Carboxylesterase 1 and Precision Pharmacotherapy

by

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Dedication

To my family, Misuk Yang, Kwangjai Her, Yongki Her, and Theresa Hyengju Her,

for their love, support, and inspiration.

With love, always.

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Abstract

Carboxylesterase 1 (CES1) is a major phase I drug-metabolizing enzyme, responsible for 80-95% of total hydrolytic activity in human livers. CES1 plays a crucial role in the metabolism of a wide range of drugs, pesticides, environmental pollutants, and endogenous compounds. The clinical relevance of CES1 has been well demonstrated in various clinical trials with methylphenidate, oseltamivir, and clopidogrel. Expression and activity of CES1 vary markedly among individuals, which is a major contributing factor to interindividual variability in the pharmacokinetics (PK) and pharmacodynamics (PD) of drugs metabolized by CES1. The loss-of-function SNP G143E (rs71647871) is the only clinically significant CES1 variant identified to date.

A multi-dose, prospective pharmacogenetics, pharmacokinetics-pharmacodynamics (PGx-PK/PD) clinical trial was conducted in healthy volunteers (n=21) to examine the impact of G143E on the PK and PD of a CES1 substrate, enalapril. Enalapril 10mg was given once daily for seven consecutive days to the G143E carriers (n=6) and the G143E non-carriers (n=15) prior to a 72h PK/PD study. The study found the CES1 G143E carriers had 30.9% lower enalaprilat C_{max} (P = 0.03), 27.5% lower enalaprilat $AUC_{0-\infty}$ (P = 0.02), and 32.3% lower enalaprilat-to-enalapril $AUC_{0-\infty}$ ratio (P = 0.003) compared to the non-carriers. The average maximum reduction of systolic blood pressure (SBP) was used as the PD surrogate marker. The non-carrier group had 12.4% lower SBP at the end of the study compared to the baseline (P = 0.001), while no statistically significant SBP reduction was observed in the G143E

carriers. The study confirmed the CES1 G143E variant significantly impacted the PK and PD of enalapril.

G143E, however, can only explain a small portion of the interindividual variability in the CES1 function. A better understanding of the regulation of CES1 expression and activity could lead to the development of a precision pharmacotherapy strategy to improve the efficacy and safety of many CES1 substrate drugs. Here, we developed a novel allele-specific protein expression (ASPE) assay to identify genetic variants regulating CES1 protein expression in human livers. The ASPE method adopted a custom-designed heavy stable isotope-labeled QconCAT internal standard and utilized a nonsynonymous variant, S75N (rs2307240), as the marker to determine the allelic expression of CES1. Two cis-acting regulatory variants (rs6499788, rs35918553) were found to be associated with CES1 ASPE and protein expression in human liver microsome (HLM) samples ($P < 0.05$). The effects of the two variants were further validated by the CES1 protein expression and activity studies in human liver S9 fractions (HLS9) ($P < 0.05$). We expect that combining all identified functional CES1 variants (e.g., G143E, rs6499788, and rs35918553) will provide a more holistic view of CES1 pharmacogenetics and allow us to better predict the PK and PD of medications metabolized by CES1.

Chapter 1: Background and Literature Review[1]

1.1 Importance of CES1 in Drug Metabolism

Carboxylesterase 1 (CES1) is a phase I drug-metabolizing enzyme (DME) responsible for 80-95% of total hydrolytic activity in the liver [2]; it metabolizes a wide range of drugs, pesticides, environmental pollutants, and endogenous compounds, including lipid esters (Table 1-1). CES1-mediated metabolism can lead to the biotransformation of a pharmacologically active drug into its inactive metabolite, as exemplified by methylphenidate hydrolysis in the liver. CES1 also plays an important role in activating prodrugs since most ester-containing prodrugs are exclusively dependent on CES1 for their activation. The clinical relevance of CES1 has been well demonstrated in various clinical trials with oseltamivir, methylphenidate, and clopidogrel [3-6]. Recent studies have also revealed that CES1 acts as a cholesteryl ester hydrolase in lipid metabolism in human macrophages and hepatocytes and suggest CES1 as a potential drug target for the treatment of metabolic diseases, such as diabetes and atherosclerosis [7-11].

CES1 plays an important role in metabolizing many clinically significant medications, especially the ester-prodrugs (Table 1-1). A prodrug refers to an inactive drug molecule that needs to be enzymatically biotransformed *in vivo* to its active metabolite to produce its intended pharmacological effect [12]. Prodrug design offers an attractive method to overcome the issue of low bioavailability for Biopharmaceutics

Classifications System (BCS) class III drug molecules. Drug molecules can be categorized into four BCS classes based on permeability and solubility, and a BCS class III substance is a hydrophilic compound with low permeability and high solubility [13]. In particular, hydrophilic compounds with –OH or –COOH functional groups usually have difficulty being absorbed into the body, and drug developers often mask these functional groups using an ester-prodrug design. The prodrug market has been growing: 20% of drugs approved in 2015 were prodrugs, compared to ~6% of all currently approved drugs [14].

Table 1-1 List of CES1 Substrates.

ACE Inhibitors	CNS Agents	Antihyperlidpidemia Agents
Enalapril*	Methylphenidate	Simvastatin*
Imidapril*	Cocaine	Lovastatin*
Benzapril*	Heroin	Clofibrate
Quinapril*	Mepridine	Fenofibrate
Ramipril*	Flumazenil	
Trandolapril*	Rufinamide	
		Chemical Warfare Agents
Antiviral Agents	Anticancer Agnet	
Oseltamivir*	Capecitabine*	Sarin
Sofosbuvir*	Irinotecan	Soman
Tenofovir alafenamide*	Telotristat etiprate*	Tabun
Endogenous Compounds	Antiplatlets/Anticoagulants	Pesticides
Cholesterol	Clopidogrel	trans-permethrin
Fatty acid ethyl esters	Dabigatran*	Para-nitrophenyl valerate
	Immunosuppressive Agents	
ARNi		Others
sacubitril*	Mycophenolate mofetil*	Dimethyl fumarate*
	Ciclesonide	Oxybutynin

*Prodrugs that need CES1 activation

Two major assumptions behind the ester-prodrug design are that prodrugs are rapidly activated via unspecific esterases in the body, and that the interindividual

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variability in activating a prodrug is clinically insignificant. These incorrect assumptions may have stemmed from the fact that many hydrolytic enzymes exist in the body, such as CES1, CES2, acetylcholinesterase, butyrylcholinesterase, paraoxonases, and arylesterase. However, these hydrolases differ in their tissue-specific expression, cellular localization, and most importantly, substrate selectivity [15]. In humans, CES1 is highly abundant in the liver and expressed to a less extent in the lung and brain; CES1 expression is considered negligible in the human intestine, kidney, and plasma. CES1 is substrate-selective towards carboxyl esters with a large ethyl group and a small alcohol group. In comparison, CES2, another major carboxylesterase in humans, is highly expressed in the intestine, kidney, and liver, and is more efficient at metabolizing compounds with a small ethyl group and a large alcohol group [16]. Numerous *in vivo* and *in vitro* studies have demonstrated the specificity of CES1, and many CES1 substrates cannot be metabolized by other esterases.

CES1 expression and activity vary significantly among individuals [17]; this variability could result in treatment failure and unexpected adverse effects of CES1 substrate drugs. A better understanding of the genetic and non-genetic factors contributing to CES1 variability will improve the design and clinical use of many drugs that are metabolized (deactivated/activated) by CES1.

1.2 Pharmacogenetics of Drug-Metabolizing Enzymes

Traditionally, fixed-dose regimens have been used for most medications. However, different individuals taking the same dose of medication do not necessarily achieve the same drug exposure and hence drug response. More individualized,

patient-centered dosing regimens have been developed based on a patient's

characteristics, such as renal clearance, liver function, body weight, and surface area [18].

In addition, genetic polymorphisms of DMEs have been found to play an important role

in response to pharmacotherapy, and pharmacogenomics has been increasingly utilized

in the clinic to improve the efficacy and safety of drug treatment. DMEs serve to

primarily detoxify digested xenobiotics through four general mechanisms: hydrolysis

(e.g., carboxylesterase), reduction (e.g., carbonyl reductase), oxidation (e.g.,

cytochrome P450), and conjugation (e.g., UDP-glucuronosyltransferase) [19]. The

expression and activity of DMEs vary significantly among individuals, and studying

pharmacogenomics of DMEs is one means of better understanding interindividual

variability in the PK and PD of a drug. For example, the active metabolite of irinotecan,

SN-38, is primarily metabolized by the enzyme UDP glucuronosyltransferase family 1

member A1 (UGT1A1 enzyme) [20]. If a patient carries the common UGT1A1*28

polymorphism, the decrease it causes in UGT1A1 enzymatic activity would impede the

metabolism of SN-38, leading towards the accrual of toxic concentrations. Accordingly,

the Food and Drug Administration (FDA) recommended that patients with

UGT1A1*28/*28 start irinotecan at a lower dose [21]. However, given that both genetic

and environmental factors contribute to DME function, we should also pay close

attention to non-genetic contributors when studying the variability of DMEs.

1.3 **CES1 Pharmacogenetics**

Although CES1 plays a critical role in the metabolism of many clinically important

medications, CES1 pharmacogenetics is understudied relative to other major DMEs

(e.g., CYP450s). CES1 is encoded by the *CES1* gene, consisting of 14 exons located

on chromosome 16q13-q22.1. *CES1 VAR* is a variation of the *CES1* gene that differs in

exon 1 DNA sequences and has an average minor allele frequency (MAF) of 17%.

Although one study claimed that *CES1 VAR* mRNA was undetectable [22], an *in vitro*

human liver study showed that the protein expressions of *CES1* and *CES1 VAR* were

not statistically different [17]. *CES1P1* is a pseudogene due to a premature stop codon in

exon 4 and lies tail-to-tail with *CES1* (Figure 1-1) [17]. Interestingly, a *CES1P1* variant

named *CES1P1 VAR* is a functional coding gene with a DNA sequence identical to

CES1 VAR. However, the transcription efficiency of *CES1P1 VAR* is only 2% of that of

CES1, due to the transcription factor Sp1, and the enhancer-binding protein C/EBPα

preferring to bind to the *CES1* promoter over the *CES1P1 VAR* promoter [23,24]. Due to

the existence of the *CES1 VAR* and *CES1P1 VAR* variants, four *CES1/CES1P1*

haplotypes can be formed (Figure 1-1). In addition to these structural variations, there

are over 7000 *CES1* SNPs registered in the NCBI SNP database, and approximately

300 of them have MAFs over 1%. These common *CES1* variants (MAF > 1%) are

distributed in various regions of the gene, including 13 in 5'-UTR and 3'-UTRs, 14 in

exons, and 308 in introns. Of the exonic SNPs, 12 are non-synonymous SNPs and two

are synonymous SNPs. In the following section, we discuss the clinical findings and

mechanistic bases of functional *CES1* variants identified to date.
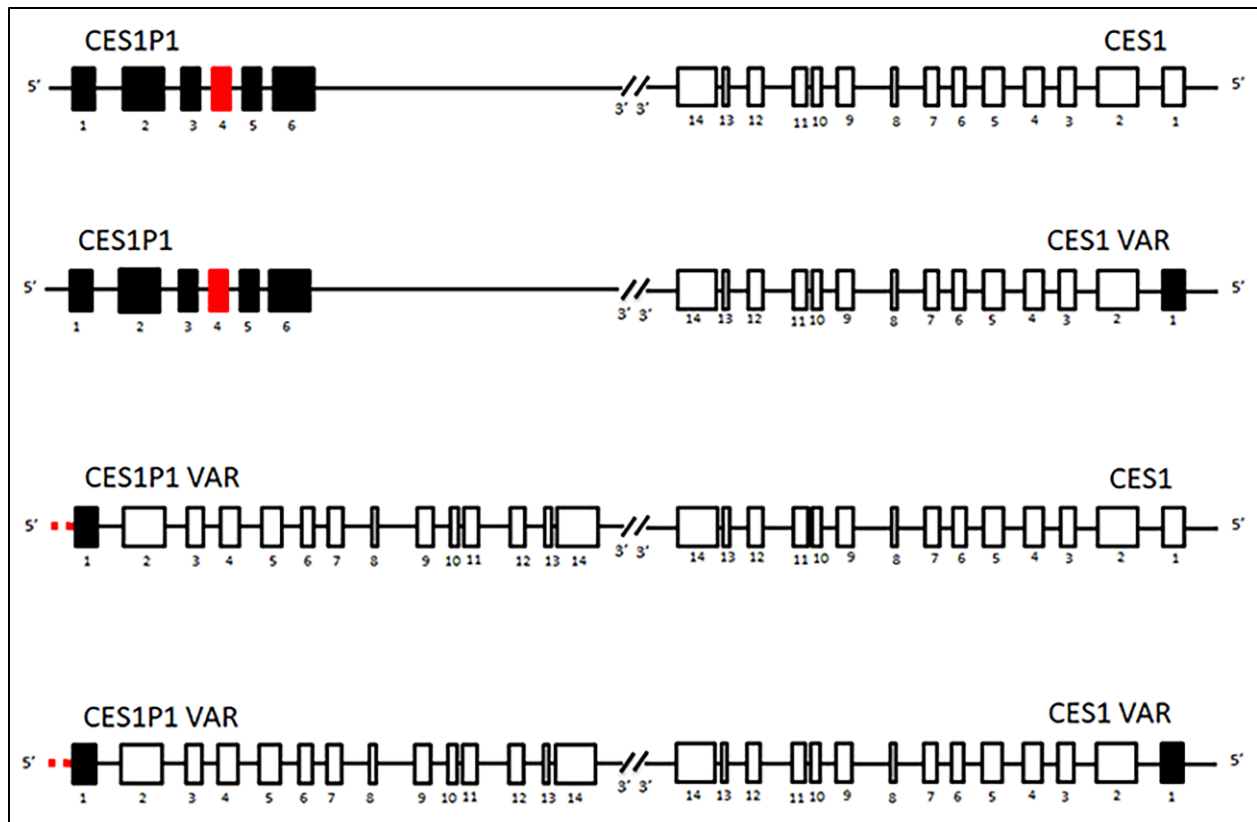
Figure 1-1 CES1 gene structure and haplotypes. CES1 gene consists of 14 exons located on chromosome 16q13-q22.1, and CES1P1 is a pseudogene, lying tail-to-tail with CES1. CES1, CES1P1 and their variants CES1 VAR and CES1P1 VAR form four major haplotypes. Red represents where the stop codon is located at. The transcription efficiency of CES1P1 VAR is approximately 2% of CES1.

1.4 **The Loss-of-Function CES1 Variant G143E (rs71647871)**

In SNP notation, G143E indicates an amino acid change from glycine to glutamic acid at amino acid position 143. G143E is also termed 428G>A, indicating that the nucleotide guanine is changed to adenine at position 428 of the *CES1* mRNA [18]. The MAF of G143E is 3.7%, 4.3%, and 2%, in White, Hispanic, and African American populations, respectively, while the SNP is extremely rare in Asian populations [5,25].

G143E is a non-conservative amino acid substitution located near the active-site triad residues of CES1 (serine 221, glutamic acid 354, and histidine 468). Serine

hydrolases share a similar catalytic mechanism involving (1) nucleophilic attack from oxygen in the serine residue on a substrate ester bond, (2) formation of a tetrahedral intermediate where the deprotonated oxygen is stabilized via an oxyanion hole, (3) formation of an acyl-enzyme intermediate, and (4) water-catalyzed hydrolysis [26]. For CES1 to maintain its enzymatic function, the catalytic triad and oxyanion hole need to be conserved [5,27]. The change from glycine (hydrophobic residue) to glutamic acid (electrostatic residue) at codon 143 disrupts the hydrophobicity needed for the oxyanion hole (Gly 141-131), resulting in a complete loss of function of CES1. The G143E is the only CES1 SNP that has been subjected to *in vitro* kinetics studies, in which the variant exhibited null catalytic activity on all tested CES1 substrates except for oseltamivir (Table 1-2). The V_{max} of G143E on oseltamivir hydrolysis was 37 nmol/min/mg with catalytic efficiency of 17.2 µl/min/mg protein — this was approximately 16% of wild type CES1 catalytic efficiency [28].

Table 1-2 In vitro kinetics of wild type CES1 in human liver S9 fractions (HLS9).

CES1 Substrates	HLS9			Reference
	Vmax (pmol/min/mg protein)	Km (µM)	Catalytic Efficiency (Vmax/Km, µl/min/mg protein)	
Clopidogrel	3558.6	62.7	56.8	Zhu[29]
2-oxoclopidogrel (clopidogrel intermediate)	158.1	2.4	65.9	
Enalapril	67.5	60.1	1.1	Wang[17]
Ramipril	18100	690.4	26.2	
Perindopril	18100	1767	23.3	
Moexipril	4400	1457	12.7	
Fosinopril	1400	471.3	3.0	
l-methylphenidate	1701	775.7	2.2	Zhu[5]
d-methylphenidate	177.2	663.5	0.3	
Oseltamivir	145000	1380	105.1	Zhu[28]
Trandolapril	103600	639.9	161.9	Zhu[30]
Dabigatran	1174	33.5	35.0	Laizure[31]

1.5 Clinical Impact of CES1 Variant G143E

1.5.1 Discovery of G143E and its Impacts on Methylphenidate PK and PD

G143E is the first loss-of-function (LOF) variant known for *CES1* and was originally discovered in a methylphenidate (Ritalin®) PK study in healthy volunteers. Methylphenidate is a central nervous system stimulant, the most commonly prescribed medication for ADHD treatment and has high abuse potential when used with alcohol [32]. Its drug product comes as a racemic mixture of d- and l-methylphenidate hydrochloride; d-methylphenidate is approximately 10 times more pharmacologically potent than l-methylphenidate [33].

Methylphenidate is metabolized by de-esterification via CES1 to ritalinic acid, an inactive metabolite which accounts for approximately 80% of the recovered dose in human urine (Figure 1-2) [32,34]. In 2007, a prospective single-dose (0.3 mg/kg) PK study was conducted in twenty healthy volunteers to examine the DDI between methylphenidate and alcohol [35]. During this study, the researchers unexpectedly found a participant that showed significantly elevated pharmacokinetic parameters (e.g., AUC, C_{max}) of methylphenidate. Specifically, dl-methylphenidate C_{max} was seven times higher, and l-methylphenidate C_{max} was 100-fold higher in this poor metabolizer compared to the rest participants. Later analysis found that this poor metabolizer carried the G143E polymorphism in *CES1* and the D260fs polymorphism in *CES1P1* [5]. This study also concluded that while CES1 metabolism is substantially stereoselective towards l-methylphenidate, d-methylphenidate metabolism is also significantly impacted by CES1 dysfunction.
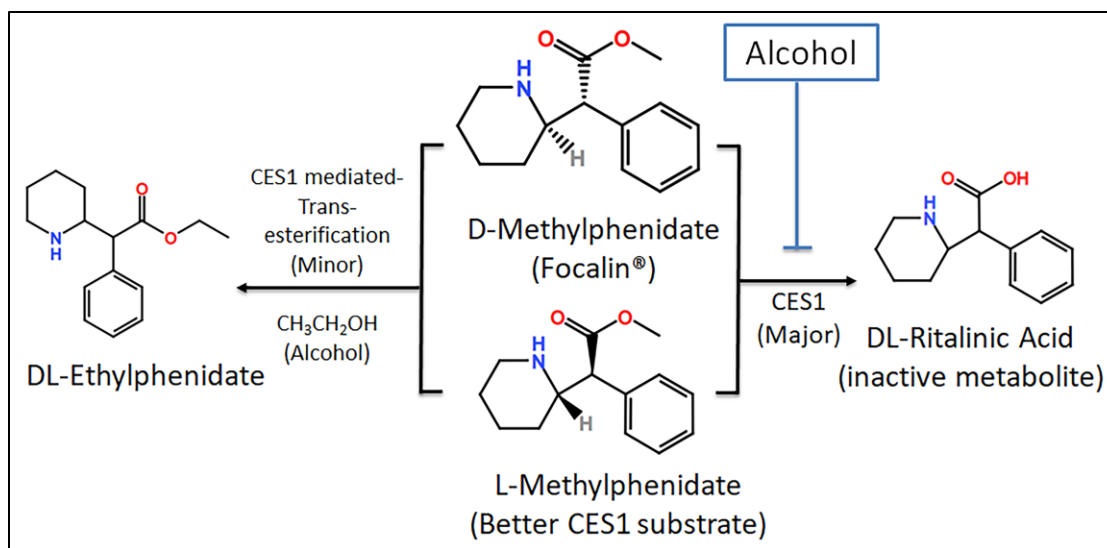
Figure 1-2 D-methylphenidate comes as a single active ingredient (Focalin®) or in combination with l-methylphenidate (racemic mixture) (Ritalin®). D-methylphenidate is approximately 10 times more pharmacologically potent than l-methylphenidate, while l-methylphenidate is a better CES1 substrate. Ethylphenidate can be formed via transesterification with ethanol.

Following the discovery of the G143E variant, a retrospective study was conducted to examine the methylphenidate response in Hungarian ADHD patients with G143E (n=7) and non-carrier patients (n=115). Even though the CES1 genotype could not explain the entire interindividual variability between responders (n=90) and non-responders (n=32), the study demonstrated an association between G143E polymorphism and methylphenidate dose reduction: five responders who had the G143E polymorphism required lower doses of methylphenidate for symptom reduction (0.410 vs 0.572 mg/kg, P=0.022) [36]. In 2017, a healthy volunteer study confirmed the significance of G143E in the PK of methylphenidate. In this open-label, prospective clinical trial (n=22), study participants carrying the G143E SNP (n=6) had approximately 152.4% higher median AUC of d-methylphenidate ($53.3 ng \times ml^{-1} \times h^{-1}$) than the non-carrier group ($21.4 ng \times ml^{-1} \times h^{-1}$) (P<0.0001) [37].

The above studies suggest that G143E carriers may be at high risk of being exposed to a toxic methylphenidate concentration. This result is clinically impactful because methylphenidate is considered the first-line pharmacotherapy for ADHD, with approximately 40 million prescriptions dispensed every year [38]. This result could potentially explain why many patients have an unsatisfactory response to the treatment. Further clinical studies in ADHD patients with larger sample sizes are needed to fully understand the effect of CES1 variants on the efficacy and toxicity of methylphenidate, and how methylphenidate doses should be adjusted based on a patient's CES1 genotypes.

