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Pharmacogenetics of Carboxylesterase 1

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Pharmacogenetics of Carboxylesterase 1

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Words are, in my not-so-humble opinion, our most inexhaustible source of magic. Capable of both inflicting injury, and remedying it.

Albus Dumbledore in Harry Potter and the Deathly Hollows
by J.K. Rowling

ABSTRACT

Carboxylesterases (CESs) catalyze the hydrolysis of a variety of ester- and amide-containing compounds to their respective free acids. The main CES isozymes involved in drug metabolism are carboxylesterases 1 and 2 (CES1 and CES2). CES1 contributes to an estimated 80 to 95% of the total hydrolytic activity in the human liver. A *CES1* c.428G>A (p.G143E, rs71647871) single nucleotide variation (SNV) markedly decreases the catalytic efficiency of CES1 *in vitro*. Possible effects of this variant on drug pharmacokinetics *in vivo* in humans, however, have not been systematically investigated. Furthermore, only a few studies have investigated the effects of *CES1* variants on its expression and/or activity. Therefore, this thesis aimed to investigate genetic variability in the *CES1* gene in the Finnish population and their effects on drug pharmacokinetics and pharmacodynamics in humans.

The frequency distribution of the *CES1* c.428G>A SNV was investigated in 860 healthy Finnish volunteers. The effects of the *CES1* c.428G>A SNV on the pharmacokinetics of oseltamivir, clopidogrel, quinapril, and enalapril were investigated in 40 healthy volunteers. The *CES1* gene and its flanking regions were sequenced in 192 healthy volunteers to identify previously unknown variants affecting *CES1* whole blood expression. The findings were replicated in another set of 88 healthy volunteers. Furthermore, the effects of the detected variants on *CES1* liver expression were investigated in 177 liver samples and on clopidogrel pharmacokinetics in 106 healthy volunteers from previous pharmacokinetic studies on clopidogrel.

The *CES1* c.428G>A variant allele was found with a minor allele frequency of 2.2%. The c.428G>A SNV reduced the hydrolysis of oseltamivir to the active oseltamivir carboxylate. The oseltamivir carboxylate to oseltamivir area under the plasma concentration-time curve (AUC) ratio was 23% smaller in heterozygous carriers than noncarriers. The c.428G>A SNV reduced the hydrolysis of clopidogrel to the inactive carboxylic acid metabolite. Consequently, the AUC of the parent clopidogrel was about 120% higher and that of the active metabolite about 70% higher in carriers than in noncarriers. Consistently, the c.428G>A SNV markedly enhanced the platelet inhibitory effect of clopidogrel. The average percentage inhibition of platelet aggregation at 0-12 hours was 19% higher in carriers than in noncarriers. The c.428G>A SNV significantly reduced the hydrolysis of enalapril to active enalaprilat. The AUC of enalaprilat was 20% lower in carriers than in noncarriers. The c.428G>A SNV had no observable effect on the pharmacokinetics of quinapril. Two intronic *CES1* rs12443580 and rs8192935 SNVs were discovered to have a major effect on *CES1* expression in whole blood, but not the liver. Moreover, these two SNVs had no effect on clopidogrel pharmacokinetics.

In conclusion, the *CES1* c.428G>A SNV reduces the bioactivation of oseltamivir, markedly increases the clopidogrel active metabolite plasma concentrations and antiplatelet effects, and reduces the bioactivation of enalapril *in vivo* in humans. The two intronic *CES1* rs12443580 and rs8192935 SNVs have tissue-specific effects on *CES1* expression. This could lead to substrate-dependent effects of these SNVs on drug biotransformation.

YHTEENVETO

Karboksyyliesteraasit hydrolysoivat erilaisia esteri- ja amidirakenteisia yhdisteitä. Lääkeaineiden hydrolyysiin osallistuvat lähinnä karboksyyliesteraasit 1 ja 2 (CES1 ja CES2). CES1 katalysoi noin 80-95 % maksassa tapahtuvasta hydrolyysistä. *CES1*-geenissä tunnetaan sen toimintaa *in vitro* heikentävä yhden nukleotidin muunnos (c.428G>A, p.G143E, rs71647871), mutta sen vaikutuksia lääkkeiden farmakokinetiikkaan ei ole aiemmin systemaattisesti tutkittu. Lisäksi muiden mahdollisten *CES1*-geenimuunnosten vaikutuksista CES1:n ilmentymiseen ja/tai aktiivisuuteen on vain vähän tietoa. Väitöskirjatyön tavoitteena oli tutkia *CES1*-geenimuunnosten esiintyvyyksiä suomalaisilla sekä näiden geenimuunnosten vaikutuksia *CES1*:n ilmentymiseen sekä lääkeaineiden farmakokinetiikkaan ja farmakodynamiikkaan ihmisillä.

CES1 c.428G>A geenimuunnoksen esiintyvyyttä tutkittiin 860 terveen vapaaehtoisen koehenkilön otoksessa. *CES1*-geenimuunnoksen vaikutusta oseltamiviirin, klopidogreelin, kinapriilin ja enalapriilin farmakokinetiikkaan tutkittiin yhteensä 40 terveellä vapaaehtoisella koehenkilöllä. *CES1*-geeni sekvensoitiin 192 terveeltä vapaaehtoiselta koehenkilöltä mahdollisten *CES1*:n ilmentymiseen kokoveressä vaikuttavien geenimuunnosten löytämiseksi. Löydettyjen geenimuunnosten vaikutus toistettiin 88 terveen vapaaehtoisen aineistossa. Lisäksi geenimuunnosten vaikutusta tutkittiin *CES1*:n ilmentymiseen maksassa 177 maksanäytteen aineistossa ja klopidogreelin farmakokinetiikkaan 106 terveellä vapaaehtoisella koehenkilöllä aiemmin tehtyjä farmakokineettisiä töitä hyödyntäen.

CES1 c.428G>A geenimuunnoksen esiintyvyys oli 2,2 %. *CES1*-geenimuunnos vähensi oseltamiviirin metaboliaa aktiiviseksi oseltamiviirikarboksylaatiksi, minkä seurauksena oseltamiviirikarboksylaatin ja oseltamiviirin pitoisuus-aikakäyrän alle jäävän pinta-alan (AUC) suhde oli 23 % pienempi kantajilla kuin ei-kantajilla. *CES1*-geenimuunnos vähensi klopidogreelin metaboliaa inaktiiviseksi karboksyylihappometaboliitiksi, minkä seurauksena klopidogreelin AUC oli noin 120 % suurempi ja aktiivisen metaboliitin AUC noin 70 % suurempi kantajilla kuin ei-kantajilla. Vastaavasti *CES1*-geenimuunnos lisäsi merkittävästi klopidogreelin antitromboottista vaikutusta; 12 tunnin keskimääräinen verihiutaleiden paakkuuntumisen esto oli 19 prosenttiyksikköä suurempi kantajilla kuin ei-kantajilla. *CES1*-geenimuunnos vähensi enalapriilin metaboliaa aktiiviseksi enalaprilaatiksi, minkä seurauksena enalaprilaatin AUC oli 20 % pienempi kantajilla kuin ei-kantajilla. *CES1*-geenimuunnoksella ei ollut vaikutusta kinapriilin farmakokinetiikkaan. Kaksi intronissa sijaitsevaa *CES1*-geenimuunnosta (rs12443580 ja rs8192935) vaikuttivat merkittävästi *CES1*:n ilmentymiseen kokoveressä, mutta eivät maksassa. Näillä kahdella geenimuunnoksella ei myöskään ollut vaikutusta klopidogreelin farmakokinetiikkaan.

Yhteenvetona voidaan todeta, että *CES1* c.428G>A geenimuunnos heikentää oseltamiviirin bioaktivaatiota, lisää klopidogreelin aktiivisen metaboliitin plasmapitoisuuksia ja antitromboottista vaikutusta sekä heikentää enalapriilin bioaktivaatiota. Kahden *CES1*-geenimuunnoksen (rs12443580 ja rs8192935) vaikutus *CES1*:n ilmentymiseen vaikuttaa olevan kudosspesifistä ja siten niiden vaikutus lääkeaineiden metaboliaan voi vaihdella eri aineiden välillä.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Tarkiainen EK, Backman JT, Neuvonen M, Neuvonen PJ, Schwab M, Niemi M. Carboxylesterase 1 polymorphism impairs oseltamivir bioactivation in humans. Clin Pharmacol Ther. 2012;92(1):68-71. doi: 10.1038/clpt.2012.13.

II Tarkiainen EK, Holmberg MT, Tornio A, Neuvonen M, Neuvonen PJ, Backman JT, and Niemi M. CES1 c.428G>A single nucleotide variation increases the antiplatelet effects of clopidogrel by reducing its hydrolysis in humans. Clin Pharmacol Ther. 2015;97(6):650-8. doi: 10.1002/cpt.101.

III Tarkiainen EK, Tornio A, Holmberg MT, Launiainen T, Neuvonen PJ, Backman JT, and Niemi M. Effect of carboxylesterase 1 c.428G>A single nucleotide variation on the pharmacokinetics of quinapril and enalapril. Br J Clin Pharmacol. 2015;80(5):1131-8. doi: 10.1111/bcp.12667.

IV Neuvonen M*, Tarkiainen EK*, Tornio A, Hirvensalo P, Tapaninen T, Paile-Hyvärinen M, Itkonen MK, Holmberg MT, Kärjä V, Männistö VT, Neuvonen PJ, Pihlajamäki J, Backman JT, and Niemi M. Effects of genetic variants on carboxylesterase 1 gene expression, and clopidogrel pharmacokinetics and antiplatelet effects. Manuscript submitted.
* Equal contribution

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ABBREVIATIONS

AADAC	Arylacetamide deacetylase
ACAT	Acyl-coenzyme A:cholesterol acyltransferase
ACTB	Actin beta
ADHD	Attention deficit hyperactivity disorder
ADP	Adenosine diphosphate
ACE	Angiotensin-converting enzyme
AChE	Acetylcholinesterase
A_e	Amount excreted into urine
AhR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
ARB	Angiotensin II receptor blocker
AUC	Area under the plasma concentration-time curve
B2M	Beta-2-microglobulin
BChE	Butyrylcholinesterase
BMI	Body mass index
c.	Nucleotide position in the coding DNA
CDA	Cytidine deaminase
cDNA	Complementary DNA
C/EBP	CCAAT/enhancer binding protein
CEH	Cholesterol ester hydrolase
CES	Carboxylesterase
CI	Confidence interval
Cl	Clearance
Cl_{renal}	Renal clearance
CMBL	Carboxymethylenebutenoledase
CNS	Central nervous system
CNV	Copy number variation
C_{max}	Peak plasma concentration
CPT-11	Irinotecan
CRP	C-reactive protein
CV	Coefficient of variation
CYP	Cytochrome P450
DECR1	Mitochondrial 2,4-dienoyl-coenzyme A reductase 1
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
f	Female
FAEE	Fatty acid ethyl ester
FDR	False discovery rate
FPGS	Folylpolyglutamate synthase
g.	Nucleotide position in the genomic deoxyribonucleic acid

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HNF4α	Hepatocyte nuclear factor 4α
IL	Interleukin
HCV	Hepatitis C virus
HepG2	Human hepatocellular carcinoma cell line
IC_{50}	Inhibitor concentration producing 50% inhibition
IV	Intravenously
k_e	Elimination rate constant
K_i	Inhibition constant
K_m	Michaelis-Menten kinetic constant
LC-MS-MS	Liquid chromatography-tandem mass spectrometry
LD	Linkage disequilibrium
lncRNA	Long noncoding RNA
m	Male
MAF	Minor allele frequency
mRNA	Messenger RNA
miRNA	MicroRNA
n.	Nucleotide position in a non-coding RNA reference sequence (gene producing an RNA transcript but not a protein)
NASH	Non-alcoholic steatohepatitis
NRF2	Nuclear factor-erythroid 2-related factor 2
OAT	Organic anion transporter
OATP	Organic anion-transporting polypeptide
p.	Amino acid position in the protein sequence
PCI	Percutaneous coronary intervention
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
PEPT	Peptide transporter
PNPA	*Para*-nitrophenyl acetate
PON	Paraoxonase
PPIB	Peptidylprolyl isomerase B
PRU	$P2Y_{12}$ reaction unit
PXR	Pregnane X receptor
qPCR	Quantitative real-time PCR
RNA	Ribonucleic acid
RPLP0	Ribosomal protein lateral stalk subunit P0
SD	Standard deviation
siRNA	Small interfering RNA
SN-38	7-ethyl-10-hydroxycamptothecin (active metabolite of irinotecan)
SNV	Single nucleotide variation
Sp1	Specificity protein 1
$t_{½}$	Elimination half-life
t_{max}	Time to peak plasma concentration
TP	Thymidine phosphorylase

TRAP1	Tumor necrosis factor receptor associated protein 1
TSS	Translation start site
UGT	Uridine 5´-diphosphate glucuronosyltransferase
UPP	Uridine phosphorylase
UTR	Untranslated region
VLDL	Very low density lipoprotein

1 INTRODUCTION

As Sir William Osler said, “If it were not for the great variability among individuals, medicine might as well be a science and not an art”. In recent years, our understanding of the influence of genes on interindividual variability has developed (Evans & McLeod 2003, Eichelbaum *et al*. 2006, Daly 2010, EMA 2011). Genetic variability can affect all aspects of a disease and its treatment, such as the rate of disease occurrence, the risk of disease progression and recurrence, the most likely beneficial drug and its therapeutic dose, the nature and extent of response to treatment, and the possibility of drug toxicity (FDA 2013). Genetic variants can potentially lead to profound effects on the efficacy and/or safety of the administered drug (EMA 2011, FDA 2013). Furthermore, genetic variants can influence the effect size of an interacting drug (perpetrator drug) (EMA 2011). In pharmacogenetics, genetic variants influencing or predicting the outcome of drug treatment are studied in relation to efficacy of drug treatment and adverse reactions (EMA 2011, FDA 2013).

Cytochrome P 450 enzymes (CYPs) contribute to the metabolism of about 70% of drugs, with most of the remaining drugs metabolized by uridine 5′-phosphate glucuronosyltransferases (UGTs) and esterases, including carboxylesterases (Oda *et al*. 2015). The clinical significance of carboxylesterases (CESs) in the metabolism of drugs is, however, not fully appreciated despite the large number of widely used drugs subject to CES-mediated hydrolysis. Concomitant with the growing number of CES1 substrate drugs increases awareness that genetic factors, as well as diseases and drug-drug interactions, may alter the activity of CESs and significantly impact the therapeutic effects of their substrate drugs (Imai 2006, Laizure *et al*. 2013).

CESs belong to an *α,β*-hydrolase-fold protein family and are expressed in many tissues, especially the liver, small intestine, and lungs. CESs catalyze the hydrolysis of a variety of ester- and amide-containing compounds to their respective free acids (Hosokawa 2008). The main CES isozymes involved in drug metabolism are carboxylesterases 1 and 2 (CES1 and CES2). CES1 contributes to an estimated 80 to 95% of the total hydrolytic activity in the human liver with the remaining hydrolytic activity being attributed to other esterases, including CES2 (Imai 2006, Zhu & Markowitz 2013).

Several sequence variations have been found in the *CES1* gene, some with functional effects on CES1 activity (Zhu *et al*. 2008, Zhu & Markowitz 2009). The *CES1* c.428G>A missense (p.G143E, rs71647871) single nucleotide variation (SNV) is associated with a reduced biotransformation of CES1 substrate drugs, such as methylphenidate (Zhu *et al*. 2008). A n.-816A>C (rs3785161) SNV residing upstream from the pseudogene *CES1P1* has been associated with an increased antiplatelet response to clopidogrel, while an association was recently found between intronic *CES1* c.1168-33A>C (rs2244613) SNV and decreased trough concentrations of active dabigatran and reduced bleeding risk. Only a few studies, however, have investigated the effects of *CES1* variants on its expression and/or activity or on drug pharmacokinetics *in vivo* in humans.

Over 90% of the anti-influenza drug oseltamivir is rapidly hydrolyzed to active oseltamivir carboxylate by CES1 in the liver (Shi *et al.* 2006). CES1 also hydrolyzes about 40-60% of the parent quinapril and enalapril to their active metabolites quinaprilat and

enalaprilat (Laizure *et al*. 2013). The angiotensin converting enzyme (ACE) inhibitors, quinapril and enalapril, are used in the treatment of hypertension and congestive heart failure. Clopidogrel, on the other hand, is widely used in the prevention and treatment of atherothrombotic diseases. Clopidogrel is a thienopyridine prodrug that is converted to an active *cis* 5-thiol metabolite through an inactive intermediate 2-oxo-clopidogrel by CYPs (Farid *et al*. 2010, Dansette *et al*. 2012). CES1, however, hydrolyzes about 90% of the parent clopidogrel to an inactive clopidogrel carboxylic acid metabolite, which is further metabolized by glucuronidation. In a previous *in vitro* study, the *CES1* c.428G>A SNV markedly affected clopidogrel metabolism (Tang *et al*. 2006). Moreover, clopidogrel active metabolite plasma concentrations were about 60% higher in seven healthy Amish *CES1* c.428G>A variant allele carriers than in non-carriers (Lewis *et al*. 2013).

Therefore, the purpose of this thesis was to investigate the frequency distribution of the *CES1* c.428G>A SNV in the Finnish population and the impact of this SNV on the pharmacokinetics of oseltamivir and on the pharmacokinetics and pharmacodynamics of clopidogrel, quinapril, and enalapril in prospective genotype panel studies. Furthermore, we investigated the effects of *CES1* genetic variants on its whole blood and liver expression and on clopidogrel pharmacokinetics and pharmacodynamics.

2 REVIEW OF THE LITERATURE

2.1 Pharmacogenetics

Although many nongenetic factors influence drug response, including age, organ function, and drug interactions, there are numerous examples of cases in which interindividual differences are due to variations in (1) genes relevant to the drug's pharmacokinetics; (2) genes encoding drug targets and other pathways related to the drug's pharmacodynamics; (3) genes that can predispose to toxicities such as immune reactions; and (4) genes that influence disease susceptibility or progression (Evans & McLeod 2003, FDA 2013). Pharmacogenetics studies the effects of DNA sequence variations on drug pharmacokinetics, pharmacodynamics, efficacy, and adverse effects (Redon *et al*. 2006, EMA 2007).

Human genetic variations range from single nucleotide changes to gains or losses of whole chromosomes. Individuals differ from each other approximately every 300-1000 nucleotides, with an estimated total of 15 million single nucleotide variations (SNVs) and thousands of deletions, insertions, duplications, and complex multi-site variants (termed copy number variations (CNVs)) in the genome (Redon *et al*. 2006, Stranger *et al*. 2007, 1000 Genomes Project Consortium *et al*. 2010). Because 95% of the genome is intergenic, most variations are unlikely to directly affect the encoded transcript or protein. Variations in noncoding regions of genes may occur in the 3′ and 5′ untranslated regions (UTRs), promoter or enhancer regions, or intronic regions essential for splice sites. Close to 45% of the human genome can be recognized as transposons, mobile DNA sequences that can migrate to different regions of the genome, although most transposons are no longer active (Burns & Boeke 2012).

Coding region SNVs are classified as nonsynonymous (or missense), if the base pair change results in an amino acid substitution, or synonymous (or sense), if the base pair substitution within a codon does not alter the encoded amino acid. Base pair substitutions that lead to a premature stop codon are termed nonsense mutations. Linkage equilibrium occurs when the genotype present at one locus is independent of the genotype at the second locus. Linkage disequilibrium (LD) occurs when the genotypes at the two loci are not independent of one another. In complete LD, genotypes at two loci always occur together (1000 Genomes Project Consortium *et al*. 2010).

Epigenetics refers to heritable patterns of gene expression that cannot be directly attributed to changes in the primary DNA sequence (Ivanov *et al*. 2012, Ivanov *et al*. 2014). The best known epigenetic mechanisms in humans are DNA methylation, posttranslational modification of histone proteins, and modulation of gene expression by noncoding RNAs (Ivanov *et al.* 2012). Approximately 60% of human genes contain CpG islands, *i.e.*, regions of the genome containing an unusually high number of CpG sites and tending to be located in gene promoters (Ivanov *et al*. 2012). The hypermethylation of these CpG islands correlates with repressed transcription (Ivanov *et al*. 2012). Histone modifications, such as acetylation, methylation, and phosphorylation, affect the overall chromatin structure and in

this way influence transcription and many other DNA processes such as repair, replication, and recombination (Bannister & Kouzarides 2011, Ivanov *et al*. 2012). MicroRNAs (miRNAs) are on average 22 nucleotides long, single-stranded, and regulate the expression of selected sets of target genes by base pairing with their transcripts (Cheng *et al*. 2005, Eulalio *et al*. 2008). Usually, the binding sites are in the 3´ UTR of target mRNA sequences, and the bound miRNA inhibits translation to down-regulate expression of the target gene (Cheng *et al*. 2005, Eulalio *et al*. 2008). Many thousands of long noncoding RNAs (lncRNAs), with lengths of over 200 nucleotides, are also thought to be involved in gene regulation (Yoon *et al*. 2013). Although the function of the great majority of the lncRNAs are unknown, some are known to be associated with chromatin modification enzymes and mediate gene activation and silencing (Yoon *et al*. 2013).

2.2 Human carboxylesterases

General aspects. Human carboxylesterases (CESs) (Enzyme Commission number 3.1.1.1) are members of the serine esterase superfamily containing an *α,β*-hydrolase-fold and comprise a multigene family (Hosokawa *et al*. 2007, Takahashi *et al*. 2008). CESs are categorized as phase 1 drug-metabolizing enzymes and are responsible for the hydrolysis of a wide variety of endogenous and exogenous compounds, including esters, thioesters, carbamates, and amides to their respective free acids and alcohols (Satoh & Hosokawa 1998, Fukami *et al*. 2008, Hosokawa 2008, Laizure *et al*. 2013). CESs can be classified into five major families (CES1-CES5) according to the homology of the amino acid sequence (Hosokawa 2008). In general, carboxylesterases exhibit approximately 80% sequence identity within a *CES* gene family (Satoh *et al*. 2002). The first carboxylesterases were purified from human intestines and liver in 1979 and 1980 (Inoue *et al*. 1979, Inoue *et al*. 1980). Later, the human liver carboxylesterase was shown to catalyze the hydrolysis of the methyl ester group of cocaine to form benzoylecgonine (Brzezinski *et al*. 1994).

Genomic organization. The human *CES* gene family consists of five protein coding genes (*CES1*, *CES2*, *CES3*, *CES4A,* and *CES5A*) and three pseudogenes (*CES1P1*, *CES1P2*, and *CES5AP1*). *CES1*, *CES5A*, *CES1P1,* and *CES1P2* are located in a cluster on chromosome 16 (16q12.2), while *CES2*, *CES3*, and *CES4A* are in a separate cluster on the same chromosome (16q22.1). *CES5AP1* is located on chromosome 22 (22q11.23). Different isoforms are generated *in vivo* for each of the *CES* genes as a result of transcriptional events, including truncation of the 5´ ends, differential presence or absence of exons, alternative splicing or retention of introns, or overlapping exons with different boundaries (Holmes *et al*. 2010).

Expression. The expression of carboxylesterases is ubiquitous with high levels in various tissues (Satoh & Hosokawa 2006, Hosokawa 2008). Typically, expression of CESs is maximal in the epithelia of most organs, suggesting that these enzymes play a protective role against xenobiotics (Imai 2006). Among various tissues, the highest hydrolytic activity

is typically found in the liver, but it can also be found in several other tissues, such as lungs, testes, kidneys, and brain (Satoh *et al*. 2002). Carboxylesterase activity in the liver is predominantly found in the microsomal fraction, although significant carboxylesterase activity is present in the lysosomal fraction, and the lysosomes contribute substantially to the general esterolytic capacity of the liver (Satoh & Hosokawa 1998, Hosokawa 2008). Smaller amounts of CESs are also present in the cytosolic fraction (Boberg *et al*. 2017). CESs are localized in the luminal side of endoplasmic reticulum (ER) (Potter *et al*. 1998, Imai 2006), through the binding of four amino acid residues at their C-terminal with the KDEL (lycine – aspartic acid – glutamic acid – leucine) ER protein retention receptor (Imai & Ohura 2010). Uridine 5´-diphosphate glucuronosyltransferases (UGTs) are colocalized with CESs and have the potential to further metabolize carboxylic acids to acyl glucuronides (Inoue *et al*. 2013). Carboxylesterase expression is highly regulated during development by nutritional status, hormonal factors, and xenobiotics (Satoh & Hosokawa 2006).

