Spain), and PCR grade water. The program conditions were: 5 min at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 56.6°C and 1 min at 72°C. PCR products were analyzed by 1.5% agarose gel electrophoresis, column purified with the High Pure PCR Product Purification Kit (Roche) and submitted to StabVida (Oeiras, Portugal) for direct sequencing on a 3730XL ABI DNA sequencer (Applied

Biosystems, Foster City, CA) using the Big Dye terminator V1.1 DNA sequencing kit.

DESIGN OF HRM PRIMERS

First, the primers used for PCR amplification were tested to ensure good genotype discrimination in a LightCycler® 480 platform (Roche) using the previously described HRM assay (Borràs et al., 2011). After optimization of the touchdown PCR annealing temperature range, the DNA samples were successfully amplified and heterozygous carriers of the c.1905+1G>A mutation could be easily identified. However, the melting profiles of mutant and wild-type homozygous samples were almost identical. Alternative primers were designed in order to obtain shorter amplicons, in which a nucleotide change would have a greater effect on the curve shape, with a single melting domain and a low level of secondary structure, according to Stitchprofiles.uio.no (http://stitchprofiles.uio.no) and DINAMelt Web Server (http://mfold.rna.albany.edu/?q=DINAMelt) predictions, but the initial results could not be improved. We therefore decided to use the above mentioned primers but to spike all samples with a known amount of wild-type DNA from a control individual to ensure differentiation of homozygous variants, as suggested in LightCycler® 480 Technical Note No. 1 (2008).

HRM ASSAY

Samples were diluted at the same concentration and spiked with 0.5 volumes of wild-type DNA, so the mutant:wild-type allele ratio (2:1 in mutant homozygous, 1:2 in heterozygous, and 0:3 in wild-type samples) maximized the ability to discriminate genotypes. Test samples were assayed in triplicate using the LightCycler® 480 system, and negative (non-template) and wild-type controls were included in each experiment. Each 10-µl reaction contained about 30 ng DNA diluted in 1.8 µl, 1x HRM mix (Roche), 3 mM MgCl2, and 200 nM HPLC-purified primers. Touchdown PCR and melting conditions were: 95°C for 10 min; 45 cycles of 95°C for 10 s, 60-53°C (1°C/cycle) for 15 s and 72°C for 10 s; 95°C for 1 min; 40°C for 1 min; a melt of 72-92°C (0.01°C/s, 45 acquisitions/°C); and 40°C for 10 s. Normalized and temperature-adjusted melting curves of test samples and wild-type controls were visualized with accompanying Gene Scanning software. Since Standards (In Run) analysis mode (grouping method) was selected, the software assigned each sample to a group based on melting standard samples included in the run (wild-type replicates). Amplicons displaying abnormal melting patterns as compared to wild-type samples could be recovered from the plate, column purified and subjected to direct sequencing as described above.

RESULTS

CLINICAL EVALUATION

The index patient was female, born in 1937, and had a history of allergic rhinitis, adenoid surgery, 2 vaginal childbirths, and hysterectomy due to uterine prolapse. In 2007, she consulted for constipation with some degree of urinary and fecal incontinence and was diagnosed with rectocele and rectal prolapse. One year later, a large and sessile serrated adenoma of the rectum was detected and the patient underwent transanal endoscopic microsurgery. The pathological examination of the surgical specimen revealed an infiltrating adenocarcinoma (T1) and colonoscopic follow-up was scheduled. After 17 months, she was found to have neoplastic recurrence (T3p N1) with multiple liver metastases and was considered for palliative chemotherapy with mFOLFOX6. At that time, she was slightly overweight (BMI of 29.2 kg/m^2: weight 64 kg, height 148 cm) without other cardiovascular risk factors, such as smoking or hypertension and showed normal liver and renal function. The first cycle, administered on January 12, 2010, involved a 2-h infusion (i.v.) of oxaliplatin (85 mg/m^2) and leucovorin (200 mg/m^2), followed by administration of a 5-FU bolus (400 mg/m^2 i.v.) and 48-h continuous infusion of 5-FU (2400 mg/m^2 i.v.) using a portable pump.

On day 6 of this treatment, the patient presented to the emergency department after 3–4 days of vomiting and diarrhea, intolerance to liquids and solids, and general malaise without fever or abdominal pain, despite having taken the prescribed ondansetron. After receiving symptomatic medication consisting of pantoprazole, metoclopramide, paracetamol, and serum therapy, she remained hemodynamically stable and was admitted to the oncology service diagnosed with grade 3 mucositis. Despite a moderate initial improvement, the oral mucositis persisted and worsened to grade 4. Over the next few days, fluconazole treatment and morphine (s.c.) analgesia were given, and total parenteral nutrition was instituted. Prophylactic filgastrim and ciprofloxacin were given due to the severity of the mucositis and the presence of afebrile grade 3 neutropenia (0.58×10^9/l), though the development of grade 4 thrombocytopenia required platelet transfusion. Of note, the diarrhea persisted during the entire admission period. After the appearance of fever and grade 4 neutropenia (0.02×10^9/l), the antibiotic coverage was extended to piperacillin/tazobactam, but the patient developed septic shock and vasoactive drugs had to be perfused. Despite all the measures taken, the patient progressed poorly and died on January 28, 16 days after the first 5-FU dose.

SEQUENCE ANALYSIS OF THE *DPYD* GENE

Direct sequencing of the 239-bp amplicon containing the *DPYD* exon 14 coding and flanking intron region revealed that the index patient was homozygous for the c.1905+1G>A mutation (II.2 in **Figures 1**, **2**). Although no functional test could be performed due to unavailability of fresh blood samples, DPD activity was assumed to be completely absent according to a prior study describing the fatal outcome of a c.1905+1G>A homozygous patient with no significant residual activity of DPD in peripheral blood mononuclear cells and fibroblasts (van Kuilenburg et al., 2001).

The family study by PCR amplification followed by direct sequencing confirmed that both sons of the index patient were obligate heterozygotes (III.2 and III.3 in **Figures 1**, **2**) and the only sister analyzed was wild type (II.5 in **Figures 1**, **2**). Of note, none of the mutation carriers of this family presented symptoms of familial pyridinemia and DPD deficiency was not discovered until the administration of 5-FU. To date, none of the other siblings of the index patient are available for testing. Nevertheless, as the members studied represent the three genotypes, their DNA samples were used to evaluate the validity of the HRM assay.

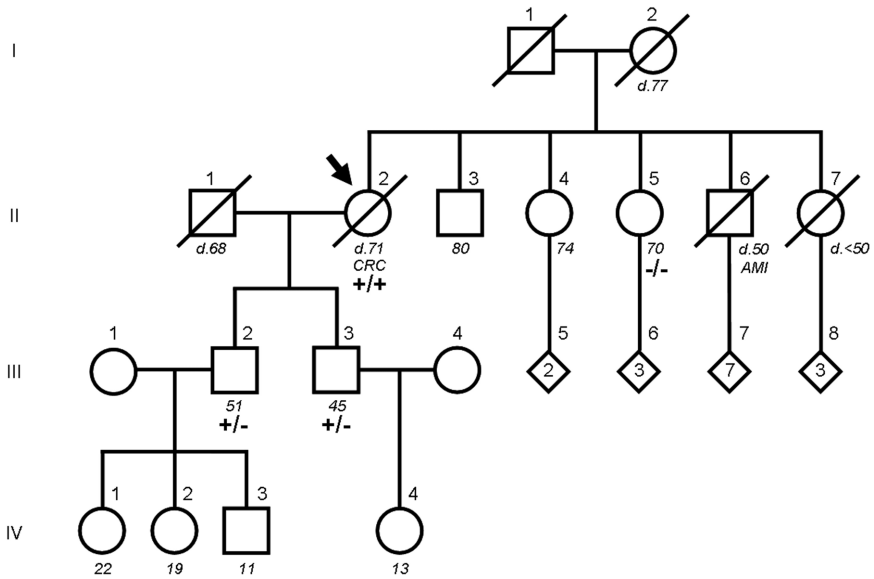

FIGURE 1 | Pedigree of the index patient and family members carrying the *DPYD* c.1905+1G>A mutation. The index patient is indicated by an arrow. For family members who were alive, age at the time of the study is shown below in italics. For deceased members, designated by a diagonal line through the symbol, age and cause of death are annotated (CRC, colorectal cancer; AMI, acute myocardial infarction). Numbers inside a diamond are children of unspecified sex. *DPYD* genotypes are wild type (−/−), heterozygous (+/−) and homozygous (+/+) carriers of the mutation.