1.5.2 **G143E and Clopidogrel (Plavix®)**

Clopidogrel is a P2Y12 inhibitor and has several clinical indications, including myocardial infarction prophylaxis, cerebrovascular accident prophylaxis, and peripheral arterial occlusive disease prophylaxis. Clopidogrel is usually considered the first-line antiplatelet agent due to its proven efficacy and cost-effectiveness [39-41]. Clopidogrel is a non-ester-prodrug that needs to be activated by two oxidation reactions via several CYPs (Figure 1-3). CYP2C19 pharmacogenetics and its impact on clopidogrel activation have been extensively studied. The Clinical Pharmacogenetics Implementation Consortium (CPIC) guideline and the FDA both recommend intermediate and poor metabolizers of CYP2C19 to use an alternative antiplatelet agent, such as ticagrelor or prasugrel [42]. Clopidogrel, its intermediate, and active metabolites are all CES1 substrates and metabolized by CES1 to inactive hydrolytic metabolites (Figure 1-3). Approximately, 85% of clopidogrel is hydrolyzed by CES1, and only 15% of clopidogrel enters the CYPs-mediated activation pathway [29]. Thus, patients with CES1 dysfunction would have a higher concentration of clopidogrel active metabolite compared to normal CES1 metabolizers when taking the same dose. However, the impact of CES1 on the PK and PD of clopidogrel is less studied than the impacts of CYPs.
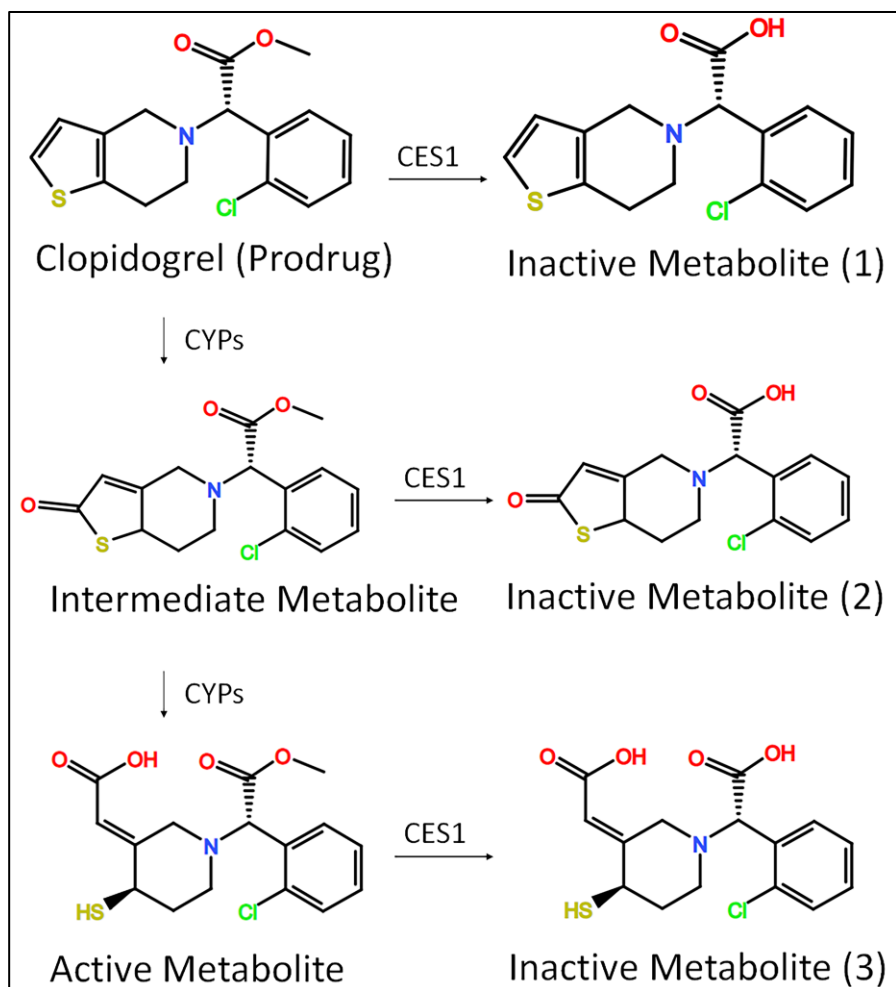
Figure 1-3 Clopidogrel metabolic pathway. Clopidogrel is a non-ester-prodrug that needs to be activated by two oxidation reactions via CYPs. Clopidogrel and its intermediate and active metabolites are all metabolized (deactivated) by CES1.

Two clinical trials support that CES1 G143E carriers have significantly higher plasma concentrations of clopidogrel active metabolite compared to non-carriers. A retrospective sub-analysis was performed on participants of the Pharmacogenomics of Antiplatelet Intervention (PAPI) Study (n=506) and on clopidogrel-treated patients at Sinai Hospital (n=350) to examine the effect of *CES1* G143E on clopidogrel metabolism. Study participants received a 300mg loading dose of clopidogrel followed by a 75mg maintenance dose for six days, and platelet aggregation was measured as a PD marker. A 50% higher active metabolite concentration was observed in G143E carriers

(n=7, 30.3 ng/ml) compared to non-carriers (n=499, 19.0 ng/mL) (P=0.001). In addition, the inhibition of adenosine diphosphate (ADP)-induced platelet aggregation was 24% higher in G143E carriers (reduced to 71% from baseline) relative to non-carriers (reduced to 57% from baseline) (P=0.003) [3,4,43]. Another prospective, single-dose, healthy volunteer (n=22) clinical study was conducted by Tarkiainen et al. to determine the effect of CES1 G143E on clopidogrel metabolism. The authors found that the $AUC_{0-\infty}$ ratio of clopidogrel carboxylic acid (inactive metabolite (1) in Figure 1-3) to clopidogrel was 53% less in G143E carriers (n=10) than non-carriers (n=12) (P=0.009). The G143E carriers also exhibited significantly higher plasma concentrations of the parent compound clopidogrel (P = 0.004) and its active metabolite (P = 0.009) compared to non-carriers. In agreement with the PK findings, the average inhibition of P2Y12-mediated platelet aggregation in the carriers was 19 percentage points higher than in non-carriers (P = 0.036) [29,44]. The findings of the above two studies are especially important for patients on triple antithrombotic therapy with a high bleeding risk [45-47]. Clopidogrel dose adjustment may be necessary to prevent potential toxicity (i.e., bleeding) in patients with CES1 dysfunction.

1.5.3 G143E and Angiotensin-Converting Enzyme Inhibitors (ACEIs)

Angiotensin-converting enzyme inhibitors (ACEIs) are generally considered to be the first-line therapy for heart failure and hypertension, and approximately 150 million ACEI prescriptions are filled in the US annually [48]. Currently, eight out of ten FDA-approved ACEIs are ester-containing prodrugs, and all ACEI prodrugs need to be activated by CES1 in order to exert their intended therapeutic effects [49,50]. The activation is essential for the pharmacological effects as the active metabolites are 10-

1,000 times more potent than their prodrug forms [51]. Therefore, patients with CES1 dysfunction would have a lower concentration of the ACEI active metabolite relative to normal CES1 metabolizers (Figure 1-4).
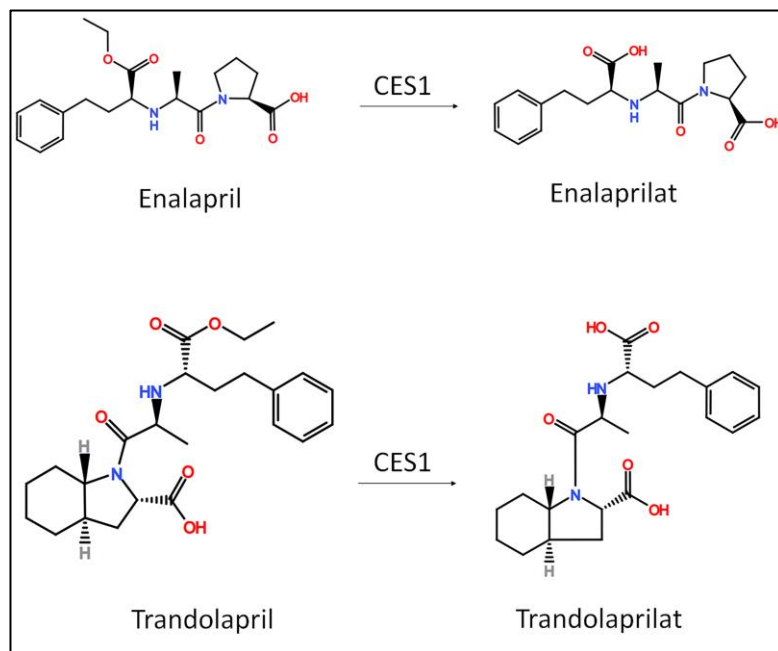
Figure 1-4 ACE inhibitors (enalapril and trandolapril) metabolism. Enalapril and trandolapril are ester-prodrugs that need to be activated by CES1.

A prospective, single-dose pharmacokinetic clinical study was conducted in healthy volunteers to examine the effect of the G143E variant on the activation of the ACEI prodrugs enalapril and quinapril. The $AUC_{0-\infty}$ of the enalapril active metabolite enalaprilat was found to be 20% lower in the G143E carriers (n=10) than in non-carriers (n=12) (P=0.049) [52]. This finding is consistent with an *in vitro* study that showed that enalapril activation was impaired in liver samples carrying the G143E variant [17]. However, the $AUCs_{0-\infty}$ of the quinapril and its active metabolite (quinaprilat) were not significantly different between carriers and non-carriers (P=0.114). A prospective, single-dose pharmacokinetic clinical trial with trandolapril also reported no statistically significant differences in PK parameters between the CES1 G143E carriers and the

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non-carrier group [53]. Further investigations are warranted to fully understand the effect of *CES1* variants on the PK and PD of ACEI prodrugs.

1.5.4 **G143E and Oseltamivir (Tamiflu®)**

Oseltamivir is an antiviral drug that has an FDA indication for influenza types A and B infections. Even though oseltamivir is rarely effective due to its specific administration requirement (i.e., this medication should be taken within 2 days of onset of symptoms in order to reduce flu duration by approximately one day), oseltamivir remains one of the most prescribed drug products due to flu epidemics [54,55]. As an ester-prodrug, oseltamivir needs to be activated by CES1 into its active metabolite, oseltamivir carboxylate [56]. An *in vitro* study based on cell lines stably transfected with CES1 variants suggested the G143E SNP markedly impaired CES1 activity in oseltamivir activation [28].

To examine the effect of G143E on oseltamivir PK and activation, a prospective, single-dose pharmacokinetic clinical study was conducted in healthy volunteers consisting of nine G143E heterozygotes, one G143E homozygote, and 12 non-carriers. The $AUC_{0-\infty}$ ratio of oseltamivir carboxylate (active metabolite) to oseltamivir (parent molecule) was 23% lower in G143E heterozygotes compared to non-carriers (P = 0.006). The one G143E homozygous individual had an $AUC_{0-\infty}$ of oseltamivir that was approximately 360% greater than the non-carriers, indicating that loss of CES1 activity could profoundly impair oseltamivir activation [6].
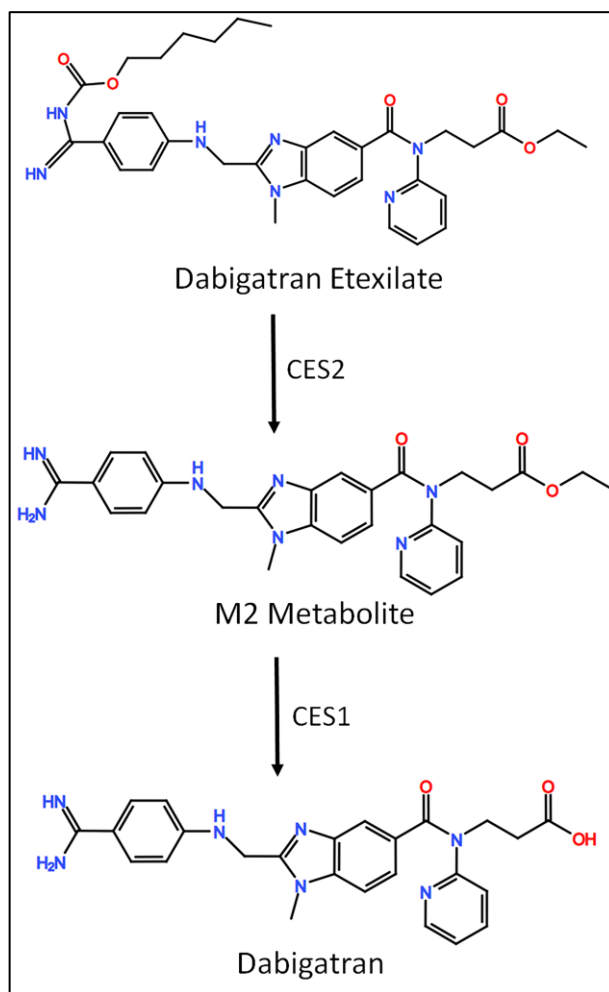
Figure 1-5 Dabigatran metabolic pathway. Dabigatran is a prodrug that activated by both CES1 and CES2.

1.5.5 **G143E and Dabigatran and Aacubitril**

Dabigatran and sacubitril are both prodrugs that need to be activated by CES1 in the liver (Figure 1-5). *In vitro* studies showed that the formation rates of the active metabolites of dabigatran and sacubitril were significantly lower in human livers carrying the G143E variant than in non-carrier samples [57,58]. However, it remains undetermined whether the variant can affect the activation and therapeutic response of these two drugs in patients.

1.6 **Pharmacogenetics of Other CES1 Genetic Variants**

In addition to G143E, many other *CES1* variants have been studied for their effects on the PK and PD of CES1 substrate drugs. However, the results were generally inconclusive, and further studies are needed to determine the clinical significance of these variants.

1.6.1 **E220G (rs200707504)**

A nonsynonymous variant E220G, commonly referred to as c.662A>G, was suggested to decrease CES1 enzymatic activity in an *in silico* analysis [59]. In agreement with that prediction, an *in vitro* study on transfected cell lines found E220G markedly decreased CES1 activity and the metabolisms of several CES1 substrates, including enalapril, clopidogrel, and sacubitril [60]. Notably, E220G has a MAF of 0.55% in East Asians but is rare in other populations. To determine the clinical impact of E220G on the PK of a CES1 substrate, a single-dose oseltamivir (75mg) PK study was conducted in 20 healthy Korean volunteers. In this study, the variant was observed to have a marginal effect on the PK of oseltamivir and its active metabolite (oseltamivir

carboxylate); however, the differences were statistically insignificant. In the E220G

carriers (n=8), the AUC_{0-48h} of oseltamivir was increased by 10% (P = 0.334), and the

AUC_{0-48h} of oseltamivir carboxylate was decreased by 5% (P = 0.513) relative to the

non-carriers (n=12) [59].

1.6.2 S75N (rs2307240)

S75N is one of the most common CES1 nonsynonymous SNP with MAFs

ranging from 2% to 7% in different populations. A retrospective pharmacodynamics

analysis was conducted to examine the effect of *CES1* S75N on the outcome of

clopidogrel therapy in patients with the coronary syndrome (n=851). The result showed

that CES1 S75N carriers (n=372) had higher incidence of cerebrovascular events (P <

0.001), acute myocardial infarction (P < 0.001), and unstable angina (P < 0.001)

compared to non-carriers. The study also found that the S75N polymorphism was more

frequent in acute coronary syndrome patients (MAF 22%) than in the general population

(MAF 5%). The authors concluded that there was a significant association between the

S75N polymorphism and the outcome of clopidogrel therapy [61]. However, this result

conflicts with another study that found the S75N variant to be not associated with the

outcomes of ADHD patients treated with methylphenidate [62]. Furthermore, an *in vitro*

study showed the S75N variant did not significantly alter the expression and activity of

CES1 in transfected cells and human livers [60].

1.6.3 -816A>C (rs3785161)

The -816A>C polymorphism is located in the promoter region of *CES1P1 VAR*

and has been suggested as a potential up-regulator of *CES1P1 VAR* expression [24]. A

prospective clinical study was conducted to examine the impact of -816A>C on the outcome of the ACEI prodrug (imidapril) therapy in hypertensive patients (n=105). The study found that after eight weeks of imidapril therapy, -816A>C homozygotes and heterozygotes (n=47) had greater systolic blood pressure reduction (24.1 mmHg) compared to non-carriers (17.6 mmHg) (P = 0.0184), indicating increased CES1 functionality in the carriers. The follow-up *in vitro* study claimed that the -816A>C SNP might have enhanced transcription of the *CES1P1 VAR* gene [63]. The -816A>C SNP was also evaluated for its impact on the outcomes of dual antiplatelet therapy (i.e., aspirin and clopidogrel) in patients with coronary heart diseases (n=162). The -816A>C carriers (n=75) had decreased vasodilator stimulated phosphoprotein-platelet reactivity index (VASP-PRI) (P=0.014), indicating increased CES1 function in the carriers [64].

However, conflicting findings were reported by other studies. A study involving the outcome of clopidogrel treatment in patients undergoing percutaneous coronary intervention, -816A>C carriers showed a lower ADP-induced maximum platelet aggregation (21.5%, n=125) compared to non-carriers (31.7%, n=124) (P=0.001), indicating decreased CES1 function [65]. Zhu et al. also performed a retrospective pharmacogenetic analysis of the INternational VErapamil SR Trandolapril (INVEST) study (n=486) and did not find an association between -816A>C and the blood pressure-lowering effect of trandolapril. The follow-up *in vitro* study also showed -816A>C genotype was not significantly associated with CES1 protein expression and trandolapril activation in human liver samples (n=100) [66]. Other researchers also noted that the *CES1P1 VAR* gene, which contains -816A>C, is considered functionally insignificant due to its low transcription efficiency [23,67].

1.6.4 **-75G>T (rs3815583)**

The -75G>T SNP is located in the promoter region of *CES1* and was suspected to alter CES1 expression in the liver; however, the findings are conflicted. A study was performed to determine the association between the variant and appetite reduction (a side effect of methylphenidate) in children with ADHD (n=213). Appetite reduction was measured by the Barkley Stimulant Side Effect Rating Scale, and methylphenidate dose was titrated up for three months as tolerable. The carrier group (n=129) had worse appetite reduction compared to non-carriers (n=76) (41% vs. 77%, P=0.01), indicating that the variant was associated with decreased CES1 function [68]. A study in patients treated with irinotecan, however, showed a contrary finding, suggesting that the -75G>T variant confers greater CES1 function [69]. CES1 is involved in the conversion of the prodrug irinotecan to its active metabolite, SN-38, and then further metabolized by UGT1As to inactive SN-38G. Following irinotecan treatment, patients who carried the T allele of this variant had higher plasma (SN-38 + SN-38G)/irinotecan AUC ratios relative to non-carriers (P=0.027) following irinotecan treatment [69].

Other CES1 substrates, isoniazid, and ACEI prodrugs were also studied in the context of -75G>T; however, no significant relationships were found between the variant and the medication responses. In one such study, the variant was evaluated for its effect on the outcomes of ACEI prodrugs in congestive heart failure patients (n=200) that underwent ACEI prodrug dose titrations. The study reported -75G>T did not significantly impact plasma ATII/ATI ratios; furthermore, the -75G>T variant was not significantly associated with fatal outcomes (i.e. cardiovascular death and all-cause

death) [70]. The study with isoniazid had similar results showing no significant association between the variant and isoniazid-induced hepatotoxicity (n=170) [71].

1.6.5 **1168-33C>A (rs2244613)**

Dabigatran (Pradaxa®) is a prodrug that needs to be activated by both CES1 and CES2 to exert its anticoagulant effect (Figure 1-5). Paré and associates conducted a genome-wide association study of dabigatran in participants (n=2944) of the Randomized Evaluation of Long-term Anticoagulation Therapy (RE-LY) clinical trial. The researchers concluded the CES1 intronic variant 1168-33C>A (rs2244613) is associated with lower trough concentrations of the active metabolite (15% decrease per allele; 95% CI 10-19%) and a lower risk of any bleeding (odds ratio, 0.67; 95% CI 0.55-0.82) compared to non-carriers [72]. However, an *in vitro* study did not find the variant to be associated with CES1 protein expression and dabigatran metabolism in human livers [73]. A prospective study also examined the impact of 1168-33C>A in ADHD patients treated with methylphenidate, and found the variant to be associated with the occurrence of sadness, a side effect of short-acting methylphenidate. However, researchers concluded this might be due to linkage disequilibrium with two SNPs of the noradrenaline transporter gene [62].

1.6.6 **Copy Number Variation (i.e., *CES1P1/CES1P1 VAR*)**

Many researchers have studied the impact of CNVs on CES1 functionality; however, the results are conflicted. Stage et al. found that participants with four functional copies of CES1 (n=5) had an increased AUC of d-methylphenidate relative to the control group with two functional copies of CES1 (n=17) (61% increase, $P = 0.011$);

participants with three copies of CES1 (n=2) had 45% increased AUC compared to the

control group (P = 0.028) [37]. Stage et al. conducted a similar study with enalapril (n=43),

however, they could not find a statistically significant correlation between CNV and

enalapril PK [74]. When Sai et al. examined the effect of CNV on irinotecan exposure,

they found patients with multiple CES1 copies (i.e., three or four) to have 1.24 fold

higher irinotecan AUC relative to patients with two copies of CES1 (P = 0.0134) [69].

Many researchers, however, did not find the relationship between CNVs and CES1

function. Suzaki et al. evaluated the relationship between CNVs of *CES1* and

oseltamivir PK parameters but did not find any correlation [75]. Nelveg-Kristensen et al.

studied the relationship between CNV and ACEI prodrugs, and again, no association

was found [70]. Moreover, an *in vitro* study showed CES1 protein expression levels to be

comparable among human livers with different copy numbers of functional *CES1* gene

[17].