Catalytic function. Carboxylesterases have a catalytic triad composed of serine, glutamate, and histidine. These three amino acids are required for hydrolytic activity, with mutation in one of these residues resulting in a catalytically inactive protein (Potter & Wadkins 2006). Carboxylesterases do not require cofactors such as inorganic ions (Liederer & Borchardt 2006). The catalytic triad employs a two-step hydrolysis mechanism (Figure 1). In the first step, a covalent acyl-enzyme intermediate is formed with the serine residue, and the alcohol product is released (Liederer & Borchardt 2006, Potter & Wadkins 2006). Histidine is required to activate the serine, and glutamate is in turn required to stabilize the histidine residue (Liederer & Borchardt 2006, Potter & Wadkins 2006). In the second step, a water molecule attacks the acyl-enzyme linkage, releasing the acyl product and regenerating the enzyme (Liederer & Borchardt 2006, Potter & Wadkins 2006).

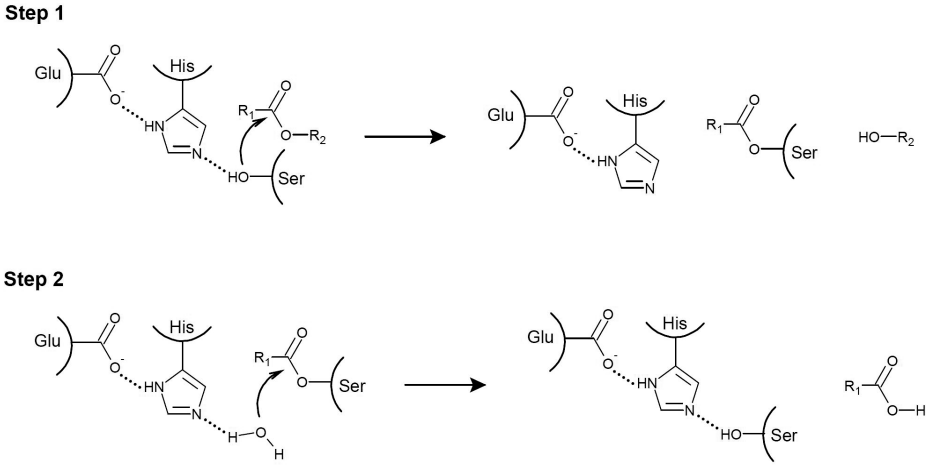

Figure 1. *Mechanism for the bioconversion of esters by carboxylesterases (adapted from Liederer & Borchardt 2006 and Satoh & Hosokawa 2006). Glu, glutamate; His, histidine; R_1, radical 1; R_2, radical 2; Ser, serine.*

Role in drug disposition. Carboxylesterases have overlapping substrate specificities, and drug substrates are often susceptible to hydrolysis by multiple carboxylesterases and other esterases. One carboxylesterase, however, usually predominates with each substrate and serves as the major pathway of hydrolysis (Laizure *et al*. 2013). The relatively high content in different tissues may also compensate for the CESs relatively low catalytic efficiency for substrates (Brzezinski *et al*. 1994). In humans, CES1 and CES2 are primarily responsible for the hydrolysis of drugs (Oda *et al*. 2015). Recent studies have shown differences between CES1 and CES2 families in terms of substrate specificity (Imai 2006). For a CES1 substrate drug that undergoes a high first-pass hydrolysis after oral administration, the parent compound that escapes first-pass metabolism in the liver will be subject to flow-dependent hepatic elimination due to the high CES1 content (Laizure *et al*. 2013). The CES2 substrate drugs that escape first-pass hydrolysis and reach systemic circulation no longer have direct access to CES2 in the small intestine (Laizure *et al*. 2013). Hydrolysis can still occur in the liver as it posseses CES2 activity, but to a diminished degree compared with the small intestine (Laizure *et al*. 2013). It is also possible that a CES2 substrate drug could gain access to intestinal CES2 through enterohepatic recirculation (Laizure *et al*. 2013).

2.3 Carboxylesterase 1

2.3.1 Genomic organization and expression

The *CES1* gene subfamily is located on the chromosome 16 and is composed of three genes, *CES1* (a functional gene), *CES1P1* (a pseudogene, previously termed *CES1A3* and *CES4*), and *CES1P2* (a pseudogene) (Figure 2). The *CES1* gene spans about 30 kb (Marsh *et al*. 2004) and consists of 14 exons encoding 567 amino acids (Yoshimura *et al*. 2008, Sai *et al*. 2010). Exon 1 encodes a signal peptide (Hosokawa 2008). The nonfunctional *CES1P1* pseudogene located in proximity with the *CES1* gene, consists of six exons, and contains a stop codon in exon 3 (Fukami & Yokoi 2012), while *CES1P2* consists of 14 exons and spans about 26 kb (Rasmussen *et al*. 2015). *CES1P1* and *CES1P2* are located in the opposite direction compared to *CES1* (Rasmussen *et al*. 2015).

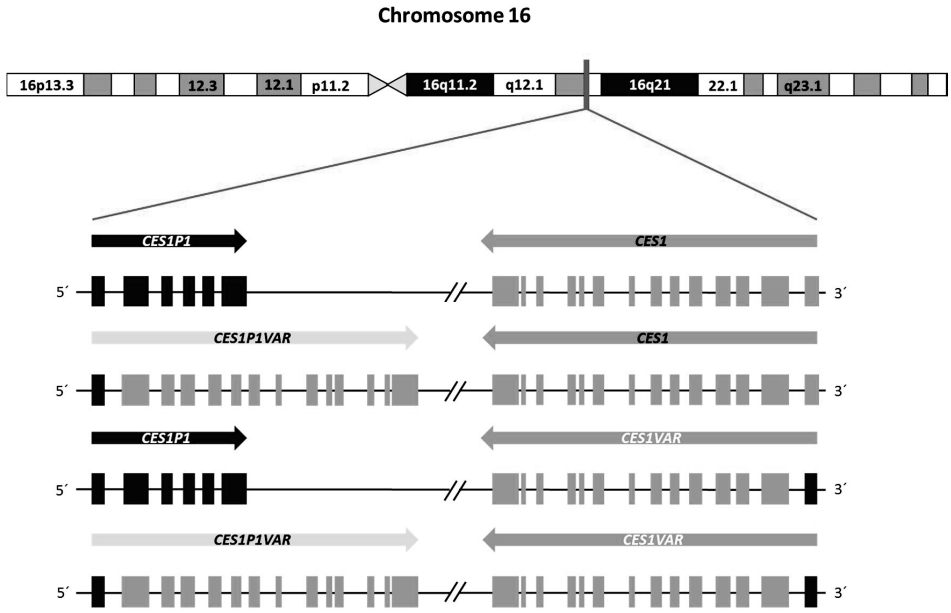

Figure 2. *CES1 and CES1P1 gene structures. CES1VAR is a variant of CES1 in which the exon 1 is converted to that of CES1P1. CES1P1VAR is a variant of CES1P1 which is identical to the CES1 gene except for the differences of five nucleotides in exon 1. CES1, carboxylesterase 1; CES1P1, carboxylesterase 1 pseudogene.*

CES1 is highly expressed in the liver and observed in other tissues, such as blood, the small intestine, lungs, heart, and testes (Satoh & Hosokawa 1998, Satoh *et al*. 2002, Redinbo *et al*. 2003, Hatfield *et al*. 2011, Kuhl *et al*. 2016), but not in plasma (Li *et al*. 2005). In accordance with the tissue expression profile of *CES1*, its promoter region is

hypomethylated in the liver and hypermethylated in the kidney (Oda *et al*. 2015). CES1 contributes to 80-95% of total hydrolytic activity in the human liver, and the residual hydrolytic activity is attributed to other esterases, such as CES2 (Imai 2006, Zhu & Markowitz 2013). CES1 has a partially overlapping tissue expression profile with CES2 (Table 1). Additionally, interindividual *CES1* expression levels seem to have greater variation than *CES2* expression levels (Liu *et al*. 2010, Boberg *et al*. 2017). *CES1* and *CES2* mRNA levels are significantly associated with the levels of respective proteins (Pope *et al*. 2005).

Table 1. *Tissue-specific gene expression profiles of CES1 and CES2 isozymes in humans.*

Tissue	*CES1*	*CES2*
Liver	+++	+
Small intestine	-	+++
Kidney	+	+++
Lung	+++	-

Adapted from (Hosokawa 2008).

-, not detected; +, weakly expressed; ++, moderately expressed; +++, strongly expressed.

CES1, carboxylesterase 1; CES2, carboxylesterase 2.

CES-mediated hydrolysis demonstrates a typical ontogeny of increasing activity as development progresses from birth to adulthood (Laizure *et al*. 2013). Adults express higher levels of CES1 and CES2 than children (<6 years) at both the mRNA and protein levels (Zhu *et al*. 2009a, Shi *et al*. 2011, Hines *et al*. 2016, Boberg *et al*. 2017). Additionally, the female sex is associated with increased *CES1* mRNA expression in adipose tissue (Friedrichsen *et al*. 2013). The *CES1* mRNA expression in adipose tissue is also positively correlated with measures of adiposity and metabolic function, such as body mass index (BMI), increased waist circumference, triglyceride level, and plasma insulin level (Jernas *et al*. 2009, Marrades *et al*. 2010, Nagashima *et al*. 2011, Friedrichsen *et al*. 2013).

Xenobiotics and pathological conditions can alter the expression of carboxylesterases. In human primary hepatocytes, dexamethasone caused a slight induction of CES1 and CES2 protein expression (Zhu *et al*. 2000). On the other hand, *CES1* mRNA expression was not induced by rifampicin or omeprazole, suggesting that pregnane X receptor (PXR) and aryl hydrocarbon receptor (AhR) are not involved in the transactivation of *CES1* (Nishimura *et al*. 2002, Oda *et al*. 2015). In human hepatocellular carcinoma (HepG2) cells, nuclear factor-erythroid 2-related factor 2 (NRF2) agonists, sulphorophane and *tert*-butylhydroquinone, induced *CES1* mRNA levels and increased CES1 protein expression and enzyme activity via an antioxidant response element at the distal promoter sequence -2025 bp from the translation start site (TSS) (Maruichi *et al*. 2010). The proinflammatory cytokine interleukin-6 (IL-6) has been implicated in decreasing the expression of CES1 and CES2 both at mRNA and protein levels by 20-60% and the decrease was comparable with the decrease in the hydrolytic activity (Yang *et al*. 2007). Furthermore, the IL-6 mediated suppression also altered cellular responsiveness to

therapeutic agents, such as clopidogrel, irinotecan, and oseltamivir *in vitro* (Yang *et al*. 2007). *CES1* expression is additionally regulated by hepatocyte nuclear factor 4α (HNF4α) via binding to the direct repeat element between -287 and -300 bp from the transcription start site (Xu *et al*. 2016). In patients with alcoholic liver steatohepatitis, CES1 expression is reduced both at the mRNA and protein levels by 75-85%, likely through ethanol-mediated inhibition of HNF4α (Xu *et al*. 2016). In patients with steatosis or nonalcoholic steatohepatitis, hepatic CES2 protein level is also reduced by about 50%-60%, likely through inhibition of HNF4α (Li *et al*. 2016). In addition, *CES1* expression is regulated by specificity protein 1 (Sp1) and CCAAT/enhancer binding protein (C/EBP). Sp1 binding sites are located at -195 bp and -84 bp from the TSS and a C/EBP element at -290 bp (Hosokawa *et al*. 2008).

2.3.2 Structure and catalytic mechanism

CES1 consists of 567 amino acid residues (Zhu *et al.* 2008). Crystal structures of human CES1 have been reported in complexes with several substrates, and the crystal structure of CES1 with tacrine is shown in Figure 3 (Bencharit *et al*. 2003). The monomeric molecular weight of CES1 is approximately 60 kDa (Brzezinski *et al*. 1994, Takai *et al*. 1997, Fukami & Yokoi 2012). It exists in a trimer-hexamer equilibrium that can be shifted toward trimer through binding of compounds to a site on the surface of the enzyme (Bencharit *et al*. 2003). The monomeric CES1 enzyme comprises of three structural domains: a central catalytic domain, an *α,β*-domain, and a regulatory domain (Staudinger *et al*. 2010). The central catalytic domain contains the serine hydrolase catalytic triad, whereas the regulatory domain contains the low-affinity surface ligand-binding Z-site, which controls the trimer-hexamer equilibrium (Potter & Wadkins 2006, Bencharit *et al*. 2006, Staudinger *et al*. 2010).

The active site of CES1 is buried at the bottom of a long gorge (Potter & Wadkins 2006). The gorge is lined with aromatic amino acids, creating a highly hydrophobic pocket and an electrostatic gradient that essentially "sucks" substrate molecules towards the catalytic amino acid residues (Bencharit *et al*. 2003, Potter & Wadkins 2006). At the base of the active site gorge, the catalytic serine (221), histidine (468), and glutamic acid (354) residues are juxtaposed so that nucleophilic attack of esterified substrates can readily take place (Bencharit *et al*. 2003, Potter & Wadkins 2006, Bencharit *et al*. 2006). Adjacent to these amino acids is an oxyanion hole (glycines 141-143) that stabilizes tetrahedral substrate-enzyme intermediates and is evolutionarily conserved across species and within related serine hydrolases (Potter & Wadkins 2006). A side door also exists within CES1 that allows rapid removal of the hydrolysis products from the active site, increasing the efficacy of substrate turnover, especially for large bulky substrates (Fleming *et al*. 2005, Potter & Wadkins 2006).

In addition to hydrolytic reactions, CES1 has been shown to catalyze transesterification (Brzezinski *et al*. 1994, Morgan *et al*. 1994). Compounds other than water can attack the covalent acyl-enzyme intermediate, leading to transesterification rather than hydrolysis, especially with hydrophobic alcohols (Dean *et al*. 1991, Bencharit *et al*. 2003, Imai 2006).

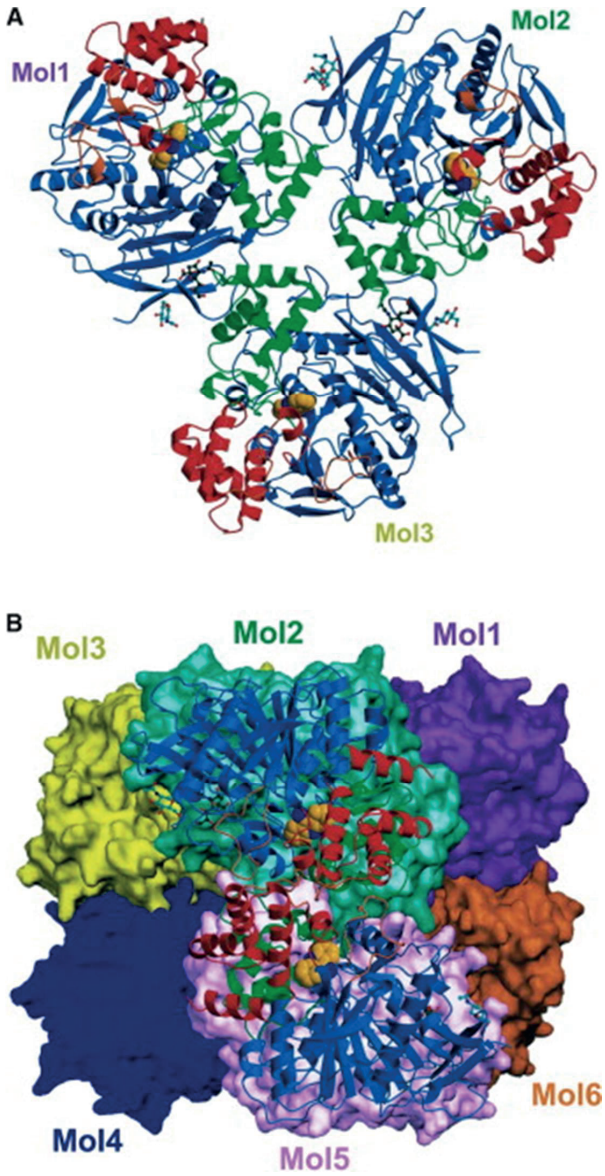

Figure 3. *Crystal structure of CES1 with tacrine (Bencharit et al. 2003). A) A trimer of CES1 in complex with tacrine viewed down the 3-fold axis of symmetry and into the catalytic gorge of each monomer. The catalytic domains of each monomer are in blue, green, and red. B) A molecular surface of the CES1 hexamer with a ribbon representation of one pair of dimers within the hexamer superimposed. Each CES1 monomer is labeled from Mol1 to Mol6. CES1, carboxylesterase 1. Reprinted with the permission of the copyright holder.*

2.3.3 Substrates

CES1 preferentially hydrolyzes compounds with a small alcohol moiety and large acyl moiety (Satoh *et al*. 2002, Imai 2006, Hatfield *et al*. 2010). In addition to the relative sizes of the alcohol and acyl moieties, the shapes of respective moieties likely contribute to the hydrolytic preference as well, particularly when the alcohol and acyl moieties have similar molecular weights (Tang *et al*. 2006). Although most CES1 substrates are esters, thioesters, amides, and carbamates are also potential substrates (Figure 4).

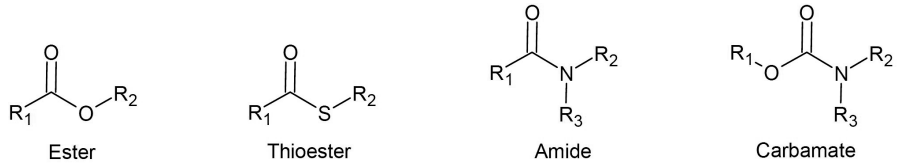

Figure 4. *Chemical structures of different types of CES1 substrates. CES1, carboxylesterase 1; R_1, radical 1; R_2, radical 2; R_3, radical 3.*

CES1 metabolizes many cardiovascular drugs, such as angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers (ARBs), antithrombotics, and statins (Table 2) (Laizure *et al*. 2013). ACE inhibitors, except captopril and lisinopril, are ester prodrugs hydrolyzed by CES1 to their corresponding active carboxylic acid metabolites. Three ARBs, candesartan, olmesartan, and azilsartan, are also ester prodrugs hydrolyzed by CES1, although the main esterase involved in their metabolism is CES2 during absorption in the small intestine (Laizure *et al*. 2013, Ishizuka *et al*. 2013). Three antiplatelet drugs aspirin, clopidogrel, and prasugrel are subject to CES-mediated hydrolysis. Clopidogrel, its inactive intermediate 2-oxo-clopidogrel, and the active *cis* 5-thiol metabolite are hydrolyzed by CES1, but aspirin and prasugrel are mainly metabolized by CES2 (Tang *et al*. 2006, Laizure *et al.* 2013). Anticoagulant dabigatran etexilate is a prodrug that is rapidly hydrolyzed by esterases, notably CES1, to active dabigatran, a reversible direct thrombin inhibitor (Pare *et al*. 2013, Shi *et al.* 2016c). The CES1 substrate drugs affecting the central nervous system (CNS) can be divided into three therapeutic categories: stimulants, opioid agonists, and anticonvulsants (Laizure *et al*. 2013). Some psychoactive synthetic cannabimimetic drugs are also metabolized by CES1 (Thomsen *et al*. 2015). The immunosuppressant drug mycophenolate mofetil and antiviral drug oseltamivir phosphate are bioactivated by CES1-catalyzed hydrolysis (Shi *et al.* 2006, Laizure *et al*. 2013). The two known CES1 carbamate substrates are oncologic drugs irinotecan and capecitabine (Laizure *et al*. 2013).

Table 2. *Drugs that are metabolized by CES1.*

Substrate	**Therapeutic use and/or drug class**	**Hydrolysis product (activity)**	**Other metabolizing enzymes**	**References**
Azilsartan medoxomil	Antihypertensive, ARB	Azilsartan (active)	CES2 CMBL	Ishizuka *et al*. 2013
Benazepril	Antihypertensive, ACE inhibitor	Benazeprilat (active)		Takai *et al*. 1997
Candesartan cilexetil	Antihypertensive, ARB	Candesartan (active)	CES2 CMBL	Ishizuka *et al*. 2013
Capecitabine	Oncologic	5'-deoxy-5-fluorocytidine (inactive)	CES2 CDA TP UPP1 UPP2	Quinney *et al*. 2005, Hamzic *et al*. 2017
Ciclesonide	Anti-inflammatoric	Des-isobutyryl-ciclesonide (active)	CYP3A4	Mutch *et al*. 2007
Clopidogrel	Antithrombotic	Clopidogrel carboxylic acid (inactive)	CYP2C19 CYP1A2 CYP2B6 CYP3As CYP2C9 UGTs	Tang *et al*. 2006, Zhu *et al*. 2013
Cocaine	Stimulant	Benzoylec-gonine (inactive)	CES2 BChE	Brzezinski *et al*. 1994
Dabigatran etexilate	Anticoagulant	Dabigatran (active)	CES2	Laizure *et al*. 2014
Enalapril	Antihypertensive, ACE inhibitor	Enalaprilat (active)		Pang *et al*. 1991, Thomsen *et al*. 2014, Holenarsipur *et al*. 2015
Flumazenil	Antidote	Flumazenil acid (inactive)	CYPs	Kleingeist *et al*. 1998
Heroine	Analgesic	6-acetyl-morphine (inactive)	CES BChE	Dean *et al.* 1991, Brzezinski *et al.* 1997, Hatfield *et al*. 2010
Imidapril	Antihypertensive, ACE inhibitor	Imidaprilat (active)		Takai *et al*. 1997, Geshi *et al*. 2005

Irinotecan (CPT-11)	Oncologic	7-ethyl-10-hydroxy-camptotecin (SN-38) (active)	CES2 UGT1A1 UGT1A7 UGT1A9 CYP3A4 CYP3A5	Takai *et al.* 1997, Humerickhouse *et al*. 2000, Fujiwara & Minami 2010
Lovastatin	Antihyperlipidemic	Dihydroxy acid metabolite (active)	CES2 CYPs	Halpin *et al*. 1993, Parker & Laizure 2010
Methylphenidate	ADHD	Ritalinic acid (inactive)		Sun *et al*. 2004
Mycophenolate mofetil	Immunosuppressant	Mycophenolate (active)	CES2 UGT1A9 UGT2B7	Fujiyama *et al.* 2010
Olmesartan medoxomil	Antihypertensive, ARB	Olmesartan (active)	CMBL CES2	Ishizuka *et al.* 2013
Oseltamivir	Antiviral	Oseltamivir carboxylate (active)		Shi *et al.* 2006
Oxybutynin	Antispasmodic, incontinence	2-cyclohexyl-2-phenylglycolic acid (inactive)	CYP3A4 CES2	Sato *et al*. 2012
Pethidine (meperidine)	Analgesic	Meperidinic acid (inactive)		Zhang *et al.* 1999
Quinapril	Antihypertensive, ACE inhibitor	Quinaprilat (active)	CES2	Takai *et al.* 1997
Ramipril	Antihypertensive, ACE inhibitor	Ramiprilat (active)		Meyer *et al*. 2015
Rufinamide	Epilepsy	Rufinamide carboxylate (inactive)		Williams *et al*. 2011b
Sacubitril	Heart failure, neprilysin inhibitor	LBQ657 (active)		Shi *et al*. 2016b
Simvastatin	Antihyperlipidemic	Dihydroxy acid metabolite (active)	CES2 CYP3A4	Vickers *et al.* 1990, Parker & Laizure 2010
Tefinostat	Oncologic	CHR-2847 (active)		Ossenkoppele *et al*. 2013

Ticlopidine	Antithrombotic	UR-4501 (active)	CYP1A2 CYP2B6 CYP2C19 CYP2D6 CYP3A4 PON1	Kim *et al*. 2014
Trandolapril	Antihypertensive, ACE inhibitor	Trandolaprilat (active)		Zhu *et al*. 2009b

ACE, angiotensin-converting enzyme; ADHD, attention deficit hyperactivity disorder; ARB, angiotensin II receptor blocker; BChE, butyrylcholinesterase; CDA, cytidine deaminase; CES1, carboxylesterase 1; CES2, carboxylesterase 2; CMBL, carboxymethylenebutenoledase; CYP, cytochrome P 450; PON1, paraoxonase 1; TP, thymidine phosphorylase; UPP1, uridine phosphorylase 1; UPP2, uridine phosphorylase 2; UGT, uridine 5´-phosphate glucuronosyltransferase

2.3.4 Pharmacogenetics of carboxylesterase 1

***CES1* variants and functional studies.** Over 1000 SNVs have been found in the *CES1* gene (Wang *et al*. 2016), with the majority of the variants located in introns. All nonsynonymous and essential splice site variants of *CES1* described in the 1000 Genomes project database are listed in the Table 3 with their continental frequencies and *in silico* predicted effects on protein function.