HRM ANALYSIS OF THE *DPYD* c.1905+1G>A MUTATION

The HRM assay developed was tested using triplicates of the above mentioned samples, adjusted to the same concentration and spiked with wild-type DNA. The homozygous mutant sample showed the lowest DNA concentration (16 ng/μl) as a result of the severe neutropenia, so the other samples were diluted accordingly. Homozygous and heterozygous carriers of the *DPYD* c.1905+1G>A mutation were successfully identified by HRM analysis, either using the adjusted melting curves (**Figure 3A**) or the differential plot (**Figure 3B**). The adjusted melting curves show a single melting domain, consistent with Stitchprofiles.uio.no predictions, but, since the melting curves of mutation carriers are a composite of both heteroduplex and homoduplex components, they dissociate more readily and shift left to a lower temperature. The difference plot calculation assigned the samples in two groups using sensitivity values from 0.2 (higher values denote high stringency and produce more groups), so the mutant samples (either homozygous or heterozygous) were distinguished from the wild-type ones (melting standards). For good HRM analysis, amplification curves were checked to produce a crossing point <30 and to reach a similar plateau height and, if replicates showed different melting patterns, the assay was repeated for that sample. Finally, the touchdown PCR and melting conditions of this *DPYD* HRM assay were suitable for analysis of *KRAS*, *BRAF*, and *EGFR* somatic hotspot mutations in tumor samples (not shown), thus enabling simultaneous analysis of relevant mutations for targeted cancer therapy.

DISCUSSION

To date, screening for the presence of DPD deficiency prior to 5-FU chemotherapy is not yet established in the daily care of cancer patients, despite the numerous studies worldwide reporting life-threatening toxicity cases. Therefore, while not specified in professional guidelines, there is general consensus that given the large number of patients treated with 5-FU and the human and economic cost of grade 3–4 toxic side effects, DPD deficiency should be tested for prior to initiation of therapy.

Several methods have been developed to assess DPD activity, such as direct assays in peripheral blood mononuclear cells, indirect evaluations by monitoring DPD substrates or metabolites (e.g., uracil/dihydrouracil plasma ratio, uracil breath test), administration of a 5-FU test dose, and measurement of DPD expression through mRNA or protein levels (Mercier and Ciccolini, 2006; Eidens et al., 2009). However, functional tests usually require special equipment and are too costly and laborious for routine implementation in clinical practice. In contrast, genotyping methods are available in most laboratories but offer an incomplete pharmacogenetic diagnosis because of the limited number of genetic variants tested and the lack of a straightforward genotype-phenotype correlation. Combining the two approaches may provide the most complete assessment of toxicity risk, although no guidelines currently exist specifying a particular testing method.

Regarding genotyping, comprehensive genetic screenings including the variation in noncoding regions result in a higher relative importance of *DPYD* variants to explain 5-FU toxicities. Current data suggest that combining information from multiple variants in this gene can identify over 20% of patients experiencing severe 5-FU toxicity (Amstutz et al., 2011). On the other hand, heterozygous carriers of deleterious variants can show normal DPD activity and only about 50% of carriers develop severe 5-FU toxicity, which suggests an allelic regulation through

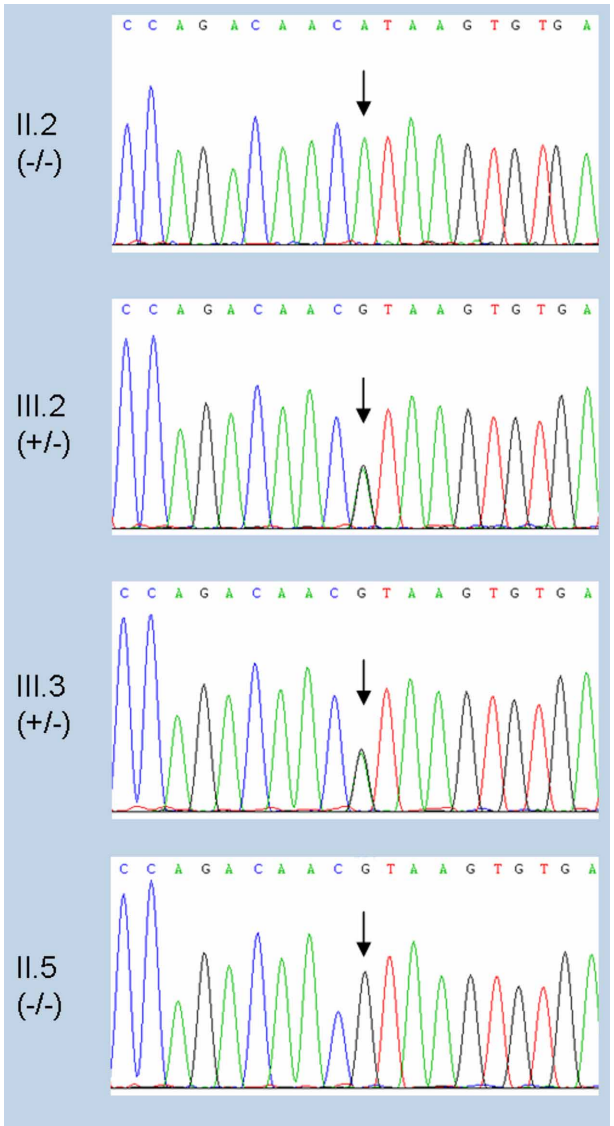

FIGURE 2 | Sequence chromatograms of the index patient and family members analyzed for the *DPYD* c.1905+1G>A mutation. Trace sequences of *DPYD* exon 14 coding and flanking intron region including position c.1905 (indicated with an arrow). From top to bottom: c.1905+1G>A homozygous (index patient, II.2), heterozygous (sons, III.2 and III.3), and wild type (sister, II.5).

an increased expression of the wild-type allele or compensation by another variant that confers above-average DPD activity (Amstutz et al., 2011). Whatever the case, extensive analyses of *DPYD* could address this issue. Moreover, genotype testing could be expanded to genetic variants in genes that may play a role in 5-FU breakdown, such as thymidilate synthase (*TYMS*) and methylenetetrahydrofolate reductase (*MTHFR*), which could modulate the impact of *DPYD* risk alleles on the overall risk of toxicity.

High-throughput sequencing technologies promise to substantially simplify this task in the future, as full sequencing of *DPYD* and other genes of potential importance for 5-FU toxicity will be achieved at reasonable costs. Further comprehensive genetic screenings in combination with phenotypic characterization of *DPYD* genotypes could help to identify the factors underlying the occurrence of normal DPD activity in carriers of risk alleles and to discern the relative contribution of individual *DPYD* variants. Meanwhile, methodologies based on genetic testing for clinically relevant variants offer the simplest way to identify patients at the highest risk of potentially life-threatening adverse drug events.

In this context, we describe an approach to detect the c.1905+1G>A mutation of *DPYD* based on HRM technology, which shows great potential for scanning germline and somatic mutations (Taylor, 2009). A HRM assay previously designed by our group (Borràs et al., 2011) had already successfully identified hotspot mutations of *KRAS*, *BRAF*, and *EGFR* with a high analytical sensitivity. Moreover, the use of a touchdown PCR and a wide melting interval allowed the simultaneous analysis of all amplicons in a single plate, saving time and cost. Although this assay was developed for FFPE tumor sections, it is also suitable for blood samples, and both DNA sources can be combined in one experiment to detect somatic and germline mutations. As DNA isolation from blood samples usually gives higher yields and better quality, lower amounts of template could be used, but the amount of starting DNA has to be standardized as much as possible to minimize reaction-to-reaction variability.

Since this test has to be validated before it is used in routine, we plan to conduct a pilot study in our institution to genotype the *DPYD* c.1905+1G>A mutation in cancer patients and to assess its importance in 5-FU toxicity. Considering that 5-FU is widely prescribed for the treatment of solid carcinomas, like those of the gastrointestinal tract, pretreatment *DPYD* genotyping could be performed together with the detection of *KRAS* and *BRAF* somatic mutations in patients with colorectal cancer to predict the response to anti-*EGFR* monoclonal antibodies, recommended by regulatory authorities (van Krieken et al., 2008; Allegra et al., 2009; NCCN Colon Cancer Guidelines, 2011).

Just as better mutation detection methods are required for stratification of patients to receive molecularly targeted treatment, tests are needed for the cost-effective screening of genes associated with drug metabolism and response. Understanding pharmacogenetic associations is especially important in cancer chemotherapy, as many chemotherapeutic agents, such as 5-FU, have a very narrow therapeutic index. Among the various techniques available to detect DPD deficiency at genotype level, including many marketed tests, HRM analysis provides a rapid, sensitive, and inexpensive method that can be easily implemented in a diagnostic setting.