1.6.7 **Other CES1 SNPs**

In addition to the polymorphisms discussed above, sporadic reports have stated

several CES1 SNPs to be associated with the outcomes of CES1 substrate

medications. For example, the SNP 1315 + 2025A>C (rs8192950) was associated with

a decreased risk of ischemic events in patients (n=64) having symptomatic extracranial

or intracranial stenosis and receiving dual antiplatelet therapy with clopidogrel for a

minimum of five days [76]. Another retrospective sub-analysis of a capecitabine clinical

study identified associations of 1168-41C>T (rs2244614), 690 + 129del (rs3217164),

95346T>C (rs7187684), -1232A>G (rs1186118) with severe early-onset of

capecitabine-induced toxicity [77]. None of these findings has been validated

independently.

A rare LOF variant, D260fs (c.70DelT), was reported in a clinical study [5]. D260fs

causes a deletion in exon 6, resulting in a frameshift and premature truncation.

Moreover, an *in vitro* study with *CES1* variants transfected cell lines examined the SNPs

proximate to the CES1 active site, and identified four LOF nonsynonymous SNPs:

G142E, G147C, Y170D, and R171C. However, these variants appear to be clinically

insignificant due to their low MAFs (< 0.4%) [78].

The above-mentioned *CES1* SNPs and their impacts on the PK and PD of CES1

substrate medications are summarized in Table 1-3.

Table 1-3 CES1 SNPs and their impacts on the PK and PD of CES1 substrate medications

AA/Nucleotide Change (db SNP ID)	Treatment	Population	Design/Outcome	Result	Conclusion
G143E (rs71647871)	Methylphenidate Single dose of 0.3mg/kg	n=20 (with 1 carrier) Healthy volunteers	Prospective study Aim: To examine the interaction between methylphenidate and alcohol	The study unexpectedly found one volunteer with elevated PK parameters of methylphenidate; C_{max} of l-methylphenidate was 100 fold higher (62 ng/mL) compared to the rest of participants	The later analysis found this volunteer had G143E and D260fs SNPs, which resulted in elevated plasma concentration of methylphenidate
	Methylphenidate Dose adjusted based on symptom reduction x 1 month	n=122 (with 7 carriers) Hungarian ADHD patients	Retrospective study Outcome: methylphenidate dose reduction	G143E carriers needed lower doses of methylphenidate for symptom reduction compared to non-carriers (0.410 vs 0.572 mg/kg , p=0.022)	G143E impaired methylphenidate metabolism *in vivo*
	Methylphenidate Single dose 10mg	n=22 (with 6 carriers) Healthy Danish Volunteers	Open labeled, prospective, PK study	G143E carriers showed 152.4% higher AUC (53.3ng×ml^{-1}×h^{-1}) compared to the non-carrier group (21.4 ng×ml^{-1}×h^{-1}) (P<0.0001)	G143E carriers had higher exposure to methylphenidate compared to non-carriers
	Clopidogrel (1) PAPI patients received 300mg LD with 75mg MD	n=506 (with 7 carriers) n=350 (with 6 carriers)	Retrospective subanalysis of two clinical studies: (1) Pharmacogenomics of Antiplatelet Intervention	(1) A 50% higher active metabolite concentration was observed in G143E carriers (n=7, 30.3 ng/ml) compared to non-carriers (n=499, 19.0 ng/mL) (P=0.001)	G143E carriers had higher plasma concentrations of clopidogrel active metabolites, and consequently had a higher antiplatelet effect

		Patient going through PCI	(PAPI) Study (2)Clopidogrel-treated patients at Sinai Hospital	(2) The inhibition of ADP-induced platelet aggregation effect was 24% higher in G143E carriers (reduced to 71% from baseline) compared to non-carriers (reduced to 57% from baseline) (P=0.003)	
	(2) Patients from Sinai Hospital received either 300 or 600 mg LD (n= 204) with MD, or just received 75mg MD		Outcome: (1) PK parameter: Clopidogrel and its active metabolite concentration (2)PD parameter: ADP-stimulated platelet aggregation		
	Clopidogrel Single-dose 600 mg	n=22 (with 10 carriers) Healthy volunteers	Prospective, PK/PD study PD outcome: Inhibition of P2Y12-mediated platelet aggregation	(1) AUC0–∞ ratios of the clopidogrel carboxylic acid to clopidogrel was 53% less in G143E carriers (P=0.009) (2) Average inhibition of P2Y12-mediated platelet aggregation in the carriers was 19 percentage points higher in non-carriers (P = 0.036)	G143E carriers had higher exposure to clopidogrel active metabolite, and consequently had a higher antiplatelet aggregation effect
	Enalapril, Quinapril Single Dose 10 mg Enalapril or Quinapril	n=22 (with 10 carriers) Healthy volunteers	Prospective PK study	(1) AUC0–∞ of the enalapril active metabolite enalaprilat was 20% lower in the G143E carriers (n=10) compared to non-carriers (n=12) (P=0.049) (2) AUCs0-∞ of the quinapril and its active metabolite (i.e., quinaprilat) were not significantly different between the non-carriers and carriers (P=0.114)	G143E carriers had a lower enalaprilat exposure compared to non-carriers

	Oseltamivir Single Dose 75mg	n=22 (with 9 G143E heterozygotes, 1 G143E homozygote) Healthy volunteers	Prospective PK Study	(1) The $AUC_{0-\infty}$ ratio of oseltamivir carboxylate (active metabolite) to oseltamivir (parent molecule) was 23% lower in G143E heterozygotes compared to non-carriers ($P = 0.006$) (2) The one G143E homozygous individual had an $AUC_{0-\infty}$ of oseltamivir that was approximately 360% greater than the non-carriers	G143E carriers had less exposure to oseltamivir active metabolite compared to non-carriers
	Dabigatran	n=102 human liver samples	*In vitro* study with human liver samples	The activation rates of DABE, M1 and M2 in G143E carriers were 53% ($P = 0.018$), 43% ($P = 0.004$), and 37% ($P = 0.001$) of normal carriers (after normalized by CES1 expression)	G143E carriers had a lower dabigatran activation rate, potentially resulting in a lower dabigatran active metabolite concentration in the carriers
	Sacubitril	n= 53 (with 5 carrier) human liver samples	*In vitro* study with human liver samples	The activation rates of sacubitril were lower in the carriers compared to the non-carriers (4.2 vs. 7.2 nmol/mg protein/min, $P = 0.025$)	G143E carriers had a lower sacubitril activation rate, potentially resulting in a lower sacubitril active metabolite plasma concentration in the carriers
E220G (rs200707504)	Oseltamivir 75mg single dose	n=20 (with 8 carriers)	Prospective, PK study	AUC_{0-48h} of oseltamivir was increased by 10% ($P = 0.334$) and AUC_{0-48h} of oseltamivir carboxylate was decreased by 5% ($P = 0.513$) in carriers	E220G appears to have no significant impact on oseltamivir activation in humans
S75N (rs2307240)	Clopidogrel 75mg x 1 year	n=851 (with 372 carriers)	Retrospective PD analysis Outcome: Cerebrovascular	CES1 S75N carriers (n=372) had more cerebrovascular events (P<0.001), acute myocardial infarction (P<0.001),	S75N appears to increase the function of CES1, resulting in the decreased efficacy of clopidogrel

				events, acute myocardial infarction, and unstable angina	and unstable angina (P<0.001) compared to non-carriers	
	Methylpheni date Weight based dosing x 6 weeks	n=44 (with 2 carriers) Children with ADHD	Naturalistic, prospective study Outcome: Side effect reported via behavioral questionnaires		No significant differences in methylphenidate side effect were found between carriers and non-carriers (p = 1)	S75N does not appear to affect the function of CES1
	Enalapril	n= 36 (with 3 carriers)	*In vitro* study with human liver samples		No statistical difference in enalapril activation rate or CES1 protein expression level between carriers and no carriers	S75N does not appear to affect the function of CES1
-816A>C (rs3785161)	imidapril 5-10 mg x 8 weeks	n=105 (with 47 carriers) Patients with hypertension	Prospective clinical study		Greater systolic blood pressure reduction (24.1 mmHg) was observed compared to non-carriers (17.6 mmHg) after 8 weeks of imidapril therapy (P = 0.0184)	-816A>C appears to up-regulate the CES1P1 VAR expression
	Clopidogrel 300 or 600 mg (LD) or 75 mg (MD) for minimum 5 days	n=162 (with 75 carriers) Patient on dual antiplatelet therapy (i.e., aspirin and clopidogrel) with coronary heart diseases	Retrospective PD analysis Outcome: Vasodilator Stimulated Phosphoprotein-Platelet Reactivity Index (VASP-PRI) to measure platelet reactivity		The carriers had decreased Vasodilator Stimulated Phosphoprotein-Platelet Reactivity Index (VASP-PRI) (45.93 vs 53.18%) (P=0.014)	-816A>C appears to up-regulate the CES1P1 VAR expression

	Clopidogrel 300mg LD + 75mg MD x 3 days	n=249 (with 108 heterozygous carrier, 17 homozygous carrier) Patient going throught PCI	Retrospective PD analysis Outcome: maximum platelet aggregation (MPA)	A lower ADP-induced maximum platelet aggregation (21.5%, n=125) was observed compared to non-carriers (31.7%, n=124) (P=0.001)	-816A>C appears to down-regulate the CES1P1 VAR expression
	Trandolapril 2-4mg x 104 weeks	(1) n=486 (with 109 heterozygous carriers, 10 homozygous carriers) (2) n=100 (*in vitro* study) (26 heterozygous carriers, 3 homozygous carriers)	(1) Retrospective analysis of the INternational VErapamil SR Trandolapril Study (2) *In vitro* study with human liver samples Outcome: Blood Pressure	(1) No association between the -816A>C and the blood pressure lowering effect of trandolapril (2) Not associated with CES1 protein expression and trandolapril activation in human liver samples	-816A>C does not appears to be associated with overall CES1 function
-75G>T (rs3815583)	Methylphenidate Dose titrated up x 3 months as tolerable	n=205 (with 129 carriers)	Retrospective PD analysis Outcome: appetite reduction - the side effect of methylphenidate	The carriers had worse appetite reduction compared to non-carriers (41% vs 77%, P=0.01)	-75G>T appears to be associated with decreased CES1 function
	Irinotecan 100 mg m−2 weekly or 150 mg m−2 biweekly	n=177 177 Japanese cancer patients	Retrospective PK analysis	The carriers had higher plasma (SN-38 + SN-38G)/irinotecan AUC ratios relative to non-carriers (P=0.027)	-75G>T appears to be associated with higher CES1 function

	ACEI	n=200 Congestive heart failure patients	Retrospective PD analysis	The -75G>T genotypes did not significantly impact the plasma ATII/ATI ratios in the study subjects or fatal outcomes (i.e. cardiovascular death and all-cause death)	-75G>T was not associated with CES1 function
1168-33C>A (rs2244613)	Dabigatran 110 or 150 mg twice daily	n=2944 (with 587 carriers) Patients with atrial fibrillation (within 6 months) and additional risk factors for stroke	Retrospective GWAS of Randomized Evaluation of Long-term Anticoagulation Therapy (RE-LY) clinical trial Outcome: Trough concentrations of dabigatran, bleeding risk	The carriers had lower trough concentrations of the active metabolite (15% decrease per allele; $P=1.2\times10^{-8}$) and a lower risk of any bleeding (odds ratio, 0.67; $P=7\times10^{-5}$) compared to non-carriers	1168-33C>A appears to be associated with lower CES1 function
	Dabigatran	n=102 (with 29 heterozygous carriers and 5 homozygous carriers)	*In vitro* study with human liver samples	No association between 1168-33C>A and dabigatran activation	1168-33C>A appears to be not associated with CES1 function

1.7 Non-Genetic Factors Affecting CES1 Expression and Activity

1.7.1 Developmental Expression of CES1

The developmental expression patterns of CES1 in human and mouse livers were similar, and many *in vitro* studies have suggested that hepatic CES1 protein expression increases with age [79-81]. An *in vitro* study with human liver samples (n=104) demonstrated the adult group (≥ 18 years of age) to have had higher CES1 expression than children (0 days–10 years); meanwhile, child group had higher CES1 expression than fetuses (82–224 gestation days). A follow-up study with liver microsomes showed that, in parallel with expression level, CES1 activity on hydrolyzing its substrate oseltamivir was also positively correlated with age [82]. The same group did a similar *in vitro* human liver study with a slightly different age bracket, in which the liver samples were divided into five age groups: 1–31 days old (group 1), 35–70 days old (group 2), 89–119 days old (group 3), 123–198 days old (group 4), and over 18 years old (group 5). Neonates (group 1) had 10% of the CES1 expression and hydrolysis levels compared to the adult group (group 5); pediatric groups (Group 2-4) had approximately 50% of the CES1 expression and hydrolysis levels compared to an adult [83]. Lastly, a similar *in vitro* study quantified CES1 protein levels in human liver samples of various ages (n=165). CES1 expression levels were 4.76 pmol/mg from birth to three weeks (n=36); 15.8 pmol/mg for those aged three weeks to six years (n=90); and 16.6 pmol/mg for ages six years to 18 years (n=36). The study team concluded that the median CES1 expression level is directly correlated with age ($P < 0.001$) [80]. Overall, CES1 expression and activity levels are lower in neonates and pediatric patients; further studies are

warranted to investigate the potential effect of CES1 maturation on the treatment outcome of CES1 substrate medications in patients in the early stages of development.

1.7.2 Sex Difference of CES1 Expression

Both *in vitro* and clinical studies have suggested that CES1 expression is higher in females than in males [35,57,79]. A PK study on healthy volunteers revealed that males had significantly higher exposure to d-methylphenidate than females [35]. Nonetheless, females experienced a more pronounced stimulant effect, despite their lower exposure. Shi et al. observed significantly higher CES1 activity in female human liver samples (n = 56) compared to male samples (n = 46). A follow-up *in vitro* study with dabigatran suggested CES1 activity was higher in females than males [57]. However, such difference was not observed in another *in vitro* study using human liver samples (n=32) and mouse liver samples (n=9) [79]. Further study is needed to examine the impact of sex on the CES1 expression level and the PK and PD of CES1 substrates.

1.8 Drug-Drug Interactions

1.8.1 CES1 Inhibitor - Alcohol

To date, ethanol is the only known CES1 inhibitor that has been confirmed in multiple *in vivo* and *in vitro* studies. The impact of ethanol on the metabolism of the CES1 substrate, methylphenidate, was tested in healthy volunteers (n=14) [84]. D-methylphenidate comes as a single active ingredient (Focalin®) or in combination with l-methylphenidate (racemic mixture, Ritalin®). D-methylphenidate is approximately ten times more pharmacologically potent than l-methylphenidate, while l-methylphenidate is a more efficient CES1 substrate (Figure 1-2). This clinical study used a pulsatile dosing regimen with methylphenidate (dl-methylphenidate 40 mg or d-methylphenidate 20 mg) and

ethanol (0.6 g/kg, four hours after methylphenidate dose) to eliminate any potential confounding effect of ethanol on methylphenidate absorption, as the methylphenidate drug products (i.e., Ritalin® and Focalin®) might undergo faster gastric dissolution in the stomach if administered with alcohol. When alcohol and d-methylphenidate (Focalin®) were co-administered, the C_{max} of d-methylphenidate was elevated by 27% (P = 0.001) and the $AUC_{4\to8h}$ was elevated by 20% (P < 0.01); when alcohol and dl-methylphenidate (Ritalin®) were co-administered, the C_{max} of d-methylphenidate was elevated by 35% (P < 0.01) and the $AUC_{4\to8h}$ was elevated by 25% (P < 0.05) [84]. These results are consistent with the previous clinical trial by Patrick et al. In that study, when alcohol and d-methylphenidate (Focalin®) were co-administered, the d-methylphenidate AUC was increased by 14%; when alcohol and dl-methylphenidate (Ritalin®) were co-administered, the d-methylphenidate AUC was increased by 21% [85]. Patrick and colleagues also showed that the co-administration of alcohol 30 min before or 30 min after methylphenidate had a similar impact on methylphenidate exposure [35]. Both authors concluded that alcohol is a strong inhibitor of CES1, and the impact of CES1 inhibition is greater for dl-methylphenidate (Ritalin®) than for d-methylphenidate (Focalin®). Additionally, the DDI between methylphenidate and ethanol produced the transesterification metabolites d-ethylphenidate and l-ethylphenidate, and the plasma concentrations of l-ethylphenidate were much higher than d-ethylphenidate due to l-ethylphehidate being a more efficient CES1 substrate [84,86]. Other *in vivo* studies with mice demonstrated similar results [87-89].

The impact of alcohol on the CES1 function was also examined in the context of a different CES1 substrate, oseltamivir. A prospective health volunteer PK study (n=18)

examined the interaction between oseltamivir 150mg (a recommended daily dose for the treatment of influenza) and alcohol. Alcohol increased the oseltamivir AUC_{0-6h} by 27% (P=0.011) and decreased the AUC_{0-6h} ratio of the active metabolite oseltamivir carboxylate to the parent compound oseltamivir by 34% (p<0.001) [90]. However, co-administration of alcohol did not significantly affect the AUC_{0-24h} of oseltamivir carboxylate. These results are consistent with *in silico* analysis of the DDI between alcohol and oseltamivir [91].

1.8.2 **Other CES1 Inhibitors: Cannabis, Protease Inhibitors, Aripiprazole, Isradipine, Tacrolimus, Valproate**

Besides alcohol, many drug products on the market have been suggested to be potent inhibitors of CES1 mainly by *in vitro* investigations (Table 1-4). A further clinical study with a validated CES1 substrate is needed to determine the clinical significance of these CES1 inhibitors.

An *in vitro* study with CES1 transfected cells suggested that cannabis (i.e., THC, CBD, and CBN) can act as a potential CES1 inhibitor. The inhibition constant (Ki) values for THC, CBD and CBN were 0.541, 0.974, and 0.263 μM (0.170, 0.306, and 0.0817 μg/ml), respectively [92]. This result could be clinically impactful as the use of cannabis is expected to increase in the next few years [93].

Several protease inhibitors (i.e., nelfinavir, amprenavir, atazanavir, ritonavir, and saquinavir) were identified as CES1 inhibitors by an *in silico* analysis and later confirmed by an *in vitro* incubation study. Among those, nelfinavir had a significantly higher inhibitory effect than the other agents. The relative CES1 activity towards PNPA (a CES1 substrate) was 5.2%, 74.2%, 51.7%, 76.9%, and 67.8% of the control after

incubation with nelfinavir, ritonavir, amprenavir, saquinavir, and atazanavir, respectively [94].

An *in vitro* study suggested aripiprazole, perphenazine, thioridazine, and fluoxetine to be potent inhibitors of CES1, and a complementary animal study (n=10) with FVB mice demonstrated that co-administration of aripiprazole and methylphenidate (CES1 substrate) significantly increased the plasma concentrations of dl-methylphenidate ($P < 0.01$) [95].

Moreover, a total of 27 cardiovascular, antiplatelet, anticoagulant and immunosuppressant drugs have been tested for CES1 inhibition using human liver microsomes and recombinant CES1. The results suggested isradipine (a dihydropyridine calcium antagonist, DHP) and tacrolimus (an immunosuppressive agent) to be potent CES1 inhibitors. CES1 activity towards PNPA was decreased to 17.6% with isradipine, and 28.4% with tacrolimus [96].

An *In vitro* study suggested valproate could inhibit CES1 function and affect rufinamide metabolism in both microsomes and cytosol. This result could be clinically significant as the two antiepileptic medications are often prescribed together when monotherapy is ineffective [97].

A combined ensemble docking and machine learning approach was utilized to identify potential CES1 inhibitors from 1114 FDA-approved drugs. Among the identified inhibitor candidates, four drugs, including diltiazem, benztropine, iloprost, and treprostinil, were found to inhibit CES1 activity *in vitro* with IC_{50} values ranging from 13.9 µM to 391.6 µM [98].

Lastly, an *in vitro* study suggested that some naturally occurring oxysterols and fatty acids might significantly inhibit CES1 activity with IC_{50} values within the micromolar range [99]. These compounds could potentially affect CES1-mediated detoxification and drug metabolism *in vivo*.

1.8.3 CES1 Inducers

Overall, CES1 inducers are understudied relative to its inhibitors. Evidence suggests that various nuclear receptors might be involved in the regulation of CES1 expression [100]. For example, several agonists of peroxisome proliferator-activated receptors (PPARs) induced the mRNA expressions of several *CES1* isoforms in mouse livers [101]. A moderate increase of *CES1* expression was observed in human hepatocytes treated with rifampicin, a prototypical human PXR-activating agent [102]. An *in vivo* study with mice suggested that glucose could induce hepatic CES1 expression by stimulating *CES1* promoter activity and increasing acetylation of histone 3 and histone 4 in the CES1 chromatin, indicating a potential role of CES1 in glucose homeostasis [103]. Moreover, phenobarbital induced CES1 expression in mouse livers, and the inducibility was more prominent in neonatal mice relative to adult mice [104]. Again, a further clinical investigation is needed to determine the impacts of *CES1* inducers on the PK and PD of CES1 substrate medications.

1.8.4 Drug-Drug Interactions between CES1 Substrates

In addition to CES1 inhibitors and inducers, concomitant use of multiple CES1 substrate drugs can theoretically impact the substrate metabolism by competitively inhibiting the CES1. This hypothesis has been tested in several studies. An *in vitro*

study suggested trandolapril and enalapril might increase clopidogrel activation [105].

Consistent with the *in vitro* study, a follow-up retrospective clinical study reported the concomitant use of ACEI prodrugs and clopidogrel increases the risk of clinically important bleeding in patients with myocardial infarction (n=70,934) (P=0.002). The clinical significance of this finding is, however, debatable as the hazard ratio of clinically significant bleeding for patients on concomitant therapy was 1.10 (95% CI 0.97-1.25) [105]. Another clinical study with the similar design did not report a significant association between the composite cardiovascular outcome and the concomitant use of ACEI prodrugs and clopidogrel in patients with myocardial infarction (n=45,918). The adjusted odds ratio (aOR) for the perindopril was 0.94 (95% CI 0.76-1.16), and for ramipril was 0.97 (95% CI 0.80-1.18), relative to lisinopril, an ACEI not metabolized by CES1 [106].