Most of the nonsynonymous variants are rare and occur at a minor allele frequency (MAF) of less than 1%. Only one of the nonsynonymous SNVs, c.34T>G (p.S12A, rs12149366), occurs at a MAF of more than 5% in all investigated populations (5% in African, 7% in European, 7% in South Asian, and 8% in East Asian populations, respectively). Two other SNVs, c.500C>G (p.T167S, rs147694791) and c.805G>T (p.A269S, rs115629050), are also found with frequencies of 6% and 9% in African population and one SNV, c.19A>G (p.I7V, rs114788146), with a frequency of 6% in South and East Asian populations. Three *CES1* SNVs lead to a premature stop codon, c.119T>G (rs151291296), c.310C>T (rs5023780), and c.1108G>T (rs185780477), and probably to a nonfunctional enzyme, but they appear to be extremely rare with a MAF below 1%. Seven predicted loss-of-function splice site variants have been reported as well. A common splice donor variant in intron 7, c.903+1G>T (rs4513095), occurs with a MAF of 5% in East Asian and South Asian populations.

The functional effects of nonsynonymous *CES1* variants have been investigated using recombinantly expressed variant proteins and human liver microsomes. The most studied nonsynonymous SNV, c.428G>A (p.G143E, rs71647871, previously rs121912777), in the *CES1* gene is located in exon 4. The glycine at position 143 constitutes a part of an oxyanion hole and genetic variations leading to an amino acid change in this position impair the catalytic function of CES1 (Fleming *et al*. 2005, Zhu *et al*. 2008). The MAF of the *CES1* c.428G>A SNV is about 2-4% in Caucasian, 4% in African-American, and 0% in Asian populations (Zhu *et al*. 2008, Walter Soria *et al*. 2010). The *CES1* c.428G>A SNV has no

effect on CES1 protein expression level in the liver (Shi *et al*. 2016c). The *CES1* c.428G>A SNV, however, markedly decreases the catalytic efficiency of CES1 *in vitro* (Zhu *et al*. 2008), resulting in a complete loss of hydrolytic activity towards methylphenidate and trandolapril (Zhu *et al*. 2008, Zhu *et al*. 2009b). The *CES1* c.428G>A SNV reduces the hydrolysis of clopidogrel, 2-oxo-clopidogrel, and the active *cis* 5-thiol metabolite to their corresponding inactive carboxylic acid derivatives *in vitro* (Hagihara *et al*. 2009, Zhu *et al*. 2013). Similarly, the *CES1* c.428G>A SNV reduced the hydrolysis of dabigatran etexilate to the active dabigatran *in vitro* (Shi *et al*. 2016c). The intrinsic clearances of the model substrate *para*-nitrophenyl acetate (PNPA) and oseltamivir have been reduced by about 80% in cell lines recombinantly expressing the p.G143E variant protein compared to the wild-type CES1 (Zhu *et al*. 2008, Zhu & Markowitz 2013).

The *CES1* c.53G>T (p.G18V, rs3826190), c.245C>T (p.S82L, rs62028647), and c.805G>T (p.A269S, rs115629050) variants have had no significant effect on the CES1 protein expression levels or bioactivation of methylphenidate, oseltamivir, clopidogrel, or enalapril (Zhu *et al*. 2013, Zhu & Markowitz 2013, Wang *et al*. 2016). In a population pharmacokinetic study, however, the c.805G>T SNV was associated with a reduced clearance of methylphenidate (Lyauk *et al*. 2016).

Two intronic *CES1* SNVs, c.1168-33A>C (rs2244613) and c.257+885T>C (rs8192935), have had no significant effect on the CES1 protein expression levels in the liver (Shi *et al*. 2016c). These SNVs also had no effect on the hydrolysis of oseltamivir or dabigatran etexilate *in vitro* (Shi *et al*. 2016a, Shi *et al*. 2016c).

A deletion in *CES1P1* exon 6, c.780delT (p.D260fs, rs71647872), results in a frameshift mutation that changes aspartic acid at position 260 to glutamic acid and alters the next 39 residues, before truncating early at a premature stop codon (Zhu *et al*. 2008). The intrinsic clearances of PNPA and trandolapril have been reduced by about 100% in cell lines recombinantly expressing the p.D260fs variant protein compared to the wild-type CES1 (Zhu *et al*. 2008).

An n.-816A>C SNV (rs3785161) in the promoter region of *CES1P1* have had no significant effect on oseltamivir hydrolysis *in vitro* (Shi *et al*. 2016a). The MAF of the n.-816A>C SNV is about 25% in Asian population (Geshi *et al*. 2005).

Table 3. *Nonsynonymous and essential splice site variants of CES1 described in the 1000 Genomes project database.*

			Variant allele frequency				*In silico* prediction	
dbSNP ID	**Nucleotide change**	**Amino acid change/ Splicing variant**	**African**	**European**	**East Asian**	**South Asian**	**SIFT**	**PolyPhen**
Exon1								
rs368050410	c.6G>T	p.W2C	-	-	-	-	tolerated	benign
rs111604615	c.11G>C	p.R4P	0.0121	0.004	0.0109	0.0174	tolerated	benign
rs201577108	c.16T>C	p.F6L	-	-	-	-	tolerated	benign
rs114788146	c.19A>G	p.I7V	0.0234	0.0348	0.0556	0.0552	tolerated	benign
rs116258771	c.31C>A	p.L11I	-	-	-	-	tolerated	benign

rs12149366	c.34T>G	p.S12A	0.0507	0.0666	0.0804	0.0726	tolerated	benign
Intron 1								
rs139063675	c.52+1G>A	Splice donor variant	0.003	0	0	0	-	-
Exon 2								
rs3826190	c.53G>T	p.G18V	-	-	-	-	deleterious	probably damaging
rs142474691	c.65C>T	p.S22L	-	-	-	-	tolerated	benign
rs3826192	c.112G>A	p.V38I	-	-	-	-	tolerated	benign
rs140555786	c.116G>T	p.S39I	-	-	-	-	tolerated	benign
rs151291296	c.119T>G	p.L40Ter	0.0061	0	0	0	-	-
rs142469038	c.131C>T	p.A44V	-	-	-	-	tolerated	benign
rs114632091	c.145A>G	p.I49V	-	-	-	-	tolerated	benign
rs3826193	c.145A>G	p.I49V	-	-	-	-	tolerated	benign
rs372817758	c.154G>A	p.G52R	-	-	-	-	deleterious	probably damaging
rs3177828	c.167C>G	p.A56G	-	-	-	-	deleterious	probably damaging
rs75463934	c.176C>T	p.P59L	-	-	-	-	deleterious	probably damaging
rs2307240	c.224G>A	p.S75N	-	-	-	-	tolerated	benign
rs373547343	c.233A>G	p.K78R	-	-	-	-	tolerated	possibly damaging
rs139250836	c.242C>T	p.T81I	-	-	-	-	deleterious	possibly damaging
rs62028647	c.245C>T	p.S82L	-	-	-	-	deleterious	possibly damaging
rs144498758	c.254C>T	p.P85L	-	-	-	-	deleterious	possibly damaging
Exon 3								
rs149261413	c.259T>C	p.C87R	-	-	-	-	deleterious	probably damaging
rs202111709	c.278C>T	p.A93V	-	-	-	-	tolerated	benign
rs375565319	c.288A>T	p.L96F	-	-	-	-	tolerated	benign
rs5023780	c.310C>T	p.R104Ter	-	-	-	-	-	-
rs201285602	c.311G>A/C	p.R104Q/P	-	-	-	-	tolerated/ deleterious	benign
rs28760313	c.338C>T	p.S113F	-	-	-	-	deleterious	probably damaging
rs368654524	c.355C>G	p.L119V	-	-	-	-	deleterious	probably damaging

Exon 4								
rs538274902	c.422G>C	p.G141A	0.0008	0	0	0	deleterious	probably damaging
rs121912777	c.425G>A/C	p.G142E/A	-	-	-	-	deleterious	probably/ possibly damaging
rs71647871	c.428G>A	p.G143E	-	-	-	-	deleterious	probably damaging
rs146456965	c.439G>T	p.G147C	-	-	-	-	deleterious	probably damaging
rs369026668	c.443C>T	p.A148V	-	-	-	-	deleterious	benign
rs187158640	c.466G>A	p.A156T	0	0	0.001	0	deleterious	benign
rs202121317	c.473C>T	p.A158V	-	-	-	-	deleterious	possibly damaging
rs372957576	c.487G>A	p.V163M	0	0	0.001	0	deleterious	probably damaging
rs147694791	c.500C>G	p.T167S	0.0613	0	0	0	tolerated	possibly damaging
rs143718310	c.506A>C	p.Q169P	-	-	-	-	deleterious	probably damaging
rs148947808	c.508T>G	p.Y170D	-	-	-	-	deleterious	probably damaging
rs201065375	c.511C>T	p.R171C	-	-	-	-	deleterious	probably damaging
rs369300055	c.512G>T	p.R171L	0.0008	0	0	0.001	deleterious	probably damaging
rs4784575	c.518G>A	p.G173D	-	-	-	-	deleterious	probably damaging
Exon 5								
rs200227274	c.556C>T	p.R186W	-	-	-	-	deleterious	probably damaging
rs60054861	c.557G>A/C	p.R186Q/P	-	-	0.0	0.0	tolerated	benign
rs373720580	c.566G>C	p.W189S	-	-	-	-	deleterious	probably damaging
rs150241462	c.568G>T	p.G190C	0.0045	0	0	0	deleterious	probably damaging
rs552379877	c.569G>T	p.G190V	0	0	0	0.001	deleterious	probably damaging
rs184487882	c.575T>C	p.L192P	-	-	-	-	deleterious	probably damaging
rs141471293	c.587C>A	p.A196D	-	-	-	-	deleterious	probably damaging

rs563293177	c.595C>T	p.R199C	-	-	-	-	deleterious	possibly damaging
rs2307243	c.596G>A	p.R199H	-	-	-	-	tolerated	benign
rs2307227	c.609C>A	p.D203E	-	-	-	-	tolerated	benign
rs541646274	c.647C>T	p.T216I	-	-	-	-	deleterious	probably damaging
rs200707504	c.659A>G	p.E220G	0	0	0.005	0	tolerated	benign
rs543499053	c.674A>G	p.E225G	0.0008	0	0	0	tolerated	possibly damaging
Exon 6								
rs201251252	c.781G>A	p.V261I	-	-	-	-	tolerated	benign
Exon 7								
rs376597833	c.799C>A	p.Q267K	-	-	-	-	tolerated	benign
rs115629050	c.805G>T	p.A269S	0.0877	0.0388	0.0149	0.046	deleterious	possibly damaging
rs373607981	c.821G>A	p.C274Y	-	-	-	-	deleterious	probably damaging
rs200489319	c.829A>G	p.T277A	0	0	0.001	0	deleterious	benign
rs114119971	c.852C>G	p.H284Q	0	0.0089	0	0	tolerated	benign
rs144078935	c.859C>T	p.R287Ter	-	-	-	-	-	-
rs560426140	c.860G>T	p.R287L	0	0	0	0.002	deleterious	possibly damaging
rs202001817	c.869C>T	p.T290M	-	-	-	-	deleterious	possibly damaging
Intron 7								
rs4513095	c.903+1G>T	Splice donor variant	0.0038	0.0109	0.0585	0.0562	-	-
rs140353865	c.904-1G>A/T	Splice acceptor variant	-	-	-	-	-	-
Exon 8								
rs537106281	c.904A>G	p.K302E	0	0	0.001	0	tolerated	benign
rs142855408	c.929G>T	p.G310V	-	-	-	-	deleterious	benign
rs201879239	c.935C>A	p.P312H	-	-	-	-	deleterious	probably damaging
Exon 9								
rs138161688	c.958G>A	p.G320S	-	-	-	-	tolerated	benign
rs368170439	c.968T>C	p.I323T	-	-	-	-	deleterious	benign
rs564847260	c.994C>A	p.P332T	-	-	-	-	deleterious	possibly damaging
rs543385969	c.1000G>A	p.E334K	0	0	0	0.001	deleterious	benign
rs576295379	c.1020T>G	p.N340K	0	0	0	0.0102	tolerated	benign
rs374924526	c.1026C>G	p.H342Q	-	-	-	-	tolerated	benign
rs145135221	c.1039A>G	p.M347V	0	0	0	0.001	deleterious	benign

rs571865256	c.1081A>G	p.M361V	0.0008	0	0	0	tolerated	benign
Intron 9								
rs112251452	c.1083+2T>A	Splice donor variant	-	-	-	-	-	-
rs368234376	c.1084-1G>T	Splice acceptor variant	-	-	-	-	-	-
rs201120164	c.1084-2A>T	Splice acceptor variant	-	-	-	-	-	-
Exon 10								
rs146595460	c.1097A>G	p.Y366C	0	0	0	0.001	tolerated	probably damaging
rs185780477	c.1108G>A/T	p.E370K/Ter	0	0	0.002	0	tolerated	benign
rs140704082	c.1135A>G	p.M379V	0	0.001	0	0	tolerated	benign
rs201004290	c.1145T>G	p.L382R	0	0	0.001	0	deleterious	probably damaging
Exon 11								
rs149663601	c.1198A>G	p.T400A	-	-	-	-	tolerated	benign
rs201990303	c.1201G>T	p.E401Ter	-	-	-	-	-	-
rs199540213	c.1205A>T	p.K402I	-	-	-	-	deleterious	benign
rs370123191	c.1227C>G	p.D409E	-	-	-	-	deleterious	benign
rs200736792	c.1238A>T	p.K413M	-	-	-	-	tolerated	benign
rs372638605	c.1288T>G	p.S430A	-	-	-	-	tolerated	benign
rs114277361	c.1295T>C	p.I432T	-	-	-	-	tolerated	benign
rs376976316	c.1303C>T	p.R435W	-	-	-	-	deleterious	possibly damaging
rs145088728	c.1304G>A	p.R435Q	0.0008	0	0	0	tolerated	possibly damaging
Intron 11								
rs74322122	c.1315+1G>A/C	Splice donor variant	-	-	-	-	-	-
Exon 12								
rs201158237	c.1405G>A	p.G469R	-	-	-	-	deleterious	probably damaging
Exon 13								
rs200368245	c.1486G>A	p.V496M	-	-	-	-	tolerated	benign

Nucleotide positions are given in relation to the full length *CES1* mRNA sequence (NM_001025194.1) and amino acid positions in relation to the respective protein sequence (NP_001020365.1). Variant allele frequency data are from the 1000 Genomes project for populations with African, European, South Asian, and East Asian ancestry (www.1000genomes.org; 1000 Genomes Project Consortium *et al*. 2012). SIFT and PolyPhen predictions of the possible impact of amino acid substitutions on protein function were obtained using the Variant Effect Predictor (Kumar *et al*. 2009, Adzhubei *et al*. 2010, McLaren *et al*. 2010). SIFT, sorting intolerant from tolerant; Ter, premature stop codon.

Effects on drug response in humans. The *CES1* c.428G>A SNV reduces the inactivation of methylphenidate to ritalinic acid (Zhu *et al*. 2008, Stage *et al*. 2017) and, therefore, reduces the required dose of methylphenidate for symptom reduction and increases the risk of adverse effects (*e.g*., worsening of appetite reduction) (Nemoda *et al*. 2009, Bruxel *et al*. 2013). In one study, the clopidogrel active *cis* 5-thiol metabolite plasma concentration, measured 1 hour after clopidogrel intake, was about 60% higher in seven healthy Amish individuals with the c.428G/A genotype than in individuals with the c.428G/G genotype (Lewis *et al*. 2013). In addition, individuals with the c.428G/A genotype had a better clopidogrel response as measured by adenosine diphosphate (ADP) stimulated platelet aggregation (Lewis *et al*. 2013).

A SNV located in the 5´-UTR of *CES1*, c.-75G>T (rs3815583), has been associated with hepatotoxicity of isoniazid in patients with latent tuberculosis infection (Yamada *et al*. 2010). Moreover, it has been associated with an increased risk of adverse effects, such as appetite reduction and sadness, in attention deficit hyperactivity disorder (ADHD) patients treated with methylphenidate (Yamada *et al*. 2010, Bruxel *et al*. 2013, Wang *et al*. 2016). The exact impact of the SNV on the CES1 enzyme is yet unknown, but, as it is located at the 5´-UTR, it could effect gene regulation (Bruxel *et al.* 2013). The allele frequency of this SNV is about 20% (Bruxel *et al.* 2013).

The *CES1* c.1168-33A>C (rs2244613) SNV has been associated with reduced dabigatran trough concentrations (by 15% per minor allele) and the c.257+885T>C (rs8192935) SNV with reduced dabigatran peak plasma concentrations (by 12% per minor allele) at genome-wide significance with a gene-dose effect (Pare *et al*. 2013). The *CES1* c.1168-33A>C SNV was also associated with a lower risk of bleeding with an odds ratio of 0.67 per minor allele (Pare *et al*. 2013). The *CES1* c. 1168-33A>C SNV is also associated with sadness as a side effect of methylphenidate treatment (Johnson *et al.* 2013). These two *CES1* SNVs are in linkage disequilibrium (r^2=0.45 and D'=1.00) (Pare *et al*. 2013).

A *CES1* c.1315+2025A>C (rs8192950) SNV in intron 1 has been associated with decreased risk of ischemic events in Chinese patients on dual antiplatelet therapy with aspirin and clopidogrel (Zhao *et al*. 2016).

An n.-816A>C SNV (rs3785161) in the promoter region of *CES1P1* has been associated with the blood pressure lowering effect of imidapril (Geshi *et al.* 2005). In one study, this SNV also associated with attenuated platelet reactivity to clopidogrel in coronary heart disease patients (Xie *et al*. 2014). This finding was not, however, replicated in another study (Zou *et al*. 2014).

A haplotype consisting of three SNVs in the *CES1* gene, c.690+129delC (rs3217164), c.1168-41C>T (rs2244614), and c.1168-33C>A (rs2244613), and two SNVs in the *CES1P1* gene, n.95+346T>C (rs7187684) and n.-1232A>G (rs11861118), was recently found to be associated with overall toxicity of capecitabine (Hamzic *et al*. 2017).

***CES1* structural variation.** A functional *CES1P1* variant gene (*CES1P1VAR*, previously termed *CES1A2*) is identical to the *CES1* gene except for differences of five nucleotides (four amino acids) in the signal peptide region in exon 1 and differences in promoter, 5´-UTR, and part of intron 1 (see Figure 2, section 2.3.1 Genomic organization and expression) (Tanimoto *et al*. 2007, Fukami & Yokoi 2012, Zhu & Markowitz 2013,

Rasmussen *et al*. 2015, Bjerre *et al*. 2017). The mature protein encoded by *CES1P1VAR* is identical to that of *CES1* (Zhu & Markowitz 2013). Within 1 kb of the upstream region, *CES1* has a C/EBP binding site and two Sp1 binding sites, whereas *CES1P1*/*CES1P1VAR* only has one Sp1 binding site and no C/EBP binding site (Oda *et al*. 2015). The exon 1 of the *CES1* gene can also be converted to that of *CES1P1* resulting in a *CES1* variant (*CES1VAR*) (Wang *et al*. 2016). The expression and activity of *CES1VAR* are similar to those of *CES1* (Wang *et al.* 2016). A rare variant of *CES1* (*CES1SVAR*) contains only the 5′-UTR of *CES1P1* (Bjerre *et al*. 2017).

In the liver, a majority of CES1 is the product of the *CES1* gene because the transcription efficiency of the *CES1P1VAR* is only about 2% of that of *CES1* (Zhu & Markowitz 2013). Therefore, only *CES1* genetic variants, and not those of *CES1P1* or *CES1P2*, are likely to significantly affect CES1 expression and activity. In line with this, the *CES1P1*/*CES1P1VAR* genotype had no significant effect on the pharmacokinetics of oseltamivir (Suzaki *et al*. 2013, Shi *et al.* 2016a) or the *CES1/CES1VAR* genotype on the pharmacokinetics of methylphenidate (Stage *et al.* 2017). Since pseudogenes can affect the mRNA levels of their protein-coding counterparts, however, *CES1P1* and *CES1P2* could potentially modulate the level of *CES1* mRNA (Friedrichsen *et al*. 2013).

2.3.5 Drug-drug interactions

Since ester derivatives are widely used as prodrugs, carboxylesterases are major determinants of the pharmacokinetic behavior of most prodrugs, and the activity can be influenced by interactions with a variety of compounds, either directly or by enzyme regulation (Satoh & Hosokawa 2006). The evidence from *in vitro* and *in vivo* studies suggests that drug-drug, drug-disease, and drug-food interactions could be important factors affecting the therapeutic activity of drugs than the substrates of CES1 and/or CES2 (Li *et al*. 2007, Yang *et al*. 2007, Laizure *et al*. 2013).

Several potent CES1 inhibitors have been found by using recombinantly expressed variant proteins, human liver s9 fractions, human liver microsomes, and human liver homogenates (Table 4). The hydrolysis of imidapril to active imidaprilat by recombinant CES1 is inhibited by several cardiovascular drugs, such as simvastatin lactone, lovastatin lactone, glibenclamide, pioglitazone, rosiglitazone, troglitazone, telmisartan, nitrendipine, diltiazem, and felodipine by as much as 80-100% *in vitro* (Fukami *et al*. 2010, Yanjiao *et al.* 2013). The HIV-protease inhibitor nelfinavir inhibits the hydrolysis of methylphenidate to inactive ritalinic acid in a concentration-dependent matter by about 50% *in vitro* (Rhoades *et al*. 2012). In addition, aripiprazole, perphenazine, thioridazine, and fluoxetine inhibit methylphenidate metabolism (Zhu *et al*. 2010, Rhoades *et al*. 2012). At equal concentrations, clopidogrel inhibits the hydrolysis of oseltamivir to active oseltamivir carboxylate by as much as 90% and at 10% concentration by as much as 55% (Shi *et al.* 2006). ACE inhibitors trandolapril and enalapril inhibit clopidogrel hydrolysis and increase the formation of 2-oxo-clopidogrel and the active *cis* 5-thiol metabolite (Kristensen *et al*. 2014). Simvastatin inhibits clopidogrel hydrolysis in a concentration dependent manner with an IC_{50} value of 18.3 μM (inhibitor concentration producing 50% inhibition), but does

not affect the formation of the active *cis* 5-thiol metabolite (Wang *et al*. 2015). Some other potential inhibitors such as ramipril, perindopril, carvedilol, verapamil, isradipine, amlodipine, rivastigmine, diclofenac acyl-β-D-glucuronide, clopidogrel acyl-β-D-glucuronide, ibuprofen acyl-β-D-glucuronide, and naproxen acyl-β-D-glucuronide have also been identified *in vitro* using the model substrate PNPA (Inoue *et al*. 2013, Tsurkan *et al*. 2013, Thomsen *et al*. 2014, Kristensen *et al*. 2014).

Though numerous drug interactions have been identified using *in vitro* methods, the clinical implications of many interactions remain unknown (Laizure *et al*. 2013). The first evidence of a clinically significant drug interaction involving a carboxylesterase substrate was an interaction between cocaine and ethanol (Bourland *et al.* 1997). CES1 catalyzes the transesterification of cocaine in the presence of ethanol producing a pharmacologically active cocaethylene metabolite (benzoylecgonine ethyl ester), which may contribute to the increased toxicity of cocaine (Brzezinski *et al*. 1994).

A metabolic interaction between methylphenidate and ethanol was first reported in 1999, when the transesterification product ethylphenidate was found in blood samples from two suicide overdoses (Markowitz *et al*. 1999). Ethanol inhibits the CES1-mediated hydrolysis of methylphenidate, resulting in increased methylphenidate exposure (Parker *et al*. 2015). The peak plasma concentration (C_{max}) and the area under the plasma concentration-time curve (AUC) of methylphenidate increased by 40% and 25% (Patrick *et al*. 2007). Ethanol co-administration also significantly inhibits oseltamivir hydrolysis to oseltamivir carboxylate, resulting in increased exposure to parent oseltamivir (Parker *et al*. 2015). The oseltamivir $AUC_{0\text{-}6h}$ increased by 27% and the oseltamivir carboxylate to oseltamivir $AUC_{0\text{-}6h}$ ratio decreased by 34% (Parker *et al*. 2015). The systemic exposure to the oseltamivir carboxylate was, however, not reduced (Parker *et al*. 2015).

In line with the *in vitro* observed interaction between ACE inhibitors and clopidogrel, co-treatment with trandolapril or enalapril and clopidogrel is associated with increased bleeding risk in patients after myocardial infarction. Hazard ratios for clinically significant bleeding in ACE inhibitor-treated patients cotreated with or without clopidogrel were 1.10 and 0.90, as compared with patients who did not receive ACE inhibitors (Kristensen *et al.* 2014). Even though clopidogrel might inhibit the CES1-mediated activation of ramipril or perindopril, their concomitant use was not associated with an elevated risk of recurrent myocardial infarction, heart failure, or death in one study (Cressman *et al.* 2015).