HRM has been applied to mutation scanning of the cytidine deaminase gene (*CDA*), involved in the catabolism of nucleoside analogs, genetic variations of which might explain the therapeutic and toxic response to gemcitabine (Evrard et al., 2007a,b). Specifically, the LightCycler® 480 platform was used to investigate variations in long PCR fragments and to genotype SNPs or mutations in short amplicons, and HRM efficiently identified single base heterozygous changes in PCR products up to 622

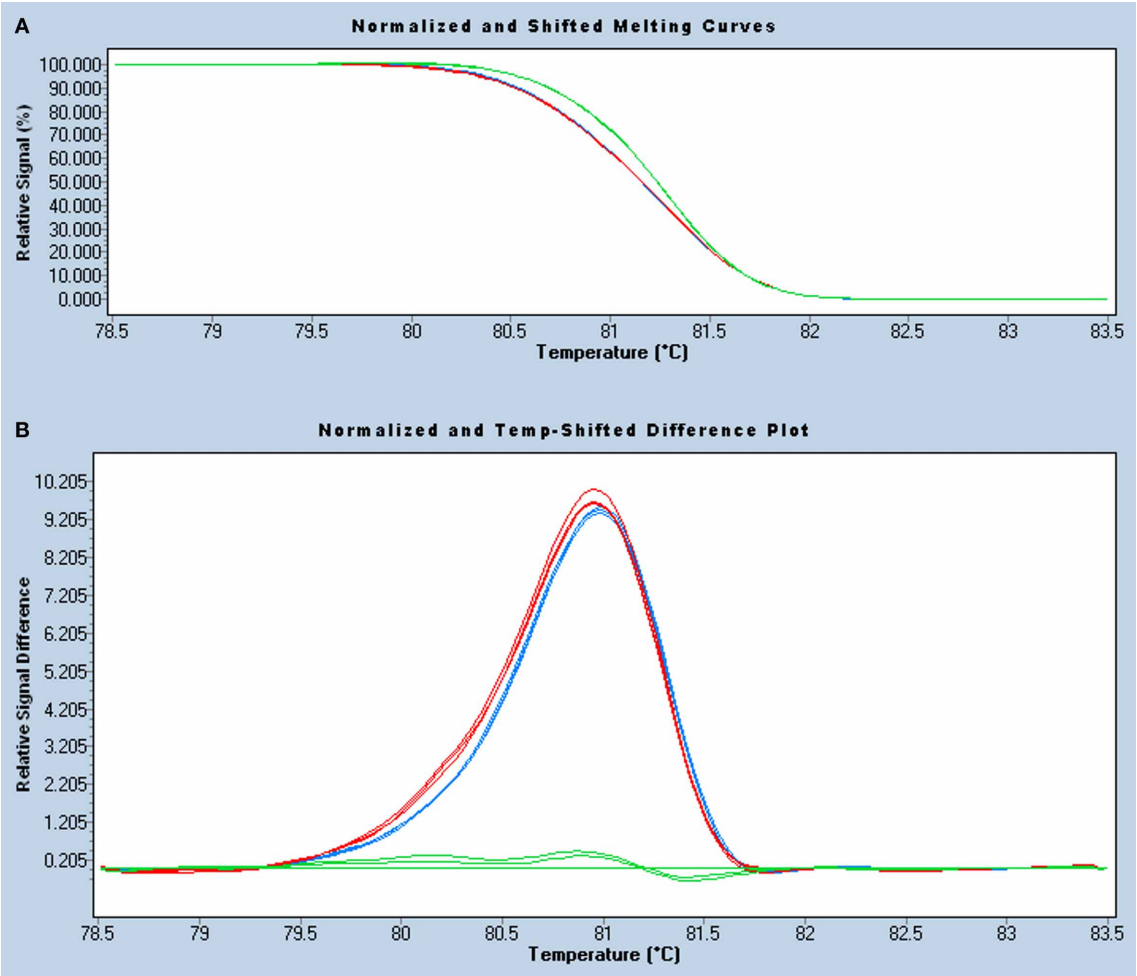

FIGURE 3 | HRM analysis of the *DPYD* c.1905+1G>A mutation in a 239-bp amplicon. Normalized and temperature-shift melting curves **(A)** and differential plot **(B)** of mutant homozygous (II.2, blue), heterozygous (III.2, red), and wild-type (II.5, green) samples, assayed in triplicate. As all samples were spiked with wild-type DNA, homozygous and heterozygous mutants show similar left-shifted melting curves and can be easily identified, especially in the differential plot.

bp. However, differentiation of homozygous variants depended on amplicon length and GC content, so the use of modified DNA is suggested. Spikes of wild-type DNA added to all samples and comparison to unspiked reactions has been shown by others to provide a valuable approach to addressing this point. Furthermore, the authors compare three methods for routine detection of c.1905+1G>A mutation in the *DPYD* and consider HRM to be a powerful tool for genotyping known SNPs or mutations in routine clinical practice (Evrard et al., 2007a,b). However, just like any screening test, HRM-identified positive samples have to be subsequently sequenced to identify the specific nucleotide alteration, which may be present in one or both alleles, and to avoid misdiagnosis due to an abnormal curve generated by a neutral variant.

An important limitation of our study is that screening for the c.1905+1G>A mutation alone may have limited effectiveness in identifying patients at risk of lethal 5-FU toxicity and could result in false-negative results for patients with rare *DPYD* variants or who might experience severe toxicity as a result of other causes. Prospective analysis of the c.1905+1G>A mutation in large numbers of toxicity cases and controls from our population is needed for a reliable estimation of the importance of this variant for the prediction of 5-FU toxicity in cancer patients and to determine the cost-effectiveness of a genetic strategy for DPD screening.

In this study, we describe the case of a woman with an unremarkable medical history before the diagnosis of colorectal cancer followed by surgery and 5-FU-based chemotherapy, with subsequent unexpected gastrointestinal and hematologic toxicity leading to death. As in other reports, most patients have no symptoms of DPD deficiency and are unaware of their condition prior to 5-FU treatment and the subsequent development of adverse side effects. In contrast, both sons of the index patient are known to be heterozygous carriers of the c.1905+1G>A mutation but the clinical implications of partial DPD deficiency are unpredictable since not everybody who carries the risk allele may actually suffer severe 5-FU side effects. In these cases, determination of the

5-FU pharmacokinetics could aid individualized therapy since the application of dose-tailored strategies based on pharmacokinetic monitoring improved the therapeutic index of 5-FU treatment, and it could be used in conjunction with genotyping to reduce toxicity and achieve maximum benefit (Saif et al., 2009; Yang et al., 2011).

As a standard practice, many authors have suggested that patients with decreased DPD activity should be monitored closely, considered for a reduced 5-FU dose, or chosen for an alternative therapy (Raida et al., 2001; Lazar and Jetter, 2008; Ciccolini et al., 2010). More recently, the Pharmacogenomics Working Group of the Royal Dutch Association for the Advancement of Pharmacy established clinical guidelines for 5-FU therapy according to *DPYD* genotype, available at the Pharmacogenomics Knowledge Base (www.pharmgkb.org). For patients carrying two inactive or decreased activity alleles, they recommend selecting an alternative drug, whereas for patients with one active and one inactive or decreased activity allele, a 50% dose reduction or selection of another drug is recommended.

With the aim of contributing toward the implementation of a pre-screening program for DPD deficiency and helping to improve the safety of 5-FU-based chemotherapy, we have developed a HRM assay for the screening of *DPYD* c.1905+1G>A mutation. This method provides a simple, robust, and inexpensive solution that can be easily implemented in diagnostic settings for pre-therapy testing. Therefore, after proper validation for routine use, we plan to include the present test in a panel of other tests for somatic cancer mutations with implication on the selection of therapy.

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Polymorphisms of phase I and phase II enzymes and breast cancer risk

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Breast cancer is a complex disease which is provoked by a multitude of exogenous and endogenous factors including genetic variations. Recent genome-wide association studies identified a set of more than 18 novel low penetrant susceptibility loci, however, a limitation of this powerful approach is the hampered analysis of polymorphisms in DNA sequences with a high degree of similarity to other genes or pseudo genes. Since this common feature affects the majority of the highly polymorphic genes encoding phase I and II enzymes the retrieval of specific genotype data requires adapted amplification methods. With regard to breast cancer these genes are of certain interest due to their involvement in the metabolism of carcinogens like exogenous genotoxic compounds or steroid hormones. The present review summarizes the observed effects of functional genetic variants of phase I and II enzymes in well designed case control studies to shed light on their contribution to breast cancer risk.

