Article type : Article

Plasma carboxylesterase 1 predicts methylphenidate exposure: a proof-of-concept study using plasma protein biomarker for hepatic drug metabolism

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Conflict of Interest statement

The authors declared no competing interests for this work.

Funding information

This work was partially supported by the National Institute on Alcohol Abuse and Alcoholism [R01AA016707, Kennerly S. Patrick] and the Eunice Kennedy Shriver National Institute of Child Health and Human Development [R01 HD093612, John S. Markowitz and Hao-Jie Zhu].

Keywords: Hepatic Drug-Metabolizing Enzymes; Plasma Protein Biomarker; Carboxylesterase 1; Methylphenidate; Pharmacokinetics

Abstract

Hepatic drug-metabolizing enzymes (DMEs) play critical roles in determining the pharmacokinetics and pharmacodynamics of numerous therapeutic agents. As such, noninvasive biomarkers capable of predicting DME expression in the liver have the potential to be used to personalize pharmacotherapy and improve drug treatment outcomes. In the present study, we quantified carboxylesterase 1 (CES1) protein concentrations in plasma samples collected during a methylphenidate (MPH) PK study. CES1 is a prominent hepatic enzyme responsible for the metabolism of many medications containing small ester moieties, including MPH. The results revealed a significant inverse correlation between plasma CES1 protein concentrations and the area under the concentration-time curves (AUCs) of plasma d-MPH (p = 0.014, r = -0.617). In addition, when plasma CES1 protein levels were normalized to the plasma concentrations of 24 liver-enriched proteins to account for potential interindividual differences in hepatic protein release rate, the correlation was further improved (p = 0.003, r = -0.703), suggesting that plasma CES1 protein could explain approximately 50% of the variability in d-

MPH AUCs in the study participants. A physiologically based pharmacokinetic (PBPK) modeling simulation revealed that the CES1-based individualized dosing strategy might significantly reduce d-MPH exposure variability in pediatric patients relative to conventional fixed dosing trial and error regimens. This proof-of-concept study indicates that the plasma protein of a hepatic DME may serve as a biomarker for predicting its metabolic function and the pharmacokinetics of its substrate drugs.

Introduction

Hepatic drug-metabolizing enzymes (DMEs) are essential determinants of the pharmacokinetics (PK) and pharmacodynamics (PD) of many medications. Hepatic DME expression varies markedly between individuals, which is a major factor contributing to interindividual variability in drug response. Accordingly, the ability to measure DME expression levels in the liver of a patient could permit the development of a personalized therapeutic regimen to improve the efficacy and safety of drug treatment. Obviously, accessing patient liver tissues in clinical practice is not an option; thus, the identification of noninvasive biomarkers capable of predicting hepatic DME levels are of substantial clinical interest.

DME genetic polymorphisms (i.e., pharmacogenomics) have been intensively studied in the past two decades.[1] Many genetic variants have been identified that are associated with variability in DME expression levels, and some polymorphisms have been adopted as genetic biomarkers to guide the clinical use of various medications.[2] However, for most DMEs, the genetic variants identified to date can only explain a modest portion of the variability in their hepatic expression. Although the majority of DMEs are primarily expressed in the liver, trace levels of many hepatic DMEs can be detected in human plasma using modern LC-MS/MS-based proteomics technologies.[3, 4] As with the hepatic enzymes (e.g., alkaline phosphatase, alanine transaminase, aspartate aminotransferase) routinely used in the clinic for evaluating liver damage or disease, these plasma-detectable hepatic DMEs are likely released into the systemic circulation from the liver. Therefore, it is plausible that plasma concentrations of hepatic DME proteins can be reflective of their abundances in the liver.

Carboxylesterase 1 (CES1) is the most abundant hydrolase in the liver and plays an important role in metabolizing numerous therapeutic agents, endogenous compounds, and environmental toxins.[5] Human CES1 is primarily retained in the endoplasmic reticulum (ER) due to its C-terminal HIEL sequences capable of binding to the KDEL receptor in the ER.[6] Interestingly, in

rats and mice, Ces1c, one of the Ces1 protein isoforms, lacks the C-terminal HXEL ER retrieval sequence and, thus, can be released from the liver into the blood, resulting in a high level of plasma Ces1 in rodents.[7, 8] Marked interindividual variability in hepatic CES1 expression has been well documented,[9-11] and is associated with significant variation in the PK and PD of CES1 substrate drugs. One such drug is methylphenidate (MPH), which is selectively metabolized by CES1 in the liver.[12] MPH is the medication most commonly used worldwide for treatment of patients with attention deficit and hyperactive disorder (ADHD) with the d-isomer responsible for efficacy. Prior studies have demonstrated that CES1 functional status significantly affects the exposure and clinical outcomes of MPH treatment.[13, 14] In a previously published study, we investigated the interaction between MPH and the CES1 inhibitor ethanol in healthy human subjects.[15] In the present study, we utilized plasma samples collected in the course of that prior work and quantified plasma CES1 protein concentrations in study participants who received dl-MPH only. Our analysis revealed a significant correlation between CES1 protein concentration and MPH exposure. To our knowledge, this proof-of-concept study represents the first attempt to demonstrate that plasma hepatic DME proteins have the potential to serve as biomarkers predicting the metabolism of their substrate drugs in the liver.