1.9 Disease States Related to CES1

A prospective clinical study was conducted in monozygotic and dizygotic twin subjects (62–83 years) with (n=48) or without (n=247) type 2 diabetes mellitus (T2DM) to examine the association of CES1 with adiposity and metabolic function. CES1 mRNA expression level in adipose tissue was positively associated with body-mass index (P<0.001), fasting glucose level (P=0.002), insulin (P=0.006), and triglycerides (P=0.003) [107]. Recent studies have also found that CES1 function was positively correlated with increased liver lipid storage and plasma lipid concentrations, indicating that CES1 might be heavily involved in lipid metabolism and is a potential drug target for the treatment of human metabolic disorders [11,108,109].

Table 1-4 Drug-Drug Interaction Summary

CES1 inhibitors	CES1 Substrates	Interaction Summary
Alcohol	Methylphenidate	Many *in vitro* and *in vivo* studies confirmed alcohol inhibits CES1 and mediates biotransformation of methylphenidate to ethylphenidate; methylphenidate plasma concentrations were increased when patients took methylphenidate with alcohol [84-90,110].
Alcohol	Oseltamivir	When alcohol was administered with oseltamivir in humans, the AUC of oseltamivir increased by 37% [91].
Cannabis	Oseltamivir	*In vitro* study with CES1-transfected cells suggested THC, CBD and CBN to be the potent CES1 inhibitors. The inhibition constant (Ki) values for THC, CBD and CBN were 0.541, 0.974, and 0.263 μM (0.170, 0.306, and 0.0817 μg/ml) [92].
Protease Inhibitors	Methylphenidate. p-nitrophenyl acetate (PNPA) and p-nitrophenol (PNP)	*In vitro* study showed that protease inhibitors (i.e., nelfinavir, amprenavir, atazanavir, ritonavir, and saquinavir) inhibited the catalytic activity of CES1 (p <0.01). Among protease inhibitors, nelfinavir had a significantly higher inhibitory effect compared to other agents [94].
Aripiprazole	Methylphenidate, p-nitrophenyl acetate (PNPA)	*In vitro* study suggested aripiprazole, perphenazine, thioridazine, and fluoxetine to be potent inhibitors of CES1. Among the medications tested, aripiprazole was the most potent inhibitor of CES1, and an *in vivo* study with FVB mouse confirmed this result [95].
Isradipine /Tacrolimus	PNPA, Trandolapril	*In vitro* study with human liver microsomes suggested isradipine (dihydropyridine calcium antagonist, DHP) and tacrolimus (immunosuppressive agent) to be potent CES1 inhibitors [96].
Valproate	Rufinamide	*In vitro* study suggested valproate could inhibit CES1 function and affect rufinamide metabolism [97].
ACEI	Clopidogrel	ACEIs and clopidogrel are often administered together as both of them are cardiovascular medications; both

		ACEIs and clopidogrel are suggested to be inhibitors of CES1. A clinical study with myocardial infarction patients (n=70,934) demonstrated concomitant use of ACEIs increased the rate of clinically significant bleeding compared to the clopidogrel monotherapy (P=0.002) [105]. Another clinical study with myocardial infarction patients (n=45,918) with clopidogrel showed that concomitant use of clopidogrel and ACEI (perindopril and ramipril) was not associated with the re-infarction, heart failure or death [106].

*This chapter was used with permission from [1].

Chapter 2: Effect of CES1 Genetic Variation on Enalapril Steady-State Pharmacokinetics and Pharmacodynamics in Healthy Subjects[111]

2.1 Abstract

Background and Objective: Enalapril is a prodrug and needs to be activated by CES1. A previous *in vitro* study demonstrated the CES1 genetic variant, G143E (rs71647871), significantly impaired enalapril activation. Two previous clinical studies examined the impact of G143E on single-dose enalapril PK (10 mg); however, the results were inconclusive. A prospective, multi-dose, pharmacokinetics, and pharmacodynamics (PK/PD) study was conducted to determine the impact of the CES1 G143E variant on enalapril steady-state PK and PD in healthy volunteers.

Methods: Study participants were stratified to G143E non-carriers (n=15) and G143E carriers (n=6). All the carriers were G143E heterozygotes. Study subjects received enalapril 10 mg daily for seven consecutive days prior to a 72h PK/PD study. Plasma concentrations of enalapril and its active metabolite enalaprilat were quantified by an established LC-MS/MS method.

Results: The CES1 G143E carriers had 30.9% lower enalaprilat C_{max} (P = 0.03) compared to the non-carriers (38.01 vs. 55.01 ng/mL). The carrier group had 27.5% lower $AUC_{0-\infty}$ (P = 0.02) of plasma enalaprilat compared to the non-carriers (374.29 vs. 515.91 ng*hr/mL). The carriers also had a 32.3% lower enalaprilat-to-enalapril $AUC_{0-\infty}$ ratio (P = 0.003) relative to the non-carriers. The average maximum reduction of systolic

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blood pressure in the non-carrier group was approximately 12.4% at the end of the study compared to the baseline (P = 0.001). No statistically significant blood pressure reduction was observed in the G143E carriers.

Conclusions: The CES1 loss-of-function G143E variant significantly impaired enalapril activation and its systolic blood pressure-lowering effect in healthy volunteers.

2.2 **Introduction**

Enalapril is an angiotensin-converting enzyme inhibitor (ACEI), and ACEI is considered to be the first-line therapy for hypertension, heart failure, and chronic kidney disease (CKD) [112-114]. More than 5 million enalapril prescriptions were dispensed in the US in 2018 [115]. Enalapril is a prodrug and needs to be enzymatically biotransformed *in vivo* to its active metabolite enalaprilat to produce its intended pharmacological effect [1]. Prodrugs (e.g., enalapril) are often designed to overcome the low bioavailability associated with the low cellular permeability of these hydrophilic compounds. In the case of enalapril, the carboxylic acid functional group was masked using an ester-prodrug design, and the ester bond needs to be cleaved by the hepatic hydrolase carboxylesterase 1 (CES1) to release its active metabolite enalaprilat (Figure 2-1) [17,30,116]. Currently, 8 out of 10 FDA approved ACEIs are ester prodrugs, and these prodrugs are all activated by CES1 [1]. Two widely perceived assumptions behind the ester-prodrug design are (1) prodrugs are activated via unspecific esterases in the body, and (2) the interindividual variability in activating a prodrug is clinically insignificant. However, a previous study showed that enalapril can only be efficiently activated CES1, but not other hydrolases [17]. In addition, significant interindividual variability in the activation of enalapril and other ester prodrugs has been consistently

observed in the clinic[117,118], suggesting that genetic variants of prodrug activating-enzymes (e.g., CES1) could be a critical factor contributing to the variability in the pharmacokinetics (PK) and pharmacodynamics (PD) of many prodrugs.
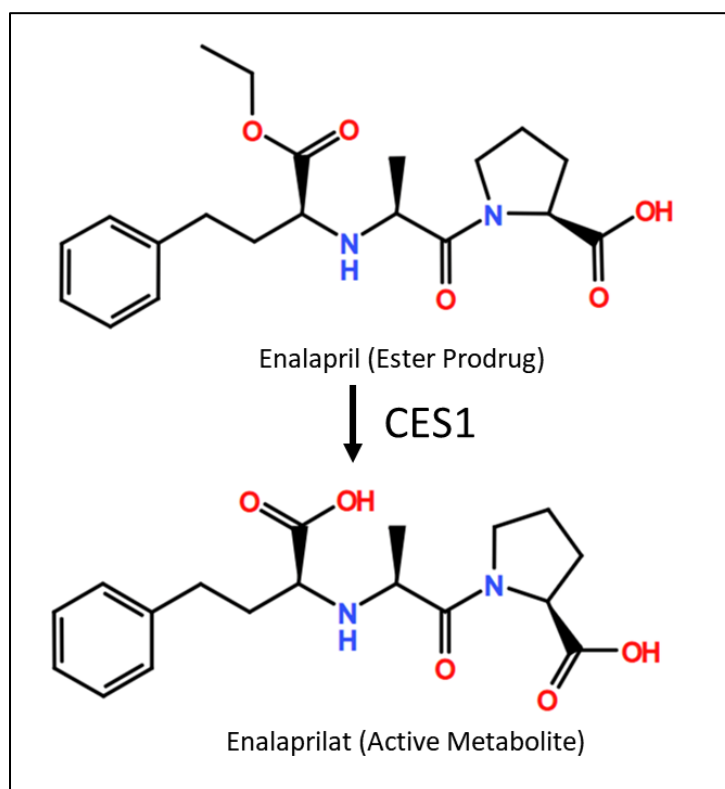
Figure 2-1 CES1-mediated enalapril activation

Previous studies have suggested enalapril monotherapy often resulted in inadequate response in patients with hypertension [119]. The interindividual variability in response to enalapril therapy is particularly concerning when treating heart failure or renal disease because there are no biomarkers such as blood pressure for monitoring the efficacy of enalapril in these patient populations. An in vitro study demonstrated that the catalytic activity of *CES1* G143E on enalapril activation was completely lost in cells transfected with the variant, suggesting G143E might be associated with the interindividual variability in response to enalapril treatment [17,120]. Two previous clinical

studies examined the impact of G143E on enalapril PK in healthy volunteers treated with a single dose of enalapril (10 mg); however, the results were inconclusive [121,122]. One study with Danish healthy volunteers (6 carriers and 16 non-carriers) showed no significant differences in enalaprilat PK between the carriers and the non-carriers (P > 0.05) [121]. The other study in Finnish subjects showed a 20% decrease for the enalaprilat $AUC_{0-\infty}$ (P = 0.049) in G143E carriers (n = 10) compared to non-carriers (n = 12) [122]. It is worth noting that long-term enalapril treatment is required in clinical practice, and it remains unexplored whether CES1 genetic variants could affect the steady-state PK and PD of enalapril. Several studies showed that steady-state PK parameters could be different from single-dose PK parameters [123,124]. In the present study, we conducted a multiple-dose PK study in healthy subjects to determine the impact of the G143E variant on enalapril steady-state PK and its blood pressure-lowering effect.

2.2.1 Pharmacokinetics Property of Enalapril

Previous studies reported that enalapril has a bioavailability of 60% and a Tmax of 1 hour; enalapril tablets have not been shown to have food effects, while enalapril suspensions did [125,126]. Enalapril has a protein binding of 50-60% [127]. Enalapril is extensively metabolized by liver CES1 and is excreted renally. The reported elimination half-life of enalapril is 1.3 hours.

2.3 Materials and Methods

2.3.1 Materials

Enalapril, enalapril-d5, and enalaprilat were purchased from Cayman Chemical (Ann Arbor, Michigan, USA), and enalaprilat-d5 was purchased from Toronto Research Chemicals (Toronto, Canada). Blank human plasma was obtained from Innovative

Research (Novi, Michigan, USA). Taq polymerase was obtained from New England Biolabs (Ipswich, MA, USA). All other chemicals and agents were of the highest analytical grade commercially available. Enalapril tablets (Vasotec®) from Merck with the same lot number were given to all participants to minimize the source of variability.

2.3.2 **Study Design**

A multi-dose enalapril PK and PD study was conducted at Michigan Clinical Research Unit, Ann Arbor, MI. All participants signed a University of Michigan Institutional Review Board (IRB) approved informed consent prior to participation (NCT03051282). Two arms in the study were the G143E carrier group and the non-carrier group based on their CES1 genotype. Participants took enalapril 10mg with 240mL room-temperature water for 7 consecutive days, and participants were instructed to fast 1 hour before and after the drug administrations to avoid potential food effects on drug absorption. Participants fasted starting 10 PM the night before the PK study, and a 72-hour PK study was initiated on the 7th day at 8 AM. Ten mL of blood was collected at the baseline (Day 1), immediately prior to the 7th dose of enalapril (Day 7, 0 hour), and 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 24, 48, and 72 hours post-dosing (Figure 2-

2). Blood samples were centrifuged at 2,000 × g for 10 min at 4 ℃, and the plasma

samples were collected, labeled, and stored at -80 ℃ until analysis.
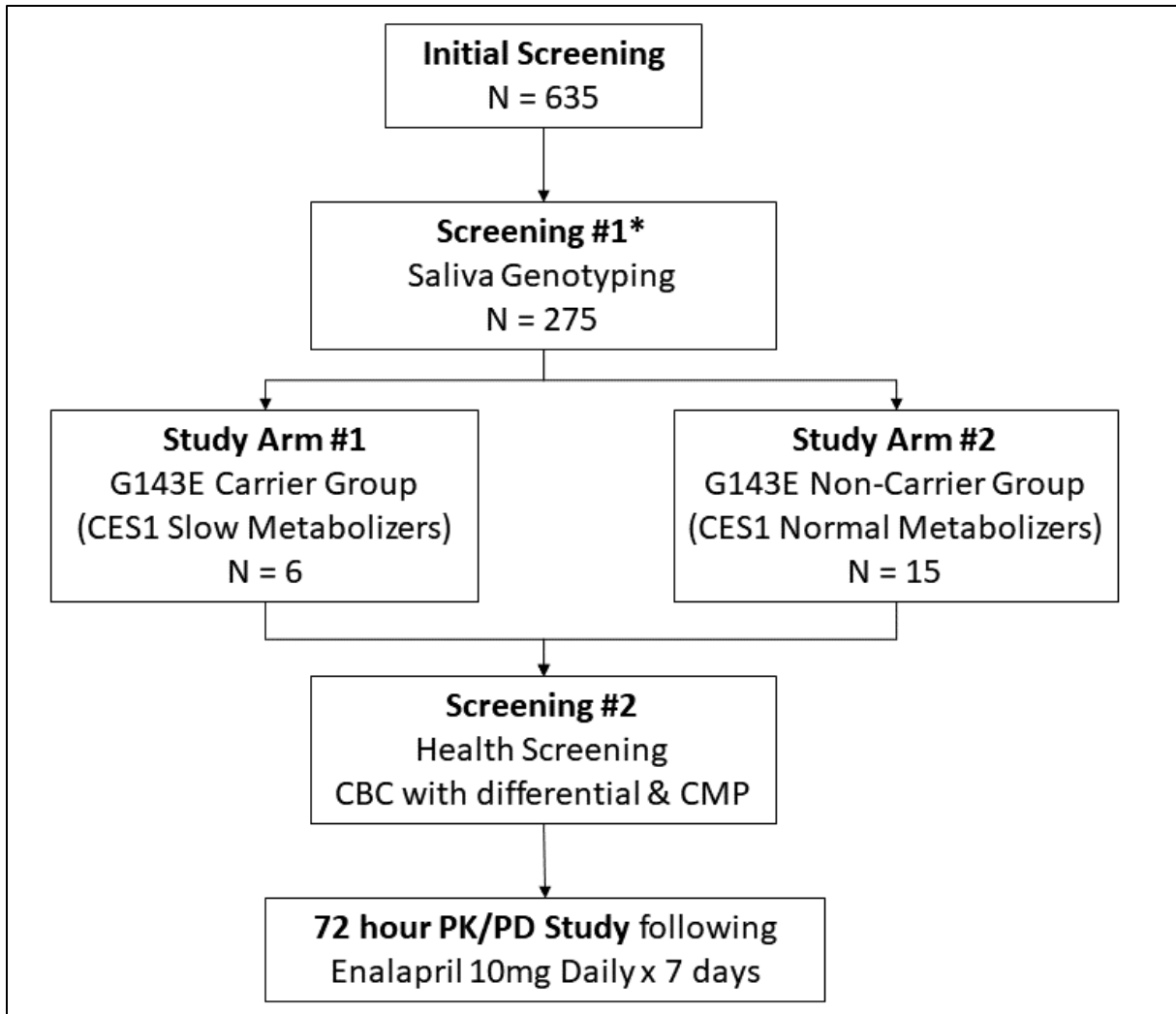
Figure 2-2 Study Design. 635 subjects were initially screened but only 275 of them met the inclusion/exclusion criteria. 275 subjects were invited to the screening visit #1 to sign the informed consent form and to provide the saliva sample for the CES1 genetic testing. Based on their CES1 G143E genotypes, subjects were stratified into the G143E carrier group (n=6) and the G143E non-carrier group (n=15). All participants in both groups completed the physical assessment and routine laboratory test to ensure their kidney and liver functions are normal. All participants took 10 mg of enalapril for 7 consecutive days, and a 72-hour PK study was conducted on the 7th day.

For the PD markers, resting systolic blood pressure (SBP), diastolic blood

pressure (DBP), and heart rate were measured at the baseline (Day 1, prior to taking

the first dose of medication) and during the PK study (Day7); the PD markers were measured immediately prior to blood draws during the PK study. The average maximum BP reduction was calculated by subtracting the lowest BP measured from Day 7 from the baseline BP (Day 1).

2.3.3 Study Participants

The major inclusion criteria were healthy volunteers between 18 and 55 years old. The major exclusion criteria were volunteers with any pre-existing condition, concurrent medication (including prescription and over-the-counter medications, herbal/vitamin supplement, and oral contraceptives), tobacco use, and excessive alcohol consumption. The detailed inclusion and exclusion criteria are listed in Table 2-1. All participants completed the physical assessment and routine laboratory tests (complete blood count with differential and comprehensive metabolic panel). The urine pregnancy test was done on all female participants. Baseline characteristics were matched between the G143E carrier (n=6) and the non-carrier groups (n=15) (Table 2-2). The sample size of 21 (6 carriers and 15 non-carriers) would provide approximately 80% power to detect the clinically meaningful AUC difference (30%) between G143E carriers and non-carriers at a 0.05 significance level when a standard deviation of 25% is assumed for each group[6,121,122,128].

Table 2-1 Inclusion and exclusion criteria of the study

Inclusion Criteria
• Healthy volunteers between the ages of 18-55 years old • Normal clinical laboratory values during the screening medical history
Exclusion Criteria
• Volunteers with any pre-existing medication condition (including pregnancy) were excluded as it might interfere with drug absorption, distribution, metabolism, or excretion. • Volunteers with any concurrent medication (including prescription and over the counter medications, birth control, herbal/vitamin supplement, and oral contraceptives), tobacco smokers, and excessive alcohol (>3 drink/day) users were excluded to avoid drug-drug interaction • No subjects weighing under 50 kg were selected • Subjects expressing inability to conform to dietary restrictions required for the study. Dietary restrictions were (1) abstaining from alcohol and grapefruit containing product starting one week prior to the study till the end, (2) fasting 1 hour before and after medication administration, and (3) fasting overnight a day before the 72-hour PK study • Asian descents were excluded as MAF of CES1 G143E is approximately 0% in the Asian population

2.3.4 Genotyping Procedure

275 healthy volunteers provided saliva samples for genotyping. Pure Link Genomic DNA Mini Kits (Life Technology, Austin, TX, USA) were used to extract DNA from saliva samples. The extracted DNA was genotyped using the genotyping method we published previously [17]. All participants in the G143E Carrier group (n=6) had a 143G/E genotype (i.e., G143E heterozygous carrier), and all participants in the non-carrier group (n=15) had 143G/G genotype (i.e., wild type) (**Supplemental Figure 1-1**).
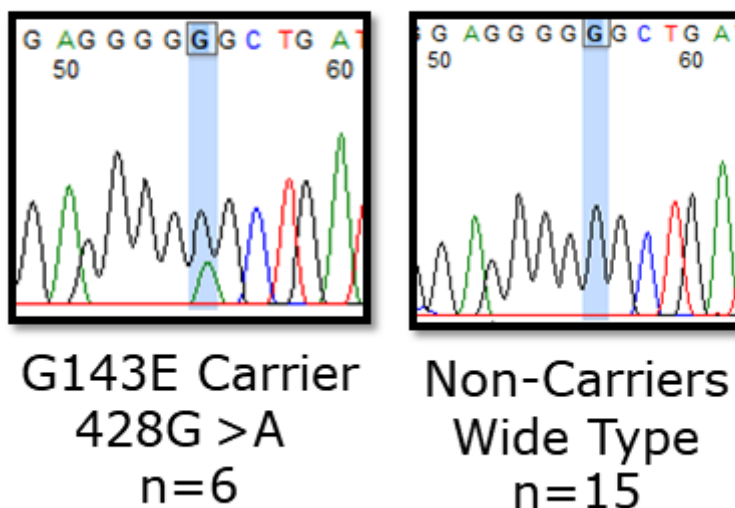
Supplemental Figure 1-1. Sanger sequencing result. All participants in the G143E Carrier group (n=6) had a 143G/E genotype (i.e., G143E heterozygous carrier). Both G and A alleles (G428G>A) were shown in the Sanger sequencing result (left panel). All participants in the non-carrier group (n=15) had 143G/G genotype (i.e., wild type) (right panel).

Enalapril and enalaprilat plasma concentrations were determined using an established LC-MS/MS method (Chapter 3). Briefly, 150 µL of plasma were prepared by mixing 30 µL trichloroacetic acid (TCA) 30% (w/v) containing the internal standards enalapril-d5 and enalaprilat-d5. The mixture was vortexed for 5 min and centrifuged for 10 minutes, and the supernatant was injected into an LC-MS/MS for the enalapril and enalaprilat quantification. The lower limit of quantification was 0.5 ng/mL for both enalapril and enalaprilat. Accuracy and precision results met the requirements in the FDA bioanalytical method validation guidance ranging from 2.1% to 9.6% for precision and from 96.9% to 114.2% for accuracy[129]. A parallel reaction-monitoring method was utilized to acquire the product ions of all four target precursors at m/z of 377.2 (enalapril), 349.2 (enalaprilat), 382.2 (enalapril-d5), and 354.2 (enalaprilat-d5). The

assay was validated in accordance with the FDA Bioanalytical Method Validation Guidance for Industry [130].

2.3.5 Data Analysis

LC-MS/MS data were analyzed using the Skyline software (version 20.1.0.76, University of Washington, Seattle, WA). The time-plasma concentration profiles of enalapril and enalaprilat were plotted using ggplot2 (R package) (Figure 2-3); enalaprilat concentrations vs. time were plotted on a semi-log scale (Figure 2-4). The PK parameters of enalapril and enalaprilat, including peak concentration (C_{max}), the area under the plasma concentration-time curve from 0 h to ∞ h ($AUC_{0-\infty}$), half-life ($t_{1/2}$), and clearance (CL) were estimated by non-compartmental analysis (NCA) using the R package PKNCA version 0.9.2. Statistical differences of PK and PD parameters between *CES1* G143E genotypes were evaluated using the one-tail student *t*-test. A P value less than 0.05 was considered statistically significant.