Keywords: breast cancer risk, tumor histo-pathology, phase I and II metabolism, polymorphisms, sequence homology

INTRODUCTION

The implementation of cost effective high-throughput genotyping methods enables the determination of genotypes at large scale and fast pace. These improvements are prerequisite of the in depth investigation of the polygenetic basis of complex diseases. Prominent examples are genome-wide association studies which led to the identification of novel breast cancer risk factors such as polymorphisms in *FGFR2*, *CCND1*, *TOX3*, *MAP3K1*, *LSP1*, *CDKN2A*, and *2B* (Easton et al., 2007; Lambrechts et al., 2012). However, a shortcoming of this comprehensive approach is the exclusion of the majority of genes encoding phase I and II enzymes, because their special genomic architecture hampers the assessment of accurate genotype data. It is necessary to overcome this limitation due to the fact that functional genetic variations in these genes are known to alter expression, activity, and stability of the encoded enzymes causing defective inactivation and excretion of hormones as well as environmental toxicants (Thompson and Ambrosone, 2000; Reszka et al., 2006). Thus, it is of high relevance to understand the potential impact of these polymorphisms in pathogenic processes such as carcinogenesis. In addition, these phase I and II genes play a pivotal role in activation and metabolism of drugs with the potential to trigger therapy response as well as occurrence of adverse side effects (Meyer et al., 2012). With respect to breast cancer pharmacogenetic investigations revealed the impact of a genetical determined poor metabolizer phenotype of the phase I enzyme cytochrome P450 (CYP) 2D6 and tamoxifen treatment outcome (Schroth et al., 2009). This finding has been a matter of debate due to reports on conflicting results that seem to be based on inaccurate genotype data (Brauch et al., 2012). Amongst others this finding underlines the need of specific genotyping methodologies for genes encoding metabolic enzymes. This review will focus on studies investigating the role of genetic variants of phase I and II enzymes in breast cancer risk that used validated genotyping methods.

BREAST CANCER RISK

Breast cancer is a multifactorial disease and it is known that the carcinogenic process is affected by several endogenous as well as exogenous factors (Rebbeck et al., 1997). In this respect, steroid hormones play a pivotal role (Key et al., 2002b). Epidemiological studies indicated an increased breast cancer risk in women with prolonged exposure to sex hormones, e.g., early menarche and late menopause (Henderson and Feigelson, 2000; Clemons and Goss, 2001). Moreover, observational studies revealed the risk effect of exogenous hormones such as postmenopausal hormone replacement therapy (HRT; Rossouw et al., 2002; Beral and Million Women Study Collaborators, 2003; Pesch et al., 2005; Flesch-Janys et al., 2008) and oral contraceptives (Collaborative Group on Hormonal Factors in Breast Cancer, 1996; Kahlenborn et al., 2006). The strong correlation between circulation steroid hormones and breast cancer risk is supported by an observation of a two-fold increased risk for women with elevated sex hormone levels (Key et al., 2002a; Eliassen et al., 2006). A functional explanation of these findings comes from *in vitro* and *in vivo* studies that indicated initiation, promotion, and progression of breast tumorigenesis by estrogens and their metabolites (Nandi et al., 1995; Yue et al., 2003; Turan et al., 2004). This effect has been attributed to estrogen-induced gene expression of factors involved in cell growth and division (Liu and Lin, 2004) as well as genotoxic action of metabolic compounds such as 4-hydroxy catechol estrogens and estrogen-3,4-quinones (Yager and Davidson, 2006). Moreover, progesterone adds to hormone-induced carcinogenesis by promotion of estrogen synthesis, estrogen receptor expression, and cell proliferation (Poutanen et al., 1995; Shyamala et al., 2002;

Moore et al., 2006; Pawlak and Wiebe, 2007). Beyond hormonal factors environmental carcinogens, e.g., tobacco smoke, or genetic factors, e.g., mutations and polymorphisms contribute to breast cancer susceptibility. A genetic basis of breast cancer has been suggested by family studies indicating a two-fold increased risk in the first-degree relatives of women with the disease (Collaborative Group on Hormonal Factors in Breast Cancer, 2001). In the 1990s, the two major breast cancer susceptibility genes *BRCA1* and *BRCA2* were identified (Miki et al., 1994; Wooster et al., 1995) revealing that harmful mutations in these genes confer to a cumulative disease risk by age 70 years of 65 and 45%, respectively (Antoniou et al., 2003). In the following years further genetic factors with different penetrance and frequency have been described. As of today less than 5% of familial breast cancer were attributed to high penetrance breast cancer genes *BRCA1*, *BRCA2*, *PTEN*, *MSH2*, *STK11*, *CDH1*, and *TP53* (Wooster and Weber, 2003; Malone et al., 2006; Walsh et al., 2006) and rare genetic variants at *ATM*, *CHEK2*, *BRIP*, *NBN*, *RAD50*, or *PALB2* that jointly confer an approximately two-fold increased risk (Meijers-Heijboer et al., 2002; The CHEK2 Breast Cancer Case-Control Consortium, 2004; Rahman et al., 2007). Recent genome-wide association studies revealed strong evidence for more than 18 common breast cancer susceptibility alleles including *FGFR2*, *CCND1*, *TNRC9*, *MAP3K1*, and *LSP1* (Cox et al., 2007; Easton et al., 2007; Lambrechts et al., 2012). Most of these genes are related to DNA repair, cell cycle control, apoptosis, cell growth, and division, representing the most important pathways for the protection of cells against carcinogenic processes. However, the lack of observed risk associations with phase I and II enzymes is potentially based on their exclusion from genome-wide association studies due to hampered assay design or poor quality data which is reflected by the low coverage of these genes in current genotyping arrays (Gamazon et al., 2012).

THE ROLE OF PHASE I AND II ENZYMES IN CARCINOGENESIS

Phase I and II enzymes are of particular interest with respect to breast cancer due to their involvement in the metabolism of steroid hormones, chemical carcinogens, and other environmental toxicants (Thompson and Ambrosone, 2000; Reszka et al., 2006). In phase I reaction substrates usually undergo reduction, oxidation, or hydroxylation yielding more polar metabolites; the predominant mediators of this phase are cytochrome P450 (CYP) enzymes (Guengerich, 1999). In most cases phase I metabolism is followed by phase II conjugation reactions. During phase II exogenous or endogenous compounds or their phase I metabolites are conjugated to a more polar molecule, a process that usually produces inactive and water soluble compounds which can be easily excreted by urine or bile (Smith et al., 1994; Turesky, 2004). Conjugating enzymes include glutathione-S-transferases (GSTs), sulfotransferases (SULTs), uridine diphosphate-glucuronosyltransferases (UGTs), *N*-acetyltransferases (NATs), and Methyltransferases. The combined phase I and II metabolism is mainly a detoxification and elimination process, however, both phases bear the risk of formation of toxic and highly reactive compounds which can induce or promote serious health problems such as cancer (Smith et al., 1994; Windmill et al., 1997). Thus, altered activity of metabolic enzyme holds the potential to increase the exposure to carcinogenic compounds and consequently the risk of tumor formation (Brockstedt et al., 2002).

