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From the Department of Laboratory Medicine,
Division of Clinical pharmacology,
Karolinska Institutet, Stockholm, Sweden

CYP2C-DEPENDENT DRUG METABOLISM *IN VIVO*

influence of genetics and drug interactions

Staffan Rosenborg

Stockholm 2010

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Ibland frågar jag mig hur vi kan leva, när
vi knappt har tid att se åt vad vi håller kärt.
Ibland så undrar jag åt vilket håll det bär,
när tid kostar mer än lycka någonsin är värd.

Om vi håller om varandra
Niklas Hillbom (Jumper)
Välkommen hit, 1998

Till mina flickor

ABSTRACT

Cytochrome P450 enzymes (CYPs) are responsible for the metabolism of the majority of therapeutic drugs. This thesis focuses on one of the CYP subfamilies, CYP2C, especially CYP2C9 and CYP2C19, which are responsible for the metabolism of 15–20% of all drugs. All CYP2C enzymes are polymorphic, i.e. there are genetic variants, which have functional consequences for drug metabolism. Individuals can be classified according to their CYP2C metabolic capacity in extensive (EMs), intermediate (IMs) and poor metabolisers (PMs). Recently, a novel variant of the *CYP2C19* gene was described in individuals with high metabolic capacity. This allele, *CYP2C19*17*, has been claimed to cause ultrarapid metabolism (UM) of CYP2C19 substrates.

The aim of this thesis is to explore some of the aspects underlying varying metabolic capacity between, and within, individuals with the focus on genetics and drug–drug interactions. The thesis is based on five published papers.

In Paper **I** we explored the influence of the genetic variant *CYP2C9*3* on the CYP2C9 dependent metabolism of the anti-inflammatory and analgesic drug celecoxib. We found a seven-fold higher median exposure at steady-state in homozygous carriers of the *CYP2C9*3* allele compared to homozygous wild-type subjects. This might be one factor behind the increased risk of cardiovascular events that has been observed in long-term users of celecoxib in a dose-dependent fashion.

Paper **II** and **III** focused on the *CYP2C19*17* allele that has been associated with extensive metabolism of CYP2C19 substrates. We showed a 52% lower exposure of omeprazole in homozygous **17* carriers compared to homozygous wild-type subjects after a single dose of 40 mg. Regarding steady-state levels of escitalopram (5 mg twice daily for a week), we noted a trend towards a 21% lower exposure in *CYP2C19*17* homozygous individuals. However, this did not reach statistical significance in this study that was powered for a 40% difference. The clinical impact (or lack of impact) of this allele for various clinically important CYP2C19 substrates will be discussed in the thesis.

A clinical consultation was the starting point for Paper **IV** in which we described eight cases of increased anticoagulant effect of warfarin in connection with concomitant use of noscapine; a cough medicine available over-the-counter. These cases were reported to the Swedish adverse drug reactions (ADR) register and we could show that they yielded a statistically significant signal worthy of further investigation. *In vitro* experiments were performed, showing that noscapine strongly inhibited CYP2C9 and CYP3A4, the key enzymes in warfarin metabolism.

Besides noscapine, another OTC drug, glucosamine, has attracted interest for suspected interaction with warfarin. In Paper **V** we addressed the pharmacokinetic aspect of these interactions by giving a cocktail of four probe drugs before and during noscapine or glucosamine. Compared to baseline phenotyping, significant inhibition of both CYP2C9 (4.9-fold increase in the urinary losartan/E3174 ratio; 95% CI 2.8 - 8.4) and CYP2C19 (3.6-fold increase in the plasma omeprazole/5-hydroxyomeprazole ratio; 95% CI 2.6 - 4.8) was seen during noscapine treatment. This is likely to explain the observed interaction with warfarin. No enzyme inhibition was seen with glucosamine and a metabolic interaction between warfarin and glucosamine seems highly unlikely.

SAMMANFATTNING

De flesta läkemedel behöver omvandlas till mer vattenlösliga substanser för att kunna utsöndras ur kroppen. Denna metabolism sker i många fall av enzymer tillhörande superfamiljen cytokrom P450 (CYP). Denna avhandling fokuserar på en underfamilj av cytokrom P450, nämligen CYP2C, särskilt de två enzymerna CYP2C9 och CYP2C19, som är ansvariga för metabolismen av 15-20 % av de kliniskt mest använda läkemedlen. Alla CYP2C-enzymer är polymorfa, d.v.s. det finns genetiska varianter som har funktionella konsekvenser för nedbrytningen av läkemedel. Utifrån sin förmåga att omvandla CYP2C-substrat kan individer klassas som snabba (extensive metabolisers, EM) eller långsamma metaboliserare (poor metabolisers, PM). Nyligen beskrevs en ny variant av *CYP2C19*-genen hos individer med hög metabol förmåga. Denna genetiska variant (*CYP2C19*17*) har hävdats orsaka ultrasnabb metabolism (UM) av läkemedel som omsätts via CYP2C19.

Målet med denna avhandling är att undersöka några av de faktorer som orsakar att förmågan att omsätta läkemedel varierar mellan och inom individer. Särskilt fokus ligger vid genetiska aspekter och läkemedelsinteraktioner. Avhandlingen bygger på fem publicerade delarbeten.

I delarbete **I** undersöktes betydelsen av den genetiska varianten *CYP2C9*3* för förmågan att bryta ned det inflammationshämmande och smärtstillande medlet celecoxib. Vi fann en sjufaldigt högre medianexponering för celecoxib hos individer med dubbel uppsättning av *CYP2C9*3* jämfört med individer med två normala *CYP2C9*-gener. Detta kan vara en av de faktorer som ligger bakom den dosberoende ökning i risken att drabbas av hjärt-kärlhändelser som observerats hos långtidsanvändare av celecoxib.

Delarbete **II** och **III** fokuserade på den genetiska varianten *CYP2C19*17* som förknippats med snabb omsättning av läkemedel som bryts ned via CYP2C19. Efter en enkeldos av magsårsmedicinen omeprazol (40 mg) uppvisade försökspersoner med dubbel uppsättning av *CYP2C19*17* i genomsnitt 52 % lägre exponering för omeprazol jämfört med individer med den vanliga varianten av *CYP2C19*-genen. Efter upprepad dosering av det antidepressiva läkemedlet escitalopram (5 mg två gånger dagligen i en vecka) noterades genomsnittligt 21 % lägre exponering hos försökspersoner med två kopior av *CYP2C19*17*, men denna skillnad var inte statistiskt säkerställd eftersom studien var dimensionerad för att påvisa en skillnad om 40 % mellan grupperna.

En klinisk frågeställning var utgångspunkten för delarbete **IV**, i vilket vi beskrev åtta fall med ökad effekt av det blodförtunnande läkemedlet warfarin vid samtidig medicinering med noskapin; en receptfri hostmedicin. Provrörsförsök visade att noskapin var en stark hämmare av både CYP2C9 och CYP3A4, två av de viktigaste enzymerna för nedbrytningen av warfarin.

