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ARTICLE

Population Pharmacokinetics of Methylphenidate in Healthy Adults Emphasizing Novel and Known Effects of Several Carboxylesterase 1 (*CES1*) Variants

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The aim of this study was to identify demographic and genetic factors that significantly affect methylphenidate (MPH) pharmacokinetics (PK), and may help explain interindividual variability and further increase the safety of MPH. *d*-MPH plasma concentrations, demographic covariates, and carboxylesterase 1 (*CES1*) genotypes were gathered from 122 healthy adults and analyzed using nonlinear mixed effects modeling. The structural model that best described the data was a two-compartment disposition model with absorption transit compartments. Novel effects of rs115629050 and *CES1* diplotypes, as well as previously reported effects of rs71647871 and body weight, were included in the final model. Assessment of the independent and combined effect of *CES1* covariates identified several specific risk factors that may result in severely increased *d*-MPH plasma exposure.

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Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
✔ Large unexplained IIV is observed in the PK of MPH. The *CES1* SNP rs71647871 GA has been reported to substantially increase MPH plasma exposure. The effect of *CES1* diplotypes on MPH metabolism is unknown.

WHAT QUESTION DID THIS STUDY ADDRESS?
✔ First, this study developed a *d*-MPH population PK model that included the effects of *CES1* variants and demographic covariates. Second, the model was used to assess the independent and combined effects of covariates on *d*-MPH plasma exposure.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE
✔ This analysis reveals the effects of rs115629050 TG and *CES1* diplotypes on *d*-MPH PK. It also confirms the effect of rs71647871 GA and body weight. Additionally, it illustrates the considerable *d*-MPH plasma exposure increases caused by the independent or combined presence of *CES1* variants.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE
✔ These findings may eventually contribute to the quantitative guidance of individual dosing recommendations based on specific *CES1* variants or variant combinations, and lead to increased safety with MPH.

Methylphenidate (MPH), a central nervous system stimulant, is the most commonly prescribed drug for the treatment of patients with attention deficit hyperactive disorder.[1,2] It is also prescribed for other conditions, such as narcolepsy in adults as well as depression in the elderly and patients with advanced illness.[3–5] A significant increase in MPH prescription has occurred in developed countries over recent years.[6–8]

The pharmacokinetics (PK) of MPH have been reported to display large unexplained interindividual variability (IIV)[9–11] and certain studies indicate a 30-fold difference in MPH serum concentrations 1 h postdose[12] and up to a sevenfold difference in maximal concentrations.[13] The study of MPH PK is relevant from a clinical perspective due to significant correlation between MPH concentration and clinical response.[14,15] Furthermore, a linear relationship between MPH dose and the development of adverse effects has been reported,[16] and, hence, MPH-related adverse effects may be concentration-dependent. Adverse effects during MPH treatment are common and are in the majority of cases categorized as mild to moderate.[17] They include insomnia, anorexia, anxiety,[16] as well as cardiovascular effects in terms of increases in heart rate and blood pressure.[18,19] However, due to their sympathomimetic activity,[20] case reports of serious adverse

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effects, such as sudden cardiac death, stroke, myocardial infarction, and hypertension, have raised concerns regarding stimulants' cardiovascular safety.[21,22] It has been hypothesized that pharmacogenetic factors may predispose certain individuals to a higher risk of harmful adverse effects during treatment and, hence, further research that may lead to increased safety with MPH has been encouraged.[17]

Human metabolism of MPH is predominantly mediated by carboxylesterase 1 (CES1), a serine esterase, which de-esterifies MPH to ritalinic acid in the liver.[23] Approximately 80% of orally administered MPH is recovered in urine as ritalinic acid, whereas minor metabolites of MPH or ritalinic acid each account for a nonsignificant amount.[24] In recent years, differences in *CES1* have been found to affect the PK of several drugs metabolized by CES1, in particular MPH.[13,25–27] A nonsynonymous single nucleotide polymorphism (SNP), rs71647871 GA (p.Gly143Glu) with a reported frequency of 3.7% in Caucasians, has been identified to significantly influence MPH PK.[13] It has been found to cause a reduction in the catalytic activity of CES1; consequently, MPH metabolism is reduced and substantially elevated MPH plasma exposure is observed in carriers.[13] Apart from the aforementioned, no studies have yet been able to identify other *CES1* SNPs that impact MPH PK. Considering the crucial role of CES1 in MPH metabolism, it is plausible that additional *CES1* SNPs that impact MPH PK may exist. In addition to *CES1* SNPs, CES1 gene structure has also been found to influence the PK of CES1-metabolized drugs. A study involving the CES1-metabolized compound, irinotecan, has shown the presence of *CES1A2* to have a significant effect on its PK,[28] and it is therefore of interest to investigate the impact of *CES1A2* on MPH metabolism. *CES1A2* is a hybrid gene with a reported allele frequency of 14.4% in Caucasians consisting of the upstream portion of the nonfunctional pseudogene *CES1P1* (also referred to as *CES1A3*) and a duplicated segment of *CES1*.[29] The latter is commonly designated *CES1A1* to distinguish it from *CES1A2*. One of three possible *CES1* diplotypes can be present in a given individual: two *CES1A1* and two *CES1P1*, which is the most frequent; two *CES1A1*, one *CES1P1*, and one *CES1A2*; or two *CES1A1* and two *CES1A2*.[30]

