2016

Effect of Disease State on Human Carboxylesterase 1 Expression and Activity

Abdullah Aljutayli
University of Rhode Island, aaljutayli@my.uri.edu

Follow this and additional works at: <https://digitalcommons.uri.edu/theses>

Recommended Citation

Aljutayli, Abdullah, "Effect of Disease State on Human Carboxylesterase 1 Expression and Activity" (2016).
Open Access Master's Theses. Paper 892.
<https://digitalcommons.uri.edu/theses/892>

This Thesis is brought to you for free and open access by DigitalCommons@URI. It has been accepted for inclusion in Open Access Master's Theses by an authorized administrator of DigitalCommons@URI. For more information, please contact digitalcommons-group@uri.edu.

EFFECT OF DISEASE STATE ON HUMAN
CARBOXYLESTERASE 1 EXPRESSION AND ACTIVITY

BY
ABDULLAH ALJUTAYLI

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN
PHARMACEUTICAL SCIENCES

UNIVERSITY OF RHODE ISLAND

2016

MASTER OF SCIENCE IN PHARMACEUTICAL SCIENCES
OF
ABDULLAH ALJUTAYLI

APPROVED:

Thesis Committee:

Major Professor   Fatemeh Akhlaghi

Sara Rosenbaum

Natallia Katenka

Nasser H. Zawia
DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND
2016

ABSTRACT

Background: Human carboxylesterase 1, CES1, is a phase I drug metabolizing enzyme that catalyzes the hydrolysis of about 20% of therapeutic agents. Factors, such as metabolic syndrome, ethnicity, gender and genetic polymorphism can influence the activity and/or the expression of drug metabolizing enzymes. Consequently, these factors can affect the pharmacokinetics of drugs leading to toxicity or therapeutic failure.

Objective: The objective of our study is to investigate the possible influence of ethnicity, gender, age, genetic polymorphism, metabolic syndrome or fatty liver on CES1 gene expression and activity.

Methods: Expression of CES1 hepatic mRNA was measured in 89 human livers using RT-PCR techniques. Enzymatic activity of CES1 was measured in S9 fractions and cytosols obtained from 40 and 96 human liver donors, respectively. The hydrolysis rates of two CES1 specific substrates clopidogrel and oseltamivir to their corresponding carboxylic acids were measured using LC-MS/MS methods. Statistical analyses were performed to compare between different groups.

Results and Conclusions: Our data showed that diabetic subjects had less CES1 hydrolytic efficiency although our data is limited by the small sample size. In addition, African American group had significantly higher CES1 activity as compared to Caucasian group. This result provides potential explanation to the clinical observation that African Americans subjects exhibit higher resistance to the CES1 substrates clopidogrel.

ACKNOWLEDGMENT

I would like to express my sincere gratitude to my advisor, Dr. Fatemeh Akhlaghi, for her support, encouragement, and guidance. The cordial atmosphere along with her kindness and patience made it easy for me to accomplish so much within a short period of time. I have been exceptionally lucky and honored to have the opportunity to work with an outstanding and great supervisor. Thanks for your time and efforts invested on me to build my scientific career. I will always be thankful.

I would also like to thank Dr. Sara Rosenbaum for her support and kindness. Sara's book and outstanding teaching skills made pharmacokinetics interesting and easy to grasp. I am grateful to also have her as part of my committee. Despite her busy schedule, Dr. Natallia Katenka, agreed to serve on my committee. I appreciate her guidance and support in statistics. Additionally, I am grateful to Dr. Koray Özpolat for accepting to be the chair of my defense committee and for taking time off his busy schedule to go through my work.

I did not know stopping by Dr. Nasser Zawia, the Dean of graduate school, to say hi at a conference would be the beginning of my entry into URI to advance my career in science. I am grateful to Dr. Zawia for his advice and help throughout my academic work in URI. I would like to thank all my fellow lab mates for their help especially Dr.

Mwlod Ghareeb who provided mentorship when I first joined the lab, and Enoch Cobbina for being an exceptional friend.

Finally, I am grateful to my mother; for her love, concern, support, and advice.

TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGMENT.....	iii
TABLE OF CONTENTS.....	v
LIST OF TABLES.....	vi
LIST OF FIGURES.....	vii
CHAPTER 1 INTRODUCTION.....	1
CHAPTER 2 REVIEW OF LITERATURE.....	3
CHAPTER 3 METHODOLOGY.....	16
CHAPTER 4 FINDINGS	22
CHAPTER 5 CONCLUSION	32
APPENDIX	35
BIBLIOGRAPHY	36

LIST OF TABLES

TABLE	PAGE
Table 1. Number of important CES1 substrates among commonly used pharmacological agents.....	2
Table 2. Differences in substrate preferences for CES1 and CES2.....	6
Table 3. Dilution factors used in the experiments of determination CES1 enzymatic activity	20
Table 4. Differences in CES1 expression levels between groups of subjects donating livers.	22
Table 5. Differences in intrinsic clearances between groups calculated based on full enzyme kinetics obtained from oseltamivir incubations with S9 fraction	22
Table 6. Individual Km, Vmax, and CLint values.....	30

LIST OF FIGURES

FIGURE	PAGE
Figure 1. Ester hydrolysis and transesterification.....	4
Figure 2. CES1 and CES2 RNA tissue expression.....	5
Figure 3. Stereo selectivity for CES1 and CES2.....	6
Figure 4. Metabolic pathways of clopidogrel.....	15
Figure 5. Chromatogram obtained from oseltamivir LC-MS/MS assay.....	18
Figure 6. Chromatogram obtained from clopidogrel LC-MS/MS assay.....	19
Figure 7. Scatter plot showing incubation time vs. metabolite formation in S9 fraction.....	24
Figure 8. Scatter plot showing incubation time vs. metabolite formation in cytosol.....	24
Figure 9. Scatter plot showing protein concentration vs. oseltamivir carboxylic acid formation rate in S9 fractions.....	25
Figure 10. Scatter plot showing protein concentration vs. oseltamivir carboxylic acid formation rate in cytosol.....	25
Figure 11. Clopidogrel incubations in pooled S9 fractions obtained from 20 African American subjects and 20 Caucasian subjects showed inhibition effect.....	26
Figure 12. Pooled Caucasians S9 fractions had slower reaction time as compared with AA	27

Figure 13. Among African Americans group, 5 subjects showed relatively higher activity.....	27
Figure 14. Boxplot showing oseltamivir carboxylic acid rates of formation of 88 Caucasian and 8 African American subjects incubation in cytosol at 10 uM.....	28
Figure 15. Dot plot showing oseltamivir carboxylic acid rates of formation of 88 Caucasian and 8 African American subjects	28
Figure 16. (A) Averaged and (B) individual oseltamivir enzyme kinetics.....	29
Figure 17. (A) Correlation between CES1 activities measured by oseltamivir and clopidogrel in African American, (B) Correlation between CES1 activities measured by oseltamivir and clopidogrel in Caucasians.....	31

CHAPTER 1

INTRODUCTION

Carboxylesterase1 (CES1) is a member of the mammalian carboxylesterases family of α/β -hydrolase fold proteins (Redinbo et al., 2003). In general, mammalian carboxylesterases (CESs) are classified based on their amino acid homology into five different groups, CES1-CES5 (Holmes et al., 2010). Among the five, CES1 and CES2 are considered to be drug metabolizing enzymes and they share 40-50% amino acid sequence identity (Imai et al., 2006). CES1 has a critical function in catalyzing the hydrolysis of a large number of endogenous as well as exogenous compounds, including drugs and toxins. Many are commonly prescribed drugs that belong to diverse therapeutic classes, including antiplatelet agents, central nervous system (CNS) stimulants, antivirals, and anti-hyperlipidemics (**Table 1**) (Casey Laizure et al., 2013). The chemical classes of CES1 substrates are ester, carbamate, thioester and amides, but the majority are ester prodrugs. In contrast to cytochrome P450 (CYP) enzymes, our knowledge about CES1 is limited (Casey Laizure et al., 2013). Inter-individual variability of CES1 has been reported to be 430-fold for mRNA, 100-fold for protein and 127-fold for activity, causing unpredicted toxicity or non-responsiveness in pharmacological agents that are activated or cleared by this pathway (Yang et al., 2009). Inter-individual variability can be attributed to many factors including genetic polymorphism, race, drug-drug interaction, age, and/or different diseases (Sanghani et al., 2009) (Xu et al., 2002). The clinical implications of such

variability can be far-reaching. Therefore, more investigation on the role of CES1 as a mediator of drug metabolism and disposition is warranted (Casey Laizure et al., 2013).