CHALLENGES OF GENOTYPING

The majority of phase I and II enzymes are encoded by related genes which constitute gene families and subfamilies depending on their degree of sequence similarities. This particular genomic architecture hampers specific genotyping due to the potential co-amplification of homolog gene sequences. Therefore, the establishment of accurate analysis methods requires primer selection by eye inspection, adapted amplification protocols, and verification of genotype calls by an independent method (Justenhoven et al., 2010). An example for the particular need of an appropriate genotyping procedure is the analysis of the *SULT1A1* 638 G > A (rs9282861) polymorphism. The human *SULT1A* subfamily comprises three genes *SULT1A1*, *SULT1A2*, and *SULT1A3* which are located in close proximity on the short arm of chromosome 16 and share sequence similarities of more than 90% (Hempel et al., 2005). Due to these remarkable homologies the selection of applicable primers which enable specific amplification of the *SULT1A1* 638 G > A region is difficult (**Figure 1**). Usually automatic assay design tools generate inappropriate primers for such sequences which lead to simultaneous amplification of all members of a gene subfamily resulting in incorrect genotype calls due to abundance of the referent allele (**Figure 2A**). Valid assays include the identification of primer binding sites in unique DNA regions of the respective gene and adapted annealing temperatures, only such highly selective amplification conditions assure correct genotype calls (**Figure 2B**). Other gene families and subfamilies with a similar degree of sequence homologies are known for *CYP3A*, *CYP2C*, *GST*s, as well as *NAT*s and *UGT*s (Salinas and Wong, 1999; Gellner et al., 2001; Tukey and Strassburg, 2001; Nelson et al., 2004; Sim et al., 2008). So far individual assays for some of these polymorphisms have been established by researchers, e.g., for *CYP3A* (Justenhoven et al., 2010; The MARIE-GENICA Consortium on Genetic Susceptibility for Menopausal Hormone Therapy Related Breast Cancer Risk, 2010), *CYP2D6* (Schaeffeler et al., 2003; Morike et al., 2008), *CYP2C19* (Justenhoven et al., 2012), *GST*, *UGT*, and *SULT1A* (The MARIE-GENICA Consortium on Genetic Susceptibility for Menopausal Hormone Therapy Related Breast Cancer Risk, 2010) as well as companies (e.g., Applied Biosystems and Third Wave Technologies)[1,2]. Moreover, particular panels and arrays for the genetic analysis of metabolic enzymes and transporters have been developed within recent years: the AmpliChip®CYP P450 Test[3], the DMET Plus Panel DNA Chip[4], VeraCode ADME Core Panel[5], and the iPLEX ADME PGx Panel[6]. These tools were initially launched to support pharmacogenomic testing in clinical research and diagnostics, however, their coverage of relevant genes is still incomplete but they provide a convenient basis for a variety of investigations dealing with diverse health issues.

[1]http://www.appliedbiosystems.com
[2]http://www.twt.com
[3]http://www.roche.com
[4]http://www.affimetrix.com
[5]http://www.illumina.com
[6]http://www.sequenom.com

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SULT1A1 TTTACTTTTCCTGAATCAGTAATCCGAGCCTCCACTGAGGGGCCCTCTGCTGCTCAGAAC
SULT1A2 TTTA-TTTTCCTGAATCAGCAATCCAAGCCTCCACTGAGGAGCCCTCTGCTGCTCAGAAC
SULT1A3 TTTACTTTTCCTGAATCAGTAATCCGAGCCTCCACTGAGGGGCCCTCTGCTGCTCAGAAC

SULT1A1 CCGAAAAGGGAGATTCAAAAGATCCTGGAGTTTGTGGGGCRCTCCCTGCCAGAGGAGACR
SULT1A2 CCCAAAAGGGAGATTCAAAAGATCCTGGAGTTTGTGGGGCGCTCCCTGCCAGAGGAGACT
SULT1A3 CCGAAAAGGGAGATTCAAAAGATCCTGGAGTTTGTGGGGCGCTCCCTGCCAGAGGAGACA

SULT1A1 GTGGACTTCYTGGTTCAGCACACGTCGTTCAAGGAGATGAAGAAGAACCCTATGACCAAC
SULT1A2 GTGGACCTCATGGTTGAGCACACGTCGTTCAAGGAGATGAAGAAGAACCCTATGACCAAC
SULT1A3 GTGGACTTCYTGGTTCAGCACACGTCGTTCAAGGAGATGAAGAAGAACCCTATGACCAAC

SULT1A1 TACACCACCGTCCCCCAGGAGCTCATGGACCACAGCATCT
SULT1A2 TACACCACCGTCCGCCGGGAGTTCATGGACCACAGCATCT
SULT1A3 TACACCACCGTCCCCCAGGAGCTCATGGACCACAGCATCT
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FIGURE 1 | Sequence homologies among the three members of the *SULT1A* gene subfamily located at chromosome 16 (NT_010393.16). A DNA fragment of 484 base pairs shows: the genetic variants *SULT1A1* 638 G > A (rs9282861, bold) and 667 A > G (rs1801030, bold/italic) as well as 100 base pairs upstream and downstream from these loci. Comparison of the DNA sequences shows that these genes differ only in a small of number of nucleotides (marked in gray) indicating sequence similarities of more than 90% between *SULT1A1*, *SULT1A2*, and *SULT1A3*.

FIGURE 2 | Amplification and genotyping of the DNA sequence comprising the *SULT1A1* 638 G > A (rs9282861) polymorphism. (A) The selection of unspecific primer binding sites lead to simultaneous amplification of *SULT1A1*, *SULT1A2*, and *SULT1A3* due to their high degree of sequence homology. This results in accumulation of amplification products carrying the referent G allele leading to an incorrect genotype call for rs9282861 (homozygous GG). **(B)** Selection of primer binding sites specific for *SULT1A1* enables amplification of the rs9282861 sequence region only resulting in correct determination of the genotype (heterozygous GA).

PHASE I AND II ENZYMES IN ASSOCIATION WITH BREAST CANCER RISK

Candidate gene approaches provide evidence for a particular role of metabolic enzymes in breast carcinogenesis. As of yet only a few studies analyzed the impact of polymorphisms in genes with high sequence homologies, whereas genes like *CYP1A1* and *CYP1B1* have been studied intensely (Economopoulos and Sergentanis, 2010; Sergentanis and Economopoulos, 2010). Therefore, this review focuses on those genes which are usually underrepresented in association studies due to technical issues. Literature search was done by PubMed[7] using the key words "breast cancer polymorphism phase I," "breast cancer polymorphism phase II," "breast cancer polymorphism CYP" "breast cancer polymorphism UGT," "breast cancer polymorphism SULT," "breast cancer polymorphism GST," and "breast cancer polymorphism NAT" in August 2012. In a next step studies analyzing associations between the respective polymorphisms and breast cancer risk factors or breast tumor characteristics were selected on the basis of study size, i.e., inclusion of more than 500 cases and 500 controls, DNA extracted from blood, validation of genotyping results by an independent method or meta analyses on summary data of at least five independent studies.

Significant associations, with $p < 0.05$ or 95% confidence interval not including 1.0, between polymorphic loci in genes encoding phase I and II enzymes and breast cancer risk are summarized in **Table 1**. It has been shown that functional genetic variants of the *CYP2C19* are associated with overall breast cancer risk and HRT-related breast cancer risk (Gan et al., 2011; Justenhoven et al., 2012). It is of note that these findings in two independent studies show similar effects. The variant *CYP2C19**3 (rs57081121) which lead to a decreased activity of the CYP2C19 has been associated

[7]http://www.ncbi.nlm.nih.gov

Table 1 | Polymorphisms in phase I and II enzymes associated with breast cancer risk.

Subgroup	Ethnicity	Gene	Polymorphism	Nucleotide exchange	Cases	Controls	Odds ratio	*p*-Value or 95% confidence interval	Reference
All	Asian	*CYP2C19*	rs57081121 (*3)	G > A	600	600	2.31	0.003	Gan et al. (2011)
	European	*UGT1A6*	rs6759892	T > G	3139	5466	1.17	0.014	The MARIE-GENICA Consortium on Genetic Susceptibility for Menopausal Hormone Therapy Related Breast Cancer Risk (2010)
	European	*UGT1A6*	rs2070959	A > G	3147	5484	1.22	0.007	The MARIE-GENICA Consortium on Genetic Susceptibility for Menopausal Hormone Therapy Related Breast Cancer Risk (2010)
	Mixed	*GSTM1* *GSTT1* *GSTP1*	gene deletion gene deletion rs1695	ins > del ins > del G > A	1052	1098	1.86	1.12–3.08	Steck et al. (2007)
Premenopausal women	European	*CYP3A*	rs10235235	T > C	4436	16393	0.91	0.03	Johnson et al. (2012)
	African-American	*GSTT1*	gene deletion	ins > del	541	635	4.07	1.12–14.8	Van Emburgh et al. (2008)
Postmenopausal women	Mixed	*SULT1A1*	rs9282861	G > A	4623	7642	1.28	0.019	Jiang et al. (2010)
Postmenopausal women with BMI > 25 kg/m^2	Asian	*SULT1A1*	rs9282861	G > A	1102	1147	3.6	1.5–8.7	Yang et al. (2005)
≥10 years use of hormone replacement therapy	European	*GSTT1*	gene deletion	del > ins	2939	5237	1.04	0.0001	The MARIE-GENICA Consortium on Genetic Susceptibility for Menopausal Hormone Therapy Related Breast Cancer Risk (2010)
	European	*GSTP1*	rs947894	C >T	2963	5269	1.05	0.022	The MARIE-GENICA Consortium on Genetic Susceptibility for Menopausal Hormone Therapy Related Breast Cancer Risk (2010)
	European	*CYP2C19*	rs12248560 (*17)	C >T	861	741	0.71	0.001	Justenhoven et al. (2012)
Smoker	European	*GSTT1*	Gene deletion	del > ins	2370	2624	1.3	1.1–1.6	Terry and Goodman (2006)
	European	*GSTM1*	Gene deletion	ins > del	2815	3170	1.4	1.1–1.9	Terry and Goodman (2006)
	African-American	*GSTP1*	rs1138272	C >T	541	635	2.12	1.02–4,41	Van Emburgh et al. (2008)
	European	*NAT2*	rs1801280	T > C	4837	6017	1.5	1.2–1.8	Terry and Goodman (2006)
			rs1799929	C >T					
			rs1208	A > G					
			rs1041983	T > C					
			rs1799930	G > A					
			rs1799931 (*5, *6, *7)	G > A					

Studies with more than 500 breast cancer cases and 500 controls were included.