Until now, studies that have reported an association between *CES1* variants and MPH PK have done so while focusing on one variant at the time through noncompartmental methods, and have therefore not considered any potential combined variant effect. This is of interest to investigate given that *CES1* variants resulting in altered drug metabolism may be due to SNPs and potentially structural *CES1* differences. Use of population PK modeling for assessing the impact of genetics on drug PK holds several advantages compared with the noncompartmental approach. These include the ability to perform a simultaneous analysis of the effects of several demographic and/or genetic covariates, as well as clinical trial simulation, which may illustrate the risk associated with a particular effect or combination of effects.[31] Furthermore, in the presence of nonlinearity or variability in drug bioavailability, model-based phenotype allows a higher probability to detect SNP effects than other phenotypes.[32] Presently, population-based analyses of MPH are few[9,33,34] and none have, to this date, included *CES1* variants as covariates, despite their reported effect on MPH metabolism[13] and the metabolism of other CES1-metabolized compounds.[28] Therefore, the aim of this work is to build on recent CES1 pharmacogenetic findings and develop an MPH population PK model, which (i) identifies significant effects of demographic covariates as well as different types of *CES1* variants on PK parameters and their IIV, and (ii) can determine the independent and potential combined effects of covariates on MPH plasma exposure.

METHODS

PK data from two previously conducted clinical studies (NCT02135263 and NCT02147535) at Bispebjerg Hospital, Copenhagen, Denmark, were aggregated for analysis. Both study protocols were reviewed and approved by an ethical review board. The studies were conducted in accordance with all applicable regulatory and Good Clinical Practice guidelines and followed the ethical principles originating in the Declaration of Helsinki. All participants were fasting overnight and MPH was administered 1 h following a standardized breakfast. Two participants did not receive the standard meal due to lactose intolerance and a food delivery issue. Study I involved 44 healthy adult volunteers who were administered a single dose of 10 mg MPH (Ritalin; Novartis, Basel, Switzerland). Plasma samples from each subject were collected predose and 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 24, and 33 h postdose. Study II involved 78 healthy adult volunteers who were administered a single dose of 10 mg MPH (Ritalin), followed by the collection of a single plasma sample from each subject 3 h postdose. All subjects were Caucasians and their demographic characteristics are presented in detail in **Supplementary Table S1**. All participants were *CES1* genotyped using DNA extracted from saliva samples. Details regarding the genetic analysis are presented in the **Supplementary Material**, along with the genetic characteristics of participants in the two studies (**Supplementary Table S2**).

d-MPH and *l*-MPH plasma concentration analysis is described in the **Supplementary Material**. Population PK modeling was performed using nonlinear mixed-effects modeling software (NONMEM, version 7.3; ICON Development Solutions, Ellicott City, MD) and the first-order conditional estimation with interaction method. In total, 503 *d*-MPH plasma concentrations collected from 122 healthy adults were analyzed and handling of data below the quantification limit is presented in the **Supplementary Material**. Model development procedure and criteria are presented in detail in the **Supplementary Material**. The following covariates were investigated: body weight, body mass index, gender, and a total of nine *CES1A1* SNPs that result in nonsynonymous amino acid substitutions in CES1, as well as *CES1* diplotypes. Covariate model development was performed using a stepwise procedure with a forward inclusion p value < 0.05 (objective function value [OFV] decrease > 3.841, 1 degree of freedom) and a backward elimination p value < 0.005 (OFV increase > 7.879, 1 degree of freedom). In order to explore the effect of *CES1* variants in which subjects in the studied population presented missing genotype data, methods for handling missing covariate data were considered. As

