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Original article

Combination of *CYP2C19* genotype with non-genetic factors evoking phenoconversion improves phenotype prediction

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ABSTRACT

Background: CYP2C19 is an important drug-metabolizing enzyme, responsible for metabolism of approximately 10% of the drugs on the market. Large inter-individual differences exist in metabolic activities, which are primarily attributed to genetic polymorphism of *CYP2C19* gene. Conflicting results have been published about the role of *CYP2C19* polymorphisms in metabolism of CYP2C19 substrates and clinical outcomes; thus, we aimed to investigate CYP2C19 genotype-phenotype associations, and we sought to elicit potential causes of discrepancies in the genotype-based prediction by incorporating the liver donors' demographic data, drug administration events and pathological conditions.
Methods: (*S*)-Mephenytoin was used to assess CYP2C19 activities in human liver microsomes derived from 114 Hungarian organ donors. *CYP2C19* genotype was determined by SNP genotyping for *CYP2C19*2*, *CYP2C19*3*, *CYP2C19*4* and *CYP2C19*17* variants, and CYP2C19 mRNA levels were measured by qPCR method. Clinical data of the donors were considered in the genotype-based phenotype prediction.
Results: CYP2C19 phenotype of 40% of the donors was well-predicted from the genotype data, whereas the phenotype of 13% was underestimated displaying higher activity, and of 47% was overestimated displaying lower activity than predicted from *CYP2C19* genotype. Among the donors with overestimated phenotype, one was treated with CYP2C19 substrate/inhibitor, 9 were on amoxicillin-clavulanic acid therapy, 7 were chronic alcohol consumers and 9 had disease with inflammatory processes.
Conclusions: *CYP2C19* genotype only partially determines the CYP2C19 phenotypic appearance; co-medication, diseases with inflammatory processes and aspecific factors, such as chronic alcohol consumption and amoxicillin-clavulanic acid therapy (or any drug therapy resulting in liver injury) seem to be potential phenotype-modifying factors.

Introduction

CYP2C19 is a clinically important member of drug-metabolizing cytochrome P450 (CYP) enzymes, and assuredly has critical role in several drug–drug interactions and adverse drug reactions [1]. Although CYP2C19 gives only a very small proportion of CYP content in human liver [2], it metabolizes about 10% of the drugs on the market, including proton pump inhibitors, antiplatelet drugs, antiepileptics, antidepressants and the antimalarial proguanil [3–5]. The metabolism of these drugs is largely variable between individuals, which is primarily attributed to the polymorphisms of *CYP2C19* gene. Populations are commonly divided into four phenotypic groups considering individuals' CYP2C19 metabolic capacity, with sub-populations of poor (PM), intermediate (IM), extensive (EM) and ultra-rapid (UM) metabolizers [6].

CYP2C19 gene is highly polymorphic, hitherto 35 allelic variants have been identified (http://www.cypalleles.ki.se/cyp2c19.htm, the last update was on 2nd May 2017), many of them have significant effect on CYP2C19 enzyme activity. For instance, the *CYP2C19*2* variant commonly occurs in Caucasian populations (allele frequency: 12–18% [7,8]), and profoundly affects CYP2C19 protein expression arising from a single nucleotide polymorphism (SNP) in exon 5 (19154G > A; rs4244285), which leads to aberrant mRNA splicing and undetectable protein level, whereas the rare *CYP2C19*3* variant (allele frequency <1%) contains a SNP that creates a premature stop codon (17948G > A; rs4986893). *CYP2C19*4* variant occurs rarely in the Caucasian population (allele

Abbreviations: CAR, constitutive androstane receptor; CI, confidence interval; CYP, cytochrome P450; EM, extensive metabolizer; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GR, glucocorticoid receptor; IM, intermediate metabolizer; PCR, polymerase chain reaction; PM, poor metabolizer; PXR, pregnane X receptor; SNP, single nucleotide polymorphism; UM, ultra-rapid metabolizer.

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frequency: <1%), and the allele carries GTG initial codon (1A > G; rs28399504), resulting in negligible protein expression and activity [3]. In contrast, the *CYP2C19*17* allele is associated with elevated mRNA expression and protein level that supposedly causes ultra-rapid metabolism of CYP2C19 substrates. The *CYP2C19*17* allele commonly occurs in Caucasians (allele frequency: 20–22%) and the variant carries a SNP in the promoter region of the gene (−806C > T; rs12248560); however, the molecular mechanism that gives rise to increased gene expression is still unclear [5,9]. Furthermore, the promoter region of the *CYP2C19* gene consists of CAR/PXR (constitutive androstane receptor/pregnane X receptor) and GR (glucocorticoid receptor) binding sites; consequently, *CYP2C19* gene expression is inducible by xenobiotics activating these nuclear receptors, such as phenobarbital, rifampicin and dexamethasone [9,10].