Table 2 | Polymorphisms in phase I and II enzymes associated with histo-pathological characteristics of breast tumor.

Subgroup	Ethnicity	Gene	Polymorphism	Nucleotide exchange	Cases		Odds ratio	*p*-Value	Reference
Grading	Europeans	*CYP3A43*	rs61469810 (*2A)	ins > delA	G1: 78	G > 1:854	1.74	0.010	Justenhoven et al. (2010)
Node status	Europeans	*CYP2C8*	rs1058930 (*4)	G > C	N0:62	*N* > 0: 16	0.18	0.002	Jernstrom et al. (2009)

Studies with more than 500 breast cancer cases and 500 controls were included.

with increased risk in Asians (Gan et al., 2011) and the variant *CYP2C19**17 (rs12248560) causing an ultra rapid metabolizer phenotype leads to a decreased HRT-related breast cancer risk in Europeans (Justenhoven et al., 2012). It is known that CYP2C19 catabolizes estrogens and progesterone (Yamazaki and Shimada, 1997; Cheng et al., 2001; Cribb et al., 2006) and the reported results suggest that increased metabolic activity of the CYP2C19 lowers endogenous hormone levels leading to a decreased risk.

The polymorphism rs10235235 located the non-coding region of the *CYP3A* locus has been associated with breast cancer risk in premenopausal women (Johnson et al., 2012). It would be of particular interest to follow-up this finding in independent case control collection and functional studies to understand the observed effect of this variant, because other genetic polymorphisms with known functional consequence located in *CYP3A4*, *CYP3A5*, *CYP3A7*, and *CYP3A43* showed no association with breast cancer risk (The MARIE-GENICA Consortium on Genetic Susceptibility for Menopausal Hormone Therapy Related Breast Cancer Risk, 2010).

Two functional genetic variants rs6759892 and rs2070959 which are located in the *UGT1A6* have been suggested to affect overall breast cancer risk. These variants did not show any association with hormonal factors (The MARIE-GENICA Consortium on Genetic Susceptibility for Menopausal Hormone Therapy Related Breast Cancer Risk, 2010), therefore, the risk effect is may be based on the role of UGT1A6 in the metabolism of exogenous compounds such as potential carcinogenic drug and food ingredients (Harding et al., 1988; Bock and Kohle, 2005).

It has been reported that the deletion of the *GSTM1* and *GSTT1* gene as well as the variant allele of the *GSTP1* rs1695 polymorphism impact overall breast cancer risk (Steck et al., 2007). Subgroup analyses showed an association of the *GSTT1* gene deletion and the *GSTP1* rs947894 variant with HRT-related breast cancer susceptibility (The MARIE-GENICA Consortium on Genetic Susceptibility for Menopausal Hormone Therapy Related Breast Cancer Risk, 2010). Moreover, the *GSTT1* deletion seems to affect breast cancer risk in premenopausal women (Van Emburgh et al., 2008). These observed effects of *GST* variants on hormone-related tumorigenesis is may be based on decreased conjugation of genotoxic estrogen quinones leading to elevated levels of DNA damage (Strange et al., 2001; Hachey et al., 2003). In addition, the *GSTM1* and *GSTT1* deletion as well as the *GSTP1* rs1138272 variant, were suggested to affect tobacco smoke-related breast cancer risk (Terry and Goodman, 2006) pointing to the potentially critical role of GSTs in the elimination of exogenous carcinogenic compounds such as polycyclic aromatic hydrocarbons (Hayes and Pulford, 1995).

The *SULT1A1* rs9282861 polymorphism has been associated with breast cancer risk in postmenopausal women, in particular with BMI > 25 kg/m^2, suggesting a modifying effect of the variant allele on endogenous sex hormone exposure (Yang et al., 2005; Jiang et al., 2010).

It has been reported that the variant *NAT2* alleles rs1801280, rs1799929, rs1208, rs1041983, rs1799930, and rs1799931 lead to an increased smoking-related breast cancer which supports the hypothesis that slow acetylators may suffer greater exposure to tobacco carcinogens (Terry and Goodman, 2006).

PHASE I AND II ENZYMES AND BREAST TUMOR CHARACTERISTICS

Only a few well designed studies investigated the association between phase I and II enzymes and histo-pathological characteristics of breast tumors (**Table 2**). One study reported an association between the rs61469810 polymorphism of *CYP3A43* (*CYP3A43**2A) and poorly differentiated breast tumors which may be explained by a potential contribution of the variant allele to increased sex hormone levels (Justenhoven et al., 2010). Another investigation suggested that the rs1058930 polymorphism of *CYP2C8* (*CYP2C8**4) affects lymph node status of breast cancer patients (Jernstrom et al., 2009). The variant allele is known to lower metabolic activity of the encoded enzyme, however, the authors stated that an impact of the *CYP2C9**2 allele which is in linkage disequilibrium with *CYP2C8**4 cannot be excluded (Jernstrom et al., 2009).

CONCLUSION

Genetic variations of phase I and II enzymes alter their activity or protein biosynthesis leading to defective detoxification and elimination of carcinogenic compounds. Due to a high degree of DNA sequence similarity among genes of subfamilies accurate genotyping requires elaborated methods and exhaustive quality control. Until now a few well designed studies give insights into the effect of polymorphisms in metabolic enzymes on breast cancer risk and point to their crucial action in steroid hormone catabolism. These finding underline the pivotal role of sex hormones in the regulation of proliferation, differentiation, and apoptosis as critical pathways for onset and progression of breast cancer (Schindler et al., 1998; Gruber et al., 2002; Seeger et al., 2003; Gadducci et al., 2005). However, a usual short coming is the publication bias related to findings without significant effect. Taken together, the prediction of breast cancer risk on polymorphisms of phase I and II enzymes is in its initial stage and prospective studies including different ethnic groups are needed in order to achieve genotyping based reliable risk determination. Recent developments of gene panels and arrays provide the technical basis for further assessment of the impact of variations in metabolic genes as well as gene–gene and gene-exposure interactions. Overall, comprehensive investigations of multiple genetic, endogenous, and exogenous factors

will promote the understanding of the molecular mechanisms of breast carcinogenesis and support the improvement of prevention strategies.

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Analysis of the functional polymorphism in the cytochrome P450 *CYP2C8* gene rs11572080 with regard to colorectal cancer risk

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In addition to the known effects on drug metabolism and response, functional polymorphisms of genes coding for xenobiotic-metabolizing enzymes (XME) play a role in cancer. Genes coding for XME act as low-penetrance genes and confer modest but consistent and significant risks for a variety of cancers related to the interaction of environmental and genetic factors. Consistent evidence supports a role for polymorphisms of the cytochrome P450 *CYP2C9* gene as a protecting factor for colorectal cancer susceptibility. It has been shown that CYP2C8 and CYP2C9 overlap in substrate specificity. Because CYP2C8 has the common functional polymorphisms rs11572080 and rs10509681 (*CYP2C8*3*), it could be speculated that part of the findings attributed to *CYP2C9* polymorphisms may actually be related to the presence of polymorphisms in the *CYP2C8* gene. Nevertheless, little attention has been paid to the role of the *CYP2C8* polymorphism in colorectal cancer. We analyzed the influence of the *CYP2C8*3* allele in the risk of developing colorectal cancer in genomic DNA from 153 individuals suffering colorectal cancer and from 298 age- and gender-matched control subjects. Our findings do not support any effect of the *CYP2C8*3* allele (OR for carriers of functional *CYP2C8* alleles = 0.50 (95% CI = 0.16–1.59; $p = 0.233$). The absence of a relative risk related to *CYP2C8**3 did not vary depending on the tumor site. We conclude that the risk of developing colorectal cancer does not seem to be related to the commonest functional genetic variation in the *CYP2C8* gene.