Table 1. A number of important CES1 substrates among commonly used pharmacological agents (Casey Laizure et al., 2013).

Therapeutic Class	Substrates	Product	Product activity
Antiplatelet/Anticoagulant	Clopidogrel	Clopidogrel carboxylate	Inactive
	2-oxo-clopidogrel	2-oxo-clopidogrel carboxylate	Inactive
	Dabigatran etexilate	Dabigatran	Active
Angiotensin-converting enzyme inhibitors	Enalapril	Enalaprilat	Active
	Imidapril	Imidaprilat	Active
Anti-hyperlipidemia agents	Simvastatin	Dihydroxy acid metabolite	Active
	Fenofibrate	Fenofibric acid	Active
CNS agents	Methylphenidate	Ritalinic acid	Inactive
Immunosuppressive agents	Mycophenolate mofetil	Mycophenolic acid	Active
Neuraminidase inhibitor	Oseltamivir	Oseltamivir carboxylic acid	Active

CHAPTER 2

REVIEW OF THE LITERATURE

2.1 First Pass Metabolism

Hydrolysis and transesterification are the two major reactions catalyzed by CES1 (Redinbo et al., 2003). Hydrolysis reactions serve as activation reactions for many prodrugs. Drug development scientists have taken advantage of the CES1 metabolism pathway to improve the oral bioavailability of many drugs by making ester prodrugs. Ester prodrugs are more lipophilic than both corresponding alcohol and carboxylic acid moieties that are part of the ester containing molecule (Merali et al., 2014). The high lipophilicity of prodrugs facilitates passive diffusion through cell membranes, resulting in higher oral bioavailability (Merali et al., 2014). For example, the active compound oseltamivir acid is a potent antiviral drug; however, due to its polarity, oseltamivir acid is not absorbed well through the gastrointestinal track (GIT). However, once synthesized as the corresponding ester (oseltamivir), the oral bioavailability reaches more than 80% (Taketani et al., 2007). CES1 mediated ester hydrolysis breaks down the ester bond forming two polar moieties, an alcohol moiety and an acyl moiety, facilitating their excretion (**Fig. 1**) (Merali et al., 2014). It should be noted that not every ester-containing drug is a prodrug. An ester functional group might also be a vital group in the structure of some drugs, such as aspirin and clopidogrel (Williams et al., 2008). For such drugs, CES1 mediated ester hydrolysis reaction is a de-activation reaction. In addition to ester hydrolysis, CES1 catalyzes

transesterification conjugation reaction, which occurs in the presence of ethyl alcohol. For example, clopidogrel in the presence of ethyl alcohol is transesterified into ethyl clopidogrel (Tang et al., 2006). Another example is the transesterification of cocaine into the ethylbenzoylecgonine metabolite that is 10 times more potent (Pennings et al., 2002).

Figure 1. Ester hydrolysis (1), and transesterification (2).

2.2 Tissue Distribution

All CES enzymes are located in the cytoplasmic and endoplasmic reticulum of the cells, and they are widely distributed in many tissues of different mammals.

According to Protein Atlas, mRNA levels of CES1 are found in its highest abundance in the liver, approximately 50% of its total distribution (www.proteinatlas.org, Last accessed June 30, 2016). The next highest is the gallbladder, making about 20% of the total mRNA tissue distribution. It should be noted that CES1 is not the only abundant carboxylesterase in the liver. Human carboxylesterase 2 (CES2) is abundant in the liver as well. However, CES2 is the predominantly found in the gastrointestinal tract, GIT, making about 60% of CES2 mRNA total expression in the whole body (Taketani et al., 2007). In contrast to CYPs, the distribution of CES2 along the entire small intestine is relatively constant (Satoh and Hosokawa, 2006). **Figure 2** shows the pattern of mRNA tissue expression of both CES1 and CES2 in various tissues.

Figure 2. CES1 and CES2 RNA tissue expression.

2.3 Substrate Specificity

Metabolism mediated by CESs usually lacks binding specificity (Holmes et al., 2010) (Hosokawa, 2008). However, specific preferences have been observed between CES1 and CES2 enzymes. It has been reported that CES1 usually prefers substrates with a large acyl group and a small alcohol group. In contrast, CES2 prefers substrates with a large bulky alcohol group and a small acyl group. As shown in **Table 2**, clopidogrel is preferably hydrolyzed by CES1 due to its relatively large acyl group, whereas irinotecan is preferably hydrolyzed by CES2 because of its relatively large alcohol group. Furthermore, CESs display stereo-selectivity as well. For example, DL-threo-methylphenidate (MPH), a central nervous system stimulant, is mainly metabolized to ritalinic acid by CES1. In addition, hydrolysis efficiency for L-isomer is 6- to 7-fold higher than the corresponding D-isomer (Sun et al., 2004). Another example is permethrin. The trans isoform is equally preferred by both CES1 and CES2, but its cis isoform is hydrolyzed only by CES2, **Figure 3** (Zhu et al., 2000).

Table 2. Differences in substrate preferences for CES1 and CES2.

Substrate	Substrate Preference
Clopidogrel	CES1
Irinotecan	CES2>>>CES1

Figure 3. Stereoselectivity for CES1 and CES2, adapted from (Zhu et al., 2000)

2.4 Species Differences

Animal models are utilized pre-clinically for prediction of pharmacology, toxicology, and pharmacokinetics properties of drugs. The accuracy of the prediction of drug metabolism/elimination is highly dependent on the resemblance between the enzyme in the human and the animal model. Generally, drug biotransformation between different species may differ in metabolic pathways and/or reaction velocity (Taketani et al., 2007). Among many species including humans, mice, rats, rabbits, dogs, and

monkeys, CESs are the most abundant hydrolases in the small intestine and the liver. Although all previously mentioned species share CES1 and CES2 families in their livers, protein levels of both enzymes vary widely (Taketani et al., 2007). In addition, protein level of CES1 family was reported to be abundant in the intestines of some species, such as monkey and rabbit, in contrast to being absent in human (Taketani et al., 2007).

Human plasma contains four esterases, three abundant and one rare, which are paraxonase, butyrylcholinesterase, albumin, and acetylcholinesterase, respectively. In contrast to other animal species, such as rodents and rabbits, none of the CESs are expressed in human plasma (Schopfer et al., 2005). A single molecule of albumin has a weak hydrolytic activity; however, albumin's significant contribution to hydrolysis is due to its high plasma concentration. The hydrolytic activity of albumin is believed to be due to Tyr411 amino acid in human albumin (Hosokawa et al., 1995).

Another important consideration is the level of protein homology between animal and human isoenzymes. Monkey CES1 showed 92.9% homology with human CES1. On the other hand, rabbit CES2 shows only 46.9 homology with human CES2. In fact, due to the differences in selectivity and activity of different species isoforms, pharmacological activity and toxicity of CES substrates are difficult to predict based on animal models and, as a result, experimental animals typically are not adequate pre-clinical models for carboxylesterases (Wood et al., 2003).