2.4 Results

Baseline characteristics were matched between the G143E carrier (n=6) and the non-carrier groups (n=15) to avoid potential confounding factors (e.g., age, sex, race, renal and liver functions)[131] (Table 2-2). Previous studies have suggested that CES1 expression is higher in females than in males[73], and African Americans tend to respond less to ACE-inhibitors due to the downregulated renin-angiotensin-aldosterone system (RAAS) pathway[132]. To avoid those potential confounding factors, age, sex, race, and renal functions were matched between the two study arms.

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Table 2-2 Baseline characteristics of study participants

	Carriers (n=6)			Non-Carriers (n=15)			P-value
Age (Year)	23.1	±	2.7	25.0	±	3.5	0.2
Sex (F/M)		1/5			2/13		1.0
BMI	25.1	±	4.0	23.0	±	2.8	0.2
CrCl (mL/min)	111.6	±	13.8	122.7	±	24.2	0.3
Race or Ethnic Group							
White		6			15		1.0

- F in Sex indicates female; M indicates male
- BMI, body mass index is calculated as the body mass divided by the square of body height
- CrCL, creatinine clearance is calculated using Cockcroft-Gault formula with participant's actual body weight
- Statistical differences in baseline characteristics between the carrier and the non-carrier group were evaluated using the two-tail student t-test

Compared to the previously reported MAF (3.7%) for the White population[133], our study showed approximately 2% MAF. The genotypes did not deviate from the Hardy-Weinberg Equilibrium. Again, all our study participants were Caucasian due to the geographical position. We have identified nine G143E heterozygotes from initial screening (n=275). Two participants declined to continue the study, and one participant was excluded due to the age limit (the participant turned 56 before proceeding to the Screening Visit #2) (Figure 2-2).
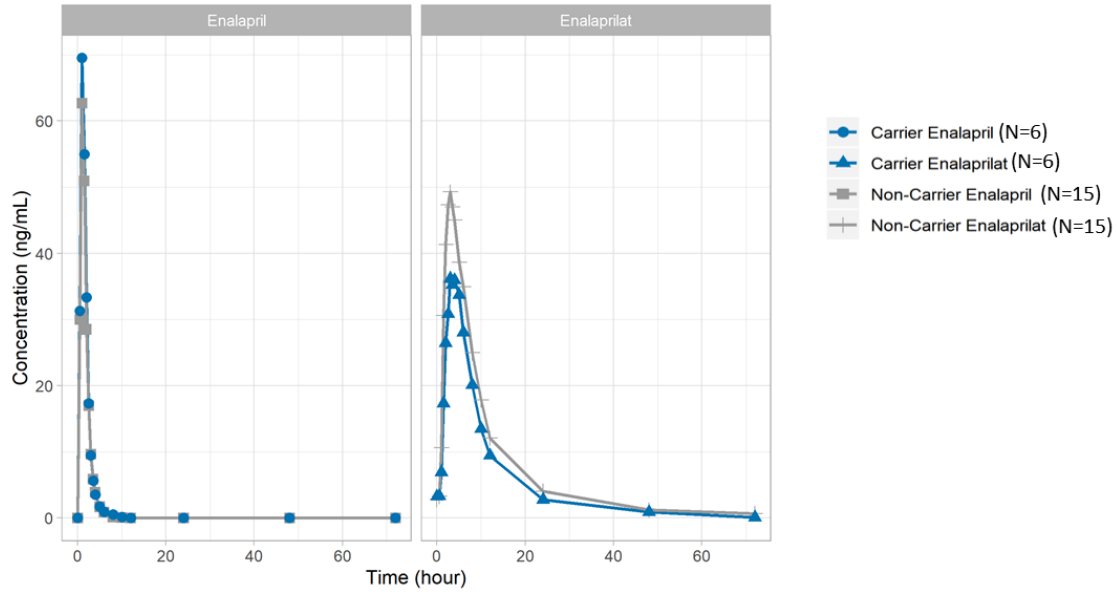
Figure 2-3 The profiles of the time-plasma concentrations of enalapril (left) and enalaprilat (right). Left panel shows enalapril concentrations (ng/mL) over time (hour), and right panel shows enalaprilat (active metabolite) concentrations (ng/mL) over time (hour). G143E carriers (i.e., CES1 slow metabolizers) are represented as a blue color, and G143E non-carriers (i.e., CES1 normal metabolizers) are represented as a gray color. Enalapril concentrations were slightly higher in the carrier group compared with the non-carrier group. Enalaprilat concentrations were significantly lower in the carrier group compared to the non-carrier group)
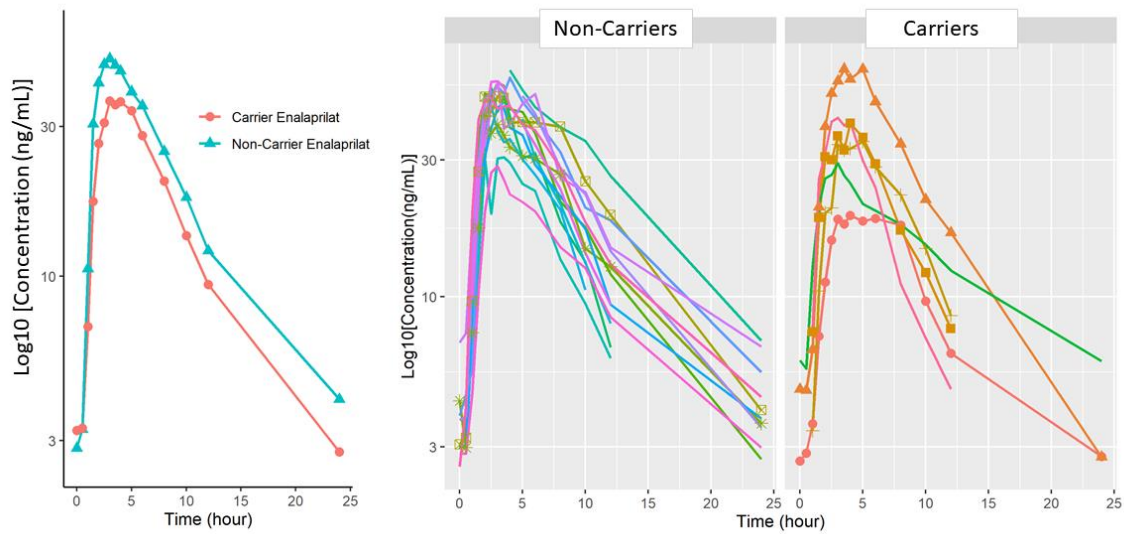
Figure 2-4 Enalaprilat concentrations (ng/mL) vs time (hour) were plotted on a semi-log scale (left panel). Red represents the average enalaprilat concentrations in the G143E carrier group (i.e., CES1 slow metabolizers), and blue represents the averages in the G143E

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2.4.1 Effect of CES1 G143E on Enalapril Pharmacokinetics

The CES1 G143E carrier group had 30.9% lower enalaprilat C_{max} (P = 0.03) and 27.5% lower $AUC_{0-\infty}$ (P = 0.027) compared to the non-carrier group (Figure 2-5). The carrier group also had 30.7% higher T_{max} (P = 0.01) of enalaprilat compared to the non-carrier group (Table 2-3). Even though statistically insignificant, the carrier group had 2.2% higher enalapril C_{max} and 9.1% higher $AUC_{0-\infty}$ compared to the non-carrier group. The elimination half-life and T_{max} of enalapril did not significantly differ between the two genotype groups.

Overall, the carrier group had a 32.3% lower enalaprilat-to-enalapril $AUC_{0-\infty}$ ratio (P = 0.003) compared to the non-carrier group (Figure 2-5). Noticeable interindividual variability in PK parameters was observed in both carrier and non-carrier groups. In non-carriers, the coefficient of variance (CV%) of the $AUC_{0-\infty}$ was 31.1% for enalapril and 29.1% for enalaprilat. In carriers, the CV% of the $AUC_{0-\infty}$ was 32.0% for enalapril and 28.0% for enalaprilat.
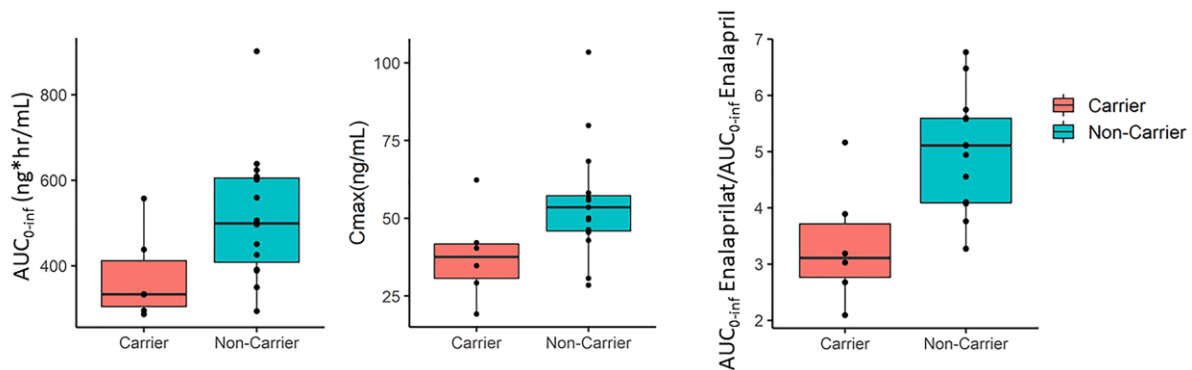
Figure 2-5 Major PK parameters comparison between the CES1 G143E carrier and non-carrier groups. The CES1 G143E carrier group (red) had 27.5% lower $AUC_{0-\infty}$ (P = 0.027) and 30.9% lower enalaprilat Cmax (P = 0.03) compared to the non-carrier group (blue). The carrier group (red) also had a 32.3% lower enalaprilat-to-enalapril $AUC_{0-\infty}$ ratio (P = 0.003) compared to the non-carrier group (blue).

2.4.2 **Effect of CES1 G143E on Enalapril Pharmacodynamics**

To minimize the potential confounding effects caused by the baseline variability in BP and heart rate among the individuals, the post-treatment BP and heart rate were normalized to the corresponding baseline values. The average maximum reduction of SBP in the non-carrier group was approximately 12.4% at the end of the study compared to the baseline (P = 0.001). There was no statistically significant SBP reduction observed in the G143E carriers (P > 0.05) (Figure 2-6). There was a statistically significant difference in the average maximum reduction of SBP between the non-carrier and the carrier groups (P = 0.016, Non-carriers: 14.6 ± 13.13 mmHg *vs.* carriers: -1.0 ± 10.68 mmHg). DBP and heart rate did not differ significantly from the baseline in both groups (P > 0.05). Overall, mean SBP reductions were found to be correlated with enalaprilat plasma concentrations.
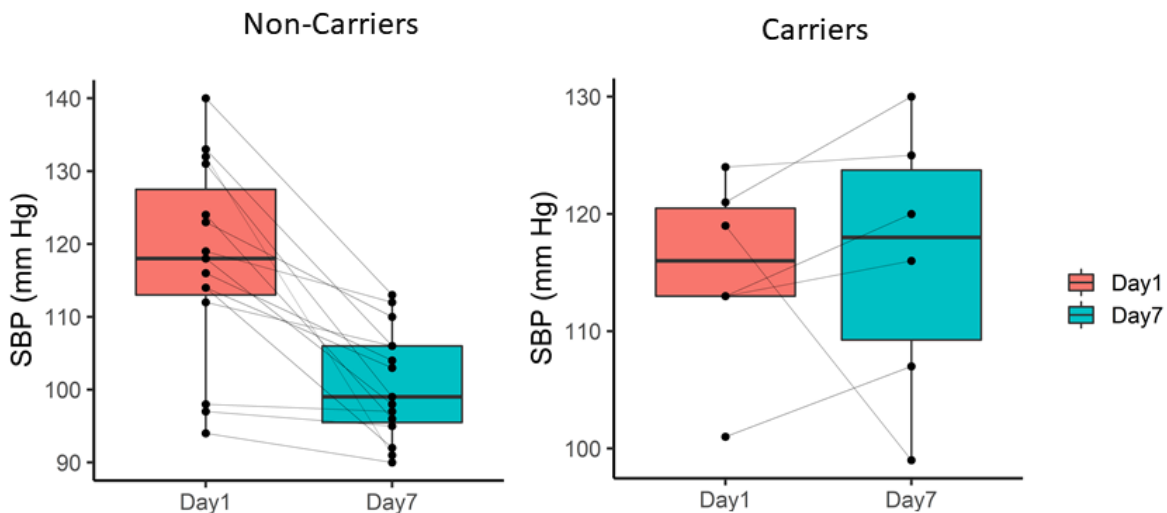
Figure 2-6 The paired sample t-test was performed to examine the difference between the baseline SBP and the SBP at the end of the study on each study arm. Blood pressure-lowering effect of enalapril in the CES1 G143E carriers and non-carriers. The non-carrier group had approximately 12.4% lower SBP at the end of the study compared to the baseline (P = 0.001). There was no statistically significant blood pressure reduction observed in the G143E carriers.

Table 2-3 Summary of PK parameter

	Non-Carriers			Carriers				Average Comparison (Carrier/Non-Carrier)	90% CI of Average Comparison	P-Value
Enalapril	**Average**		**Sd**	**CV (%)**	**Average**		**Sd**	**CV (%)**			
AUC $_{0-72h}$ (ng*hr/mL)	106.5	±	33.1	31.1	115.8	±	36.2	31.3	1.09	0.85-1.42	0.29
AUC $_{0-inf}$ (ng*hr/mL)	108.0	±	33.6	31.1	117.8	±	37.7	32.0	1.09	0.84-1.43	0.28
C_{max} (ng/mL)	68.0	±	22.2	32.7	69.5	±	20.0	28.7	1.02	0.80-1.34	0.44
T_{max} (hr), median (range)	1.0 (0.5-1.5)			22.3	1.0 (1.0-1.0)			0.00	0.99	0.89-1.11	0.36
T1/2 (hr)	1.3	±	1.4	105.7	2.1	±	2.1	101.5	1.35	0.62-2.93	0.18
Enalaprilat											
AUC $_{0-72h}$ (ng*hr/mL)	502.0	±	151.3	30.1	363.2	±	104.6	28.8	0.72	0.58-0.92	0.03*
AUC $_{0-inf}$ (ng*hr/mL)	515.9	±	150.0	29.1	374.3	±	104.7	28.0	0.73	0.58-0.92	0.02*
C_{max} (ng/mL)	55.0	±	18.6	33.7	38.0	±	14.5	38.2	0.69	0.49-0.96	0.03*
T_{max} (hr), median (range)	2.9 (1.5-4.0)			23.3	3.8 (3.0-5.0)			1.3	1.32	1.10-1.59	0.01*
T1/2 (hr)	16.5	±	6.3	38.1	10.9	±	4.9	44.9	0.66	0.46-0.95	0.03*
AUC /AUC $_{0-72h}$ (Enalaprilat/Enalapril)	4.9	±	1.1	21.6	3.3	±	1.0	29.4	0.67	0.52-0.85	0.002*
AUC/AUC $_{0-inf}$ (Enalaprilat/Enalapril)	4.9	±	1.1	21.9	3.3	±	1.1	32.0	0.68	0.51-0.87	0.003*

* indicates the statistically significant difference between the G143E non-carrier (i.e., CES1 normal metabolizers) and the G143E carrier group (i.e., CES1 slow metabolizers).
- Statistical differences of PK and PD parameters between *CES1* G143E genotypes were evaluated using the one-tail student *t*-test; geometric Mean and 90% CIs were included

2.5 **Discussion**

Numerous CES1 genetic polymorphisms have been investigated for their potential impact on CES1 function, and the G143E variant is the only one that has been consistently shown to significantly affect both PK and PD of CES1 substrate drugs [1]. A previous *in vitro* study showed enalapril is selectively activated by hepatic CES1, and CES1 G143E genetic variant completely impairs enalapril activation [17]. In this multi-dose healthy volunteer enalapril PK study, we demonstrated that CES1 G143E carriers had significantly lower enalaprilat exposure compared to the non-carriers. We also observed an appropriate trend of an increased plasma concentration of the prodrug enalapril in the carriers, although the differences did not reach the level of statistical significance. In addition, the blood pressure-lowering effect was only observed in the non-carrier group, which is consistent with the higher plasma concentrations of enalaprilat observed in the non-carrier group. Previous literature reported healthy volunteers responded less to the blood pressure-lowering medication (e.g., enalapril) compared to patients with hypertension due to the downregulated renin-angiotensin-aldosterone system (RAAS) pathway [134,135]. In clinical practice, the impact of G143E on the therapeutic effect of enalapril could be more pronounced in a patient population.

Two previous clinical studies examined the impact of G143E on single-dose enalapril PK, and the results were inconclusive [121,122]. One study reported that the mean enalaprilat $AUC_{0-\infty}$ in the carriers (n=6) was 6% lower than that in the non-carriers (n = 16) after study subjects were orally administered a single dose of 10 mg enalapril, however, the difference was statistically insignificant. It is worth noting that, in addition to G143E (6 variant carriers), several other CES1 variations were included in the

analysis (e.g., 15 individuals with different gene copy variations and 16 controls) [121].

Another single dose (10 mg enalapril) PK study in Finnish subjects (10 G143E carriers

and 12 non-carriers) showed a modest 20% decrease for the enalaprilat $AUC_{0-\infty}$ in the

G143E carriers compared to the non-carriers with a borderline significance (P = 0.049)

[122]. As a comparison, in the present multi-dose enalapril PK/PD study, despite the

smaller sample size (n=6 G143E carriers), a 27.5% reduction in enalaprilat $AUC_{0-\infty}$ was

observed in the G143E carriers (Figure 2-5). The underlying mechanism of the greater

effect of G143E in this multiple-dose study relative to the previous single-dose trials

remains elusive. We speculate that the impact of G143E on reducing enalaprilat

formation may have accumulated following each dose, and the maximum effect was

achieved after the PK reached a steady-state. Overall, this finding indicates that the

CES1 G143E variant may have a more significant impact on the steady-state PK; our

results (steady-state PK) are more indicative of the effects of this variant on real-world

patient populations. However, it should be noted that, given the small sample size and

large inter-individual PK variabilities in both the present investigation and the two

previous single-dose studies, the observed differences might not be statistically

significant.

In line with previous reports, significant intragroup variability of enalapril PK was

observed. Even though CES1 genetic polymorphisms are an important factor

contributing to CES1 variability, it is worth noting that all CES1 genetic variants

identified to date can only explain a small portion of interindividual variability of the

CES1 function [1]. Considering the low CES1 G143E MAF (0 to 4%), it can be therefore

assumed that CES1 G143E variation can only explain a limited portion of enalapril

response variation in clinical practice. Previous in vitro studies observed a marked variability of CES1 protein expression in human liver samples [17,136]. Considering G143E only affects the catalytic efficiency of CES1 without altering its expression [73,133,137], the main source of interindividual variability in the current study may have resulted from the different expression levels of CES1 in individual participants. Therefore, reliable CES1 biomarkers capable of predicting hepatic CES1 expression could be used to further improve the therapeutic outcomes of enalapril and other CES1 substrate drugs. As an excretory protein, CES1 can be released into the blood from tissues with high levels of CES1 expression (i.e., the liver). Indeed, we recently detected CES1 protein in human plasma using a highly sensitive LC-MS/MS proteomics assay [138]. Although plasma CES1 is insignificant for drug metabolism due to its extremely low plasma concentration, there is potential for plasma CES1 protein to be served as a biomarker to predict the PK and PD of enalapril and other CES1 substrate drugs. Alternatively, plasma exosomes are extracellular vesicles and contain functional proteins and nucleic acids derived from cells of different origins. A recent study showed high correlations between exosomal mRNA expressions and hepatic protein levels for several hepatic drug-metabolizing enzymes [139], but CES1 was not included in the study. It is plausible that CES1 genetic variants and plasma and exosomal CES1 could be used as complemental biomarkers allowing for the prediction of a large portion of CES1 variability and the development of an individualized pharmacotherapy strategy to improve the effectiveness and safety of drugs metabolized by CES1.

The main limitation of this study is its small sample size, which was mainly due to the low MAF of the G143E variant. In particular, given this small sample size and that

the study was done in healthy volunteers, the blood pressure-lowering effect needs to be further evaluated to better understand the true effect especially in a patient population. Importantly, we were not able to evaluate the effect of homozygous G143E on enalapril activation as all participants in the carrier group were G143E heterozygotes. It can be hypothesized that the magnitude of difference in PK and PD parameters between non-carries and homozygous G143E carriers may even be greater than what was observed with the G143E heterozygotes group.

This multi-dose enalapril healthy volunteer PK study demonstrated that the CES1 G143E variant significantly reduced enalapril activation and its blood pressure-lowering effect in healthy volunteers. Assuming enalapril follows linear kinetics[140-142], a 27.5% reduction in enalapril AUC might require a 38.3% increase in enalapril dose in G143E carriers. This is especially important for the treatment of heart failure or CKD as there is no biomarker (such as blood pressure) to adjust the dose or to switch the medication. Future studies are warranted to investigate the effects of the G143E variant on the activation and clinical outcomes of enalapril and other ACEI prodrugs in patients with hypertension, heart failure, and CKD.

*This chapter was used with permission from [111]

Chapter 3: A Liquid Chromatography - Parallel Reaction Monitoring Mass Spectrometry Method for the Quantification of Enalapril and its Active Metabolite Enalaprilat in Human Plasma[143]

3.1 Abstract

A simple, sensitive, and robust liquid chromatography-parallel-reaction monitoring (PRM) mass spectrometry method was developed and validated to quantify enalapril and enalaprilat in human plasma. This assay utilized trichloroacetic acid for plasma protein precipitation and stable isotope-labeled analytes (enalapril-d5 and enalaprilat-d5) as internal standards (IS), enabling precise and accurate quantification of enalapril and enalaprilat. The analytes were separated on a Waters Acquity UPLC C18 column using a five-minute gradient. The product ions from the precursors of enalapril (m/z 377.21), enalaprilat (m/z 349.18), enalapril-d5 (m/z 382.24, IS for enalapril), and enalaprilat-d5 (m/z 354.21, IS for enalaprilat) were detected in the positive ion mode using a PRM data acquisition method on a Sciex TripleTOF 5600+ LC-MS/MS system. The lower limit of quantification (LLOQ) was 0.5 ng/mL for both enalapril and enalaprilat, and the linear range was validated over 0.5 ng/mL to 200 ng/mL. The intra-batch and inter-batch variability, matrix effect, and stability were evaluated. This assay was successfully applied to an enalapril pharmacokinetics study in humans.