In general terms, subjects carrying two loss-of-function *CYP2C19* alleles (e.g. *CYP2C19*2*, *CYP2C19*3* or *CYP2C19*4*) are considered to be PMs with deficient enzyme activity. Individuals with single loss-of-function allele and a wild-type allele (*CYP2C19*1*) are IMs, whereas those with two wild-type alleles are EMs. Subjects homozygous or heterozygous for *CYP2C19*17* allele are typically considered to be UMs, supposing loss-of-function alleles are not present. Phenotypic classification of individuals carrying the *CYP2C19*17* gain-of-function allele in combination with a loss-of-function allele is ambiguous; however, limited data suggest that *CYP2C19*17* allele may not compensate for the loss-of-function allele; thus, such subjects mostly classified as IMs [11].

Up to now, several investigations have been implemented to apply genotype-based therapies by utilizing *CYP2C19* genetic data. A well-established example is the bioactivation of the antiplatelet clopidogrel by CYP2C19. A number of studies have demonstrated that *CYP2C19*2* carriers are at increased risk for recurring cardiovascular events under clopidogrel therapy, comparing to those patients homozygous for the wild-type allele [4,12]. *In vivo* clinical studies observed increases in drug area under the concentration-time curve of the proton-pump inhibitor omeprazole or lansoprazole in association with *CYP2C19* genotype [13]. *CYP2C19* genotype also has an effect on the pharmacokinetics of some antidepressants, such as citalopram [14] and amitriptyline [15]. On the other hand, a recent *in vitro* study using human liver microsomes reported that *CYP2C19*1/*2* and *CYP2C19*1/*3* genotypes have almost no effect on CYP2C19 enzyme activity (omeprazole 5-hydroxylation) when compared with the homozygous wild-type genotype (*CYP2C19*1/*1*) [16]. Significant effect of the *CYP2C19*17* variant on the therapy of CYP2C19 substrate citalopram and clopidogrel is not demonstrated unequivocally by *in vivo* trials [14,17]. Although no statistically significant difference in CYP2C19 enzyme activity has been demonstrated between hepatic microsomes from subjects carrying heterozygous or homozygous *CYP2C19*17* genotype (*CYP2C19*1/*17* or *CYP2C19*17/*17*) and microsomes from those with homozygous wild-type genotype, the subjects with *CYP2C19*17/*17* and *CYP2C19*1/*17* genotype exhibited modestly higher expression of CYP2C19 mRNA and protein [18,19]. Additional contradictory results were published about the bioactivation of the antimalarial proguanil and chlorproguanil into their respective major active metabolite cycloguanil and chlorcycloguanil. CYP2C19 has been proposed to be the main enzyme catalysing cycloguanil formation by *in vitro* inhibition studies [20]. Moreover, relationship was found between *CYP2C19* genotype and variation in proguanil and cycloguanil plasma concentrations *in vivo*; however, there was no association with clinical outcome [21].

The present work attempted to clarify the conflicting literature of the CYP2C19 phenotypic appearance. We used human liver microsomes and (*S*)-mephenytoin as probe substrate to characterize inter-individual variability of CYP2C19 enzyme activity, and aimed to delineate associations between the measured phenotype and the corresponding *CYP2C19* genotype. We also sought to elicit the potential causes of discrepancies in the genotype-based prediction by incorporating the donors' demographic data, drug administration events as well as pathological conditions.

Materials and methods

Human liver microsomes

Human liver tissues (n = 114) were obtained from organ transplant donors at the Department of Transplantation and Surgery, Semmelweis University (Budapest, Hungary) (Table 1). Human livers were perfused with Euro-Collin's solution (Fresenius AG, Bad Homburg vdH, Germany) and excised. The tissues were homogenized in 0.1 M Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 154 mM KCl. Microsomes were prepared by differential centrifugation as described by van der Hoeven and Coon [22]. Microsomal protein content was determined by the method of Lowry et al. [23] with bovine serum albumin as the standard. Approximately 50 mg of liver tissues were homogenized in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the total RNA was extracted according to the manufacturer's instructions. The RNA was precipitated by using ethanol and stored at −80 °C for further analyses.

CYP2C19 enzyme assay

Published method was followed to determine mephenytoin 4′-hydroxylation selective for CYP2C19 [24]. The incubation mixture contained NADPH-generating system (1 mM NADP, 10 mM glucose 6-phosphate, 5 mM $MgCl_2$ and 2 units/ml glucose 6-phosphate dehydrogenase), human liver microsomes (1 mg/ml) and mephenytoin (1 mM). After 20-min incubation, the reaction was terminated by ice-cold methanol and the incubation mixture was centrifuged for 10 min at 10.000*g*. High-performance liquid chromatographic analysis was performed according to published method [24]. CYP2C19 enzyme assay for each donor was performed in triplicate, and median (min, max) was calculated.