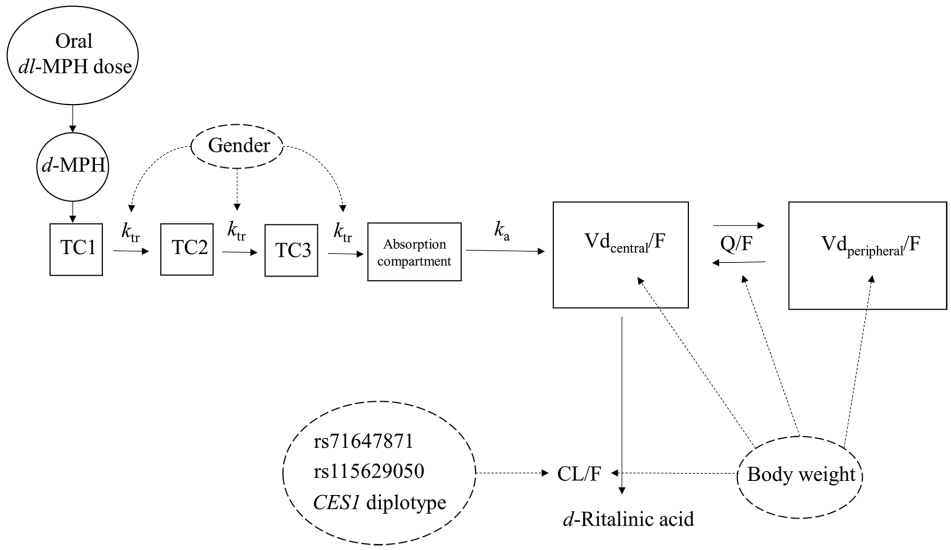

Figure 1 Schematic diagram of the structural *d*-methylphenidate (MPH) pharmacokinetic model along with model-incorporated covariates. *d*-MPH passes through three transit compartments (TCs) into an absorption compartment, where it is absorbed into the central compartment and distributed to the peripheral compartment. *d*-MPH undergoes elimination from the central compartment to *d*-ritalinic acid (CL/F). k_{tr} is the transit rate constant and is defined as (number of TCs + 1) divided by mean transit time. k_a is the absorption rate constant. $Vd_{central}/F$ and $Vd_{peripheral}/F$ represent apparent central and peripheral volumes of distribution, respectively. Q/F is the apparent inter-compartmental clearance. Dotted circles and arrows indicate covariates and the parameters they affect, respectively. CES1, carboxylesterase 1.

a first step, the nature of the missing data were investigated. It was examined whether the missing data were missing completely at random, missing at random,[35] or missing not at random.[36] Based on the nature of the missing data, a method for handling missing data was chosen. Permutation testing was performed for each included covariate to determine actual significance levels and account for type I error (**Supplementary Material**). The individual contribution of each incorporated covariate on IIV reduction and explanation of parametric variability was also assessed (**Supplementary Material**).

The final fixed-effect and variability parameter estimates of the model were used to perform Monte-Carlo simulations ($n = 5{,}000$) for each model-included covariate and assess its independent effect on *d*-MPH plasma exposure together with the 95% prediction interval, following oral administration of a 10 mg MPH dose. The same procedure was used to determine the effect of physiologically plausible covariate combinations. A change of $\pm$ 30% in *d*-MPH plasma exposure compared with a reference population was considered clinically significant in this study. Last, the model was used to obtain a preliminary estimate of the incidence of specific covariates and plausible simultaneous combination in individuals within a large virtual population ($n = 100{,}000$) and their relation to changes in *d*-MPH plasma exposure.

RESULTS

The final *d*-MPH PK model was a two-compartment model with absorption transit compartments and first-order elimination from the central compartment. The parameter estimation process and covariance step both converged successfully using the first-order conditional estimation method with interaction. Three significant digits were requested in the parameter estimates. The structural model along with PK parameters and covariates that influence them are shown in **Figure 1**. The final parameter estimates are shown in **Table 1**, along with nonparametric 95% confidence intervals obtained through bootstrapping. The estimated number of transit compartments was originally 3.05 (data not shown) but was fixed to the value of 3.0 in subsequent models to aid model stability and successful convergence. No significant changes to the model fit and parameter estimates were observed doing so. IIV was estimated for apparent oral clearance (CL/F), central volume of distribution ($Vd_{central}/F$), and mean transit time (MTT) in a full variance-covariance matrix, containing both diagonal and nondiagonal elements. A proportional error model was used to describe unexplained residual variability.

The final model included allometrically scaled body weight using a fixed value of 0.75 for CL/F and apparent intercompartmental clearance (Q/F) and 1 for $Vd_{central}/F$ and apparent peripheral volume of distribution ($Vd_{peripheral}/F$). Estimation of allometric exponents for these parameters was evaluated; however, no significant change in OFV or parameter estimates was obtained. The effects of the CES1 genetic covariates rs71647871, rs115629050, and *CES1* diplotypes were included on CL/F. Genotype information regarding *CES1* diplotypes, rs71647871, and rs115629050 was missing in 0.8%, 5%, and 42%, respectively, of the total studied population. Missing data were considered as being missing not at random and was handled using the EXTRA (or EST) method.[37,38] Gender was included on MTT due to the improvement of model fit assessed through decrease of OFV and improvement of visual diagnostics. Incorporation of the aforementioned covariates reduced the OFV by 104.3 points, reduced unexplained CL/F IIV by 15.0%, and increased the proportion of explained CL/F IIV to total CL/F IIV from zero