Ett annat receptfritt läkemedel, glukosamin (som används mot ledbesvär), har misstänkts kunna interagera med, och förstärka effekten av, warfarin. För att undersöka om dessa misstänkta läkemedelsinteraktioner berodde på hämmad metabolism av andra läkemedel gavs i delarbete **V** en cocktail av fyra olika markörläkemedel före och under behandling med noskapin eller glukosamin. Under behandling med noskapin sågs en påtaglig hämning av både CYP2C9 och CYP2C19. Detta förklarar sannolikt den observerade interaktionen med warfarin. Under glukosaminbehandling noterades ingen enzymhämning och en metabol interaktion mellan warfarin och glukosamin förefaller således osannolik.

LIST OF PUBLICATIONS

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I. Lundblad MS, **Ohlsson S**, Johansson P, Lafolie P, Eliasson E. *Accumulation of celecoxib with a 7-fold higher drug exposure in individuals homozygous for CYP2C9*3*. Clin Pharmacol Ther. 2006;**79**(3):287-8.

II. Baldwin RM, **Ohlsson S**, Pedersen RS, Mwinyi J, Ingelman-Sundberg M, Eliasson E, Bertilsson L. *Increased omeprazole metabolism in carriers of the CYP2C19*17 allele; a pharmacokinetic study in healthy volunteers*. Br J Clin Pharmacol. 2008;**65**(5):767-74.

III. **Ohlsson Rosenborg S**, Mwinyi J, Andersson M, Baldwin RM, Pedersen RS, Sim SC, Bertilsson L, Ingelman-Sundberg M, Eliasson E. *Kinetics of omeprazole and escitalopram in relation to the CYP2C19*17 allele in healthy subjects*. Eur J Clin Pharmacol. 2008;**64**(12):1175-9.

IV. **Ohlsson S**, Holm L, Myrberg O, Sundström A, Yue QY. *Noscapine may increase the effect of warfarin*. Br J Clin Pharmacol. 2008;**65**(2):277-8.

V. **Rosenborg S**, Stenberg M, Otto S, Östervall J, Masquelier M, Yue QY, Bertilsson L, Eliasson E. *Clinically significant CYP2C inhibition by noscapine but not by glucosamine*. Clin Pharmacol Ther. 2010;**88**(3):343-6.

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LIST OF ABBREVIATIONS

ADR	Adverse drug reaction
AE	Adverse event
AUC	Area under the (concentration–time) curve
BCPNN	Bayesian confidence propagation neuronal network
BMI	Body mass index (weight divided by height-squared)
CI	Confidence interval
Cl	Clearance
C_{max}	Maximal concentration during the dosing interval
COX-2	Cyclooxygenase type 2 (Prostaglandin G/H synthase type 2)
CPTU	Clinical Pharmacology Trial Unit
CRF	Case Report Form
CV	Coefficient of variation
CYP	Cytochrome P450
DDI	Drug-drug interaction
df	Degree(s) of freedom
DNA	Deoxyribonucleic acid
ECG	Electrocardiogram
EM	Extensive metaboliser
F	Bioavailability
GCP	Good Clinical Practice
HLM	Human liver microsome
HPLC	High-performance liquid chromatography
IC	Information component
IC_{50}	Inhibitory concentration, 50% (see footnote on p. **Fel! Bokmärket är inte definierat.**)
ICH	International (Tripartite) Conference on Harmonisation
IM	Intermediate metaboliser
INR	International normalized ratio
i.v.	Intravenous(ly)
LC-MS	Liquid chromatography with mass spectrometric detection
LC-MS/MS	Liquid chromatography with mass spectrometric selection and detection (tandem mass spectrometry)
LLOQ	Lower limit of quantification
LOD	Limit of detection
M	Molar, moles/liter of solution, mol/L
MPA	Medical Products Agency (Läkemedelsverket)
NSAID	Non-steroidal anti-inflammatory drug
OR	Odds ratio
OTC	Over-the-counter
PCR	Polymerase chain reaction
PD	Pharmacodynamics
PK	Pharmacokinetics
PM	Poor metaboliser
PPI	Proton pump inhibitor

PRR	Proportional reporting ratio
mRNA	Messenger ribonucleic acid
SD	Standard deviation
SNP	Single nucleotide polymorphism (SNP is pronounced “snip”)
SPC	Summary of product characteristics
SPE	Solid phase extraction
SSRI	Selective serotonin re-uptake inhibitor
SWEDIS	Swedish drug information system (ADR register)
τ	Dosing interval
$T_{½}$ or $t_{½}$	Half-life (elimination half-life unless otherwise specified)
TDM	Therapeutic drug monitoring
UM	Ultrarapid metaboliser
UV	Ultraviolet
V_d	Volume of distribution (or apparent volume of distribution)
WMA	World Medical Association

1 INTRODUCTION

1.1 THE QUESTION

On Valentine's Day 2006, E., an 82-year-old retired physician with recurrent respiratory infections, once again got a bad cough. He was prescribed the OTC[*] cough syrup Nipaxon in a normal dose equivalent to 150 mg noscapine daily. E. had suffered a stroke two years earlier and had paroxysmal atrial fibrillation. Hence, he was treated with the anticoagulant[†] warfarin in a weekly dose of 20–20.5 mg. His INR[‡] had been stable within the therapeutic range. However, after six days of concomitant noscapine treatment, E.'s INR was 7.2, i.e. he was at great risk of bleeding. The same day his doctor called the Drug Information Centre at the Karolinska University Hospital and asked the question "Is noscapine known to interact with warfarin?" The answer to this question was "no", but in science it all depends on how you put the question. By asking the right question, the limits of knowledge can be extended. "Does noscapine interact with warfarin?" was the question that made this thesis become a unity and Papers **IV** and **V** are parts of the answer. First, however, a few other things need to be clarified.

1.2 CLINICAL PHARMACOLOGY

1.2.1 Introduction

The field of Clinical Pharmacology is about the safe and rational use of therapeutic drugs. To be able to prescribe and use therapeutic drugs safely and rationally, one has to know something about pharmacokinetics[§] (what the body does to the drug) and pharmacodynamics[**] (what the drug does to the body). These are the basics for all other branches of clinical pharmacology, including therapeutic drug monitoring (TDM), pharmacoepidemiology (the science of therapeutic drug use in the community), pharmacovigilance[††], drug interactions, clinical trials, and drug development. The following subsections on pharmacokinetics and pharmacodynamics are, unless otherwise stated, freely based on text books in pharmacology and clinical pharmacology, such as Rang & Dale, Rowland & Tozer, and Gabrielsson & Weiner [1-3].

---

[*] OTC is an abbreviation of over-the-counter, i.e. available without a doctor's prescription

[†] Anticoagulant, from Greek *anti-* (against) and Latin *coagulare* (to clot), refers to a substance that prevents the blood from clotting

[‡] INR (International Normalized Ratio) is a measure of anticoagulant effect. Normal INR is 1.0, therapeutic INR is 2.0–3.0.

[§] Pharmacokinetics (PK), from Greek *phármakon* (drug) and *kīnētikós* (moving), the branch of pharmacology concerned with the way drugs are taken into, move around, and are eliminated from, the body.

[**] Pharmacodynamics (PD), from Greek *dynamikós* (relating to the force), the branch of pharmacology dealing with the action and effect of drugs.

[††] Pharmacovigilance, from Greek *phármakon* (drug) and French *vigilance* (attentiveness), the science and activities relating to the detection, assessment, understanding and prevention of adverse effects or any other drug-related problem.