Methods

Reagents

Dynabeads® M-280 Tosylactivated were purchased from Thermo Fisher Scientific (Vilnius, Lithuania). Anti-CES1 antibody (ab45957) was purchased from Abcam (Waltham, MA). Ammonium sulfate, boric acid, bovine serum albumin (BSA), and trifluoroacetic acid were purchased from Sigma-Aldrich (Saint Louis, MO). Acetonitrile, acetone, dithiothreitol, urea, and water with 0.1% formic acid were purchased from Fisher Scientific (Pittsburgh, PA). Iodoacetamide and ammonium bicarbonate were the products of Acros Organics (Morris Plains, NJ). Acetonitrile with 0.1% formic acid was obtained from J.T. Baker Chemical Company (Phillipsburg, NJ). TPCK-treated trypsin was obtained from Worthington Biochemical Corporation (Freehold, NJ). Lysyl endopeptidase was purchased from Wako Chemicals (Richmond, VA). The Oasis HLB columns were from Waters Corporation (Milford, MA). Pierce™ BCA protein assay kits were purchased from Thermo Fisher Scientific (Waltham, MA). Human recombinant CES1 was purchased from R&D Systems (Minneapolis, MN). Blank human plasma

was obtained from Innovative Research (Novi, MI). All other chemicals were of analytical grade and commercially available.

Study subjects

Plasma samples remaining from a previous healthy volunteer study of racemic MPH and ethanol interaction were utilized in this investigation.[15] The original drug-drug interaction study was a crossover design that included four groups: dl-MPH (40 mg, modified-release) or d-MPH (20 mg, modified-release), with or without ethanol (0.6 g/kg). Only samples collected from subjects who received modified-release dl-MPH alone (n = 15) were utilized in this investigation because other groups had limited sample availability. The formulation was a 50:50 mixture of d-threo-(R:R)-MPH and l-threo-(S:S)-MPH isomers (Figure S1). Study participants were all Caucasian, with eight males and seven females. The study design of the original investigation was detailed in the previous publication.[15]

LC-MS/MS-based proteomics analysis

Immunoprecipitation

Dynabeads® M-280 Tosylactivated (Invitrogen) were coated with anti-CES1 antibody (Abcam ab45957) according to the manufacturer's instructions. The procedure described below is based on 5 mg beads and 100 µg antibody and can be scaled up as required. Briefly, 165 µL beads (5 mg) were washed with 1 mL of 0.1 M borate buffer, pH 9.5 (buffer A), and the supernatant was discarded after the tube was placed on a magnet for 1 min. The beads were resuspended in 165 µL buffer A and were transferred to a new tube. The supernatant was again removed using a magnet. The beads were then resuspended in 100 µL anti-CES1 antibody (100 µg), 50 µL buffer A, and 100 µL 3 M ammonium sulfate in buffer A (buffer C). The mixture was incubated on a roller mixer at 37 °C for 12-18 h, after which the tube was placed on a magnet for 2 min, and the supernatant was removed. The beads were then blocked by phosphate-buffered saline (PBS), pH 7.4, containing 0.5% (w/v) BSA (buffer D) on a roller at 37 °C for 1 h. The antibody-coated beads were washed twice using 1 mL PBS, pH 7.4, with 0.1% (w/v) BSA (buffer E) and resuspended in 240 µL buffer E for future use.

A stable isotope labeling with amino acids cell culture (SILAC) internal standard was utilized to ensure the robustness and accuracy of the assay. Each plasma sample (1 mL) was mixed with 6.68 µg S9 fraction proteins prepared from SILAC CES1-expressing HEK293 cells as described

previously.[9] For standard curve samples, different amounts of recombinant CES1 (0, 10, 25, 50, 100, 200, 500 ng) and 6.68 µg SILAC CES1 S9 fraction proteins were spiked into 1 mL blank plasma. After a brief vortex, 250 µg of the antibody-coated beads solution was added to the mixture. Following incubation on a roller at 37 °C for 2 h, the beads were washed three times with buffer E. Finally, the CES1 proteins were eluted twice from the beads using 75 µL PBS (pH 2.8).

Proteomics sample preparation and LC-MS/MS analysis

A ten-fold volume of pre-cooled acetone was added to the plasma (containing 80 µg total protein) and the immunoprecipitation samples to precipitate proteins. The precipitated proteins were subjected to proteomic sample preparation based on a previously reported method.[16] The extracted peptides were analyzed on an LC-MS/MS system consisting of a TripleTOF 5600+ mass spectrometer (AB Sciex, Framingham, MA) and an Eksigent 2D plus LC system (Eksigent Technologies, Dublin, CA). A parallel reaction monitoring (PRM) method was utilized to analyze the CES1 immunoprecipitation samples, with LC separation performed via a trap-elute configuration.[17] The MS parameters of the PRM acquisition are listed in Table S1. Digested plasma samples were analyzed using a data-dependent acquisition (DDA) approach detailed in a recent plasma proteomics study.[18]

Comparison of d-MPH exposure between fixed dosing and CES1-based individualized dosing regimens using physiologically based pharmacokinetic (PBPK) modeling