Keywords: *CYP2C8*, colorectal cancer risk, polymorphisms, xenobiotic-metabolizing enzymes, biomarkers

INTRODUCTION

In addition to the known effects on drug metabolism and response, functional polymorphisms of genes coding for xenobiotic-metabolizing enzymes (XME) play a modest but consistent role in cancer risk. Genes coding for XME act as low-penetrance genes and confer significant risks for a variety of cancers related to the interaction of environmental and genetic factors (Agundez, 2004). Cytochrome P450 (CYP) enzymes are involved in the metabolism of drugs and other xenobiotics. Two enzymes of the CYP2C subfamily, CYP2C8 and CYP2C9, have received special attention because they are involved in the metabolism of several commonly used drugs and are capable of activating carcinogens and mutagens. Consistent evidence supports a role for polymorphisms of the cytochrome P450 *CYP2C9* gene that produce decreased enzymatic activity as a protecting factor for colorectal cancer susceptibility (Martinez et al., 2001; Chan et al., 2004, 2009; Cleary et al., 2010; Northwood et al., 2010).

One of the mechanisms proposed for this protecting effect is based on the effect of non-steroidal anti-inflammatory drugs (NSAIDs). Inflammation is a known risk factor for colorectal cancer, and the use of NSAIDs constitute a potential means of decreasing inflammation in the colonic epithelium (Ulrich et al., 2006). For this reason, interindividual variability in the metabolism of NSAIDs may play a role in colorectal cancer risk (Bosetti et al., 2012). Two cytochrome P450 enzymes, namely CYP2C9 and, to a lesser extent, CYP2C8, play a major role in the metabolism of NSAIDs (for a review, see Agundez et al., 2009). CYP2C enzymes are expressed in human colon and in colorectal cancer cells (Martinez et al., 2002; Bergheim et al., 2005b; Garcia-Martin et al., 2006b). In addition CYP2C8 expression in colorectal cancer cells is inducible (Garcia-Martin et al., 2006b), and it has been postulated that altered expression of CYP2C enzymes might contribute to the development of colon cancer (Bergheim et al., 2005a).

Although it is known that CYP2C9 y CYP2C8 have common substrates including NSAIDs (Garcia-Martin et al., 2004; Martinez et al., 2005; Totah and Rettie, 2005), a putative association between *CYP2C8* polymorphisms and colorectal cancer risk has not been analyzed in detail. Only the effect of *CYP2C8* polymorphisms as modifying factors for the protective effect of regular NSAID use has been analyzed (McGreavey et al., 2005). This study

indicated a lack of association of *CYP2C8* polymorphisms, but also of *CYP2C9* polymorphisms as modifiers of the protective effect of regular NSAID use on the risk of colorectal carcinoma. Nevertheless, the potential of *CYP2C8* polymorphisms as potential modifiers of colorectal cancer risk remains to be analyzed in detail.

In the present study we analyzed the association of *CYP2C8* 3* with colorectal cancer in a Spanish population. CYP2C8 is involved in the metabolism of arachidonic and retinoic acid. It is the main enzyme involved in the metabolism of R-ibuprofen and it has been shown to make a significant contribution to the metabolism of many other NSAIDs (Martinez et al., 2006). Several variant alleles have been described for the *CYP2C8* gene, *CYP2C8* 3* being the most common variant allele among Caucasian individuals (Garcia-Martin et al., 2006a). The effect *in vivo* of this mutation is a decrease in the metabolism and altered pharmacokinetics of CYP2C8 substrates (Martinez et al., 2005).

MATERIALS AND METHODS

The study group consisted of 153 unrelated consecutive patients (82 men and 71 women) with colorectal cancer, and 298 healthy subjects (**Table 1**). These subjects have participated in previous studies (Garcia-Martin et al., 2001; Martinez et al., 2001). All the participants were white Spanish individuals, living in the same areas as the patients (Madrid and surrounding areas), and were included in the study after giving informed written consent. The diagnosis was based on histology analyses of endoscopic biopsies and/or surgical resection specimens. Data regarding known previous digestive diseases, alcohol, and tobacco consumption, serum tests for hepatitis B and C virus and other diseases were collected. Heavy drinkers were defined as individuals drinking more than 50 g of alcohol per day. All the patients were requested to participate in the study, and all of them agreed to do so. Control samples were obtained from medical students, University, and Hospital staff. A medical examination was carried out to identify subjects in good health. Over 95% of the healthy subjects requested agreed to participate in the study. The protocol was approved by the Ethics Committee of the San Carlos University Hospital, Madrid. A possible confounding factor in the present study is that, within a study group, the frequency of individuals carrying a determined variant allele may change with age in the event that the presence of such an allele would be related to severe diseases. If a determined genotype has a "protecting effect" against any disease, it may be expected that in populations consisting of older subjects there is an increased frequency of such a protecting genotype. Therefore we included within the control group a selected subgroup of 41 healthy subjects with ages ranging from 90 to 95 years (Martinez et al., 2001). Genotype analysis indicates frequencies that were identical to those of younger healthy subjects (see Results). Another possible confounder is related to the fact that the control subjects are highly educated people, and differences in lifestyle as compared to cancer patients may be expected. Since digestive cancers are partly related to diet, these changes in lifestyle may be relevant. However, it should be stated that patients and controls were interviewed to assure that diet and lifestyle did not differ between patients and control subjects. Blood samples from all participants were stored at −80°C until analysis and genomic DNA was prepared from peripheral leukocytes. The analyses for the *CYP2C8* 3* gene variants were carried out by amplification-restriction procedures. Briefly, the pair of primers 5′-CTTCCGTGCTACATGATGACG-3′ and 5′-CTGCTGAGAAAGGCATGAAG-3′ was used for a mismatch PCR-RFLP test. The 117 bp PCR product was purified and digested with the endonuclease *Xmn*I, that produced two fragments of 92 and 25 bp in the *CYP2C8* 1* allele product, and did not digest the *CYP2C8* 3* PCR product. Further details are described elsewhere (Martinez et al., 2005). Twenty DNA samples with every genotype (*CYP2C8* 1/* 1* and ** 1/* 3*) and all samples with the *CYP2C8* 3/* 3* genotypes were cross-tested with TaqMan probes designed to detect the *CYP2C8* 3* variant allele (rs11572080; C__25625794_10; Applied Biosystems, Madrid, Spain) and in all cases the genotypes obtained by amplification-restriction and by the TaqMan analyses fully corresponded.

STATISTICAL ANALYSIS

The intergroup comparison values were calculated using chi-square or Fisher's exact tests when appropriate. Logistic regression analyses were performed using the SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA) to test for confounders, including gender, age at diagnosis, smoking and drinking habits, and tumor localization. The 95% confidence intervals were also calculated. The statistical power calculated for the sample size of this study, determined from allele frequencies with a genetic model analyzing the frequency of the risk gene with RR = 2.0; $p = 0.05$) for two-tailed and one-tailed associations of the risk with the variant allele is 98.1 and 99.2%, respectively. The minimum odds ratio detectable

Table 1 | Characteristics of the individuals included in the study.

	Patients		Controls	
	Male (*n* = 82)	**Female (*n* = 71)**	**Male (*n* = 160)**	**Female (*n* = 138)**
Age years (mean ± SD)	66.9 ± 9.6	65.6 ± 12.9	65.8 ± 10.2	66.0 ± 11.7
Non-smokers/smokers	53/29	69/2	108/52	129/9
Non-drinkers/drinkers	60/22	71/0	141/19	138/0
TUMOR SITE				
Non-sigmoid	33	22	–	–
Sigmoid	26	22	–	–
Rectum	23	27	–	–

with this sample size is 1.55, with a statistical power for one-tailed association equal to 80%.

RESULTS

Table 2 shows the CYP2C8*1/*3 genotypes and allele frequencies in colorectal cancer patients and healthy subjects. Differences noted by the comparison of genotype frequencies among them were not statistically significant. Odds ratio for carriers of the functional *CYP2C8* 1* allele = 0.50 95% C.I. = 0.160–1.59; $\chi^2 = 1.42$; $p = 0.233$. These findings were not influenced by gender, previous surgical therapy, or chemotherapy and Dukes' stage of the tumor. Genotypes in both patients and control subjects are in Hardy–Weinberg's equilibrium. Deviation test from Hardy–Weinberg's equilibrium (Pearson): $p = 0.172$ (patients); $p = 0.265$ (controls). Trend test with the number of variant *CYP2C8* 3* alleles: $p = 0.584$.