1.2.2 Pharmacokinetics

1.2.2.1 ADME

Drugs that are given orally need to be absorbed (A) in the gut to reach the systemic circulation for further distribution (D) to the site of action before being metabolised (M) and excreted (E). Absorption can be by passive diffusion or by active transport. Many drugs are even actively extruded back into the gut lumen once taken up into the epithelial* cells of the gut wall. The blood from the gut passes the liver before being distributed to the rest of the body. Absorption and distribution often involves active transport (in or out) through the gut wall, blood–brain barrier, blood–placenta barrier, and individual cell membranes [4]. Drugs that are not soluble in water need to be made more soluble before being excreted, mainly through bile and/or urine. For some drugs, this metabolism takes place already in the gut wall and upon first passage through the liver (first-pass metabolism). The liver is the main drug metabolising organ in the body. About 73% of clinically used drugs are cleared via metabolism, 25% by renal excretion, and a few per cent are excreted unchanged into the bile [5].

1.2.2.2 Clinical pharmacokinetics

When a tablet or capsule is taken orally, absorption and metabolism occurs simultaneously as shown in Figure 1. Initially absorption dominates and later elimination prevails the concentration–time curve. The fraction of an orally given drug that reaches the systemic circulation is called the bioavailability (F). The total exposure of the drug can be described by the area under the curve from intake to infinity ($AUC_{0-\infty}$). The rate of elimination is described by the clearance* of the drug. Clearance (Cl) is defined as the systemically given dose (D) divided by the AUC, or for an orally given drug,

$$Cl = F \times D / AUC_{0-\infty}$$

As the bioavailability cannot be determined without intravenous administration (F = 100% when the drug is given i.v.), the expression oral clearance (Cl/F) is often used. An alternative way of expressing the rate of elimination is the elimination half-life ($t_{½}$), which is the time needed for eliminating half the amount of drug in the body.

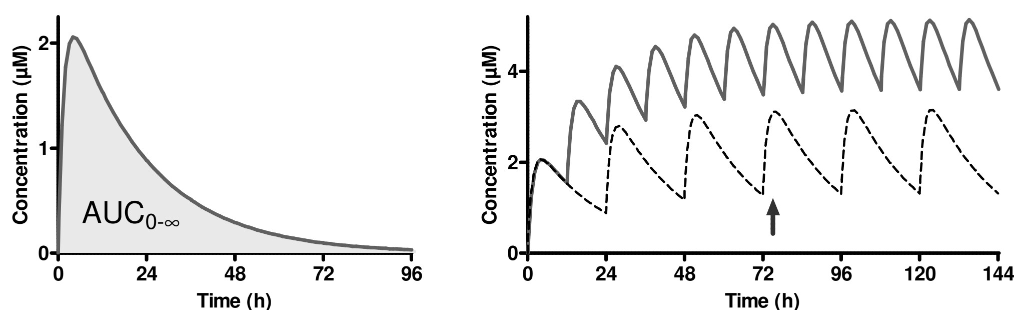

Figure 1 Plasma concentration plotted against time after single and repeated dosing. The shaded area in the left diagram is the $AUC_{0-\infty}$. The arrow in the right diagram indicates five half-lives, i.e. the time to reach steady-state. Solid line: dosing interval 12 hours, dashed line: dosing interval 24 hours.

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* Epithelial, from Greek *epi-* (above) and *thēlē* (teat, nipple), refers to the epithelium, the outer cell lining of any gland or gland duct, including the gastro-intestinal canal.

Upon repeated dosing with a constant dosing interval (τ), the drug concentration will gradually increase with every new dose until a steady state is achieved. During steady-state, the mean drug concentration (C_{ss}) is constant and the amount of drug absorbed during the dosing interval equals the amount of drug that is eliminated. The apparent volume of distribution (V_d) is the imaginary volume that would be needed to dissolve the given dose to achieve the same concentration as in plasma. The apparent volume of distribution is important for the pharmacokinetic profile after a single dose, but does not influence the steady-state concentration.
These parameters relate to one another according to the following equations:

$$C_{ss} = \frac{F \times D}{Cl \times \tau} \quad \text{and} \quad t_{½} = \ln 2 \times V_d / Cl$$

It follows that the steady-state concentration will increase with decreasing clearance, unless the dose is reduced or the dose interval prolonged. It can also be noted in the right diagram of Figure 1 that changing the dose interval will change the steady-state concentration, but not the time to steady-state, which (as a rule of thumb) is achieved after four to five half-lives.
Clearance is largely dependent on individual factors. Clearance of drugs that are excreted by the kidneys is highly dependent on renal function. Liver metabolism is mainly genetically determined as shown by, among others Alexanderson *et al.*, who could show that steady-state plasma concentrations of the antidepressant nortriptyline varied considerably more between fraternal than between identical twins [6].

1.2.3 Pharmacodynamics

Pharmacodynamics is a wide field and deals with all aspects of how drugs affect organisms, including mechanisms of actions and concentration–effect relationships. The following description will be limited to the latter aspects.

1.2.3.1 Concentration–effect relationship

Most concentration–effect relationships are sigmoid (i.e. S-shaped) when the effect or response is plotted against the logarithm of concentration. This means that if there is no or very little drug in the body, there will be no drug effect. Once the concentration rises, a threshold will be reached and there will be a steep increase in effect with increasing concentration. Upon further increase in concentration, the effect will level off to reach a maximum. When the maximal effect is reached, there will be no further increase in effect no matter how high the concentration. However, most drugs have more than one effect, sometimes wanted but often unwanted. These side-effects ideally occur at higher concentrations than the desired effect as shown in Figure 2.

1.2.3.2 Therapeutic interval

As a result of the sigmoid shape of the concentration–effect relationship and the occurrence of side-effects at high concentrations, it is tempting to define a therapeutic interval for any given drug. At therapeutic concentrations there should be a decent

---

[*] Clearance is a key pharmacological quantity that describes the capacity of drug elimination and is expressed in volume of cleared plasma (or blood) per unit time (e.g. L/h or mL/min).

beneficial effect and minimal side-effects. A therapeutic interval could therefore be defined as the concentration range between the level of minimal effect and the level of minimal side-effects. In Figure 2 this corresponds to about 1–10, where the upper limit depends on the tolerability of the side-effect. Dosing recommendations usually aim at reaching a steady-state concentration within the therapeutic interval.

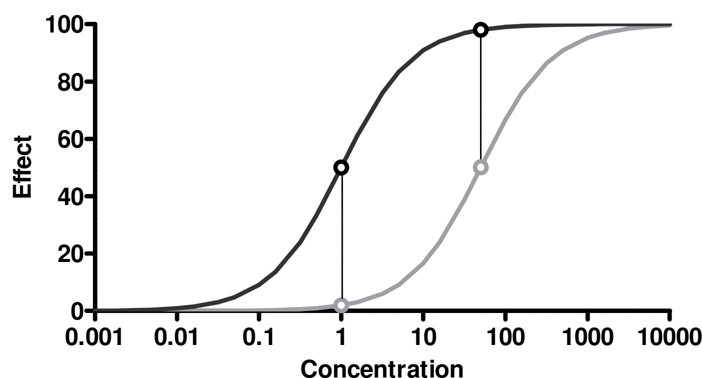

Figure 2 Schematic representation of concentration–effect relationships of a wanted (black) and an unwanted (grey) effect. Symbols represent half-maximal effects and the position on the other curve at the corresponding drug concentrations.