Table 1 Final *d*-MPH population PK model parameter estimates

Model parameter	NONMEM estimate (RSE%)	Bootstrap estimate (RSE%)	Nonparametric (95% CI)
Structural model			
θ_1: MTT (/h)	0.505 (13.7)	0.521 (16.3)	(0.371–0.707)
θ_2: No. of transit compartments (fixed)	3	–	–
θ_3: k_a (/h)	0.418 (18.8)	0.430 (134.0)	(0.370–1.304)
θ_4: CL/F (L/h)	233.0 (3.6)	231.7 (4.5)	(214.0–251.8)
θ_5: $Vd_{central}$/F (L)	97.6 (28.6)	101.8 (63.7)	(47.6–288.7)
θ_6: Q/F (L/h)	70.1 (47.8)	74.3 (38.7)	(49.9–175.3)
θ_7: $Vd_{peripheral}$/F (L)	252 (30.9)	260.7 (64.5)	(198.2–453.2)
Covariate effects			
θ_8: Female gender on MTT	0.925 (44.3)	0.852 (42.7)	(0.297–1.831)
θ_9: rs71647871 (GA) on CL/F	−0.587 (6.9)	−0.585 (8.8)	(−0.677 to −0.473)
θ_{10}: Missing data rs71647871 on CL/F	−0.157 (46.9)	−0.161 (54.6)	(−0.330 to −0.00164)
θ_{11}: One *CES1A2* on CL/F	−0.182 (31.9)	−0.184 (36.8)	(−0.307 to −0.0475)
θ_{12}: Two *CES1A2* on CL/F	−0.410 (22.5)	−0.408 (25.6)	(−0.583 to −0.192)
θ_{13}: Missing data *CES1A* diplotype on CL/F	−0.535 (9.0)	−0.535 (11.3)	(−0.663 to −0.440)
θ_{14}: rs115629050 (TG) on CL/F	−0.403 (26.1)	−0.399 (31.4)	(−0.714 to −0.180)
θ_{15}: Missing data rs115629050 on CL/F	0.090 (79.6)	0.0913 (86.8)	(−0.0623 to 0.262)
Weight exponent on CL/F (fixed)	0.75	–	–
Weight exponent on Vdcentral/F (fixed)	1	–	–
Weight exponent on Q/F (fixed)	0.75	–	–
Weight exponent on Vdperipheral/F (fixed)	1	–	–
IIV (%CV)			
MTT	62.1 (27.7)	60.8 (29.3)	(43.1–78.8)
CL/F	21.6 (15.4)	21.0 (17.6)	(17.3–24.4)
$Vd_{central}$/F	90.1 (27.1)	87.7 (38.5)	(54.7–123.5)
Residual variability			
Proportional error	0.184 (9.6)	0.181 (10.2)	(0.144–0.218)

CES1, carboxylesterase 1; CI, confidence interval; CL/F, apparent oral clearance; CV, coefficient of variation; IIV, interindividual variability; MPH, methylphenidate; MTT, mean transit time; PK, pharmacokinetic; Q/F, apparent intercompartmental clearance; RSE, relative standard error; Vdcentral/F, central volume of distribution. k_a, absorption rate constant; NONMEM, non-linear mixed effects modeling software. 2,000 nonparametric bootstrap CI were generated.

to 50.1% compared with the base model without covariates. OFV drop, CL/F IIV reduction, and proportion of explained CL/F IIV to total CL/F IIV increase for the stepwise addition of each covariate is shown in **Table 2**. The two model-included *CES1A1* SNPs, rs71647871 and rs115629050, were not correlated as observed in a linkage disequilibrium analysis (**Supplementary Figure S1**) and both could therefore be incorporated in the model. Permutation testing results for the model-inclusion of covariates are shown in **Supplementary Table S3**.

The equations that describe the typical values of the final model parameters are:

$$\mathrm{MTT} = \theta_1 * (1 + \mathrm{Gender} * \theta_8)$$

$$\frac{\mathrm{CL}}{\mathrm{F}} = \theta_4 * (1 + \mathrm{rs71647871} * \theta_9) * (1 + \mathrm{One\ CES1A2} * \theta_{11}) * (1 + \mathrm{Two\ CES1A2} * \theta_{12}) * (1 + \mathrm{rs115629050} * \theta_{14}) * \left(\frac{\mathrm{WT}}{70}\right)^{0.75}$$

$$\mathrm{Vd_{central}/F} = \theta_5 * \left(\frac{\mathrm{WT}}{70}\right)$$

$$\mathrm{Q/F} = \theta_6 * \left(\frac{\mathrm{WT}}{70}\right)^{0.75}$$

$$\mathrm{Vd_{peripheral}/F} = \theta_7 * \left(\frac{\mathrm{WT}}{70}\right)$$

The variables *Gender*, *rs71647871*, *rs115629050*, *one CES1A2*, and *two CES1A2* are equal to zero and take on the value of one for individuals that possess the gender or genotype reported in **Table 1**. WT represents the body weight of the individual subject. Goodness of fit plots and a visual predictive check for the final model are shown in **Figures 2** and **3**, respectively.

Results of the Monte-Carlo simulations highlighting the independent effects of each model-incorporated covariate on *d*-MPH plasma exposure are shown in **Figure 4**. Significant increases in *d*-MPH plasma exposure compared with a wild-type reference population were observed for carriers of 71647871 GA (143%), two *CES1A2* (70%), as well as rs115629050 TG (68%), whereas a minor increase was seen for carriers of one *CES1A2* (22%). Populations consisting of 50 kg as well as 100 kg individuals were simulated to

Table 2 Effect of each included covariate on the objective function value, decrease in unexplained *d*-MPH apparent clearance (CL/F) IIV, and increase in explained *d*-MPH CL/F IIV

Model	OFV decrease	Unexplained CL/F IIV decrease (% CV)	Explained CL/F IIV increase (%)
Model A: Base model + body weight	20.068	1.6	10.2
Model B: Model A + rs71647871	38.874	8.6	19.3
Model C: Model B + rs115629050	15.888	2.3	10.5
Model D: Model C + gender	12.693	0.40	1.5
Final model: Model D + *CES1* diplotype	16.820	2.1	8.6

CL/F, apparent oral clearance; CV, coefficient of variation; IIV, interindividual variability; MPH, methylphenidate; OFV, objective function value. Base model represents the final structural model without covariates. Unexplained CL/F IIV was assessed through NONMEM's OMEGA output. Explained CL/F IIV was calculated as explained CL/F IIV divided by total CL/F IIV (total CL/F IIV being the sum of unexplained and explained CL/F IIV and is subject to change during model development), and explained and total parametric variability estimates were obtained from *pvar* in Perl-Speaks-NONMEM (PsN).