CYP2C19 genotyping

Hydrolysis SNP analysis for *CYP2C19*2, CYP2C19*3, CYP2C19*4* and *CYP2C19*17* was performed by polymerase chain reaction (PCR) with TaqMan probes (Metabion, Planegg/Steinkirchen, Germany) using a CFX96 real-time detection system (Bio-Rad Laboratories). Primers and probes (Table 2) were designed based on the reference SNP sequences in the National Center for Biotechnology Information reference assembly. Genomic DNA was isolated from liver samples by Quick-DNA™ Universal Kit (Zymo Research, Irvine, CA, USA). PCR was carried out with 30 ng of genomic DNA by using Luminaris Color Probe qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). To confirm the results of *CYP2C19* genotyping, a sequence analysis was performed. The PCR products were sequenced directly in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) by the Sequencing Service of Biomi Ltd. (Gödöllő, Hungary).

Analysis of CYP2C19 mRNA levels with quantitative real-time PCR

Total RNA (3 μg) was reverse-transcribed into single-stranded cDNA by using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules CA, USA), and then real-time PCR with human cDNA was

Table 1
Demographic data of the human organ donors.

Demographic data			
Donor number			114
Age (year)		median (min; max)	45 (13; 74)
Gender		Male/female	60/52
Cause of death			
	Cerebral hemorrhage/hematoma		61
		Subarachnoid hemorrhage	30
		Subdural hemorrhage	5
		Epidural hematoma	1
		Intraventricular hemorrhage	5
		Ruptured cerebral aneurysm	2
		Unknown	18
	Stroke		7
		Ischemic stroke	6
		Hemorrhagic stroke	1
	Tumour		11
	Accident		28
		Car/motor/bike accident	5
		Seizure induced cerebral injury	1
		Suicide	4
		Asphyxia	1
		Unknown cerebral injury	17
	Unknown		7
Anamnesis			
		Alcohol consumption	7
		Medication with CYP2C19 substrate/inhibitor	1
		Medication with agent which cause potential liver dysfunction	9
		Inflammation-induced liver dysfunction	7

Table 2
Sequences of PCR primers and probes for *CYP2C19* genotyping and mRNA quantitation.

	Primer	Sequence	Probe	Sequence
SNP genotyping				
*CYP2C19*2*	Forward	5′-CTTAGATATGCAATAATTTTCCCAC-3′	Wild	FAM-TGATTATTTCCCGGGAACCCATAAC-BHQ1
	Reverse	5′-GAAGCAATCAATAAAGTCCCGA-3′	Mutant	CalRed610-TGATTATTTCCCAGGAACCCATAAC-BHQ2
*CYP2C19*3*	Forward	5′-AGATCAGCAATTTCTTAACTTGATG-3′	Wild	FAM-ACCCCCTGGATCCAGG-BHQ1
	Reverse	5′-TGTACTTCAGGGCTTGGTC-3′	Mutant	CalRed610-ACCCCCTGAATCCAGG-BHQ2
*CYP2C19*4*	Forward	5′-CAAAGAGGCACACACACTTA-3′	Wild	FAM-CAAGAGGAGAAGGCTTCAATGGAT-BHQ1
	Reverse	5′-CCAGATTGAAAGGAGAAGCA-3′	Mutant	HEX-CAAGAGGAGAAGGCTTCAGTGGAT-BHQ1
*CYP2C19*17*	Forward	5′-ATGAACAGGATGAATGTGGTAT-3′	Wild	FAM-TCTGTTCTCAAAGCATCTCTGATGT-BHQ1
	Reverse	5′-TCTTCTGATGCCCATCGT-3′	Mutant	HEX-TCTGTTCTCAAAGTATCTCTGATGT-BHQ1
mRNA quantitation				
CYP2C19	Forward	5′-TGAAGGTGGAAATTTTAAGAAAAGTAA-3′	Probe	FAM-5′-CAGCAGGA-3′-BHQ1
	Reverse	5′-CCCTCTCCCACACAAATCC-3′		

FAM, CalRed610, HEX, fluorescent labelling; BHQ, black hole quencher.

performed by using FastStart Taq DNA polymerase (LightCycler 480 Probes Master; Roche Diagnostics) and UPL probes for CYP2C19 (Roche Diagnostics). The sequences of primers and probe used for the real-time PCR analyses are shown in Table 2. The quantity of target RNA relative to that of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was determined.

