PHARMACOKINETIC INTERACTIONS AND PHARMACOGENETICS OF CLOPIDOGREL, PRASUGREL AND TICAGRELOR

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ACADEMIC DISSERTATION

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<tbody>
<tr>
<td>ABC</td>
<td>Adenosine triphosphate binding cassette superfamily</td>
</tr>
<tr>
<td>ACS</td>
<td>Acute coronary syndrome</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AhR</td>
<td>Aryl hydrocarbon receptor</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>AU</td>
<td>Aggregation units</td>
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<tr>
<td>AUC</td>
<td>Area under the plasma concentration-time curve</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast cancer resistance protein</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CAR</td>
<td>Constitutive androstane receptor</td>
</tr>
<tr>
<td>CES</td>
<td>Carboxylesterase</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>C_{\text{max}}</td>
<td>Peak plasma concentration</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>f</td>
<td>Female</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
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<tr>
<td>HR</td>
<td>Hazard ratio</td>
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<tr>
<td>IC_{50}</td>
<td>Inhibition concentration producing 50% inhibition</td>
</tr>
<tr>
<td>IPA</td>
<td>Inhibition of platelet aggregation</td>
</tr>
<tr>
<td>k_{e}</td>
<td>Elimination rate constant</td>
</tr>
<tr>
<td>K_{\text{ow}}</td>
<td>Octanol/water partition ratio</td>
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<tr>
<td>m</td>
<td>Male</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
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</tr>
<tr>
<td>VKORC1</td>
<td>Vitamin K epoxide reductase complex subunit 1</td>
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<tr>
<td>vWF</td>
<td>Von Willebrand factor</td>
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ABSTRACT

The antiplatelet drugs clopidogrel, prasugrel and ticagrelor are used to treat and prevent atherothrombotic events. They inhibit platelet activation by blocking the platelet P2Y₁₂ adenosine diphosphate (ADP) receptor. Considerable variation exists in the pharmacodynamics and therapeutic response to clopidogrel treatment due to pharmacogenetic factors and drug-drug interactions. The newer platelet inhibitors, prasugrel and ticagrelor, have been shown to be superior to clopidogrel, both in terms of efficacy and consistency of therapeutic response.

Clopidogrel is a pro-drug that requires the formation of an active metabolite for platelet inhibition. Up to 90% of clopidogrel is hydrolyzed to an inactive metabolite by carboxylesterase 1 (CES1), and cytochrome P450 (CYP) enzymes CYP1A2, CYP2B6, CYP2C19, and CYP3A4 are responsible for the bioactivation process. Of these, CYP2C19 seems to be the most important in vivo according to pharmacogenetic data from patient studies and known drug-drug interactions. Prasugrel requires metabolic activation for therapeutic efficacy, as well. CES2 catalyzes the formation of a primary, inactive metabolite, which in turn is metabolized by CYP3A4 and CYP2B6 to a secondary, active metabolite. Data from pharmacogenetic and drug-drug interaction studies suggest that the pharmacokinetics of prasugrel is not as susceptible to CYP activity variation as that of clopidogrel. Ticagrelor differs from clopidogrel and prasugrel in that it does not require bioactivation. Most of ticagrelor is metabolized by CYP3A4 and CYP3A5, and drug-drug interactions with CYP3A4 inhibitors have been shown to increase ticagrelor exposure significantly. Like prasugrel, ticagrelor can be used to treat patients with high on-clopidogrel treatment platelet activity.

In addition to the liver, considerable CYP3A4 and some CYP2C19 activity exist in the small intestinal wall, where they have an important role in first-pass metabolism. Grapefruit juice has been shown to inhibit intestinal CYP3A4 in vivo and affect the pharmacokinetics of several drugs. Furthermore, grapefruit juice constituents have been shown to inhibit other CYPs, e.g. CYP2C19, in vitro. CYP3A4 and CYP3A5 have generally overlapping substrate specificity and they both contribute to the total CYP3A activity. Functionally significant variants in the CYP3A4 gene include the CYP3A4*22 allele, which results in a significant decrease in CYP3A4 expression both in heterozygous and homozygous carriers. The allelic frequency of CYP3A4*22 is around 5% in Caucasians. The loss-of-function allele CYP3A5*3 is a much more common variant in the CYP3A5 gene with an allelic frequency of 94% in Caucasians. Consequently, the proportion of Caucasians with a functional copy of the CYP3A5 gene (the CYP3A5*1 allele) is low and only 10 to 15% express a significant amount of CYP3A5.

This thesis comprises three randomized, controlled, cross-over pharmacokinetic and pharmacodynamic drug interaction studies, and one prospective genotype panel study in healthy volunteers. The effect of grapefruit juice-mediated inhibition of intestinal CYP3A4 and possibly CYP2C19 on the pharmacokinetics and pharmacodynamics of clopidogrel, prasugrel, and ticagrelor was studied. The effect of genetic CYP3A activity variation on the pharmacokinetics and antiplatelet
effect of clopidogrel, prasugrel, and ticagrelor was studied by comparing carriers of a single
CYP3A4*22 allele and carriers of a CYP3A5*1 allele to controls with CYP3A4*1/*1 and
CYP3A5*3/*3 genotypes. The plasma concentrations of the study drugs were measured from timed
blood samples and pharmacokinetic variables were calculated. The pharmacodynamic response was
studied with ex vivo platelet function tests in blood samples.

Grapefruit juice markedly inhibited the bioactivation of clopidogrel. The clopidogrel active
metabolite area under the plasma concentration-time curve (AUC) was decreased to 14% of that in
controls and consequently the platelet inhibitory effect was markedly reduced. In contrast, prasugrel
pharmacokinetics and antiplatelet effect were only modestly altered by grapefruit juice consumption.
The prasugrel active metabolite AUC was decreased to 74% of the control by grapefruit juice and
only a minor reduction in platelet inhibitory activity was seen. Ticagrelor exposure was significantly
increased by grapefruit juice. Ticagrelor AUC was increased to 221% of the control by grapefruit
juice consumption and accordingly the interaction resulted in enhanced platelet inhibition. The
CYP3A4*22 allele was associated with an impaired ticagrelor elimination, but not with a significant
effect on the bioactivation of clopidogrel or prasugrel. The CYP3A5*3 genotype did not affect the
pharmacokinetics of any of the study drugs. Ticagrelor AUC was 89% higher in carriers of a
CYP3A4*22 allele compared to control and the antiplatelet effect was enhanced.

In conclusion, the bioactivation of clopidogrel was severely impaired by inhibition of intestinal
metabolism by grapefruit juice. Considering the magnitude of the effect, the mechanism of the
interaction may have involved inhibition of intestinal CYP2C19, in addition to inhibition of CYP3A4.
Compared to clopidogrel, prasugrel bioactivation was much less sensitive to the effect of grapefruit
juice, consistent with previous drug-drug interaction studies, and probably due to the contribution of
CYP2B6 in prasugrel metabolism. Ticagrelor elimination was markedly inhibited by grapefruit juice,
underlining the importance of both intestinal metabolism and CYP3A4 in ticagrelor
pharmacokinetics. Furthermore, the CYP3A4*22 allele was associated with significantly higher
ticagrelor exposure. The concomitant use of grapefruit juice with clopidogrel and ticagrelor is best
avoided due to risk of poor therapeutic efficacy and adverse effects, respectively. Genotyping for
CYP3A4*22 could be used to predict ticagrelor pharmacokinetics, which could be particularly
important in patients with increased risk of bleeding.
INTRODUCTION

Atherothrombotic cardiovascular diseases (CVDs), such as coronary artery disease (CAD), peripheral artery disease (PAD), and ischemic stroke are major sources of morbidity and mortality throughout the world. Treatment and prevention of CVDs have advanced significantly in recent decades. Yet, CVDs caused over 17 million deaths worldwide in 2012 and are expected to cause over 20 million deaths by the year 2030. Acute coronary syndrome (ACS) and ischemic stroke were the leading causes of CVD-related mortality, accounting for 7.3 and 6.2 million deaths in 2012, respectively (Laslett et al. 2012). The pathophysiologic mechanisms of atherothrombotic diseases include inflammation, dysfunction of arterial endothelium, lipid accumulation leading to growth of atherosclerotic lesions, plaque rupture, platelet activation, and thrombus formation. This, in turn, can result in reduced or occluded blood flow and mismatch between oxygen supply and demand (Koo 2015, Krishna, Moxon & Golledge 2015, Yahagi et al. 2016).

Clopidogrel, prasugrel, and ticagrelor are drugs that inhibit platelet activation and are used to treat and prevent atherothrombotic events. They inhibit the platelet P2Y_{12} adenosine diphosphate (ADP) receptor and, by this mechanism, reduce platelet activation and aggregation. Combination therapy with aspirin and an ADP-receptor inhibitor has been shown to be particularly useful in preventing stent thrombosis after coronary artery stent implantation in ACS (Schomig et al. 1996). Considerable inter-individual variation exists in the pharmacodynamic response to clopidogrel treatment due to pharmacogenetic factors and drug-drug interactions (Simon et al. 2009). Prasugrel and ticagrelor have been shown to offer improvement, both in terms of efficacy and more consistent antiplatelet effect compared to clopidogrel in ACS patients. Prasugrel and ticagrelor associate with increased risk of bleeding compared to clopidogrel. However, the reduction in major cardiac events is considered to outweigh the increased bleeding risk (Weerakkody et al. 2007, Alexopoulos et al. 2011, Wallentin et al. 2009).

Clopidogrel is a pro-drug that requires the formation of an active thiol metabolite for its therapeutic antiplatelet effect. Around 90% of the orally administered clopidogrel is rapidly hydrolysed to an inactive carboxylic acid metabolite by carboxylesterase-1 (CES1) (Tang et al. 2006, Farid, Kurihara & Wrighton 2010). Cytochrome P450 (CYP) enzymes catalyse the bioactivation process of the remaining clopidogrel in two steps with 2-oxo-clopidogrel as an intermediate. CYP1A2, CYP2B6, CYP2C19, CYP2C9, CYP3A4 and CYP3A5 are involved in the metabolic activation in vitro (Dansette et al. 2012). CYP2C19 has an important role in the bioactivation in vivo, as carriers of an inactivating variant of the CYP2C19 gene have reduced clopidogrel active metabolite formation and increased mortality in acute coronary syndrome when treated with clopidogrel (Simon et al. 2009, Hulot et al. 2006, Hulot et al. 2011). Furthermore, the CYP2C19 inhibitor omeprazole has decreased the area under the plasma concentration-time curve (AUC) of clopidogrel active metabolite by over 40% (Angiolillo et al. 2011).
Like clopidogrel, prasugrel requires bioactivation for its platelet inhibitory effect. CES2 catalyzes the formation of prasugrel’s primary, inactive metabolite R-95913. CYP3A4, CYP2B6, and to a lesser extent CYP2C9 and CYP2C19 then convert R-95913 to the secondary, active metabolite R-138727 (Sugidachi et al. 2001, Rehmel et al. 2006). Compared to clopidogrel, the antiplatelet activity and therapeutic efficacy of prasugrel do not seem as susceptible to CYP activity variation and drug-drug interactions (Mega et al. 2009b, Mega et al. 2010a). The CYP2B6 and CYP3A4 inhibitor ritonavir reduced the prasugrel active metabolite AUC by 38% (Ancrenaz et al. 2013) and the CYP3A4 inhibitor ketoconazole inhibited clopidogrel metabolism but did not affect prasugrel bioactivation (Farid et al. 2007b).

Ticagrelor differs from clopidogrel and prasugrel in that it does not require metabolic activation and it binds to the P2Y<sub>12</sub> receptor reversibly. Ticagrelor is extensively metabolised by CYP3A4 and CYP3A5 to two major metabolites, C124910XX and C133913XX (Wallentin et al. 2009, van Giezen et al. 2009, Zhou, Andersson & Grimm 2011). Both ticagrelol and prasugrel can be used to overcome high on-clopidogrel treatment platelet activity seen in loss-of-function CYP2C19 allele carriers. (Wallentin et al. 2010). Ticagrelor elimination is susceptible to CYP3A4 inhibition and ketoconazole has increased the AUC of ticagrelor by over 600% (Teng, Butler 2013a).

The liver is the most important organ for CYP-mediated metabolism and clearance of drugs. However, the small intestinal wall has significant levels of CYP activity and has been shown to be important in the first-pass metabolism of several drugs (Yang, Tucker & Rostami-Hodjegan 2004, Thummel et al. 1996, Thelen, Dressman 2009). CYP3A4 is the most abundant enzyme in the enterocytes, but CYP2C9 and CYP2C19 activity is also present (Läpple et al. 2003, Paine et al. 2006, Galetin, Houston 2006). Grapefruit juice inhibits intestinal CYP3A4 in a mechanism-based manner, with furanocoumarin constituents as the primary inhibitors (Lown et al. 1997, Schmiedlin-Ren et al. 1997). Since the discovery of the CYP3A4 inhibitory effect of grapefruit juice, multiple significant interactions between CYP3A4 substrate drugs and grapefruit juice have been discovered. The effect of grapefruit juice on simvastatin metabolism is particularly strong (Lilja, Kivisto & Neuvonen 2000, Lilja, Kivisto & Neuvonen 1998). Grapefruit juice has also been shown to impair the platelet inhibitory effect of clopidogrel. The effect of grapefruit juice on clopidogrel pharmacokinetics has not been investigated, but the pharmacodynamic interaction suggests impaired clopidogrel bioactivation during grapefruit juice consumption (Campbell et al. 2014). In addition to CYP3A4, grapefruit juice constituents have been shown to inhibit other CYPs, including CYP2C19, P-glycoprotein (P-gp), and various organic anion-transporting polypeptides (OATPs) in vitro (Tassaneeyakul et al. 2000, Dresser et al. 2002, Satoh et al. 2005a, Bailey et al. 2007, Mandery et al. 2012). In vivo, significant interactions have been found between certain OATP substrates and grapefruit juice, such as aliskiren (Tapaninen, Neuvonen & Niemi 2010) and celiprolol (Lilja et al. 2003). The clinical relevance of the other non-CYP3A4-mediated effects remains unknown.

Genetic polymorphisms in the CYP3A4 and CYP3A5 genes can modulate CYP3A activity and have clinical consequences on drug metabolism. The single nucleotide variation (SNV) CYP3A4*22 c.522-
191C>T (rs35599367), with an allelic frequency of 5% in Caucasians, reduces the hepatic expression of CYP3A4 both in heterozygous and homozygous carriers of the allele (Wang et al. 2011, Klein et al. 2012). Patients carrying the CYP3A4*22 allele required 40-80% lower atorvastatin, simvastatin, or lovastatin doses for optimal lipid control (Wang et al. 2011). The CYP3A5*3 c.219-237A>G (rs776746) SNV is a common variant of the CYP3A5 gene, resulting in aberrant splicing and decreased CYP3A5 expression. The allelic frequency of the CYP3A5*3 is over 90% in Caucasians and consequently only 10 to 15% express CYP3A5 at significant levels. CYP3A4 and CYP3A5 have generally overlapping substrate specificity and they both contribute to the total CYP3A activity with CYP3A4 as the primary enzyme in most Caucasians. However, genetic variations in CYP3A5 activity have been shown to affect the pharmacokinetics of certain drugs, e.g. tacrolimus (Hesselink et al. 2003, Anglicheau et al. 2007, Staatz, Goodman & Tett 2010, Elens et al. 2011b), verapamil (Jin et al. 2007), and saquinavir (Josephson et al. 2007).

CYP3A-activity contributes to the bioactivation of clopidogrel and prasugrel, and is essential to the metabolic elimination of ticagrelor. Previous studies have shown that the potential for drug-drug interactions with CYP3A4 inhibitors differs between these antiplatelet drugs. Based on current pharmacogenetic data, CYP3A5 genotype does not seem to impact the pharmacokinetics or pharmacodynamics of clopidogrel, prasugrel, or ticagrelor but the effect of the CYP3A4*22 reduced-function allele is unknown (Hulot et al. 2006, Mega et al. 2009b, Kim, Park & Park 2008, Li et al. 2017). The purpose of this thesis was to study the significance of intestinal CYP3A4-mediated metabolism and the effect of grapefruit juice on the pharmacokinetics and pharmacodynamics of clopidogrel, prasugrel, and ticagrelor. Furthermore, the impact of variability in CYP3A activity caused by the CYP3A5*3 and the CYP3A4*22 alleles on the pharmacokinetics and antiplatelet effect of these drugs was investigated.
1. REVIEW OF THE LITERATURE

1.1. Pharmacokinetics

Pharmacokinetics describes in quantitative terms how the body handles a drug. Several distinct processes occur in the passage of a drug through the body: absorption, distribution, metabolism, and excretion. The relationship of a drug dose and its concentration in different tissues and time points depends on these pharmacokinetic processes. The effect of a drug is usually related to its concentration at the site of action. Therefore, pharmacokinetics has an essential role in the therapeutic use and possible toxicity of a drug (Rowland 2011).

Drug metabolism is an important part of pharmacokinetics. Over two thirds of clinically used drugs are mainly eliminated by metabolism in the body (Pelkonen et al. 2008). Liver and intestine are the most important sites for drug metabolism. Hydrophilic drugs are often excreted unchanged. However, most drugs need to be metabolized to become hydrophilic and to facilitate their excretion (Brodie, Gillette & La Du 1958). Drug metabolism can be divided into phase I and phase II reactions. Phase I reactions, including oxidation, reduction, and hydrolysis, introduce or expose a functional group in the drug molecule. This can result in loss of therapeutic function, or in the case of prodrugs, gain of function. Phase I enzymes include cytochrome P450 enzymes (CYPs), aldehyde dehydrogenases, carboxylesterases, and others. In phase II reactions, the functional group of a drug or drug metabolite is conjugated to a hydrophilic substance, such as glucuronic acid, sulphate, or glutathione. Some drugs undergo several metabolic pathways, while others only phase I or phase II reactions, and some are excreted completely unchanged (Testa, Kramer 2006).

To reach the therapeutic site of action, a drug often needs to pass several biological membranes. The passage can occur via either a paracellular or transcellular path. The transcellular path involves passive diffusion and active or facilitated mechanisms, such as channels and membrane transporters. Membrane transporter proteins transport both endogenous and exogenous substances, such as drugs, into and out from cells. An increasing amount of data is implicating the importance of membrane transporters in the pharmacokinetics of drugs (International Transporter Consortium 2010).

Pharmacokinetic interactions can occur when a drug is co-administered with another drug or substance capable of affecting some part of the pharmacokinetic process. The relevance of the interaction depends on the magnitude of the change in the affected drug plasma concentration and whether this leads to clinically important changes in therapeutic effect and/or toxicity. The interactions range from negligible to serious, or even life threatening (EMA 2013). Any part of the pharmacokinetics of a drug can be affected by an interaction. Changes in absorption can occur, for example in the gastrointestinal tract. Metabolism-based interactions usually originate from inhibition.
or induction of CYP enzymes, and membrane transporter activity can also be inhibited or induced. (Rowland 2011).

There are several ways to describe the pharmacokinetics of a drug, for example, depending on the site of measurements, such as plasma, serum, blood, urine, breath, milk, saliva, and solid tissues, and the properties of the drug, such as tissue distribution and systemic or local use. Plasma concentrations are commonly measured to quantitate the systemic pharmacokinetics of a drug. The highest plasma concentration following a single drug dose is called the peak plasma concentration (C_max). The time to the occurrence of C_max (T_max) is also commonly reported. After the peak plasma concentration is reached, the elimination of a drug can be quantified by following the decline in concentration in repeated samples. The time it takes for the drug concentration to halve is called the elimination half-life (t_1/2) and several distinct elimination phases can be observed in plasma, depending on the tissue distribution properties of a drug. Usually, the terminal half-life is reported as it describes the elimination of a drug from the body. Drug clearance describes the volume of plasma that is depleted of a drug per unit of time. The maximum clearance depends on the blood flow to the organ responsible for drug elimination (e.g. the liver, kidneys, lungs), and the organ’s ability to eliminate the drug. Theoretically, if the fraction of the drug eliminated from the plasma by the organ (extraction ratio, ER) is 1, the drug clearance equals the blood flow to the organ. A common way to quantitate the systemic exposure of a drug is to calculate the area under the plasma concentration-time curve (AUC), which accounts for both the concentration levels and the time under the possible effect of a drug. Finally, the variability of the pharmacokinetic parameters is commonly described by coefficient of variation (CV). CV is the ratio of standard deviation (SD) to the sample mean expressed as percentage. CV for AUC of 10% or less is typically considered low variability, 25 to 40% moderate and over 40% high variability. High variability in pharmacokinetics does not always imply high variability in drug response as the relation of a drug concentration and effect varies considerably between drugs. Consequently, variability in pharmacokinetics is of greatest concern when the drug has a narrow therapeutic window, that is, the difference between the low concentrations causing inadequate effect and the high concentrations causing adverse or excessive effects (Rowland 2011).