2.5 Effect of Gender

Gender can influence the activity and expression of drug metabolizing enzymes (DMEs). For example, CYP3A4 activity is reported to be two-times higher in female subjects as compared with male subjects (Patrick et al., 2007). Although the CES1 gender differences are not well established, some evidence has been reported supporting the effect of gender on metabolism of some drugs. For example, one study reported that the CES1 substrates lovastatin and simvastatin, are hydrolyzed more efficiently in women than in men (Patrick et al., 2007). Similarly, another clinical study showed differences in response and area under the concentration-time curve (AUC) for a dose of 0.3 mg/kg of methylphenidate administered to men and women subjects. The value of AUC (SD), in average, was much higher in men as compared with women [93.4 (25.3) ng h/ml vs. 73.5 (12.8) ng h/ml respectively] (Patrick et al., 2007; Casey Laizure et al., 2013).

2.6 Age-dependent Expression and Activity

Age can potentially influence many drug metabolizing enzymes including cytochrome P450 (CYP) enzymes (Koukouritaki et al., 2004). The activity of some CYPs (i.e. CYP2D6, CYP2C9, and CYP2E1) increase gradually with age (Stevens et al., 2003). In contrast, CYP3A7 protein level showed a drastic decrease by more than 60-fold by the end of their first birthday (Mehrotra et al., 2016). The influence of age on CES1 have been reported by Yang et al. and Zhu et al. (Yang et al., 2009)(Zhu et al, 2009). The average expression of CES1 in subjects younger than one year was significantly lower in comparison with pooled samples. For example, only 20.3% and 11.1 % of the

activity of the pooled samples were observed in 13-day and 30-day old subjects. Studies conducted using human liver microsome showed 4-fold and 10-fold higher adult CES1 activity compare to children and fetal microsomes, respectively (Yang et al., 2009). Furthermore, the correlation between growth hormone (GH) and CESs age-related expression in mice models was studied (Zhu et al., 2009). However, no correlation was observed neither after a short-term treatment with GH (1 day) nor after a longer term treatment of 9 days, indicating no contribution of GH to reduced CESs expression in young mice.

The results of Yang et al. were consistent with these findings (Yang et al., 2009). In their study, they grouped livers from 104 subjects into three groups, fetal, child, and adults. The expression and the activity were age related in such that it was higher in adults than in children, which was, also, higher than the expression in the fetal group.

2.7 Lipid Metabolism

The role of CES1 in lipid metabolism is very significant. For example, CES1 metabolizes endogenous substrates, such as cholesteryl esterase, triacylglycerol, and 2-arachidonylglycerol through a mechanism of forming a fatty acyl-enzyme intermediate and releasing an alcohol substituent (Merali et al., 2014). The intermediate acyl-enzyme, then, reacts with water releasing an acyl containing-substrate. In addition, CES1 possesses cholesterol transferase activity that helps with de-esterification and trans-esterification of lipids and forms cholesteryl esters in the presence of free cholesterol (Friedrichsen et al., 2013). Friedrichsen et al. reported a correlation between CES1 expression in adipose tissue and obesity, in which CES1

expression was higher in obese subjects (Friedrichsen et al., 2013). Furthermore, diet induced weight loss decreased CES1 expression in obese subjects. In fact, some speculate that CES1 expression in adipose tissue is connected to measures of metabolic function and obesity, such as plasma insulin level, triglycerides level, waist circumference, and homeostasis model assessment-insulin resistance, and, therefore, it may contribute to obesity and type-2 diabetes mellitus (Merali et al., 2014).

2.8 Effect of Pathological Conditions

Disease conditions are another factor that can alter CES1 expression or activity. Severe health conditions are accompanied by an elevation in the concentration of interleukin 6 (IL-6), an inflammatory biomarker, which has a suppression effect on the CES1 expression leading to decreases in CES1 activity (Yang et al., 2007). Hepatic dysfunction, such as cirrhosis and hepatitis, reduces the hydrolysis activity of CES1 (Thiollet et al., 1992; Eriksson et al., 2003). However, CES1 expression in mice infected with hepatitis C virus was up-regulated, which is believed to help the formation of lipid droplets that may be necessary for viral proliferation (Zhu et al., 2000).

Type 2 diabetes mellitus was reported to decrease CES1 protein level (Valle et al., 2012). Diabetes is characterized by many metabolism disorders including hyperglycemia, hyperinsulinemia, and abnormalities in fat, carbohydrates and protein metabolism (Valle et al., 2012). Moreover, steatosis and NASH are associated with diabetes and obesity (Fabbrini et al., 2010; Leite et al., 2011). Non-alcoholic fatty liver disease (NAFLD) is characterized by steatosis, a condition that is defined as an

abnormal retention of lipids in hepatocytes (Merali et al., 2014). Steatosis *per se* is an independent and important marker for multi-organ insulin resistance (Vega et al., 2007; Korenblat et al., 2008). NAFLD can progress to severe stages such as non-alcoholic steatohepatitis (NASH), cirrhosis or hepatocellular carcinoma (8,9).

2.9 Enzyme Inhibition

Competitive inhibition is likely to occur between CES1 substrates; especially some of CES1 substrates that are commonly prescribed together (Zhu et al., 2000). For example, it is reported that clopidogrel inhibits the conversion of oseltamivir to its active metabolite by 90% (Goel et al., 2007). A clinical trial reported that severe mania episode occurred as a result of concurrent administration of aripiprazole and the CES1 selective substrate DL-threo-methylphenidate (MPH) (Holmes et al., 2010). This effect might be as a result of the aripiprazole potential inhibitory effect on CES1 metabolism reported *in vitro* (Zhu et al., 2010).

2.10 Pharmacogenomics and Genetic Polymorphism of CESs

The highly polymorphic CES1 gene is located on chromosome 16q12.1, and it consists of 14 exons (Merali et al., 2014). Six CES1 single nucleotide polymorphisms (SNP) have been identified to be associated with alterations in drug disposition (Hines et al., 2016). The CES1 alleles rs3785161 and rs2241409 are associated with increase promoter activity and decreased transcript levels (Marsh et al., 2004; Geshi et al., 2005). CES1 rs71647871 results in a total loss-of-activity due to a substitution in amino acid number 143 from glycine to glutamic acid in the protein product. This

substitution is a result of a nonsynonymous transition of G to A at cDNA position 428 of CES1 in exon 4 (Hines et al., 2016). The frequency of this variant is reported to be around 4.3% in African Americans, 3.7% in Caucasians, and 2% in Hispanics, and 1% in Asians (Casey Laizure et al., 2013).

2.11 Oseltamivir and Clopidogrel

In our study, we were interested on the role of CES1 in oseltamivir and clopidogrel metabolism. Therefore, both pharmacological agents are reviewed below.

2.11.1 *Oseltamivir*

The World Health Organization (WHO) recommends use of oseltamivir during influenza pandemics as a treatment and prevention for influenza A and B virus infections (Merali et al., 2014). Oseltamivir carboxylate, a CES1 metabolism product, is the active form of the prodrug oseltamivir. Oseltamivir carboxylate is a neuraminidase inhibitor that prevents viral reproduction. *In vivo* and *in vitro* response variability has been reported. Rs71647871 mutant cell lines showed 25% lower activity than the corresponding wild type (Merali et al., 2014). The average increase in the AUC of oseltamivir was 360% and 18% for heterozygotes (428AA) and homozygote (428GA) alleles, respectively, compare to the wild type (428GG) allele (Taketani et al., 2007). In healthy volunteers, heterozygous rs121912777 SNP carriers showed 18% increase in oseltamivir AUC, and 23% decrease in metabolite-to-drug AUC compare to non-carriers (Merali et al., 2014).

2.11.2 *Clopidogrel*

Clopidogrel is a prodrug that undergoes a cascade of biotransformation by several CYP enzymes including CYP1A2, CYP3A4, CYP2B6, CYP2C9, and CYP2C19 (**Fig. 4**) (Zhu et al., 2013) (Rehmel et al., 2006; Kazui et al., 2010). However, only 15% of the oral dose is metabolized to 2-oxo-clopidogrel and the remaining 85%, undergoes hydrolysis by CES1 to the corresponding inactive metabolite (Hagihara et al., 2009). The metabolite, 2-oxo-clopidogrel undergoes further metabolism to the pharmacologically active 2-thiol clopidogrel (clopidogrel-AM), which is a bio reactive compound that irreversibly blocks P2Y₁₂ adenosine diphosphate (ADP) receptor on platelet membrane by forming a disulfide bridge (Bouman et al., 2011). CES1 dominate the metabolism of both 2-oxo- and 2-thiol-clopidogrel to the inactive corresponding carboxylic acids (Bouman et al., 2011).