Table 4. *Drugs, drug metabolites, and other compounds that are CES1 inhibitors.*

Inhibitor	**K_i**	**IC_{50}**	**Test system**	**Substrate**	**C_{max} ** (Dose)**	**References**
Aripiprazole		61.7μM	s9 fraction	Methylphenidate	0.07μM (6mg)	Kubo *et al.* 2007, Zhu *et al.* 2010
Clopidogrel	5μM		rCES1	Oseltamivir	0.01μM (600mg)	Shi *et al.* 2006, Tarkiainen *et al.* 2015a
Clopidogrel	*	*	HLM, rCES1	Oxybutynin	0.01μM (600mg)	Sato *et al.* 2012, Tarkiainen *et al.* 2015a

Digitonin		25.8μM	rCES1	Lidocaine	n/a	Shimizu *et al*. 2014
Diltiazem	9.0μM		rCES1	Trandolapril	0.1μM (60 mg)	Caille *et al*. 1991, Thomsen *et al*. 2014
Enalapril	*	*	s9 fraction	Clopidogrel	0.23μM (10mg)	Kristensen *et al*. 2014, Tarkiainen *et al*. 2015b
Ethanol	*	*	s9 fraction	Clopidogrel	20mM (55g)	Jones 1984, Tang *et al*. 2006
Ethanol		23mM	rCES1	Oseltamivir	20mM (55g)	Jones 1984, Parker *et al.* 2015
Fluoxetine		58.9μM	s9 fraction	Methylphenidate	0.04μM (20mg)	Zhu *et al.* 2010, Shi *et al.* 2010
Loperamide		0.44mM	rCES1	Capecitabine	0.01μM (16mg)	Doser *et al.* 1995, Quinney *et al.* 2005
Nelfinavir		6.6μM	s9 fraction	Methylphenidate	5.6μM (800mg)	Kaldor *et al*. 1997, Rhoades *et al.* 2012
Nitrendipine	1.1μM		rCES1	Imidapril	0.1μM (20mg)	Kirch *et al*. 1984, Yanjiao *et al.* 2013
Nitrendipine	1.2μM		HLM	Imidapril	0.1μM (20mg)	Kirch *et al.* 1984, Yanjiao *et al.* 2013
Perphenazine		65.0μM	s9 fraction	Methylphenidate	2.2nM (7mg)	Özdemir *et al.* 1997, Zhu *et al*. 2010
Physostigmine	*	*	HLM	Irinotecan	0.6nM (2mg)	Walter *et al.* 1995, Slatter *et al*. 1997
Procainamide	29.3μM		HLM	Imidapril	0.02μM (1000mg)	Martin *et al.* 1996, Takahashi *et al.* 2009
Procainamide	*	*	Human liver homogenate	Meperidine	0.02μM (1000mg)	Martin *et al.* 1996, Bailey & Briggs 2003
Quinidine	*	*	Human liver homogenate	Meperidine	6.7μM (250mg)	Rao & Rambhau 1995, Bailey & Briggs 2003
Simvastatin (lactone)		18.3μM	s9 fraction	Clopidogrel	0.02μM (40mg)	Keskitalo *et al*. 2009, Wang *et al*. 2015

Simvastatin (lactone)	0.1μM		rCES1	Imidapril	0.02μM (40mg)	Keskitalo *et al.* 2009, Fukami *et al.* 2010
Simvastatin (lactone)	0.8μM		HLM	Imidapril	0.02μM (40mg)	Keskitalo *et al.* 2009, Fukami *et al.* 2010
Telmisartan	0.5μM		rCES1	Imidapril	0.3μM (40mg)	Zhang *et al.* 2006, Yanjiao *et al.* 2013
Telmisartan	1.7μM		HLM	Imidapril	0.3μM (40mg)	Zhang *et al.* 2006, Yanjiao *et al.* 2013
Thioridazine		58.3μM	s9 fraction	Methylphenidate	1.0μM (100mg)	Chakraborty *et al.* 1989, Zhu *et al.* 2010
Troglitazone	0.6μM		rCES1	Imidapril	2.7μM (400mg)	Ott *et al.* 1998, Fukami *et al.* 2010
Troglitazone	5.6μM		HLM	Imidapril	2.7μM (400mg)	Ott *et al*. 1998, Fukami *et al.* 2010
Valproate		2.5mM	HLM	Rufinamide	0.2μM (300mg)	Rha *et al.* 1993, Williams *et al.* 2011b
Verapamil	16.0μM		rCES1	Trandolapril	0.1μM (80mg)	McAllister & Kirsten 1982, Thomsen *et al.* 2014

* K_i or IC_{50} values not reported.

** Oral dose.

CES1, carboxylesterase 1; CES2, carboxylesterase 2; HLM, human liver microsome; HJM, human jejunum microsome; IC_{50}, inhibitor concentration producing 50% inhibition; K_i, inhibition constant; n/a, not available; rCES1, recombinant carboxylesterase 1; rCES2, recombinant carboxylesterase 2.

2.3.6 Physiological role

In addition to its role in xenobiotic metabolism, CES1 has been shown to catalyze reactions involved in cholesterol homeostasis and fatty acid metabolism (Satoh & Hosokawa 1998, Fleming et al. 2005). CES1 possesses acyl-coenzyme A:cholesterol acyltransferase (ACAT) activity, which generates cholesterol esters from fatty-acyl coenzyme A and free cholesterol (Becker *et al.* 1994, Bencharit *et al.* 2003, Fleming *et al*. 2005). It has been also found to catalyze the reverse reaction, acting as a cholesterol ester hydrolase (CEH) (Bencharit *et al*. 2003, Redinbo *et al*. 2003, Fleming *et al.* 2005). These actions are important for cholesterol trafficking both within cells and between tissues throughout the body (Bencharit *et al*. 2003). For example, overexpression of CES1 *in vitro* induces the efflux of cholesterol from the cell (Ghosh & Natarajan 2001, Redinbo *et al.* 2003).

Furthermore, CES1 can produce fatty acid ethyl esters (FAEEs) via the transesterification of short-chain and long-chain fatty acids with ethanol (Fleming *et al.* 2005, Friedrichsen *et al.* 2013).

CES1 appears to be critical to protein retention and release from the ER (Bencharit *et al*. 2003, Redinbo et al. 2003). It contains ER targeting and retention sequences at its N and C termini and associates with the ER lumen (Bencharit *et al*. 2003). CES1 also complexes with the UGTs to hold them in the ER lumen (Bencharit *et al*. 2003) and is involved in binding to and retaining the C-reactive protein (CRP) in the ER lumen of human hepatocytes before its release into the plasma (Yue *et al*. 1996, Bencharit *et al*. 2003, Redinbo *et al*. 2003). Reduction in CES1 affinity for CRP is accountable for the release of CRP at levels up to 1000-fold higher than normal (Macintyre *et al*. 1994, Satoh & Hosokawa 1998, Redinbo *et al*. 2003). CRP responds to infection and cellular damage throughout the body and is a highly sensitive early marker for the development of atherosclerosis (Labarrere *et al*. 2002, Bencharit *et al*. 2003, Redinbo *et al*. 2003).

In addition, CES1 seems to have a role in hepatitis C virus (HCV) replication, translation, and virion release (Blais *et al*. 2010). The knockdown of *CES1* with small interfering RNA resulted in lower levels of HCV replication and correspondingly upregulation of *CES1* favored HCV propagation (Blais *et al*. 2010).

2.4 Other carboxylesterases

Carboxylesterase 2. The *CES2* gene contains 12 exons and spans about 11 kb (Wu *et al.* 2003, Marsh *et al*. 2004). It lies approximately 11 Mb downstream from the *CES1* gene (Marsh *et al*. 2004). The close proximity and homology (73% coding region homology and 48% amino acid sequence homology) of these genes imply an ancestral gene duplication event at this chromosomal region (Marsh *et al*. 2004, Staudinger *et al*. 2010). *CES2* has three transcription start sites at -74, -629, and -1187, all of which are transcriptionally active in HepG2 cells (Wu *et al.* 2003). *CES2* appears to have very little genetic variation, with the majority of SNVs occurring in intronic regions (Marsh *et al*. 2004).

CES2 is expressed in the small intestine, colon, kidney, spleen, liver, heart, brain, and testis and is essentially absent in all other organs (Schwer *et al*. 1997, Xu et al. 2002, Satoh *et al*. 2002, Imai 2006, Kuhl *et al.* 2016). Duodenal and jejunal CES2 expression levels correlate with each other, but not with the hepatic expression levels (Imai & Ohura 2010, Chen *et al*. 2015). In the kidney, CES2 contributes to about 95% of the hydrolytic activity (Hatfield *et al*. 2011). CES2 is an about 60 kDa monomer (Pindel *et al*. 1997, Imai & Ohura 2010, Fukami & Yokoi 2012) and its catalytic triad consists of serine (228), glutamic acid (345), and histidine (457) (Schiel *et al*. 2007).

The preferential substrates for CES2 are compounds esterified with relatively large alcohol moieties (Table 5) (Satoh *et al*. 2002, Imai 2006, Laizure *et al*. 2013). The most investigated CES2 substrate is irinotecan (CPT-11), a topoisomerase I inhibitor used for the treatment of a variety of malignancies. CES2 catalyzes the hydrolysis of irinotecan to its active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38), which is further

metabolized to an inactive glucuronide metabolite (SN-38G) (Humerickhouse *et al*. 2000). Irinotecan appears to be ineffective in tumors lacking CES2 expression, such as lymphomas, suggesting that local CES2 expression in tumors may influence treatment outcome (Schiel *et al*. 2007). Interestingly, it is overexpressed in pancreatic ductal adenocarcinoma tissue both at the mRNA and protein level and its overexpression associates with a longer overall survival (Capello *et al*. 2015). CES2 also hydrolyzes several cardiovascular drugs, such as candesartan cilexetil and simvastatin, and antiplatelet drugs, such as acetylsalicylic acid and prasugrel (Williams *et al.* 2008, Laizure *et al.* 2013).

In vitro, telmisartan, vinblastine, diltiazem, and verapamil inhibit the hydrolysis of irinotecan (Table 6) (Yanjiao *et al*. 2013, Shimizu *et al.* 2014). The hydrolytic activity of recombinant CES2 is also inhibited by timolol, cilnidipine, simvastatin, lovastatin, and fenofibrate (Fukami *et al*. 2010, Yanjiao *et al*. 2013). In human liver microsomes, orlistat inhibits the hydrolysis of the model substrate PNPA (Xiao *et al*. 2013).

Table 5. *Drugs that are metabolized by CES2.*

Substrate	**Therapeutic use and/or drug class**	**Hydrolysis product (activity)**	**Other metabolizing enzymes**	**References**
6-monoacetyl-morphine	Heroin metabolite	Morphine (active)		Dean *et al.* 1991, Brzezinski *et al*. 1997, Hatfield *et al*. 2010
Acetylsalicylic acid	Antiplatelet, analgesic	Salicylate (active)		Takai *et al.* 1997, Tang *et al*. 2006
Capecitabine	Oncologic	5'-deoxy-5-fluorocytidine (inactive)	CES1 CDA TP UPP1 UPP2	Quinney *et al.* 2005
Candesartan cilexetil	Antihypertensive, angiotensin II receptor blocker	Candesartan (active)	CES1 CMBL	Ishizuka *et al*. 2013
Cocaine	Analgesic	Ecgonine methyl ester (inactive)	CES2 BChE	Brzezinski *et al*. 1994
Dabigatran etexilate	Anticoagulant	Dabigatran (active)	CES1	Laizure *et al.* 2014
Flutamide	Oncologic	5-amino-2-nitro-benzotrifluoride (inactive)	CYP1A2 CYP3A4 AADAC	Kobayashi et al. 2012
Heroin	Analgesic	6-monoacetyl-morphine (active)	CES1 BChE	Dean *et al.* 1991, Brzezinski *et al.* 1997, Hatfield *et al.* 2010

Irinotecan (CPT-11)	Oncologic	7-ethyl-10-hydroxycampto-thecin (SN-38) (active)	CES1 UGT1A1 UGT1A7 UGT1A9 CYP3A4 CYP3A5	Takai *et al*. 1997, Humerickhouse *et al.* 2000
Mycophenolate mofetil	Immunosuppressant	Mycophenolate (active)	CES1	Fujiyama *et al*. 2010
Olmesartan medoxomil	Antihypertensive, angiotensin II receptor blocker	Olmesartan (active)	CES1 CMBL	Ishizuka *et al.* 2010
Oxybutynin	Antispasmodic, incontinence	2-cyclohexyl-2-phenylglycolic acid (inactive)	CES1 CYP4A4	Takai *et al.* 1997
Prasugrel	Antiplatelet	Thiolactone metabolite (inactive)	CYP3A CYP2B6 AADAC	Williams *et al*. 2008, Kurokawa *et al*. 2016
Simvastatin	Antihyperlipidemic	Dihydroxy acid metabolite (active)	CES1 CYP3A4	Vickers *et al.* 1990, Parker & Laizure 2010

AADAC, arylacetamide deacetylase; BChE, butyrylcholinesterase; CDA, cytidine deaminase; CES1, carboxylesterase 1; CMBL, carboxymethylenebutenoledase; CYP, cytochrome P450; TP, thymidine phosphorylase; UPP1, uridine phosphorylase 1; UPP2, uridine phosphorylase 2; UGT, uridine 5′-phosphate glucuronosyltransferase.

Table 6. *Drugs, drug metabolites, and other compounds that are CES2 inhibitors.*

Inhibitor	**K_i**	**IC_{50}**	**Test system**	**Substrate**	**C_{max} ** (Dose)**	**References**
Clopidogrel		50μM	rCES2	Oxybutynin	0.01μM (600mg)	Sato *et al*. 2012, Tarkiainen *et al*. 2015a
Diltiazem	0.3μM		rCES2	Irinotecan	0.1μM (60mg)	Caille *et al.* 1991, Yanjiao *et al*. 2013
Diltiazem	2.9μM		HLM	Irinotecan	0.1μM (60mg)	Caille *et al.* 1991, Yanjiao *et al*. 2013
Diltiazem	4.7μM		HJM	Irinotecan	0.1μM (60mg)	Caille *et al.* 1991, Yanjiao *et al.* 2013
Fenofibrate	0.04μM		rCES2	Irinotecan	13.3μM (160mg)	Jones *et al*. 2004, Fukami *et al*. 2010
Fenofibrate	87.7μM		HLM	Irinotecan	13.3μM (160mg)	Jones *et al.* 2004, Fukami *et al*. 2010
Fenofibrate	0.5μM		HJM	Irinotecan	13.3μM (160mg)	Jones *et al*. 2004, Fukami *et al.* 2010

Loperamide	*	*	Human liver homogenate	6-acetyl-morphine	0.01μM (16mg)	Doser *et al.* 1995, Andersson *et al*. 2015
Loperamide	*	*	HJM	Flutamide	0.01μM (16mg)	Doser *et al.* 1995, Kobayashi *et al*. 2012
Loperamide	5μM		rCES2	Oxybutynin	0.01μM (16mg)	Doser *et al.* 1995, Sato *et al.* 2012
Physostigmine	*	*	HLM	Irinotecan	0.6μM (2mg)	Walter *et al*. 1995, Slatter *et al.* 1997
Physostigmine	3.μM		HJM	Irinotecan	0.6μM (2mg)	Walter *et al*. 1995, Takahashi *et al*. 2009
Simvastatin (lactone)	0.7μM		rCES2	Irinotecan	0.02μM (40mg)	Keskitalo *et al*. 2009, Fukami *et al*. 2010
Simvastatin (lactone)	1.9μM		HLM	Irinotecan	0.02μM (40mg)	Keskitalo *et al*. 2009, Fukami *et al*. 2010
Simvastatin (lactone)	3.7μM		HJM	Irinotecan	0.02μM (40mg)	Keskitalo *et al*. 2009, Fukami *et al*. 2010
Telmisartan	0.4μM		rCES2	Irinotecan	0.3μM (40mg)	Zhang *et al*. 2006, Shimizu *et al*. 2014
Telmisartan	0.5μM		HLM	Irinotecan	0.3μM (40mg)	Zhang *et al*. 2006, Shimizu *et al*. 2014
Verapamil	3.8μM		rCES2	Irinotecan	0.1μM (80mg)	McAllister & Kirsten 1982, Yanjiao *et al.* 2013
Verapamil	11.5μM		HLM	Irinotecan	0.1μM (80mg)	McAllister & Kirsten 1982, Yanjiao *et al*. 2013
Verapamil	15.8μM		HJM	Irinotecan	0.1μM (80mg)	McAllister & Kirsten 1982, Yanjiao *et al.* 2013
Vinblastine	37.4μM		HLM	Irinotecan	0.01μM ($3mg/m^2$)	Chong *et al.* 1988, Shimizu *et al*. 2014
Vinblastine	2.0μM		rCES2	Irinotecan	0.01μM ($3mg/m^2$)	Chong *et al.* 1988, Shimizu *et al*. 2014

* K_i or IC_{50} values not reported.

** Oral dose, except for vinblastine which is admistered IV.

CES1, carboxylesterase 1; CES2, carboxylesterase 2; HLM, human liver microsome; HJM, human jejunum microsome; IC_{50}, inhibitor concentration producing 50% inhibition; IV, intravenously; K_i, inhibition constant; rCES1, recombinant carboxylesterase 1; rCES2, recombinant carboxylesterase 2.

Carboxylesterases 3, 4, and 5. CES3 is expressed in the liver, brain, and colon, but at markedly lower levels than CES1 and CES2 (Sanghani *et al*. 2004, Sun *et al*. 2004, Imai 2006, Williams *et al*. 2011a). CES3 is more highly expressed in brain endothelial cells than in the liver and has been suggested to function at the blood-brain barrier (Walter Soria et

al. 2010). *In vitro*, CES3 participates in the hydrolysis of irinotecan, but its catalytic efficiency is markedly lower than those of CES1 and CES2 (Sanghani et al. 2004).

Little is known about the expression of CES4 and CES5 in humans. They are apparently expressed in the brain (Holmes et al. 2010) but their metabolic roles are not yet known (Holmes *et al.* 2010).

2.5 Other esterases

Esterases can be divided into three categories according to the Aldridge esterase classification (Aldridge 1953). Esterases that efficiently hydrolyze organophosphates and require an inorganic ion as a cofactor are classified as A-esterases, such as paraoxonases (Staudinger *et al*. 2010, Williams *et al*. 2011b). Esterases that are inhibited by organophosphates, carbamates, and organosulphur compounds are classified as B-esterases, such as carboxylesterases (Satoh & Hosokawa 2006, Staudinger *et al*. 2010). Esterases that do not hydrolyze organophosphates and are inhibited by them are classified as C-esterases, such as carboxymethylenebutenoledase (Staudinger *et al*. 2010). In addition, multiple other enzymes such as carboxypeptidase A, aldehyde dehydrogenase, carbonic anhydrases B and C, lipase, trypsin, and CYPs have esterase activity and could contribute to the biotransformation of ester-based prodrugs (Liederer & Borchardt 2006). Characteristically, carboxylic acid ester hydrolysis by CYPs leads to an aldehyde and an acid rather than an alcohol and an acid (Liederer & Borchardt 2006).

Albumin. Albumin is abundantly present in plasma and extracellular fluids (Bahar *et al*. 2012). Although albumin is a carrier protein rather than an esterase, it reportedly has esterase-like activity with some substrates, such as PNPA and acetylsalicylic acid (Liederer & Borchardt 2006, Bahar *et al*. 2012). The hydrolytic activity of albumin is significantly lower than that of carboxylesterases, and typically it contributes to less than 20% of the overall esterase activity (Liederer & Borchardt 2006, Bahar *et al*. 2012).

Acetylcholinesterase. Acetylcholinesterase (AChE) rapidly hydrolyzes the neurotransmitter acetylcholine at cholinergic synapses and neuromuscular junctions (Liederer & Borchardt 2006), as well as some esters, amides, and anilides (Liederer & Borchardt 2006). Depending on its molecular form, AChE is present mainly in the brain and red blood cells and at neuromuscular junctions (Li *et al*. 2005, Liederer & Borchardt 2006, Bahar *et al.* 2012).

Arylacetamide deacetylase. Arylacetamide deacetylase (AADAC) is a microsomal serine esterase that is expressed in the liver and gastrointestinal tissues at both the mRNA and protein expression levels (Fukami & Yokoi 2012, Oda *et al*. 2015). AADAC is localized on the luminal side of the ER, similar to carboxylesterases (Shimizu *et al*. 2014). It was first identified as the enzyme that catalyzes the deacetylation of the carcinogen 2-acetylaminofluorene (Fukami & Yokoi 2012), but it also hydrolyzes flutamide,

phenacetin, indiplon, rifamycins, and prasugrel (Fukami & Yokoi 2012, Kurokawa *et al*. 2016). The AADAC-mediated hydrolysis of flutamide is associated with hepatotoxicity and nephrotoxicity and that of phenacetin with hematologic toxicities (Shimizu *et al.* 2014).

Butyrylcholinesterase. Butyrylcholinesterase (BChE, also known as pseudocholinesterase) is present in plasma as well as the rough ER of various tissues, such as liver, lung, brain, muscle, pancreas, and kidney (Liederer & Borchardt 2006, Fukami & Yokoi 2012, Oda *et al.* 2015), although the enzyme activity was undetectable in the liver (Shimizu et al. 2014). The physiological function of BChE is not completely understood. In the absence of BChE no harmful physiological effects are observed (Liederer & Borchardt 2006). Several drugs, such as succinylcholine, mivacurium, cocaine, and heroine are substrates of BChE (Fukami & Yokoi 2012). Inherited deficiency of BChE leads to prolonged succinylcholine-induced paralysis and apnea, for up to 3-4 hours instead of the usual 5-15 minutes (Gardiner & Begg 2006).

Carboxymethylenebutenoledase. Carboxymethylenebutenoledase (CMBL) is predominantly expressed in the liver, intestine, and kidney (Ishizuka *et al*. 2013). CMBL hydrolyzes olmesartan medoxomil to its active metabolite olmesartan (Fukami & Yokoi 2012, Ishizuka *et al*. 2013).

Paraoxonases. Paraoxonases (PONs, also known as arylesterases) have a catalytic dyad consisting of two histidine residues (at positions 115 and 134), which are activated by the binding of divalent calcium (Bahar *et al*. 2012). PON1 is expressed in the liver and plasma and it plays a role in the lipid transfer and assembly of very low density lipoproteins (VLDLs) (Liederer & Borchardt 2006). PON2 is ubiquitously expressed in human tissues, except plasma (Oda *et al.* 2015). PON3 is expressed in the liver and plasma (Liederer & Borchardt 2006). PON1 and PON3 are the main plasma esterases responsible for the hydrolysis of pesticides possessing an aryl group or organophosphorus esters, such as paraoxon, sarin, and soman (Bahar *et al*. 2012). PONs also catalyze the hydrolysis of a broad spectrum of compounds such as aromatic carboxylic acid esters, lactones, and cyclic carbonates (Satoh *et al*. 2002, Liederer & Borchardt 2006). Several drugs, including pilocarpine, simvastatin, lovastatin, and spironolactone are hydrolyzed by PON1 and PON3, whereas seemingly no drugs are hydrolyzed by PON2 (Satoh *et al.* 2002, Oda *et al*. 2015). Phenobarbital and mercurial compounds increase paraoxonase activity while smoking decreases paraoxonase activity (Liederer & Borchardt 2006).

Valacyclovir hydrolase. Valacyclovir hydrolase (also known as valacyclovirase and biphenyl hydrolase-like protein) is an α-amino acid ester hydrolase and highly effective in catalyzing the hydrolysis of valacyclovir to the active antiviral drug acyclovir (Liederer & Borchardt 2006, Lai *et al*. 2008). Otherwise, valacyclovir hydrolase shows relatively low activity towards characteristic substrates of the serine esterases, and its physiological role is unknown (Liederer & Borchardt 2006). Valacyclovir hydrolase is expressed in the liver, kidney, heart, intestine, and skeletal muscle (Kim *et al*. 2004).

2.6 Drugs studied

2.6.1 Oseltamivir

Oseltamivir ($C_{16}H_{28}N_2O_4$, 312.40 g/mol) is widely used in the treatment and prophylaxis of influenza A and B virus infections. Oseltamivir reduces the duration and severity of influenza (Widmer *et al.* 2010). It is an ethyl ester prodrug that is rapidly hydrolyzed in the liver to active oseltamivir carboxylate by CES1 (Figure 5), no other metabolites have been identified. The active metabolite oseltamivir carboxylate exerts its antiviral effects via inhibition of the viral surface glycoprotein neuraminidase (Massarella *et al.* 2000, Davies 2010). Neuraminidase is the enzyme responsible for the release of newly formed viral particles from the surface of infected cells, and it increases the mobility of the virus through the mucus of the respiratory tract (Massarella *et al*. 2000, Widmer *et al.* 2010). Since replication of the influenza virus in the respiratory tract reaches its peak between 24 and 72 hours after the onset of illness, oseltamivir must be administered as early as possible (Widmer *et al.* 2010).