### 1.1.1. Drug metabolism and CYP enzymes

Cytochrome P450 was discovered in the 1950s during studies on carbon monoxide-mediated inhibition of adrenal and liver microsome reactions. CYP bound to carbon monoxide and in reduced form had a characteristic peak absorption wavelength of 450 nm. This substance was identified as haemoprotein and named P-450 (“Pigment-450”). (Omura, Sato 1962). Soon after that, the drug metabolizing ability of CYP was found (Estabrook, Cooper & Rosenthal 1963, Cooper et al. 1965), different forms of CYPs were identified, and several studies were initiated to characterize these forms.

Enzymes in the CYP superfamily catalyze most of the phase I oxidation reactions in drug metabolism (Wrighton, Stevens 1992). The catalytic mechanism is similar in all CYPs (Lin, Lu 1998), and in
addition to the role in drug and xenobiotic metabolism, they participate in several important endogenous functions, such as bile acid synthesis, cholesterol metabolism, and both synthesis and metabolism of steroids and vitamin D₃ (Nebert, Russell 2002). The most important sites of CYP activity and drug metabolism are the liver and intestine (Pelkonen et al. 2008), but CYPs are expressed in other tissues as well, such as lung and skin epithelia. In general, CYP activity is high at biological interfaces, which is indicative of the important role that CYPs play in xenobiotic and drug metabolism. At the cellular level, CYPs participating in drug metabolism are located in the endoplasmic reticulum, with their active sites at the cytosolic side of the membrane (Cribb et al. 2005).

The human CYP enzyme superfamily includes 57 genes, which are divided into 18 families and 44 subfamilies based on their similarities in amino acid sequence (Nelson et al. 1996, Nelson 2009). CYP1, CYP2 and CYP3 families are responsible for the majority of drug metabolism in humans (Zanger et al. 2008) (Table 1). CYP3A is the single most important drug-metabolizing enzyme subfamily in the liver and intestine, contributing to the metabolism of about half of the clinically used drugs, and over 30% of total CYP protein content in the liver and over 70% in the intestine. In the CYP3A subfamily, CYP3A4 is the major enzyme in most individuals. (Thelen, Dressman 2009, Pelkonen et al. 2008). Other important drug-metabolizing CYPs in the liver include CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A5 (Zanger, Schwab 2013).

The liver is considered the most important organ for both CYP-mediated first-pass metabolism and CYP-mediated overall clearance of drugs. However, the intestine also has drug-metabolizing ability and is an important extrahepatic site for drug biotransformation. The CYP3A protein contents (pmol/mg) in microsomal preparations are comparable in hepatic and intestinal samples but the total CYP3A content in the intestine is only about 1% of that of the liver when accounting for the organ masses (human liver and small intestine) (Yang, Tucker & Rostami-Hodjegan 2004). The extraction ratio of midazolam in the intestine, however, was found to be similar to that in the liver, and several other studies have also indicated the importance of intestine in the first-pass metabolism of orally administrated CYP3A substrates (Thummel et al. 1996, Thelen, Dressman 2009). Apart from CYP3A, a very limited amount of CYP expression is found in the intestine compared with that in the liver. CYP2C is the second most abundant subfamily with 15-20% contribution to the total CYP content in both liver and intestine. In the intestine, CYP2C9 is the most important enzyme in the CYP2C subfamily, followed by CYP2C19. Some CYP2D6 activity can also be found in the small intestine (Drozdzik et al. 2017). The average microsomal protein content (pmol/mg) in the intestine was only about 10% of the hepatic levels in the CYP2C subfamily (Läpple et al. 2003, Paine et al. 2006). However, after correcting for the activity loss in intestinal microsomes due to enterocyte isolation method, comparable levels of hepatic and intestinal CYP2C activity have been proposed (Galetin, Houston 2006). There are inconsistencies in the reports of intestinal CYP activity. In addition to methodological problems, large inter-individual variation in the expression of both CYP3A and CYP2C subfamilies exists in the intestine, which could explain the varying results (Thelen, Dressman 2009). Of the other CYP subfamilies, CYP1A, CYP1B, CYP2J, CYP2D, CYP2E,
CYP2S, and CYP4F have been found to be expressed in the intestine, but generally with low protein concentrations, and their clinical significance is unclear (Thelen, Dressman 2009).

CYP activity is affected by various factors, such as age, sex, disease, and ethnicity. CYP activity in newborns is undeveloped, and while the specific fetal form CYP3A7 exists at birth, it disappears soon after (Lacroix et al. 1997, Tanaka 1998). CYP maturation occurs during the first year of life. The elimination of many drugs is faster in infants and young children than in adults due to the higher relative size of liver (Rowland 2011, Tanaka 1998). The hepatic clearance of many drugs decreases with advanced age, but this doesn’t seem to be due to reduced hepatic CYP activity, rather it reflects the decrease in liver blood flow and volume (Parkinson et al. 2004). Gender may have a clinically relevant effect on drug metabolism in certain situations. A genome-wide gene expression study in 112 male and 112 female liver samples from surgery patients identified more than 1300 genes whose mRNA expression was affected by sex, with majority of them showing higher expression in females. CYP1A2, CYP3A4 and CYP7A1 showed female bias, and CYP3A5 and CYP27B1 showed male bias (Zhang et al. 2011). Particularly, CYP3A4 substrates antipyrine, alfentanil, erythromycin, midazolam, and verapamil seem to be affected by gender (Cotreau, von Moltke & Greenblatt 2005).

The regulation of CYP enzymes involves hormones from the pituitary, adrenal and thyroid glands, pancreas and gonads, and differences in hormonal status could explain some of the inter-individual variation in CYP activity and drug metabolism (Gibson, Skett 2001). Certain disease states can influence CYP activity as well. Cytokines released in inflammation can inhibit CYP activity which could result in elevated drug concentrations (Gandhi, Moorthy & Ghose 2012, Raaska et al. 2002, Zanger, Schwab 2013). Liver cirrhosis can also result in decreased CYP activity in microsomal samples (Parkinson et al. 2004). Lastly, genetic polymorphism and ethnicity are both significant factors in inter-individual variation in specific CYP activity (Zanger et al. 2008, Zanger, Schwab 2013). The factors affecting selected clinically important CYP enzymes are summarized in Table 1.

1.1.2. CYP-related pharmacokinetic interactions

Besides being substrates for CYP enzymes, several drugs and other substances can either inhibit or induce the activity of a certain CYP or CYPs. Co-administration of drugs, or drug and another substance, may lead to clinically relevant interaction if the metabolism and elimination is significantly affected. The inhibition of a drug metabolizing CYP enzyme can result in increased drug exposure (usually defined by the AUC), which can lead to enhanced efficacy or even adverse effects and toxicity. In the case of prodrugs, the bioactivation and thereby therapeutic efficacy may be reduced by CYP inhibition. CYP enzyme induction may increase drug elimination to the extent that efficacy is compromised (Pelkonen et al. 2008). Table 1 summarizes selected important CYP-mediated interactions.

The inhibition of CYP enzymes involves direct interaction between the inhibitor and enzyme. The inhibition can be either reversible or irreversible (mechanism-based). Reversible inhibition is
characterized by non-covalent binding of the inhibitor to the enzyme and the enzyme-inhibitor complex retains the ability to dissociate. The enzyme inhibition ends after the removal of inhibitor, and the enzyme is not permanently destroyed. Reversible inhibition can be divided into competitive, non-competitive, and uncompetitive inhibition, depending on whether the inhibitor and the substrate binding site on the enzyme is the same, the inhibitor and the substrate binding sites are different, or the inhibitor binds to the enzyme-substrate complex, respectively. Mixed-type inhibition has elements of both competitive and non-competitive inhibition. Irreversible inhibitors require metabolic activation to form intermediates that inactivate the enzyme, usually by forming covalent bonds. This leads to permanent inhibition of the enzyme molecule and resynthesis of the enzyme is required before the activity is restored (Hollenberg 2002). The induction of CYP enzymes occurs typically by mechanism of transcription factor activation and increased protein synthesis (Handschin, Meyer 2003). The intracellular receptors involved in CYP induction include aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR), glucocorticoid receptor (GR), and constitutive androstane receptor (CAR) (Waxman 1999).

1.1.3. CYP3A

CYP3A4, which is the major isoform of CYP3A enzymes, is the most important CYP enzyme in both liver and intestine. It is estimated that CYP3A4 is involved in the metabolism of approximately 50% of clinically used drugs (Pelkonen et al. 2008, Wrighton, Stevens 1992) and it has an important role in the metabolism of clopidgrel, prasugrel, and ticagrelor (Kazui et al. 2010, Sugidachi et al. 2001, van Giezen et al. 2009). In liver microsomal samples, its expression level is around 60-150 pmol/mg and it contributes to about 25% of total hepatic CYP content (Guengerich 1989, Kolars et al. 1992, Ohtsuki et al. 2012, Achour, Barber & Rostami-Hodjegan 2014). The majority of individuals express CYP3A4 abundantly, but population variability is very high. Complete absence of CYP3A4 expression has been documented in only one case report (Werk et al. 2014). The contribution of the otherwise minor isoform CYP3A5 to CYP3A substrate metabolism may be substantial in CYP3A5 expressing individuals with low CYP3A4 expression as CYP3A4 and CYP3A5 have overlapping substrate specificity (Zanger, Schwab 2013). Only a minority of Caucasians express significant levels of CYP3A5, however, due to the common loss-of-function allele CYP3A5*3 c.219-237A>G (rs776746). CYP3A5 has a particularly important role in the metabolism of some drugs, such as tacrolimus. Accordingly, CYP3A5 pharmacogenetic variation has been associated with clinically significant effects in tacrolimus pharmacokinetics (Hesselink et al. 2003). PXR, CAR, and GR are involved in CYP3A4 regulation. The enzymatically active site of CYP3A4 is large and flexible, leading to a broad substrate specificity (Ekroos, Sjogren 2006). CYP3A4 substrates vary in size and structure and include both endogenous compounds, such as steroid hormones, and several clinically important drugs such as midazolam, cyclosporine, calcium-channel blockers, statins, and antiretroviral drugs (Dresser, Spence & Bailey 2000, Wrighton et al. 2000).

CYP3A4 can be induced by many substances including rifampicin, phenobarbital, phenytoin, carbamazepine, and St. John's wort (Kolars et al. 1992, Combalbert et al. 1989, Backman et al. 1996,

1.1.4. CYP2C19

CYP2C19 contributes to the hepatic CYP content to a much lesser extent (<5% of total CYP protein) compared to CYP3A4. However, it is involved in the metabolism of many clinically important drugs, such as citalopram, diazepam, and proton pump inhibitors (Chiba et al. 1993, Andersson et al. 1994, Pearce et al. 1996, Olesen, Linnet 1999). Furthermore, CYP2C19 has a key role in the bioactivation of clopidogrel (Dansette et al. 2012, Hulot et al. 2006, Kazui et al. 2010). Clopidogrel is also known to inhibit CYP2C19 activity (Richter et al. 2004, Hagihara et al. 2008). Other inhibitors of CYP2C19 include fluvoxamine, omeprazole, and certain antifungal drugs such as miconazole, voriconazole, and fluconazole (Chiba et al. 1993, Jeppesen et al. 1996, Ko et al. 1997, Niwa, Shiraga & Takagi 2005). The CYP3A4-inhibiting effect of grapefruit juice is well established. Nootkatone and bergamottin, both present in grapefruit juice, have also been shown to inhibit CYP2C19 activity in vitro (Tassaneeyakul et al. 2000). The nuclear receptors CAR, PXR, and GR are important in controlling the expression of CYP2C19 (Chen et al. 2003). Via these mechanisms, several drugs can also induce CYP2C19 activity, including rifampicin and phenobarbital (Chen, Goldstein 2009, Rana et al. 2010, Uppugunduri et al. 2012).
Table 1. Selected substrates, inhibitors and inducers of the most important CYP enzymes. The relative abundance of CYPs in liver is shown (%), as well as various other factors affecting the enzyme activity (↑, increased activity; ↓, decreased activity; f, female; m, male; factors with evidence for clinical relevance are underlined). Adapted from (Pelkonen et al. 2008, Zanger et al. 2008, Zanger, Schwab 2013, Achour, Barber & Rostami-Hodjegan 2014, Neuvonen 2012).

<table>
<thead>
<tr>
<th>CYP1A2 (&gt;10%)</th>
<th>CYP2C8 (&gt;5%)</th>
<th>CYP2C9 (&gt;15%)</th>
<th>CYP2C19 (&lt;5%)</th>
<th>CYP2D6 (&lt;5%)</th>
<th>CYP3A4 (25%)</th>
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<tbody>
<tr>
<td>↓ with age</td>
<td>Inflammation (↓)</td>
<td>Polymorphism (↑)</td>
<td>Inflammation (↓)</td>
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**SUBSTRATES**

| Agomelatine | Caffeine | Clozapine | Duloxetine | Olanzapine | Theophylline | Tizanidine | Zolmitriptan | Cerivastatin | Diclofenac | Fluvastatin | Glibenclamide | Ibuprofen | Losartan | Naproxen | Phenyttoin | S-warfarin | Citalopram | Clomipramine |
|-------------|----------|-----------|------------|------------|-------------|------------|-------------|--------------|------------|-------------|---------------|-------------|-----------|-----------|------------|------------|-----------|-----------|-------------|
|             |          |           |            |            |              |            |             |              |            |             |               |              |           |           |             |            |           |            |            |

**INHIBITORS**

| Ciprofloxacin | Fluvoxamine | Oral contraceptives | Gemfibrozil | Tramethoprim | Amiodarone | Fluconazole | Metronidazole | Voriconazole | Clopidogrel | Fluconazole | Fluvoxamine | Omeprazole | Buprobion | Fluoxetine | Paroxetine | Quinidine | Terbinafine | Amiodarone | Clarithromycin | Diltiazem | Erythromycin | Grapefruit juice | Indinavir | Itraconazole | Ketoconazole | Nelfinavir | Ritonavir | Saquinavir | Tekithromycin | Verapamil | Voriconazole |
|---------------|-------------|---------------------|-------------|--------------|-------------|-------------|---------------|-------------|-------------|-------------|-------------|-------------|-----------|-----------|------------|-----------|----------|------------|-------------|---------------|-------------|--------------|----------------|--------|-------------|---------------|-----------|-----------|-------------|-------------|-----------|-------------|
|               |             |                     |             |              |             |             |               |             |             |             |             |             |           |           |             |           |          |             |             |             |             |              |           |             |               |           |           |             |             |           |             |               |           |           |             |             |           |             |

**INDUCERS**

<table>
<thead>
<tr>
<th>Broccoli</th>
<th>Carbamazepine</th>
<th>Polyaromatic hydrocarbons (grilled food, cigarette smoke)</th>
<th>Rifampicin</th>
<th>Carbamazepine</th>
<th>Phenobarbital</th>
<th>Phenyttoin</th>
<th>Rifampicin</th>
<th>St. John’s wort</th>
<th>Phenobarbital</th>
<th>Rifampicin</th>
<th>St. John’s wort</th>
<th>Non-inducible</th>
<th>Bosentan</th>
<th>Carbamazepine</th>
<th>Dexamethasone</th>
<th>Phenobarbital</th>
<th>Phenyttoin</th>
<th>Rifampicin</th>
<th>St. John’s wort</th>
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1.1.5. Drug metabolism and non-CYP enzymes

CYP enzymes contribute to the primary metabolic clearance of two-thirds of clinically used drugs. In additions to CYPs, monoamine oxidases, arylacetamide deacetylase, paraoxonase, and esterases such as carboxylesterases (CES) and butyrylcholinesterase participate in phase I of drug metabolism. Transferases such as uridine diphosphate glucuronosyltransferase (UGT), N-acetyltransferase, and sulfotransferases mediate phase II reactions (conjugation reactions). (Williams et al. 2004, Fukami, Yokoi 2012, Oda et al. 2015).

1.1.5.1. Carboxylesterases

Carboxylesterases catalyze the hydrolysis of several endogenous and exogenous substances, such as toxins and drugs. Ester hydrolysis results in the formation of corresponding carboxylic acid and alcohol, and these have generally increased water solubility compared to the original ester, which can facilitate renal elimination. CESs are found in the cytoplasm and endoplasmic reticulum of various tissues, including the liver, small intestine, kidney, and lungs. CESs are expressed most abundantly in the liver and small intestine, where they can contribute significantly to the first-pass metabolism of many drugs (Taketani et al. 2007, Laizure et al. 2013). CES-mediated hydrolysis is involved in the metabolism of a wide array of drugs, including clopidogrel, prasugrel, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, statins, central nervous system stimulants, narcotic analgesics, antiviral agents, immunosuppressants, and oncology agents (Laizure et al. 2013, Satoh, Hosokawa 2006).

The relative importance of CESs can be high in the elimination pathway of a drug. Up to 90% of absorbed clopidogrel is rapidly hydrolyzed by CES1 to an inactive carboxylic acid metabolite and this is further conjugated to a glucuronic acid (Tang et al. 2006). CESs can also have a key role in the bioactivation of a prodrug. For example, CES1 is important in the bioactivation of oseltamivir to active oseltamivir carboxylate, which was demonstrated with the CES1 reduced-function single nucleotide variation (SNV) c.428G>A (rs121912777) (Zhu, Markowitz 2009, Tarkiainen et al. 2012). CESs can also be prone to drug-drug interactions. CES1 substrates and inhibitors include such commonly used drugs as enalapril, simvastatin, diltiazem, and carvedilol (Fukami et al. 2010, Kristensen et al. 2014, Thomsen et al. 2014). The CES1 substrate clopidogrel was shown to inhibit oseltamivir hydrolytic activation in microsomal models (Shi et al. 2006) and grapefruit juice flavonoids inhibited CES1 in vitro and in rat models (Li et al. 2007).
1.1.5.2. Drug transporters

Membrane transporter proteins mediate the transport of both endogenous and exogenous substances into and out from cells. Drugs may cross cell membranes via passive or facilitated diffusion and by active transport. An increasing amount of evidence from *in vitro*, drug-drug interaction, and pharmacogenetic studies involving transporter protein substrates, suggests that transporters play a clinically important role in the distribution of drugs across biological barriers (Dobson, Kell 2008, Giacomini, Sugiyama 2012, Giacomini *et al*. 2013). The drug transporters are mostly located in the intestinal, renal, and hepatic epithelia, and based on their function, can be classified as either influx or efflux transporters. The influx transporters mediate drug entry into cells and belong to the solute carrier (SLC) superfamily of transporters. Examples of influx transporters include organic anion transporters (OATs), organic cation transporters (OCTs), and organic anion-transporting polypeptides (OATPs). The efflux transporters mediate drug exit from the cell and most belong to the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily. Examples of efflux transporters include P-glycoprotein (P-gp), the multi-drug resistance-associated protein family (MRP), and the breast cancer resistance protein (BCRP). In addition to mediating drug distribution in specific tissues, transporters may also act as protective barriers to organs. For example, P-gp has a role in the blood-brain barrier. Drug transporters may also contribute to drug resistance, e.g. overexpression of P-gp in tumour cells may result in lack of therapeutic effect due to increased efflux of anticancer drugs (Giacomini, Sugiyama 2012).