1.3 GENETICS

1.3.1 Cytochrome P450 in general

Many drugs are hydrophobic, i.e. poorly soluble in water, and need to be made hydrophilic before being excreted in urine or bile. This conversion is in many cases catalyzed by a superfamily of haeme-containing enzymes called cytochrome* P450 because they give colour to the liver (and kidney) cell and absorbs light at a peak of 450 nm in their reduced carbon monoxide binding state [7, 8]. Cytochrome P450s (CYPs) are divided into families designated with numbers and subfamilies designated with letters based on their aminoacid sequence homologies. The individual enzymes are then numbered, e.g. CYP1A2, CYP2C9, CYP2D6, CYP3A4, and so on. The genes that encode these enzymes are called the same, but are written in italics (*CYP1A2*, *CYP2D6*). Cytochrome P450 enzymes are responsible for the clearance of about three quarters of the drugs that are cleared via metabolism (i.e. about 55% of all drugs) [5]. Genetic variants have been shown to largely influence the metabolic activity of cytochrome P450 enzymes. Polymorphic† drug metabolism of CYP substrates was first reported for the tricyclic antidepressants desipramine and nortriptyline [9], the antiarrhythmic drug sparteine [10] and the antihypertensive agent debrisoquine [11]. This polymorphism was explained by loss-of-function variants of the *CYP2D6* gene [12-17]. Initially, subjects were divided into extensive metabolisers (EM) and poor metabolisers (PM), the latter of which are more prone to side-effects [12]. Later, ultrarapid metabolisers (UM) were discovered, who metabolise CYP2D6 substrates more rapidly than EMs and who are at risk of a subtherapeutic response at standard doses. UM phenotype‡ was shown to be associated with duplication of the *CYP2D6* gene [18] and up to 13 gene copies have been found on the same chromosome [19]. Figure 3 shows the population distribution of CYP2D6 phenotypes.

* Cytochrome, from Greek *kýtos* (container, body, cell) and *chrôma* (color), i.e. a cellular pigment

† Polymorphic, from Greek *polýs* (many) and *morphos* (form), refers to the simultaneous occurrence of different variants of a genetically determined trait in a population.

‡ Phenotype, from Greek *phaínein* (to shine) and *typos* (class, character), the observable or measurable constitution or trait of an individual, e.g. eye colour or CYP2D6 activity.

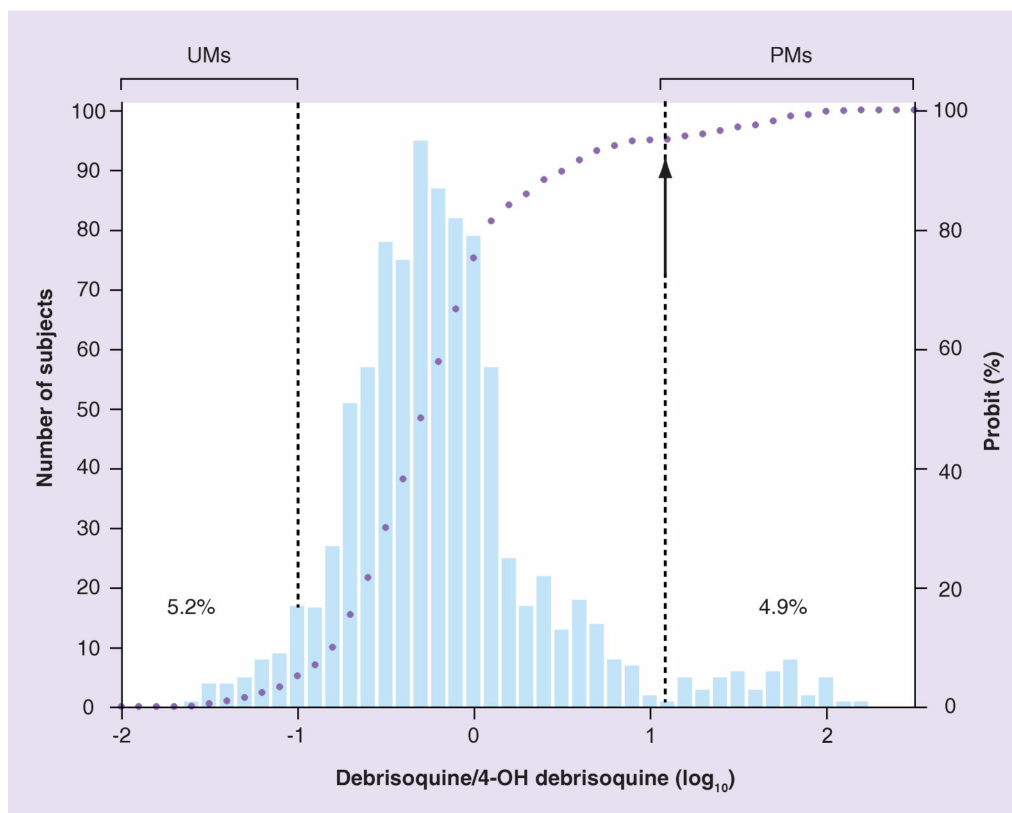

Figure 3 Distribution of CYP2D6 phenotypes measured as the metabolic ratio of debrisoquine. Note that the ultrarapid metabolisers do not form a distinct group, but rather a tail to the left. Reproduced from Llerena *et al.*, Pharmacogenomics 2009;10(1):17-28.

This thesis will focus on enzymes of another polymorphic cytochrome P450 subfamily (CYP2C), whose members are involved in the metabolism of 15-20% of clinically used drugs (or 25–30% of drugs metabolised by CYP enzymes) [5].

1.3.2 The *CYP2C* locus

All members of the CYP2C family are encoded by genes that, in humans, cluster together on the long arm of chromosome 10 (10q24) [20]. The human *CYP2C* locus contains four genes: *CYP2C8*, *2C9*, *2C18* and *2C19*. All of these are polymorphic and the enzymes encoded by these genes are important for the metabolism of several drugs and other xenobiotics* [20-22]. The enzyme CYP2C18 has, however, not been unequivocally shown in human tissues [21]. Some authors claim an association between variants of *CYP2C18* and different pharmacological effects [23, 24], but this is probably due to linkage disequilibrium† between different point mutations (SNPs) within the *CYP2C* locus. The *CYP2C18* gene can, however, be expressed *in vitro*‡ and

* Xenobiotic, from Greek xénos (foreign) and *biōtikós* (concerning life), refers to a substance not generated within the body.

† Linkage disequilibrium (LD) refers to the situation when two (or more) traits occur together more often than would be expected from their frequency in the population, i.e. when the traits are non-randomly associated. Complete LD means that the traits are inherited dependently on each other.

‡ *in vitro* (within the glass): a procedure taking place in a test tube or Petri dish involving cultivated cells or cell preparations.

the gene product has enzymatic activity. Some substrates of the CYP2C enzymes are shown in Table 1.