3.2 **Introduction**

Several liquid chromatograph-tandem mass spectrometry (LC-MS/MS) methods have been published for enalapril and enalaprilat quantification in human plasma[144-147]. These previously published assays met used the multiple-reaction monitoring methods on triple quadrupole mass spectrometers. Parallel-reaction monitoring (PRM) is a new data acquisition method originally developed for targeted proteomics analyses on a high-resolution and accurate mass (HRAM) mass spectrometer (e.g., Quadruple Time-of-Flight (Q-TOF) and Q-Orbitrap) [148]. Similar to MRM assays, PRM methods exhibit excellent sensitivity, dynamic range, and reproducibility for peptide and protein quantification. Moreover, PRM has two major advantages over MRM [149], including that 1) PRM is more specific because the MS/MS data is acquired in a high-resolution mode on an HRAM mass analyzer; 2) unlike MRM methods, PRM does not require a priori selection of product ions, thus, simplifying the assay development. In this study, we developed and validated a PRM method for quantifying plasma concentrations of enalapril and enalaprilat and successfully applied the method to an enalapril pharmacokinetics (PK) study in human subjects.

3.2.1 **Physicochemical Property of Enalapril**

Enalapril maleate is a Biopharmaceutics Classification System class III substance [150]. It has a human intestinal permeability of 1.57×10^{-4} cm/s [151], solubilities of 25 mg/mL (at pH 3.5) and 200 mg/mL (at pH 7.0) [152] and a partition coefficient (the log P, octanol/water) value of 2.45 [153]. The key pKa of enalapril subgroups are at 2.97 (carboxylic acid group) and 5.35 (amine group) at 25°C [154,155].

3.3 Materials and Methods

3.3.1 Reagents

Enalapril, enalapril-d5, and enalaprilat were purchased from Cayman Chemical (Ann Arbor, Michigan, USA), and enalaprilat-d5 was purchased from Toronto Research Chemicals (Toronto, Canada). Blank human plasma was obtained from Innovative Research (Novi, Michigan, USA). All other chemicals and agents were of the highest analytical grade commercially available.

3.3.2 Solution Preparation

All stock solutions were prepared in methanol. The working solution containing both enalapril and enalaprilat was prepared by diluting enalapril and enalaprilat stock solution at the following concentrations: 10, 20, 50, 100, 200, 500, 1000, 2000, 4000 ng/mL. To prepare calibrator samples, 142.5 uL of blank plasma was mixed with 7.5 uL of working solution (total of 150uL), and the final concentrations of calibration standards were 0.5, 1, 2.5, 5, 10, 25, 50, 100, and 200 ng/mL. Quality control (QC) samples' concentrations for both analytes were 1 ng/mL (low), 25 ng/mL (medium), and 200 ng/mL (high).

3.3.3 Human PK Study

A multiple-dose enalapril PK study was performed in humans. Briefly, study subjects received enalapril tablet 10 mg daily for seven consecutive days prior to a 72 h PK study. Ten mL of blood was collected at the baseline (Day 1), immediately prior to the 7th dose of enalapril (Day 7, 0 hour), and 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12,

24, 48, and 72 hours post-dosing. Blood samples were centrifuged at 2,000 × *g* for 10 min at 4 ºC, and the plasma samples were collected, labeled, and stored at -80 ºC until analysis.

3.3.4 Plasma Sample Preparation

150 µL of plasma sample was mixed with 30 µL trichloroacetic acid (TCA) solution (30%, w/v) containing the internal standards (IS) enalapril-d5 (20 ng/mL) and enalaprilat-d5 (100 ng/mL) for the quantification of enalapril and enalaprilat, respectively. The mixture was vortexed at 1,500 rpm for 5 min, followed by centrifugation at 21,000 × *g* for 10 min at 4 ºC. Fifteen µL of the supernatant were injected into an LC-MS/MS system for the determination of enalapril and enalaprilat concentrations.

3.3.5 LC-MS/MS Conditions

The LC-MS/MS system consisted of a Sciex TripleTOF 5600+ mass spectrometer (Sciex, Framingham, MA) coupled with a Shimadzu LC system (Shimadzu, Tokyo, Japan). Analytes were separated on an analytical column (ACQUITY UPLC BEH C18-CL, 130 Å, 50 × 2.1 mm, 1.7 µm, Waters, Milford, MA). Mobile phase A was water with 0.1% formic acid, and mobile phase B was acetonitrile with 0.1% formic acid. The mobile phase was delivered at a flow rate of 0.22 mL/min for 5 min, and the gradient is described in Supplemental Table 3-1. The mass spectrometer was operated in a positive ion mode with an ion spray voltage floating at 5,500 V, ion source gas one at 30 psi, ion source gas two at 40 psi, curtain gas at 25 psi, and source

temperature at 550 ºC. The PRM acquisition consisted of a 200 ms TOF-MS scan from

300-400 Da and subsequent 120 ms product ions scans from 50 Da to 400 Da for each

target precursors at m/z of 377.21 (enalapril), 349.18 (enalaprilat), 382.24 (enalapril-d5),

and 354.21 (enalaprilat-d5). The MS parameters were set the same for all four

compounds with declustering potential at 50 v, collision energy at 30 v and collision

energy spread at 5 v.

Supplemental Table 3-1 The LC gradient conditions for enalapril and enalaprilat
LC-MS/MS analysis

Time (min)	B (%)	A (%)
0	3	97
2.5	20	80
2.8	90	10
3.4	90	10
3.8	3	97
5	Stop	

Mobile phase:
A: water containing 0.1% formic acid
B: acetonitrile containing 0.1% formic acid

3.3.6 **Data Analysis**

LC-MS/MS data were analyzed using the Skyline software (version 20.1.0.76,

University of Washington, Seattle, WA). The peak area sum of the top 3 product ions for

each analyte was used for quantification. Specifically, the product ions of 303.1703,

234.1498, and 160.1121 were used for enalapril; 303.1703, 206.1176, and 160.1121

were used for enalaprilat; 308.2040, 239.1816, and 165.1447 were used for enalapril-

d5; 308.2049, 211.1503, and 165.1552 were used for enalaprilat-d5 (Figure 3-1). The

enalapril/enalapril-d5 and enalaprilat/enalaprilat-d5 peak ratios were calculated for

quantifying enalapril and enalaprilat plasma concentrations. The R package ggplot2 was used to draw a plasma concentration-time profile for the preliminary PK study.
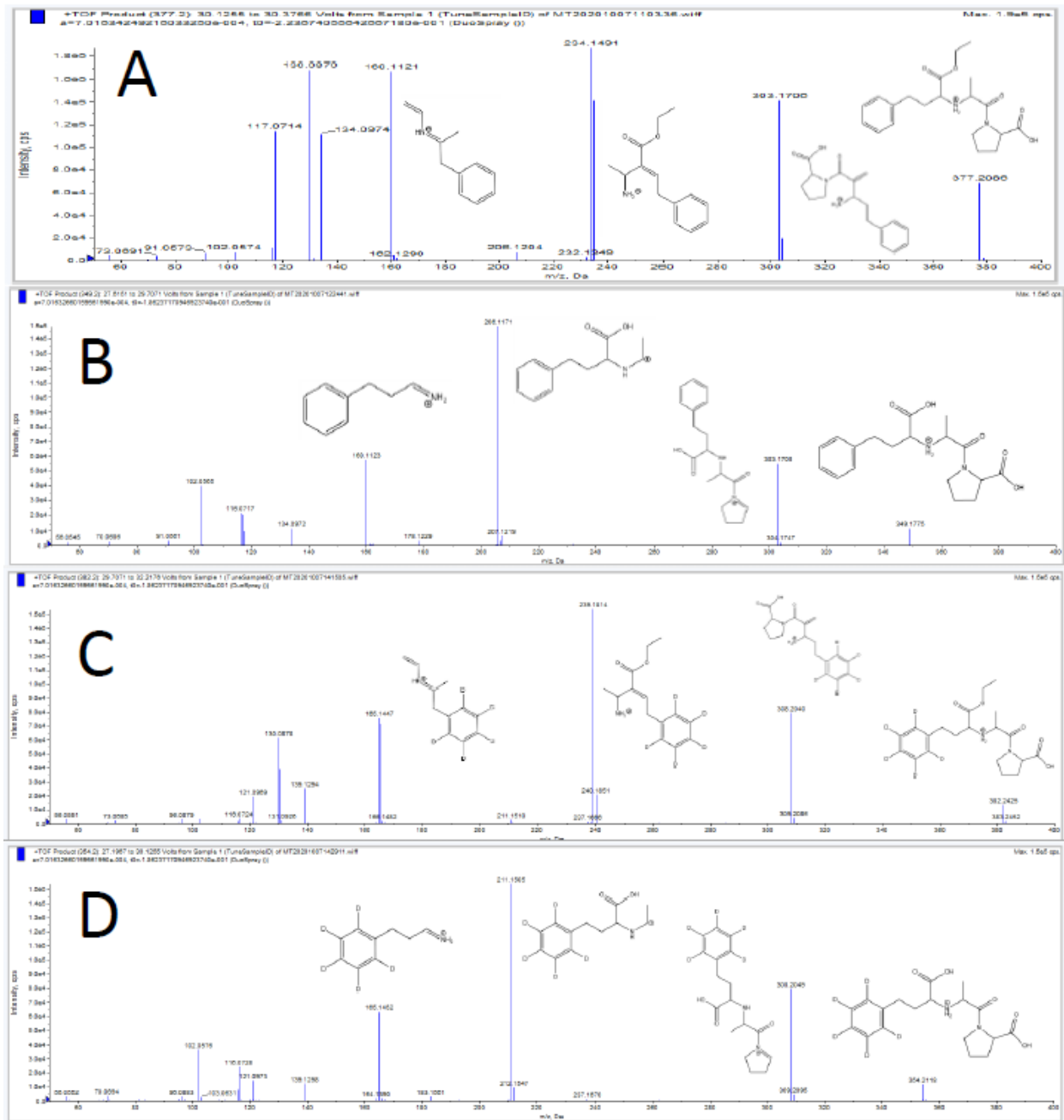
Figure 3-1 Mass spectra showing both the precursor and product ions of enalapril (A), enalaprilat (B), and their isotope-labeled internal standard enalapril-d5 (C) and enalaprilat-d5 (D).

3.4 **Results**

3.4.1 **Method Validation**

The bioanalytical method for enalapril and enalaprilat was validated for specificity, linearity, precision, accuracy, and stability based on the FDA Bioanalytical Method Validation Guidance for Industry.

3.4.2 **Selectivity**

Selectivity was evaluated by analyzing blank plasma samples and blank plasma spiked with enalapril and enalaprilat at the lower limit of quantification (LLOQ) (0.5 ng/mL). The blank human plasma samples were obtained from six different sources. No significant interference peaks were observed in any blank samples (Figure 3-2). The heights of background peaks were less than 10% of enalapril and enalaprilat at the LLOQ.
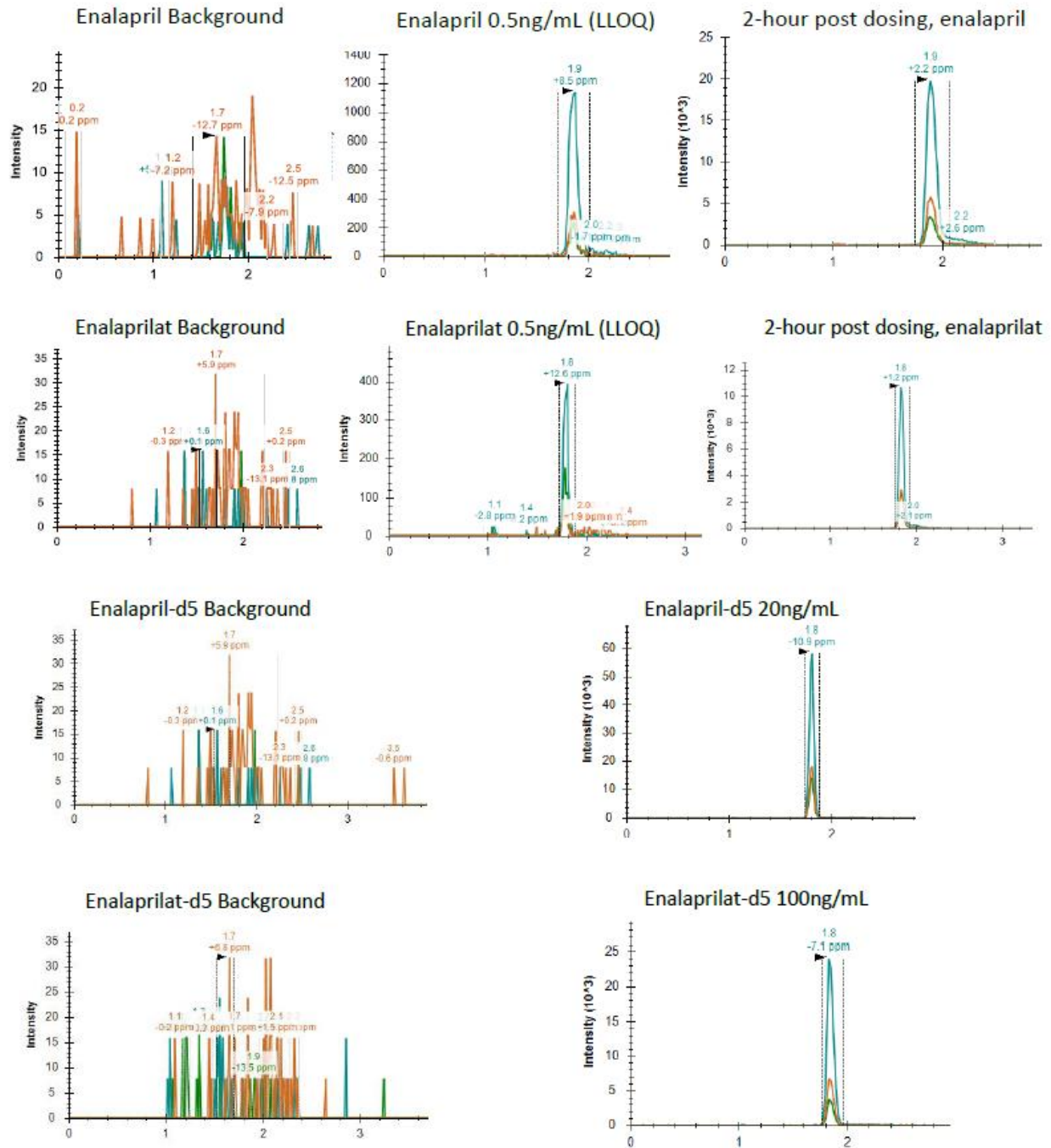
Figure 3-2 Representative LC-MS/MS chromatograms of blank plasma, plasma at the lower limit of quantifications (LLOQ, 0.5ng/mL) and plasma samples from an enalapril PK study in humans (2-hour post-dosing of 10mg enalapril). Enalapril-d5 and Enalaprilat-d5 chromatograms are also included.

3.4.3 Linearity and LLOQ

The assay showed excellent linearity ($R^2 > 0.99$) over the concentration range of 0.5-200 ng/mL for both enalapril and enalaprilat (Figure 3-3). The LLOQ was 0.5 ng/mL for both enalapril and enalaprilat in plasma at a signal-to-noise ratio >10. The evaluation results of accuracy and precision at the LLOQ were provided in the section below.
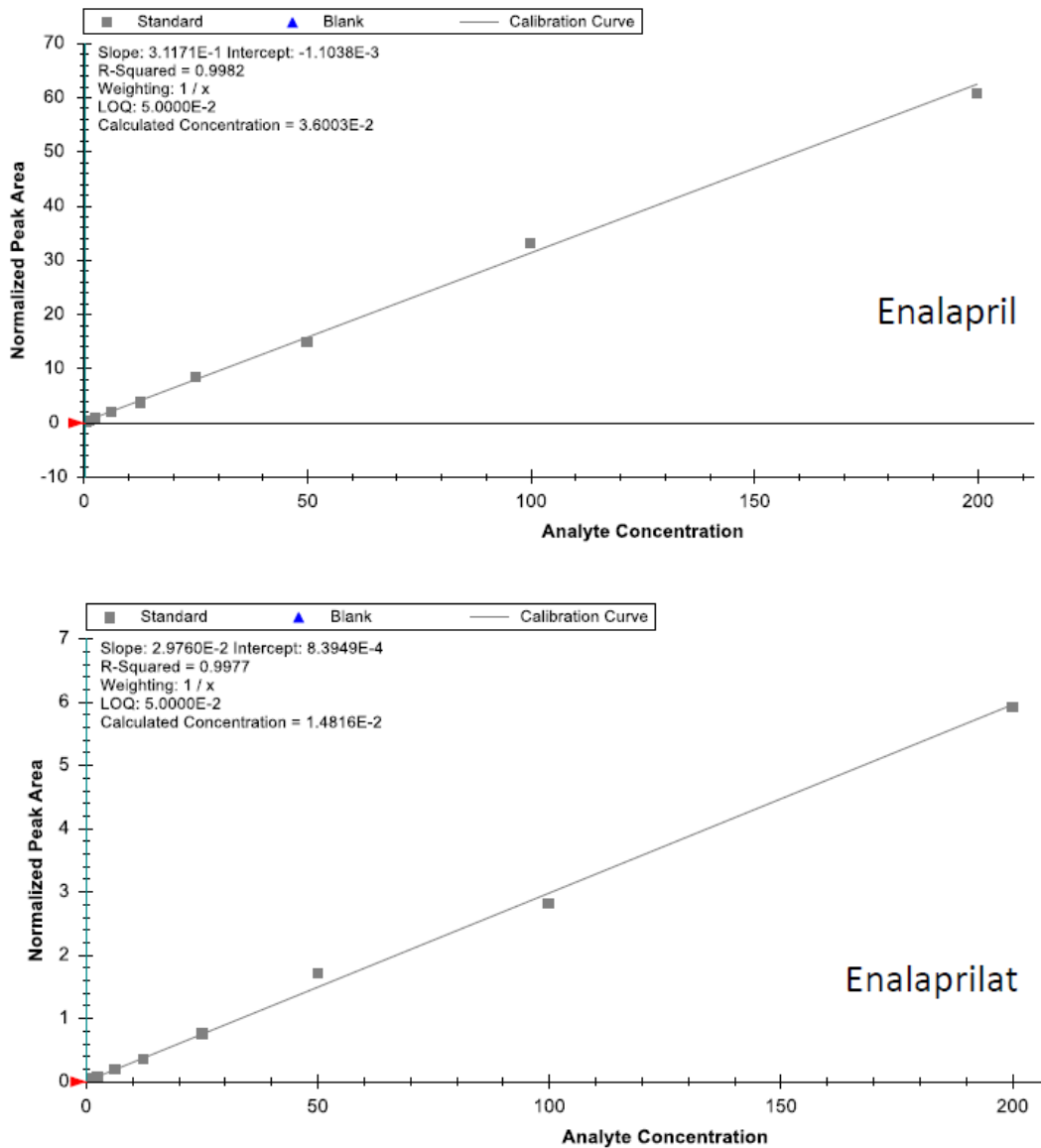
Figure 3-3 Calibration curves for enalapril and enalaprilat with the concentrations ranging from 0.5 ng/mL to 200 ng/mL in human plasma.

3.4.4 **Accuracy and Precision**

Intra-batch and inter-batch accuracy and precision for enalapril and enalaprilat were evaluated by analyzing three QC samples (Table 3-1) with three replicates for each sample. The concentrations of the QC samples were 1 (Low-QC), 25 (Mid-QC), 200 (High-QC) ng/mL. Accuracy and precision were also determined at the LLOQ for both analytes. Accuracy and precision results met the requirements in the FDA bioanalytical method validation guidance with the range of 0.01-9.62% (precision) and 96.89-114.80% (accuracy)[129].

Table 3-1 Summary of intra-day and inter-day accuracy and precision of LC-MS/MS assay for the determination of enalapril and enalaprilat in human plasma (n=3).

Concentration	Inter-batch		Intra-batch	
	Accuracy (%)	**Precision (%)**	**Accuracy (%)**	**Precision (%)**
Enalapril				
LLOQ	111.18	4.72	101.21	9.62
Low-QC	100.22	3.43	106.76	1.87
Mid-QC	107.56	2.07	114.80	3.30
High-QC	107.57	6.35	103.11	7.99
Enalaprilat				
LLOQ	109.87	8.68	114.15	1.30
Low-QC	99.50	2.80	97.06	0.01
Mid-QC	106.10	4.25	110.56	6.04
High-QC	105.27	7.96	96.89	2.22

3.4.5 **Matrix Effects**

Six blank human plasma samples from different sources were used to assess the matrix effects. Two sets of QC samples were prepared. Set A: QC samples (1, 200 ng/mL) prepared with blank human plasma (n=6). Set B: QC samples (1, 200ng/mL) prepared with water (n=3). The percentage of the matrix effect was calculated by dividing the peak area obtained from Set A by Set B. As shown in Table 3-2, the mean

matrix effect for both analytes ranged from 37.47% to 74.00% with CV ranged from

1.36% to 12%.

Table 3-2 Matrix effect of enalapril and enalaprilat in human plasma (n=3 with water, n=6 with plasma).

Analytes and Matrix	Concentration (nM)	Matrix effect (%, Mean ± S.D.)	CV (%)
enalapril	0.5	58.37 ± 7.00	12.00
	200	74.00 ± 2.40	3.24
enalaprilat	0.5	37.47 ± 5.10	1.36
	200	41.70 ± 1.82	4.37

3.4.6 **Stability**

The stability of enalapril and enalaprilat in human plasma was tested under

different conditions, including benchtop, autosampler, and long-term (-80 °C) stability;

three replicates were prepared for each condition. Freeze-thaw stability was not

evaluated given that freeze-thaw stability has been studied in previous investigations

and is independent of analytical assays[144,145,147]. Benchtop stability was tested after

exposing the QC samples at room temperature for 12 hours. The autosampler stability

was tested after storing the processed QC samples in autosampler (4 °C) for 7 days.