Pharmacokinetic interactions involving transporter proteins can lead to alterations in drug plasma concentrations and tissue distribution. The location and type of transporter determines the outcome of the interaction. Inhibition of intestinal influx transporters, such as certain OCTs and OATPs located in the enterocyte basolateral membrane, may lead to decreased drug absorption and consequently exposure. Inhibition of an efflux transporter, such as P-gp in the same location, however, may lead to increased drug exposure (Giacomini, Sugiyama 2012). Transporter induction is also a possible mechanism causing interactions but the number of known induction-mediated interactions is low compared to transporter inhibition (Konig, Muller & Fromm 2013). Many transporters and CYPs have overlapping substrate specificities and are regulated by the same nuclear receptors, which can make the evaluation of the role of a single transporter or CYP difficult (Yoshida, Maeda & Sugiyama 2012). Furthermore, transporter interactions may change the concentration of a drug within a specific tissue without altering its plasma concentration, because the amount of drug distributing to the tissue is small compared to that found in the whole body, or the drug is eliminated mainly by metabolism, not by transport (Endres *et al*. 2006). Examples of clinical interactions include itraconazole-mediated inhibition of renal P-gp which increased the exposure of aliskiren (Tapaninen *et al*. 2011) and digoxin (Jalava, Partanen & Neuvonen 1997). Similarly, the P-gp inhibitors cobicistat and verapamil increased dabigatran exposure (Kumar *et al*. 2017, Hartter *et al*. 2013). Induction of intestinal P-gp was also shown to decrease digoxin exposure (Greiner *et al*. 1999). Grapefruit juice is known to inhibit OATP1A2 and OATP2B1 *in vitro* (Satoh *et al*. 2005b, Bailey *et al*. 2007) and grapefruit juice, as well as orange and apple juice, has also reduced the oral bioavailabilities of some OATP1A2 or
1.2. Pharmacodynamics

Pharmacodynamics describes in quantitative terms how the drug affects the body. A drug produces its therapeutic effect when there is adequate concentration and exposure of the drug (or in the case of a prodrug, active metabolite) at the target site in the body. This is how pharmacokinetics and pharmacodynamics intertwine. The actual correlation of concentration and effect, however, is often complex and non-linear, and varies considerably from drug to drug. Pharmacokinetic-pharmacodynamic (PK/PD) modeling attempts to predict drug responses at given concentrations with statistical models based on empirical data (Rowland 2011). The target site in the body can be an enzyme or receptor, or it can be a gene or DNA itself. Sometimes the mechanism of action is unknown or can include multiple different components. (Mager, Wyska & Jusko 2003). Pharmacodynamic drug interactions can be caused by a variety of mechanisms and these can lead to antagonistic, synergistic, or additive effects (Danhof et al. 2007).

1.3. Pharmacogenetics

Pharmacogenetics is the study of genetic variations and their relation to interindividual variation in drug response. The clinical aim of pharmacogenetics is to facilitate the personalization of drug therapy through genotype-specified drug and dose selection in order to maximize drug efficacy and minimize adverse drug reactions (Eichelbaum, Ingelman-Sundberg & Evans 2006). The variations (polymorphism) in DNA nucleotide sequence include nucleotide substitutions (single-nucleotide variation, SNV), insertions, deletions, duplications, short sequence repeats, and gene copy-number variations. Drug-metabolizing enzymes, drug transporters and proteins involved in the pharmacodynamics of a drug can be affected by functionally significant polymorphisms leading to variations in drug responses (Eichelbaum, Ingelman-Sundberg & Evans 2006, Turner, Pirmohamed 2014). The amino acid sequence, and consequently the structure and function of the translated protein may be altered by polymorphism in the coding regions of the gene (non-synonymous variants). Synonymous polymorphisms that do not alter the amino acid sequence can affect the secondary structure, stability or translation efficiency of messenger ribonucleic acid (mRNA), causing altered protein expression levels. Variations in the regulatory and non-coding regions of the gene can influence gene expression and thus the quantity of the translated protein. Polymorphisms in splice sites and splicing regulatory sites can alter RNA splicing, affecting the produced mRNA. The polymorphisms can be further classified as cis-acting, which are located close to the target genes, or trans-acting, which are far from the target genes, even on other chromosomes (Pastinen, Ge & Hudson 2006, Cheung, Spielman 2009).
A wide array of different polymorphisms with suggested clinical importance have been discovered. While the impact on appropriate drug dose and risk of adverse effects is well characterized in many of the pharmacogenetic determinants, there is a controversy over the clinical utility of this knowledge. Inadequate levels of research evidence, as well as financial and logistical problems, hinder the widespread clinical use of pharmacogenetic data (Turner, Pirmohamed 2014). Nevertheless, numerous examples exist with the potential for meaningful personalization of drug therapy. Warfarin pharmacodynamics is altered by a relatively common (minor allelic frequency, MAF, of 40% in Caucasians) decreased activity variant in its target, the vitamin K epoxide reductase complex subunit 1 \( VKORC1 \). \( VKORC1 \) \(-1639G>A\) (rs9923231) SNV leads to reduced warfarin dose requirements and explains about 20–25% of the variance in warfarin maintenance dose in Caucasian and Asian populations (Owen \textit{et al.} 2010, Johnson \textit{et al.} 2011). \( CYP2C9*2 \) (rs1799853) and \( CYP2C9*3 \) (rs1057910) variant alleles lead to decreased CYP2C9 function and reduced warfarin dose requirements (Johnson \textit{et al.} 2011). With \( CYP2C9*3 \), an increased risk of bleeding in warfarin users has been suggested by meta-analysis (Yang \textit{et al.} 2013). The loss-of-function allele \( CYP2C19*2 \) c.681G>A (rs4244285) has been strongly associated with impaired clopidogrel bioactivation and risk of stent thrombosis as discussed later. Finally, the \( SLCO1B1*5 \) (rs4149056) SNV prevents the normal localization of OATP1B1 to the hepatic cell membrane, impairing its transport capacity. This variant has been associated with higher plasma concentrations of simvastatin acid, and increased risk for adverse effects, such as statin induced myopathy (Pasanen \textit{et al.} 2006, SEARCH Collaborative Group \textit{et al.} 2008, Voora \textit{et al.} 2009, Carr \textit{et al.} 2013). There are several other examples of potentially significant polymorphisms affecting drug pharmacokinetics and pharmacodynamics and significant effort is put into elucidating their possible clinical impact (Eichelbaum, Ingelman-Sundberg & Evans 2006, Turner, Pirmohamed 2014, Niemi, Pasanen & Neuvonen 2011).

### 1.3.1 CYP3A4

There is a high degree of heritability in CYP3A4 drug metabolizing capacity towards several of its substrates but the genetic basis for these observations have been difficult to elucidate. Attempts to associate CYP3A4 enzymatic activity to specific genotypes have resulted in the discovery of numerous functionally significant, but rare CYP3A4 variants and much of the observed functional variability remains unexplained (Ozdemir \textit{et al.} 2000, Sadee 2012). The exonic variants \( CYP3A4*2 \), \( *7 \), \( *8 \), \( *11 \), \( *12 \), \( *13 \), \( *16A \), \( *16B \), and \( *20 \) lead to decreased or lost enzymatic activity but their minor allelic frequencies (MAFs) are very low (Werk, Cascorbi 2014). Polymorphism in \( FoxA2 \), \( HNF4a \), \( FoxA3 \), \( PXR \), \( ABCB1 \) genes, and the \( CYP3A4 \) promoter explained about 25% of the variation in hepatic \( CYP3A4 \) mRNA expression in one study (Lamba \textit{et al.} 2010). Others identified SNPs in the Ah-receptor nuclear translocator (ARNT), GR, progesterone receptor membrane component 2 \( (PGRMC2) \), and peroxisome proliferator activated receptor alpha \( (PPAR\alpha) \) to be associated with CYP3A4 phenotype. In multivariate analysis, two linked \( PPAR\alpha \) SNVs explained about 8–9% of the atorvastatin hydroxylase activity variation, whereas all genetic and nongenetic factors together explained about 33% of the atorvastatin metabolic variation (Klein \textit{et al.} 2012).
A relatively common variant allele CYP3A4*1B c.-392G>A (rs2740574) occurs at over 50% frequency in African populations and 3-5% in Caucasians. CYP3A4*1B was initially associated with higher tumor grade and stage in prostate cancer and showed higher nifedipine oxidase activity in human livers (Rebbeck et al. 1998). However, later studies have failed to demonstrate any effect of this variant on CYP3A4 activity (Klein et al. 2012). In contrast, the CYP3A4*22 intron 6 c.522-191C>T (rs35599367) variant allele is well characterized as having impact on CYP3A4 function. This SNV leads to decreased expression of CYP3A4 mRNA in cultured cells (Wang et al. 2011) and decreased protein content in human livers (Klein et al. 2012). There was also a significant association of CYP3A4*22 with decreased 2-OH-atorvastatin/atorvastatin AUC₀–∞ ratio in atorvastatin-treated volunteers (Klein et al. 2012) and the metabolism of both midazolam and erythromycin was decreased in CYP3A4*22 carriers (Elens et al. 2013). Clinical studies support the functional significance of CYP3A4*22, as well (Werk, Cascorbi 2014). CYP3A4*22 allele carrying patients treated with atorvastatin, simvastatin, or lovastatin required 1.7- to 5-fold reduced statin doses compared to non-carriers for optimal lipid control (Wang et al. 2011). Others had similar results for simvastatin pharmacodynamics (Elens et al. 2011a), and renal transplant recipients who were carriers of the reduced-function CYP3A4*22 allele had a 33% reduced mean daily-dose requirement to reach the same tacrolimus blood concentration compared to homozygotes of the wild type CYP3A4*1 allele (Elens et al. 2011b) and 1.6 to 2.0-fold higher dose-adjusted plasma concentrations of tacrolimus and cyclosporine in stable renal transplant patients (Elens et al. 2011). In one study, CYP3A*22 has also been shown to associate to higher risk of delayed graft function in cyclosporine treated kidney transplant patients. The mechanism of this observation is unclear, however, as cyclosporine exposure did not differ between the genotype groups (Elens et al. 2012). Despite convincing evidence for the functional significance of CYP3A4*22, its contribution to the overall variability of CYP3A4 activity is limited as the allele is rare: 5% MAF in Caucasians and less than 1% in Africans and Asians (Zhou, Ingelman-Sundberg & Lauschke 2017).

1.3.2 CYP3A5

CYP3A5 expression is markedly polymorphic. Only 5-10% of Caucasians, but over 60% of Africans and Asians have a significant amount of CYP3A5 in the liver. The ethnic differences are mostly explained by two alleles that result in aberrant splicing and deficient expression of the functional transcript. The intronic variant CYP3A5*3 is very common in Caucasians (94% allelic frequency). The frequency in Asian populations is lower, at around 70%, and markedly so in Africans at 18% (Zhou, Ingelman-Sundberg & Lauschke 2017). The CYP3A5*6 exon variant (rs10264272) also leads to an aberrantly spliced mRNA and is only present in populations of African origin (Kuehl et al. 2001). Due to CYP3A5*22, the proportion of Caucasians with a functional copy of the CYP3A5 gene (CYP3A5*1 allele) is considerably lower than in Asian and African populations.

CYP3A5 and CYP3A4 have generally overlapping substrate specificity (Daly 2006). However, metabolism by CYP3A5 is preferred over CYP3A4 in the elimination of certain drugs, such as tacrolimus, and CYP3A5 polymorphism may have a clinically significant effect on the
pharmacokinetics of these drugs. Associations between pharmacokinetics and CYP3A5 genotype were reported for tacrolimus (Hesselink et al. 2003, Anglicheau et al. 2007, Staatz, Goodman & Tett 2010, Elens et al. 2011b), cyclosporine (Hu et al. 2006), verapamil (Jin et al. 2007), and saquinavir (Josephson et al. 2007). The median tacrolimus through concentration in CYP3A5 expressors (carriers of at least one CYP3A5*1 allele) was 61 ng/mL compared to 94 ng/mL in non-expressors (the CYP3A5*3/*3 genotype). No difference was found between heterozygous and homozygous carriers of the CYP3A5*1 allele (Hesselink et al. 2003). The median cyclosporine through concentration in CYP3A5 expressors with the CYP3A5*1/*1 genotype was 14.8 ng/mL, 23.7 ng/mL in patients with the CYP3A5*1/*3 genotype, and 26.4 ng/mL in non-expressors (Hu et al. 2006). The median AUCs of both verapamil and saquinavir were 50% higher in healthy CYP3A5 non-expressors compared to expressors (carriers of at least one CYP3A5*1 allele) (Jin et al. 2007, Josephson et al. 2007).

In a large cohort of kidney transplant recipients (n=446), CYP3A5*3 alone explained 39%, and clinical covariates and CYP3A5*3 together explained 46% of the variability of tacrolimus blood concentration to dose ratio at steady state (Birdwell et al. 2012). In another trial, a predictive model including age, ethnicity, and concomitant use of medications explained around 30% of the variability in tacrolimus dosing. Combined with the inclusion of CYP3A5*3, this model explained 58% of the variability (Wang et al. 2010).

1.3.3. CYP2C19

Numerous variants of the ancestral CYP2C19*1 allele have been found. However, most of the loss-of-function alleles (e.g., *2-*8) are rare. The exception is the CYP2C19*2 SNV with an estimated minor allelic frequency of 18% in both Africans and Caucasians, and around 30% in Asians. The CYP2C19*2 c.636G>A (rs4986893) variant is present in East Asian populations with a frequency of 7%, but in Africans and Caucasians, this SNV is extremely rare (Zhou, Ingelman-Sundberg & Lauschke 2017). The impact of CYP2C19*2 and CYP2C19*3 is particularly well characterized in the bioactivation of clopidogrel. Both alleles are associated with reduced clopidogrel active metabolite plasma concentrations and for CYP2C19*2, a meta-analysis supports its per-allele association to high on-treatment platelet activity (Holmes et al. 2011). Numerous other studies and meta-analyses report its association to stent thrombosis after percutaneous coronary intervention (PCI) in patients using clopidogrel (Turner, Pirmohamed 2014, Mega et al. 2009a, Mega et al. 2010b). At least one systematic review has questioned the associations of the loss-of-function alleles with major cardiovascular events, with the suggestion that publication bias and/or heterogeneity of the original studies may explain much of the observed risk increase (Osnabrugge et al. 2015). Furthermore, genotyping PCI patients routinely for CYP2C19 is not supported by the European Society of Cardiology, as randomized clinical trials have not demonstrated any clinical benefit of genotype guided dual antiplatelet therapy (Valgimigli et al. 2018). The impact of CYP2C19 genotype in patients receiving clopidogrel therapy for other indications (e.g. ischemic stroke or peripheral artery disease)
is somewhat uncertain. However, a recent meta-analysis suggests that the risk of stroke is indeed increased in clopidogrel-treated carriers of the *CYP2C19* loss-of-function alleles (Pan *et al.* 2017).

The gain of function (enhanced transcription) *CYP2C19*17 c.-806C>A (rs12248560) SNV has been associated with a decreased incidence of cardiovascular events and increased bleeding risk in patients treated with clopidogrel in some studies (Li *et al.* 2012, Li-Wan-Po *et al.* 2010, Tiroch *et al.* 2010). Others, however, have suggested that the observed effect of this variant is due to linkage disequilibrium with the *CYP2C19*2 loss-of-function variant and that the effect of *CYP2C19*17 alone is negligible on clopidogrel active metabolite formation (Lewis *et al.* 2013b). The MAFs of *CYP2C19*17 are estimated at 24%, 1.5%, 14%, and 22% in Africans, East Asians, South Asians and Caucasians, respectively (Zhou, Ingelman-Sundberg & Lauschke 2017).

### 1.3.4. Carboxylesterase 1

Several sequence variations have been found in the CES genes, some with functional effects (Marsh *et al.* 2004, Zhu *et al.* 2008). Of these, the CES1 c.428G>A SNV, which results in decreased enzymatic activity, is particularly well characterized. The minor allelic frequency of this SNV was estimated to be 4.3% in African-Americans, 3.7% in Caucasians, 2.0% in Hispanics, and 0% in Asians (Zhu *et al.* 2008). There is increasing evidence that polymorphism in the CES genes may have clinically relevant effects on drug pharmacokinetics. The CES1 c.428G>A SNV decreased the enzymatic activity of CES1 and impaired oseltamivir bioactivation to active oseltamivir carboxylate *in vivo* (Tarkiainen *et al.* 2012). The majority of orally administered clopidogrel is rapidly hydrolyzed to an inactive carboxylic acid metabolite by CES1 (Tang *et al.* 2006). Supporting the hypothesis that reduced clopidogrel hydrolysis by this SNV could lead to an increase in clopidogrel bioactivation, the active metabolite plasma concentrations were found to be about 60% higher in seven healthy Amish individuals with the CES1 variant genotype than in individuals with the CES1 wild type genotype (Lewis *et al.* 2013a). Furthermore, in addition to increased active metabolite concentrations, the clopidogrel antiplatelet effect was enhanced in healthy volunteers with the CES1 c.428G>A SNV. (Tarkiainen *et al.* 2015)

### 1.4. Grapefruit juice

#### 1.4.1 Grapefruit juice and CYP3A4

Grapefruit juice was first accidentally discovered to cause pharmacokinetic interactions when it was used to mask the taste of ethanol in a study involving felodipine. Co-administration of felodipine with grapefruit juice resulted in significant increase in the plasma concentrations of felodipine (Bailey *et al.* 1991, Bailey *et al.* 1989). Afterwards, mechanism-based inhibition of intestinal CYP3A4 by

Numerous in vivo studies have been conducted since the discovery of the CYP3A4 inhibiting activity of grapefruit juice and it has been shown to significantly increase the oral bioavailability of various CYP3A4 substrate drugs. AUC increases as high as 1500% have been reported (Table 2). Inhibition of intestinal CYP3A4 by grapefruit juice constituents has been shown to occur rapidly; 6’7’-dihydroxybergamottin reached maximum inhibitory effect in 30 minutes, whereas bergamottin acted slower (maximum inhibition within 3 hours) (Paine, Criss & Watkins 2005). The expression of CYP3A4 protein in the duodenum has been shown to decline by 30% within 1-2 hours after ingestion of 300 ml of grapefruit juice (Glaeser et al. 2007). In another study, grapefruit juice was ingested three times a day for six days, resulting in 62% reduction in the concentration of CYP3A4 in the duodenal epithelia. The CYP3A4 activity in the liver was unaffected (determined using a ¹⁴C-N-methyl-erythromycin breath test), as were the duodenal P-gp, CYP1A1, and CYP2D6 concentrations and the CYP3A5 content in colon (Lown et al. 1997). The inhibition of felodipine metabolism has been shown to reach its maximum already after the first glass of grapefruit juice co-administered with the drug (Lundahl et al. 1998). On the other hand, double-strength grapefruit juice taken three times a day for two days had a greater effect on the AUC of triazolam (increased to 240% of control) than single doses of normal-strength or double-strength grapefruit juice (both increased to 150% of control). Multiple doses also prolonged the t½ of triazolam by 53%, suggesting inhibition of both intestinal and hepatic CYP3A4 (Lilja et al. 2000). Repeated consumption of grapefruit juice has been shown to prolong the t½ of several other CYP3A4 substrates, including atorvastatin, buspirone, cisapride, midazolam, and oxycodone (Lilja et al. 1998, Kivisto et al. 1999, Lilja, Kivisto & Neuvonen 1999, Nieminen et al. 2010). Furthermore, one study reported a decrease in the amount of exhaled ¹⁴C-N-methyl-erythromycin after repeated consumption of double strength grapefruit juice over several days, also suggesting inhibition of hepatic CYP3A4 (Veronese et al. 2003). Grapefruit juice has not inhibited the hepatic metabolism of several intravenously administered CYP3A4 substrates, however, including midazolam, cyclosporine, felodipine, or saquinavir (Ducharme, Warbasse & Edwards 1995, Kupferschmidt et al. 1995, Lundahl et al. 1997, Kupferschmidt et al. 1998b). The inhibition of CYP3A4 by grapefruit juice has been found to last at least 24 hours and in some cases residual effect was seen up to three days after the juice ingestion (Greenblatt et al. 2003, Lundahl et al. 1995, Lilja, Kivisto & Neuvonen 2000, Takanaga et al. 2000, Culm-Merdek et al. 2006). No effect on the pharmacokinetics of simvastatin was seen seven days after the ingestion of grapefruit juice (Lilja, Kivisto & Neuvonen 2000).
1.4.2 Grapefruit juice and other CYPs

Grapefruit juice is best characterized as an inhibitor of CYP3A4. However, grapefruit juice constituents nootkatone, bergamottin, 6’7’-dihydroxybergamottin, and furanocoumarin dimers GF-I-1 and GF-I-4 affected the function of other CYPs as well in an *in vitro* study with human liver microsomes (Tassaneeyakul *et al.* 2000). In addition to CYP3A4, bergamottin and 6’7’-dihydroxybergamottin inhibited CYP1A2, CYP2C9, CYP2C19, and CYP2D6 with IC₅₀ values for corresponding oxidations ranging from 0.19 to 0.32 μmol/l with bergamottin and 0.6 to 4 μmol/l with 6’7’-dihydroxybergamottin. Nootkatone inhibited CYP2A6 and CYP2C19, with an IC₅₀ value of 11.5 μmol/l for coumarin 7-hydroxylation and 22.5 μmol/l omeprazole 5-hydroxylation, respectively. GF-I-1 and GF-I-4 were the most potent inhibitors of CYP3A4; both had 0.003 μmol/l IC₅₀ for nifedipine oxidation compared to the IC₅₀ values of 1.5 μmol/l and 0.65 μmol/l found for bergamottin and 6’7’-dihydroxybergamottin, respectively. However, the furanocoumarin dimer concentrations in the juice were 20-200 fold lower compared to those of bergamottin and 6’7’-dihydroxybergamottin (Tassaneeyakul *et al.* 2000). There is a considerable variation in the concentrations of bergamottin, 6’7’-dihydroxybergamottin in grapefruit juice, depending on the study and juice brand investigated. Bergamottin is found at concentrations ranging from 3 to 33 μmol/l, and 6’7’-dihydroxybergamottin levels range from 1-44 μmol/l. Depending on the juice brand, both furanocoumarins could affect CYP1A2, CYP2C9, CYP2C19, and CYP2D6, at least based on the IC₅₀-values. Nootkatone concentrations have been reported at 2-3 μmol/l which are low compared to the IC₅₀-values for CYP2A6 and CYP2C19 inhibition (Schmiedlin-Ren *et al.* 1997, Edwards, Bellevue & Woster 1996, He *et al.* 1998, Guo *et al.* 2000, Kakar *et al.* 2004, Kirchner, Miller 1953, Bailey *et al.* 2000, Messer *et al.* 2011). Few *in vivo* studies suggest grapefruit juice-mediated inhibition of CYPs other than CYP3A4, however. Furthermore, the metabolism of lansoprazole, a substrate of CYP2C19, was unaffected by grapefruit juice (Uno *et al.* 2005).