Data analysis

Hepatic CYP2C19 activities were determined individually in each donor, the frequency distribution of the CYP2C19 activities were recorded for 114 donors, and four categories (low, intermediate, high, ultra-high) were distinguished for poor, intermediate, extensive and ultra-rapid metabolizers. Approximately 10–15% of the donors displaying low CYP2C19 activity (<8 pmol/(mg protein*min)) were designated as phenotypic PMs, whereas 10–15% of the donors with ultra-high activities (>75 pmol/(mg protein*min)) were designated as phenotypic UMs. The donors displaying CYP2C19 activities between 8 and 75 pmol/(mg protein*min) were considered to be phenotypic IMs and EMs. The cut-off value between IMs and EMs was the median of CYP2C19 activities (23 pmol/(mg protein*min)). The donors' CYP2C19 phenotypes were also predicted from their *CYP2C19* genotypes, and the accuracy of prediction was evaluated on the basis of the activity-based CYP2C19 categories. The donors carrying two loss-of-function alleles (*CYP2C19*2, CYP2C19*3, CYP2C19*4*) were considered to be PMs, those with one functional (*CYP2C19*1*) and one loss-of-function alleles were designated as IMs, the subjects with two functional alleles (*CYP2C19*1/*1*) were EMs, whereas the donors carrying the *CYP2C19*17* allele (both homozygous and heterozygous) were UMs. Considering the ambiguous character of the *CYP2C19*2/*17* genotype, both IM and EM phenotypes were accepted in the presence of such diplotype. Underestimation of CYP2C19 phenotype occurred when the CYP2C19 activity-based phenotypic category was higher than the corresponding genotype-based predicted phenotypic category. Conversely, overestimation was established when the CYP2C19 activity-based phenotypic category was lower than the corresponding genotype-based predicted phenotypic category. The nonparametric Mann–Whitney test was performed for the comparison of mRNA expression levels between diplotype groups.

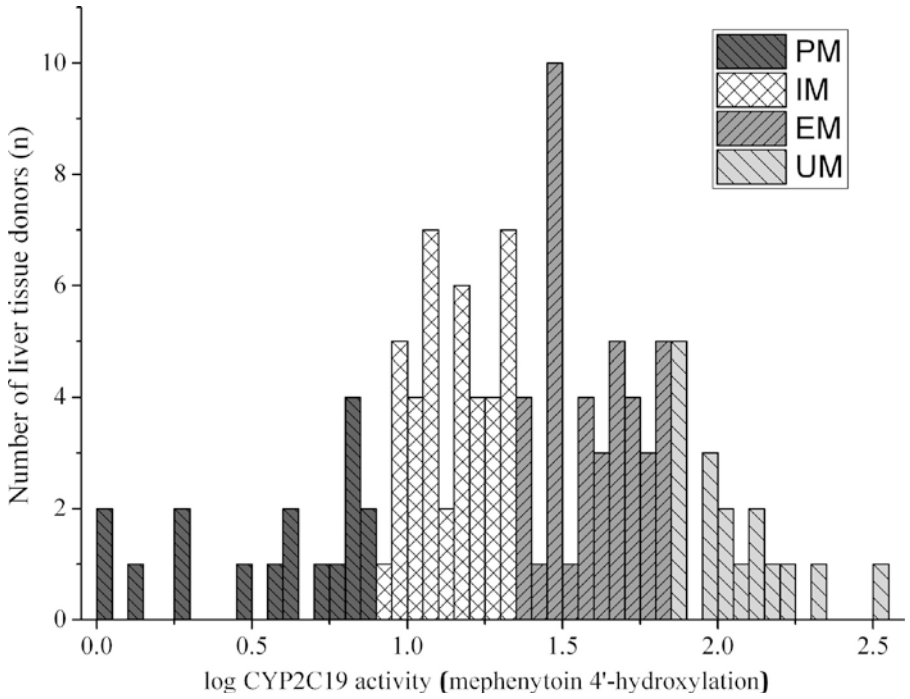

Fig. 1. Frequency distribution of hepatic mephenytoin 4′-hydroxylase activity selective for CYP2C19 in human organ donors (n = 114).

Results

Hepatic CYP2C19 activities

CYP2C19 metabolic activities were measured using microsomes from 114 human liver tissues and (*S*)-mephenytoin as probe substrate [24]. Formation of 4′-hydroxymephenytoin by CYP2C19 was quantified based on "per mg microsomal protein and per minute". Inter-individual variations of mephenytoin 4′-hydroxylase activities ranged from non-detectable to rather high values, and showed skewed distribution (Fig. 1). The cut-off values (CI = 95%) between categories of PMs, IMs, EMs and UMs were 8, 23 and 75 pmol/(mg*min), respectively. CYP2C19 activities were not affected by demographics, respecting donors' age and sex; furthermore, we found no associations between causes of death and metabolic activities (data not shown). In the anamnesis of 7 donors, chronic alcohol consumption was reported, one donor was chronically treated with CYP2C19 substrate/inhibitor (ranitidine, carbamazepine), and 9 of the 114 donors were treated with amoxicillin-clavulanic acid. Diseases with inflammatory processes, such as epilepsy, rheumatoid arthritis and gastrointestinal perforation were diagnosed at 9 individuals.