Substrate class	CYP2C8	CYP2C9	(CYP2C18)	CYP2C19
Anti-epileptics	(phenytoin)	phenytoin	(phenytoin [25]) (*R*-mephenytoin)	phenytoin *S*-mephenytoin
Anti-diabetics	(tolbutamide) repaglinide [26] rosiglitazone [26] pioglitazone [26]	tolbutamide glibenclamide [26]		
NSAIDs		diclofenac ibuprofen piroxicam celecoxib [27]		
Anti-coagulants	(*R*-warfarin)	*S*-warfarin acenocoumarol [28] phenprocoumon [29]	(*R* and *S*-warfarin)	*R*-warfarin
Anti-microbials	amodiaquine chloroquine [30]	(voriconazole [31])		proguanil voriconazole [31]
Proton pump inhibitors			(lansoprazole [32])	omeprazole pantoprazole lansoprazole
Anti-depressants				citalopram imipramine chlomipramine
Various (endogenous and exogenous)	arachidonic acid [33] retinol retinoic acid benso(*a*)pyrene montelukast [34] paclitaxel	arachidonic acid [33] Δ^1-tetrahydro-cannabinol		diazepam clopidogrel [35] propranolol

Table 1 Substrate specificity of different CYP2C enzymes according to Goldstein *et al.* [21, 22], unless otherwise specified. Weak effects or effects only shown *in vitro* have been put within parentheses.

1.3.3 *CYP2C8*

To date (October 2010) 14 different alleles* have been described for CYP2C8 [36]. Some of them have been found to result in abolished or decreased enzyme function [36,

* Allele, from Greek *allelos* (each other), refers to a specific variant of a gene carrying one or more point mutations (SNPs). Each individual carries two alleles (one of maternal and one of paternal origin), which may or may not be the same. The first allele variant ever reported is designated **1* and is also called *wild-type*.

37]. *CYP2C8*3* that encodes an enzyme with reduced *in vitro* catalytic activity has an allele frequency[*] of 10-23% in Caucasian populations [30]. The results of clinical studies of the *in vivo* effect of this allele are inconsistent; It has been associated with increased clearance of the antidiabetics rosiglitazone and repaglinide [38]. However, in patients treated with the anticancer drug paclitaxel somewhat lower clearance [39] and higher risk of neurotoxicity [40] has been observed. The clinical impact of CYP2C8 polymorphisms remains to be clarified [21, 41].

1.3.4 *CYP2C9*

The polymorphism of CYP2C9 was first described in the late 1970s for the metabolism of the oral antidiabetic agent tolbutamide [21]. This polymorphism was later discovered to be due to a rare allele now called *CYP2C9*3* that carries a functional SNP. To date 34 different *CYP2C9* alleles have been described, all of them associated with decreased or abolished metabolic capacity [36]. Recently a case of ultrarapid CYP2C9 metabolism was described but the genetic background remains to be elucidated [42].

1.3.4.1 *CYP2C9*2*

The *CYP2C9*2* allele was described by Rettie *et al.* in 1994 [43]. It has been shown to encode an enzyme with somewhat changed substrate specificity and a lower catalytic capacity than the wild-type enzyme, possibly because of a lower affinity for the cytochrome P450 oxidoreductase [44]. For tolbutamide, *CYP2C9*2* has been associated with a somewhat decreased clearance only in homozygous[†] individuals [26]. An epidemiological study showed no significant difference in the dose needed for glycaemic control in *CYP2C9*2* carriers treated with oral antidiabetics [45]. In contrast, the clearance of warfarin is reduced to the extent that a 20% lower maintenance dose is required in heterozygous[‡] individuals and 36% lower in homozygous individuals [46]. In a Swedish population, the allele frequency of *CYP2C9*2* is 9-13% [47].

1.3.4.2 *CYP2C9*3*

As described above, the *CYP2C9*3* allele encodes an enzyme with a prominent reducetion in enzymatic capacity for all investigated substrates. On average, *CYP2C9*3* heterozygotes require 34% and homozygotes 78% less warfarin than wild-type carriers [46]. Carriers of *CYP2C9*3* also have a greater risk of over-anticoagulation during warfarin treatment, both during initial and maintenance therapy [48, 49]. *CYP2C9*3* carriers need dose adjustments more often and still have a tendency to have a larger hypoglycaemic effect of oral antidiabetics than non-carriers [45, 50]. The allele frequency is about 7,5% in a Swedish population [47].

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[*] Allele frequency for a variant allele is the number of variant alleles found divided by the total number of alleles investigated. A homozygous individual contributes with two alleles and a heterozygous with one allele. If one homozygote and ten heterozygotes are found among 100 subjects, the allele frequency will be (2+10)/(100×2) = 6%.

[†] Homozygous, from Greek *homós* (one and the same) and *zygón* (yoke), refers to the carriage of two identical alleles of maternal and paternal origin. A homozygous individual is said to be a homozygote.

[‡] Heterozygous, from Greek *héteros* (the other of two, different) and *zygón* (yoke), refers to carrying different alleles on the maternal and paternal chromosomes.

1.3.5 *CYP2C19*

The polymorphism of this enzyme was first described for the metabolism of the anti-epileptic drug mephenytoin [51]. Hence the enzyme is also known as mephenytoin hydroxylase. To date 28 alleles of *CYP2C19* have been described [36]. Most of them are rare, however, and the following description will be limited to the most common alleles. Some rare alleles are common in special populations and may have clinical relevance in certain ethnic groups [52].

1.3.5.1 *CYP2C19*2* and *CYP2C19*3*

*CYP2C19*2* and **3* were originally described by de Morais *et al.* in 1994 [53, 54]. *CYP2C19*2* results in a splicing defect of mRNA and *CYP2C19*3* results in the insertion of a premature stop codon [21]. Thus no enzyme is produced from neither of these alleles. The allele frequency varies between different populations and in a Caucasian population poor metaboliser phenotype occurs at a frequency of 3-5%, while 12-23% of most Asian populations are PMs. However, some Polynesian and Micronesian populations have a frequency of PM genotype of up to 79% [55].

1.3.5.2 *CYP2C19*17*

*CYP2C19*17* was first described in 2006 and consists of two coupled SNPs in the upstream regulatory region of the *CYP2C19* gene. The mutation at position -806 (C>T) results in the binding of nuclear proteins, which leads to increased expression of the gene and higher than average enzyme activity, whereas the other mutation (position -3402) is silent [56]. The two SNPs are in complete linkage disequilibrium in Caucasians and Ethiopians [56], but not always in Sub-Saharan African populations [52]. Carriers of *CYP2C19*17* are rapid extensive metabolisers but, as will be discussed in further detail in relation to Papers **II** and **III**, not ultrarapid metabolisers. The allele frequency of *CYP2C19*17* has been estimated to 18-32% in European and Ethiopian populations but only 4% in Chinese [56, 57].

1.3.6 *CYP2C* haplotypes*

The *CYP2C* alleles are in linkage disequilibrium with each other. As suggested in Paper **II**, the *CYP2C19*17* allele is almost always inherited together with *CYP2C8*1* and *CYP2C9*1*, at least in Nordic populations. Only one of 896 Nordic subjects carried the *CYP2C19*17* allele together with *CYP2C8*3* [58]. *CYP2C19*2* is in complete linkage disequilibrium with *CYP2C9*2* or *CYP2C9*3*. *CYP2C8*3* is often inherited together with *CYP2C9*2* [58, 59]. Pedersen *et al.* described ten different *CYP2C* haplotypes, of which 6 occured in 99% of Nordic subjects [58], see Table 2.