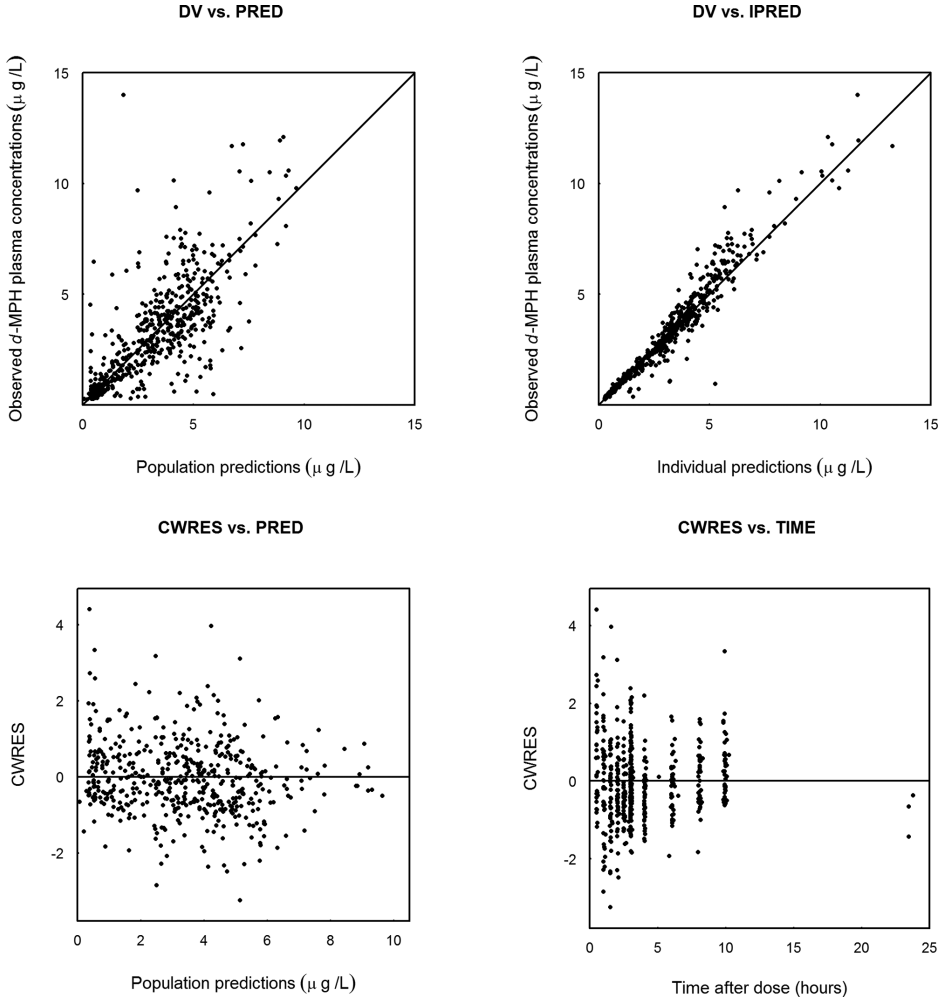

Figure 2 Diagnostic plots for the final *d*-methylphenidate (MPH) population pharmacokinetic model. Top left corner: Observed *d*-MPH plasma concentrations (DV) vs. population predictions (PRED). Top right corner: Observed *d*-MPH plasma concentrations (DV) vs. individual predictions (IPRED). Bottom left corner: Conditionally weighted residuals (CWRES) vs. PRED. Bottom right corner: CWRES vs. time after dose.

explore the effect of low or high body weight, respectively, on *d*-MPH exposure. A 29% *d*-MPH exposure increase and 24% *d*-MPH exposure decrease was observed for individuals of 50 and 100 kg, respectively. However, differences in body weight did not alter the effects of the *CES1* variants to a significant extent compared with their independent effects in 70 kg individuals (**Supplementary Table S4** and **Supplementary Figure S2**).

The simulation results investigating the effect of the physiologically plausible simultaneous presence of two or more *CES1* variants on *d*-MPH plasma exposure are shown in **Figure 5**. Twofold to sevenfold plasma *d*-MPH exposure increases resulting from different variant combinations were observed. In order to estimate the frequency of occurrence of *CES1* variants as well as their plausible combined presence in individuals, a simulation of a large virtual population