We developed a d-MPH PBPK model to simulate d-MPH exposure ($AUC_{0\text{-}24h}$) following dl-MPH treatment in a pediatric population using PK-Sim® (Open Systems Pharmacology Suite, Version 9.0). The simulation included two groups: one group received a fixed single oral dose of immediate-release dl-MPH (20 mg), and another group was treated with individualized doses of dl-MPH based on the ratios of individual hepatic CES1 protein abundance to the mean CES1 level in the population. Given that human plasma CES1 is insignificant for drug metabolism because of its extremely low plasma level, hepatic CES1 level was utilized to individualize dl-MPH dose during the PBPK model development, assuming that plasma CES1 concentrations are reflective of hepatic CES1 abundances. The mean CES1 protein concentration and its standard deviation (SD) in the human liver (22.9 ± 8.0 µmol/L liver volume) were derived from a previously published proteomics study.[19] Other key parameters of the PBPK model are listed in Table S2.

Data analysis

DDA data generated from the plasma samples were searched against a human reference proteome using the MaxQuant software (Version 1.6.12.0, Max Planck Institute of Biochemistry, Germany) with default settings. The reference human proteome fasta file was downloaded from Uniprot on 4/1/2020, which contains 20,350 protein entries. Trypsin/P was the digestion enzyme.

PRM data from the CES1 immunoprecipitation samples were analyzed using the Skyline software (University of Washington) with automatic MS/MS chromatographic peak matching against a spectral library generated from an in-house human liver S9 fraction DDA data set. All chromatographic peaks were checked manually following the automated matches. The resolution powers of MS1 and MS/MS filtering were 30,000 and 15,000, respectively, and the "Targeted" acquisition method was used in MS/MS filtering.

Plasma CES1 protein abundances were determined based on the light-to-heavy ratios of three CES1 surrogate peptides (Table S1). The calibration curve exhibited excellent linearity in the range of 10 - 500 ng/mL (R^2 > 0.99, Figure S2).

Given that plasma CES1 concentrations could be affected by both CES1 abundance in the liver and the hepatic protein release rate, differences in release rates among individuals were accounted for by normalizing plasma CES1 concentrations to the average plasma concentrations of liver-enriched proteins using the following equation:

$$CES1_{i\_normalized} = CES1_i \,/\, \left(\sum_{j=1}^{m} \left(LEP_{ij} \Big/ \left(\sum_{i=1}^{n} LEP_{ij} \Big/ n\right)\right) / m\right) \quad \text{(Equation 1)}$$

$CES1_{i\_normalized}$: plasma CES1 concentration in subject i normalized by the plasma concentrations of liver-enriched proteins (LEP); $CES1_i$: plasma CES1 concentration in subject i as measured by LC-MS/MS assay; LEP_{ij}: plasma level of the liver-enriched protein j in subject i. Liver-enriched proteins are those having at least four-fold higher mRNA levels in the liver compared to any other tissue;[20] a total of 242 liver-enriched proteins are registered in the Human Protein Atlas database (Table S3).[20] It is assumed that the liver-enriched proteins detected in the plasma are mainly released from the liver, and thus, the average interindividual differences in plasma concentrations of these liver-enriched proteins should reflect differences in hepatic protein release rates among the study subjects. The normalization procedure includes two steps: 1) normalization of LEP_j in subject i to the mean LEP_j concentration of all 15 study participants (i.e.,

$LEP_{ij} / (\sum_{i=1}^{n} LEP_{ij} / n)$); and 2) normalization of CES1 in subject i to the mean value of normalized LEP concentrations in subject i (i.e., $CES1_i$ / ($\sum_{j=1}^{m} (LEP_{ij} / (\sum_{i=1}^{n} LEP_{ij} / n)) / m$)). The overall study design is illustrated in Figure 1.

Results

Plasma proteomics analysis

We quantified CES1 protein concentrations in plasma samples collected from a previous MPH PK study[21] using an antibody immunoprecipitation-based targeted proteomics method. The plasma CES1 protein concentrations were found to vary markedly among study participants, ranging from 55.4 to 182.2 ng/mL (Figure 2). We also determined the global proteomes of the plasma samples using an unlabeled DDA method and quantified a total of 179 protein groups (Table S4), in which 24 are liver-enriched proteins,[22] including HPX, FGA, APOH, APOA2, AHSG, ITIH2, A1BG, ITIH1, ALB, VTN, HP, CFB, CP, ITIH4, ORM1, SERPINA1, GC, TF, PLG, C3, KNG1, SERPINA3, CFH, APOA1 (Figure 3). Plasma CES1 concentrations were then normalized to the plasma levels of those 24 liver-enriched proteins (Equation 1) to account for the differences in hepatic protein release rates between individuals (Figure 2).

Correlation between plasma CES1 protein and d-MPH AUCs

We found a significant negative correlation between plasma CES1 protein concentrations and the area under the concentration-time curve from time 0 to the last measurement ($AUC_{0\text{-last}}$) of plasma d-MPH (Figure 4A, p = 0.014, r = -0.617). A more significant correlation was observed after plasma CES1 protein concentrations were corrected for the hepatic protein release rates (Figure 4B, p = 0.003, r = -0.703), indicating that plasma CES1 alone could explain approximately 50% of the interindividual variability in d-MPH exposure among human subjects treated with a single dose of dl-MPH.