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* Haplotype, from Greek *haploûs* (onefold, single), refers to a set of alleles inherited by an individual from a single parent.

Haplo-type	*CYP2C19*17*	*CYP2C19*2*	*CYP2C9*2*	*CYP2C9*3*	*CYP2C8*3*	No of haplotypes	PHASE frequency	STATA frequency
1	–	–	–	–	–	869	0.4862	0.4866
2	+	–	–	–	–	342	0.1901	0.1902
3	–	+	–	–	–	286	0.1589	0.1595
4	–	–	+	–	–	39	0.0218	0.0219
5	–	–	–	+	–	102	0.0562	0.0558
6	–	–	–	–	+	4	0.0023	0.0023
7	+	–	–	–	+	1	0.0006	8.3E-30
8	–	+	–	–	+	5	0.0026	0.0027
9	–	–	+	–	+	142	0.0787	0.0791
10	–	+	+	–	+	2	0.0012	0.0001

Table 2 *CYP2C* haplotypes in a Nordic population (276 Danish, 309 Norwegian and 311 Faroese subjects). Allele frequencies calculated with two different methods (PHASE and STATA). Note that each individual carries two haplotypes, thus the sum is 896×2. Reprinted from Pedersen *et al.* [58].

1.4 DRUG INTERACTIONS

This thesis will focus on drug interactions in the context of drug-drug interactions (DDIs), but food-drug and herb-drug interactions are not to be forgotten, as we have learnt from history.

1.4.1 History

Drug interactions were first recognized in the early 1960's. Dramatic food-drug interactions (severe headaches, hypertensive crises, intracranial haemorrhages and even deaths) were first described in 1963 following cheese consumption in patients treated with the antidepressant tranylcypromine and other irreversible monoamine oxidase inhibitors [60-62]. It could be shown that this was likely due to the tyramine content in cheese [63].
The same year a Danish group published a case series and a clinical study of the drug-drug interaction between the sulphonamide antibiotic sulphaphenazole and tolbutamide [64] (Figure 4). This was the first description of a drug interaction mediated by CYP2C9 inhibition. Sulphaphenazole is now the standard CYP2C9 inhibitor used in *in vitro* systems [65].

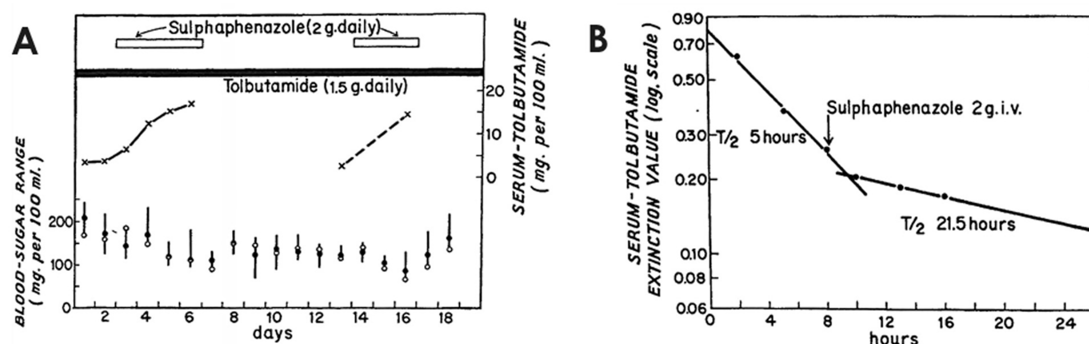

Figure 4 (A) Sulphaphenazole-induced increase in serum tolbutamide and decrease in blood glucose. (B) Effect of i.v. sulphaphenazole on the elimination half-life of tolbutamide. Reproduced from Christensen *et al.*, Lancet, 1963;2(7321):1298-301.

It was soon realised that drugs (and other exogenous compounds) may both induce and inhibit the metabolism of other drugs, thus complicating pharmacotherapy [66]. Extensive and ultrarapid metabolisers are generally more sensitive to inhibitory drug interactions than poor metabolisers [67], a fact that still isn't fully realised by the medical community (e.g. [68]). In some instances, a metabolic pathway can play a minor role in EMs, but act as an escape route in PMs. Inhibition of such an escape route may have a stronger impact in poor metabolisers. An example of this is the anti-depressant venlafaxine that is mainly metabolised by CYP2D6 and to a lesser extent by CYP3A4. In some CYP2D6 PMs, the co-administration of the CYP3A4 inhibitor keto-conazole causes a major inhibition of venlafaxine metabolism [69]. Another exception is when poor metabolisers have a low, but not absent, enzymatic capacity. One example of this is the interaction between the oral anticoagulant agent acenocoumarol and NSAIDs metabolised by CYP2C9. None of these drugs inhibit CYP2C9, but given concomitantly they increase the risk of overanticoagulation in *CYP2C9*2* and *CYP 2C9*3* carriers, but not in homozygous wild-type carriers [70].

1.4.2 CYP2C8-mediated drug interactions

Only a few CYP2C8-mediated drug interactions of clinical importance have been described. Concomitant treatment with sulfamethoxazole/trimethoprim and the oral antidiabetic agent repaglinide resulted in symptomatic hypoglycaemia [71], probably due to trimethoprim inhibiting CYP2C8. Gemfibrozil, an antilipaemic[*] agent, also inhibits CYP2C8 and affects the pharmacokinetics of repaglinide [72], ibuprofen [73] and montelukast [34], an oral antiasthmatic agent, that is a well established *in vitro* inhibitor of CYP2C8 [65], but that does not seem to inhibit CYP2C8 *in vivo*[†] [74]. There has been concern that the anticancer drug sorafenib (kinase inhibitor) may inhibit the CYP2C8 dependent metabolism of paclitaxel (also known as taxol, a cytostatic agent derived from the Pacific yew-tree, *Taxus brevifolia*), but this has not been shown in pharmacokinetic studies [75, 76], although higher mortality rates were observed when combining the two drugs in patients with advanced squamous cell lung cancer [77].

One of the probably most clinically significant CYP2C8-mediated inhibitory drug-drug interactions might have been the interaction between the lipid-lowering agents gemfib-rozil (a potent inhibitor of CYP2C8 *in vivo* [41]) and cerivastatin. The latter of these drugs was withdrawn from the market in August 2001 due to an unexpectedly high number of rhabdomyolysis[‡] cases, many of which were associated with cerivastatin used in a high dose or in combination with gemfibrozil [41, 78].

CYP2C8 is also readily induced by certain drugs, especially rifampicin [41], but clinical evidence is lacking about the impact on drug metabolism *in vivo*.

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[*] Antilipaemic, from Greek *antí* (opposite), *lípos* (fat) and *haîma* (blood), refers to the ability to lower blood fat levels.

[†] *in vivo*, Latin (in the living): an experiment involving the whole, intact organism.

[‡] Rhabdomyolysis, Latin from Greek *rhábdos* (rod, stick), *mŷs* (muscle), and *lýsis* (loosening or breakage), rapid breakdown of skeletal muscle, often associated with acute renal failure.

1.4.3 CYP2C9-mediated drug interactions

In contrast to CYP2C8-mediated drug interactions, there is a voluminous literature on CYP2C9-mediated drug interactions. Some examples are given below.