The long-term stability was tested after the QC samples were stored at -80 °C for 30

days. The results were compared with the freshly prepared QC samples. The assay

was stable in all conditions, and enalapril and enalaprilat concentrations were within

84.04-111.86% of the freshly prepared QC samples (Table 3-3).

Table 3-3 Stability of enalapril and enalaprilat in human plasma under different conditions (n=3).

	Accuracy (%)		
Enalapril	Bench-Top Stability (After 12 Hours)	AutoSampler (4 °C for 7 days)	30 days at −80 °C
QC-L	95.35 ± 8.45	94.73 ± 9.61	103.97 ± 2.67
QC-H	111.86 ± 6.37	103.09 ± 7.02	105.45 ± 4.16
Enalaprilat			
QC-L	100.75 ± 0.72	101.01 ± 1.63	97.08 ± 11.51
QC-H	84.04 ± 8.00	93.14 ± 4.24	99.98 ± 9.42

3.4.7 Application in a Human PK Study

The validated method was successfully applied to a preliminary PK study in humans after oral administration of enalapril tablet 10 mg for 7 consecutive days. Although limited to one subject (Figure 3-4), the method enabled accurate quantification of enalapril and enalaprilat in human plasma for up to 72 hours.
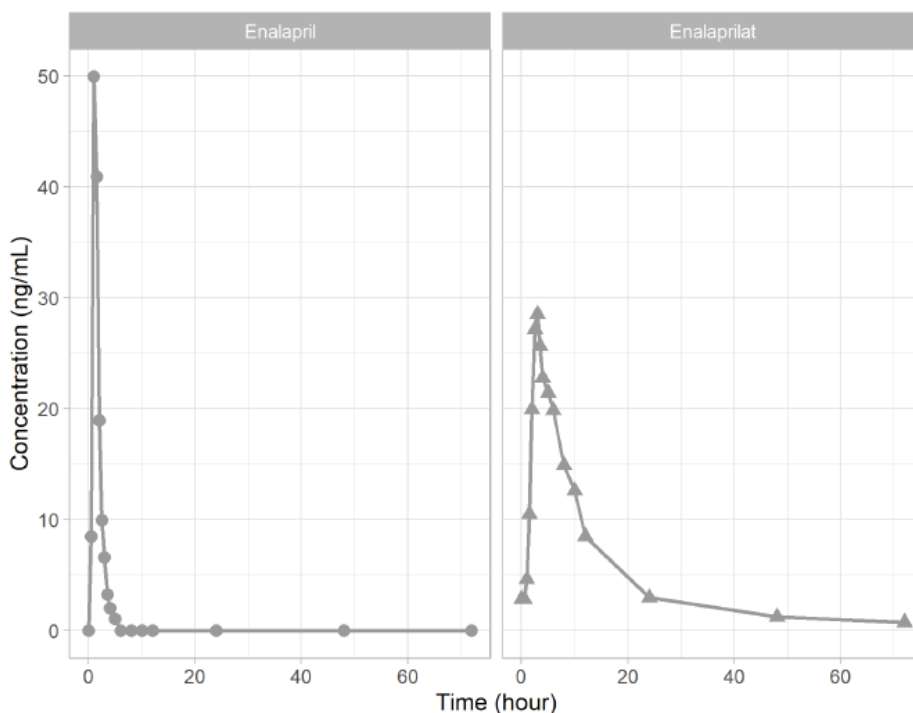
Figure 3-4 A plasma concentration-time profile of enalapril and enalaprilat in a healthy human subject after 10 mg enalapril oral administration for 7 consecutive days (a preliminary PK study).

3.4.8 Comparison of the Current Method with Previously Published LC-MS/MS Methods

To the best of our knowledge, this is the first PRM assay to quantify enalapril and enalaprilat in human plasma, whereas previously published methods used the MRM-based data acquisition [144-147]. PRM was originally developed for targeted proteomics analysis and has been increasingly used for small molecule quantification in the past decade [148,156]. MRM methods are performed on low-resolution triple quadrupole mass spectrometers. In contrast, PRM utilizes HRAM instruments, such as Q-TOF and Q-Orbitrap, enabling higher specificity and selectivity [148]. In addition, different from MRM, pre-selecting product ions are not required for PRM, which simplifies the assay development procedure. The sensitivity of our PRM assay was found to be comparable to that reported in previous studies (LLOQ: 0.5 ng/mL vs. 0.638-1 ng/mL), although a lower LLOQ can be achieved based on the signal-to-noise ratios observed at the current LLOQ (Figure 3-2). Moreover, the IS used in the previous methods were compounds with chemical structures similar to enalapril and enalaprilat. As a comparison, the IS in our method were stable-isotope labeled analytes (i.e., enalapril-d5 and enalaprilat-d5), which are more reliable and robust than other types of IS. Finally, a TCA protein precipitation method was developed for plasma sample preparation in the present study, while the previous studies utilized either acetonitrile protein precipitation or solid-phase extraction methods. The TCA method results in less sample dilution (i.e., higher sensitivity) compared to acetonitrile protein precipitation methods and is more efficient and cost-effective than solid-phase extraction.

3.5 **Conclusion**

In this present study, we have developed and validated a sensitive and robust PRM LC-MS/MS method for the analysis of enalapril and enalaprilat in human plasma. The unique PRM approach, the TCA protein precipitation method, and the stable-isotope labeled IS enabled precise and accurate quantification of enalapril and enalaprilat in human plasma with LLOQ at 0.5 ng/mL. A preliminary PK study showed that this assay is readily applicable to human enalapril PK study.

Chapter 4: Identification of CES1 Regulatory Variants Using an Allele-Specific Protein Expression (ASPE) Method

4.1 **Abstract**

Identifying genetic variants responsible for regulating the expression of drug-metabolizing enzymes is important and can lead to the optimization of pharmacotherapy of many medications. In the present study, we developed an allele-specific protein expression (ASPE) assay using a heavy stable isotope-labeled QconCAT internal standard to identify the CES1 regulatory variants. The CES1 nonsynonymous variant S75N (rs2307240) was utilized as a marker to differentiate the protein expressions of the two CES1 alleles. The method was successfully applied to the determination of the ASPE of CES1 in 30 S75N heterozygous liver samples. Two cis-acting regulatory variants were found to be significantly associated with CES1 ASPE and protein expression in the human liver microsome samples ($P < 0.05$). The association was confirmed by the studies of CES1 expression and catalytic activity on enalapril hydrolysis in human liver S9 fraction samples. Compared to conventional gene expression-based approaches (e.g., GWAS), ASPE has a better statistical power to detect a regulatory variant with a small effect size since non-genetic regulators (e.g., inducers) would not alter the allelic expression ratios. Relative to mRNA allele-specific expression assays, ASPE also accounts for the genetic variants regulating gene

expression at the post-transcriptional level. Thus, the ASPE approach is expected to be widely used to identify cis-regulatory variants.

4.2 **Introduction**

The expression of a drug-metabolizing enzyme can vary markedly, which can be a major contributing factor to the interindividual variability in pharmacokinetics and pharmacodynamics of its substrate medications. The CES1 gene is highly polymorphic with numerous genetic variants in both regulatory and coding regions. CES1 nonsynonymous variants have been extensively studied in the past decade. Among those identified CES1 nonsynonymous variants, the loss-of-function variant G143E (rs71647871) markedly altered the pharmacokinetics and clinical outcomes of several CES1 substrates, such as methylphenidate and clopidogrel [128,133]. However, these nonsynonymous variants can only explain a small portion of CES1 interindividual variability because of their low frequencies and do not account for the expression variation. Although much effort has been devoted to identifying CES1 regulatory variants [23,24,59-65,67,69,71], none of the studied variants showed consistent effects on CES1 expression or clinical outcomes across different studies.

Regulatory genetic variants can be classified into two classes: cis- and trans-acting variants. A cis-acting regulatory variant is located in the proximity of the gene being regulated, such as the promoter of the gene, while a trans-variant and the regulated gene are usually located on different chromosomes [157,158]. As a consequence, cis-variants affect gene expression in an allele-specific manner, whereas trans-variants regulate gene expression in both alleles. Trans-variants typically have weaker effects on gene expression than cis-variants [158-160]. Gene expression can be heavily influenced by

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non-genetic factors (e.g., disease and age), which can impair the statistical power when the gene expression level is used as a phenotype for genetic variant identification. Accordingly, measuring gene allele-specific expression (ASE) has been increasingly used as a powerful means to identify cis-acting polymorphisms because non-genetic regulators do not alter the allelic expression ratios.

ASE was traditionally studied at the mRNA level. However, recent studies with various DMEs, including CES1, showed that mRNA expression was poorly correlated with the protein expression and enzymatic activity, whereas the correlations between protein levels and the corresponding enzymatic activity were high [161,162]. The discordant mRNA and protein expression are likely caused by post-transcriptional processes, such as protein translation, post-translational modification, and degradation. Genetic variants affecting such post-transcriptional processes could not be identified by conventional mRNA expression and ASE approaches. Thus, a new strategy is needed to study gene expression regulation at the protein level to identify all functional genetic variants, including those affecting gene expression at the post-transcriptional level. Accordingly, we recently developed a novel targeted proteomics method to precisely quantify the allele-specific protein expression (ASPE) of a gene using QconCAT internal standards (Figure 4-1) [163]. This ASPE approach is superior to the conventional ASE method because it has the potential to detect genetic variants that regulate gene expression at the post-transcriptional level. The ASPE assay is also advantageous over conventional protein expression-based assays because allelic protein expression ratios are not affected by non-genetic regulators. In this study, we used this ASPE assay to identify cis-acting variants of the CES1 gene and revealed two single nucleotide polymorphisms

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(SNPs) that were significantly associated with CES1 protein expression and activity in human livers.
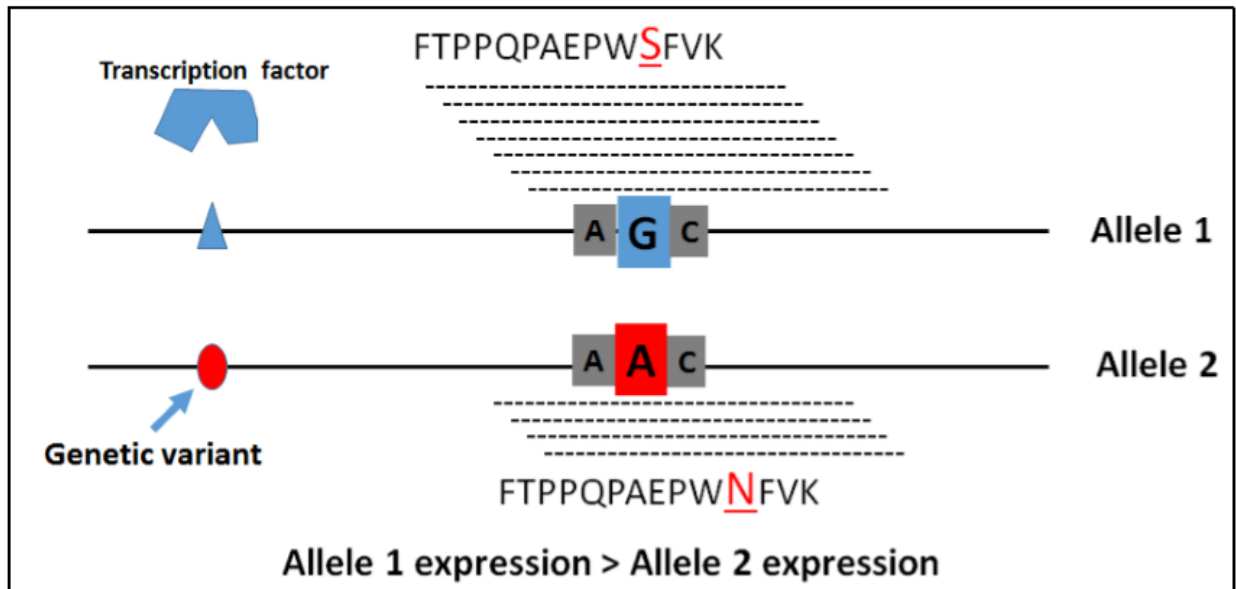
Figure 4-1 ASPE Concept

4.3 **Materials and Methods**

4.3.1 **Materials**

Amino acids, acetonitrile, benzonase nuclease, calcium chloride hexahydrate, formic acid, glucose, M9 salts, magnesium sulfate, imidazole, isopropyl-D-1-thiogalactopyranoside disodium phosphate, sodium chloride, thiamine, and trifluoroacetic acid were purchased from Sigma-Aldrich (Saint Louis, MO). $^{13}C_6$ arginine and $^{13}C_6$ and $^{15}N_2$ lysine were products from Cambridge Isotope Laboratories (Tewksbury, MA). Lysyl endopeptidase was purchased from Wako Chemicals (Richmond, VA). TPCK-treated trypsin was purchased from Worthington Biochemical Corporation (Freehold, NJ). Urea and dithiothreitol were purchased from Fisher Scientific Co. (Pittsburgh, PA). Iodoacetamide and ammonium bicarbonate were

purchased from Acros Organics (Morris Plains, NJ). Water Oasis HLB columns were from Waters Corporation (Milford, MA). Synthetic iRT standards solution was from Biognosys AG (Cambridge, MA). *Escherichia coli* strain BL21(DE3) and BugBuster protein extraction reagent were products of EMD Millipore (Burlington, MA). HisTrap HP histidine-tagged protein purification columns were from GE Healthcare (Pittsburgh, PA). Lysozyme solution (50 mg/mL), slide-A-Lyzer G2 dialysis cassettes (3.5K MWCO), and Pierce^{TM} BCA protein assay kit were obtained from ThermoFisher Scientific (Waltham, MA). 287 normal human liver samples were obtained from XenoTech LLC (Lenexa, KS, USA), the University of Minnesota Liver Tissue Cell Distribution System, and the Cooperative Human Tissue Network (CHTN) (Supplemental Table 4-1) [13].

Supplemental Table 4-1 Gender and ethnicity of human liver samples

Characteristics		Number of Samples	Percentage (%)
Gender	Male	114	39.7
	Female	156	54.4
	Not available	17	5.9
Ethnicity	Caucasian	172	59.9
	Black	29	10.1
	Other	3	1.1
	Not available	83	28.9

4.3.2 **Human Liver Sample Preparation Method**

Human liver microsomes (HLM) and human liver S9 fractions (HLS9) were prepared using previously published methods [17,164]. Briefly, a 200 mg human liver tissue was cut into approximately one by one mm pieces and homogenized in 600 μL PBS buffer (pH 7.4) using a tissue grinder. The sample was centrifuged at 10,000 x g for 30 min, and the supernatant (i.e., HLS9) was collected. To prepare HLM, the supernatant was transferred to Beckman ultracentrifuge tubes and centrifuged at 300,000 g for 20 min. The pellets were resuspended in PBS using a tissue grinder and collected (i.e.,

HLM). Protein concentrations were determined using a Pierce™ BCA protein assay kit.

Both HLS9 and HLM samples were stored at −80 °C until use. Of note, the same set of

liver samples were used for the preparation of the HLM and HLS9 samples; however,

different proteomics methods were used for CES1 protein quantifications (i.e., heavy

stable isotope internal standard-based assay for HLM vs. label-free quantification

method for HLS9) [136].

4.3.3 QconCAT Internal Standard Preparation

A QconCAT DNA construct was synthesized de novo to generate the heavy

isotope-labeled internal standard for the CES1 ASPE analysis [163]. The construct

contains the DNA sequences encoding for both the wild-type CES1 tryptic peptide

FTPPQPAEPW**S**FVK and the S75N (rs2307240) variant FTPPQPAEPW**N**FVK. The

S75N is a benign nonsynonymous variant without significant effects on CES1

expression and activity [60,62] and was chosen as a biomarker to differentiate CES1 allelic

expression. The minor allele frequency (MAF) S75N is around 5% across different

populations [60]. Both peptides are flanked by 15 native amino acids to ensure the same

trypsin digestion efficiency [163]. The DNA construct also includes three CES1 surrogate

peptides (AISESGVALTSVLVK, TAMSLLWK, and ELIPEATEK) for total CES1 protein

quantification.

The QconCAT DNA construct was transformed to *Escherichia coli* strain BL2L

and cultured in the medium supplemented with $^{13}C_6$ arginine, $^{13}C_6$, and $^{15}N_2$ lysine.

QconCAT protein expression was induced via adding 1 mM isopropyl-D-1-

thiogalactopyranoside. After 5 hours of growth at 37°C, the cells were lysed, and

QconCAT proteins were extracted using affinity chromatography followed by three rounds of dialysis against 50 mM ammonium bicarbonate containing 1 mM dithiothreitol.

4.3.4 **Proteomic Sample Preparation**

HLS9 and HLM samples were prepared for proteomics analysis using the method we previously published [165]. Briefly, 80 μg HLS9 or HLM protein was mixed with 0.2 μg bovine serum albumin. For the HLM samples, the QconCAT internal standard (172 ng) was also added. One ml of precooled acetone was added, and the mixture was briefly vortexed and stored at −20°C for at least 2 hours to precipitate proteins. The mixture was then centrifuged at 17,000g for 15 minutes at 4°C, the supernatant was removed, and the pellet (i.e., precipitated proteins) was air-dried. The pellet was resuspended in 100 μl of 4 mM dithiothreitol/8 M urea solution/100 mM NH_4HCO_3 solution for reduction at 37°C for 45 minutes. A 100 μl of 20 mM iodoacetamide/8 M urea solution/100 mM NH_4HCO_3 was added, and the mixture was incubated at room temperature in the dark for 30 minutes for alkylation. Following the incubation, 56.6 μl of 50 mM NH_4HCO_3 was added to adjust the urea concentration to 6 M.

A two-step protease digestion protocol was used to digest the proteins. The first digestion was with lysyl endopeptidase (protein: lysyl endopeptidase = 100:1) in an orbital incubator shaker at 220 rpm and 37°C for 6 hours. Then, 733 μl of 50 mM NH_4HCO_3 was added to further adjust the urea concentration to 1.6 M. The second digestion was carried out with tosyl phenylalanyl chloromethyl ketone-treated trypsin (protein: trypsin = 50:1) at 220 rpm and 37°C for overnight. One μl trifluoroacetic acid was added to terminate the digestion. Waters Oasis HLB columns were utilized to clean and extract the digested peptides. The eluted peptides were dried in a SpeedVac

SPD1010 vacuum concentrator and resuspended in 3% acetonitrile solution with 0.1%

formic acid. The eluted peptides were then centrifuged, and half of the supernatant was

transferred to an autosampler vial and mixed with 1 µl of the synthetic iRT standards

solution prior to LC-MS/MS analysis.

4.3.5 LC-MS/MS-Based Proteomics Analysis

The Proteomic analysis was conducted using the previously published method

[136]on a TripleTOF 5600+ mass spectrometer (AB Sciex, Framingham, MA) coupled with

an Eksigent 2D plus LC system (Eksigent Technologies, Dublin, CA). A trap-elute

configuration was adopted for the analysis, which included a trapping column (ChromXP

C18-CL, 120 Å, 5 µm, 0.3 mm cartridge, Eksigent Technologies, Dublin, CA) and an

analytical column (ChromXP C18-CL, 120 Å, 150 × 0.3 mm, 5 µm, Eksigent

Technologies, Dublin, CA). Six µg of digested proteins were injected, and peptides were

trapped and cleaned on the trapping column with the mobile phase A (water with 0.1%

formic acid) at a flow rate of 10 µl/min for 3 min before being separated on the analytical

column with gradient elution at a flow rate of 5 µl/min. The gradient time program was

set as follows for the phase B (acetonitrile containing 0.1% formic acid): 0–68 min: 3%–

30%, 68–73 min: 30%–40%, 73–75 min: 40%–80%, 75–78 min: 80%, 78–79 min: 80%–

3%, and finally 79–90 min at 3% for column equilibration. A blank sample was injected

between each analysis to prevent carryover. The mass spectrometer was operated in a

positive ion mode with an ion spray voltage floating at 5500 v, ion source gas one at 28

psi, ion source gas two at 16 psi, curtain gas at 25 psi, and ion source temperature at

280 °C.

Both HLS9 and HLM samples were analyzed using a DIA method we previously reported, which included a 250-ms TOF-MS scan from 400 to 1250 Da and MS/MS scans from 100 to 1500 Da [166]. The MS/MS scans of all precursors were performed in a cyclic manner using a 100-variable isolation window scheme. The accumulation time was 25 ms per isolation window, resulting in a total cycle time of 2.8 s.

For the S75N heterozygous HLM samples, the expression levels of two alleles of CES1 (i.e., ASPE) were determined based on the ratios of the light peptides FTPPQPAEPWNFVK (S75N mutant peptide) and FTPPQPAEPWSFVK (S75N wild-type peptide) to the corresponding heavy internal standards. Total CES1 protein expression levels in the HLM were determined based on the ratios of the peak areas of the CES1 signature peptides AISESGVALTSVLVK, TAMSLLWK, and ELIPEATEK to their heavy isotope-labeled internal standard counterparts. The Skyline software (University of Washington, Seattle, WA) was used for the HLM proteomics data analysis. The HLS9 DIA data were analyzed by the Spectronaut™ Pulsar software (version 11.0; Biognosys AG, Schlieren, Switzerland) with default settings (precursor Q value < 0.01, protein Q value < 0.01) and its internal reference spectral library "Human - Liver (fractionated)".

4.3.6 **CES1 Activity Measurements**

Enalapril is a selective substrate of CES1, and enalapril hydrolysis was determined in the HLS9 samples as a surrogate maker for CES1 activity [17]. Briefly, 100 μl of enalapril solution (0.5mg/mL in PBS) was mixed with 100 μl of 0.2 mg/ml of HLS9. After incubation at 37ºC for 10 min, the reactions were terminated by adding a 4-fold volume of methanol containing the analytical internal standard 5-hydroxyomeprazole

(10 ng/ml). Then, the samples were vortexed and centrifuged at 13,200 rpm at 4ºC for 20 min to remove the precipitated proteins. The supernatant was collected and analyzed for the concentrations of the hydrolytic metabolite enalaprilat utilizing an LC-MS/MS method [17].