CES1-based individualized dosing regimen reduced d-MPH exposure variability

We simulated d-MPH exposure in pediatric patients who received a fixed dose or an individualized dose of dl-MPH based on the individual's hepatic CES1 protein level. While the

mean d-MPH $AUC_{0\text{-}24h}$ values were comparable between the two dosing regimens, the coefficient of variation of d-MPH $AUC_{0\text{-}24h}$ in patients who received individualized doses was reduced by approximately 50% compared to those treated with a fixed dose of dl-MPH ($AUC_{0\text{-}24h}$: 104.8 ± 134.7 versus 84.8 ± 58.9 ng×h/mL, Figure 5).

Discussion

The present study demonstrated a significant negative correlation between plasma CES1 protein concentration and d-MPH AUC in healthy subjects, indicating the utility of measuring plasma proteins of liver-enriched DMEs for predicting the hepatic metabolism and PK of their substrate drugs. Our PBPK modeling simulation revealed that a CES1-guided individualized dosing regimen might significantly reduce d-MPH exposure variability in pediatric patients. The findings suggest that plasma CES1 protein has the potential to serve as a biomarker to individualize the dosing regimen of CES1 substrate medications.

The modified-release dl-MPH formulation utilized in the study incorporates both immediate-release and delayed-release components, and the two components exhibit distinct MPH release and absorption profiles, resulting in two plasma d-MPH peaks appearing at 1.5 h and 6 h after drug administration.[15] Therefore, plasma C_{max} is not a reliable indicator of d-MPH exposure. In fact, we observed a trend of negative correlation between d-MPH C_{max} and plasma CES1 protein concentration; however, the correlation is not statistically significant. Notably, relative to d-MPH, the pharmacologically inactive isomer l-MPH is more rapidly metabolized by CES1.[12] Consequently, plasma l-MPH concentrations were below the limit of quantification in many samples in the present study, preventing an accurate determination of l-MPH AUC. Thus, d-MPH AUC is the parameter of choice for estimating MPH exposure in this investigation.

As one of the most abundant hepatic DMEs, CES1 plays a critical role in metabolizing various endogenous and exogenous compounds.[5, 23] CES1 enzymatic function varies markedly between individuals,[10, 11, 13, 24] which is associated with interindividual variability in the PK and

PD of many medications metabolized by CES1. Therefore, biomarkers capable of predicting CES1 function could enable the optimization of pharmacotherapy regimens for drugs metabolized by the enzyme. Considerable efforts have been devoted to identifying CES1 biomarkers such as genetic variants and non-genetic regulators.[5, 25-27] In particular, many investigations have explored the effects of genetic polymorphisms on the activity and expression of CES1 and ensuing impacts on the PK and clinical outcomes of CES1 substrate drugs. However, while several CES1 genetic variants have been found to affect CES1 activity and expression, only the nonsynonymous variant G143E showed consistent effects on both the PK and PD of CES1 substrates such as MPH, clopidogrel, and enalapril.[13, 14, 28-32] It should be noted that the G143E variant impairs CES1 catalytic activity without altering CES1 expression, and hepatic CES1 expression varies markedly among individuals having the same G143E genotype.[10, 11] Similarly, the PK of CES1 substrate drugs differed significantly among subjects in the same G143E genotype group.[29, 31-33] Therefore, beyond the G143E variant, the identification of a reliable biomarker of hepatic CES1 protein expression is of significant clinical interest. Our findings suggest that plasma CES1 protein has the potential to be developed as a noninvasive biomarker for estimating CES1 protein abundance and metabolic function in the liver.

The plasma proteome is dominated by several highly abundant proteins (e.g., albumin) and has a large dynamic range in protein concentration,[34, 35] making it challenging to quantify low-abundance plasma proteins. We found it difficult to reliably quantify plasma CES1 using conventional targeted and global proteomics with our experimental settings. In the present study, we adopted an antibody-based immunoprecipitation method to enrich plasma CES1 prior to LC-MS/MS analysis. Moreover, the assay included stable heavy isotope-labeled SILAC CES1 protein as an internal standard to ensure the robustness and accuracy of quantification. Our study revealed significant interindividual variability in plasma CES1 protein concentrations (55.4 ~ 182.2 ng/mL). These concentrations are substantially lower than that in the liver, and in fact, plasma CES1 was insignificant for drug metabolism because of its low abundance.[36] In addition, we recently developed an antibody-free, two-dimensional LC-based proteomics assay for the analysis of low-abundance proteins in human plasma and successfully applied it to quantify plasma CES1 protein.[18] It should be noted that, although being highly sensitive and specific, the LC-MS/MS-based CES1 quantification method is technically demanding. We envision that a more clinically feasible assay such as ELISA could be developed in the future to measure plasma CES1 protein concentrations after the clinical utility of plasma CES1 is fully validated.