Grapefruit juice impaired the platelet inhibitory activity of clopidogrel in healthy volunteers. The effect of grapefruit juice on the antiplatelet effect of clopidogrel was studied in both after a loading dose and seven days maintenance therapy of clopidogrel. The *ex vivo* platelet aggregation during grapefruit juice consumption was higher on average, and more frequently over the predefined high on-treatment platelet reactivity level compared to control (Campbell *et al.* 2014). The interaction was statistically significant in the loading dose protocol, and a similar trend was observed in the maintenance dose protocol. The pharmacokinetics of clopidogrel was not investigated in the study, but the pharmacodynamic interaction suggests impaired clopidogrel bioactivation, possibly due to CYP3A4 or CYP2C19 inhibition by grapefruit juice.

Both CYP2C19 and CYP3A4 contribute significantly to the metabolism of diazepam (Andersson *et al.* 1994). Grapefruit juice has been shown to inhibit the metabolism of diazepam, resulting in a 3.2 fold increase in diazepam AUC compared to control (Ozdemir *et al.* 1998). Considering the magnitude of the interaction, it is possible that CYP2C19 inhibition was involved as well.
Grapefruit juice inhibited the metabolism of deuterium-labeled nicotine to cotinine and increased the renal clearance of both nicotine and cotinine in healthy volunteers. Grapefruit juice consumption reduced cotinine AUC to 85% compared to controls. The oral clearance of nicotine was unaffected by grapefruit juice, however, as the metabolic effect was offset by an increase in the renal clearance of nicotine (Hukkanen et al. 2006). Grapefruit juice has also been shown to inhibit the CYP2A6-mediated metabolism of coumarin to 7-hydroxycoumarin, observed as decreased 7-hydroxycoumarin excretion in urine (Merkel et al. 1994, Runkel et al. 1997 and Bourian et al. 1999).

1.4.3. Grapefruit juice and drug transporters

Besides CYPs, grapefruit juice seems to inhibit drug transporters as well. This was first discovered because grapefruit, orange, and apple juices were shown to decrease the AUC of fexofenadine by 60-70% (Dresser et al. 2002). The metabolism of fexofenadine is negligible (Russell, Stoltz & Weir 1998) and it is a substrate of P-gp, OATP1A2, OATP1B1, OATP1B3 and OATP2B1 (Dresser et al. 2002, Glaeser et al. 2007, Cvetkovic et al. 1999, Nozawa et al. 2004, Niemi et al. 2005, Shimizu et al. 2005). Both grapefruit and orange juices were shown to inhibit OATP1A2-mediated fexofenadine uptake in vitro in human cervical carcinoma (HeLa) cells. Accordingly, the suggested mechanism of the in vivo fruit juice-fexofenadine interactions was inhibition of OATP1A2-mediated uptake of fexofenadine in the small intestine by these juices (Dresser et al. 2002). Furthermore, grapefruit juice has been shown to reduce the AUC of celiprolol and talinolol, which are substrates of both OATP1A2 and OATP2B1 (Lilja et al. 2003, Schwarz et al. 2005). The AUC of aliskiren, a substrate of CYP3A4, OATB1A2 and OATP2B1, was also reduced significantly by grapefruit juice (Tapaninen, Neuvonen & Niemi 2010a). In vitro studies have been carried out to identify the grapefruit constituents responsible for drug transporter interactions. The grapefruit flavonoid naringin was identified as a potent inhibitor of OATP1A2 (Bailey et al. 2007), and it also inhibited OATP2B1 (Satoh et al. 2005a). Naringenin inhibited OATP1B1 and OATP1B3 (Mandery et al. 2012). Naringenin and the other grapefruit juice constituents quercetin, bergamottin, and 6’7’-dihydroxybergamottin inhibited OATP2B1 (Satoh et al. 2005a) and quercetin inhibited OATP1A2 as well (Mandery et al. 2010). Nonetheless, the clinical relevance of these findings is unclear and grapefruit juice has not affected the pharmacokinetics of the OATP2B1 substrate glibenclamide, in humans (Lilja et al. 2007). The duration of OATP inhibition by grapefruit juice appears to be shorter compared to CYP3A4 inhibition. The grapefruit juice-fexofenadine interaction was significant up to two hours after grapefruit juice ingestion, but no effect was seen when fexofenadine was taken four hours after grapefruit juice (Glaeser et al. 2007). No change in the intestinal OATP1A2 expression was seen 1-2 hours after grapefruit juice ingestion (Glaeser et al. 2007) and repeated juice consumption had an effect comparable to a single glass of grapefruit juice on talinolol pharmacokinetics (Schwarz et al. 2005). These findings suggest a different mechanism of OATP inhibition by grapefruit juice compared to CYP3A4.
The grapefruit juice constituents 6’7’-dihydroxybergamottin and naringin have been shown to inhibit P-gp, with IC\textsubscript{50} values of 33 μmol/l and 3000 μmol/l, respectively (Dresser \textit{et al.} 2002). Bergamottin, by contrast, failed to inhibit P-gp activity at concentrations of up to 50 μmol/l. However, 6’,7’-dihydroxybergamottin and naringin are typically found in grapefruit juice at low concentrations in relation to IC\textsubscript{50} values to P-gp-inhibition, 1-44 μmol/l and 130-1230 μmol/l, respectively (Schmiedlin-Ren \textit{et al.} 1997, Edwards, Bellevue & Woster 1996, Guo \textit{et al.} 2000, Kakar \textit{et al.} 2004). Accordingly, grapefruit juice has not been shown to affect the pharmacokinetics of the P-gp model substrate drug digoxin (Becquemont \textit{et al.} 2001, Parker \textit{et al.} 2003) nor the expression level of P-gp in the small intestine (Lown \textit{et al.} 1997, Glaeser \textit{et al.} 2007). When substrates of both P-gp and OATPs have been studied, the OATP-inhibiting effect appeared to be dominant (Dresser \textit{et al.} 2002, Glaeser \textit{et al.} 2007, Cvetkovic \textit{et al.} 1999, Nozawa \textit{et al.} 2004, Lilja \textit{et al.} 2003, Schwarz \textit{et al.} 2005).
Table 2. The effect of co-administration of grapefruit juice on the AUC of various drugs. AUC ratio (grapefruit juice / control, typically water) is reported. *Cotinine AUC ratio. **Possibly involving inhibition of CYP2C19. ***Bioavailability ratio (grapefruit juice / control). ****Through concentration ratio (grapefruit juice / control)

<table>
<thead>
<tr>
<th>Drug investigated</th>
<th>AUC ratio (grapefruit juice / water)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CYP2A6-inhibition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotine</td>
<td>0.85*</td>
<td>(Hukkanen et al. 2006)</td>
</tr>
<tr>
<td><strong>CYP3A4-inhibition</strong></td>
<td></td>
<td></td>
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<tr>
<td>Benzodiazepines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Midazolam</td>
<td>1.5</td>
<td>(Kupferschmidt et al. 1995)</td>
</tr>
<tr>
<td>Triazolam</td>
<td>1.5</td>
<td>(Hukkanen et al. 1995)</td>
</tr>
<tr>
<td>Diazepam</td>
<td>3.2**</td>
<td>(Ozdemir et al. 1998)</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amiodipine</td>
<td>no effect</td>
<td>(Vincent et al. 2000)</td>
</tr>
<tr>
<td>Felodipine</td>
<td>2.8***</td>
<td>(Bailey et al. 1991)</td>
</tr>
<tr>
<td>Nifedipine (+/- enantiomers)</td>
<td>1.4/1.9</td>
<td>(Uno et al. 2000)</td>
</tr>
<tr>
<td>Nimodipine</td>
<td>1.3****</td>
<td>(Bailey et al. 1991)</td>
</tr>
<tr>
<td>Nisoldipine</td>
<td>1.8</td>
<td>(Bailey et al. 1993)</td>
</tr>
<tr>
<td>Nitrendipine</td>
<td>2.3</td>
<td>(Soons et al. 1991)</td>
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<tr>
<td>Verapamil (S/R enantiomers)</td>
<td>1.4/1.3</td>
<td>(Ho et al. 2000)</td>
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<tr>
<td><strong>Statins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Others</strong></td>
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<td></td>
</tr>
<tr>
<td>Amiodarone</td>
<td>1.5</td>
<td>(Libersa et al. 2000)</td>
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<tr>
<td>Buspirone</td>
<td>9.2</td>
<td>(Lilja et al. 1998)</td>
</tr>
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<td>Carbamazepine</td>
<td>1.4</td>
<td>(Garg et al. 1998)</td>
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<tr>
<td>Cisapride</td>
<td>2.4</td>
<td>(Kivisto et al. 1999)</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>1.4, 1.3****</td>
<td>(Yee et al. 1995, Ducharme et al. 1993)</td>
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<tr>
<td>Erythromycin</td>
<td>1.5</td>
<td>(Kanazawa, Ohkubo &amp; Sugawara 2001)</td>
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<td>(Varis, Kivisto &amp; Neuvonen 2000)</td>
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<td>Nilotinib</td>
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<td>(Yin et al. 2010)</td>
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<td>(Nieminen et al. 2010)</td>
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<td>(Liu et al. 2009)</td>
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<td>Tolvaptan</td>
<td>1.6</td>
<td>(Shoaf, Mallikaarjun &amp; Bricmont 2012)</td>
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<td><strong>OATP-inhibition</strong></td>
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<td>(Tapaninen, Neuvonen &amp; Niemi 2010)</td>
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<td>(Lilja et al. 2007)</td>
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<td>Talinolol</td>
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<td>(Schwarz et al. 2005)</td>
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<td><strong>P-gp-inhibition</strong></td>
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<td></td>
</tr>
<tr>
<td>Digoxin</td>
<td>no effect</td>
<td>(Becquemont et al. 2001, Parker et al. 2003)</td>
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</table>
1.5. Drugs investigated

1.5.1. Cardiovascular diseases

Coronary artery disease (CAD) and other atherothrombotic cardiovascular diseases (CVDs) are major causes of morbidity and mortality throughout the world. There have been notable advances in the treatment and prevention of CVDs, yet they accounted for 17.3 million deaths worldwide in the year 2012 and the number is expected to grow to well over 20 million by the year 2030. Among CVDs, acute coronary syndrome (ACS) and stroke accounted for 7.3 and 6.2 million deaths, respectively in 2012 (Laslett et al. 2012). The pathophysiology of atherothrombotic diseases, including CAD, peripheral artery disease (PAD) and ischemic stroke, is characterized by reduced or occluded blood flow in the diseased artery and thus mismatch between oxygen supply and demand in tissues. The narrowing of an artery lumen is caused by a gradual growth of atherosclerotic lesions in the blood vessel. A complex network of mechanisms is involved in this process, including inflammation, dyslipidemia, and hypertension. The disruption of an atherosclerotic lesion leads to platelet activation and thrombus formation resulting in complete or near complete occlusion of the artery (an acute atherothrombotic event) (Koo 2015, Krishna, Moxon & Golledge 2015, Yahagi et al. 2016).

1.5.2. Platelet activation and atherothrombotic events

Platelets play a key role in the physiological response to a blood vessel injury and arresting bleeding. The subendothelial matrix in blood vessels is highly prothrombotic, and when the luminal endothelium is disrupted in injury, the circulating platelets are exposed to proteins such as von Willebrand factor (vWF), collagen, and vitronectin. The platelets are initially tethered to the site of injury by adhesive receptors and interactions between vWF and collagen (Jackson, Nesbitt & Kulkarni 2003). Subsequently, the adhesion is consolidated by additional receptors and activation of the platelets is initiated. The activated platelets release substances such as adenosine diphosphate (ADP), factor V (F-V), fibrinogen, P-selectin, and thromboxane A2 (TxA2) causing further platelet activation (Jackson, Nesbitt & Kulkarni 2003). The coagulation system is also activated by released F-V and exposed subendothelial tissue factor. ADP mediates its platelet activating effect via P2Y1 and P2Y12 receptors (Kunapuli et al. 2003), and TxA2 via thromboxane-prostanoid receptor on the platelet surface (Jackson, Nesbitt & Kulkarni 2003). Proteinase-activated receptor-1 (PAR-1) and PAR-4 also contribute to the platelet activation. Thrombin, produced by the coagulation system, acts as a ligand for PAR-1 and PAR-4 (Kahn et al. 1999). Interactions of the platelets with collagen, vWF, ADP, TxA2, and thrombin cause intracellular platelet signaling that leads to the activation of an integrin (glycoprotein IIb/IIIa) functioning as fibrinogen receptor and binding of fibrinogen. This leads to cross-linking of platelets by fibrinogen, platelet accumulation and aggregation at the site of injury, and the arrest of bleeding (Brass et al. 1997, Shattil, Kashiwagi & Pampori 1998).
Many of the mechanisms involved in platelet aggregation are either dependent on or enhanced by P2Y12 receptor activation. The P2Y12 receptor is coupled with the Gi family of G proteins, primarily to Gαi2. After binding with ADP, the P2Y12 receptor initiates signaling pathways that lead to inhibition of adenyl cyclase and activation of PI3K, Akt, Rap1b, and potassium channels. Eventually this results in fibrinogen receptor activation and aggregation of platelets. Furthermore, the P2Y12 receptor promotes a positive feedback loop of platelet activation, for example by degranulation, ADP release, and P-selectin expression (Dorsam and Kunapuli 2004).

Pathophysiologic conditions, such as atherosclerotic plaque rupture, or the endothelial damage and introduction of foreign material in coronary artery stent implantation, can lead to platelet activation and blood flow occluding intra-arterial thrombus formation, which can cause an acute atherothrombotic event, such as myocardial infarction (Shattil, Kashiwagi & Pampori 1998). The P2Y12 receptor is important not only in physiological, but also in pathophysiological platelet activation. This has been demonstrated by drugs that inhibit the P2Y12 receptor (Kunapuli et al. 2003) and by patients with dysfunctional P2Y12 receptors (Cattaneo, Gachet 1999). The P2Y12 receptor inhibitors ticlopidine and clopidogrel were shown to be more effective in the prevention of myocardial infarction, ischemic stroke, and other vascular events compared to aspirin (an inhibitor of TxA2 synthesis) (CAPRIE Steering Committee 1996). Combination therapy with clopidogrel and aspirin was shown to further reduce the risk of acute coronary events in patients with unstable angina pectoris (Mitka 2001).

1.5.3 Clopidogrel

Clopidogrel is a widely prescribed P2Y12 receptor antagonist drug and its indications include ACS, recent ischemic stroke, and established PAD. (EMA 2016, FDA 2016). It replaced ticlopidine, an earlier P2Y12 receptor antagonist, after large controlled trials demonstrated its safety and efficacy, both alone (CAPRIE Steering Committee 1996) and with additional benefits when combined to aspirin (Mitka 2001). Clopidogrel has less adverse effects compared to ticlopidine (Moussa et al. 1999).

Clopidogrel belongs to the thienopyridine class of antiplatelet drugs. It has a molecular formula of C16H16ClNO2S and a molecular mass of 321.82 g/mol. Clopidogrel dissociation constant (pKa) is 5.3 and its octanol/water partition ratio logarithm (log Kow) is 3.8, not putting it in the extremes of water solubility (HSDB 2016). The chemical structure and main metabolites are shown in Figure 1. Clopidogrel is a prodrug and its therapeutic effects rely on the formation of the active cis 5-thiol metabolite. The active metabolite binds to the platelet P2Y12 receptor irreversibly and by this mechanism inhibits ADP-mediated platelet activation (Farid, Kurihara & Wrighton 2010, Mills et al. 1992, Savi et al. 2000).
1.5.3.1 Pharmacokinetics and platelet inhibitory effect

Clopidogrel is rapidly absorbed and extensively metabolized. In a study with radio-labelled clopidogrel, the inactive carboxylic acid metabolite accounted for 71% of the $^{14}$C concentration in plasma one hour after a 75 mg oral dose of $^{14}$C-clopidogrel and the total $^{14}$C concentration peak in plasma was reached in one hour (median). Cumulatively over 120 hours, 41% and 46% of $^{14}$C was excreted in urine and 35-57% and 39-59% was excreted in feces after single dose and in steady state, respectively (Lins et al. 1999). The active metabolite concentrations appear proportional to clopidogrel in doses between 50 mg and 150 mg. In higher doses, the exposure to active metabolite increases less than linearly; increasing clopidogrel dose from 75 mg to 300 mg resulted in three times greater active metabolite AUC, but an increase from 300 mg to 600 mg raised the AUC by only 44% (Farid, Kurihara & Wrighton 2010). Ex vivo platelet aggregation tests have become the standard for measuring the pharmacodynamic responses of antiplatelet drugs (Jakubowski et al. 1985). Furthermore, the on-treatment platelet activity in these point-of-care assays have been shown to correlate to therapeutic efficacy and predict mortality and morbidity after coronary artery stent implantation (Geisler et al. 2006, Blieden et al. 2007, Buonamici et al. 2007, Price et al. 2008, Sharma et al. 2012). The steady state in clopidogrel platelet inhibitory effect is generally reached after three to seven days when the 75 mg daily dose is used and during the first 24 hours the platelet function is usually intact (Cadroy et al. 2000). A loading dose of 600 mg clopidogrel has been recommended since it has been shown to be effective in producing rapid response in platelet inhibition and superior to a 300 mg dose. No further benefit has been shown with a 900 mg loading dose (von Beckerath et al. 2005). The recovery of platelet function is complete after one week, on average, when clopidogrel therapy is discontinued from steady state (Thebault et al. 1999, Storey et al. 2011). Marked inter-individual variation has been observed in the platelet response, however, both in the initiation of clopidogrel therapy and in continuous use (Geisler et al. 2006, Blieden et al. 2007, Buonamici et al. 2007, Angiolillo et al. 2007).