4.3.7 Data Analysis

The genotype data of the human liver samples (n=287) were retrieved from a study recently published by our group [167], which contained 1,779,819 genetic markers. The subsequent quality control analysis was performed to remove SNPs with MAF < 0.01 or deviating from Hardy-Weinberg equilibrium (p < 0.0001) [167]. The genotype data were phased during genotype imputation [167] in order to detect the allele-specific effects of cis-regulatory variants. Previous literature suggested that cis-acting regulatory variants are usually located approximately 5,000 base pairs (bp) upstream and downstream of the gene [168,169]. Thus, a total of 856 SNPs located 5,000 bp upstream and downstream of the CES1 gene were included in this study. SNPs were removed if their MAFs were less than 10% in the 30 S75N heterozygous liver samples. Moreover, when several SNPs were in complete linkage disequilibrium, only one SNP was used as the tag SNP for data analysis to alleviate the multiple testing burden. Overall, 72 SNPs were tested in this study.

The CES1 ASPE ratios of 75S to 75N were log2 transformed to normalize the effect size of regulatory SNPs, given that the SNPs can reside on either the S or the N allele. For each of the 72 SNPs, two linear models were created to identify CES1 cis-regulatory variants. The first model used the S75N ASPE ratios as the phenotype to test

the associations between the genotypes and the ASPE ratios in the S75N heterozygous

samples (n=30). The second model used the total CES1 expression (i.e., the CES1

expression from both alleles) to test the associations between the genotypes and the

CES1 expression in all 287 liver samples. P-values from the two linear models were

then combined using a Fisher's combined probability test, and the Benjamini-Hochberg

method was used for the multiple testing correction (Table1)

Table 4-1 Statistical Analysis

SNP	Test 1 (ASPE)		Test 2 (Total Expression)		Joint	Adjusted
	ASE Beta	P-value	TE Beta	P-value	P-Value	P-Value
rs6499788	-0.18	0.0078	-0.31	0.0077	0.00064	0.033*
rs35918553	-0.18	0.0078	-0.3	0.0114	0.00092	0.033*

- Linear regression t-test was performed for Test 1 and Test 2.
- Test 1 and Test 2 were performed independently, and p-values from Test 1 and Test2 were combined using Fisher's combined probability test for each SNP (i.e., Joint P-Value)
- Joint P-value was corrected using Benjamini-Hochberg method to correct multiple testing bias (i.e., adjusted P-value)

The conventional genome-wide association study (GWAS) was performed using

the previously published method [167]. The HLM and HLS9 expression data and genotype

datasets from the same human liver samples were used. After QC of the genomic data

[170], the GWAS for both S9 and CES1 datasets including gender as a covariate for

analysis.

4.4 Results

4.4.1 CES1 ASPE and CES1 Protein Expression in HLM and HLS9

We measured the CES1 ASPE in 30 S75N heterozygous liver samples using a QconCAT internal standard. The ASPE ratios of 75S to 75N ranged from 0.80 to 1.47 (log2 transformed values: -0.3 to 0.5) (Figure 4-2), indicating the presence of cis-regulatory genetic variants of the CES1 gene. Total CES1 expressions varied markedly in both HLM and HLS9 samples. Neither HLM nor HLS9 CES1 levels were significantly correlated with the ASPE ratios in the 30 S75N heterozygous samples.
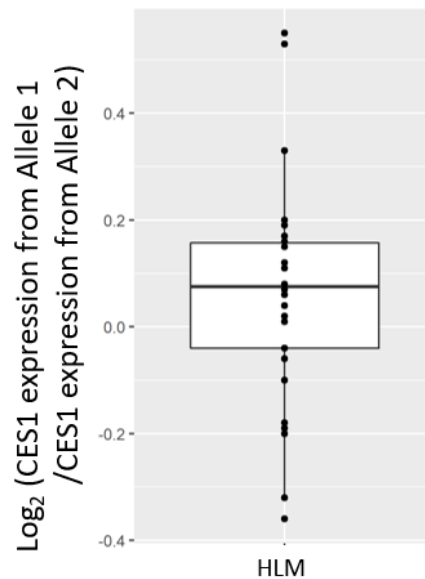
Figure 4-2 CES1 Expression

4.4.2 GWAS Result

Conventional GWAS could not detect any regulatory variant affecting the CES1 protein expression level in both HLM and HLS9, probably due to the heavy background noise (Figure 4-3).
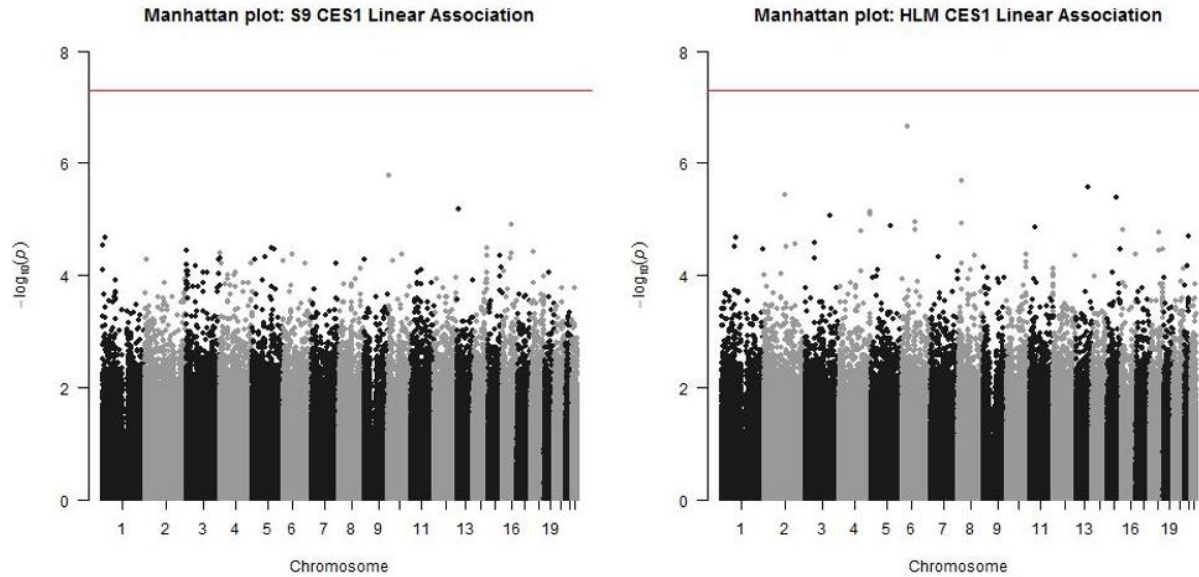
Figure 4-3 Conventional GWAS could not detect any CES1 regulatory variant affecting the expression level detect, probably due to the heavy background noise.

4.4.3 Identification of Cis-Acting CES1 Regulatory Variants

The ASPE-based statistical model revealed two CES1 regulatory variants (rs6499788, rs35918553) significantly associated with CES1 ASPE in the HLM samples (Figure 4-4). Moreover, rs6499788 and rs35918553 were associated with 15.9% (P = 0.01) and 14.9% (P = 0.01) reductions of total CES1 expressions in HLM, respectively. The effect of the two variants on CES1 protein expression was also evaluated in HLS9 prepared from the same human liver samples. Consistent with the findings from HLM, rs6499788 and rs35918553 were associated with 10.3% and 11.8% reductions, respectively, in CES1 protein expression in HLS9 (Figure 4-5). The activity study confirmed that rs6499788 and rs35918553 reduced CES1 activity on hydrolyzing the CES1 selective substrate enalapril by 27.9% (P = 0.04) and 26.6% (P = 0.08), respectively, in the HLS9 samples. (Figure 4-5).

84
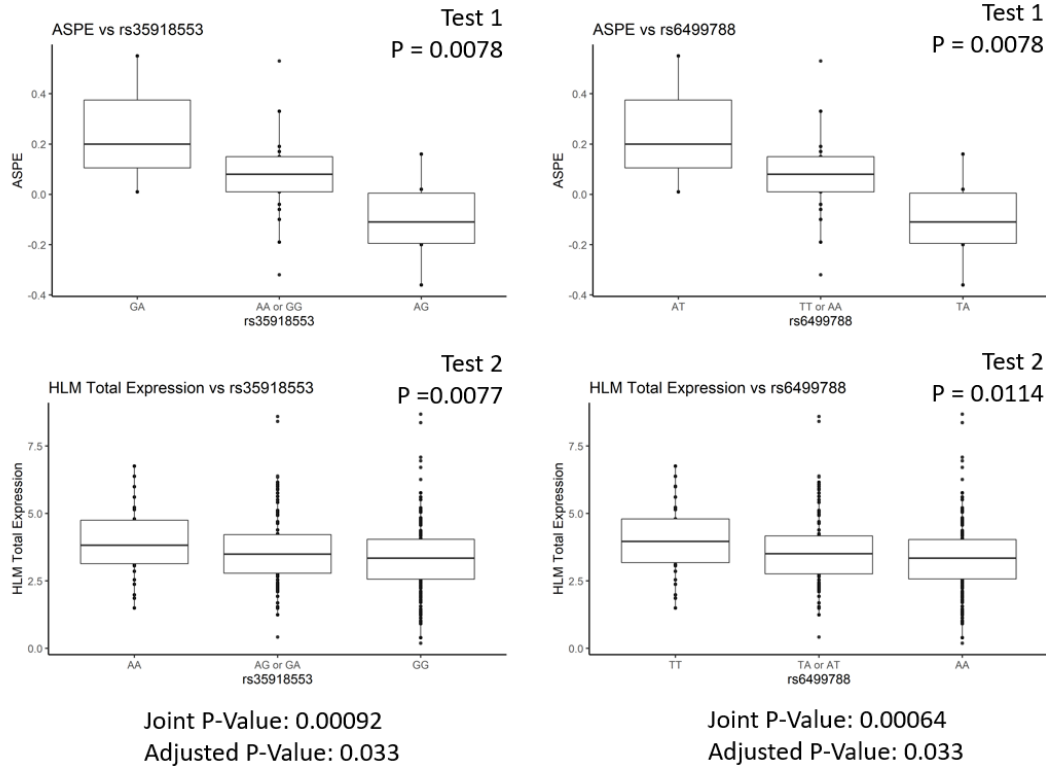
Figure 4-4 Statistical Analysis; impact of rs6499788 and rs35918553 on CES1 ASPE and total CES1 protein expression
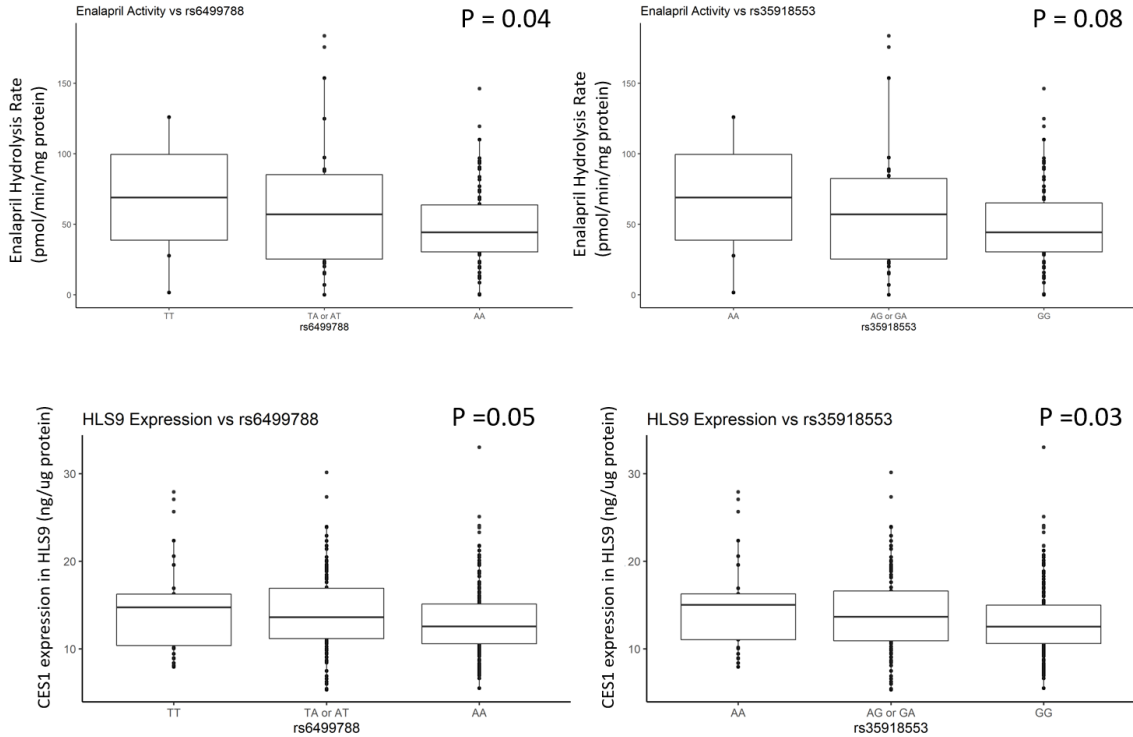
Figure 4-5 Validation; impact of rs6499788 and rs35918553 on CES1 protein expression and catalytic activity on enalapril hydrolysis.

rs6499788 and rs35918553 are located within 5000 bp upstream of the CES1

gene (Table 4-2). Both SNPs are common variants (MAF: rs6499788: 27.7% and

rs35918553: 27.2%) and are in high linkage disequilibrium (D = 1, Ensembl genome

database). The impact of rs6499788 and rs35918553 on CES1 protein expression and

catalytic activity on enalapril hydrolysis on Table 4-3 and Table 4-4.

Table 4-2 Summary of the SNP

SNP	Allele Change	Position	BP from CES1	MAF	P-Value
rs6499788	T>A	16:55871837	4127	0.27	0.033*
rs35918553	A>G	16:55871135	4829	0.27	0.033*

Table 4-3 Impact of rs6499788 on CES1 protein expression and catalytic activity
on enalapril hydrolysis

rs6499788	TT			TA or AT			AA			R^2	P-Value
Average relative CES1 expression in HLM	4.01	±	1.34	3.66	±	1.42	3.37	±	1.31	0.02	0.01
Average absolute CES1 expression in HLS9 (ng/ug protein)	14.99	±	5.46	14.24	±	4.89	13.44	±	4.24	0.01	0.05
Enalapril Hydrolysis Rate (pmol/min/mg protein)	69.08	±	44.70	64.94	±	50.55	49.77	±	29.82	0.03	0.04

- Relative CES1 expression was quantified using QconCAT IS.
- Absolute CES1 expression was quantified using a label-free quantification method. [136]
- Enalapril hydrolysis rate was used as a surrogate marker for CES1 activity. Enalapril is a selective substrate of CES1.[17]

Table 4-4 Impact of rs35918553 on CES1 protein expression and catalytic activity on enalapril hydrolysis

rs35918553	AA			AG or GA			GG			R^2	P-Value
Average relative CES1 expression in HLM	3.95	±	1.31	3.69	±	1.44	3.37	±	1.31	0.02	0.01
Average absolute CES1 expression in HLS9 (ng/ug protein)	15.23	±	5.39	14.20	±	4.93	13.43	±	4.22	0.01	0.03
Enalapril Hydrolysis Rate (pmol/min/mg protein)	69.08	±	44.70	63.01	±	49.36	50.68	±	31.14	0.02	0.08

- Relative CES1 expression was quantified using QconCAT IS.
- Absolute CES1 expression was quantified using a label-free quantification method. [136]
- Enalapril hydrolysis rate was used as a surrogate marker for CES1 activity. Enalapril is a selective substrate of CES1.[17]

4.5 Discussion

For the first time, a novel ASPE approach was utilized to identify cis-regulatory genetic variants, resulting in the identification of two cis-acting CES1 genetic variants associated with CES1 ASPE and protein expression in HLM. The findings were further validated with the CES1 protein expression and CES1 activity data obtained from HLS9 samples. This novel ASPE method enabled us to detect regulatory variants with a small effect size (Figure 4-4) that the conventional genome-wide association study (GWAS) was not able to detect (Figure 4-3), probably due to that GWAS is more prone to the influence of the expression variability caused by non-genetic regulators. These small-effect-size yet common variants (MAF 0.277) could play an important role in building a comprehensive model to better predict the PK and PD of CES1 substrate drugs.

Importance of studying CES1 regulatory variant

CES1 is an important drug-metabolizing enzyme responsible for 80-95% of total hydrolytic activity in the liver [2]. CES1 plays a key role in the first-pass metabolism of endogenous compounds, environmental toxins, and numerous therapeutic agents [17,171] CES1 expression and activity vary significantly among individuals, which is a major factor contributing to inter-individual variability in response to medications metabolized by CES1. Though considerable efforts have been devoted to the study of functional CES1 genetic variants, to date, the nonsynonymous variant G143E (rs71647871) is the only clinically significant loss-of-function variant identified for CES1 [23,24,59-65,67,69,71]. However, considering the MAF of G143E is 2–4% (carrier frequency 4-8%),[17] G143E can only explain a small portion of inter-individual variability of CES1 function.

While nonsynonymous polymorphisms (nsSNP) can directly affect the catalytic function of an enzyme by altering amino acid sequences in regions critical to the protein function, regulatory variants can affect the function of an enzyme by regulating the gene expression level.[172] To the best of our knowledge, the two CES1 regulatory variants identified in the present study are the first found to be associated with both CES1 protein expression level and CES1 activity. Sanford et al. reported a translocation variant CES1VAR reduces mRNA expression of CES1 by 30%. However, this regulatory variant identified by a conventional ASE method was not associated with CES1 protein expression or CES1 activity [173].

Novelty and significance of the ASPE assay

The main advantage of this assay is an improved statistical power to detect even a small effect size variant by filtering out non-genetic regulators (e.g., diseases and inducers). In addition, the ASPE method accounts for the genetic variants regulating gene expression at the post-transcription level. To date, the most common method to identify functional regulatory genetic variants involves the association study between the mRNA expression level and genetic variations. Unfortunately, drug-metabolizing enzymes in particular have a poor correlation between mRNA and protein expression levels as various regulatory elements can affect post-transcriptional processing of mRNA (RNA silencing and sequestration, for instance), resulting in high false positive or false negative rates.[78,173-175] Recently, protein expression level has been increasingly used as the phenotype to identify regulatory genetic variants,[175,176] however, the power of this approach is compromised by the fact that protein expression may also be influenced by non-genetic factors in addition to genetic variants.

Instead of measuring total protein expression from both alleles of a gene, ASPE enables accurate quantification of protein expression from each allele of the gene. Since *cis*-regulatory variants influence gene expression in an allele-specific manner while *trans*-acting regulatory elements and environmental factors affect gene expression on both alleles, the observation of an allelic expression imbalance would suggest the existence of *cis*-acting regulatory elements. Thus, ASPE is more accurate, sensitive, and robust than conventional approaches (e.g., GWAS), given that the expression of each of the two alleles is measured simultaneously in the same individual under the same experimental conditions, and each allele can serve as a control for the other.

Although the present study focused on CES1 genetic variants, this ASPE assay could potentially be widely used to identify regulatory variants of other genes.

Some limitations of the study involve a small sample size of the S75N heterozygous HLM samples (n=30) and a lack of true biological replicates for the validation study. Future studies are warranted to examine the effect of rs6499788 and rs35918553 on the PK and PD of drugs metabolized by CES1. In addition, future investigations involving more S75N heterozygous liver samples method might allow researchers to detect more cis-acting CES1 regulatory variants.

Chapter 5: Conclusions and Future Directions

The work presented in this thesis focuses on CES1 pharmacogenetics and precision medicine. We have examined and confirmed the impact of a loss-of-function G143E variant on CES1 catalytic efficiency on CES1 substrate, enalapril, in healthy volunteers. We have also identified two regulatory variants that impacted the CES1 protein expression and activity in human livers.

In the near future, the CES1 pharmacogenetics testing will likely be implemented in the clinic prior to prescribing a CES1 substrate medication. Considering the signficant impact of G143E on the PK of CES1 substrates and the low cost of genetic testing[177], it is cost-effective to check the CES1 genetic variants before prescribing CES1 substrate medication. The prospective PGx-PK/PD clinical study reported the CES1 G143E genetic variant significantly impacted the enalapril PK in healthy volunteers. To translate this information into clinical practice, a more defined and structured dose adjustment suggestion is needed. A future clinical study with the patient population can give us the information necessary on how we should adjust the dose of enalapril (and other CES1 substrates).

The previously reported PGx research tended to focus on one genetic variant with a noticeable effect size. However, common variants with a small effect size can work together to create a clinically significant impact. The ASPE assay has identified two CES1 regulatory variants with a small effect size (R^2 range 0.01-0.03) with MAF

over 27%. CES1 nonsynonymous variants (e.g., G143E) combined with common regulatory variants (i.e., rs6499788, rs35918553) will give us a more holistic picture and accurate prediction of the PK and PD of CES1 substrates.

All CES1 genetic variants identified to date can only explain a small portion of interindividual variability of the CES1 function [1]. The CES1 G143E variation can only explain a limited portion of enalapril response variation in clinical practice (MAF 0-4%); two regulatory variants identified had a very small effect size. Previous in vitro studies observed a marked variability of CES1 protein expression in human liver samples [17,136]. Given the G143E only affects the catalytic efficiency of CES1 without altering its expression [73,133,137], the main source of interindividual variability in the current study may have resulted from the different expression levels of CES1 in individual participants. Reliable CES1 biomarkers capable of predicting hepatic CES1 expression could be used to further improve the therapeutic outcomes of enalapril and other CES1 substrate drugs. As an excretory protein, CES1 can be released into the blood from tissues with high levels of CES1 expression (i.e., the liver). Our lab recently detected CES1 protein in human plasma using a highly sensitive LC-MS/MS proteomics assay [138]. Although plasma CES1 is insignificant for drug metabolism due to its extremely low plasma concentration, there is potential for plasma CES1 protein to be served as a biomarker to predict the PK and PD of enalapril and other CES1 substrate drugs. Alternatively, plasma exosomes are extracellular vesicles and contain functional proteins and nucleic acids derived from cells of different origins. A recent study showed high correlations between exosomal mRNA expressions and hepatic protein levels for several hepatic drug-metabolizing enzymes [139], but CES1 was not included in the study.

It is plausible that CES1 genetic variants and plasma and exosomal CES1 could be used as complemental biomarkers allowing for the prediction of a large portion of CES1 variability and the development of an individualized pharmacotherapy strategy to improve the effectiveness and safety of drugs metabolized by CES1.

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