Plasma exosomes and small extracellular vesicles (sEVs) have recently attracted considerable interest in the field of precision pharmacotherapy.[37] Achour and colleagues showed that plasma exosomal mRNA concentrations of many DMEs and transporters were highly correlated to their protein levels in matched liver tissue samples and advocated the use of plasma exosomes as a means of "liquid biopsy" for biomarker discovery for precision pharmacotherapy.[38] However, poor correlations of hepatic mRNA and protein expression have been found for many DMEs,[39, 40] including CES1.[41] Our previous investigation showed that neither CES1 protein expression nor its activity was significantly correlated to CES1 mRNA expression in the liver,[41] indicating that exosomal CES1 mRNA is unlikely to be a valid biomarker for the PK of CES1 substrates. In addition to exosomal DME mRNA, DME proteins in plasma exosomes and sEVs may also have the potential to serve as biomarkers for hepatic DME function. Two recently published studies showed that CYP3A4 and CYP2D6 protein expression in plasma exosomes/sEVs correlated with the hepatic clearances of midazolam and dextromethorphan, respectively[42, 43]. However, while the mRNA and protein contents of plasma exosomes and sEVs are promising biomarkers of corresponding DMEs in the liver, the significant effort involved in exosome/sEVs preparation and the additional variability introduced during that preparation process could hinder the application of these biomarkers in clinical practice.

CES1 is highly enriched in the liver relative to other organs: in the human liver, its protein abundance is about ten-fold higher than that in the next most abundant tissue – the lung.[44] Therefore, it can reasonably be assumed that most plasma CES1 protein is released from the liver, and consequently, plasma CES1 protein concentration should be reflective of hepatic CES1 protein expression. CES1 protein released from extrahepatic tissues is expected to affect plasma CES1 levels to a far less extent. Moreover, it could be advantageous for plasma CES1 protein concentration to be able to collectively reflect overall CES1 abundance in various organs in the body since CES1 substrates could also be metabolized by extrahepatic CES1.

Besides the interindividual variability in hepatic CES1 expression, the potential differences in hepatic protein release rates between individuals could also affect CES1 protein levels in plasma. In a previously published study examining correlations between exosomal and hepatic DMEs and transporters, investigators applied an exosome shedding factor to correct for interindividual variability in liver exosome shedding.[38] In the present study, we performed an untargeted proteomics analysis of the plasma samples and quantified a total of 24 liver-enriched proteins.[22] We re-calculated plasma CES1 protein concentrations by normalizing them to the abundances of the liver-enriched proteins to account for variability in hepatic protein release.

This normalization led to a modest increase in the correlation between plasma CES1 protein levels and d-MPH AUCs (from r = -0.617 to r = -0.703). A total of 242 liver-enriched proteins have been reported in human plasma, but only 24 of those proteins were quantified in the present study, which is due to our study having adopted a non-fraction and non-enrichment method to ensure assay reproducibility and robustness at the expense of protein coverage. Since plasma concentrations of liver-enriched proteins are also affected by their hepatic expression and the interindividual variability in hepatic protein release rate, we expect that including more liver-enriched proteins in the normalization could further enhance the predictive power of plasma CES1 protein on the PK of CES1 substrate drugs. Of note, hepatic protein release rate and pattern could differ between healthy subjects and individuals with liver diseases. Thus, the utility of liver-enriched proteins in normalizing plasma CES1 protein levels warrants further investigations in patients with hepatic dysfunction.

In sum, this study demonstrated a significant association between plasma CES1 and MPH exposure, with plasma CES1 protein concentration alone explaining approximately 50% of the variability in d-MPH AUCs in study participants suggesting its viability as a DME biomarker. For the first time, it has been demonstrated that the plasma protein level of a hepatic enzyme is indictive of its metabolic function and can be used to predict the PK of its substrate drugs. Notably, low levels of many other clinically important hepatic DMEs such as sulfotransferase 1A1, catechol-*O*-methyltransferase, and aldehyde dehydrogenase have also been detected in human plasma, and it is anticipated that still more will be detected as the sensitivity of proteomics assays improves. Therefore, future investigations could be directed to explore whether plasma proteins of those hepatic DMEs could be utilized as biomarkers to improve personalized pharmacotherapy and predict clinical outcomes.

Acknowledgements

We dedicate this work to the memory of Kennerly S. Patrick, Ph.D., scientist, scholar, mentor, and beloved friend. His enthusiasm for life and the natural world and his wit and humor will be sorely missed.

Study Highlights

What is the current knowledge on the topic?

Hepatic drug-metabolizing enzymes (DMEs) are a determinant of pharmacokinetics and pharmacodynamics of many medications. Varied expression levels of DMEs in the liver are associated with interindividual variability in response to pharmacotherapy. Carboxylesterase 1 (CES1) is one of the most abundant DMEs in the liver and plays an important role in metabolizing ester-containing drugs. CES1 hepatic expression affects the pharmacokinetics and clinical outcomes of its substrate drugs.

What question did this study address?

The study was to determine if plasma CES1 protein could serve as a protein biomarker to predict the exposure of its substrate drug methylphenidate in human subjects.

What does this study add to our knowledge?

The study demonstrated a significant correlation between plasma CES1 protein concentration and the area under the concentration-time curve of d-methylphenidate, indicating that plasma levels of a hepatic DME could be reflective of its protein expression and metabolic function in the liver.

How might this change clinical pharmacology or translational science?

The findings suggest that plasma proteins of hepatic DMEs have the potential to serve as biomarkers to individualize pharmacotherapy.