Prolonged dual antiplatelet therapy with aspirin and clopidogrel, an antiplatelet thienopyridine inhibitor, is recommended to reduce ischemic events in atherothrombotic patients. Dual antiplatelet therapy is pivotal in conjunction with the use of drug-eluting stents (DES) to prevent stent thrombosis, ST, which is associated with high rates of morbidity and mortality (Anderson et al., 2007; Antman et al., 2008). (Yusuf et al., 2001; Sabatine et al., 2005). DES is a widely used technique making about 75% of the total percutaneous coronary intervention, PCI. Although the dual therapy with clopidogrel and aspirin decreases the occurrence of ST, several studies reported rare yet life threatening STs in some patients (Vaknin-Assa et al., 2007).

A significant inter-individual variability in the response to clopidogrel has been reported (Yusuf et al., 2001; Steinhubl et al., 2002; Sabatine et al., 2005). This variability has an impact in a large proportion of the population and increases the risk of cardiovascular events in case of hypo-responsiveness (Wang et al., 2006; Angiolillo and Alfonso, 2007; Angiolillo et al., 2007; Gurbel et al., 2007). Many studies have associated CYP2C19 reduced-function alleles (*2, *3, *4, *5) with lower levels of clopidogrel-AM and consequently lower platelet inhibition and higher rate of adverse cardiovascular events, such as ST (Mega et al., 2009; Simon et al., 2009). However, it has been suggested that black race is an independent predictor of definite drug-eluting stent thrombosis, and its potential mechanism of influence on ST has been provoked for further investigation (Collins et al., 2010). CES1 is believed to play a major role in clopidogrel inter-individual variability and it might explain the mechanism of higher cardiovascular events among African American (AA) patients.

Figure 4. Metabolic pathways of clopidogrel (Hall et al., 2011).

CHAPTER 3

METHODOLOGY

3.1 Chemicals and Supplies

Oseltamivir, oseltamivir acid, and oseltamivir-d3-acid were obtained from TRC (Toronto, ON, Canada). Clopidogrel and clopidogrel carboxylic acid were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Naproxen was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sections of human livers were obtained from a commercial source (Xenotech LLC, Lenexa, KS). Unless otherwise indicated, all other reagents were purchased from Fisher Scientific (Pittsburgh, PA).

3.2 Tissue Preparation

Human liver samples and their demographic data, i.e. gender, age, height, weight, smoking and drinking habits, were obtained from XenoTech LLC. Thawed liver samples, 0.5 g each, were homogenized using automated Omni Bead Rutor 24 bead mill homogenizer (Omni International, Kennesaw, GA). The homogenates were further centrifuged at 10,000 g for 20 min at 4 °C using Eppendorf centrifuge (Hamburg, Germany). The supernatant, S9 fractions, were aliquoted and stored for further experiments at -80 °C. For microsome and cytosols preparations, the supernatants were subjected to ultracentrifugation at 100,000 g for 60 min at 4 °C. The pellets were washed and re-suspended in glycerol buffer (20% glycerol, 20 uM BHT, 0.5 mM EDTA, 50mM Tris-HCL, and 0.25 M sucrose) and stored in -80 °C.

3.3 Total Protein Quantification

Total protein concentration in S9, microsomes, and cytosols samples were quantified using Bicinchoninic Acid Protein Assay kit (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer's protocol.

3.4 LC-MS/MS Analyses

3.4.1 *Oseltamivir Assay*

A tandem mass spectrometry technique was used. Acquity Xevo TQ MS mass spectrometry (Waters Corp.) equipped with an electrospray ionization source (ESI). Analytes detection was performed in the positive ion mode, and the mass transitions used were m/z 313.11 to 120 with retention time 0.83 minutes for oseltamivir, and m/z 285.13 to 197 with retention time of 0.75 minutes for oseltamivir acid. The desolvation gas flow rate was 650 L/h at 350 °C. Cone gas flow rate was set at 2 L/h and the source temperature was 150°C. The column was Acquity UPLC BEH C18 column, 2.1 x 50 mm, 1.7 μ m (Waters Corp., Milford, MA) and was maintained at 45°C. Mobile phases were (A) 0.05% formic acid and 5% acetonitrile in deionized water, and (B) acetonitrile sustained at a flow rate 0.5 ml/min during the entire run of 2 minutes. The elution mode was 95:5 (A:B) for 0.1 min. From 0.1 to 1.0 minutes, there was continuous gradient change to A: B 5:95 (v/v). The composition was kept constant for 1.5 minutes. The mobile phase composition was reversed back with a fast gradient to A:B 95:5 (v/v), and the column was re-equilibrated for 2 minutes.

The linearity was established from 5 ng/ml to 1000 ng/ml for both oseltamivir and its metabolite. All standards and quality control points were prepared in Tris-HCl buffer (50 mM, pH 7.4). Then, they were mixed with 1:2 acetonitrile containing the internal standard, oseltamivir-d3-acid, before injecting in the system.

Figure 5. Chromatogram obtained from oseltamivir LC-MS/MS assay.

3.4.2 Clopidogrel Assay

For clopidogrel LC-MS/MS analysis, the mass transitions used were m/z 322.0 to 211.85 with retention time 1.6 minutes for clopidogrel, m/z 307.7 to 197 with retention time 1.2 minutes clopidogrel carboxylate, and 228.89 to 168.95 for the IS naproxen. . Mobile phases were (A) 0.05% formic acid, and 5% acetonitrile in deionized water, and (B) acetonitrile sustained at a flow rate 0.5 ml/min during the entire run of 3 minutes. The elution mode was 95:5 (A:B) for 0.2 min. From 0.1 to 1.0 minutes, there was continues gradient change to A: B 5:95 (v/v). The composition of mobile phase was kept constant to 2.0 minutes. It retained back with a fast gradient

to A:B 95:5 (v/v), and re-equilibrated until 3 minutes. The instrument used and its parameters were the same as described earlier using the same analytical column. The linearity was established from 0.4 ng/ml to 300 ng/ml clopidogrel carboxylic acid. All standards and quality control points were prepared as described above with oseltamivir.

Figure 6. Chromatogram obtained from clopidogrel LC-MS/MS assay.

3.5 Determination of Enzymatic Activity

Human carboxylesterase 1, CES1, activity was assessed by the measuring the rate of CES1 hydrolysis of the specific substrate, oseltamivir to its carboxylic acid metabolite. Oseltamivir hydrolysis was carried out by incubation with human liver cytosols. With 30 ul of the reaction buffer, Tris-HCl (50 mM, pH 7.4), containing 0.2 mg/ml protein were pipetted into 1 mL Waters 96/well microplates. This was mixed with an equal volume of the substrate at concentrations ranged from 9.15 uM to 12.55 mM for S9 fraction, 10 and 40 uM for cytosols. After 10 minutes of incubation at 37°C, the

reaction was terminated using 120 μ l of ice-cold acetonitrile containing the internal standard, oseltamivir-d3-acid. Then, the mixture was centrifuged at 4000 g and 4°C for 15 minutes. Reactions that were performed without protein, served as controls. The concentrations of metabolites were measured using the LC-MS/MS method described above.

Clopidogrel incubations with S9 fractions followed the same protocol. However, incubation time was 20 min and [S] ranged from 2.24 μ M to 605 μ M for enzyme kinetics and 8 μ M for determination of incubation time curves.

For all enzyme kinetics experiments, samples were diluted according to dilution factors shown in **Table 3** to fit in the range of analysis calibration curve.