CYP2C19 allele frequencies

The donors were genotyped for *CYP2C19* allele variants most frequently occurring in Caucasian populations. *CYP2C19*2* (19154G > A), *CYP2C19*3* (17948G > A), *CYP2C19*4* (1A > G) and *CYP2C19*17* (−806C > T) alleles were identified, all of which have functional impact on CYP2C19 enzyme activity. Wild-type allele was assessed when none of these mutations was detected. Allele frequencies of the 114 Hungarian organ donors were compared to those of other Caucasian populations (Table 3). The most frequent loss-of-function allele was *CYP2C19*2* (allele frequency: 18.4%), which is in accordance with the literature data, whereas none of the donors carried the *CYP2C19*3* and *CYP2C19*4* variants. The prevalence of the *CYP2C19*17* allele was modestly higher (26.3%) as compared with Caucasians. Homozygous loss-of-function (*CYP2C19*2/*2*) and homozygous gain-of-function (*CYP2C19*17/*17*) diplotypes occurred only at one and eight individuals, respectively, whereas 12 of the donors carried the ambiguously classifiable *CYP2C19*2/*17* genotype. In accordance with previous observations [25], namely that the 19154G > A (*CYP2C19*2*) and the −806C > T (*CYP2C19*17*) SNPs are in complete linkage disequilibrium, none of the donors in the study population carried these two mutations on the same haplotype.

Table 3
Frequency of *CYP2C19* alleles in the study population (n = 114) and in Caucasians.

CYP2C19 allele	Allele frequency (%)	
	Subjects	*Caucasians* [7,8]
**2*	18.4	12–18
**3*	0	<1
**4*	0	<1
**17*	26.3	20–22

Association between CYP2C19 genotype and CYP2C19 enzyme activity

Human organ donors were categorized as PM (poor), IM (intermediate), EM (extensive) and UM (ultra-rapid) metabolizers based on their corresponding *CYP2C19* genotypes, and the genotype-based predicted phenotype was compared to the CYP2C19 activity-based phenotype (Fig. 2). Altogether, one individual with two loss-of-function alleles (*CYP2C19*2/*2*) displayed low metabolic activity (7.6 pmol/(mg*min)) and accordingly PM phenotype. Mephenytoin 4′-hydroxylase activities of the donors carrying one wild-type and one loss-of-function alleles varied between wide ranges (2–110 pmol/(mg*min)); less than half of the donors (13/27) showed the predicted IM phenotype, a few of them (4/27) were PMs. Furthermore, 10 heterozygous donors exhibited higher CYP2C19 activity than it could be predicted from their genotypes. 36 of the 114 donors carried homozygous wild genotype; however, only 14 showed extensive metabolism; 11 and 7 of them were IMs and PMs, respectively. Four donors, despite

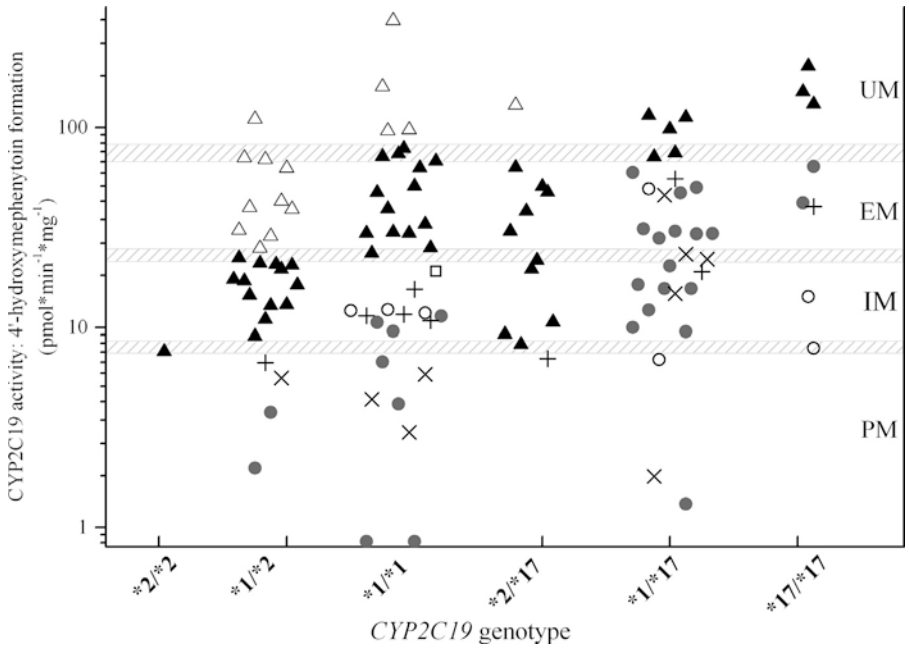

Fig. 2. Hepatic CYP2C19 activity (mephenytoin 4′-hydroxylation) in subjects carrying various *CYP2C19* genotypes.
▲ acceptable prediction from genotype, △ underestimated phenotype from genotype, ● overestimated phenotype from genotype, ◦ chronic alcohol consumption, + liver dysfunction caused by disease, □ subjects under CYP2C19 substrate/inhibitor drug therapy, × subjects under amoxicillin-clavulanic acid therapy.