Author Contributions

J.S., B.E.B, J.S.M., K.S.P., and H.J.Z. wrote the manuscript; J.S.M., K.S.P., and H.J.Z. designed the research; J.S., J.X., X.W., and S.M.J. performed the research; J.S. and H.J.Z. analyzed the data.

Figure legends

Figure 1. Overall study design. Plasma samples were collected from 15 healthy subjects who received a single dose of modified-release dl-MPH (40 mg). d-MPH PK analysis was performed in a previously published study. Plasma samples were subjected to the targeted quantification of CES1 proteins and global untargeted proteomics analysis. Plasma CES1 concentrations were

furthered normalized by the plasma levels of liver-enriched proteins. The correlations between d-MPH AUCs and both unnormalized and normalized CES1 plasma concentrations were determined.

Figure 2. Plasma concentrations of CES1 protein before (left panel) and after normalization (right panel) to the plasma concentrations of liver-enriched proteins.

Figure 3. Heatmap of plasma concentrations of the 24 liver-enriched proteins used for CES1 plasma concentration normalization in individual subjects.

Figure 4. Correlations between d-MPH AUCs and plasma CES1 protein concentrations (A) and plasma CES1 concentrations normalized to liver-enriched proteins (B) in subjects who received a single dose of modified-release dl-MPH (40 mg) (n = 15).

Figure 5. PBPK modeling simulation of d-MPH plasma concentration-time profiles in pediatric patients who received a single oral fixed or individualized dose of immediate-release dl-MPH (20 mg). The solid lines and shaded areas represent the mean values and the 95% confidence intervals, respectively, of the simulated plasma concentrations in the virtual pediatric populations.

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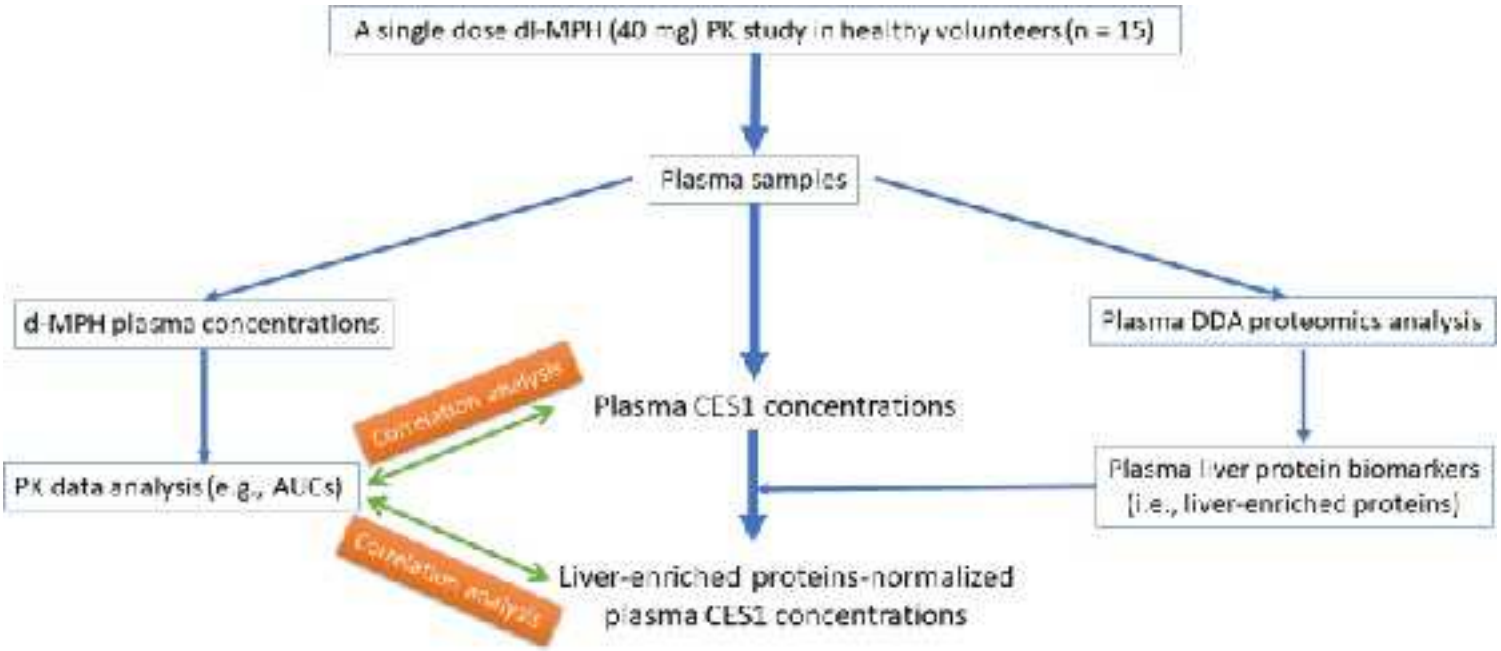
Supplementary Files