Table 3. Dilution factors used in the experiments of determination CES1 enzymatic activity.

#	[Oseltamivir] μ M	Dilution Factor	[Clopidogrel] μ M	Dilution Factor
1	12556	1371.7	605.9	4285
2	3766.5	411.5	393.8	2785
3	1130	123.4	256.	1810
4	339	37	166.4	1176
5	101.7	11.11	83.2	588
6	30.51	3.3	24.9	176.5
7	9.153	1	7.48	53
8	-	-	2.2	15.8

Ahead of all experiments, pilot studies were performed using pooled S9 fractions and cytosols from 6 livers of healthy donors to optimize incubation conditions and to maintain the reactions in the linear range. Incubation period (0 to 60 min) and protein concentration (0.035 to 1 mg/ml) experiments were performed as described earlier; however, using 1.5 ml Eppendorf vials.

3.6 Real-Time PCR

Isolation of total RNA was performed with High Pure RNA Paraffin Kit (Roche Diagnostics, Indianapolis, IN, USA). For cDNA preparation we used QIAamp DNA Mini Kit (QIAGEN Sciences, Maryland). Messenger RNA (mRNA) levels were measured using TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA, USA) on a Vii7 real-time PCR machine from Applied Biosystems (Foster City, CA). The results were based on Ct values, which were normalized to B-actin mRNA level, and corrected based on 16s rRNA.

3.7 Statistical Analysis

The experimental results were analyzed using IBM SPSS Statistics software (Version 23, Chicago, USA). Results are expressed as the mean $\pm$ standard deviation (SD) or geometric mean $\pm$ SEM. Distribution normality of the variables were confirmed using Kolmogorov-Smirnov's test. Differences between groups were determined using either independent samples t-test or nonparametric Kruskal-Wallis test was used as specified in each case. P values of 0.05 or less was considered statistically significant.

CHAPTER 4

RESULTS

4.1 Differences in CES1 Expression and Intrinsic Clearances between Groups of Subjects Donating Livers

Differences in CES1 expression and intrinsic clearances between groups of subjects donating livers are presented in **Table 4** and **Table 5**, respectively. Data for the factors including fibrosis, NASH or alcohol consumption in **Table 5** was not available for all subjects. Subjects with diabetes showed significantly higher intrinsic clearance (Cl_{int}) compare to non-diabetic subjects, $P=0.007$. Despite the limited sample size of 6 subjects with probable NASH, it seems that NASH might affect as compare to subjects with no NASH, $P=0.099$.

Table 4. Differences in CES1 expression levels between groups of subjects donating livers.

Factor	Variables (N)	Mean (SD) $\Delta\Delta$ CT	<i>P</i> Value (Kruskal-Wallis test)
Gender	Female (43) Male (46)	1.23 (1.04) 1.82 (2.6)	0.521
Race	Caucasian (81) African American (8)	1.60 (2.1) 0.88 (0.52)	0.521
Alcohol Consumption	Yes (45) No (44)	1.66 (1.73) 1.40 (2.28)	0.030
Smoker	Yes (47) No (42)	1.55 (1.79) 1.51 (2.22)	0.475
Diabetes	No (37) Yes (52)	1.44 (1.82) 1.59 (2.15)	0.603
Fibrosis	No (77) Yes (12)	1.58 (2.14) 1.20 (0.7)	0.867
NASH according to Kleiner <i>et al.</i> classification system (Kleiner et al., 2005)	Not NASH (50) Borderline NASH (26) NASH (13)	1.505 (1.76) 1.58 (2.7) 1.5 (1.19)	0.513

Table 5. Differences in intrinsic clearances between groups calculated based on full enzyme kinetics obtained from oseltamivir incubations with S9 fraction as determined in **Fig. 17**.

Factors	Variables (N)	Mean (SD) ul/min*mg	P Values
Gender	Female (18)	160.9 (125.1)	0.338 ^b
	Male (21)	189.9 (129)	
Race	Caucasians (19)	135.33 (98.5)	0.047 ^{*a}
	African Americans (20)	215.5 (193.4)	
Alcohol Consumption	Yes (13)	130.2 (65.7)	0.772 ^a
	No (16)	150.2 (104.1)	
Smoker	Yes (27)	180.2 (133)	0.903 ^b
	No (12)	168.2 (115.3)	
Diabetes	No (27)	204.5 (183.1)	0.007 ^{**a}
	Yes (12)	113.5 (62)	
Fibrosis	No (26)	144.9 (91.9)	0.523 ^b
	Yes (3)	109.4 (35.7)	
NASH according to Kleiner <i>et al.</i> classification system (Kleiner <i>et al.</i> , 2005)	Not NASH (22) Probable NASH (6)	158.2 (90.2) 94.7 (60.2)	0.099 ^b

^a Independent samples t-test

^b Kruskal-Wallis test

4.2 Experiment Conditions

4.2.1 Time-dependent carboxylesterase 1 activity

Oseltamivir experiments conditions were determined to be linear in the range of experiments as shown in **(Fig. 7)** for S9 and **(Fig. 8)** for cytosols pooled from 6 different subjects.

Figure 7. Scatter plot showing incubation time vs. metabolite formation in S9 fraction.

Figure 8. Scatter plot showing incubation time vs. metabolite formation in cytosol.

4.2.2 Establishment of the linearity between protein concentration and hydrolysis activity

Oseltamivir experiments conditions were determined to be linear (**Fig. 9**) for S9 and (**Fig. 10**) for cytosols in the range of the experiments. Cytosol and S9 fractions were pooled from 6 different subjects.

Figure 9. Scatter plot showing protein concentration vs. oseltamivir carboxylic acid formation rate in S9 fractions.

Figure 10. Scatter plot showing protein concentration vs. oseltamivir carboxylic acid formation rate in cytosol.

4.3 Clopidogrel Enzyme Kinetics in S9 fraction

Pooled liver S9 fractions of 20 AA and 20 Caucasian subjects were used to compare CES1 activity in metabolizing clopidogrel to clopidogrel carboxylic acid.

The pooled AA group had significantly higher CL_{int}, $P=0.036$ (Fig. 11). Substrate inhibition was observed and the data were fitted using equation 1. At high substrate concentrations, clopidogrel inhibited its own metabolism to clopidogrel carboxylic acid *in vitro*. Clopidogrel was reported to inhibit oseltamivir hydrolysis *in vitro* (Tang et al., 2006). Organic Solvents used to dissolve clopidogrel did not exceed 1%.

$$\text{Equation 1: } Y = V_{max} * \frac{X}{Km + X * \left(1 + \frac{X}{Ki}\right)}$$

Figure 11. Clopidogrel incubations in pooled S9 fractions obtained from 20 African American subjects and 20 Caucasian subjects showed inhibition effect.

4.4 Clopidogrel Hydrolysis Activity as a Function of Time

Incubations with 8 uM clopidogrel (**Fig.12**) shows that although both groups were able to form 550 nM clopidogrel carboxylic acid, pooled S9 fractions from Caucasians had slower reaction time as compared with AA. This confirms that CES1 activity in AA is more efficient even at low substrate concentrations.

Figure 12. Pooled Caucasians S9 fractions had slower reaction time as compared with AA.

4.5 Higher CES1 activity in African Americans is Highly Influenced by CES1 Activity in Five Subjects

Among the 20 African American subjects, 5 subjects showed relatively and constantly higher enzyme activity in metabolizing both clopidogrel and oseltamivir. When removing these subjects, African American group loses the significant level as shown in plot with Clint, $P < 0.0001$ (one way ANOVA comparing the three groups) (**Fig. 13**).

Figure 13. Among African Americans group, 5 subjects showed three times higher Clint than the other groups, $P < 0.001$ (one way ANOVA).