their homozygous wild genotype, showed rather high mephenytoin 4′-hydroxylase activity, and therefore were categorized as phenotypic UMs. Almost all of the donors (10/12) with *CYP2C19*2/*17* genotype were phenotypic IMs and EMs, except for two donors, one with slightly higher and one with lower activity, respectively. Donors with *CYP2C19*1/*17* and *CYP2C19*17/*17* genotypes are generally considered to be UMs. In our study, mephenytoin 4′-hydroxylase activity of the *CYP2C19*17* carriers (both homozygous and heterozygous) showed enormous differences, and only a small proportion of the donors (8/38) exhibited ultra-rapid metabolism. Although CYP2C19 activities of the donors with *CYP2C19*1/*17* genotype showed no difference (37.17 ± 31.12 pmol/(mg*min) *vs.* 42.20 ± 62.41 pmol/(mg*min), $p = 0.4209$), the donors with *CYP2C19*17/*17* genotype exhibited modestly, but not significantly higher mean activity value when compared to the donors with homozygous wild genotype (81.77 ± 71.48 pmol/(mg*min) *vs.* 42.20 ± 62.41 pmol/(mg*min), $p = 0.0697$).

*Associations between CYP2C19 mRNA levels and CYP2C19*17 allele*

Mutation in the promoter region of *CYP2C19*17* allele has been reported to increase the expression of *CYP2C19* gene [5,19]; therefore, the relative CYP2C19 mRNA levels in human liver tissues were investigated as the function of *CYP2C19* genotype. No significant difference was found between CYP2C19 expression levels of each genotype group (Fig. 3A). Non-genetic factors (e.g. co-medication, nutrition, disease) have been demonstrated to substantially modify CYP2C19 expression and activity due to phenoconversion [26]; therefore, those individuals whose anamnesis contained CYP2C19 substrate/inhibitor therapy, amoxicillin-clavulanic acid therapy, chronic alcohol consumption or inflammatory processes, were excluded from the comparison analysis (Fig. 3B). Although no difference was observed in CYP2C19 expression between the donors with *CYP2C19*1/*17, CYP2C19*1/*2, CYP2C19*2/*17* genotypes and the donors with *CYP2C19*1/*1* genotype, *CYP2C19*17/*17* homozygous subjects showed significantly higher mRNA levels compared to the individuals with homozygous wild genotype ($p = 0.0227$).

Discussion

The aim of this work was to elucidate the role of *CYP2C19* genetic polymorphisms in CYP2C19 phenotypic appearance. In the present study, human liver microsomes isolated from Hungarian organ donors were characterized using mephenytoin 4′-hydroxylase activity as phenotyping assay and using *CYP2C19* SNP genotyping platform for the allelic variants occurring most frequently in the Caucasian populations. *CYP2C19* allele frequencies of 114 Hungarian donors were consistent with previously described frequencies of Caucasian populations [8], and were also consistent with a pilot study on a Hungarian cohort published by Rideg et al. [27]. In the latter study by Rideg et al., the prevalence of the *CYP2C19*2* variant was nearly identical compared to our results (16.5% *vs.* 18.4%); however, detection of the *CYP2C19*17* variant was not included in the trial by Rideg et al. In our study population, functional defect of *CYP2C19* gene was attributed solely to the presence of the *CYP2C19*2* variant, since neither the *CYP2C19*3* nor the *CYP2C19*4* alleles occurred. The prevalence of the gain-of-function *CYP2C19*17* allele was slightly higher compared to Caucasians (26.3% *vs.* 20–22%); however, the difference was not significant, owing to the relatively low number of the cohort.

Up to now, clinical significance of the *CYP2C19*2/*2* genotype in the metabolism of CYP2C19 substrates, such as clopidogrel, diazepam, omeprazole, proguanil, was unequivocally demonstrated by a number of *in vivo* trials [3,21]. Since only one donor carried *CYP2C19*2/*2* genotype in the current cohort, although this donor showed low mephenytoin 4′-hydroxylase activity and was categorized as PM, the genotype group was inappropriate to compare with other diplotypes. The phenotype of the individuals with one loss-of-function and one wild-type alleles proved to be poorly predictable (13/27). Nine donors showed extensive and one donor displayed ultra-rapid metabolism, despite the lack of the −806C > T SNP in the promoter region of the gene or of medication of any pharmaceutics with CYP2C19 inducing capabilities that potentially could increase the transcription of *CYP2C19* gene *via* activating CAR/PXR or GR nuclear receptors. Four of the 36 donors