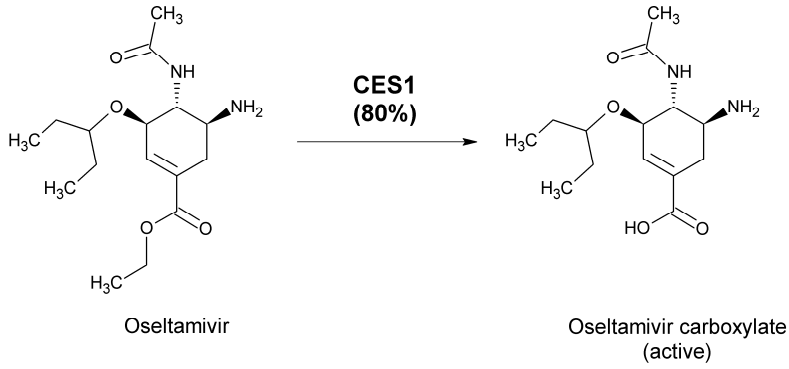

Figure 5. *Biotransformation of oseltamivir. CES1, carboxylesterase 1.*

The usual dosage of oseltamivir is 75 mg orally twice daily for 5 days. Oseltamivir is well tolerated at single doses of up to 1000 mg and twice-daily doses of up to 500 mg (He *et al.* 1999, Massarella *et al.* 2000). In clinical trials, approximately 10-20% of patients reported adverse effects and the most common adverse effects were upper gastrointestinal disturbances such as nausea, vomiting, and abdominal pain (Widmer *et al*. 2010). In adolescents, however, oseltamivir has also been associated with neuropsychiatric reactions including delirium, behavioral changes, convulsions, and even suicides (Izumi *et al*. 2007). Oseltamivir has a relatively high lipophilicity and passive permeability through cell membranes, although P-glycoprotein (P-gp) transports it from the CNS (Holodniy *et al.* 2008, Wattanagoon *et al.* 2009). Oseltamivir carboxylate poorly penetrates the blood-brain barrier. High concentrations of the parent oseltamivir in plasma may result in increased concentrations in the CNS and therefore individuals with a reduced P-gp or CES1 activity

may be at increased risk for neurotoxicity (Izumi *et al*. 2007, Wattanagoon *et al*. 2009, Ito *et al.* 2017).

Following oral administration, oseltamivir is rapidly absorbed from the gastrointestinal tract (Davies 2010). The oral bioavailability of oseltamivir is more than 75% (Table 7) (He *et al*. 1999, Holodniy *et al*. 2008). The time to reach peak plasma concentration (t_{max}) of oseltamivir carboxylate is about 4 hours, and it is eliminated primarily by renal excretion that includes glomerular filtration and tubular secretion by organic anion transporters 1 and 3 (OAT1 and OAT3) (He *et al*. 1999, Massarella *et al*. 2000, Holodniy *et al*. 2008). Approximately 60-70% of an oral dose of oseltamivir is excreted into the urine as the active metabolite, and less than 5% is excreted as the parent oseltamivir (He *et al*. 1999). Small amounts appear in feces (<20%, 50% as oseltamivir and 50% as oseltamivir carboxylate) (He *et al*. 1999, Widmer *et al*. 2010). The elimination half-lifes ($t_{½}$) of oseltamivir and oseltamivir carboxylate are about 1-3 hours and 6-10 hours (Davies 2010). Administration of oseltamivir with food does not significantly affect the C_{max} or $AUC_{0-\infty}$ of the active metabolite (Widmer *et al.* 2010). Also, altered gastric pH, such as induced by antacids, does not affect the absorption of oseltamivir (He *et al*. 1999). Oseltamivir carboxylate is minimally bound to plasma proteins (<3%), compared to the parent oseltamivir (approximately 42%) (He *et al*. 1999, Widmer *et al*. 2010). Plasma concentrations of oseltamivir and oseltamivir carboxylate are linear and proportional at doses of up to 500 mg twice daily (He *et al*. 1999, Massarella *et al*. 2000). Multiple-dose exposure is predictable from single-dose data, and steady-state plasma concentrations are achieved within 2-3 days (Massarella et al. 2000).

Children usually express low levels of carboxylesterases and the hydrolysis of oseltamivir is delayed (oseltamivir carboxylate t_{max} 6 hours versus 4 hours) in children (1-2 years) (Shi *et al*. 2006, Yang *et al.* 2009, Widmer *et al*. 2010, Shi *et al.* 2011). Optimal exposure to oseltamivir carboxylate is achieved by using adjusted weight-based dosing (*e.g.*, 3-3.5mg/kg) (Kimberlin *et al*. 2013). The overall exposures to oseltamivir and oseltamivir carboxylate at steady state have been approximately 25% larger in elderly patients than in younger patients, likely due to decreased renal function (Massarella *et al*. 2000, Widmer *et al.* 2010). The AUC of oseltamivir carboxylate increases 100%, 200%, and 1000% in patients who have mild, moderate, and severe renal impairment (He *et al*. 1999, Widmer *et al.* 2010), though the increased drug exposure has not been associated with reduced tolerability (He *et al*. 1999). In patients with hepatic impairment, the oseltamivir and oseltamivir carboxylate C_{max} values were 6% and 19% lower, and their AUC values 33% higher and 19% lower than in healthy subjects (Snell *et al*. 2005). The oseltamivir carboxylate to oseltamivir AUC ratio was also slightly lower in patients with hepatic impairment (Snell *et al*. 2005).

Oseltamivir and oseltamivir carboxylate are not substrates of and do not interact with CYPs or UGTs. Therefore, no drug-interactions are expected to occur via these metabolic pathways (He *et al*. 1999, Widmer *et al.* 2010). The low protein binding of both compounds also suggests that drug interactions involving displacement from proteins are unlikely (He *et al.* 1999). Oseltamivir is a substrate of peptide transporter 1 (PEPT1, *SLC15A1*) and P-gp (*ABCB1*), the latter of which could limit its absorption from the gastrointestinal tract (Widmer *et al*. 2010). The OAT1 inhibitor probenicid effectively inhibits the renal

excretion of oseltamivir, increasing the AUC of oseltamivir carboxylate by 150% and decreasing its renal clearance by 30-50% (He *et al*. 1999, Holodniy *et al.* 2008, Wattanagoon *et al.* 2009). *In vitro*, the antiplatelet drug clopidogrel inhibits the hydrolysis of oseltamivir by as much as 90% when assayed at equal concentrations (Shi *et al*. 2006), though the assessed drug concentrations were hundreds of times higher than those observed in clinical use. Dexamethasone slightly reduced the AUC of oseltamivir carboxylate and increased the renal clearance of oseltamivir, but the interaction was not clinically significant (Jang *et al.* 2017). Coadministration of oseltamivir with paracetamol, aspirin, cimetidine, and amoxicillin had no clinically significant effect on the pharmacokinetics of these compounds or oseltamivir itself (Hill *et al.* 2002, Oo *et al*. 2002, Snell *et al*. 2002, Davies 2010).

2.6.2 Clopidogrel

Clopidogrel ($C_{16}H_{16}ClNO_2S$, 321.81 g/mol) is widely used in the treatment and prophylaxis of atherothrombotic and thromboembolic diseases, such as stroke and myocardial infarction. It is a thienopyridine prodrug that is converted to a pharmacologically active *cis* 5-thiol metabolite through an inactive intermediate 2-oxo-clopidogrel, catalyzed by CYP enzymes, mainly CYP2C19, CYP3As, CYP2B6, CYP1A2, and CYP2C9 (Figure 6) (Farid *et al*. 2010, Dansette *et al.* 2012). The active *cis* 5-thiol metabolite irreversibly binds to the platelet $P2Y_{12}$ subtype of the ADP receptor and thereby inhibiting aggregation (Mills *et al*. 1992). About 90% of the absorbed clopidogrel is, however, rapidly hydrolyzed by CES1 to an inactive carboxylic acid metabolite, which is further metabolized by glucuronidation (Caplain *et al*. 1999, Lins *et al.* 1999, Tang *et al.* 2006, Hagihara *et al*. 2009, Silvestro *et al*. 2011). In addition, CES1 hydrolyzes 2-oxo-clopidogrel and the active metabolite to their corresponding carboxylic acid derivatives (Hagihara *et al.* 2009). Ultimately, only a small proportion of clopidogrel is converted to the active metabolite (Farid *et al*. 2010).

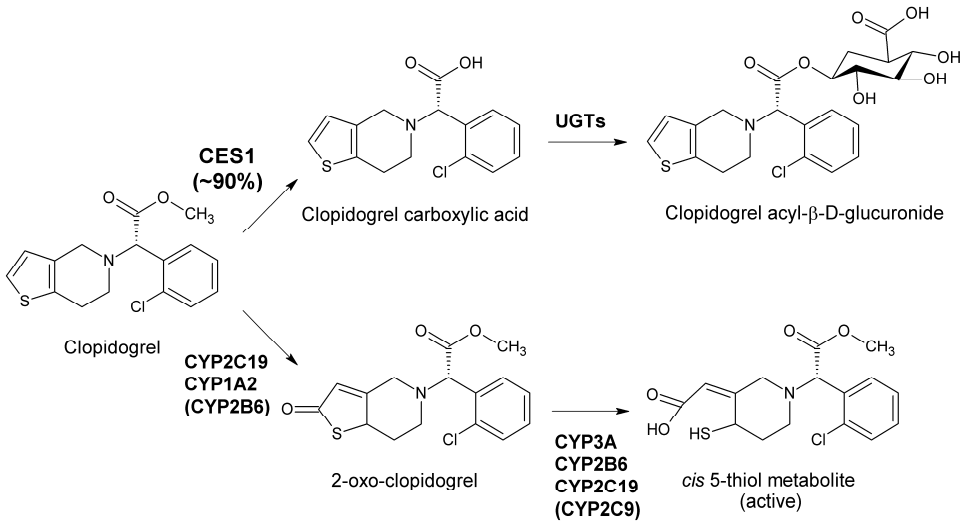

Figure 6. *Biotransformation pathways of clopidogrel. CES1, carboxylesterase 1; CYP, cytochrome P 450; UGT, uridine 5′-diphosphate glucuronosyltransferase.*

Marked interindividual variability exists in the pharmacokinetics, platelet inhibitory effects, and clinical efficacy of clopidogrel (Caplain *et al.* 1999, Gurbel *et al.* 2003, Matetzky *et al.* 2004, Cuisset *et al*. 2006). A significant percentage (5-40%) of individuals treated with clopidogrel do not receive the anticipated therapeutic benefit, which is associated with an increased risk of adverse outcomes (Zhu *et al*. 2013). Mechanisms of variability in the pharmacodynamics of clopidogrel are likely due to several factors including poor compliance to treatment, variable absorption of the prodrug, variable clearance of the active metabolite, drug-drug interactions, and ability of thrombin to bypass complete ADP-inhibition and still induce platelet aggregation (Malinin *et al.* 2007). The $P2Y_{12}$ receptor variability, genetic polymorphisms of platelet receptors, and differences in platelet signal transduction pathways may also contribute to variability in clopidogrel response (Malinin *et al*. 2007).

Genetic factors explain an estimated 70% of the interindividual variance in clopidogrel pharmacokinetics and pharmacodynamics (Shuldiner *et al*. 2009). In particular, the loss-of-function of *CYP2C19* c.681G>A (**2*, rs4244285) and c.636G>A (**3*, rs4986893) SNVs impair clopidogrel bioactivation and efficacy (Kim *et al.* 2008, Simon *et al*. 2009, Mega *et al.* 2009, Scott *et al.* 2011, Scott *et al.* 2013, Samant *et al* 2017, Backman *et al* 2017). Carriers of the *CYP2C19*2* allele show lower active metabolite levels, higher platelet reactivity, and poorer outcomes (Windecker *et al.* 2014). *CYP2C19* loss-of-function alleles account for about 12% of the variability in clopidogrel response (Shuldiner *et al*. 2009). Some studies implicate variants in other genes associated with clopidogrel response (*e.g.*, *ABCB1*, *CES1*, *CYP2B6*, *CYP2C9*, *P2Y12*, and *PON1*) (Scott *et al*. 2011).

The usual dosage of clopidogrel is 75 mg once daily. A loading dose of 300-600 mg reduces the time required to achieve maximal platelet aggregation inhibition (Jarvis &

Simpson 2000, Windecker *et al*. 2014), and is therefore recommended if rapid onset of the antiplatelet effect is needed, such as before percutaneous coronary intervention (PCI). The effect of 75 mg daily doses of clopidogrel on platelet aggregation typically reaches a steady state within 3 to 7 days (Farid *et al.* 2010). The most common adverse effects are gastrointestinal bleeding, headache, abdominal pain, rash, dizziness, dyspepsia, diarrhea, pruritus, constipation, vertigo, gastritis, ulcer, and hemorrhage, each occurring in less than 8% of patients (Jarvis & Simpson 2000). During postmarketing surveillance of clopidogrel, hypersensitivity reactions, including angioedema, bronchospasm, anaphylactoid reactions, arthritis, and thrombotic thrombocytopenic purpura have been reported (Jarvis & Simpson 2000, Balamuthusamy & Arora 2007, Beavers *et al*. 2015).

The bioavailability of clopidogrel is approximately 50% (Table 7) (Jarvis & Simpson 2000). *In vitro*, P-gp limits the uptake of clopidogrel, suggesting that P-gp may affect the intestinal absorption and oral bioavailability of clopidogrel (Jiang *et al*. 2015). Very low levels of the inactive parent clopidogrel are present after oral administration of clopidogrel, since it is rapidly metabolized in the liver (Jarvis & Simpson 2000). The t_{max} of the main inactive metabolite of clopidogrel, clopidogrel carboxylic acid, is about 1 hour and the $t_{½}$ about 7-8 hours (Caplain *et al*. 1999). About 50% of clopidogrel dose is excreted into the urine and about 46% into feces, presumably as metabolites (Lins *et al*. 1999). The renal clearance (Cl_{renal}) of clopidogrel is approximately 0.25 l/h (Caplain *et al.* 1999). Administration with food does not significantly affect the pharmacokinetics or pharmacodynamics of clopidogrel (Jarvis & Simpson 2000). The exposure to clopidogrel metabolites increase in a dose-dependent, but less than dose-proportional manner, likely due to saturable absorption, metabolism, or both (Farid *et al.* 2010). Clopidogrel and its carboxylic acid metabolite are 98% and 94% bound to plasma proteins (Farid *et al.* 2010).

Plasma concentrations of clopidogrel carboxylic acid were higher in the elderly than in young patients, but this does not appear to be of clinical relevance since bleeding times remained unaffected (Jarvis & Simpson 2000). Exposure to clopidogrel carboxylic acid was lower in patients with severe versus moderate renal dysfunction (Jarvis & Simpson 2000). Mild to moderate hepatic impairment slowed the clopidogrel metabolite formation (Jarvis & Simpson 2000).

Since the pharmacological effect of clopidogrel is closely linked to its bioactivation via CYPs, other concomitant medications or food constituents that suppress the activity of relevant CYPs (*e.g.*, CYP2C19, CYP3As, CYP2C9, CYP2B6, or CYP1A2) may interrupt the antiplatelet activity of clopidogrel and thus negatively impact the clinical outcome (Jiang *et al.* 2015). For example, grapefruit juice impairs the bioactivation and antiplatelet effects of clopidogrel by inhibiting CYP3A4 and CYP2C19. Grapefruit juice reduced the C_{max} and $AUC_{0\text{-}3h}$ of the active *cis* 5-thiol metabolite by more than 80% and markedly decreased the antiplatelet effect of clopidogrel (Holmberg *et al.* 2014). It has also been shown, that concomitant use of omeprazole and ketoconazole with clopidogrel reduces the bioactivation and antiplatelet effect of clopidogrel (Farid *et al.* 2007, Gilard *et al*. 2008, Zahno *et al*. 2010, Angiolillo *et al.* 2011). In addition, treatment with CES1-inhibiting ACE inhibitors may increase bleeding risk in clopidogrel-treated patients (Kristensen *et al.* 2014, Cressman *et al.* 2015). Clopidogrel acyl-β-D-glucuronide is a potent time-dependent inhibitor of CYP2C8 (Tornio *et al*. 2014). In a crossover study with 9 healthy volunteers,

the $AUC_{0-\infty}$ of repaglinide was increased by 5-fold with a 300-mg loading dose of clopidogrel and by 4-fold with continued daily administration of 75 mg clopidogrel (Tornio *et al*. 2014). *In vitro*, clopidogrel reduces the hydrolytic efficiency of CES1 (Shi *et al.* 2006).

2.6.3 Quinapril and enalapril

The ACE inhibitors quinapril ($C_{25}H_{30}N_2O_5$, 438.52 g/mol) and enalapril ($C_{20}H_{28}N_2O_5$, 376.45 g/mol) are widely used in the treatment of hypertension and congestive heart failure. Both are ethyl ester prodrugs that are rapidly hydrolyzed in the liver to the active diacid metabolites, quinaprilat and enalaprilat (Klutchko *et al*. 1986, Todd & Heel 1986). This bioactivation is catalyzed mainly by CES1 (Figure 7) (Takai *et al*. 1997, Thomsen *et al*. 2014). Parent quinapril and enalapril are relatively weak inhibitors of ACE. The active metabolites quinaprilat and enalaprilat are, however, potent inhibitors of ACE thereby preventing the conversion of angiotensin I to the vasoactive peptide angiotensin II. These effects are mediated by binding to both tissue and plasma ACE (Kaplan *et al*. 1989, Culy & Jarvis 2002).

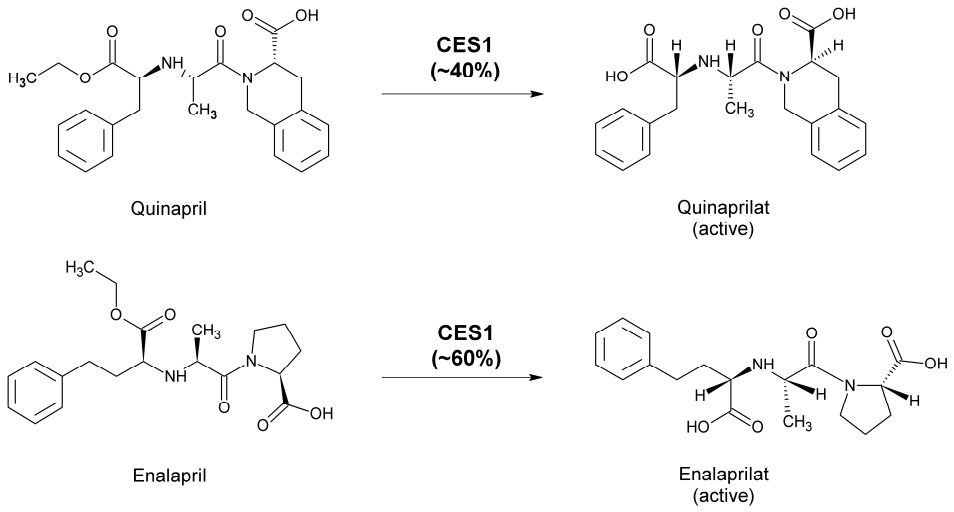

Figure 7. *Biotransformations of quinapril and enalapril. CES1, carboxylesterase 1.*

Large interindividual differences in the effects of ACE inhibitors on blood pressure and the renin-angiotensin system exist, particularly after the first dose. Several studies have investigated the effects of genetic variants on the pharmacodynamics of ACE inhibitors (Hingorani *et al*. 1995, Todd *et al*. 1995, Voors *et al*. 2004, Hannila-Handelberg *et al*. 2010). For example, some studies show an association of variants in ACE, angiotensinogen, angiotensin receptor 1, and α-adducin 1 with a response to ACE inhibitors (Arnett *et al.* 2006). In addition, polymorphisms in the *SLCO1B1* gene, encoding the hepatic influx

transporter organic anion-transporting polypeptide 1B1 (OATP1B1), may influence the pharmacokinetics of enalapril and temocapril (Maeda *et al.* 2006, Niemi *et al.* 2011, Tian *et al*. 2011).

The usual, generally well tolerated, dosage of quinapril and enalapril is 5-40 mg orally once a day (McFate Smith *et al*. 1984, Culy & Jarvis 2002). The most common adverse effects are dizziness, headache, cough, fatigue, nausea, and vomiting, each occurring in less than 8% of patients (Irvin & Viau 1986, Frank *et al*. 1989, Culy & Jarvis 2002). Angioedema has been seen in 0.1% of patients using quinapril or enalapril (Culy & Jarvis 2002).

Following oral administration, quinapril and enalapril are hydrolyzed to the active diacid metabolites, quinaprilat (about 40% of an oral dose) and enalaprilat (about 60% of an oral dose) (Table 7) (Davies *et al.* 1984, Kaplan *et al*. 1989, Olson *et al.* 1989, Wadworth & Brogden 1991, MacFadyen *et al*. 1993). Quinapril also has two minor inactive diketopiperazine metabolites (Olson *et al*. 1989, Wadworth & Brogden 1991), but enalapril has no further metabolites (Todd & Heel 1986). The t_{max} of quinaprilat and enalaprilat is about 2 hours and 2-4 hours, and they are eliminated primarily by renal excretion with elimination half-lifes of about 2 hours and 2-6 hours (Todd & Heel 1986, Olson *et al.* 1989, Kaplan *et al*. 1989, MacFadyen *et al*. 1993). Quinaprilat and enalaprilat have long terminal elimination half-lives (about 25 hours and 36 hours), likely because of slow release of quinaprilat and enalaprilat from ACE (MacFadyen *et al.* 1993, Culy & Jarvis 2002). Approximately 30-40% of an oral dose of quinapril and enalapril is excreted into the urine as the active metabolites (Ulm 1983, Todd & Heel 1986, Olson *et al.* 1989). The remainder of quinapril and approximately 30% of enalapril are eliminated in the feces as unabsorbed parent prodrugs or by biliary excretion of parent prodrugs and their metabolites (Todd & Heel 1986, Olson *et al*. 1989). The Cl_{renal} of quinapril is about 2.5 l/h and that of enalapril about 18 l/h (Todd & Heel 1986, Halstenson *et al.* 1992). Both quinapril and quinaprilat are highly bound to plasma proteins, about 97% (Olson *et al.* 1989). Enalaprilat is about 50% bound to plasma proteins (MacFadyen *et al*. 1993). The bioavailability of quinapril or enalapril is not affected by food (MacFadyen *et al.* 1993, Plosker & Sorkin 1994). Plasma concentrations of quinapril and enalapril are linear and dose-proportional at doses up to 80 mg once daily and 40 mg once daily (Todd & Heel 1986, Olson *et al.* 1989).

Renal and hepatic impairment affects the pharmacokinetics of quinapril and quinaprilat. Quinapril and quinaprilat $t_{½}$ increased by about 5-fold, C_{max} by about 3-fold, and AUC by about 10-fold in patients with renal impairment (Culy & Jarvis 2002). Markedly elevated enalaprilat plasma concentrations were also seen in patients with severe renal impairment. Quinaprilat C_{max} and AUC are decreased by about 70% and 50% in patients with liver cirrhosis (Culy & Jarvis 2002). A delay in the appearance of enalaprilat in plasma after a single dose of enalapril was noted in patients with hepatic impairment (MacFadyen *et al.* 1993).

The pharmacokinetics of quinapril are not affected by concomitant administration of hydrochlorothiazide, propranolol, or cimetidine, and quinapril does not affect the pharmacokinetics of digoxin, hydrochlorothiazide, or warfarin (Culy & Jarvis 2002). Quinapril can reduce the oral absorption of tetracycline, possibly due to binding of tetracycline with magnesium in the quinapril tablet formulation (Culy & Jarvis 2002).

There is no pharmacokinetic interaction between enalapril and furosemide, hydrochlorothiazide, or digoxin (Todd & Heel 1986). *In vitro*, enalapril increases the formation of the clopidogrel active *cis* 5-thiol metabolite due to inhibition of the CES1-mediated hydrolysis of clopidogrel (Kristensen *et al*. 2014). In a pharmacoepidemiological study with over 70000 patients with a myocardial infarction, concomitant use of ACE inhibitors increased bleeding risk (Kristensen *et al*. 2014). This finding was, however, not replicated in another study with over 45000 patients with a myocardial infarction (Cressman *et al*. 2015).