1. Supplemental Material.docx
2. Supplemental Table S3.pdf
3. Supplemental Table S4.pdf



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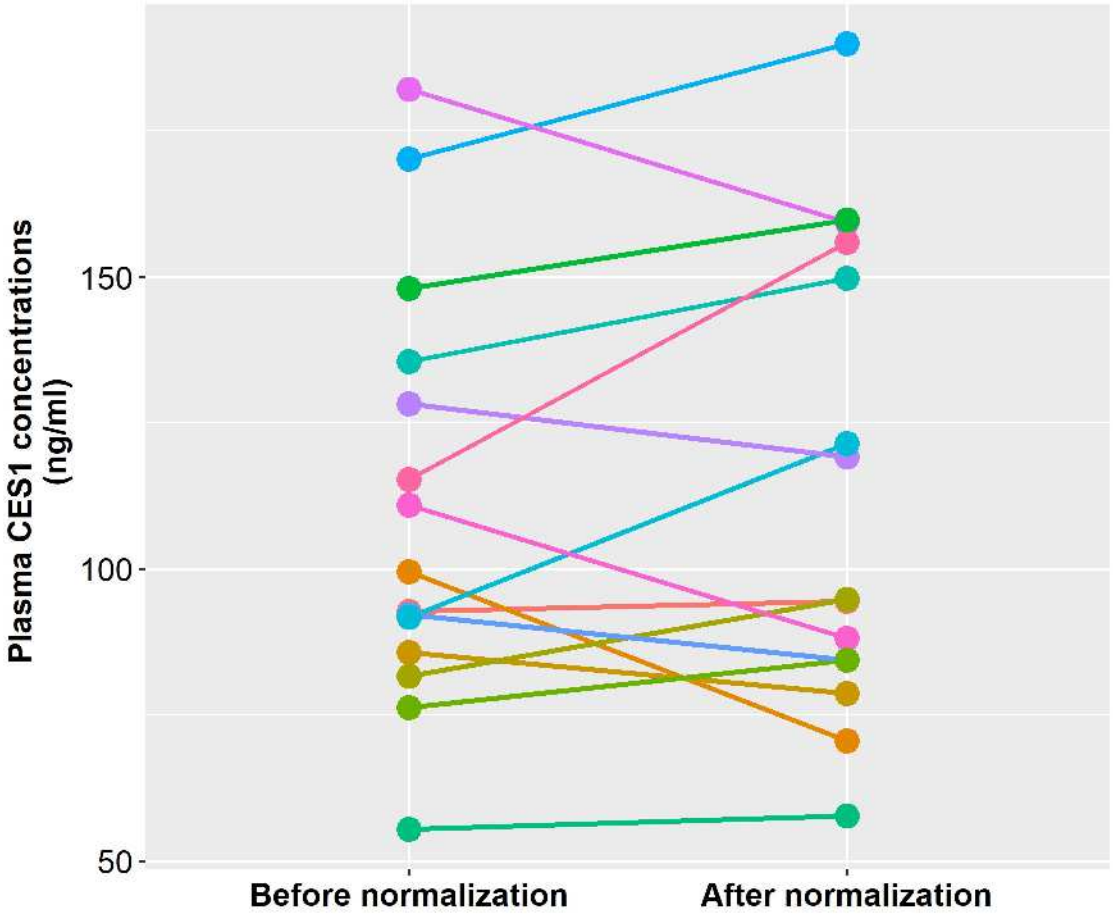

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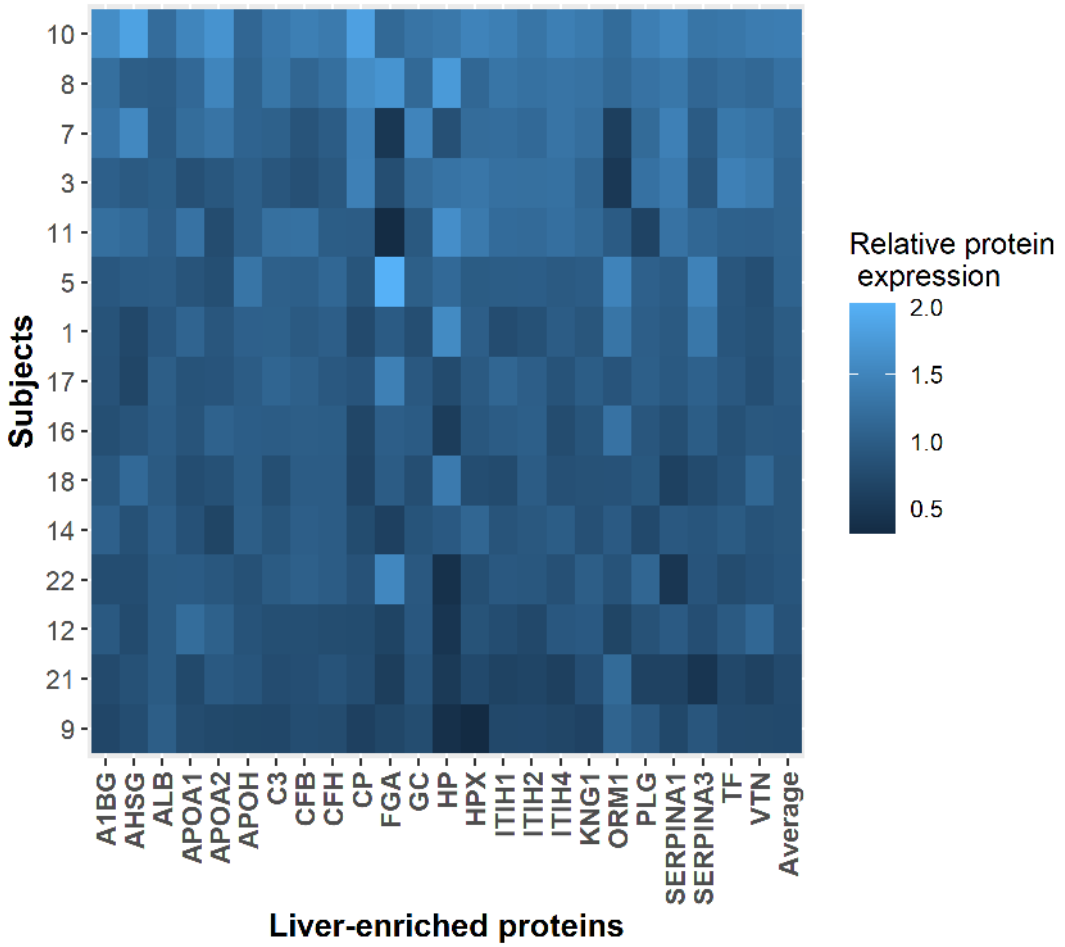