The majority of orally administered clopidogrel is rapidly hydrolyzed to an inactive carboxylic acid metabolite by CES1 and this metabolite is subsequently glucuronidated (Tang et al. 2006). CYP enzymes are responsible for the oxidative metabolic pathway leading to the formation of an active cis 5-thiol metabolite (Figure 1). Only a small proportion of clopidogrel is ultimately converted to the active form (Farid, Kurihara & Wrighton 2010). In vitro studies have revealed that the first step in the bioactivation process is catalyzed by CYP1A2, CYP2B6, and CYP2C19 leading to the thiolactone intermediate metabolite, 2-oxo-clopidogrel. This metabolite is then converted to the active cis 5-thiol form by CYP2B6, CYP2C9, CYP2C19, and CYP3A4 (Dansette et al. 2012, Kazui et al. 2010). The estimated contributions of CYP1A2, CYP2B6, and CYP2C19 in the first step were 35.8, 19.4, and 44.9%, respectively. CYP2B6, CYP2C9, CYP2C19, and CYP3A4 had 32.9, 6.76, 20.6, and 39.8% estimated contributions to the second step, respectively (Kazui et al. 2010). In addition, another study suggested that paraoxonase 1 (PON1) has a critical role in the bioactivation of clopidogrel, and found evidence that the loss-of-function polymorphism PON1 c.575A>G (rs662) is associated with increased risk for stent thrombosis in clopidogrel users (Bouman et al. 2011). Later studies, however, have generally failed to replicate these results (Hulot et al. 2011, Lewis et al. 2011, Mega et al. 2015).
Furthermore, a study using human liver microsomes characterized the metabolism of 2-oxo-clopidogrel in detail and found that PON1 is responsible for an alternative but minor pathway leading to the cis 5-thiol metabolite and CYPs accounted for most of the conversion (Dansette et al. 2012).

1.5.3.2. Pharmacogenetics

Of the CYPs participating in the bioactivation of clopidogrel, CYP2C19 appears to be particularly important in vivo. Several studies and meta-analyses have found an association between CYP2C19 genetic polymorphism and clopidogrel therapeutic efficacy. CYP2C19*2 is a relatively common (18% frequency in Africans and Caucasians, over 30% in Asians) SNV that leads to reduced enzymatic activity. This variant allele associates to both clinical outcome (Turner, Pirmohamed 2014) and to the ex vivo measured platelet activity in patients (Hulot et al. 2006, Holmes et al. 2011, Li et al. 2016). Carriers of the CYP2C19*2 variant allele using clopidogrel as the P2Y12 receptor antagonist after PCI appear to have a higher risk for stent thrombosis compared to homozygous CYP2C19*1 allele (wild type) carriers (Simon et al. 2009, Turner, Pirmohamed 2014, Mega et al. 2009a, Mega et al. 2010b, Scott et al. 2013). A corresponding meta-analysis has found a per-allele association to high on-treatment platelet activity (Holmes et al. 2011). The risk for stent thrombosis varies between studies. A meta-analysis with conservative estimates reported hazard ratios (HR) of 2.67 and 3.97 for carriers of one or two loss-of-function alleles, respectively, compared to non-carriers (Mega et al. 2010b). However, while the association of CYP2C19*2 to the clopidogrel platelet inhibitory response is robust, there are several meta-analyses that have cast doubt on the association to cardiovascular events, in addition to those that support the clinical relevance. A study of several meta-analyses suggested that differences in conclusions result from different assessment and interpretation of between-study heterogeneity and publication bias that were evident in many of the original studies (Osnabrugge et al. 2015). Personalized antiplatelet therapy based on genotype was therefore not supported by the authors. Furthermore, as the clinical benefit of genotype guided dual antiplatelet therapy is uncertain, the European Society of Cardiology does not recommend genotyping for CYP2C19 loss-of-function alleles before clopidogrel treatment (Valgimigli et al. 2018). Nevertheless, a recent study suggests that CYP2C19 genotyping could indeed predict clinical outcome and guide the decision between clopidogrel and an alternative P2Y12 inhibitor therapy in CAD patients (Cavallari et al. 2018). One rationale for genotype-guided choice between clopidogrel, and the more potent P2Y12 inhibitors ticagrelor and prasugrel would be reduction of bleeding complications in dual antiplatelet therapy. The effect of CYP2C19 loss-of-function alleles in patients receiving clopidogrel therapy for other indications (e.g. ischemic stroke or peripheral artery disease) is not as thoroughly characterized as in CAD patients. However, a recent meta-analysis suggests that the risk of stroke is increased in clopidogrel-treated carriers of the CYP2C19 loss-of-function alleles (Pan et al. 2017).

CYP2C19*3 is a loss-of-function variant allele that occurs with a frequency of 7% in East Asian populations, but is extremely rare in Africans and Caucasians. Similar to CYP2C19*2, it has been associated with impaired clopidogrel bioactivation and therapeutic efficacy (Holmes et al. 2011).
CYP2C19*17, on the other hand, leads to enhanced transcription and enzymatic activity and is relatively common in African and Caucasian populations (23% MAF). This variant allele has been associated with reduced risk of cardiovascular events and increased risk of bleeding in patients treated with clopidogrel (Li et al. 2012, Li-Wan-Po et al. 2010, Tiroch et al. 2010). However, the observed effect has been suggested to result from linkage disequilibrium with the CYP2C19*2 loss-of-function variant (Lewis et al. 2013b). Studies on genetic polymorphism in other CYPs have generally not revealed associations to clopidogrel metabolism or response. In a study with PCI patients, the following CYP polymorphisms were investigated for associations to platelet activity or stent thrombosis risk: CYP1A2*1F (rs762551), CYP2B6*9 (rs3745274), CYP2C8*3 (rs10509681), CYP2C9*2 (rs1799853), CYP2C19*2 (rs4244285), CYP2C19*3 (rs4986893), CYP2C19*4 (rs28399504), CYP2C19*5 (rs56337013), CYP2C19*17 (rs12248560), CYP3A4*3 (rs4986910), CYP3A4*16B (rs2242480), and CYP3A5*3 (rs776746). Only CYP2C19*2 was found to have an impact on the clopidogrel response (Viviani Anselmi et al. 2013). While CYP3A appears to have an important role in the clopidogrel bioactivation process, the CYP3A5*3 loss-of-function variant has not affected clopidogrel pharmacodynamics in studies performed in healthy subjects (Hulot et al. 2006), pharmacokinetics and pharmacodynamics in healthy subjects (Kim, Park & Park 2008), nor pharmacodynamics and clinical outcome in PCI patients (Viviani Anselmi et al. 2013). However, an association to the clopidogrel platelet response was found in a study with Tamilian CAD patients (Priyadharsini et al. 2014) and in another study with Japanese PCI patients (Hokimoto et al. 2014). In the latter population, CYP3A5*3 allele associated with reduced clopidogrel platelet inhibitory response in patients with homozygous CYP2C19*2 genotype (poor metabolizers of CYP2C19 substrates). Furthermore, co-administration of amlodipine with clopidogrel led to an attenuated clopidogrel response in CYP3A5*3 carriers. CYP3A4 inhibition by amlodipine and the lack of compensation by CYP3A5 was suggested as the mechanism (Park et al. 2012). The effect of CYP3A4*22 on clopidogrel response has been studied in one study with PCI patients, where 12 heterozygous carriers of the reduced-function allele were compared to 199 controls; no difference in the light transmittance aggregometry was observed between the genotypes (Kreutz et al. 2013). Finally, two studies have suggested that CYP2C9 polymorphism associates to high on-treatment platelet activity (Harmsze et al. 2010a) and stent thrombosis risk (Harmsze et al. 2010c). CYP2C9 has only a minor role in vitro in the conversion of 2-oxo-clopidogrel to the active metabolite but CYP2C9*3 loss-of-function allele was associated with clinical outcome (OR 3.3 for subacute stent thrombosis), as was CYP2C19*2 (OR 2.5 for subacute stent thrombosis) in this study (Harmsze et al. 2010c).

CES1 rapidly hydrolyzes most of clopidogrel to inactive carboxylic acid metabolite. Reduced-function polymorphism in CES1 could therefore increase active metabolite formation. The CES1 c.428G>A SNV was found to associate with high active metabolite plasma concentrations (60% increase compared to control) in seven healthy Amish individuals, supporting this hypothesis (Lewis et al. 2013a). Furthermore, the CES1 c.428G>A SNV decreased clopidogrel carboxyl acid to clopidogrel AUC ratio, increased clopidogrel active metabolite concentrations and enhanced the antiplatelet effect in healthy volunteers compared to non-carriers (Tarkiainen et al. 2015). Of the other non-CYP polymorphisms affecting clopidogrel metabolism, P-gp (ABCB1 gene) is the best characterized. This efflux transporter is widely distributed in various tissues and mediates the exit of
several drugs from cells. P-gp can therefore act as a rate limiting factor in drug absorption in tissues, such as the intestine and the central nervous system (Giacomini, Sugiyama 2012). Polymorphism in the \textit{ABCB1} have been characterized and a synonymous SNV in exon 26 (c.3435T>C; rs1045642) alters the expression of P-gp in the intestine (Sakaeda, Nakamura & Okumura 2003). Higher P-gp expression due to this polymorphism has been associated with reduced clopidogrel absorption (Taubert \textit{et al.} 2006, Karazniewcz-Lada \textit{et al.} 2015) and to increased stent thrombosis risk (Mega \textit{et al.} 2010a). A meta-analysis did not find any association between \textit{ABCB1} rs1045642 and on treatment platelet activity or stent thrombosis risk, however, an increased risk for early (< 30 days) major cardiovascular events (OR, 1.77 between homozygous variants and nonvariants) and decreased risk for bleeding was found in ASC patients (OR 0.51) (Su \textit{et al.} 2012).

\subsection*{1.5.3.3. Pharmacokinetic interactions}

Consistent with the pharmacogenetic studies, the CYP2C19-inhibiting proton pump inhibitors (PPIs) omeprazole and esomeprazole, and to lesser extent, lansoprazole reduced the clopidogrel active metabolite formation and antiplatelet activity, however, pantoprazole and rabeprazole had no effect (Angiolillo \textit{et al.} 2011, Frelinger \textit{et al.} 2012). Furthermore, fluoxetine decreased clopidogrel active metabolite concentrations and antiplatelet effect by inhibiting CYP2C19, CYP2C9, and CYP3A4 (Delavenne \textit{et al.} 2013). Despite the data on pharmacokinetic interactions between clopidogrel and PPIs, randomized clinical trials fail to support the hypothesis that concomitant use of PPIs with clopidogrel would increase the risk of atherothrombotic events (Valgimigli \textit{et al.} 2018). The role of CYP3A4 in clopidogrel bioactivation supported by pharmacokinetic interaction studies. The CYP3A4 inhibitor ketoconazole inhibited clopidogrel bioactivation (Farid \textit{et al.} 2007b) and amlodipine reduced clopidogrel antiplatelet response in \textit{CYP3A5*3} carriers, likely by CYP3A4 inhibition (Park \textit{et al.} 2012). The CYP2C9, CYP2C19, and CYP3A4 inducer St. John’s wort has been shown to enhance clopidogrel antiplatelet effect and CYP3A4 activity in poor responders of clopidogrel therapy (Lau \textit{et al.} 2011). Rifampicin, an inducer of CYP1A2, CYP2C8, CYP2C9, CYP2C19, and CYP3A4, also increased clopidogrel active metabolite concentrations and platelet inhibitory activity (Judge \textit{et al.} 2010). P-gp polymorphism has been shown to affect clopidogrel absorption, however, the P-gp inhibiting calcium channel blocker verapamil did not affect clopidogrel pharmacokinetics or response (Harmsze \textit{et al.} 2010b). Many angiotensin-converting enzyme inhibitors (ACEIs) are metabolized by CES1, which in turn hydrolyzes most of orally administered clopidogrel into inactive form. An inhibition of CES1 \textit{in vitro} by ACEIs and an increase in bleeding risk in clopidogrel-treated myocardial infarction patients using ACEIs has been shown (Kristensen \textit{et al.} 2014). However, the clinical importance of this interaction remains unclear, as others have shown that ACEIs did not associate to increased bleeding risk in patients treated with clopidogrel. (Cressman \textit{et al.} 2015).

Grapefruit juice impaired the platelet inhibitory effect of clopidogrel in a study with healthy volunteers (Campbell \textit{et al.} 2014). Inhibition of intestinal CYP3A4 or possibly CYP2C19 by
grapefruit juice may have been involved, however, the pharmacokinetics of the interaction was not studied.

Besides being a substrate of CYP2C19, clopidogrel is a mechanism-based inhibitor of CYP2C19 (Nishiya et al. 2009) and has been shown to increase exposure to omeprazole (Chen et al. 2009). Clopidogrel is also an inhibitor of CYP2B6 (Nishiya et al. 2009) and therefore it can reduce the elimination of efavirenz, particularly in reduced-function CYP2B6*6 allele carriers (Jiang et al. 2013). Clopidogrel acyl glucuronide was identified as a mechanism-based inhibitor of CYP2C8 (Tornio et al. 2014) and has been shown to increase the plasma concentrations of CYP2C8 substrates, e.g. repaglinide and pioglitazone (Tornio et al. 2014, Itkonen et al. 2016). In addition to CYP2C8 inhibition, clopidogrel, 2-oxo clopidogrel and clopidogrel acyl glucuronide inhibit OATP1B1-mediated drug uptake, which has been proposed as a contributing mechanism to the elevated rhabdomyolysis risk seen with clopidogrel and cerivastatin co-medication (Tamraz et al. 2013). However, clopidogrel had no effect on simvastatin pharmacokinetics (Itkonen et al. 2015).

1.5.4. Prasugrel

The beneficial effect of P2Y12 receptor antagonists is established in CVD patients and clopidogrel has been widely used in the treatment and secondary prevention of CAD, PAD, and ischemic stroke. However, there are several limitations in clopidogrel therapy, including slow onset of action and highly variable and often inadequate platelet inhibition response (Geisler et al. 2006, Bliden et al. 2007, Buonamici et al. 2007, Thebault et al. 1999, Angiolillo et al. 2007). This has led to the development of novel antiplatelet drugs targeting the P2Y12 receptor. The antiplatelet activity and therapeutic efficacy of prasugrel is not as variable and susceptible to CYP activity as those of clopidogrel (Mega et al. 2009b, Mega et al. 2010a). Prasugrel can be used to inhibit platelet activation in poor responders of clopidogrel therapy (Alexopoulos et al. 2011).

The molecular formula of prasugrel is C20H20FNO3S and its molecular mass is 409.9 g/mol. The prasugrel log Kow is 3.9 (HSDB 2016). The chemical structure and main metabolites are shown in Figure 1. Like clopidogrel, prasugrel belongs to the thienopyridine class of antiplatelet drugs and requires metabolic activation for therapeutic efficacy. The active metabolite R-138727 binds to the platelet P2Y12 receptor irreversibly and inhibits ADP-mediated platelet activation. (Sugidachi et al. 2001, Rehmel et al. 2006).

1.5.4.1. Pharmacokinetics and platelet inhibitory effect

Prasugrel is rapidly absorbed and undergoes extensive first-pass metabolism following oral administration. The peak plasma concentrations of the active metabolite occur in the first hour after
ingestion. In a mass-balance study in healthy subjects, 70% of radioactivity was excreted in the urine and 25% in the feces following $^{14}$C-labelled prasugrel administration and the terminal $T_{1/2}$ of plasma radioactivity was 8 days. The terminal $T_{1/2}$ of R-138727 was 7 hours. A 60 mg loading dose of prasugrel results in near maximal platelet inhibition at on average one hour after ingestion and a 10 mg daily maintenance dose usually produces a consistent antiplatelet effect. Recovery of platelet function occurs in 4 to 7 days (Farid, Kurihara & Wrighton 2010, Asai et al. 2006, Farid et al. 2007c). CES2 catalyzes the formation of prasugrel primary, inactive metabolite R-95913, which in turn is metabolized to the secondary, active metabolite R-138727 by CYP3A4, CYP2B6, and to a lesser extent CYP2C9 and CYP2C19. (Sugidachi et al. 2001, Rehmel et al. 2006, Asai et al. 2006, Farid et al. 2007c).

1.5.4.2. Pharmacogenetics

Compared to clopidgrel, prasugrel antiplatelet activity and therapeutic efficacy do not seem as susceptible to CYP activity variation. The pharmacokinetics and pharmacodynamic response of prasugrel has been investigated in healthy carriers of at least one loss-of-function allele of CYP2C19, CYP2C9, CYP2B6, CYP3A5, and CYP1A2, and none of these polymorphisms was associated with impairment of bioactivation or antiplatelet efficacy (Mega et al. 2009b). The loss-of-function variants of CYP2C19 and gain-of-function SNV c.3435T>C in ABCB1 (P-gp) did not associate to adverse outcomes in acute coronary syndrome (ACS) patients with coronary artery stent (Mega et al. 2010a). One study, however, found a statistically significant association between the loss-of-function allele CYP2C9*2 and low antiplatelet efficacy of prasugrel in ACS patients. A significant association to reduced antiplatelet effect was also seen with body mass index and a trend with CYP2B6*6 in the same study (Franken et al. 2013).

1.5.4.3. Pharmacokinetic interactions

Consistent with the pharmacogenetic evidence, known drug-drug interactions of prasugrel are limited in number and magnitude. Ritonavir inhibits both CYP2B6 and CYP3A4 and was shown to reduce prasugrel active metabolite $C_{\text{max}}$ by 45% and AUC by 38% (Ancrenaz et al. 2013). However, another well-known CYP3A4 inhibitor, ketoconazole, inhibits clopidogrel metabolism but does not affect prasugrel bioactivation (Farid et al. 2007b). Similarly, the CYP2C19 inhibitor lansoprazole impairs clopidogrel, but not prasugrel bioactivation (Collet et al. 2014). Rifampicin strongly induces CYP3A4 but does not significantly affect prasugrel active metabolite pharmacokinetics (Farid et al. 2009).
1.5.5. Ticagrelor

Ticagrelor is an antiplatelet drug that offers improvement in efficacy in the treatment of ACS patients compared to clopidogrel (Wallentin et al. 2009). Like prasugrel, ticagrelor can be used to overcome high on-clopidogrel treatment platelet activity in loss of-function CYP2C19 allele carriers (Wallentin et al. 2010).

The ticagrelor molecular formula is C_{23}H_{28}F_{2}N_{6}O_{4}S and its molecular mass is 522.27 g/mol. The ticagrelor log Kow is 1.98, thus it is less lipid soluble than clopidogrel and prasugrel (HSDB 2016). The chemical structure and main metabolites are shown in Figure 1. Similar to clopidogrel and prasugrel, ticagrelor binds to the platelet P2Y_{12} receptor and inhibits ADP-mediated platelet activation. However, in contrast to the thienopyridines, ticagrelor does not require metabolic activation and its mechanism of action is reversible. (van Giezen et al. 2009).

1.5.5.1. Pharmacokinetics and platelet inhibitory effect

Ticagrelor is rapidly absorbed after oral administration, extensively metabolized in the first-pass and has an oral bioavailability of 36%. Ticagrelor C_{max} occurs between 1 to 2 hours after ingestion and its T_{1/2} is 7 h. 27% of radioactivity appeared in urine and 58% in feces after 14C-labelled ticagrelor administration (Teng et al. 2010). The platelet inhibitory activity of ticagrelor was investigated in single ascending doses up to 400 mg. Near maximal inhibition of platelet aggregation (IPA) was achieved in doses starting from 100 mg and the peak effect occurred 2 hours after ticagrelor administration in healthy volunteers. A 30 mg dose resulted in partial antiplatelet effect, and smaller doses did not significantly affect platelet function (Teng, Butler 2010). At 24 h after a single dose, the platelet inhibitory effect of ticagrelor was declining in doses up to 400 mg (Teng, Butler 2010). After discontinuation of antiplatelet therapy from steady state, recovery of platelet function was faster in ticagrelor-treated patients than in those using clopidogrel. An average of 30% IPA was seen at 58 h post-dose with ticagrelor and 115 h post-dose with clopidogrel, and 10% IPA at 108 h and 225 h, respectively (Storey et al. 2011).