Table 7. *Pharmacokinetic properties of the study drugs' carboxylic acid metabolites.*

	Oseltamivir carboxylate	**Clopidogrel carboxylic acid**	**Quinaprilat**	**Enalaprilat**
Proportion of the parent drug metabolized by CES1 (%)	80	90	40	60
Distribution volume (l)	267	n/a	n/a	n/a
Protein binding (%)	<3	94	97	50
t_{max} (h)	4	1	2	2-4
$t_{½}$ (h)	7	8	25	36
Clearance (l/h)	25	10	13	8-10
Renal excretion (%)	60-70	50	30	40
Metabolite activity	Active	Inactive	Active	Active

Adapted from (Olson *et al.* 1989, MacFadyen *et al*. 1993, FDA 1997, Culy & Jarvis 2002, Widmer *et al*. 2010).
CES1, carboxylesterase 1; n/a, not available; $t_{½}$, elimination half-life; t_{max}, time to peak plasma concentration.

3 AIMS OF THE STUDY

The specific aims were as follows:

Study I To investigate the frequency distribution of the *CES1* c.428G>A SNV in the Finnish population.

Studies I-III To investigate the effects of the *CES1* c.428G>A SNV on the pharmacokinetics of oseltamivir, clopidogrel, quinapril, and enalapril and on the pharmacodynamics of clopidogrel, quinapril, and enalapril in prospective genotype panel studies in healthy volunteers.

Study IV To investigate the effects of sequence variations in close proximity to or within the *CES1* gene on its expression in whole blood and the liver, and on the pharmacokinetics and pharmacodynamics of clopidogrel.

4 MATERIALS AND METHODS

4.1 Population genetic study

In total, 860 young (18-40 years) healthy white Finnish volunteers participated in the population genetic study. The healthy volunteers were recruited from the University of Helsinki student mailing lists during a time period of eight years. Following a written informed consent, a 12-ml blood sample was drawn into an ethylenediaminetetraacetic acid (EDTA) containing tube for genotyping and stored at -20°C until DNA extraction. The health of the volunteers was ascertained by medical history before they entered the study.

DNA preparation and genotyping. Genomic DNA was extracted using standard methods (QIAamp DNA Blood Mini Kit, Qiagen, Hilden, Germany or Maxwell® 16 LEV Blood DNA Kit on a Maxwell 16 Research automated nucleic acid extraction system, Promega, Madison, WI, USA). DNA concentration and absorbance 260/280 ratio (A_{260}/A_{280}) were determined with the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The extracted DNA was stored at -20°C or -80°C until genotyping. The subjects were genotyped for the *CES1* c.428G>A SNV (rs71647871) by allelic discrimination with a TaqMan® 5'-nuclease assay on an Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) or a QuantStudio™ 12K Flex Real-Time PCR system (Life Technologies, Carlsbad, CA, USA) (forward primer: 5´-TGATGGGAGTGTCCTCCCGAAG-3´ and reverse primer: 5´-GGGTAGGTAGTGTGTCCAATTAC-3´). The PCR volume was 8 µl in the 7300 Real-Time PCR system and 5 µl in the QuantStudio™ system. Pre- and post-PCR fluorescence measurements and genotype calls were made with the 7300 Real-Time PCR system or QuantStudio™ 12K Flex Real-Time PCR system. The PCR cycling conditions were as follows: 1 cycle at 95°C for 10 minutes, followed by 40 cycles of melting at 95°C for 15 seconds, and annealing and extending at 60°C for 1 minute. For the purpose of Study II, the subjects were also genotyped for the *CYP2C19*2* (c.681G>A, rs4244285), **3* (c.636G>A, rs4986893), and **17* (c.-806C>T, rs12248560) SNVs using the same reaction volumes and PCR cycling conditions as above. About 10% of the samples were genotyped in duplicate.

4.2 Pharmacokinetic and pharmacodynamic studies

4.2.1 Subjects

A total of 40 healthy Finnish volunteers participated in Studies I-III (14 females and 26 males) (Table 8). Four volunteers participated in all three studies. The subjects were recruited from the pool of healthy genotyped subjects (see Materials and methods, section

4.1) and selected based on the *CES1* c.428G>A genotype. Before entering the studies, all participants were given both oral and written information, and they gave written informed consent.

In Study I, the subjects were allocated into one of three groups according to the genotype: the *CES1* c.428A/A group consisted of one participant, the c.428G/A group of nine, and the c.428G/G (control) group of 12. The number of participants in Study I was estimated to be sufficient to detect a 30% smaller oseltamivir carboxylate $AUC_{0-\infty}$ between different genotypes with a statistical power of 80% at an alpha level of 5%. In Studies II and III, the subjects were allocated into one of two groups according the genotype: the *CES1* c.428G/A group consisted of 10 participants and the c.428G/G (control) group of 12. In Study II, the subjects were balanced in two groups based on the *CYP2C19*2*, **3*, and **17* genotypes to minimize variability in clopidogrel pharmacokinetics due to these genetic variants. In Studies II and III, the number of participants was estimated to be sufficient to detect a 30% smaller metabolite to parent drug $AUC_{0-\infty}$ ratio between different genotypes (power 80%, alpha 5%).

The volunteers were ascertained to be healthy by medical history, physical examination, and routine laboratory tests before entering the studies. Female subjects had a negative pregnancy test before entering the studies. None of the participants used any continuous medication, *e.g.*, oral contraceptives, and no one was a tobacco smoker. Use of other drugs was prohibited for one week before and three days after the study drug admistration. The participants were instructed to abstain from consuming grapefruit products for three days before and after study drug administration. Use of alcohol was prohibited the day before, on the days of study drug administration, and on the following blood sampling days. Participation in any other trial or blood donation within three months before and after each study was also prohibited. Forceful physical exercise was prohibited on the day of clopidogrel ingestion and on the following three days in Study II. Use of acetylsalicylic acid, non-steroidal anti-inflammatory drugs, and omega-3-supplements was also prohibited for one week before and after clopidogrel ingestion in Study II. Subjects with a systolic blood pressure of more than 145 mmHg and less than 100 mmHg were not included in the Study III. There were no withdrawals.

Study IV included whole blood DNA and RNA samples from 212 (whole blood discovery cohort) and 106 (whole blood replication cohort) healthy volunteers, and whole blood DNA and liver RNA samples from 201 patients (liver sample cohort). Good quality RNA and gene expression data were obtained from 192 subjects in the whole blood discovery cohort, 88 in the whole blood replication cohort, and 177 in the liver sample cohort (Table 8). In addition, Study IV included pharmacokinetic data on clopidogrel from all 106 healthy volunteers in the whole blood replication cohort (49 women and 57 men; mean ± SD: age 24 ± 4 years, height 175 ± 9 cm, weight 71 ± 13 kg, and BMI 23 ± 3 kg/m^2).

The whole blood discovery and replication cohort volunteers were ascertained to be healthy by medical history, physical examination, and routine laboratory tests before entering the studies. Female subjects had a negative pregnancy test before entering the studies. None of the participants used any continuous medication, *e.g.*, oral contraceptives, and no one was a tobacco smoker. The liver samples were divided into the following three

histological diagnosis categories: 1) normal liver without any steatosis, inflammation, ballooning, or fibrosis, 2) simple steatosis without inflammation, ballooning, or fibrosis, and 3) non-alcoholic steatohepatitis (NASH). The degree of steatosis was graded from 0 to 3 and that of lobular inflammation from 0 to 2. In the liver sample cohort, 70 patients had type 2 diabetes, 29 had non-alcoholic fatty liver, 30 had NASH, and 54 used lipid-lowering mediation. Before entering the studies, all participants were given both oral and written information, and they gave a written informed consent. The studies were approved by the Coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa and the Finnish Medical Agency Fimea (whole blood discovery and replication cohorts) and the Ethics Committee of the Northern Savo Hospital District (liver sample cohort).

Table 8. *Subject characteristics.*

Study no	Genotype	Sex	Age (years)	Height (cm)	Weight (kg)	BMI (kg/m^2)
I	*CES1* c.428G/G	3 (f)	23 ± 4	170 ± 9	64 ± 3	22 ± 1
	(*n*=12)	9 (m)	23 ± 2	183 ± 8	81 ± 9	24 ± 3
	CES1 c.428G/A	4 (f)	26 ± 3	164 ± 8	53 ± 5	20 ± 2
	(*n*=9)	5 (m)	26 ± 4	183 ± 9	71 ± 7	21 ± 2
	CES1 c.428A/A	1 (m)	23	186	83	24
	(*n*=1)					
II and III	*CES1* c.428G/G	5 (f)	24 ± 6	170 ± 6	68 ± 15	23 ± 4
	(n=12)	7 (m)	24 ± 4	180 ± 8	80 ± 14	25 ± 3
	CES1 c.428G/A	4 (f)	28 ± 3	166 ± 9	56 ± 7	20 ± 2
	(*n*=10)	6 (m)	25 ± 5	183 ± 7	76 ± 8	23 ± 2
IV Whole blood	n/a	99 (f)	24 ± 4	167 ± 6	62 ± 8	22 ± 2
(discovery)	(*n*=192)	93 (m)	23 ± 3	181 ± 6	77 ± 11	23 ± 3
IV Whole blood	n/a	41 (f)	24 ± 4	168 ± 7	63 ± 9	22 ± 3
(replication)	(*n*=88)	47 (m)	24 ± 4	182 ± 6	78 ± 11	24 ± 3
IV Liver	n/a	122 (f)	48 ± 9	n/a	n/a	44 ± 6
	(*n*=177)	55 (m)	50 ± 8	n/a	n/a	43 ± 5

Data are given as mean ± SD unless otherwise indicated.

BMI, body mass index; CES1, carboxylesterase 1; f, females; m, males; n/a, not available; SD, standard deviation.

4.2.2 Study design

Studies I-III were prospective genotype panel studies, Study I with one study drug and Studies II and III with a fixed order crossover design and three phases. Studies were carried out at the Department of Clinical Pharmacology (University of Helsinki and Helsinki University Hospital, Helsinki, Finland). Following an overnight fast, the subjects ingested a single oral dose of oseltamivir, clopidogrel, quinapril, or enalapril with 150 ml of water at 8 AM (Table 9). A standardized warm meal was served 4 hours and a standardized light meal 7 and 10 hours after study drug ingestion. The subjects were under medical

supervision for 12 hours after the study drug ingestion. In Studies II and III, a washout period of at least one week was between the phases and food intake was identical in the different study phases.

In Study IV, whole blood DNA and RNA samples from healthy volunteers were obtained during eight pharmacokinetic studies (whole blood discovery and replication cohorts) carried out at the Department of Clinical Pharmacology (Holmberg *et al*. 2014, Tornio *et al*. 2014, Itkonen *et al*. 2015, Tarkiainen *et al*. 2015a, Itkonen et al. 2016, P.H. *et al*. unpublished results, M.T.H. *et al*. unpublished results, M.K.I. et al. unpublished results). Whole blood DNA and liver RNA samples were obtained from patients undergoing laparoscopic gastric bypass operation, as part of the Kuopio Obesity Surgery Study (liver sample cohort; Kuopio University Hospital, Kuopio, Finland; (Pihlajamäki *et al*. 2012, Männisto et al. 2014, Nilsson *et al*. 2015). In addition, blood samples from 106 healthy volunteers were obtained during seven pharmacokinetic studies on clopidogrel carried out at the department of Clinical Pharmacology (whole blood replication cohort) (Holmberg *et al*. 2014, Tornio *et al*. 2014, Itkonen *et al*. 2015, Tarkiainen *et al*. 2015b, Itkonen *et al*. 2016, M.T.H. *et al*. unpublished results, M.K.I. *et al*. unpublished results). In these pharmacokinetic studies following an overnight fast, the subjects ingested a single oral dose of 300 mg (*n*=49) or 600 mg (*n*=57) of clopidogrel with 150 ml of water at 8 or 9 AM (Table 9). A standardized warm meal and light meals were served at prespecified time points after clopidogrel ingestion. The subjects were under medical supervision for 12 hours after clopidogrel ingestion.

Table 9. *Details of the drugs used in the studies.*

Study	Drug substance	Dose (mg)	Drug product	Manufacturer
I	Oseltamivir	75	Tamiflu tablet	Roche Pharma AG, Grenzach-Wyhlen, Germany
II	Clopidogrel	600	Plavix tablet	Sanofi Pharma Bristol-Myers Squibb SNC, Paris, France
III	Quinapril	10	Accupro tablet	Pfizer, Freiburg, Germany
III	Enalapril	10	Renitec tablet	Merck Sharp & Dohme B.V., Haarlem, Netherlands
IV	Clopidogrel	300 (*n*=49) or 600 (*n*=57)	Plavix tablet	Sanofi Pharma Bristol-Myers Squibb SNC, Paris, France

4.2.3 Sampling

In the prospective genotype panel studies (Studies I-III), a forearm vein of each participant was cannulated for blood sampling on the days of study drug administration. Timed EDTA blood samples (8 ml or 9 ml) were drawn prior to and for up to 12 hours after clopidogrel ingestion, for up to 24 hours after quinapril ingestion, and for up to 48 hours after oseltamivir and enalapril ingestion for the determination of study drug plasma

concentrations. In Study II , part of the samples (4 ml) were derivatized immediately after drawing with 25 µl of 500 mM 2-bromo-3'-methoxyacetophenone in acetonitrile to stabilize the active *cis* 5-thiol metabolite, as previously described (Delavenne *et al*. 2010). The sample tubes were then immediately placed on ice. Plasma was separated within 30 minutes by cold centrifugation (2100 g, 11 minutes). Urine was collected for up to 12 hours after study drug ingestion. Plasma and urine aliquots were stored at -70°C or -80°C until analysis.

In Study II, timed sodium citrate blood samples (2.7 ml each, containing 3.2% of sodium citrate) were drawn prior to and for up to 12 hours after clopidogrel ingestion for pharmacodynamic measurements. The tubes were inverted three to five times and stored in room temperature for at least 10 minutes, as recommended by the manufacturer (Accumetrics, San Diego, CA, USA). Whole blood platelet function tests were performed within 4 hours.

In Study III, systolic and diastolic blood pressures and heart rates were measured twice from the forearm with an automatic oscillometric blood pressure monitor (Omron M6W, Omron Healthcare Co., Ltd., Kyoto, Japan), with the subjects in a sitting position, prior to and at 4 and 12 hours after quinapril and enalapril ingestion.

In Study IV, fasting 2.5-ml whole blood RNA samples (PAXgene® Blood RNA tube; PreAnalytiX GmbH, Hombrechtikon, Switzerland) and 9-ml whole blood DNA samples were drawn prior to study drug ingestion during eight pharmacokinetic studies. The PAXgene® Blood RNA sample tubes were stored at -70°C or -80°C until RNA extraction and the whole blood DNA samples at -20°C or -80°C until DNA extraction. Fasting whole blood DNA samples and liver biopsies were obtained from patients undergoing laparoscopic gastric bypass operation. During the seven pharmacokinetic studies on clopidogrel, a forearm vein of each participant was cannulated for blood sampling on the days of study drug administration. Timed EDTA blood samples (4 ml or 9 ml) were drawn prior to and for up to 4-24 hours after clopidogrel ingestion. The samples were handled as in the Study II, including derivatization, plasma separation, and storing. Timed sodium citrate samples (2.7 ml each, containing 3.2% of sodium citrate) were drawn prior to and for up to 12-24 hours after clopidogrel ingestion in a subpopulation of 46 participants. The samples were handled as in Study II.

4.2.4 RNA preparation and reverse transcription quantitative real-time PCR

In Study IV, RNA was extracted by standard methods using Maxwell® 16 LEV simplyRNA Kit (Promega, Madison, WI, USA), including DNase I treatment, on the Maxwell 16 Research system (whole blood discovery and replication cohorts) or miRNeasy Mini Kit (liver sample cohort; Qiagen). Extracted RNA was stored at -80°C until complementary DNA (cDNA) synthesis.

The RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (whole blood discovery cohort; Thermo Fisher Scientific) or a SuperScript® VILO cDNA Synthesis Kit (whole blood replication and liver sample cohorts; Thermo Fisher Scientific). Before quantitative real-time PCR (qPCR), the whole blood replication and liver sample cohort cDNA samples were preamplified with a custom TaqMan pre amp pool containing assays for the *CES1* and reference genes (14 cycles; Thermo Fisher Scientific).

The qPCR was carried out using OpenArray technology on the QuantStudio™ 12K Flex Real-Time PCR System. The custom OpenArray plate contained *CES1* and reference gene assays (Table 10), allowing the within-sample normalization with multiple reference genes.

Table 10. *Assays used in quantitative real-time PCR.*

Cohort	Gene	Assay ID
Whole blood (discovery)	*CES1*	Hs00275607_m1
	DECR1	Hs00154728_m1
	PPIB	Hs00168719_m1
	FPGS	Hs00191956_m1
	TRAP1	Hs00212474_m1
Whole blood (replication)	*CES1*	Hs00275607_m1
	DECR1	Hs00154728_m1
	PPIB	Hs00168719_m1
	FPGS	Hs00191956_m1
	B2M	Hs00941230_m1
Liver	*CES1*	Hs00275607_m1
	ACTB	Hs01060665_g1
	RPLP0	Hs99999902_m1
	GAPDH	Hs02758991_g1
	B2M	Hs00941230_m1

ACTB, actin beta; B2M, beta-2-microglobulin; CES1, carboxylesterase 1; DECR1, mitochondrial 2,4-dienoyl-coenzyme A reductase 1; FPGS, folylpolyglutamate synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PPIB, peptidylprolyl isomerase B; RPLP0, ribosomal protein lateral stalk subunit P0; TRAP1, tumor necrosis factor receptor associated protein 1.

4.2.5 DNA preparation, sequencing and genotyping

In Study IV, genomic DNA was extracted using the Maxwell 16 LEV blood DNA Kit on the Maxwell Research system (whole blood discovery and replication cohorts) or DNeasy Blood & Tissue Kit (liver sample cohort; Qiagen).

Targeted resequencing of the *CES1* gene ± 20 kb (chr16: 55,816,763-55,887,075; genome build GRCh37) was performed in all whole blood discovery cohort participants (n=212). For library preparation, genomic DNA was processed according to the NEBNext DNA Sample Prep protocol (New England BioLabs, Ipswich, MA, USA). Target enrichment capture was performed using the NimbleGen SeqCap EZ Choice capture

protocol (Roche sequencing, Pleasanton, CA). Sequencing was done on the Illumina HiSeq2000 platform with 100 bp paired-end reads (Illumina, San Diego, CA, USA). Quality control, short read alignment, and variant calling and annotation were carried out using a pipeline developed at the Technology Center at the Institute for Molecular Medicine Finland (FIMM; Helsinki, Finland). The sequencing and bioinformatics pipelines were carried out at the FIMM.

The whole blood and liver samples were genotyped for the *CES1* c.52+579A>G (rs12443580) and c.257+885T>C (rs8192935) SNVs with custom TaqMan® 5´-nuclease assays using OpenArray technology on the QuantStudio™ 12K Flex Real-Time PCR System. Call identity with sequencing data was 99.4% and 100% for the rs12443580 and rs8192935 SNVs. The replication cohort samples were also genotyped for the *CES1* c.428G>A (p.G143E, rs71647871) and *CYP2C19*2* (c.681G>A, rs4244285), **3* (c.636G>A, rs4986893), and **17* (c.-806C>T, rs12248560) SNVs with TaqMan 5´-nuclease assays using OpenArray technology on the QuantStudio™ 12K Flex Real-Time PCR System.

4.3 Determination of drug concentrations

Oseltamivir. Prior to analysis, a simple protein precipitation of plasma samples by acetonitrile was performed, except for the very low oseltamivir plasma concentrations (24, 34, and 48-h samples) that were prepared by a MCX solid phase extraction (Waters, Milford, MA, USA). The plasma oseltamivir and oseltamivir carboxylate concentrations were measured using an API 3000 liquid chromatography-tandem mass spectrometry system (AB Sciex, Toronto, Canada) (Table 11). Deuterated oseltamivir and deuterated oseltamivir carboxylate served as internal standards.

Clopidogrel. The plasma concentrations of clopidogrel, clopidogrel active *cis* 5-thiol metabolite, clopidogrel carboxylic acid, and clopidogrel acyl-β-D-glucuronide were measured with a Nexera X2 liquid chromatography instrument (Shimadzu, Kyoto, Japan) coupled to a QTRAP 5500 tandem mass spectrometer (AB Sciex, Toronto, ON, Canada) (Table 11), according to a previously described method with slight modifications (Tornio *et al.* 2014, Holmberg *et al*. 2014). Plasma sample preparation was automated using a 96-well Phree phospholipid removal plate (Phenomenex, Torrance, CA, USA) conducted on a Freedom EVO automated liquid handling system (Tecan Group, Männedorf, Switzerland). Deuterium labeled forms of clopidogrel, the active metabolite methylphenacyl derivative, and clopidogrel carboxylic acid served as internal standards. No other clopidogrel metabolite interfered with the assay.

Quinapril and enalapril. Plasma samples were prepared for analysis by protein precipitation in acetonitrile containing the internal standards, deuterated quinapril, deuterated enalapril, and deuterated enalaprilat. Deuterated quinapril served as an internal standard for both quinapril and quinaprilat. Urine samples were diluted with the acetonitrile

internal standard solution and centrifuged prior to analysis. The drug concentrations were determined by using an Agilent 1100 series liquid chromatography system (Agilent Technologies, Waldbronn, Germany) coupled to an API 2000 tandem mass spectrometer (AB Sciex, Toronto, Ontario, Canada) (Table 11). In urine, the lower limit of quantification was 10 ng/ml for all analytes and the intra-day coefficients of variation were below 3.5% at relevant concentrations for all analytes (*n*=6).

Table 11. *Summary of the plasma quantification methods.*

Study	Analyte	LC-MS-MS system	Lower limit of quantification (ng/ml)	Interday CV
I	Oseltamivir	AB Sciex API 3000	0.04	<10% (*n*=5)
	Oseltamivir carboxylate	AB Sciex API 3000	1.0	<10% (*n*=5)
II	Clopidogrel	Shimadzu Nexera X2 - AB Sciex 5500 Qtrap	0.05	<6% (*n*=4)
	Active *cis* 5-thiol metabolite	Shimadzu Nexera X2 - AB Sciex 5500 Qtrap	0.05	<10% (*n*=4)
	Clopidogrel carboxylic acid	Shimadzu Nexera X2 - AB Sciex 5500 Qtrap	100	<7% (*n*=4)
	Clopidogrel acyl-β-D-glucuronide	Shimadzu Nexera X2 - AB Sciex 5500 Qtrap	100	<10% (*n*=4)
III	Quinapril	Agilent 1100 - AB Sciex API 2000	0.5	<3% (*n*=8)
	Quinaprilat	Agilent 1100 - AB Sciex API 2000	1.0	<5% (*n*=8)
III	Enalapril	Agilent 1100 - AB Sciex API 2000	0.5	<4% (*n*=7)
	Enalaprilat	Agilent 1100 - AB Sciex API 2000	1.0	<7% (*n*=7)
IV	Clopidogrel	Shimadzu Nexera X2 - AB Sciex 5500 Qtrap	0.01-0.1	<15% (*n*=21)
	Active *cis* 5-thiol metabolite	Shimadzu Nexera X2 - AB Sciex 5500 Qtrap	0.01-0.1	<15% (*n*=21)
	Clopidogrel carboxylic acid	Shimadzu Nexera X2 - AB Sciex 5500 Qtrap	20-100	<15% (*n*=19)

CV, coefficient of variation; LC-MS-MS, liquid chromatography-tandem mass spectrometry.

4.4 Pharmacokinetic analysis

In Study I, the pharmacokinetic variables of oseltamivir and oseltamivir carboxylate were calculated by conventional non-compartmental methods using MK-Model, version 5.0 (Biosoft, Cambridge, UK). The terminal log-linear part of each concentration-time curve was identified visually, and the elimination rate constant (k_e) was determined from logarithmically transformed data with linear regression analysis. The $t_{½}$ was calculated with the equation $t_{½} = \ln 2/k_e$. The $AUC_{0-\infty}$ was calculated by a combination of the linear and log-linear trapezoidal rules (linear up and logarithmic down), with extrapolation to infinity by division of the last measured concentration by k_e.

In Studies II-IV, the C_{max}, t_{max}, $t_{½}$, and $AUC_{0-\infty}$ values were calculated for clopidogrel, the active *cis* 5-thiol metabolite, clopidogrel carboxylic acid, clopidogrel acyl-β-D-glucuronide, quinapril, quinaprilat, enalapril, and enalaprilat with standard non-compartmental methods using log-linear trapezoidal rules with Phoenix WinNonlin, version 6.3 (Certara, St. Louis, MO, USA). The amount excreted into urine from 0 to 12 h (A_e) and renal clearance (Cl_{renal}) of quinapril and enalapril were calculated using Phoenix WinNonlin, version 6.3. All the $AUC_{0-\infty}$, C_{max}, and A_e values were adjusted for a 70 kg body weight.