To elucidate whether the CYP2C8 genotype could be related to a particular type of colorectal cancer, patients were divided into three subgroups according to the anatomical site of the tumor (rectum, sigmoid colon, and non-sigmoid colon). **Table 3** shows that none of the intergroup comparisons or comparisons with control subjects were significant. Deviation test from Hardy–Weinberg's equilibrium (Pearson): $p = 0.603$ (non-sigmoid); $p = 0.167$ (sigmoid); $p = 0.587$ (rectum). Logistic regression analyses did not indicate any influence of confounders such as gender, age at diagnosis, smoking and drinking habits, or tumor localization in the negative findings obtained in this study.

DISCUSSION

The clinical use of genetic biomarkers for the identification of high risk subpopulations is a major goal which scientists have long been pursuing. In the case of colorectal cancer, the search for risk biomarkers by means of genome wide association studies (GWAs) has provided relevant information, pointing in particular to the single nucleotide polymorphism rs3802842 at 11q23.1 and five additional loci. When SNPs in these loci are combined, the risk increases with an increasing number of variant alleles (Pittman et al., 2008; Tomlinson et al., 2008). Nevertheless, the biomarkers identified in GWA studies explain only a minor percentage of the risk and so these studies should be completed with additional research.

Various candidate gene association studies (CGAs) have been carried out in an attempt to identify low-penetrance genes capable of increasing the risk of developing colorectal cancer. With regard to cytochrome P450 enzymes, two mechanisms have been proposed to explain a putative association between the *CYP2C9* polymorphism and colorectal cancer risk. A number of xenobiotics that are ingested as pyrolysis products have been related to colorectal cancer risk. These include polycyclic aromatic hydrocarbons and heterocyclic aromatic amines. Several of these mutagenic substances are CYP2C substrates (Snyderwine et al., 1997; Jonsdottir et al., 2012) and therefore a genetically determined alteration in the metabolism of carcinogens and mutagens may underlie the observed association. This first hypothesis would imply that, besides CYPs, polymorphisms in other enzymes involved in the metabolism of polycyclic aromatic hydrocarbons and heterocyclic aromatic amines would influence the risk. This hypothesis is partly supported by the influence of *NAT2* polymorphisms in colorectal cancer risk (revised in Agundez, 2008). The second mechanism proposed to explain the association between the *CYP2C9* polymorphism and colorectal cancer risk is the key role that CYP2C9 plays in the metabolism of NSAIDs. This, together with the protective effect that NSAIDs play in human colorectal cancer, leads to the theory that in individuals with high CYP2C9 enzyme activity the protective effect of NSAIDs could be diminished. Because CYP2C8 and CYP2C9 show partial overlapping in substrate specificity, particularly related to some NSAIDs such as ibuprofen, celecoxib, diclofenac, or tenoxicam (Tang, 2003; Garcia-Martin et al., 2004; Martinez et al., 2005; Daly et al., 2007; Daily and Aquilante, 2009), it could be speculated that the presence of defect *CYP2C8* variant alleles may influence the risk of developing colorectal cancer.

To date, only the effect of polymorphisms in CYP2C8 as modifiers of the protective effect of regular NSAID use has been analyzed. (McGreavey et al., 2005) In this study, *CYP2C8* or *CYP2C9* polymorphisms did not influence the protective effect of regular NSAID use on the risk of colorectal cancer. Because *CYP2C8* and *CYP2C9* polymorphisms show a great interethnic and intraethnic variability (Martinez et al., 2006) and because positive association of *CYP2C9* genotypes with colorectal cancer risk was observed in some populations (Bigler et al., 2001; Martinez et al., 2001; Tranah et al., 2005; Hubner et al., 2006; Samowitz et al., 2006; Liao et al., 2007; Cotterchio et al., 2008; Cross et al., 2008; Chan et al., 2009; Siemes et al., 2009; Cleary et al., 2010) but not in other populations, and particularly in UK individuals (Sachse et al., 2002; Landi et al., 2005; Hubner et al., 2006; Kury et al., 2007). It could be speculated that the association of low-penetrance genes with colorectal cancer risk may vary from one population to another. Diet is a key

Table 2 | *CYP2C8* 1/* 3* genotypes and allele frequencies in colorectal cancer patients and healthy controls.

	Patients ($n = 153$, 306 alleles)	Controls ($n = 298$, 596 alleles)	OR (95% CI), p
GENOTYPES rs11572080 C/T (*1/*3)			
*CYP2C8*1/*1*	111 (72.5; 65.5–79.6)	202 (67.8; 62.5–73.1)	1.26 (0.80–1.98), 0.299
*CYP2C8*1/*3*	36 (23.5; 16.8–30.3)	90 (30.2; 25.0–35.4)	0.71 (0.44–1.14), 0.135
*CYP2C8*3/*3*	6 (3.9; 0.8–7.0)	6 (2.0; 0.4–3.6)	1.99 (0.56–7.09), 0.233
ALLELE FREQUENCIES			
*CYP2C8*1*	258 (84.3; 80.2–88.4)	494 (82.9; 79.9–85.9)	1.11 (0.75–1.64), 0.586
*CYP2C8*3*	48 (15.7; 11.6–19.8)	102 (17.1; 14.1–20.1)	0.90 (0.61–1.33), 0.586

The values in each cell represent: number (percentage) and [95% confidence intervals].

Table 3 | *CYP2C8* 1/* 3* genotypes of patients with colorectal cancer according to tumor site.

	Non-sigmoid	Sigmoid	Rectum
GENOTYPES rs11572080 C/T (*1/*3)			
*CYP2C8*1/*1*	39 (70.9; 58.9–82.9)	37 (77.1; 65.2–89.0)	35 (70.0; 57.3–82.7)
*CYP2C8*1/*3*	14 (25.5; 13.9–37.0)	9 (18.8; 7.7–29.8)	13 (26.0; 13.8–38.2)
*CYP2C8*3/*3*	2 (3.6; 0–8.6)	2 (4.2; 0–9.8)	2 (4.0; 0–9.4)
HAPLOTYPES			
*CYP2C8*1*	92 (83.6; 76.7–90.5)	83 (86.5; 79.6–93.3)	83 (83.0; 75.6–90.4)
*CYP2C8*3*	18 (16.4; 9.5–23.3)	13 (13.5; 6.7–20.4)	17 (17.0; 9.6–24.4)

The values in each cell represent: number (percentage) and (95% confidence intervals).

factor in colorectal cancer risk, and because dietary determinants of colorectal cancer may vary greatly in different geographic locations, the contribution of low-penetrance genes to the overall risk may vary across populations.

For the above-mentioned reasons we decided to undertake the study in a population in which a positive effect of *CYP2C9* polymorphism in colorectal cancer risk had already been identified. Because *CYP2C9* 2* and *CYP2C8* 3* variant alleles are at linkage disequilibrium (Yasar et al., 2002; Martinez et al., 2005, 2007), it could be expected that the association observed between the CYP2C9 genotypes and colorectal cancer risk (Martinez et al., 2001; Chan et al., 2004, 2009; Cleary et al., 2010; Northwood et al., 2010), could be partly related to the presence of the CYP2C8*3 variant allele. However, the findings obtained in the present study indicate that no major association of the *CYP2C8* 3* variant allele and colorectal cancer risk is present in the population analyzed.

A limiting factor in this study is that no other defect *CYP2C8* alleles were analyzed. However, it should be emphasized that we tested for *CYP2C8* 3* which is the most common defect *CYP2C8* allele in Caucasian individuals (see the website http://www.cypalleles.ki.se/cyp2c8.htm), whereas other variant alleles such as *CYP2C8* 2*, *CYP2C8* 4*, or *CYP2C8* 5* are extremely rare or no conclusive evidence for their deleterious effect on drug metabolism *in vivo* is available, revised in (Martinez et al., 2006). Another putative limiting factor in this study is the sample size. It should however be emphasized that in the study population the positive association of colorectal cancer risk with *CYP2C9* genotype was identified with a smaller sample size (Martinez et al., 2001) and hence, in the event that a major linkage between *CYP2C8* 3* and colorectal cancer risk might exist in the population studied, the sample size would be enough to identify it. In conclusion, our findings, with sufficient statistical power to detect an odds ratio equal to 1.6, indicate that the *CYP2C8* 3* variant allele does not show a major association with colorectal cancer risk.

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