Ticagrelor is metabolized by CYP3A4 and CYP3A5 to two major metabolites, C124910XX and C133913XX; C124910XX retains the platelet inhibitory effect (van Giezen et al. 2009, Zhou, Andersson & Grimm 2011, Teng et al. 2010). The major components resulting from ticagrelor metabolism in plasma and feces are ticagrelor and C124910XX, and in urine those are C133913XX and its glucuronide conjugate (Teng et al. 2010).
1.5.5.2. Pharmacogenetics

As ticagrelor metabolism is highly dependent on CYP3A activity, functional polymorphisms in CYP3A4 and CYP3A5 could be expected to influence ticagrelor pharmacokinetics. Two rare SNVs, CYP3A4*7 (rs56324128) and rs62471956, in the CYP3A4 gene have been shown to associate with ticagrelor pharmacokinetics, but not with clinical outcomes in a genome-wide association study involving ASC patients treated with ticagrelor (Varenhorst et al. 2015). Furthermore, a SNV (rs113681054) in SLCO1B1 was shown to associate with ticagrelor pharmacokinetics in the same study. This SNV is in linkage disequilibrium with SLCO1B1*5, a reduced-function allele in the OATP1B1 coding gene. However, another study with healthy volunteers investigating the effects of SLCO1B1*5, CYP3A4*1G, and CYP3A5*3 found no association of these variant alleles to ticagrelor pharmacokinetics and pharmacodynamics (Li et al. 2017), and the SLCO1B1 variants did not affect clinical outcomes in patients treated with ticagrelor (Varenhorst et al. 2015). Like prasugrel, ticagrelor is effective in patients that respond poorly to clopidogrel therapy due to loss-of-function CYP2C19 polymorphism (Wallentin et al. 2010). Polymorphisms affecting P-gp activity have not been shown to affect the therapeutic efficacy of ticagrelor (Wallentin et al. 2010).

1.5.5.3. Pharmacokinetic interactions

Ticagrelor pharmacokinetic interactions with CYP3A4 inhibitors has been studied in healthy volunteers. Diltiazem increased ticagrelor C_{max} by 69% and AUC by 174% compared to control (Teng, Butler 2013a). Ketoconazole increased ticagrelor C_{max} by 135% and AUC by over 600% (Teng, Butler 2013a, AstraZeneca 2011). The effect of ticagrelor on other drugs has also been investigated. Ticagrelor increased atorvastatin AUC by 36% (with ticagrelor dose 90 mg twice daily) and simvastatin AUC by 56% (with ticagrelor dose 180 mg twice daily) in a study with healthy volunteers. Ticagrelor pharmacokinetics was not affected by co-administration with statins and CYP3A4 inhibition by ticagrelor was proposed as the underlying mechanism for increased statin exposure (Teng, Mitchell & Butler 2013b). However, ticagrelor has not been shown to markedly affect CYP3A activity in vitro (Zhou, Andersson & Grimm 2011). CYP3A induction has been shown to affect ticagrelor pharmacokinetics. Rifampicin significantly decreased ticagrelor C_{max} and AUC, and while the maximal inhibition of platelet aggregation caused by ticagrelor was unaffected, the recovery of platelet function occurred more rapidly with rifampicin co-administration (Teng, Mitchell & Butler 2013a). Ticagrelor has been shown to be a substrate and inhibitor of P-gp in vitro (AstraZeneca 2011) and in 400 mg once daily dose it increased digoxin steady state C_{max} by 75% and AUC by 28% (Teng, Butler 2013b). Co-administration of the CYP3A4, OATP1B1, and P-gp inhibitor cyclosporine with ticagrelor in healthy volunteers resulted in 183% increase in ticagrelor AUC and 33% increase in C124910XX AUC, but did not affect the pharmacokinetics of cyclosporine (Teng, Kujacic & Hsia 2014).
Figure 1. The structure and metabolism of clopidogrel, prasugrel, and ticagrelor (Farid, Kurihara & Wrighton 2010, Dansette et al. 2012).
2. AIMS OF THE STUDY

Clopidogrel pharmacokinetics varies considerably between individuals and poor pharmacodynamic response in clopidogrel therapy has been associated with an increased risk for atherothrombotic events. CYP2C19 and CYP3A4 have been shown to have an important role in clopidogrel bioactivation. Current data suggests that prasugrel, compared to clopidogrel, has less interindividual pharmacokinetic and pharmacodynamic variation and less potential for drug-drug interactions. Prasugrel bioactivation relies on CYP3A4 and CYP2B6. Ticagrelor is eliminated primarily by CYP3A4 and drug-drug interaction studies have shown that CYP3A4 activity is important for ticagrelor pharmacokinetics and can be affected by enzyme inhibition or induction.

These studies were conducted to examine the significance of first-pass metabolism and genetic variation in CYP3A activity in the metabolism of clopidogrel, prasugrel, and ticagrelor. These studies investigated the effect of intestinal CYP3A4 inhibition by grapefruit juice in the pharmacokinetics and pharmacodynamics of clopidogrel, prasugrel and ticagrelor. Furthermore, the effects of the reduced-function CYP3A4*22 and loss-of-function CYP3A5*3 variant alleles on the bioactivation of clopidogrel and prasugrel, and in the elimination of ticagrelor were investigated.

Specific aims:

Study I To investigate the effect of intestinal CYP3A4 inhibition by grapefruit juice on the pharmacokinetics and pharmacodynamics of ticagrelor.

Study II To investigate the effect of intestinal CYP3A4 inhibition by grapefruit juice on the pharmacokinetics and pharmacodynamics of clopidogrel.

Study III To investigate the effect of intestinal CYP3A4 inhibition by grapefruit juice on the pharmacokinetics and pharmacodynamics of prasugrel.

Study IV To investigate the effect of the CYP3A4*22 and CYP3A5*3 variant alleles on the pharmacokinetics and pharmacodynamics of clopidogrel, prasugrel, and ticagrelor.
3. MATERIALS AND METHODS

3.1. Subjects

The subjects in the studies were healthy volunteers. Before entering the studies, each was given oral and written information and written informed consent was required. The health of the subjects was confirmed by medical history, clinical examination, and routine laboratory tests. All participants had normal plasma creatinine and alanine aminotransferase values, blood platelet counts, and hematocrit values. Female subjects were screened for pregnancy before entering the studies. None was on continuous medication, nor was a tobacco smoker. The use of alcohol, all other drugs, other grapefruit products, and apple and orange juice was prohibited during the study and one week before and three days after administration of the study drugs. Participation in any other clinical trial on a medicinal product or blood donation was prohibited for three months before and after each study. The characteristics of the subjects are shown in Table 3.

A total of 25 women and 33 men participated in the studies. Two subjects in Study III withdrew before first administration of study drug and one was excluded from analysis due to non-compliance with the prohibition of the use of other drugs. In Study IV, one subject withdrew due to personal reasons after participating in the ticagrelor phase, and another after participating in the ticagrelor and clopidogrel phases. In Studies I-III, the number of subjects was estimated to be sufficient to detect a 30% difference in the relevant AUC of ticagrelor, the active metabolite of clopidogrel and the active metabolite of prasugrel, respectively, between the water and grapefruit juice phases, with a power of at least 80% (α level 5%). In Study IV, the number of subjects was estimated to be sufficient to detect a 50% difference in the relevant AUC of ticagrelor, the active metabolite of clopidogrel and the active metabolite of prasugrel, between the different genotype groups with a power of at least 80% (α level 5%).

Subjects in the pharmacogenetic Study IV were recruited from a pool of 1109 genotyped individuals. The participants were selected and divided into three groups, according to their CYP3A4 and CYP3A5 genotypes. The reduced-function CYP3A4*22 and loss-of-function CYP3A5*3 (the common variant) alleles were investigated. The subject genotype in the first group (control) was CYP3A4*1/*1, CYP3A5*3/*3; the genotype in the second group (CYP3A5 expressors) was CYP3A5*1/*3, CYP3A4*1/*1; and the genotype in the third group (CYP3A4*22 carriers) was CYP3A5*3/*3, CYP3A4*1/*22. To avoid the possible confounding effect of the CYP2C19*2 loss-of-function allele in clopidogrel pharmacokinetics, heterozygous carriers of this allele were balanced between the groups and homozygotes were excluded from the study.
Table 3. Characteristics of the subjects.

<table>
<thead>
<tr>
<th>Study (n)</th>
<th>Genotype (n)</th>
<th>Sex (f, m)</th>
<th>Age (years)</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
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<tbody>
<tr>
<td>I (10)</td>
<td>n/d</td>
<td>4, 6</td>
<td>22 ± 3 (19-27)</td>
<td>177 ± 9 (157-187)</td>
<td>75 ± 14 (57-95)</td>
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<tr>
<td>II (14)</td>
<td>All (14)</td>
<td>6, 8</td>
<td>21 ± 2 (20-25)</td>
<td>174 ± 6 (164-183)</td>
<td>69 ± 10 (54-88)</td>
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<td></td>
<td>CYP2C19 *1/*1 (7 of 14)</td>
<td>3, 4</td>
<td>21 ± 1 (20-22)</td>
<td>174 ± 7 (164-183)</td>
<td>71 ± 10 (60-88)</td>
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<tr>
<td></td>
<td>CYP2C19 *1/*2 (5 of 14)</td>
<td>2, 3</td>
<td>22 ± 2 (20-25)</td>
<td>174 ± 6 (167-180)</td>
<td>65 ± 9 (54-75)</td>
</tr>
<tr>
<td></td>
<td>CYP2C19 *2/*2 (2 of 14)</td>
<td>1, 1</td>
<td>23 (22, 24)</td>
<td>174 (168, 180)</td>
<td>73 (61, 85)</td>
</tr>
<tr>
<td>III (7)</td>
<td>n/d</td>
<td>4, 3</td>
<td>21 ± 1 (19-22)</td>
<td>179 ± 5 (171-187)</td>
<td>73 ± 5 (65-79)</td>
</tr>
<tr>
<td>IV (27)</td>
<td>All (27)</td>
<td>14, 13</td>
<td>25 ± 4 (19-37)</td>
<td>175 ± 9 (156-190)</td>
<td>71 ± 13 (54-100)</td>
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<tr>
<td></td>
<td>Controls (13 of 27)</td>
<td>6, 7</td>
<td>24 ± 4 (19-31)</td>
<td>175 ± 9 (161-186)</td>
<td>72 ± 10 (54-84)</td>
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<td></td>
<td>CYP3A5 expressors (8 of 27)</td>
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<td>25 ± 5 (21-36)</td>
<td>177 ± 9 (166-190)</td>
<td>75 ± 18 (54-100)</td>
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<tr>
<td></td>
<td>CYP3A4*22 carriers (6 of 27)</td>
<td>4, 2</td>
<td>26 ± 6 (20-37)</td>
<td>172 ± 12 (156-185)</td>
<td>64 ± 8 (56-78)</td>
</tr>
</tbody>
</table>

Data is presented as mean ± standard deviation with range. f is female, m is male. n is number of subjects. n/d: not determined.
3.2. Study designs

The pharmacokinetic studies (I-III) with grapefruit juice as CYP3A4 inhibitor were carried out with a randomized crossover design. The study drugs were ticagrelor, clopidogrel, and prasugrel in Studies I, II and III, respectively. The studies had two phases in random order, both including a pre-treatment with either grapefruit juice or water, and a washout period of two weeks. The subjects ingested 200 ml of grapefruit juice or water three times a day for three days. On the third day, after an overnight fast, they ingested a single dose of the study drug with an additional 200 ml of grapefruit juice or water. The dose was 90 mg for ticagrelor, 600 mg for clopidogrel and 10 mg for prasugrel. Standardized meals were served at 4, 7, and 10 hours after study drug ingestion. During this study day, the subjects were under medical supervision for 12 hours after study drug administration.

The pharmacogenetic Study IV had a prospective genotype panel design. The subjects in each genotype group completed three phases in fixed order with ticagrelor, clopidogrel, and prasugrel as a study drug in individual phases. A washout period of at least two weeks was used between the phases. On the study days, following an overnight fast, the subjects ingested a single dose of either 90 mg ticagrelor, 600 mg clopidogrel, or 10 mg prasugrel. Standardized meals were served at 4, 7, and 10 hours after study drug ingestion. During this study day, the subjects were under medical supervision for 12 hours after study drug administration.

The study drugs were supplied by the Pharmacy of Helsinki University Central Hospital. Ticagrelor (Brilique, 90 mg tablet; AstraZeneca, London, UK), clopidogrel (Plavix, two 300 mg tablets; Sanofi-Aventis, Paris, France) and prasugrel (Efient, 10 mg tablet; Eli Lilly, Nederland BV, Houten, The Netherlands) were administered and their ingestion supervised by the researchers. Grapefruit juice was purchased from the local grocery stores and for each study, the same production batch was used whenever possible. The grapefruit juice brand was Valio Greippitäysmehu (Valio, Finland) in Studies I and II and Tropicana Golden Grapefruit (Tropicana Looza, France) in Study III. Juice ingestion was supervised during the study days. Pre-treatment by grapefruit juice before the study day was performed by the subjects at home, unsupervised, but with pre-bottled doses of juice from the researchers.

3.3. DNA preparation and genotyping

Genomic DNA was extracted using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) or Maxwell 16 LEV Blood DNA Kit (Promega, Madison, USA). Genotyping was performed for the CYP3A4*22 (rs35599367), CYP3A5*3 (rs776746), CYP2C19*2 (rs4244285), and CYP2C19*17 (rs12248560) SNVs by allelic discrimination with TaqMan 5’-nuclease assays on an Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems, Foster City, USA) or a Life Technologies QuantStudio 12K Flex Real-Time PCR system (Life Technologies, Carlsbad, USA).
3.4. Blood sampling and platelet activity measurements

Timed blood samples were drawn from a cannulated forearm vein of the subjects. Ethylenediaminetetraacetic acid (EDTA) and sodium citrate were used as an anticoagulant for the samples. In Study I, hirudin anticoagulated samples were also drawn. A sample volume of 9 ml was used for the EDTA tubes in Studies I-II and 8 ml (2 x 4 ml) in studies III and IV. A 2.7 ml sample volume was used for the citrate, and 3 ml for the hirudin tubes. Plasma was separated within 30 minutes in EDTA tubes and stored at -70°C until analysis. The blood samples were drawn as follows: in Study I prior to and 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 and 34 hours after ticagrelor administration; in Study II prior to and 1, 2, 4 and 12 hours after clopidogrel administration; in Study III prior to and 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 and 48 hours after prasugrel administration; in Study IV prior to and 0.5, 1, 1.5, 2, 3, 4, 5, 7, 9, 12 and 24 hours after the study drug administration.

3.4.1. Platelet aggregation assays

Platelet function tests were performed from citrate anticoagulated whole blood samples within 2 hours of sampling in Studies I-IV. The antiplatelet activity of ticagrelor, clopidogrel, and prasugrel was tested with a turbidimetric optical detection system (VerifyNow® P2Y12; Accumetrics, San Diego, CA, USA). The ADP–activated platelets aggregate on fibrinogen-coated microbeads in the test channel and the resultant change in optical signal is measured and expressed as P2Y12 reaction units (PRUs). In the control channel, platelets are activated with thrombin receptor (PAR-1 and PAR-4) -activating peptides, and the maximal aggregation signal is measured. Comparing the aggregation signal in the test channel to the signal in the control channel, the drug-dependent inhibition of P2Y12-mediated platelet aggregation can be expressed as percentage (Varenhorst et al. 2009). The average platelet inhibition values were calculated by dividing the area under the effect vs. time curve from time zero to the last blood sampling time point by the corresponding time interval. The platelet function assays were unavailable during the first pharmacokinetic experiments in Study II. The effect of grapefruit juice on the antiplatelet activity of clopidogrel was studied in two subjects after the majority of the other experiments, with similar protocol. The initial platelet function tests were performed in two parallel samples. As the variations between samples were found acceptable and within the values provided by the manufacturer, single samples were used afterwards.

Hirudin anticoagulated samples were also used in platelet function testing in Study I, in which two additional platelet aggregation assays were used. The first system is based on an impedance method (Multiplate®; Dynabyte, Munich, Germany). Whole blood samples were collected in hirudin tubes at 0, 4, and 24 h after ticagrelor dosing. ADP-activated platelets (ADP at 6.5 mM and 2.0 mM) aggregate on sensor surfaces and increase the electrical resistance which is continuously measured and converted into aggregation units (AU) (Velik-Salchner et al. 2008). The area under the AU–time
curve is calculated for each sample (AU*min). The second system is based on blood flow obstruction by platelet aggregation (PFA-100®; Siemens, NY, USA, with Innovance® PFA P2Y cartridges) (Koessler et al. 2011). The closure time is measured to determine collagen- and shear force-induced platelet aggregation in the presence of ADP. Samples for this test were collected 0, 4, 24, and 34 h after administration of ticagrelor.

3.5. Determination of drug concentrations

3.5.1. Clopidogrel

In Study II, the plasma samples were stablized using a method adapted from Bouman et al. (Bouman et al. 2011). A 1-ml volume of plasma was mixed with a 100-μl volume of freshly prepared solution with an equal amount of sodium sulfite as an antioxidant and boric acid as a complex-forming agent. In addition, blood samples from two healthy volunteers were collected and divided into two separate sample sets. The first set was stabilized using the Bouman method, and the second set was immediately derivatized with an alkylating reagent, 2-bromo-3-methoxyacetophenone (MP), as previously described (Tuffal et al. 2011).

MP was added into the previously underivatized plasma samples to 10 mmol/l concentration before analysis, and a simple protein precipitation by acetonitrile was performed for all samples. The concentrations of clopidogrel and 4b’cis-clopidogrelmethoxy phenacyl derivative, the less polar form, were analyzed with an AB Sciex 5500 Qtrap liquid chromatography–tandem mass spectrometry system (AB Sciex, Toronto, Canada). The mobile phase consisted of 10 mmol/l ammonium acetate (pH 2.9 or 5.2) adjusted with 98% formic acid (channel A) and acetonitrile (channel B); an enantioselective chromatography was performed on a Sun FireC18 column (150 × 2.1 mm, 3 μm; Waters, Milford, USA) using gradient elution. The gradient programming was as follows: 1 min at 45% B, 5 min linear increase to 55% B, and 2 min at 90% B, followed by equilibration at 45% B. The mass spectrometer was operated in positive turbo ion spray mode at the transitions m/z 322 to 155 and m/z 504 to 354 for clopidogrel and 4b’cis-clopidogrel-MP, respectively. Deuterated clopidogrel and deuterated 4b’cisclopidogrel-MP served as internal standards. The limits of quantification of plasma clopidogrel and 4b’cis-clopidogrel-MP were 0.01 and 0.25 ng/ml, respectively, and the day-to-day coefficient of variation was <15% at the relevant concentrations for both analytes. All sample sets were analyzed with two different mobile phase conditions, pH 2.9 and pH 5.2, to exclude possible interference caused by known or unknown clopidogrel metabolites. Concentrations of the active metabolite of clopidogrel (4b’cis-clopidogrel-MP, less polar form) could be reliably quantified only in the time period of 0–3 h. The concentrations measured with the Bouman and the direct derivatization methods in the samples of two of the subjects showed a strong correlation (Pearson correlation r = 0.983; P < 0.001). In Study II, the pharmacokinetic calculations were performed using concentrations measured with the Bouman method. In Study IV, the direct derivatization method was used for all samples as this was considered technically more
straightforward. (S)-(+)‐clopidogrel hydrogen sulfate, cis‐clopidogrel‐MP derivate (pair of enantiomers), racemic clopidogrel‐d4 hydrogen sulfate, and cis‐clopidogrel‐MP‐13C, d3 derivate were purchased from Toronto Research Chemicals (North York, Canada).

3.5.2. Prasugrel

Plasma concentrations of prasugrel inactive metabolite R‐95913 and active metabolite R‐138727 were determined form MP‐derivatized samples, as previously described (Farid et al. 2007a), with minor modifications. Liquid chromatography–tandem mass spectrometry measurements were conducted using a Shimadzu Nexera LC system (Shimadzu, Kyoto, Japan) coupled to a 5500 Qtrap mass spectrometer (AB Sciex, Toronto, Canada) with a TurboIonSpray ionization interface. The mobile phase consisted of 0.1% formic acid (channel A) and acetonitrile (channel B), and the chromatographic separation was achieved on a Kinetex C18 column (100mm × 2.1mm i.d., 2.6μm; Phenomenex, Torrance, USA) using gradient elution. The mobile phase gradient profile was set with linear increase from 40% B to 55% B over 2.7 min, followed by 1 min at 90% B and equilibration at 40% B. The mass spectrometer was operated in positive ion mode, and measurements were performed using multiple reaction monitoring of the m/z 332 to 109 transition for the MP derivate of R‐95913 and m/z 498 to 248 transition for the MP derivate of R‐138727. Deuterium‐labelled internal standards R‐95913‐D4 and R‐138727‐MP‐D3 (Clearsynth Labs Ltd, Mumbai, India) and commercially available reference compounds R‐95913 and R‐138727‐MP and were used for quantification. The lower limits of quantification of R‐95913 and R‐138727 were 0.2 and 0.1 ng/ml, respectively. The day‐to‐day coefficients of variation were below 15% at relevant concentrations for both analytes.