1.4.3.1 Antidiabetics

The first CYP2C9-mediated drug-drug interaction was described in the late 1950's between tolbutamide and isoniazid [79], although the mechanism was not known at the time. The concomitant medication with oral antidiabetics and CYP2C9 inhibitors is still a major issue, as illustrated in a Finnish study where 20% of patients treated with glibenclamide (glyburide in the US), glimepiride or glipizide received a known CYP2C9 inhibitor during admission to hospital, resulting in exaggerated pharmacodynamic effect [80]. It seems that drug interactions that inhibit the metabolism of oral antidiabetics are more important clinically than interactions with drugs that induce the metabolic capacity of antidiabetic agents, as both rifampicin [81] and St John's wort [82] have been shown to substantially decrease exposure to glimepiride and gliclazide, respectively, but the investigators have been unable to show a corresponding change in pharmacodynamics in healthy volunteers. On the other hand, a diabetic patient had to double his gliclazide dose while concomitantly treated with rifampicin [83] and Park *et al.* could show both pharmacokinetic and pharmacodynamic effects in healthy volunteers given single doses of gliclazide before and after 6 days of rifampicin [84]. Possible reasons for these discrepancies are 1) differences in study design (with or without oral glucose tolerance testing), 2) rifampicin being a more potent inducer than St John's wort, 3) differences in the pharmacokinetic/pharmacodynamic relationship in diabetics and healthy volunteers. The reason for the relative lack of case reports of therapeutic failure in diabetic patients treated with oral agents may be that there are many other reasons for suboptimal therapeutic response in type-2 diabetes than DDIs and that hypoglycaemia is more likely to be symptomatic than hyperglycaemia [85].

1.4.3.2 Warfarin

One of the most studied CYP2C9 substrates in relation to drug interactions is warfarin. Warfarin is an important drug that has revolutionised the treatment of thromboembolic diseases [86]. The most active warfarin enantiomer*, *S*-warfarin, is mainly metabolised by CYP2C9 [43, 48, 87, 88]. Since warfarin has a narrow therapeutic interval, it is particularly prone to drug interactions. To date (October 2010) 219 drug-drug or herb-

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* Enantiomer, from Greek *enantíos* (opposite) and *méros* (part), one of a pair of optical isomers that mirror each other. When mixed in a 1:1 proportion the mixture of the two enantiomers (or stereoisomers) is called a racemate, from Latin *racemus* (bunch of grapes) over French *acid racémique* (tartaric acid). There are three different systems to depict stereoisomers: 1) optical activity: (+) or *d* for dextrorotatory and (-) or *l* for levorotatory, 2) comparison with glyceraldehyde: D or L, or 3) arranged according to atomic number prioritization: *R* and *S* for Latin *rectus* (right) and *sinister* (left). None of these systems have any fixed relation to the others. The *R*/*S* system is more general as it can be used to specify several optical centers in the same molecule, and has the advantage of not confusing the sometimes opposite meaning of a lower case letter (*l*/*d*) with a small capital letter (L/D), e.g. D-fructose is *l*-fructose and has four optical centers, thus (2*R*,3*S*,4*R*,5*R*)-2,5-bis(hydroxymethyl)oxolane-2,3,4-triol.

drug interactions are listed in the Swedish-Finnish Interaction X-referencing Database (SFINX) [89], most of them pharmacokinetic, but also many pharmacodynamic interactions. Inhibitory interactions include those with the antibiotic sulfamethoxazole/ trimethoprim, the antifungal drug fluconazole, and the antiarrhythmic amiodarone. Substances that can induce the metabolism of warfarin include St John's wort, the tuberculostatic drug rifampicin and the anti-HIV-drug nevirapine [89].

1.4.3.3 Phenytoin

Another important CYP2C9 substrate is the antiepileptic drug phenytoin. Phenytoin has a narrow therapeutic interval and has the pharmacokinetically interesting property to saturate its own metabolism even at concentrations within the therapeutic range, thus making plasma concentrations rise disproportionately to an increase in dose [1]. The SFINX interaction database lists 239 known interactions with phenytoin. Most of them are due to the ability of phenytoin to induce CYP3A4 and thus decrease the plasma levels of many other drugs [89]. However, amiodarone, isoniazid, sulfamethoxazole/ trimethoprim, fluconazole, and the antidepressant fluoxetine have been associated with inhibition of phenytoin metabolism, whereas nelfinavir and rifampicin can decrease phenytoin plasma levels by inducing CYP2C9 [89].
A recent case report of ultrarapid phenytoin metabolism illustrates that extensive and ultrarapid metabolisers are prone to inhibitory drug interactions. Despite being treated with high doses of phenytoin (600–700 mg daily) the patient had very low plasma levels. On two separate occasions she received fluconazole for fungal infections and was intoxicated by phenytoin (Figure 5) [42].

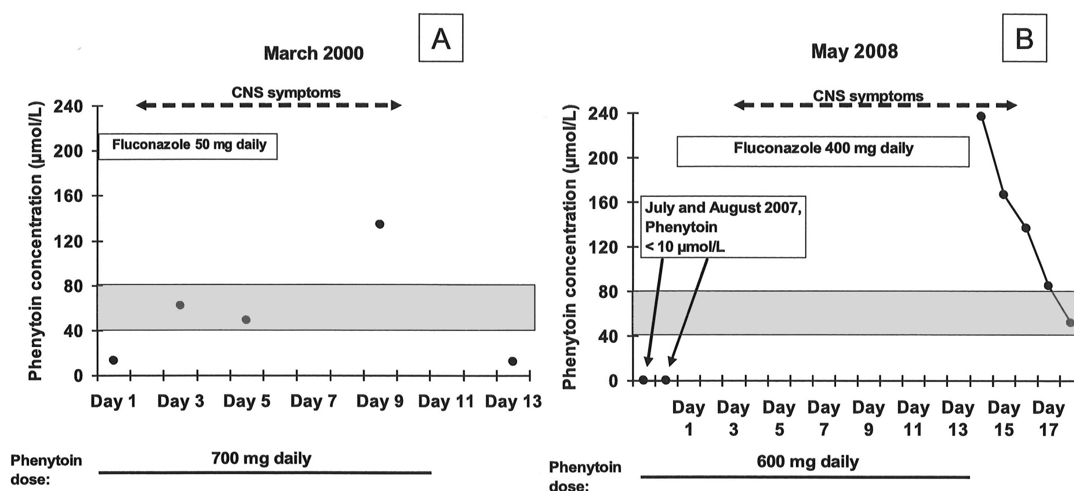

Figure 5 Schematic illustration of two phenytoin–fluconazole interaction episodes. (A) In March 2000, the patient showed central nervous system (CNS) symptoms a few days after the initiation of fluconazole treatment, despite phenytoin concentrations within therapeutic range (40–80 µmol/L, shaded area). The CNS symptoms were probably caused by the rapid increase in phenytoin plasma levels. (B) In May 2008, the patient developed severe signs of phenytoin toxicity after a few days of concomitant fluconazole treatment. From Helldén *et al.*, Eur J Clin Pharmacol., 2010; 66(8):791-5.