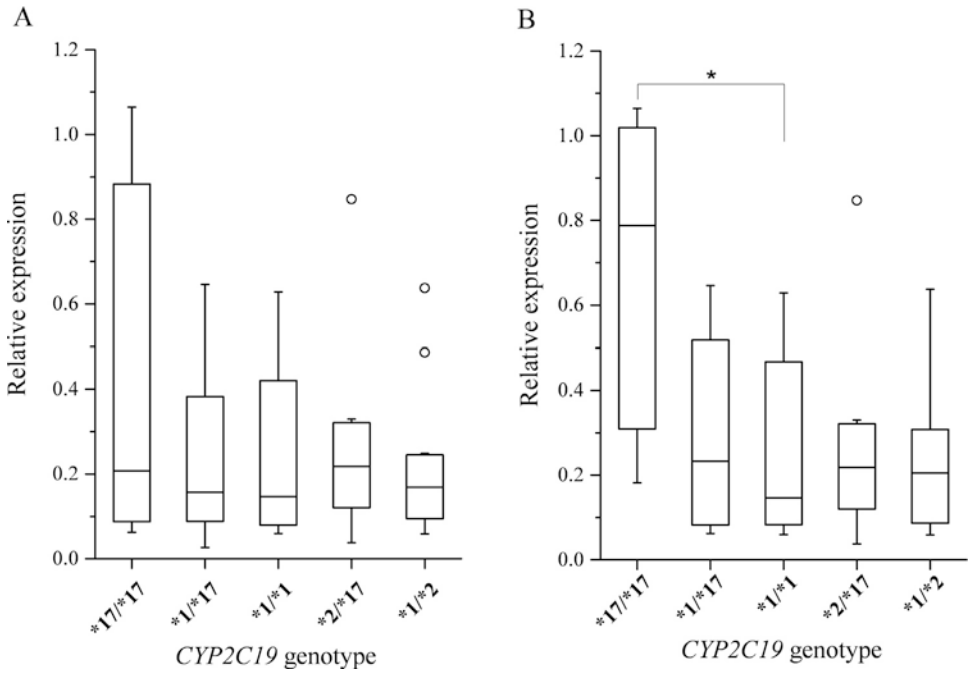

Fig. 3. Association between *CYP2C19* genotype and mRNA expression.
Data represents the relative mRNA expression values of 56 donors (A) and expression values of the donors after exclusion of individuals with potential phenotype-modifying non-genetic factors (chronic alcohol consumption, amoxicillin-clavulanic acid therapy, CYP2C19 substrate/inhibitor therapy, disease with inflammation processes) (B). Data are presented as a box plot and whisker plot (box shows median with the 25th and 75th percentiles), and minimum and maximum values are shown by whiskers. Outliers that do not fall within the bounds of the plot are indicated with ◦.
Nonparametric Mann-Witney test was performed for the comparison of expression levels between the diplotype groups; $^{*}p < 0.05$ value was considered to be statistically significant.

with homozygous wild genotype were categorized as UMs based on their mephenytoin 4′-hydroxylase activity; however, individuals with wild diplotype are generally expected to be EMs. Underestimation of individuals' phenotype could be explained by medication which is accountable for the activation of nuclear receptors (CAR/PXR or GR) and, hereby, for increased CYP2C19 transcription [10], and was not listed in the clinical history of the donors.

The phenotype of 53 of the 114 individuals was overestimated based on their corresponding genotype, 16 of them exhibited very low CYP2C19 metabolic activity and were categorized as phenotypic PMs similarly to the donor carrying two loss-of-function alleles. This observation is in agreement with previous population studies, which demonstrated that the prevalence of phenotypic PM status exceeds the prevalence of genotypic PMs (data reviewed by Fricke-Galindo et al. [7]). Since a number of *in vivo* trials showed that PMs are endangered during CYP2C19 substrate drug therapy (e.g. clopidogrel, omeprazole, citalopram) [4,11–15], it is essential to identify potential factors that could be responsible for the phenotype-genotype discrepancies, and could evoke phenoconversion and phenotypic poor metabolism despite the presence of one or two functional *CYP2C19* alleles. In the present study, non-genetic factors, which are assuredly able to modify CYP2C19 activity or liver metabolic function, were considered in the phenotype prediction. Co-administration of CYP2C19 substrates or inhibitors could lead to a decrease in metabolic capacity of CYP2C19 enzyme and to phenotypic poor metabolism, hence it could be the source of various drug–drug interactions [28,29]. In the study population, one individual with homozygous wild genotype showed lower mephenytoin 4′-hydroxylase activity and was categorized as phenotypic IM. Regarding clinical data, this individual was on chronic ranitidine and carbamazepine therapy. Ranitidine is an H_2-receptor antagonist, and is widely used to treat duodenal and stomach ulcers. Demethylation of ranitidine is principally catalysed by CYP2C19, CYP1A2 and CYP2D6 [30]. Although carbamazepine, an anti-epileptic agent, is a well-established inducer of various CYPs *via* CAR and PXR regulatory pathways [31], it has been shown that carbamazepine and its metabolites have the potential to inhibit the metabolism of CYP2C19 substrates [32]. Although both carbamazepine and ranitidine are competitive inhibitors and do not bind CYP2C19 enzyme covalently, phenotypic intermediate metabolism of the individual with homozygous wild-type alleles might be evolved due to the moderate phenoconversion caused by the supposed presence of the CYP2C19 substrate ranitidine and the CYP2C19 inhibitor carbamazepine and their metabolites in the surroundings of CYP2C19 enzyme. Chronic alcohol consumption and amoxicillin-clavulanic acid treatment could also be responsible for phenotype conversion. Amoxicillin is an antibiotic drug applied in combination with clavulanic acid for treatment of various bacterial infections. Hepatotoxic effect of both amoxicillin-clavulanic acid and alcohol is well-described [33]; however, their impact on CYP2C19 activity is hardly investigated. Our results suggested that amoxicillin-clavulanic acid treatment and chronic alcohol consumption are likely to have an aspecific effect and might contribute to the reduction of CYP2C19 activity, leading to discrepancies in the genotype-based phenotype prediction. Sixteen donors under chronic alcohol consumption or amoxicillin-clavulanic acid treatment exhibited lower CYP2C19 metabolic activity than it was predicted from their genotype data; moreover, 7 of them were PMs in spite of the presence of one or two either wild-type or gain-of-function *CYP2C19*17* alleles. Ultimately, chronic diseases with inflammatory processes were also considered in the phenotype prediction. Inflammation-induced liver dysfunction is also a potential candidate that could have crucial role in phenoconversion. A number of trials investigated the