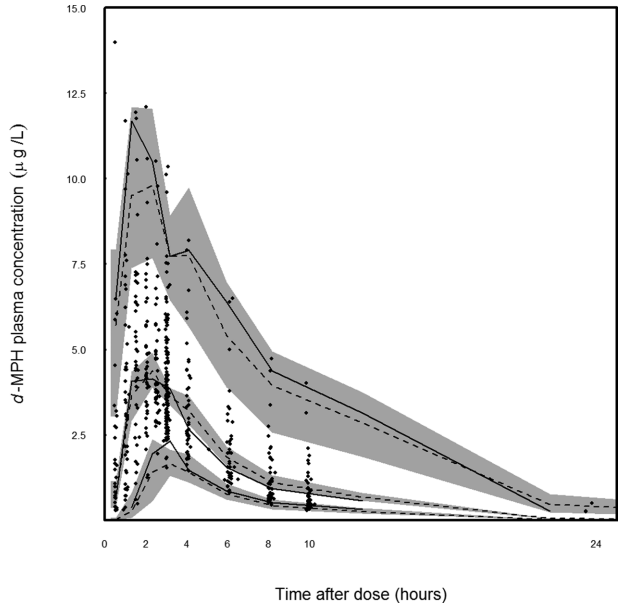

Figure 3 Visual predictive check for the final *d*-methylphenidate (MPH) population pharmacokinetic model. Solid lines represent the 5th, 50th, and 95th percentiles of the observed data. Dashed lines represent the 5th, 50th, and 95th percentiles of the simulated data. Shaded areas represent 95% confidence intervals around the 5th, 50th, and 95th percentiles of the simulated data.

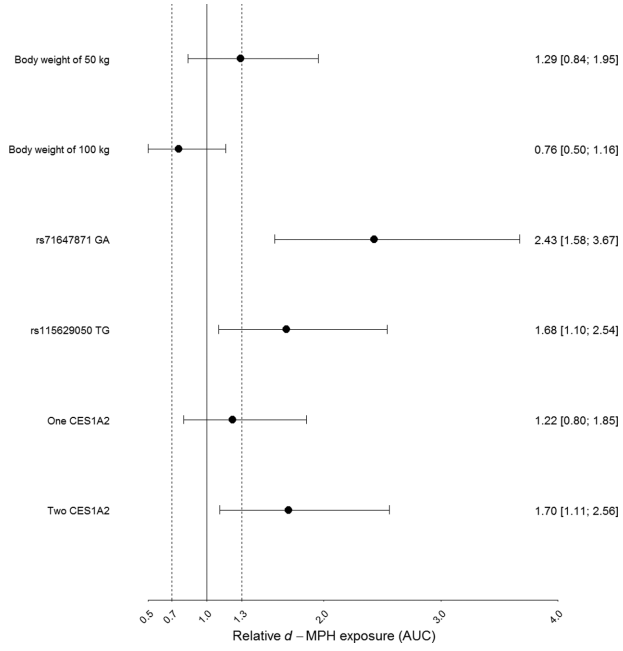

Figure 4 Forest plot of the simulated independent effect of each model-included covariate. Effects are expressed as *d*-methylphenidate (MPH) plasma exposure (area under the curve $[AUC]_{0\text{-inf}}$) relative to a reference population (70-kilogram male adults, carrying the wild-type carboxylesterase 1 [*CES1*] variants rs71647871 GG, rs115629050 GG, and no *CES1A2*). The independent effect of each covariate is investigated, keeping all other characteristics similar to the reference population. Median $AUC_{0\text{-inf}}$, followed by 95% prediction intervals in brackets are reported for each population, consisting of 5,000 virtual individuals. Dotted lines indicate $AUC_{0\text{-inf}}$ changes of ± 30%, which was considered clinically significant in this study.

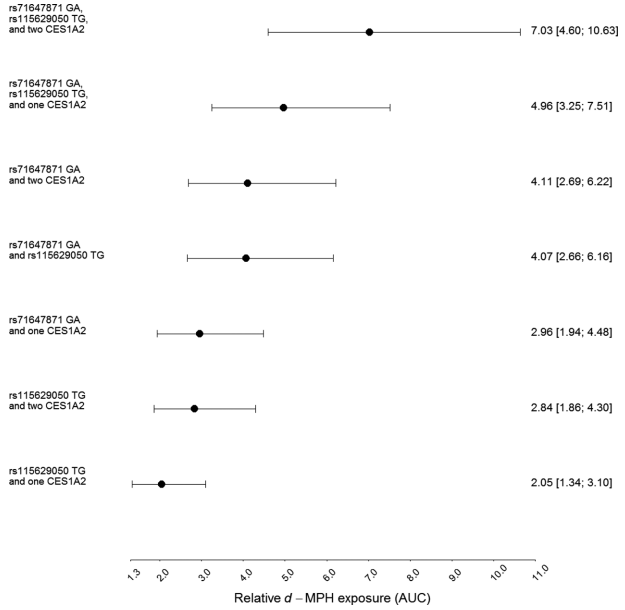

Figure 5 Forest plot of the physiologically plausible simulated combined effects of model-included covariates. Effects are expressed as *d*-methylphenidate (MPH) plasma exposure (area under the curve $[AUC]_{0\text{-inf}}$) relative to a reference population (70-kilogram male adults, carrying the wild-type carboxylesterase 1 [*CES1*] variants rs71647871 GG, rs115629050 GG, and no *CES1A2*). The combined effects of each covariate are investigated, keeping all other characteristics similar to the reference population. Median $AUC_{0\text{-inf}}$, followed by 95% prediction intervals in brackets, are reported for each population, consisting of 5,000 virtual individuals.