4.5 Pharmacodynamic analysis

Platelet function testing. In Studies II and IV, antiplatelet activity was tested with a turbidimetric optical detection system (VerifyNow P2Y12 Test, Accumetrics, San Diego, CA) in the sodium citrate anticoagulated whole blood samples. In the test device, ADP-activated platelets aggregate on fibrinogen-coated microparticles in one channel. The resultant change in light transmittance is measured and expressed as $P2Y_{12}$ reaction units (PRU). In a second channel of the test device, platelets are activated with a thrombin receptor-activating peptide and protease-activated receptor 4 activating peptide, and the baseline platelet function is measured. The percentage of drug-induced inhibition of $P2Y_{12}$-mediated platelet aggregation is measured by comparing the aggregation in the two channels (Malinin *et al*. 2007, Lordkipanidze *et al*. 2008, Jeong *et al*. 2012).

Blood pressure and heart rates. In Study III, the pharmacodynamics of quinapril and enalapril were characterized by average systolic and diastolic blood pressures and average heart rates (the mean value of the two measurements was used), calculated by dividing the area under the effect-time curve from 0 to 12 h by 12 h.

4.6 Statistical analysis

Population genetic study (Study I). The SNV frequency is given with 95% confidence intervals (CIs). Deviation from Hardy-Weinberg equilibrium was tested using the Fisher's exact test. We considered *P* values below 0.05 statistically significant.

Pharmacokinetic and pharmacodynamic studies (Studies I-IV). In Studies I-III, the data were analyzed using the statistical program IBM SPSS 19.0 for Windows (Chicago, IL, USA). The $AUC_{0-\infty}$, C_{max}, and A_e values were adjusted for a 70-kg body weight. Except for t_{max}, the pharmacokinetic variables were logarithmically transformed before analysis. Differences in the pharmacokinetic (except t_{max}) and pharmacodynamic variables between the genotypes were investigated using analysis of variance (ANOVA). The t_{max} values were compared using the Mann-Whitney U test. Differences were considered statistically significant when *P* was below 0.05.

In Study IV, the data were analyzed using the statistical programs JMP Genomics 7.0 (SAS Institute Inc., Cary, NC) and IBM SPSS 22.0 for Windows (Armonk, NY). Possible effects of demographic covariates on *CES1* whole blood and liver expression were investigated using a forward stepwise linear regression analysis. For the whole blood discovery cohort, age, sex, and body weight were used as demographic variables and for the liver sample cohort, age, sex, BMI, type 2 diabetes, degree of lobular inflammation and steatosis, and use of lipid-lowering medication were used. The *P* value thresholds of 0.05 and 0.10 were employed for entry into and removal from the model. The effects of *CES1* genetic variants on its whole blood expression in the discovery cohort were then investigated using a forward stepwise linear regression analysis adjusting for demographic covariates as necessary. *P* values were adjusted with the Benjamini-Hochberg false discovery rate (FDR) method. A *P* value threshold of 0.05 was employed for entry of genetic variants into the model. For the whole blood replication and liver sample cohorts, the analysis was carried out using linear regression analysis adjusting for demographic covariates as necessary. The $AUC_{0-\infty}$ and C_{max} values were adjusted for a 70-kg body weight. Except for the t_{max}, the pharmacokinetic variables were logarithmically transformed before analysis. Differences in the pharmacokinetic (except t_{max}) and pharmacodynamic variables were investigated using a forward stepwise linear regression analysis, with demographic covariates, *CES1* and *CYP2C19* genotypes, and clopidogrel dose as independent variables. The t_{max} values were compared using Kruskal-Wallis 1-way ANOVA. Differences were considered statistically significant when the *P* was below 0.05.

5 RESULTS

5.1 Population genetic study (Study I)

Among 860 healthy white Finnish volunteers, the *CES1* c.428G>A variant allele was found with a frequency of 2.2% (95% CI, 1.6-3.0%). This is slightly less than seen in a previous study of 455 white subjects (3.7%) and similar to that in 299 Hispanics (2.0%) (Zhu *et al*. 2008). Only 1 of the 860 subjects showed a homozygous variant genotype (0.05%, 95% CI, 0.02-0.7%), while 4.3% (95% CI, 3.0-5.7%) were heterozygous carriers. We found no deviation from the Hardy-Weinberg equilibrium (P>0.999).

5.2 Effects of the *CES1* c.428G>A variant on drug pharmacokinetics (Studies I-III)

Oseltamivir. The $AUC_{0-\infty}$ of oseltamivir and oseltamivir carboxylate varied 6.8-fold and 1.8-fold between individual subjects (n=22). The mean $AUC_{0-\infty}$ of oseltamivir was 18% larger in subjects with the *CES1* c.428G/A genotype (n=9) than those with the c.428G/G genotype (n=12) (P=0.025) (Figure 8). Subjects with the c.428G/A genotype also had a 15% lower C_{max} of oseltamivir carboxylate than subjects with the c.428G/G genotype (P=0.011). The oseltamivir carboxylate to oseltamivir $AUC_{0-\infty}$ ratio was 23% smaller in subjects with the *CES1* c.428G/A genotype than in those with the c.428G/G genotype (P=0.006). The CES1-catalyzed bioactivation of oseltamivir to oseltamivir carboxylate was reduced to a much greater extent in the one *CES1* c.428A/A homozygote than in the c.428G/A heterozygotes (Figure 8). The $AUC_{0-\infty}$ of oseltamivir was 360% or 290% larger in the one subject with the *CES1* c.428A/A genotype than in those with the c.428G/A or c.428G/G genotype. Consistently, the $AUC_{0-\infty}$ of oseltamivir carboxylate was 20% or 27% smaller in the one subject with the *CES1* c.428A/A genotype than in those with the c.428G/A or c.428G/G genotype. Furthermore, in the one subject with the *CES1* c.428A/A genotype, the oseltamivir carboxylate to oseltamivir $AUC_{0-\infty}$ ratio was 79% or 84% smaller than in those with the c.428G/A or c.428G/G genotype. The $t_{½}$ and t_{max} of oseltamivir and oseltamivir carboxylate were not affected by the *CES1* c.428G>A SNV.

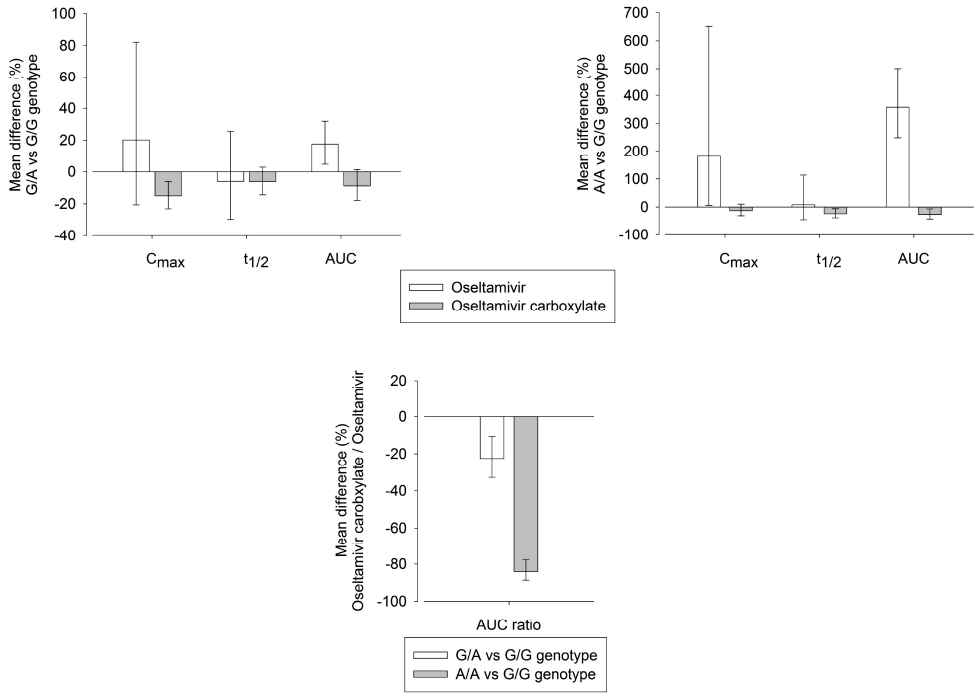

Figure 8. *Effect of the CES1 c.428G>A SNV on the pharmacokinetic variables of oseltamivir. Bars represent geometric mean ratios and whiskers 90% CIs. $AUC_{0-\infty}$, area under the concentration-time curve from 0 hours to infinity; CES1, carboxylesterase 1; CI, confidence interval; C_{max}, peak plasma concentration; $t_{½}$, elimination half-life.*

Clopidogrel. The $AUC_{0-\infty}$ of the parent clopidogrel varied 13-fold between individual subjects (n=22). The geometric mean $AUC_{0-\infty}$ and C_{max} of clopidogrel were 123% and 109% higher in subjects with the *CES1* c.428G/A genotype than in those with the c.428G/G genotype (P=0.004 and P=0.035) (Figure 9). The $AUC_{0-\infty}$ of the active metabolite varied 6.3-fold between individual subjects (n=22). The $AUC_{0-\infty}$ and C_{max} of clopidogrel active *cis* 5-thiol metabolite were 67% and 63% higher in subjects with the *CES1* c.428G/A genotype than in those with the c.428G/G genotype (P=0.009 and P=0.017). The *CES1* c.428G>A SNV had no significant effect on the plasma concentrations of carboxylic acid or glucuronide metabolites. The clopidogrel carboxylic acid to clopidogrel and clopidogrel acyl-β-D-glucuronide to clopidogrel $AUC_{0-\infty}$ ratios were 53% and 58% smaller in subjects with the *CES1* c.428G/A genotype than in those with the c.428G/G genotype (P=0.009 and P=0.007). In contrast, the active metabolite to clopidogrel and clopidogrel acyl-β-D-glucuronide to clopidogrel carboxylic acid $AUC_{0-\infty}$ ratios were not significantly affected by the *CES1* c.428G>A SNV. The $t_{½}$ and t_{max} of clopidogrel or any of its metabolites were not affected by the *CES1* c.428G>A SNV. Exclusion of the

loss-of-function *CYP2C19*2* allele and gain-of-function *CYP2C19*17* allele carriers from the analysis did not change the results regarding the effects of the *CES1* c.428G>A SNV.

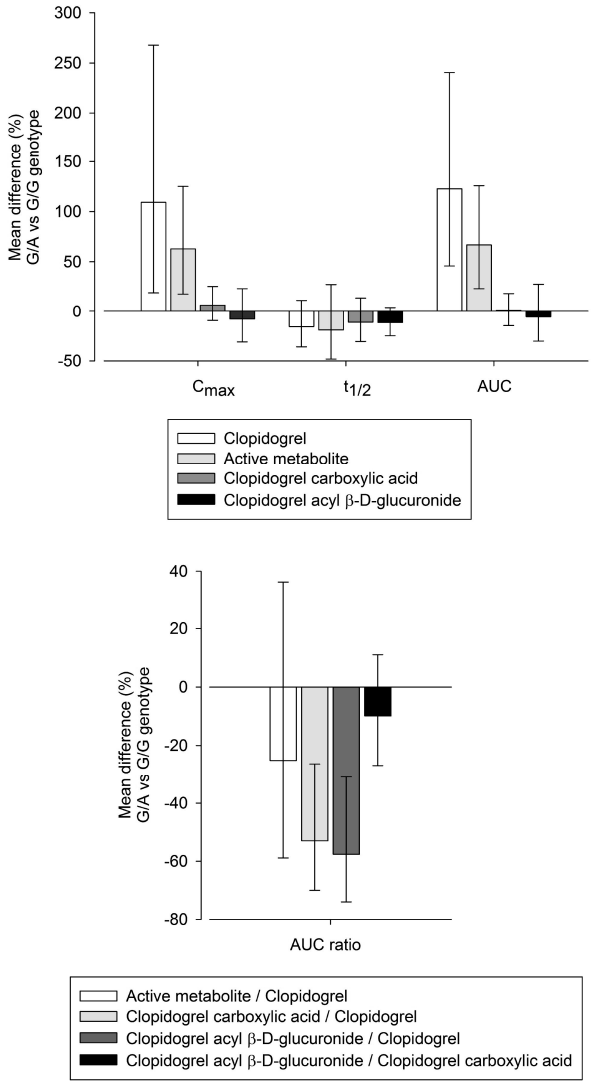

Figure 9. *Effect of the CES1 c.428G>A SNV on the pharmacokinetic variables of clopidogrel. Bars represent geometric mean ratios and whiskers 90% CIs. $AUC_{0-\infty}$, area under the concentration-time curve from 0 hours to infinity; CES1, carboxylesterase 1; CI, confidence interval; C_{max}, peak plasma concentration; $t_{½}$, elimination half-life.*

In line with the pharmacokinetic effects, the *CES1* genotype significantly affected the platelet inhibitory effect of clopidogrel (Figure 10). The average platelet aggregation activity at 0-12 hours after clopidogrel dosing was 61 $P2Y_{12}$ reaction units (PRUs) lower in subjects with the *CES1* c.428G/A genotype than those with the c.428G/G genotype (*P*=0.015). In subjects with the *CES1* c.428G/A genotype, the average percentage inhibition of $P2Y_{12}$-mediated platelet aggregation at 0-12 hours was 19 percentage points higher than in those with the c.428G/G genotype (*P*=0.036). The maximum observed platelet inhibition was 19 percentage points higher and the minimum observed platelet aggregation activity was 59 PRUs lower in subjects with the *CES1* c.428G/A genotype than in those with the c.428G/G genotype (*P*=0.041 and *P*=0.026). Exclusion of the *CYP2C19*2* allele carriers from the analysis did not change the results.

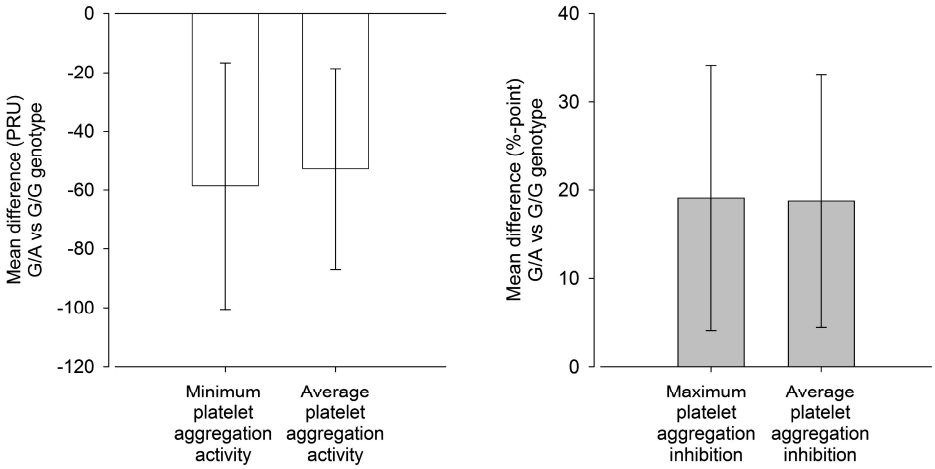

Figure 10. *Effect of the CES1 c.428G>A SNV on the antiplatelet functions of clopidogrel. Bars represent geometric mean ratios and whiskers 90% CIs.CES1, carboxylesterase 1; CI, confidence interval; PRU, $P2Y_{12}$ reaction unit.*

Quinapril and enalapril. The $AUC_{0-\infty}$ of quinapril and that of quinaprilat varied 3.5-fold among all subjects (*n*=22). The *CES1* c.428G>A genotype had no significant effect on the pharmacokinetic or pharmacodynamic variables of quinapril (Figure 11).

The $AUC_{0-\infty}$ of enalapril and that of enalaprilat varied 2.8-fold and 2.7-fold among all subjects (*n*=22). The $AUC_{0-\infty}$ of enalaprilat was 20% lower in subjects with the *CES1* c.428G/A genotype than in those with the c.428G/G genotype (*P*=0.049) (Figure 11). Accordingly, enalaprilat A_e was 35% smaller in subjects with the *CES1* c.428G/A genotype than those with the c.428G/G genotype (*P*=0.044). Otherwise, the *CES1* genotype had no significant effect on any other pharmacokinetic or pharmacodynamic variable of enalapril.

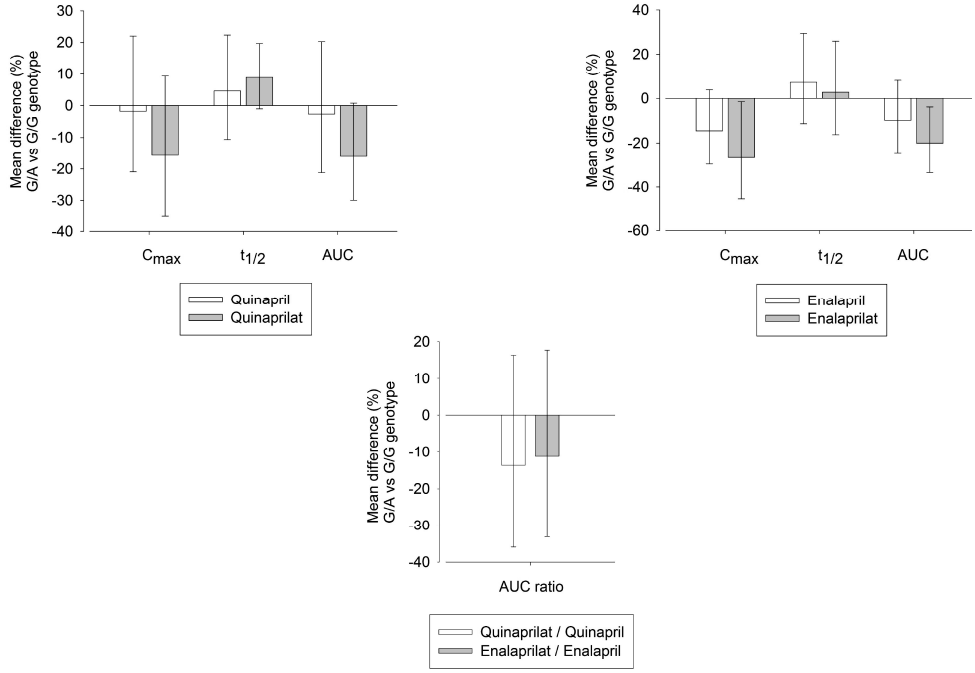

Figure 11. *Effect of the CES1 c.428G>A SNV on the pharmacokinetic variables of quinapril and enalapril. Bars represent geometric mean ratios and whiskers 90% CIs. $AUC_{0-\infty}$, area under the concentration-time curve from 0 hours to infinity; CES1, carboxylesterase 1; CI, confidence interval; C_{max}, peak plasma concentration; $t_{½}$, elimination half-life.*

5.3 Effects of *CES1* genetic variants on its expression, and clopidogrel pharmacokinetics and pharmacodynamics (Study IV)

Massively parallel sequencing. A total of 279 sequence variants with MAFs of at least 1% were discovered within 20 kb upstream and downstream of the *CES1* gene among the 192 participants. Seven of the variants were located in the coding sequence, 100 in the upstream region, 128 in the introns, and 44 downstream.

***CES1* expression.** *CES1* gene expression in whole blood was on average 11% relative to the mean expression of the endogenous control genes and varied 81-fold among the study participants (n=192). Of the 279 sequence variants, a total of 140 variants were associated with *CES1* expression with FDR-corrected P values below 0.05. In a forward stepwise linear regression analysis, demographic covariates (age, sex, and body weight) were not associated with *CES1* expression. Two SNVs, however, showed independent associations

with *CES1* expression (Figure 12). The SNV with the strongest observed effect, rs12443580 (c.52+579T>C, MAF of 35%) located in intron 1, was associated with a 39% reduction in *CES1* expression by each copy of the minor allele (P=4.0 x 10^{-13}). The rs8192935 (c.257+885G>A; MAF of 31%) SNV, located in intron 2, was associated with an additional 31% reduction in *CES1* expression per copy of the minor allele (P=2.5 x 10^{-8}). In a replication cohort of 88 whole blood samples, rs12443580 was associated with a 28% reduction and rs8192935 a 25% reduction of *CES1* expression per copy of the minor allele (P=0.0015 and 0.0091).

CES1 expression in the liver was on average 209% relative to the mean expression of endogenous control genes, and varied 9-fold between the samples (n=177). In a forward stepwise linear regression analysis, demographic covariates (age, sex, BMI, type 2 diabetes, degree of lobular inflammation or steatosis, and the use of lipid-lowering drugs) were not associated with *CES1* expression. Moreover, the *CES1* rs12443580 and rs8192935 SNVs showed no significant association with *CES1* expression in the liver samples.

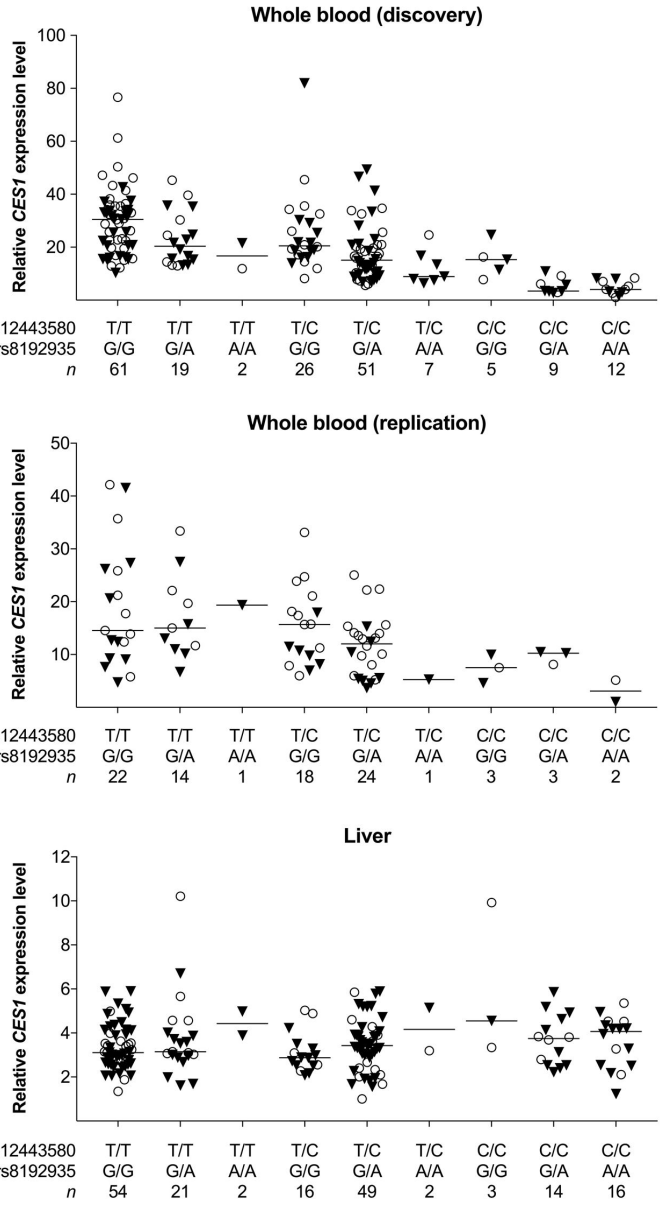

Figure 12. *Scatter plots of the effects of rs12443580 and rs8192935 SNVs on CES1 expression in whole blood and the liver. The lowest expression level within the discovery samples (A), replication samples (B), and liver samples (C) was set as 1. The horizontal lines represent the median. Individual data points are given as circles for men and as triangles for women. CES1, carboxylesterase 1; SNV, single nucleotide variation.*

Pharmacokinetics and pharmacodynamics of clopidogrel. In a forward stepwise linear regression analysis, the clopidogrel carboxylic acid to clopidogrel $AUC_{0-\infty}$ ratio was 58% lower per copy of the *CES1* c.428G>A variant allele (90% CI, 38-72%; *P*=0.0004), 74% higher following a 600 mg dose (*n*=57) than 300 mg dose (*n*=49) of clopidogrel (90% CI, 35-124%; *P*=0.0005), and 1.9% lower per 1 kg body weight (90% CI, 0.9-2.8%; *P*=0.001) (Figure 13). The $AUC_{0-\infty}$ of clopidogrel was 163% (90% CI, 76-293%; *P*=0.0001) and that of the active *cis* 5-thiol metabolite 60% (90% CI, 22-110%; *P*=0.005) higher per copy of the *CES1* c.428G>A variant allele. The *CES1* rs12443580 and rs8192935 or *CYP2C19* SNVs were not significantly associated with any pharmacokinetic variable, but the $AUC_{0-\infty}$ of the active *cis* 5-thiol metabolite was 13% lower per copy of the *CYP2C19* c.681G>A variant allele (**2*, rs4244285; *P*=0.058).