alterations of CYP expression or metabolic capacities in different pathological conditions, indicating substantial reduction of CYP2C activities in patients with advanced cancer [34], congestive heart failure [35], epilepsy [36] as well as with liver disease [28,37]. In pathological conditions, down-regulation of *CYP2C19* gene expression was supposedly evoked by inflammatory cytokines; however, the regulatory mechanism is still unclear [26,35,38]. Diseases with inflammatory processes (epilepsy, rheumatoid arthritis and gastrointestinal perforation) were diagnosed at 9 individuals in our study population, all of them showed lower CYP2C19 activity than it was expected from their genotype data, and 2 of the 9 donors were categorized as phenotypic PMs. In the anamnesis of 27 donors, we were unable to explain the overestimated phenotype with any factors, including genetic and non-genetic factors. Morbid obesity leading to inflammatory processes and lipid accumulation in hepatocytes can be considered to be risk factors that can modify CYP2C19 expression and enzyme activity; however, histopathological evaluation of the liver tissues did not indicate steatotic status except for those with excessive alcohol intake. Supposedly, other factors not listed in the clinical history of the donors might affect the phenotypic appearance. Our results are in accordance with a recently published research using human liver microsomes and S-mephenytoin as probe substrate [18]. Shirashaka et al. established that *CYP2C19* genotype only partially contributed to the variability of CYP2C19 enzyme activity and expression. Furthermore, no association with clinical or demographic data was found; however, clinical information, such as drug treatment or disease, was not available at appreciable number of the donors [18]. Similar results were published by Gao et al., who concluded that the genotype and protein content of some CYPs had only limited effects on phenotypic appearance, which implied other important factors influencing inter-individual variations [16]. A potential source of inconsistencies can be the overlapping CYP2C19 metabolizer categories. There is a wide range of metabolic activity assigned to be extensive metabolizers, and still seems to overlap with the activities of intermediate metabolizers increasing the number of genotype-phenotype discrepancies.

Effect of the *CYP2C19*17* allele on gene expression was evaluated by analysing the association between CYP2C19 mRNA levels and *CYP2C19* genotype data. We found no significant difference between the CYP2C19 mRNA levels of each genotype group; however, after excluding individuals with co-medication, chronic alcohol consumption, amoxicillin-clavulanic acid treatment and inflammation-induced liver dysfunction, as potential factors evoking phenoconversion and discrepancies in genotype-based prediction, donors with *CYP2C19*17/*17* diplotype showed significantly higher relative CYP2C19 expression compared to homozygous wild-type individuals. This finding is partially concordant with the results published by Sanford et al., who demonstrated that not only the donors with *CYP2C19*17/*17*, but the subjects with *CYP2C19*1/*17* diplotype showed significantly higher mRNA levels compared to donors with homozygous wild-type diplotype; however, these differences were not reflected by CYP2C19 enzyme activities [19].

In conclusion, *CYP2C19* genotype is not the only determinant factor in CYP2C19 metabolizer status, but co-medication with CYP2C19 substrate or inhibitor, diseases with inflammatory processes and aspecific factors, such as chronic alcohol consumption and amoxicillin-clavulanic acid therapy (or any drug therapy resulting in liver injury) might have the potential to modify CYP2C19 metabolic activity leading to genotype-phenotype mismatches. Furthermore, *CYP2C19*17/*17* genotype determines the potential for increased gene expression and for ultra-rapid metabolism; however, phenotypic appearance seems to strongly depend on several non-genetic factors that may modify CYP2C19 metabolic capacity.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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