4.6 African Americans had Significantly Higher Oseltamivir Hydrolysis

Rates Compare to Caucasians in Human Liver Cytosol

The efficiency of hydrolysis 10 uM and 40 uM oseltamivir to oseltamivir carboxylic acid expressed as formation rates (pmol/min/mg protein) in human liver cytosols of 8 African Americans were significantly higher than the rates in cytosols of 88 Caucasian subjects indicating higher CES1 enzymatic activity in African Americans, $P=0.006$, $P=0.002$ (Kruskal-Wallis test) for incubations with 10 uM and 40 uM, respectively. Data plotted in (Fig. 14) compares the hydrolysis rates of 10 uM oseltamivir in African Americans and Caucasians, while (Fig. 15) shows the frequency of distribution for incubations with 40 uM.

Figure 14. . Boxplot showing oseltamivir carboxylic acid rates of formation of 88 Caucasian and 8 African American subjects incubation in cytosol at 10 uM.

Figure 15. Dot plot showing oseltamivir carboxylic acid rates of formation of 88 Caucasian and 8 African American subjects (incubation in cytosol at 40 uM).

4.7 Oseltamivir Enzyme Kinetics Using S9 fractions showed Higher Clint in African American Livers

Averaged and Individual (**Fig. 16 A and B**) enzyme kinetic results obtained from individual incubations in S9 fractions of 20 African Americans showed significantly higher intrinsic clearance (Clint) in AA compare to averaged 19 Caucasians, $P=0.047$. Intrinsic clearance was calculated for each subject according to the equation: $Clint = V_{max}/K_m$. Parameters K_m ($p=0.05$) and V_{max} ($p=0.87$) were calculated using Prism (**Table 6**).

Figure 16. (A)Averaged and (B) oseltamivir enzyme kinetics.

Table 6. Individual Km ,Vmax, and Clint values.

ID AA	Km (uM)	Vmax (nmol/min/mg)	Clint (ul/min*mg)		ID Caucasian	Km (uM)	Vmax (nmol/min/mg)	Clint (ul/min*mg)
H0331	1085.7	497.3	458.1		H0415	1310	505.49	385.86
H0339	2303.7	532.9	231.3		H0423	2644	365.38	138.19
H0379	995.1	335.2	336.9		H0436	4683.1	558.86	119.33
H0510	4449.9	1044.2	234.7		H0447	11229.8	552.68	49.22
H0533	2132.6	296.8	139.2		H0453	3189.5	294.89	92.45
H0561	3496	680	194.5		H0466	8183	381.88	46.67
H0566	1233.6	680.3	551.5		H0473	NA	NA	NA
H0568	4155.8	286.3	68.9		H0481	6279.9	47.23	7.52
H0717	1258	333	264.8		H0484	11998.1	1550.81	129.25
H0737	2376.5	296.4	124.7		H0715	1736.7	561	323.07
H0749	1555.2	270	173.6		H0741	5143.9	461.62	89.74
H0753	3312.5	231.1	69.8		H0745	2811.4	530.63	188.74
H0762	1864.4	328.2	176		H0758	1329.4	303.6	228.37
H0780	2957.4	1049.2	354.8		H0761	2609.8	206.3	79.05
H0803	1120.7	133.8	119.4		H0763	3653.9	606.23	165.92
H0805	1490	260	174.6		H0768	2006.8	341	169.96
H0828	6824	427.7	62.7		H0791	1993	379.26	190.30
H0839	3755	506.4	134.8		H0798	17702	289.78	16.37
H0886	2412	945.9	392.2		H0812	3204.2	249.12	77.75
H0899	26540.4	1282.8	48.3		H0879	8707	641.48	73.67

4.10 Inter Individual Variability in Hydrolysis of Oseltamivir

Human Carboxylesterase shows high inter-individual variability in both cytosol (CV= 66%) and S9 fractions (CV= 73%) as shown in **Figures 16 (a) and (b)**.

4.8 Establishment of the correlation between CES1 activities measured by oseltamivir and clopidogrel

Positive correlation was observed between CES1 activities measured using oseltamivir and clopidogrel hydrolysis in S9 fractions (**Fig. 17 (a) and (b)**). The data used in these correlations are oseltamivir enzyme kinetics data above and clopidogrel enzyme kinetics data mentioned in the appendix, which defined the metabolism phase by seven [S] points in contrast to 3 [S] points in **Figure 11**.

Figure 17 (A). Positive correlation between CES1 activities measured by oseltamivir and clopidogrel in African American subjects.

Figure 17 (B). Positive correlation between CES1 activities measured by oseltamivir and clopidogrel in Caucasian subjects.

CHAPTER 5

CONCLUSION

The goal of this study was to investigate variables influencing CES1 expression and activity, and subsequently the PK/PD of drugs undergo CES1 hydrolysis. Metabolism efficiency of oseltamivir mediated by CES1 appeared to be lower in diabetic subjects compare to non-diabetic subjects. Despite the limited sample size of 6 subjects with probable NASH, it is likely that NASH might reduce the CES1 hydrolysis efficiency. However, the effect of both diabetes and NASH should be studied with a larger sample size. Also, among many variables, subjects who consume alcohol had higher level of CES1 gene expression although it was not translated into higher enzymatic activity.

Another important and clinically relevant finding is that African Americans had a significantly higher CES1 activity compare to non-African Americans. This finding can provide potential mechanism explaining the clinical observations of African Americans developing nearly three times stent thrombosis compare to non-blacks after implantation with drug-eluting stent (DES) (Collins et al., 2010). Our data support that black race *per se* is an independent strong predictor for developing ST after DES (Collins et al., 2010). Our results indicate that CES1 activity in human liver S9 fractions and Cytosols were significantly higher in Africans American group compare to Caucasians using two different CES1 probes, oseltamivir and clopidogrel.

In case of clopidogrel metabolism, a two-step metabolism process by CYP1A2 and CYP2B6 to clopidogrel thiolacton, and then by CYP2B6, CYP2C9, and CYP2C19 are necessary for forming clopidogrel AM. However, clopidogrel and its metabolites are subject to a parallel competitive inactivation pathway. The inactivation pathway is predominant since more than 85% of clopidogrel and 49% of clopidogrel thiolacton is inactivated by CES1 to the corresponding carboxylic acid metabolite. Hydrolysis pathway by CES1 is a very efficient pathway compare to CYP450 pathways involved in clopidogrel metabolism. For example, Hagihara *et al.* reported the mean (activity pmol/mg/min) in 20 human liver microsome of CYP1A2 to be (39.0), CYP2B6 (2.0), CYP2C9 (54.8), CYP2C19 (10.1), CYP2D6 (27.3), CYP2E1 (1522.4), CYP3A4 (2084.8), and CES1 to be (20,299.7) (Hagihara et al., 2009). In addition to CES1 efficiency over other CYPs, CES1 has a combined effect on clopidogrel metabolism such that CES1 is involved in inactivation of clopidogrel and all its metabolites. Therefore, due to CES1 efficiency and combined effect, it is very likely that CES1 activity correlate inversely with clopidogrel AM formed and any variability in CES1 activity might lead to variation in clopidogrel AM, and consequently its plate inhibition activity. However, clopidogrel resistant appears to be multifactorial, and some factors, such as CYP2C19 polymorphisms have been reported as contributors.

Clopidogrel inhibited its own metabolism *in vitro* (**Fig. 11**). It was reported that clopidogrel inhibited oseltamivir hydrolysis (Shi et al., 2006). Substrate inhibition

was reached at a relatively high concentration and does not seem to have a clinical significant.

In contrast to clopidogrel, variability in CES1 activity might not be of a clinical implication for drugs that follow sequential non-competitive CES1 metabolism pathways, such as oseltamivir. Assuming enough presentation time to CES1 in the liver, CES1 will eventually metabolize all available substrate owing to its high efficiency and low *in vivo* concentration of most drugs. Such variability in CES1 activity is likely to be counterbalanced although it might influence some pharmacokinetic parameters such as T_{max}.

In conclusion, this study gives a potential explanation of the clinical observation and strongly supports the conclusion of *Collines et al.* that black populations are exposed to more risk incidence of stent thrombosis (Collins et al., 2010). Our data support that black race *per se* is a strong predictor of stent thrombosis.