($n = 100{,}000$) was performed (**Supplementary Tables S5** and **S6**). It was found that approximately 4.1% of the total simulated population showed a *d*-MPH plasma exposure increase between twofold and threefold while carrying *CES1* variants or variant combinations. It is to be noted that 5.4% of individuals (234 of 4,339 subjects) presenting a plasma exposure increase between two to threefold were carriers of the wild-type CES1 genotype (**Supplementary Table S6**). Furthermore, approximately 1.3% of the virtual population consisting of 100,000 individuals showed a larger than threefold plasma exposure increase while carrying *CES1* variants or combinations. In comparison, only 0.3% of the total simulated population showed a larger than threefold elevated *d*-MPH exposure while carrying the wild-type CES1 genotype (**Supplementary Table S6**). Thus, these results indicate that a small minority of a given Caucasian population undergoing treatment with MPH may be predisposed to experience substantially higher *d*-MPH plasma exposure owing directly to specific *CES1* variants or variant combinations. Subsequently, such patients may potentially have a higher risk of adverse effects during MPH treatment.

DISCUSSION

This study is the first to examine the population PK of *d*-MPH while including effects of both demographic covariates and *CES1* variants. Model-based analyses investigating the PK

of MPH are limited,[9,34] and none have included information concerning *CES1*.

Rs71647871 GA (p.Gly143Glu) was found to significantly reduce *d*-MPH apparent oral CL/F and this is in accordance with previously reported findings.[13] Its effect has also been identified on the PK of several other CES1-metabolized drugs.[25–27] Incorporation of rs71647871 was found to decrease *d*-MPH CL/F unexplained IIV and increase explained CL/F IIV to a greater extent than inclusion of other covariates. This indicates that rs71647871 is a very important covariate in determining MPH PK interindividual differences. The simulated independent presence of rs71647871 GA resulted in a 2.4-fold increase in *d*-MPH plasma exposure, and, hence, this variant could potentially be considered an adverse effect risk factor during MPH treatment.

This study found that rs115629050 TG (p.Ala270Ser) significantly reduces *d*-MPH CL/F and this is, to our knowledge, the first time this has been reported. Model incorporation of this variant was found to reduce unexplained and increase explained *d*-MPH CL/F IIV to a reasonable level compared with the inclusion of other model-included covariates. Simulations showed that rs115629050 TG resulted in approximately 68% higher *d*-MPH plasma exposure in carriers compared with the wild-type genotype. The only current evidence, which supports the observed effect of this *CES1A1* SNP, are *in silico* CES1 functional annotation predictions, performed using different software packages, and a total of nine different scoring methods (**Supplementary Table S7**). For rs115629050 TG, the scores were equal to or greater than those of rs71647871 GA in six of the nine approaches (**Supplementary Table S8**). However, *in vitro* studies regarding the impact of *CES1* variants on a series of CES1-metabolized angiotensin-converting enzyme inhibitors have not been able to identify the effect of rs115629050 TG on drug metabolism.[39] These conflicting results may arise from differences in CES1 affinity to the aforementioned angiotensin-converting enzyme inhibitors and MPH, resulting in a lack of effect of rs115629050 TG. Furthermore, it should be noted that extrapolation of *in vitro* results to clinical study observations is difficult. Additional studies need to be performed to confirm the effect of rs115629050 TG on CES1 activity and MPH PK.

CES1 diplotypes were associated with significantly lower estimates of *d*-MPH CL/F. Furthermore, model-inclusion of *CES1* diplotypes decreased unexplained and increased explained *d*-MPH CL/F IIV to a reasonable level compared with the other model-included covariates. Simulations indicated that two *CES1A2* copies resulted in approximately 70% higher *d*-MPH plasma exposure in carriers compared with the wild-type genotype, whereas one *CES1A2* copy yielded an approximately 22% increase. These findings are in conflict with those from a study with CES1-metabolized irinotecan, in which presence of *CES1A2* resulted in an increase in systemic clearance.[28] Additionally, a clinical PK study involving oseltamivir found no effect associated with *CES1* diplotypes.[40] Therefore, additional studies need to be performed to confirm the effect of *CES1* diplotypes on CES1 activity and MPH PK.

Body weight was not found to alter the observed effects of model-included *CES1* variants to a significant extent. This may imply that when adjusting dose according to weight, carriers of plasma exposure-increasing *CES1* variants, included in the current study, may still experience significantly increased MPH plasma exposures, and potentially be in higher risk of adverse effects. A preliminary study investigating the body weight-based daily dose required for symptom reduction in patients with attention deficit hyperactive disorder, has shown that significantly lower MPH doses were needed in patients carrying rs71647871 GA compared with noncarriers.[41] The completion of similar studies focusing on *CES1* variants identified in this study is of interest to establish the clinical relevance of the findings.