3.5.3. Ticagrelor

Ticagrelor and its C124910XX metabolite plasma concentrations were measured using an API 3000 LC/MS/MS system (AB Sciex, Toronto, Canada) (Beaudry et al. 1999, Mistri, Jangid & Shrivastav 2008). Acetonitrile was used for protein precipitation of plasma samples and chromatographic separation was achieved on an ACE 3 C‐18 PFP column (100 × 2.1mm i.d.) using a mixture of 10mM ammonium formate (channel A) and acetonitrile (channel B) as mobile phase. The flow rate was set at 190 ml/min and the gradient profile was set as follows: linear increase from 40% B to 90% B over 4 min followed by 1.5 min at 90% B, and 14 min re‐equilibrium to starting mobile phase composition. The mass spectrometer was operated in negative multiple reaction monitoring mode using mass‐to‐charge ratio (m/z) 521 to 361 and m/z 477 to 361 transitions for ticagrelor and C124910XX, respectively. The lower limit of quantification was 1.0 ng/ml for ticagrelor and the assay was linear over the standard curve range of 1–2500 ng/ml. Sample dilutions were used to confirm the linearity of the C124910XX detector response and a signal‐to‐noise ratio of 10:1 was used as the lower limit of quantification for C124910XX. The between‐day coefficient of variation was <10% at relevant concentrations (n = 4).
3.6. Pharmacokinetics

In Studies I-III, the pharmacokinetic variables were calculated with noncompartmental methods using MK-Model software, version 5.0 (Biosoft, Cambridge, UK). In Study IV, Phoenix WinNonlin software, version 6.3 (Certara, St. Louis, USA), was used. Pharmacokinetics was characterized in all studies by $C_{\text{max}}$, $T_{\text{max}}$, $t_{1/2}$, and AUC. The elimination rate constant ($k_e$) was calculated by linear regression analysis of the terminal log-linear part of the plasma drug concentration–time curve. The $t_{1/2}$ was calculated by the equation $t_{1/2} = \ln 2 / k_e$. AUC was calculated from the time of study drug ingestion (time 0) to infinity ($AUC_{0-\infty}$) in all studies by a combination of the linear and log-linear trapezoidal rules, with extrapolation to infinity by division of the last measured concentration by $k_e$. In addition, AUC was calculated from time 0 to various time points: from 0 to 3 ($AUC_{0-3}$) and 12 ($AUC_{0-12}$) hours in Study II, and from 0 to 34 ($AUC_{0-34}$) hours in Study I. $AUC_{0-3}$ was investigated in addition to $AUC_{0-\infty}$ in study II due to the high variation and rapid decline of clopidogrel active metabolite concentrations to values below low limit of quantification. Similarly, due to high variation in low concentrations, the $k_e$ in clopidogrel pharmacokinetics was calculated including $C_{\text{max}}$ in the linear regression in Study IV. The AUC and $C_{\text{max}}$ data in Study IV was calculated and presented in 70 kg weight adjusted form (observed AUC or $C_{\text{max}}$ * subject weight (kg)/70 kg).

3.7. Statistical analysis

Statistical analysis was performed with SPSS 20 and SPSS 22 software (IBM SPSS Statistics, Chicago, USA). The $C_{\text{max}}$, $t_{1/2}$, and AUC data were logarithmically transformed before statistical analysis. In Study I, the $C_{\text{max}}$, $t_{1/2}$, and AUC results were expressed as geometric means with geometric CV and geometric mean ratios (grapefruit juice phase / water phase) with 95% confidence intervals (CI). The PRU, inhibition percentage, and AU*min data were expressed as means ± SD. In Study II, the $C_{\text{max}}$, $t_{1/2}$, and AUC results were expressed as geometric means with geometric CV and geometric mean ratios (grapefruit juice phase / water phase) with 90% CIs. In both studies I and II, the plasma concentration-time curves were expressed as geometric means with 90% CIs. In Study III, $C_{\text{max}}$, $t_{1/2}$, and AUC data were expressed as both geometric means with geometric CV and geometric mean ratios (grapefruit juice phase / water phase) with 95% CIs and arithmetic means ± SD. The plasma concentration-time curves were expressed as means ± standard error of mean (SEM) and platelet inhibition-time curves as means ± SD. In Studies I-IV, $T_{\text{max}}$ data was expressed as medians with range. In Studies I-III, the $C_{\text{max}}$, $t_{1/2}$, AUC, and platelet inhibition data of the grapefruit juice and water phases were statistically compared by repeated measures analysis of variance (ANOVA) with treatment phase and treatment sequence as factors. In Study IV, the $C_{\text{max}}$, $t_{1/2}$, AUC and platelet inhibition data of the CYP3A5 expressors and CYP3A4*22 carriers were statistically compared to the controls by ANOVA. A covariate analysis was performed to investigate the effect of the CYP2C19*2 and CYP2C19*17 SNVs. The $T_{\text{max}}$ data were analyzed with the Wilcoxon signed rank test in Studies I-
III and Mann Whitney \( U \) test in Study IV. The AUC and \( C_{\text{max}} \) data in Study IV was statistically analyzed in 70 kg weight-adjusted form. Differences were considered statistically significant when \( P < 0.05 \), and corresponding CIs were calculated for the ratio to control or the difference to control.

### 3.8. Ethical considerations

The study protocols of Studies I and II were approved by the Coordinating Ethics Committee of the Helsinki and Uusimaa Hospital District, and the Finnish Medicines Agency, Fimea. The study protocol of study III was approved by the Finnish National Committee on Medical Research Ethics TUKIJA and the Finnish Medicines Agency Fimea. The study protocol of study IV was approved by the Coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa.
4. RESULTS

4.1. Effect of grapefruit juice on the pharmacokinetics and platelet inhibitory activity of ticagrelor (Study I)

Ten healthy volunteers ingested a single 90 mg dose of ticagrelor with either water (control) or grapefruit juice in a randomized crossover study. Grapefruit juice increased the plasma concentrations of ticagrelor. The interaction resulted in enhanced platelet inhibitory effect of ticagrelor in the VerifyNow® and the Multiplate® platelet function tests.

Intake of grapefruit juice resulted in 65% (P<0.001) increase in the mean ticagrelor C\text{max} and 121% (P<0.001) increase in the mean AUC\text{0-\infty} compared to control. The ticagrelor T\text{1/2} was increased by 7% (P=0.036) and T\text{max} from 1.5 h to 3 h (P=0.011) by grapefruit juice intake. The mean C\text{max} of ticagrelor active metabolite C124910XX was decreased by 45% (P<0.001) and the mean AUC\text{0-34} was decreased by 14% (P=0.027) in the grapefruit juice phase compared to control. However, the C124910XX AUC\text{0-\infty} remained unaffected. The mean C124910XX to ticagrelor AUC\text{0-34} ratio was 60% (P<0.001) lower in the grapefruit juice phase than in the control phase. The mean C124910XX T\text{1/2} was increased by 45% (P=0.001) and the T\text{max} from 2 to 3 h (P=0.004) by grapefruit juice (Figure 2).

In accordance with the pharmacokinetic results, grapefruit juice enhanced the antiplatelet effect of ticagrelor. In the VerifyNow® P2Y12 test, the average platelet inhibition at 0 to 34 h after ticagrelor ingestion was 42% in the control phase and 59% in the grapefruit juice phase (P<0.001). There was no significant difference in the maximum inhibition between the phases. In addition to optical detection, the platelet aggregation was also investigated with an impedance-based system (Multiplate®) and a blood flow-based system (PFA-100® with Innovance® PFA P2Y cartridges). The average platelet aggregation in the impedance-based test was 28 aggregation units (AU) in the control phase and 21 AU in the grapefruit juice phase (P=0.027). The maximum inhibition of platelet aggregation did not differ between the phases. In the blood flow-based system, the platelet inhibition exceeded measuring range (>300 s blood flow closure time) in both phases at 4 h after ticagrelor ingestion and did not recover until the last sample time point (34 h, data not shown) (Figure 2).
Figure 2. Ticagrelor and ticagrelor active metabolite geometric mean concentrations and platelet inhibitory effect (VerifyNow®) after single 90 mg dose of ticagrelor in grapefruit juice and control phases. Error bars represent 90% confidence intervals (concentrations) and standard deviations (platelet inhibition). CU, concentration units.

4.2. Effect of grapefruit juice on the pharmacokinetics and platelet inhibitory activity of clopidogrel (Study II)

Fourteen healthy volunteers ingested a single 600 mg dose of clopidogrel with either water (control) or grapefruit juice in a randomized crossover study. Grapefruit juice inhibited the bioactivation of clopidogrel. The effect of the pharmacokinetic interaction on the antiplatelet effect of clopidogrel was afterwards studied in two subjects. The interaction resulted in impaired platelet inhibitory effect of clopidogrel in the VerifyNow® platelet function test.

The mean $C_{\text{max}}$ of clopidogrel active metabolite was decreased by 87% ($P<0.001$) in the grapefruit juice phase compared to the control phase. The active metabolite AUC$_{0-3}$ was reduced by 86%
(P<0.001), and the active metabolite AUC$_{0-\infty}$ by 84% (P<0.001) by grapefruit juice. The active metabolite to clopidogrel AUC$_{0-3}$ ratio was reduced by 89% (P<0.001) in the grapefruit juice phase. Grapefruit juice had no significant effect on the T$_{1/2}$ and T$_{\text{max}}$ of clopidogrel active metabolite or the pharmacokinetics of the parent drug clopidogrel (Figure 3).

The magnitude of the grapefruit juice-clopidogrel interaction varied considerably between individual subjects. However, all had markedly reduced active metabolite C$_{\text{max}}$ (range: 56-92% decrease), active metabolite AUC$_{0-3}$ (range: 61-91% decrease) and active metabolite to clopidogrel AUC$_{0-3}$ ratio (range: 74-94% decrease) in the grapefruit juice phase. Two subjects were homozygous and five were heterozygous carriers of the loss-of-function CYP2C19*2 allele. However, no significant differences between genotypes were observed in the AUC$_{0-3}$ of the active metabolite of clopidogrel or the grapefruit juice-clopidogrel interaction.

The platelet inhibitory activity of clopidogrel was investigated in two subjects with an optical platelet aggregation detection system (VerifyNow® P2Y12 test). Consistent with the pharmacokinetic results, both had significantly decreased anti-platelet response during grapefruit juice intake. The average platelet inhibition, expressed as percentage of maximum inhibition of platelet aggregation, at 0 to 12 hours after clopidogrel ingestion was 47% and 90% in the control phase and 12% and 20% in the grapefruit juice phase. The highest observed platelet inhibition was similarly decreased from 56% and 98% in the control phase to 22% and 24% in the grapefruit juice phase (Figure 3).
Figure 3. Clopidogrel and the clopidogrel active metabolite geometric mean concentrations and platelet inhibitory activity in optical platelet aggregation test (VerifyNow®) after single 600 mg dose of clopidogrel in grapefruit juice (GFJ) and control phases. Error bars represent 90% confidence intervals.

4.3. Effect of grapefruit juice on the pharmacokinetics and platelet inhibitory activity of prasugrel (Study III)

Seven healthy volunteers ingested a single 10 mg dose of prasugrel with either water (control) or grapefruit juice in a randomized crossover study. Grapefruit juice reduced the bioactivation of prasugrel modestly. The interaction did not result in a significant effect on the platelet inhibitory activity of prasugrel in the VerifyNow® platelet function test.
The prasugrel primary, inactive metabolite (R-95913) AUC$_{0-\infty}$ increased by 64% ($P=0.008$) during grapefruit juice intake compared to control. The $C_{\text{max}}$, $T_{1/2}$, and $T_{\text{max}}$ of R-95913 were unaffected by grapefruit juice consumption. The prasugrel secondary, active metabolite (R-138727) AUC$_{0-\infty}$ was decreased by 26% ($P=0.014$) and $C_{\text{max}}$ was decreased by 49% ($P=0.017$) in the grapefruit juice phase. The R-138727 $T_{1/2}$ and $T_{\text{max}}$ remained unchanged during grapefruit juice intake. The reduction in active metabolite concentrations by grapefruit juice was seen in six of the seven subjects in the study (Figure 4).

The antiplatelet effect of prasugrel was investigated with the VerifyNow® P2Y12 test. The average platelet inhibitory activity at 0 to 24 h after prasugrel ingestion was five percentage points lower in the grapefruit juice phase than in the control phase ($P=0.034$). There was no statistically significant difference in the maximum platelet inhibition between the grapefruit juice and control phases (Figure 4).

![Prasugrel active and inactive metabolite geometric mean concentrations and mean platelet inhibitory effect (VerifyNow®) after a single 10 mg dose of prasugrel in grapefruit juice (GFJ) and control phases. Error bars represent 90% confidence intervals (concentrations) and standard deviations (platelet inhibition).](image-url)
4.4. Effects of CYP3A4*22 and CYP3A5*3 variant alleles on the pharmacokinetics and platelet inhibitory activity of clopidogrel, prasugrel and ticagrelor (Study IV)

Altogether 27 healthy volunteers were recruited according to their CYP3A4 and CYP3A5 genotypes. The control group (13 subjects) had CYP3A4*1/*1 and CYP3A5*3/*3 genotype, the CYP3A4*22 carrier group (six subjects) had CYP3A4*1/*22 and CYP3A5*3/*3 genotype and the CYP3A5 expressor group (eight subjects) had CYP3A4*1/*1 and CYP3A5*1/*3 genotype. The subjects ingested single doses of clopidogrel (600 mg), prasugrel (10 mg) and ticagrelor (90 mg) on separate occasions. The C\textsubscript{max} and AUC data were expressed and analyzed in weight-adjusted form.

The reduced-function CYP3A4*22 variant allele increased the plasma concentrations of ticagrelor. A modestly increased ticagrelor platelet inhibitory activity was seen in carriers of the CYP3A4*22 allele in the VerifyNow® platelet function test. However, CYP3A4*22 did not affect the bioactivation of clopidogrel or prasugrel and the CYP3A5 genotype had no significant effect on the pharmacokinetics of any of the drugs investigated.

4.4.1 Clopidogrel

The mean C\textsubscript{max}, AUC\textsubscript{0-\infty}, metabolite to clopidogrel AUC\textsubscript{0-\infty} ratio, and T\textsubscript{1/2} of clopidogrel, clopidogrel active metabolite and clopidogrel acyl glucuronide were not significantly affected by the CYP3A4 and CYP3A5 genotypes. The mean AUC\textsubscript{0-\infty} of clopidogrel carboxyl acid was 35% ($P=0.026$) higher in the CYP3A4*22 carrier group than in control. Statistically significant differences were not observed with the other pharmacokinetic variables of clopidogrel carboxyl acid. The platelet inhibitory effect of clopidogrel was investigated with the VerifyNow® P2Y12 test. The maximum and average platelet inhibition at 0 to 24 h after clopidogrel ingestion was not significantly affected by the CYP3A4*22 and CYP3A5*1 alleles (Figure 5).

A covariate analysis was performed to investigate the effects of the CES1 c.428G>A, CYP2C19*2, and CYP2C19*17 variant alleles on the differences in the clopidogrel pharmacokinetic variables between the genotype groups. No statistically significant covariate effect was found.
4.4.2 Prasugrel

The mean $C_{\text{max}}$, AUC$_{0-\infty}$, active to inactive metabolite AUC$_{0-\infty}$ ratio, and $T_{1/2}$ of prasugrel active (R-138727) and inactive (R-95913) metabolites were not significantly affected by the $CYP3A4*22$ and $CYP3A5*I$ alleles. Statistically significant differences between the genotype groups were not observed in the average or maximum platelet inhibition activity of prasugrel, as assessed with the VerifyNow® P2Y12 test (Figure 5).

4.4.3 Ticagrelor

The mean AUC$_{0-\infty}$ of ticagrelor was 89% ($P=0.004$) higher in $CYP3A4*22$ carriers than in controls. The AUC$_{0-\infty}$ of ticagrelor active metabolite (C124910XX) was also increased by 30% ($P=0.028$) in $CYP3A4*22$ carriers, but the C124910XX to ticagrelor AUC$_{0-\infty}$ ratio was decreased by 31% ($P=0.042$). Observed ticagrelor $C_{\text{max}}$ was 30% higher in the $CYP3A4*22$ carrier group than in the control but the difference was not statistically significant ($P=0.089$). Ticagrelor and C124910XX $T_{1/2}$ and C124910XX $C_{\text{max}}$ were not significantly affected by the $CYP3A4*22$ allele. The mean $C_{\text{max}}$, AUC$_{0-\infty}$, C124910XX to ticagrelor AUC$_{0-\infty}$ ratio and $T_{1/2}$ of ticagrelor and C124910XX were not significantly affected by the $CYP3A5*I$ allele. In a covariate analysis, the $SLCO1B1$ c.521T>C SNV did not change the statistical conclusions.

The antiplatelet effect of ticagrelor was investigated with the VerifyNow® P2Y12 test. In accordance with the pharmacokinetic results, the average inhibition of platelet aggregation at 0-24 h after ticagrelor administration was 68% in $CYP3A4*22$ carriers and 59% in controls, but the difference was not statistically significant ($P=0.172$). The maximum inhibition was unaffected by the $CYP3A4$ genotype. However, the platelet inhibition at 24 h after ticagrelor ingestion was 43% in the $CYP3A4*22$ carrier group compared to 21% in the control group ($P=0.029$). The $CYP3A5*I/*3$ genotype did not affect the average or maximum platelet inhibitory activity of ticagrelor (Figure 5).
Figure 5. Weight-adjusted geometric mean clopidogrel active metabolite, prasugrel active metabolite, and ticagrelor concentrations; and mean clopidogrel, prasugrel, and ticagrelor platelet inhibitory effect in CYP3A5 expressors, CYP3A4*22 carriers, and controls after single doses of 600 mg clopidogrel, 10 mg prasugrel, and 90 mg ticagrelor. Error bars represent 90% confidence intervals (concentrations) and standard deviations (platelet inhibition).
4.5. Summary

Grapefruit juice markedly inhibited the bioactivation of clopidogrel. The interaction resulted in significantly reduced active metabolite concentrations and impaired platelet inhibitory activity. Grapefruit juice had a modest inhibitory effect on the bioactivation of prasugrel, but it did not significantly affect the antiplatelet activity. Grapefruit juice markedly increased the plasma concentrations of ticagrelor. The interaction resulted in enhanced platelet inhibitory activity.

Carriers of a single *CYP3A4*\(^*\)\(^{22}\) variant allele had markedly increased ticagrelor plasma concentrations and the recovery of platelet function was delayed. *CYP3A4* genotype did not significantly affect the bioactivation of clopidogrel of prasugrel and *CYP3A5* genotype did not affect the pharmacokinetics of any of the drugs investigated.