APPENDIX

I. Results of clopidogrel Incubation, Experiment # 2.

BIBLIOGRAPHY

- Anderson JL, Adams CD, Antman EM, Bridges CR, Califf RM, Casey DE, Chavey WE, Fesmire FM, Hochman JS, and Levin TN (2007) ACC/AHA 2007 guidelines for the management of patients with unstable angina/non–ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non–ST-Elevation Myocardial Infarction) developed in collaboration with the American College of Emergency Physicians, the Society for Cardiovascular Angiography and Interventions, and the Society of Thoracic Surgeons endorsed by the American Association of Cardiovascular and Pulmonary Rehabilitation and the Society for Academic Emergency Medicine. *Journal of the American College of Cardiology* **50**:e1-e157.
- Angiolillo DJ and Alfonso F (2007) Platelet function testing and cardiovascular outcomes: steps forward in identifying the best predictive measure. *Thrombosis and haemostasis* **98**:707.
- Angiolillo DJ, Bernardo E, Sabaté M, Jimenez-Quevedo P, Costa MA, Palazuelos J, Hernández-Antolin R, Moreno R, Escaned J, and Alfonso F (2007) Impact of platelet reactivity on cardiovascular outcomes in patients with type 2 diabetes mellitus and coronary artery disease. *Journal of the American College of Cardiology* **50**:1541-1547.
- Antman EM, Hand M, Armstrong PW, Bates ER, Green LA, Halasyamani LK, Hochman JS, Krumholz HM, Lamas GA, and Mullany CJ (2008) 2007 Focused Update of the ACC/AHA 2004 Guidelines for the Management of Patients With ST-Elevation Myocardial Infarction A Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines: Developed in Collaboration With the Canadian Cardiovascular Society Endorsed by the American Academy of Family Physicians: 2007 Writing Group to Review New Evidence and Update the ACC/AHA 2004 Guidelines for the Management of Patients With ST-Elevation Myocardial Infarction, Writing on Behalf of the 2004 Writing Committee. *Circulation* **117**:296-329.

- Bouman HJ, Schömig E, van Werkum JW, Velder J, Hackeng CM, Hirschhäuser C, Waldmann C, Schmalz H-G, ten Berg JM, and Taubert D (2011) Paraoxonase-1 is a major determinant of clopidogrel efficacy. *Nature medicine* **17**:110-116.
- Casey Laizure S, Herring V, Hu Z, Witbrodt K, and Parker RB (2013) The role of human carboxylesterases in drug metabolism: have we overlooked their importance? *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy* **33**:210-222.
- Collins SD, Torguson R, Gaglia MA, Lemesle G, Syed AI, Ben-Dor I, Li Y, Maluenda G, Kaneshige K, and Xue Z (2010) Does black ethnicity influence the development of stent thrombosis in the drug-eluting stent era? *Circulation* **122**:1085-1090.
- Eriksson A, Gretzer C, and Wallerstedt S (2003) Elevation of cytokines in peritoneal fluid and blood in patients with liver cirrhosis. *Hepato-gastroenterology* **51**:505-509.
- Fabbrini E, Sullivan S, and Klein S (2010) Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. *Hepatology* **51**:679-689.
- Friedrichsen M, Poulsen P, Wojtaszewski J, Hansen PR, Vaag A, and Rasmussen HB (2013) Carboxylesterase 1 gene duplication and mRNA expression in adipose tissue are linked to obesity and metabolic function. *PloS one* **8**:e56861.
- Geshi E, Kimura T, Yoshimura M, Suzuki H, Koba S, Sakai T, Saito T, Koga A, Muramatsu M, and Katagiri T (2005) A single nucleotide polymorphism in the carboxylesterase gene is associated with the responsiveness to imidapril medication and the promoter activity. *Hypertension research* **28**:719.
- Goel S, Desai K, Karri S, Gollamudi R, Chaudhary I, Bulgaru A, Kaubisch A, Goldberg G, Einstein M, and Camacho F (2007) Pharmacokinetic and safety study of weekly irinotecan and oral capecitabine in patients with advanced solid cancers. *Investigational new drugs* **25**:237-245.
- Gurbel PA, Becker RC, Mann KG, Steinhubl SR, and Michelson AD (2007) Platelet function monitoring in patients with coronary artery disease. *Journal of the American College of Cardiology* **50**:1822-1834.

- Hagihara K, Kazui M, Kurihara A, Yoshiike M, Honda K, Okazaki O, Farid NA, and Ikeda T (2009) A possible mechanism for the differences in efficiency and variability of active metabolite formation from thienopyridine antiplatelet agents, prasugrel and clopidogrel. *Drug Metabolism and Disposition* **37**:2145-2152.
- Hall HM, Banerjee S, and McGuire DK (2011) Variability of clopidogrel response in patients with type 2 diabetes mellitus. *Diabetes and Vascular Disease Research* **8**:245-253.
- Hines RN, Simpson PM, and McCarver DG (2016) Age-Dependent Human Hepatic Carboxylesterase 1 (CES1) and Carboxylesterase 2 (CES2) Postnatal Ontogeny. *Drug Metabolism and Disposition*:dmd. 115.068957.
- Holmes RS, Wright MW, Laulederkind SJ, Cox LA, Hosokawa M, Imai T, Ishibashi S, Lehner R, Miyazaki M, and Perkins EJ (2010) Recommended nomenclature for five mammalian carboxylesterase gene families: human, mouse, and rat genes and proteins. *Mammalian Genome* **21**:427-441.
- Hosokawa M (2008) Structure and catalytic properties of carboxylesterase isozymes involved in metabolic activation of prodrugs. *Molecules* **13**:412-431.
- Hosokawa M, Endo T, Fujisawa M, Hara S, Iwata N, Sato Y, and Satoh T (1995) Interindividual variation in carboxylesterase levels in human liver microsomes. *Drug metabolism and disposition* **23**:1022-1027.
- Imai T, Taketani M, Shii M, Hosokawa M, and Chiba K (2006) Substrate specificity of carboxylesterase isozymes and their contribution to hydrolase activity in human liver and small intestine. *Drug metabolism and disposition* **34**:1734-1741.
- Kazui M, Nishiya Y, Ishizuka T, Hagihara K, Farid NA, Okazaki O, Ikeda T, and Kurihara A (2010) Identification of the human cytochrome P450 enzymes involved in the two oxidative steps in the bioactivation of clopidogrel to its pharmacologically active metabolite. *Drug Metabolism and Disposition* **38**:92-99.
- Korenblat KM, Fabbrini E, Mohammed BS, and Klein S (2008) Liver, muscle, and adipose tissue insulin action is directly related to intrahepatic triglyceride content in obese subjects. *Gastroenterology* **134**:1369-1375.

- Koukouritaki SB, Manro JR, Marsh SA, Stevens JC, Rettie AE, McCarver DG, and Hines RN (2004) Developmental expression of human hepatic CYP2C9 and CYP2C19. *Journal of Pharmacology and Experimental Therapeutics* **308**:965-974.
- Leite NC, Villela-Nogueira CA, Pannain VLN, Bottino AC, Rezende GFM, Cardoso CRL, and Salles GF (2011) Histopathological stages of nonalcoholic fatty liver disease in type 2 diabetes: prevalences and correlated factors. *Liver International* **31**:700-706.
- Marsh S, Xiao M, Yu J, Ahluwalia R, Minton M, Freimuth RR, Kwok P-Y, and McLeod HL (2004) Pharmacogenomic assessment of carboxylesterases 1 and 2. *Genomics* **84**:661-668.
- Mega JL, Close SL, Wiviott SD, Shen L, Hockett RD, Brandt JT, Walker JR, Antman EM, Macias W, and Braunwald E (2009) Cytochrome p-450 polymorphisms and response to clopidogrel. *New England Journal of Medicine* **360**:354-362.
- Mehrotra A, Boberg M, Vrana M, Gaedigk A, Pearce RE, Leeder S, and Prasad B (2016) Age-dependent Expression Analysis of Major Drug Metabolizing Enzymes in Human Liver. *The FASEB Journal* **30**:713.711-713.711.
- Merali Z, Ross S, and Paré G (2014) The pharmacogenetics of carboxylesterases: CES1 and CES2 genetic variants and their clinical effect. *Drug metabolism and drug interactions* **29**:143-151.
- Patrick K, Straughn A, Minhinnett R, Yeatts S, Herrin A, DeVane C, Malcolm R, Janis G, and Markowitz J (2007) Influence of ethanol and gender on methylphenidate pharmacokinetics and pharmacodynamics. *Clinical Pharmacology & Therapeutics* **81**:346-353.
- Pennings EJM, Leccese AP, and Wolff FAd (2002) Effects of concurrent use of alcohol and cocaine. *Addiction* **97**:773-783.
- Redinbo MR, Bencharit S, and Potter PM (2003) Human carboxylesterase 1: from drug metabolism to drug discovery. *Biochemical Society Transactions* **31**:620-624.