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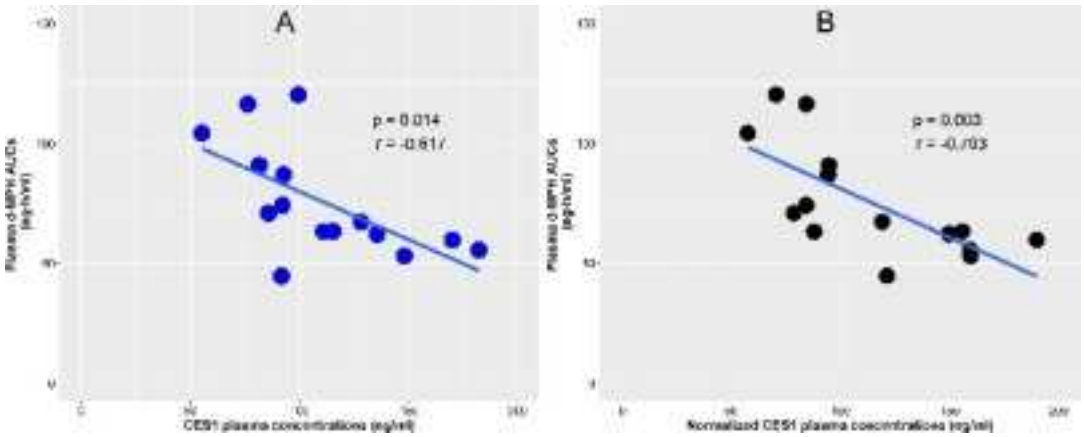

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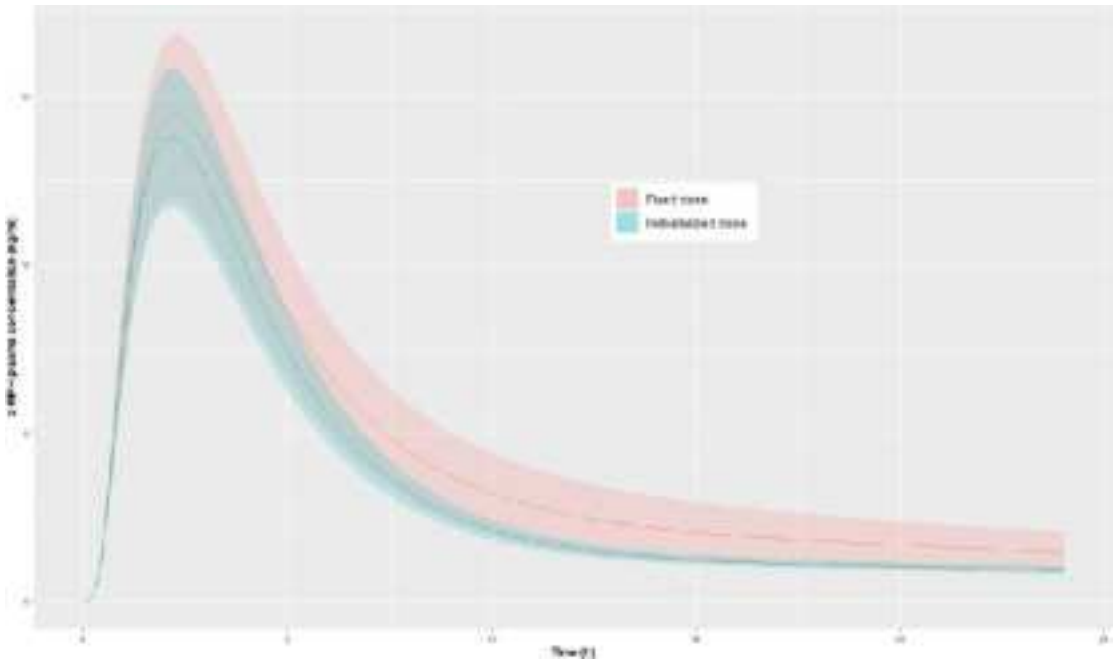

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