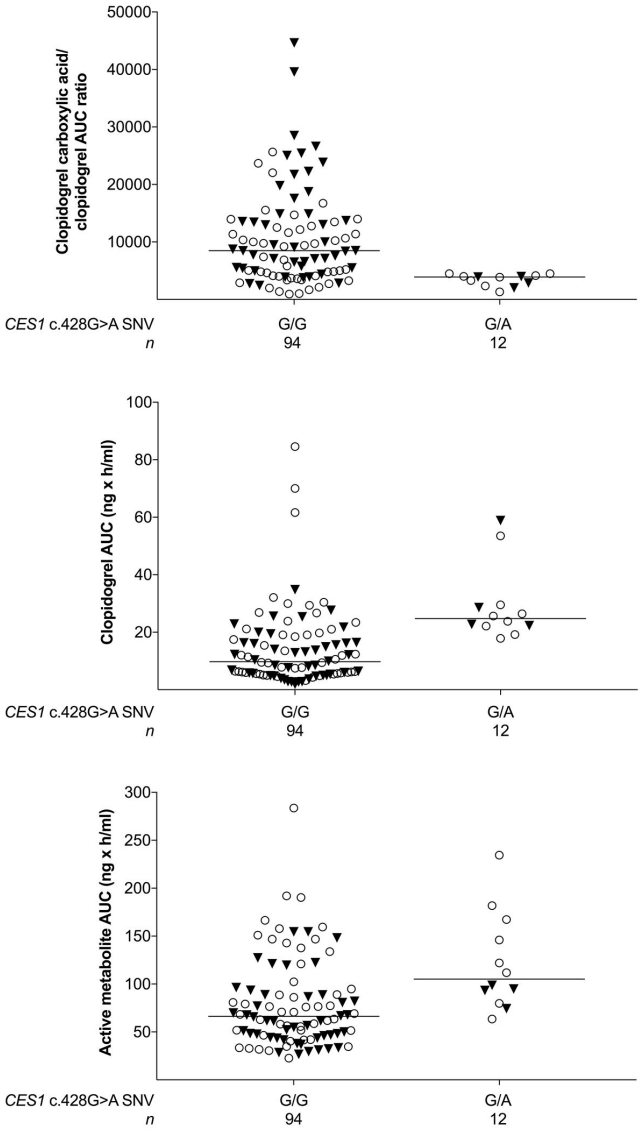

Figure 13. *Scatter plots of the effects of CES1 c.428G>A SNV (rs71647871) on dose-adjusted clopidogrel carboxylic acid to clopidogrel $AUC_{0-\infty}$ ratio (A), dose- and weight-adjusted clopidogrel $AUC_{0-\infty}$ (B), and dose- and weight-adjusted active metabolite $AUC_{0-\infty}$ (C). The horizontal lines represent the median. Individual data points are given as circles for men and as triangles for women. $AUC_{0-\infty}$, the area under the plasma concentration-time curve from 0 hours to infinity; CES1, carboxylesterase 1; SNV, single nucleotide variation.*

The average and minimum platelet aggregation activities at 0-12 hours after clopidogrel dosing were 68 PRUs and 64 PRUs lower per copy of the *CES1* c.428G>A variant allele (90% CIs, 39-97 PRUs and 35-93 PRUs, *P*=0.0003 and *P*=0.0006). Both the average and maximum percentage inhibition of $P2Y_{12}$-mediated platelet aggregation at 0-12 hours after clopidogrel dosing were 21 percentage points higher per copy of the *CES1* c.428G>A variant allele (90% CIs, 11-31% and 11-32%; *P*=0.009 and *P*=0.001; Figure 14). In comparison, the average and maximum percentage inhibition of $P2Y_{12}$-mediated platelet aggregation at 0-12 hours were 12 and 13 percentage points lower per copy of the *CYP2C19* c.681G>A variant allele (90% CIs, 5-20% and 5-21%; *P*=0.01 and *P*=0.01). The *CES1* rs12443580 and rs8192935 SNVs had no significant effect on the pharmacodynamics of clopidogrel.

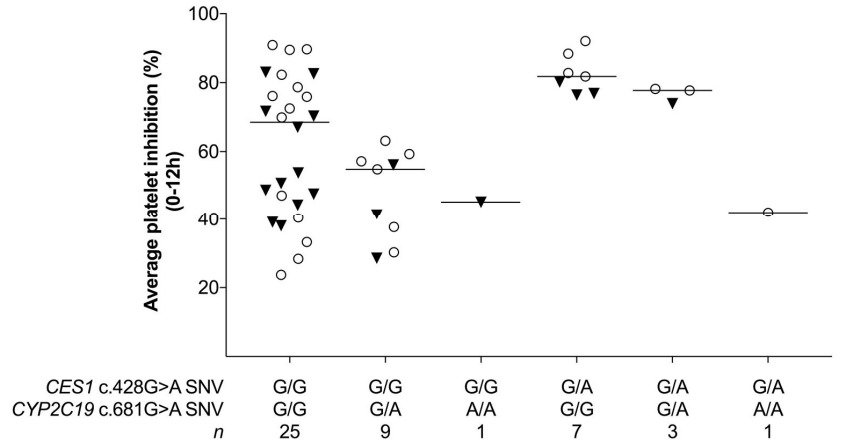

Figure 14. *Scatter plots of the effects of CES1 c.428G>A (rs71647871) and CYP2C19 c.681G>A (*2, rs4244285) SNVs on the average platelet inhibition of a 600 mg oral dose of clopidogrel. The horizontal lines represent the median. Individual data points are given as circles for men and as triangles for women. CES1, carboxylesterase 1; CYP, cytochrome P450; SNV, single nucleotide variation.*

6 DISCUSSION

6.1 Methodological considerations

Population genetic study (Study I). In the population genetic study, 860 DNA samples were genotyped from healthy white Finnish volunteers. The investigated SNV was selected based on a previous *in vivo* study, in which the *CES1* c.428G>A SNV markedly reduced the hydrolysis of methylphenidate (Zhu *et al*. 2008). The large number of participants reduced random sampling error affecting the results of allele frequency. The investigated allele frequency was in Hardy-Weinberg equilibrium. The genotype data was analyzed in a coded form. The information on the genotypes and the volunteers was kept in secured files with limited access. There were no reported adverse effects and no study withdrawals

Pharmacokinetic and pharmacodynamic studies. The prospective genotype panel studies (Studies I-III) were carried out with 40 healthy volunteers with different *CES1* c.428G>A SNV genotypes. The *CES1* c.428G>A SNV and especially the homozygous A/A genotype are rare in Caucasians; about 0.04-0.2% are homozygous for the *CES1* c.428G>A variant allele. Therefore, the number of possible heterozygous and homozygous individuals to be considered for participation in the Studies I-III was small. Study I included only one homozygous individual and 9 heterozygous individuals, and Studies II and III had no homozygous individuals and only 10 heterozygous. In Study II, the subjects were also genotyped for the *CYP2C19*2*, **3*, and **17* alleles, because it was not possible to target subjects who had the *CES1* c.428G/A SNV and were without altered function *CYP2C19* alleles. The two *CES1* genotype groups, however, were balanced with respect to the reduced function *CYP2C19* alleles. In these prospective genotype panel studies, the subjects were not genotyped for any other variants, and therefore it is not possible to exclude the affect other *CES1* variants or variants in some other genes might have on the pharmacokinetics and pharmacodynamics of the study drugs. Study IV included samples obtained from seven pharmacokinetic studies on clopidogrel carried out at the Department of Clinical Pharmacology (22 samples from Study II and 84 samples from the other studies). Therefore, in Study IV, the *CES1* c.428G>A variant allele was overrepresented, as the study included participants from a genotype panel study focusing on this variant (Study II). This overrepresentation could have overshadowed the effects of *CYP2C19* variants.

In the prospective genotype panel studies (Studies I-III), no significant differences in age, height, weight, or BMI were observed between the groups. In Study IV, the demographic variables (age, sex, and BMI) were different between the two whole blood and liver sample cohorts. The *CES1* whole blood or liver expression was, however, not associated with these variables. This diverges from previous studies with smaller sample sizes, which suggested possible effects of age and sex on *CES1* liver expression (Hosokawa *et al.* 1995, Zhu *et al*. 2009a, Xu *et al.* 2016). Age, sex, and BMI were associated with *CES1* expression in adipose tissue in elderly twins (Friedrichsen *et al*. 2013). When

appropriate, the pharmacokinetic variables were adjusted for body weight. To further reduce variation, restrictions were imposed on the use of other drugs, alcohol, grapefruit juice, and supplements before and during the pharmacokinetic studies. In addition, the study drugs were administered after an overnight fast and food intake during the study days was standardized and controlled. In Study IV, 11 individuals with a non-Caucasian background or excess relatedness (siblings) were excluded from the analysis.

In clinical practice, oseltamivir is usually administered for a course of 5 days, and clopidogrel, quinapril, and enalapril on a regular basis. The steady-state plasma concentrations of oseltamivir, clopidogrel, quinapril, and enalapril have been predictable from their pharmacokinetic variables obtained after single doses. The relative differences in the steady-state AUCs between individuals with different *CES1* c.428G>A SNV genotypes should be similar to the AUC differences observed after a single dose. Therefore, in the prospective genotype panel studies, a single dose was chosen for safety and practical reasons. The doses of study drugs corresponded to their normal therapeutic doses. In Study IV, the clopidogrel dose varied from 300 mg to 600 mg between the seven different pharmacokinetic studies. The clopidogrel dose was therefore treated as an independent variable in the linear regression analysis. Six healthy volunteers participated in more than one of the pharmacokinetic studies on clopidogrel (Study IV), and only samples from one of these studies were included in the pharmacokinetic and statistical analyses.

In the genotype panel studies, the sampling schedule was selected to cover the plasma concentration-time curve sufficiently in order to provide a reliable estimate of the extent of exposure. Timed EDTA blood samples were drawn for up to 12-48 hours after study drug ingestion, due to varying elimination kinetics of the drugs. Based on the study drugs' elimination half-lives, a washout period of at least one week in Studies II and III was sufficient to minimize possible carry-over effects . Urine was collected only during the time spent at the Clinical Research Unit to ensure compliance.

In Study IV, the sampling schedule varied from 4 hours to 24 hours between the seven different pharmacokinetic studies. Only timed EDTA blood samples drawn for up to 4 hours and 12 hours were used in the pharmacokinetic analysis. The sodium citrate anticoagulated whole blood samples were obtained from only 46 participants and these blood samples were drawn for up to 12-24 hours after clopidogrel ingestion. All of these participants, however, had ingested a 600 mg dose of clopidogrel and only samples drawn for up to 12 hours were used in the pharmacodynamic analysis.

In the pharmacokinetic studies, there were no reported adverse effect and no study withdrawals. The liver biopsies were obtained during laparoscopic gastric bypass surgery minimizing procedure-related complications (such as risk of bleeding and injury to a nearby tissue) in comparison to percutaneously obtained liver biopsies.

6.2 Population genetics (Study I)

Interethnic differences are common in the frequencies of genetic variants affecting drug pharmacokinetics (Li *et al*. 2011). The *CES1* c.428G>A variant allele was less common in the Finnish population (2.2%) than in European-Americans (4%) (Zhu *et al*. 2008). This difference may be explained by a founder effect of the Finnish population or population admixture in the European-American population.

6.3 Effects of the *CES1* c.428G>A SNV on drug pharmacokinetics and pharmacodynamics (Studies I-III)

Oseltamivir. This was the first study showing that the *CES1* c.428G>A SNV affects the pharmacokinetics of oseltamivir, indicative of the important role that CES1 plays in the bioactivation of oseltamivir *in vivo*. Subjects with the *CES1* c.428G/A genotype had a significantly smaller oseltamivir carboxylate to oseltamivir $AUC_{0-\infty}$ ratio, a larger $AUC_{0-\infty}$ of oseltamivir, and lower C_{max} of oseltamivir carboxylate than subjects with the c.428G/G genotype. Notably, in the one subject with the *CES1* c.428A/A genotype, the hydrolysis of oseltamivir to oseltamivir carboxylate was even more impaired. In that subject, the oseltamivir carboxylate to oseltamivir $AUC_{0-\infty}$ ratio was about 80% to 85% smaller, the $AUC_{0-\infty}$ of oseltamivir about 300% to 400% larger, and the $AUC_{0-\infty}$ of oseltamivir carboxylate 20% to 25% smaller than in the subjects with the c.428G/A or c.428G/G genotype.

The results are in good agreement with a previous *in vitro* study, in which the c.428G>A variant allele markedly decreased the enzymatic activity of CES1, and with a recent *in vitro* study, in which the c.428G>A variant reduced the bioactivation of oseltamivir by about 60% (Zhu & Markowitz 2013, Shi *et al.* 2016a).

Clopidogrel. The *CES1* c.428G>A SNV affected the pharmacokinetics and pharmacodynamics of clopidogrel, demonstrating that CES1 plays an important role in the formation of the inactive clopidogrel carboxylic acid metabolite *in vivo*. Clopidogrel carboxylic acid to clopidogrel $AUC_{0-\infty}$ ratio was about 50% less in subjects with the *CES1* c.428G/A genotype than in subjects with the c.428G/G genotype, indicating reduced hydrolysis of parent clopidogrel. Consequently, the $AUC_{0-\infty}$ of clopidogrel was about 120% larger and the $AUC_{0-\infty}$ of active metabolite was about 70% larger in subjects with the *CES1* c.428G/A genotype than in subjects with the c.428G/G genotype. The AUCs of the clopidogrel carboxylic acid and acyl-β-D-glucuronide metabolites remained unchanged. Of note, the plasma exposures to the carboxylic acid and glucuronide metabolites are more than 1000-fold compared to the exposures to the parent clopidogrel and its active metabolite. Therefore, even a small shift from the hydrolytic pathway towards the oxidative activating pathway should result in a significant increase in exposures to the parent clopidogrel and the active metabolite, but only a small increase with carboxylic acid and glucuronide metabolites exposure.

Consistent with the pharmacokinetic findings, the *CES1* c.428G>A variant allele markedly enhanced the platelet inhibitory effect of clopidogrel. The average platelet aggregation activity was 61 PRUs lower and the minimum platelet aggregation activity 59 PRUs lower in subjects with the *CES1* c.428G/A genotype than those with the c.428G/G genotype. In addition, both the average and maximum observed percentage platelet aggregation inhibitions were 19 percentage points higher in subjects with the *CES1* c.428G/A genotype.

In comparison, healthy volunteers carrying one or two well-established loss-of-function *CYP2C19* c.681G>A (**2*, rs4244285) or c.636G>A (**3*, rs4986893) alleles have been shown to have about 30-45% lower systemic exposure to the active metabolite and about 10-20 percentage point reduction in the antiplatelet effect of clopidogrel in *ex vivo* light transmission aggregometry (Brandt *et al*. 2007, Mega *et al.* 2009). Study II did not include any *CYP2C19* c.636G>A variant allele carriers and this exclusion did not change the results. Furthermore, *CYP2C19* c.681G>A allele carriers tended to have only a slightly reduced exposure to the active metabolite. The overrepresentation of the *CES1* variant allele carriers, however, may have overshadowed the effects of the *CYP2C19* c.681G>A SNV.

The results are in good agreement with previous *in vitro* studies and a recent physiology-directed population pharmacokinetic/pharmacodynamic model indicating a major role for CES1 in the biotransformation of clopidogrel (Tang *et al.* 2006, Zhu *et al*. 2013, Jiang *et al*. 2016). They also provide a mechanistic explanation for the previously observed association between the *CES1* c.428G>A SNV and clopidogrel active metabolite plasma concentrations (Lewis *et al*. 2013). In addition, the effect of the c.428G>A SNV on clopidogrel pharmacokinetics and pharmacodynamics seems to be similar in size to that of well-established loss-of-function *CYP2C19* alleles (Kim *et al*. 2008, Mega *et al.* 2009, Shuldiner *et al*. 2009, Scott *et al*. 2013), but with an opposite direction.

Clopidogrel could serve as a probe substrate for CES1 in drug interaction and pharmacogenetic studies in humans despite its weaknesses (*e.g.*, metabolized by several CYPs) since its hydrolysis is highly sensitive to altered CES1 activity.

Quinapril and enalapril. The *CES1* c.428G>A SNV had no observable effect on the pharmacokinetics or pharmacodynamics of quinapril. The $AUC_{0\text{-}\infty}$ of active enalaprilat was 20% smaller in subjects with the *CES1* c.428G/A genotype than in subjects with the c.428G/G genotype. Consequently, the A_e of enalaprilat was 35% smaller in subjects with the *CES1* c.428G/A genotype than those with the c.428G/G genotype. Otherwise, the *CES1* polymorphism had no significant effect on the pharmacokinetics or pharmacodynamics of enalapril.

The lack of effect on quinapril biotransformation and the smaller than expected effect on enalapril biotransformation can be partially explained by overlapping substrate specificities of carboxylesterases. Quinapril is also hydrolyzed by CES2 (Takai *et al*. 1997), and therefore, the significance of CES1 in the bioactivation of quinapril is likely to be smaller than in that of enalapril. The higher fraction of oral enalapril (60%) bioactivated compared to that of quinapril (40%) can also contribute to the different effect size of the c.428G>A SNV on their pharmacokinetics.

These results are in good agreement with previous *in vitro* and *in vivo* studies on the effects of *CES1* c.428G>A variant allele on the pharmacokinetics of other widely used CES1 substrate drugs (Patrick *et al*. 2007, Wang *et al*. 2016). In these studies, the effect size has been of the same magnitude or larger than seen on enalapril.

Since the *CES1* c.428G>A SNV had no observable effect on the pharmacokinetics of quinapril and only a modest effect on the pharmacokinetics of enalapril, they are likely not sensitive probe drugs for CES1 activity in humans. This suggests that ACE inhibitors differ in their liability to the effects of the *CES1* c.428G>A SNV. Consistently, in a recent *in vitro* study, CES1 hydrolyzed ramipril about 10 times more efficiently than trandolapril or enalapril (Thomsen *et al*. 2014).

6.4 Effects of *CES1* genetic variants on its expression, and clopidogrel pharmacokinetics and pharmacodynamics (Study IV)

Sequence variations in close proximity to or within the *CES1* gene affect its expression. Using massively parallel sequencing, two intronic *CES1* c.52+579A>G (rs12443580; MAF of 35%) and c.257+885T>C (rs8192935; MAF of 31%) SNVs were discovered to be strongly and independently associated with *CES1* whole blood expression in two independent whole blood sample cohorts. The mechanisms underlying these associations may be that the SNVs directly affect *CES1* transcription or that they are in LD with other variants affecting *CES1* expression. No upstream variants in LD with the rs8192935 SNV (r^2>0.30) and none of the upstream variants in LD with the rs12443580 SNV (r^2>0.30), however, were located at previously identified transcription factor binding sites (Hosokawa *et al*. 2008, Maruichi *et al.* 2010, Xu *et al*. 2016).

The lack of effect of rs12443580 and rs8192935 variants on *CES1* liver expression, despite strong effects on whole blood expression, suggests tissue-specific transcriptional regulation of *CES1*. It should be kept in mind that our whole blood samples were from healthy volunteers and the liver samples were from patients with variable degrees of hepatic steatosis and inflammation. Neither these characteristics, nor age, sex, BMI, type 2 diabetes, or use of lipid-lowering medication, however, affected *CES1* liver expression. It was not possible to investigate whether *CES1* liver expression levels correlated with *CES1* whole blood expression, since the study did not include whole blood RNA samples from the liver sample cohort patients.

The two SNVs affecting *CES1* whole blood expression had no effect on the pharmacokinetics or pharmacodynamics of clopidogrel, consistent with the lack of effect on *CES1* liver expression and the hypothesis that the CES1-mediated hydrolysis of clopidogrel mainly takes place in the liver. In contrast, the *CES1* c.428G>A missense SNV significantly impaired the hydrolysis of clopidogrel, increasing the exposure to clopidogrel active metabolite and enhancing its platelet inhibitory effects. The *CYP2C19* c.681G>A loss-of-function allele was also significantly associated with reduced clopidogrel antiplatelet effects, but it had no observable effect on exposure to the active metabolite.

Notably, the effect of the *CES1* c.428G>A SNV on clopidogrel antiplatelet effects is opposite to, and stronger than that caused by the *CYP2C19* c.681G>A variant allele.

6.5 Ethical considerations

All participants received both oral and written information and gave written informed consent before entering the studies. The study protocols were approved by the Coordinating Ethics Committee of the Helsinki and Uusimaa Hospital District (Studies I-IV) and by the Ethics Committee of the Northern Savo Hospital District (liver sample cohort; Study IV). The pharmacokinetic studies were also approved by the Finnish Medicines Agency Fimea (Studies I-IV).

6.6 Clinical implications

The identification and characterization of the *CES1* genetic variations affecting its expression and activity may improve drug efficacy and reduce the risk of adverse effects. The impaired bioactivation of oseltamivir in the *CES1* c.428G>A variant allele carriers could be important to the antiviral efficacy and toxicity of oseltamivir. It is possible that the lower concentrations of oseltamivir carboxylate in c.428G>A variant allele carriers translate into an increased risk of drug resistance. Moreover, oseltamivir treatment is associated with neuropsychiatric reactions and even suicides in adolescents (Izumi *et al.* 2007). The parent oseltamivir has a relatively high lipophilicity and passive permeability through cell membranes. It is possible that the increased plasma concentrations of oseltamivir in *CES1* c.428G>A allele carriers, and especially in homozygotes, could result in an increased penetration of oseltamivir into the brain and increased risk of CNS toxicity.

The *CES1* c.428G>A SNV markedly increased the clopidogrel active metabolite plasma concentrations and antiplatelet effects by reducing the hydrolysis of parent clopidogrel to the inactive carboxylic acid metabolite. Therefore, the *CES1* c.428G>A allele might increase clopidogrel efficacy and bleeding risk. Based on the more than 2-fold increased exposure to the parent clopidogrel in *CES1* c.428G>A carriers, it can be estimated that their average clopidogrel dose requirement could be about 50% smaller than in noncarriers.

Of note, the pharmacokinetic studies on clopidogrel (Studies II and IV) were performed in healthy young volunteers and not in the target population with atherothrombotic diseases. Patients using clopidogrel are also likely to use other drugs and the effects of the *CES1* c.428G>A SNV on clopidogrel could be modulated by CES1-mediated drug-drug interactions. Several drugs used by patients on clopidogrel are known substrates or inhibitors of CES1, including simvastatin, diltiazem, and carvedilol (Fukami *et al.* 2010, Yanjiao *et al*. 2013, Thomsen *et al*. 2014). Use of ACE inhibitors was associated with an increased risk of clinically significant bleeding in patients co-treated with ACE inhibitors and clopidogrel, suggesting a possible CES1-mediated drug-drug interaction (Kristensen *et al*. 2014), but this finding was not replicated in another study (Cressman *et al*. 2015). It is

reasonable to assume that the *CES1* c.428G>A SNV can also increase the antiplatelet effects of clopidogrel in patients.

The *CES1* c.428G/A SNV also decreases active enalaprilat plasma concentrations, but has no observable effect on quinapril. Furthermore, it is possible that the hydrolysis of enalapril is reduced to an even greater extent in *CES1* c.428A/A homozygotes, potentially leading to a reduced antihypertensive efficacy.

7 CONCLUSIONS

The following conclusions can be made based on the studies in this thesis:

1. The *CES1* c.428G>A SNV occurs at a minor allele frequency of 2.2% in the Finnish population.

2. The *CES1* c.428G>A SNV affects the pharmacokinetics of oseltamivir by impairing the bioactivation of oseltamivir to its active oseltamivir carboxylate metabolite. The *CES1* c.428G>A SNV significantly affects the pharmacokinetics and pharmacodynamics of clopidogrel by shifting its metabolism from the hydrolytic pathway to the oxidative activating pathway. Therefore, the *CES1* c.428G>A SNV may increase the efficacy and risk of adverse effects of clopidogrel. The *CES1* c.428G>A SNV significantly reduces the hydrolysis of enalapril to active enalaprilat, but has no observable effect on quinapril hydrolysis. This suggests that ACE inhibitors differ in their liability to the effects of the *CES1* c.428G>A SNV.

3. Clopidogrel pharmacokinetics are highly sensitive to *CES1* c.428G>A variant, indicating that it can potentially be used as a CES1 probe substrate in humans.

4. The *CES1* rs12443580 and rs8192935 variants have a major effect on *CES1* expression in whole blood, but not in the liver, suggesting tissue-specific effects of these SNVs. This could lead to substrate-dependent effects of these SNVs on drug biotransformation.

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