- Rehmel JLF, Eckstein JA, Farid NA, Heim JB, Kasper SC, Kurihara A, Wrighton SA, and Ring BJ (2006) Interactions of two major metabolites of prasugrel, a thienopyridine antiplatelet agent, with the cytochromes P450. *Drug Metabolism and Disposition* **34**:600-607.
- Sabatine MS, Cannon CP, Gibson CM, López-Sendón JL, Montalescot G, Theroux P, Claeys MJ, Cools F, Hill KA, and Skene AM (2005) Addition of clopidogrel to aspirin and fibrinolytic therapy for myocardial infarction with ST-segment elevation. *New England Journal of Medicine* **352**:1179-1189.
- Sanghani SP, Sanghani PC, Schiel MA, and Bosron WF (2009) Human carboxylesterases: an update on CES1, CES2 and CES3. *Protein and peptide letters* **16**:1207-1214.
- Satoh T and Hosokawa M (2006) Structure, function and regulation of carboxylesterases. *Chemico-biological interactions* **162**:195-211.
- Schopfer LM, Champion MM, Tamblyn N, Thompson CM, and Lockridge O (2005) Characteristic mass spectral fragments of the organophosphorus agent FP-biotin and FP-biotinylated peptides from trypsin and bovine albumin (Tyr410). *Analytical biochemistry* **345**:122-132.
- Simon T, Verstuyft C, Mary-Krause M, Quteineh L, Drouet E, Méneveau N, Steg PG, Ferrières J, Danchin N, and Becquemont L (2009) Genetic determinants of response to clopidogrel and cardiovascular events. *New England journal of medicine* **360**:363-375.
- Steinhuibl SR, Berger PB, Mann III JT, Fry ET, DeLago A, Wilmer C, Topol EJ, and Investigators C (2002) Early and sustained dual oral antiplatelet therapy following percutaneous coronary intervention: a randomized controlled trial. *Jama* **288**:2411-2420.
- Stevens JC, Hines RN, Gu C, Koukouritaki SB, Manro JR, Tandler PJ, and Zaya MJ (2003) Developmental expression of the major human hepatic CYP3A enzymes. *Journal of Pharmacology and Experimental Therapeutics* **307**:573-582.
- Sun Z, Murry DJ, Sanghani SP, Davis WI, Kedishvili NY, Zou Q, Hurley TD, and Bosron WF (2004) Methylphenidate is stereoselectively hydrolyzed by human

carboxylesterase CES1A1. *Journal of Pharmacology and Experimental Therapeutics* **310**:469-476.

Taketani M, Shii M, Ohura K, Ninomiya S, and Imai T (2007) Carboxylesterase in the liver and small intestine of experimental animals and human. *Life sciences* **81**:924-932.

Tang M, Mukundan M, Yang J, Charpentier N, LeCluyse EL, Black C, Yang D, Shi D, and Yan B (2006) Antiplatelet agents aspirin and clopidogrel are hydrolyzed by distinct carboxylesterases, and clopidogrel is transesterificated in the presence of ethyl alcohol. *Journal of Pharmacology and Experimental Therapeutics* **319**:1467-1476.

Thiollet M, Funck-Brentano C, Grange J, Midavaine M, Resplandy G, and Jaillon P (1992) The pharmacokinetics of perindopril in patients with liver cirrhosis. *British journal of clinical pharmacology* **33**:326-328.

Vaknin-Assa H, Assali A, Ukabi S, Lev EI, and Kornowski R (2007) Stent thrombosis following drug-eluting stent implantation. A single-center experience. *Cardiovascular Revascularization Medicine* **8**:243-247.

Valle A, Catalán V, Rodríguez A, Rotellar F, Valentí V, Silva C, Salvador J, Frühbeck G, Gómez-Ambrosi J, and Roca P (2012) Identification of liver proteins altered by type 2 diabetes mellitus in obese subjects. *Liver International* **32**:951-961.

Vega GL, Chandalia M, Szczepaniak LS, and Grundy SM (2007) Metabolic correlates of nonalcoholic fatty liver in women and men. *Hepatology* **46**:716-722.

Wang TH, Bhatt DL, and Topol EJ (2006) Aspirin and clopidogrel resistance: an emerging clinical entity. *European heart journal* **27**:647-654.

Williams ET, Jones KO, Ponsler GD, Lowery SM, Perkins EJ, Wrighton SA, Ruterbories KJ, Kazui M, and Farid NA (2008) The biotransformation of prasugrel, a new thienopyridine prodrug, by the human carboxylesterases 1 and 2. *Drug Metabolism and Disposition* **36**:1227-1232.

Wood AJ, Kearns GL, Abdel-Rahman SM, Alander SW, Blowey DL, Leeder JS, and Kauffman RE (2003) Developmental pharmacology—drug disposition, action,

and therapy in infants and children. *New England Journal of Medicine* **349**:1157-1167.

- Xu G, Zhang W, Ma MK, and McLeod HL (2002) Human carboxylesterase 2 is commonly expressed in tumor tissue and is correlated with activation of irinotecan. *Clinical Cancer Research* **8**:2605-2611.
- Yang D, Pearce RE, Wang X, Gaedigk R, Wan Y-JY, and Yan B (2009) Human carboxylesterases HCE1 and HCE2: ontogenic expression, inter-individual variability and differential hydrolysis of oseltamivir, aspirin, deltamethrin and permethrin. *Biochemical pharmacology* **77**:238-247.
- Yang J, Shi D, Yang D, Song X, and Yan B (2007) Interleukin-6 suppresses the expression of carboxylesterases HCE1 and HCE2 through transcriptional repression. *Mol Pharmacol* **72**:686-694.
- Yusuf S, Björkstén N, and Moons A (2001) Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation: the Clopidogrel in Unstable Angina to Prevent recurrent Events Trial Investigators. *The New England journal of medicine* **345**:494-502.
- Zhu H-J, Appel DI, Jiang Y, and Markowitz JS (2009) Age- and sex-related expression and activity of carboxylesterase 1 and 2 in mouse and human liver. *Drug Metabolism and Disposition* **37**:1819-1825.
- Zhu H-J, Appel DI, Peterson YK, Wang Z, and Markowitz JS (2010) Identification of selected therapeutic agents as inhibitors of carboxylesterase 1: potential sources of metabolic drug interactions. *Toxicology* **270**:59-65.
- Zhu H-J, Wang X, Gawronski BE, Brinda BJ, Angiolillo DJ, and Markowitz JS (2013) Carboxylesterase 1 as a determinant of clopidogrel metabolism and activation. *Journal of Pharmacology and Experimental Therapeutics* **344**:665-672.
- Zhu W, Song L, Zhang H, Matoney L, LeCluyse E, and Yan B (2000) Dexamethasone differentially regulates expression of carboxylesterase genes in humans and rats. *Drug metabolism and disposition* **28**:186-191.