**Table 4.** Summary of the pharmacokinetic and pharmacodynamic results of key variables in studies I-IV.

<table>
<thead>
<tr>
<th>Investigated drug/metabolite PK/PD variable</th>
<th>Effect of grapefruit juice</th>
<th>Effect of <em>CYP3A4</em>(^*)(^{22}) variant allele</th>
<th>Effect of <em>CYP3A5</em> expressor genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clopidogrel active metabolite</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(C_{\text{max}})</td>
<td>↓ 87% (Study II)</td>
<td>no effect (Study IV)</td>
<td>no effect (Study IV)</td>
</tr>
<tr>
<td>AUC</td>
<td>↓ 86% (0-3 h)</td>
<td>no effect</td>
<td>no effect</td>
</tr>
<tr>
<td>IPA</td>
<td>↓</td>
<td>no effect</td>
<td>no effect</td>
</tr>
<tr>
<td>Prasugrel active metabolite</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(C_{\text{max}})</td>
<td>↓ 49% (Study III)</td>
<td>no effect (Study IV)</td>
<td>no effect (Study IV)</td>
</tr>
<tr>
<td>AUC</td>
<td>↓ 26% (0-(\infty))</td>
<td>no effect</td>
<td>no effect</td>
</tr>
<tr>
<td>IPA</td>
<td>(†)</td>
<td>no effect</td>
<td>no effect</td>
</tr>
<tr>
<td>Ticagrelor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(C_{\text{max}})</td>
<td>↑ 65% (Study I)</td>
<td>no effect (Study IV)</td>
<td>no effect (Study IV)</td>
</tr>
<tr>
<td>AUC</td>
<td>↑ 121% (0-(\infty))</td>
<td>↑ 89% (0-(\infty))</td>
<td>no effect</td>
</tr>
<tr>
<td>IPA</td>
<td>↑</td>
<td>(†)</td>
<td>no effect</td>
</tr>
</tbody>
</table>

Data is presented as geometric mean (\(C_{\text{max}}\) and AUC). The effect direction is expressed as symbols; ↑, increase; ↓, decrease; († or ‡), modest but statistically significant increase or decrease. ‘No effect’ denotes the lack of statistically significant effect. PK, pharmacokinetics; PD, pharmacodynamics. \(C_{\text{max}}\), peak plasma concentration; AUC, area under the plasma concentration-time curve. IPA, inhibition of platelet aggregation.
5. DISCUSSION

5.1. Methodological considerations

Healthy, young adults were recruited as subjects in these studies to avoid confounding factors such as chronic diseases and medications. The pharmacokinetic and pharmacodynamic variables of clopidogrel, ticagrelor, and prasugrel observed in clinical studies with coronary artery disease patients have been comparable to those in previous studies involving healthy volunteers. However, steady state pharmacokinetics and antiplatelet activity were not investigated in the studies in this thesis. Single doses were used instead for practical and safety reasons. Thus, the results of our studies cannot be directly extrapolated to clinical practice but according to pharmacokinetic theory, the $AUC_{0-\infty}$ of a single dose equals the dose interval $AUC$ in steady state with linear pharmacokinetics (Rowland 2011). Furthermore, the effect magnitude seen with certain variables in these studies suggests potential for clinical importance.

The 600 mg dose of clopidogrel used in Studies II and IV is the usual loading dose in clinical use, and it has been shown to result in rapid active metabolite formation, for which the maintenance dose of 75 mg is insufficient (von Beckerath et al. 2005). The 10 mg dose of prasugrel used in Studies III and IV is the usual maintenance dose, and it has been shown to result in measurable active metabolite concentrations but not maximum platelet inhibitory effect in response to a single dose (Asai et al. 2006). As the antiplatelet effect of prasugrel is irreversible and generally less variable than that of clopidogrel, the 10 mg dose was chosen to limit the bleeding risk in the healthy volunteers. The 90 mg dose of ticagrelor used in Studies I and IV is the usual maintenance dose, and it has been shown to result in marked but rapidly reversible platelet inhibition in single dose (Teng, Butler 2010).

The pharmacokinetic interaction Studies I-III were controlled randomized cross-over studies with a washout period of two weeks. The cross-over design, where subjects served as their own controls, was chosen to reduce the number of participants and the variation between control and test phases. Washout periods and randomization limited the risk of carry-over effects. The pharmacogenetic Study IV was a prospective genotype panel study. The different genotype groups in this study were balanced according to sex and there were no significant differences in age between the groups. Average weight varied moderately; the genotype group mean weights were 64, 72, and 75 kg. To minimize the effect of weight as a confounding factor, the $C_{max}$ and $AUC$ variables were adjusted for a standard weight of 70 kg. The $CYP2C19*2$ allele, a potentially confounding factor particularly in the clopidogrel experiments, was accounted for by excluding homozygous carriers of the variant and attempting to balance heterozygous carriers in the genotype groups. The $CYP3A5$ expressor group had only one carrier of a $CYP2C19*2$ allele compared to 3 out of 6 in the $CYP3A4*22$ carrier group and 6 out of 13 in the control group. However, no apparent covariate effect of $CYP2C19*2$ or $CYP2C19*17$ alleles on clopidogrel pharmacokinetics was found in statistical analysis. Similarly, as $CES1$ c.428G>A is associated with clopidogrel metabolism and $SLCO1B1$ c.521T>C may have a minor influence in
ticagrelor metabolism (Varenhorst et al. 2015), these SNVs were included in covariate analyses. No covariate effects were found.

The participants of the studies were healthy volunteers without medications to minimize the risk of confounding factors and to improve participant safety during the studies. Overnight fasting before study drug administration and standardized meals during the study served to limit the effect of food on the pharmacokinetics of the study drugs. Furthermore, the participants were prohibited from using other drugs, grapefruit products other than those used in the study protocol, and alcohol before and during the studies. None was a tobacco smoker. In addition to controlling confounding factors for pharmacokinetics, these prohibitions reduce the intra-individual variation in platelet function tests considerably (Peace et al. 2009).

In Studies I-III, the number of subjects was estimated to be sufficient to detect a 30% difference in the AUC of ticagrelor, the active metabolite of clopidogrel and the active metabolite of prasugrel, respectively, between the water and grapefruit juice phases. In Study IV, the number of subjects was estimated to be sufficient to detect a 50% difference in the AUC of ticagrelor, the active metabolite of clopidogrel and the active metabolite of prasugrel, respectively, between the different genotype groups. The observed variances in the main pharmacokinetic variables were comparable to the estimated variances on which the statistical power calculations were based.

5.2. Effects of grapefruit juice on the pharmacokinetics and platelet inhibitory activity of ticagrelor, clopidogrel, and prasugrel

Grapefruit juice affected the pharmacokinetics of all the drugs investigated in these studies. The strongest interaction was seen with clopidogrel: grapefruit juice reduced the active metabolite exposure to 14-16% of control, resulting in significantly impaired platelet inhibitory effect. Ticagrelor elimination was also significantly affected. The AUC of ticagrelor was more than doubled by grapefruit juice compared to control, and the interaction enhanced the inhibition of platelet aggregation (IPA). The grapefruit juice–prasugrel interaction was modest compared to the effects of grapefruit juice on clopidogrel and ticagrelor, and the effect on prasugrel IPA was negligible. Nevertheless, a statistically significant reduction in prasugrel active metabolite formation was seen with grapefruit juice consumption.

Grapefruit juice has been shown to affect the pharmacokinetics of several drugs (Table 2). The magnitude of the interaction varies but is particularly strong for statins that are subject to CYP3A4-mediated first-pass metabolism. Grapefruit juice intake has been shown to increase the AUC of atorvastatin, lovastatin and simvastatin by 3.3 to 16 fold (Lilja, Kivisto & Neuvonen 1999, Lilja, Kivisto & Neuvonen 2000, Kantola, Kivisto & Neuvonen 1998, Lilja, Kivisto & Neuvonen 1998). A comparable effect was shown in Study II on clopidogrel active metabolite formation. Considering
that the effect of grapefruit juice on intestinal CYP3A4 is well established, these results underline the significance of first-pass metabolism in clopidogrel bioactivation. However, in repeated intake, grapefruit juice has been shown to prolong the $t_\frac{1}{2}$ of several drugs, suggesting that inhibition of hepatic CYP3A4 could be involved as well (Lilja et al. 2000, Lilja et al. 1998, Kivisto et al. 1999, Lilja, Kivisto & Neuvonen 1999, Nieminen et al. 2010).

The interaction between grapefruit juice and clopidogrel in Study II was particularly strong compared to previous studies on CYP3A4 inhibitors and clopidogrel. Ketoconazole has inhibited clopidogrel active metabolite formation, but only by 22-29% (AUC) (Farid et al. 2007b). In addition to CYP3A4, CYP2C19 has an important role in clopidogrel metabolism. CYP2C19 is present in human small intestine (Läpple et al. 2003, Paine et al. 2006, Galetin, Houston 2006) and grapefruit juice constituents have been shown to inhibit CYP2C19 (Tassaneeyakul et al. 2000). Furthermore, according to in vitro studies, the first step in clopidogrel bioactivation is catalyzed predominantly by CYP2C19 and CYP1A2 (with minor contribution from CYP2B6), CYP3A having primary role only in the second step (Farid, Kurihara & Wrighton 2010). Taken together, while grapefruit juice is best known for its CYP3A4 inhibitory effect, intestinal and/or hepatic CYP2C19 inhibition cannot be ruled out in the grapefruit juice–clopidogrel interaction.

The metabolism of ticagrelor is dependent on CYP3A activity (van Giezen et al. 2009, Zhou, Andersson & Grimm 2011, Teng et al. 2010) and previous studies have demonstrated that CYP3A4 inhibitors impair ticagrelor elimination. Ketoconazole, for example, has increased ticagrelor AUC by over 600% (Teng, Butler 2013a). Consistent with these results, grapefruit juice more than doubled the AUC of ticagrelor in Study I. Prasugrel bioactivation, in contrast, is not dependent on a single CYP family and prasugrel pharmacokinetics seems relatively resistant to drug-drug interactions. CES2 is required for formation of the primary metabolite but CYP2B6 and CYP3A4 both have major roles in the formation of the secondary, active metabolite (Sugidachi et al. 2001, Rehmel et al. 2006, Asai et al. 2006, Farid et al. 2007c). Ketoconazole has not affected prasugrel bioactivation (Farid et al. 2007b). Ritonavir, an inhibitor of both CYP2B6 and CYP3A4 has reduced the prasugrel active metabolite AUC, but only by 38% (Ancrenaz et al. 2013). In agreement with previous studies, grapefruit juice had only modest effect on prasugrel active metabolite pharmacokinetics and prasugrel IPA was unaffected in Study III.

5.3. Effects of CYP3A4*22 and CYP3A5*3 variant alleles on the pharmacokinetics and platelet inhibitory activity of clopidogrel, prasugrel, and ticagrelor

The reduced-function CYP3A4*22 allele had a significant effect on ticagrelor metabolism in Study IV. Carriers of a single variant allele had 89% increased ticagrelor AUC compared to controls and the effect was also seen in the residual IPA at 24 h after ticagrelor ingestion. In contrast, the CYP3A4 genotype did not affect clopidogrel or prasugrel pharmacokinetics or pharmacodynamics in this study. The CYP3A5 genotype did not affect the pharmacokinetics or pharmacodynamics of any of the drugs.
investigated in this study. The small sample size and relatively high variances in some of the pharmacokinetic variables, however, prevented statistically reliable detection of possible modest effects.

Ticagrelor is metabolized to the active (C124910XX) and the inactive (C133913XX) metabolite by CYP3A4 and CYP3A5 (Zhou, Andersson & Grimm 2011). A marked impairment in ticagrelor elimination was seen in heterozygous carriers of the reduced-function CYP3A4*22 allele. However, the CYP3A5 genotype did not affect ticagrelor pharmacokinetics, when heterozygous carriers of the CYP3A5*1 allele were compared to homozygous carriers of the common loss-of-function allele CYP3A5*3. The results of Study IV are in agreement with previous pharmacokinetic interaction studies and Study I, and they suggest a critical role of CYP3A4 and minor role of CYP3A5 in ticagrelor metabolism.

In contrast to the pharmacokinetic interaction between clopidogrel and grapefruit juice in Study II, carriers of a single reduced-function CYP3A4*22 allele did not exhibit significantly altered clopidogrel pharmacokinetics in Study IV. Clopidogrel active metabolite formation and antiplatelet effect were unaffected. These results suggest that the partial loss of CYP3A4 activity was compensated by the other CYPs participating in clopidogrel metabolism. CYP2B6 and CYP2C19 both have a role in the formation of clopidogrel active metabolite from 2-oxo-clopidogrel, in addition to CYP3A (Figure 1). Similarly, CYP3A5 activity variation did not affect clopidogrel pharmacokinetics, probably due to the redundancy in CYPs participating in the metabolism. Furthermore, the difference in the effects of grapefruit juice and CYP3A4*22 allele in the pharmacokinetics of clopidogrel is in accordance with a study showing that CYP3A4*22 impairs CYP3A4 expression in the liver, but not in the intestine (Wang, Sadee 2016).

Prasugrel pharmacokinetics and pharmacodynamics were unaffected by the CYP3A4 and CYP3A5 genotypes in Study IV. These results are in agreement with previous pharmacokinetic interaction studies and Study III. Compared to ticagrelor and clopidogrel, prasugrel does not seem sensitive to CYP3A activity variation.

5.4. General discussion and clinical implications

Clopidogrel bioactivation and antiplatelet activity were significantly reduced by grapefruit juice in Study II. Considering the magnitude of the effect, these results strongly suggest that grapefruit juice consumption during clopidogrel treatment could result in poor therapeutic efficacy. Stent thrombosis in acute coronary syndrome patients is an example of a critical consequence of impaired clopidogrel platelet inhibitory activity. The impairment of clopidogrel active metabolite formation and antiplatelet effect by grapefruit juice in Study II is in accordance with another study showing that the on-clopidogrel-treatment platelet reactivity is higher during grapefruit juice consumption compared
to control (Campbell et al. 2014). Thus, grapefruit juice is best avoided during clopidogrel therapy. The strong interaction between grapefruit juice and clopidogrel suggests that intestinal first-pass metabolism has an important role in clopidogrel metabolism. However, the reduced-function CYP3A4*22 and loss-of-function CYP3A5*3 alleles did not impair clopidogrel bioactivation in Study IV. In contrast, previous studies have demonstrated that functional polymorphisms in the CYP2C19 gene affect clopidogrel bioactivation in healthy volunteers as well as in the clinical setting. Considering that grapefruit juice constituents have been shown to inhibit CYP2C19, it is thus plausible that intestinal and/or hepatic CYP2C19 inhibition by grapefruit juice may have been part of or even the primary mechanism in the interaction in Study II.

Ticagrelor exposure was markedly increased by concomitant grapefruit juice consumption in Study I. The interaction resulted in enhanced platelet inhibitory activity and could increase the risk of adverse effects such as bleeding in the clinical setting. These results suggest that CYP3A4-mediated first-pass metabolism is important in ticagrelor pharmacokinetics. The AUC of ticagrelor in carriers of the CYP3A4*22 allele was 89% higher than in controls, further supporting the role of CYP3A4 in ticagrelor metabolism. While the frequency of CYP3A4*22 is low, a marked reduction in ticagrelor elimination was seen even in heterozygous carriers of the allele. Genotyping for CYP3A4*22 could therefore be used to predict ticagrelor response and estimate the probability of ticagrelor adverse effects, particularly in patients with elevated bleeding risk.

In Study III, prasugrel bioactivation was modestly reduced by grapefruit juice consumption, but prasugrel IPA was unaffected. Consistent with these results, the CYP3A4 and CYP3A5 genotypes did not affect prasugrel pharmacokinetics or pharmacodynamics in Study IV. CYP3A activity variation did not significantly affect prasugrel metabolism in either studies. These results, and previous studies suggest that prasugrel is less sensitive to CYP3A-mediated drug-drug interactions or functional polymorphisms in CYP genes compared to clopidogrel and ticagrelor.

The inhibition of platelet aggregation (IPA) following ticagrelor ingestion was investigated using three different methods in Study I. All three assays measured the ADP-dependent and P2Y12 receptor-specific platelet aggregation ex vivo. The optical-(VerifyNow®) and the impedance-(Multiplate®) based systems demonstrated individual variability in the antiplatelet effect of ticagrelor. These two assays are therefore suitable for monitoring platelet activity and pharmacodynamic response in ticagrelor treatment. The blood flow-based system (PFA-100®) was sensitive in detecting small changes in platelet aggregation at 34 h after ticagrelor ingestion. However, the closure times (the time to platelet aggregation-induced occlusion of blood flow in the test) exceeded the measuring range in most subjects prior to the 34 h post-dose time point and no individual variability could be demonstrated. This assay could therefore be used in testing the recovery of platelet function after discontinuation of ticagrelor treatment, for example before invasive interventions. The disadvantages of the PFA-100® -system in this setting include limited experience with P2Y12 inhibitors compared to the other platelet function tests and its sensitivity to changes in hematocrit (Lenk and Spannagl 2014).
VerifyNow® was chosen as the platelet function test for Studies II-IV after comparisons to Multiplate® and PFA-100® in Study I. The vasodilator-stimulated phosphoprotein-phosphorylation (VASP-P) assay, while considered as the standard of measuring P2Y12-mediated platelet function (Mingant et al. 2018), was unavailable for the studies in this thesis. Of the three tests used in Study I, however, VerifyNow® was considered to be the most suitable method for comparisons of submaximal platelet inhibition. The variations between initial parallel samples were acceptable. The platelet inhibitory activity of clopidogrel in the VerifyNow® test has been shown to correlate to clopidogrel active metabolite concentrations, VASP-P and to certain clinical outcomes (Good et al. 2015, Price et al. 2008, Varenhorst et al. 2009). The Multiplate® test has been shown to guide clinical decision making in dual antiplatelet therapy in one study (Sibbing et al. 2017). However, the correlation between the impedance based, blood flow-based and light transmission aggregometry platelet function tests has been modest at best in other studies (Good et al. 2015, Helten et al. 2018, Mingant et al. 2018) and the clinical benefit of platelet function testing is still uncertain (Helten et al. 2018). Therefore, the European Society of Cardiology does not recommend platelet function testing in dual antiplate therapy (Valgimigli et al. 2018).

The number of subjects in our studies limits the detection of small differences. Furthermore, our studies were performed with single drug doses in healthy individuals in non-clinical setting. However, the magnitude and consistency of the effects of grapefruit juice on ticagrelor and clopidogrel pharmacokinetics in Studies I and II suggest clinical importance. Modest effects of CYP3A4*22 carrier and CYP3A5 expressor genotypes cannot be ruled out in clopidogrel and prasugrel pharmacokinetics in Study IV. More accurate assessment of the magnitude and possible clinical importance of the CYP3A4*22 variant allele on ticagrelor pharmacokinetics would require further studies. However, the CYP3A4*22 variant allele had a marked effect on ticagrelor elimination in study IV. Considering that the effect was seen in heterozygous carriers of CYP3A4*22, it seems likely that the allele has an impact in ticagrelor pharmacokinetics in the clinical setting as well.
6. CONCLUSIONS

The conclusions based on the results of these four studies:

**Study I**
Grapefruit juice significantly increased the plasma concentrations of ticagrelor and enhanced its antiplatelet effect. Grapefruit juice is known to inhibit intestinal CYP3A4 both *in vitro* and *in vivo* and CYP3A4 is the primary enzyme responsible for ticagrelor metabolic elimination. Therefore, the mechanism of the interaction was most likely inhibition of intestinal CYP3A4 by grapefruit juice.

These results indicate that intestinal first-pass metabolism is important in ticagrelor pharmacokinetics and suggest that grapefruit juice consumption during ticagrelor treatment is best avoided.

**Study II**
Grapefruit juice markedly impaired clopidogrel bioactivation. The interaction resulted in reduced clopidogrel active metabolite concentrations and antiplatelet effect. Besides CYP3A4, grapefruit juice has been shown to inhibit CYP2C19 *in vitro* and both CYP3A4 and CYP2C19 are important in clopidogrel bioactivation. In previous studies, the magnitude of interaction between CYP3A4 inhibitors and clopidogrel has been modest compared to the grapefruit juice-clopidogrel interaction in this study.

These results indicate that intestinal first-pass metabolism is important in clopidogrel pharmacokinetics and suggest that grapefruit juice may impair clopidogrel bioactivation by inhibition of CYP2C19, in addition to CYP3A4. Due to the large impairment in antiplatelet effect, grapefruit juice consumption during clopidogrel treatment should be avoided.

**Study III**
Grapefruit juice had a modest inhibitory effect on prasugrel bioactivation, but the interaction did not result in impaired antiplatelet effect.

Prasugrel bioactivation is mediated by CYP3A4 and CYP2B6. These results suggest that inhibition of CYP3A4 alone does not significantly alter prasugrel pharmacokinetics, and that prasugrel is not sensitive to intestinal CYP activity variation.
Carriers of a single reduced-function *CYP3A4*22 allele had markedly increased ticagrelor plasma concentrations and modestly enhanced ticagrelor antiplatelet activity. The *CYP3A4*22 allele did not significantly alter the pharmacokinetics of clopidogrel or prasugrel, and the common loss-of-function *CYP3A5*3 allele did not affect the pharmacokinetics of ticagrelor, clopidogrel, nor prasugrel.

These results underline the importance of CYP3A4 in ticagrelor metabolism and suggest that genotyping for the *CYP3A4*22 allele could be used to predict ticagrelor pharmacokinetics. Genetic variation in CYP3A4 activity was probably compensated for by other CYPs in clopidogrel and prasugrel metabolism. Genetic variation in CYP3A5 activity was likely compensated by CYP3A4 in the metabolism of all the investigated drugs.
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