To date, only rs71647871 GA has been reported to have significant impact on MPH PK,[13] yet these findings have revealed that significant increases in *d*-MPH exposure may also occur due to other *CES1* variants. Within a large population, it is possible for several genetic and/or demographic covariates to co-occur in individuals. Through simulation, it was observed that the simultaneous presence of several different *CES1* variants may result in severe increases in *d*-MPH plasma exposure, ranging from twofold to sevenfold. Such elevated exposures may put patients in high risk of developing adverse effects, which could potentially be serious as well as sudden. In this context, diagnosed or undiagnosed preexisting conditions (e.g., congenital heart disease) may further contribute to the occurrence of such aforementioned events.[17] In adults, a significant increase in the use of stimulants to treat attention deficit hyperactive disorder has been observed.[42,43] Compared with children, adult patients may present risk factors that are more prevalent (e.g., coronary atherosclerosis, hypertension, smoking, or concomitant use of other drugs)[22] and may, therefore, be predisposed to a higher risk of serious complications during treatment. A recent study has indicated a significant increase in risk of ventricular arrhythmia and sudden death in adults treated with MPH compared with nonusers, although it was not possible to determine a causal effect due to lack of a dose-response relationship.[43] However, in light of the current study's findings, it could hypothetically be more appropriate to consider a dose-concentration-response relationship, given that individuals with different genotypes may experience markedly different concentrations with the same dose. Despite the preliminary low incidence estimate of *CES1* variant combinations that result in substantial exposure increases, it is important to consider the widespread use of MPH. Hence, the reported variants and combinations have cause to be taken into clinical consideration, along with the individual patient's possible preexisting clinical risk or family history. It is important to note, however, that improvement of clinical outcome as well as cost-effectiveness need to be evaluated before routine pretreatment screening of patients' CES1 genotype can be implemented in MPH treatment. Considering the high cost of genetic sequencing and reported low incidence estimates of risk factor *CES1* variants in a large population, implementation of *CES1* screening is not currently expected to be cost-effective; however, with evolving technology and decreasing prices of genetic assays, *CES1* screening may be a viable tool in the future. Clinical implementation of *CES1* testing could on the other hand potentially occur in specific cases (e.g., should specific patients

exhibit unexpected dose-dependent adverse effects at standard therapeutic doses). In such cases, *CES1* genotyping could be used as a diagnostic tool to clarify if patients are carriers of risk factor *CES1* variants, and thereby help guide MPH dose adjustment.

In this study, large amounts of missing data were observed for several *CES1A1* SNPs (**Supplementary Table S2**). Missing genotype data were in a large part due to a limitation of the genetic analysis, which is not able to provide sequence information downstream exon 5 in *CES1A1* if *CES1A2* is present. To the best of our knowledge, no assays currently exist that can overcome the limitation of the current method. The high sequence homology among *CES1A1*, *CES1P1*, and *CES1A2* renders it difficult to develop methods that can differentiate between the three genes. Taking the aforementioned limitation of the genetic analysis into account, as well as the proportion of different missing genotypes, in this study, missing data were considered as being missing not at random. The EXTRA (or EST) method, which quantifies the effect of missing covariate data through full maximum likelihood modeling and adds a parameter for each covariate containing missing data, was therefore chosen due to its precise and unbiased parameter estimates when dealing with missing not at random data, as well as its ease of implementation in stepwise covariate procedures.[37,38] Genotype information regarding rs115629050, located in *CES1A1* exon 7, was found to be missing in 42% of the study population, which was high compared with the amount of missing data observed in the other final model-included *CES1* variants. However, for low (10%) and high (50%) levels of missing categorical covariate data, the EXTRA method has been shown to perform similarly well, yielding estimates of PK parameters and covariate effects with adequately low bias and imprecision.[37] Other limitations possibly include that all carriers of rs115629050 TG as well as a large part of single *CES1A2* carriers were present in the very sparsely sampled study II (**Supplementary Table S2**), and this may have influenced accurate estimation of the effect of these variants on *d*-MPH CL/F. When analyzing sparse data containing missing covariate data, a higher bias/imprecision for the covariate effect magnitude may be observed.[37] This may have implications for the simulation results in this study, and these should therefore be viewed as preliminary. However, concerning type I errors associated with final model-inclusion of these covariates, the actual significance levels for their exclusion from the final model were found to be lower than the threshold applied in this study (**Supplementary Table S3**). Therefore, rs115629050 and *CES1* diplotypes were ultimately kept in the final model. Furthermore, despite having applied methods for dealing with missing covariate data, it cannot be ruled out that the currently reported *CES1A* diplotype effect on *d*-MPH CL/F is caused by the presence of SNPs downstream *CES1A1* exon 5 that affect MPH PK. Therefore, due to the previously mentioned conflicting findings with other CES1-metabolized drugs, possibly inaccurate/imprecise reported covariate effect magnitude, and possible presence of unidentifiable *CES1A1* SNPs downstream exon 5 in subjects carrying *CES1A2*, additional studies are needed to confirm the effects of rs115629050 TG and *CES1* diplotypes on CES1 activity and MPH PK. Last, in light of the presented results, the need for novel genetic assays that are able to determine SNPs in all *CES1A1* exons yet still distinguish *CES1A1* from *CES1A2*, is greater than ever before. Additional study limitations are described in the **Supplementary Material**.

To conclude, the presented work has developed a population *d*-MPH PK model that includes the effects of *CES1* variants. Novel effects of *CES1* variants on MPH PK were revealed and require further investigation. This study has contributed to a better understanding of factors, which may be determinants of interindividual differences in MPH PK. Through additional studies, it may also contribute to the accurate estimation of variability in MPH response, as well as the risk of adverse effects, which in rare cases may be serious. Last, the need for novel and more informative *CES1* assays has also been made clear.

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