1.4.4 CYP2C19-mediated drug interactions

1.4.4.1 The clopidogrel–PPI interaction

There has been considerable scientific controversy in recent years about the interaction between the irreversible platelet aggregation inhibitor clopidogrel and proton-pump inhibitors (PPIs), especially omeprazole; first whether there is an interaction or not, and second whether this interaction is of clinical relevance. Clopidogrel is used in cardiovascular diseases for preventing myocardial and cerebral (re-)infarction. PPIs are often given to the same patients to prevent gastrointestinal haemorrhage due to aggressive antithrombotic treatment, which often include aspirin, clopidogrel, low molecular-weight heparin, and sometimes even antagonists of the platelet glycoprotein IIb/IIIa receptor [90]. Currently, there are over 200 articles in PubMed* regarding the clopidogrel–PPI combination. The adverse interaction was first noted in 2006 by Gilard *et al.* who could show higher platelet aggregability in omeprazole users compared to non-users when treated with clopidogrel [91]. These preliminary *ex vivo*† findings have since been reproduced by several investigators [92-94]. Hulot *et al.* showed an association between *CYP2C19*2* and clopidogrel resistance [95], and Mega *et al.* could show a relation between *CYP2C19* genotype, plasma levels of the active thiol metabolite of the inactive prodrug clopidogrel, and clinical outcome [35]. Omeprazole (or its sulphone metabolite) is an inhibitor of CYP2C19 [96, 97] and hence possibly of clopidogrel bioactivation. An increased risk of a new cardiovascular event has been observed in carefully performed epidemiological studies in patients receiving concomitant therapy with clopidogrel and PPIs [98, 99]. Indeed, if the entire difference in the observed risk of a new cardiovascular event were ascribed to the interaction, this would correspond to an abolished clopidogrel effect [98]. However, these findings could not be reproduced in a retrospective re-analysis of two randomized controlled trials [100]. To further complicate matters, clopidogrel has been shown to inhibit the metabolism of omeprazole [101], possibly further increasing the CYP2C19 inhibition by shunting omeprazole to the CYP3A4-generated sulphone metabolite. In conclusion, omeprazole inhibits the bioactivation of clopidogrel, diminishes clopidogrel response *ex vivo*, but the clinical implication of this is still unclear. It has been suggested that this potential interaction may be avoided by separating the intake of clopidogrel and omeprazole, but this has not been investigated. Indeed, although omeprazole acts as a competitive‡ inhibitor *in vitro* [97], omeprazole administration has been shown to inhibit CYP2C19 *in vivo* in a time-dependent§ manner [96, 102, 103].

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* PubMed is a free database of biomedical citations supplied by the US National Library of Medicine.

† *ex vivo*, Latin (out of the living), refers to an experiment or measurement done on cells or tissues taken out of the living organism.

‡ Competitive inhibition: A situation when the inhibitor competes with the substrate for the same binding site. Competitive inhibition can be overcome by increasing the concentration of the substrate in relation to the inhibitor. Cf non-competitive inhibition: when the inhibitor binds to a site different from the substrate, thus changing the conformation of the enzyme (or transporter) and the affinity for the substrate.

§ Time-dependent inhibition: Any type of inhibition that involves a dimension of time, often mechanism-based inhibition, i.e. when the inhibitor forms a (relatively) stable enzyme-inhibitor complex, thus inactivating the enzyme.

1.4.4.2 Omeprazole and diazepam

It is fascinating how some scientific information can disseminate into population groups far from academia. When I was at the Detox Unit in Falköping in the south-west of Sweden during my internship, I heard from the patients that you could get a better rush from diazepam (Valium®) if you took omeprazole (Losec®) concomitantly. The pharmacokinetic background to this phenomenon was elucidated in the late 1980's and early 1990's, when it could be shown first that diazepam metabolism was related to mephenytoin hydroxylase (CYP2C19) phenotype [104]. It was soon shown in interaction studies that the clearance of diazepam was decreased in a dose-dependent manner by 25-50% after seven days of omeprazole [105, 106]. This raised the idea that omeprazole was metabolised by CYP2C19, too. A clinical trial confirmed this in 1990 [107]. No studies have been found that correlate this interaction to an increased pharmacodynamic effect, but it might be clinically relevant, especially as it seems to be common knowledge among substance abusers.

1.4.4.3 Oral contraceptives

Combined oral contraceptives (OCs) have been shown to significantly inhibit the CYP2C19-mediated metabolism of mephenytoin and omeprazole [108, 109]. In one study healthy volunteers, previously phenotyped for CYP2C19 activity with mephenytoin and/or omeprazole, were stratified according to sex and OC use. The ratios of *S*/*R*-mephenytoin and omeprazole/5-hydroxyomeprazole were 2.5- and 2-fold higher, respectively, in women taking OCs compared to men and women not taking OCs [108]. This has also been reproduced in a placebo-controlled trial showing very similar results [109]. No general sex difference in CYP2C19 activity has been consistently found [108, 110]. However, during pregnancy, decreased CYP2C19 activity has been observed [111]. The mechanism for female sex steroids to affect drug metabolism has not been fully clarified. Palovaara *et al.* could show that the effect was mediated by the oestrogen component rather than the progestin of combined OCs [112]. Ethinyloestradiol (the oestrogen component of most combined oral contraceptive pills) has been shown to have some inhibitory effect on CYPs *in vitro*, but the IC_{50}[*] for CYP2C19 is some 500-fold greater than the ethinyloestradiol concentrations achieved *in vivo* [113]. Recently, however, it could be shown that the *CYP2C19* gene contains an oestrogen responsive element in its promoter[†] region and this was shown to be able to mediate down-regulation of gene transcription[‡] at oestrogen concentrations of physiological magnitude [114].

1.4.4.4 Phenytoin

Phenytoin is mainly metabolised by CYP2C9 but CYP2C19 contributes to a lesser degree (approximately 15-20% of total clearance as estimated from *in vitro*

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[*] IC_{50}, inhibitory concentration 50%, the concentration needed to achieve half maximal inhibition.

[†] Promoter: a regulatory region of DNA located upstream of a gene, providing a control point for regulated gene transcription.

[‡] The central dogma of molecular biology: DNA is *transcribed* to mRNA, which is *translated* into polypeptides (called proteins if they are larger than or equal in size to insulin). The whole process including post-translational modification is called gene *expression*.

studies [115]) (Table 1). Phenytoin intoxications have been noted in patients prescribed CYP2C19 inhibitors, such as the SSRI fluvoxamine [116] and the platelet aggregation inhibitor ticlopidine [117, 118]. In the fluvoxamine case, phenytoin levels were increased from 65 to 194 µmol/L (therapeutic range 40-80 µmol/L) upon concomitant therapy with fluvoxamine 50 mg daily in a patient not carrying any mutant allele of *CYP2C9* or *CYP2C19* [116]. Fluvoxamine has been shown to be a moderate inhibitor of several CYPs, including CYP2C9 and CYP2C19 [119, 120]. There are two cases of phenytoin intoxication after the addition of ticlopidine resulting in severe intoxication symptoms and phenytoin plasma levels around 180 µmol/L [117, 118]. In the American case, CYP2C9 and CYP2C19 genotypes were found to be wild-type [118]. Ticlopidine has been shown to be a selective mechanism-based inhibitor (see footnote on page 13) of CYP2C19 [121], but a weak inhibitor of CYP2C9 [118]. This indicates that CYP2C19 may play a greater role in phenytoin metabolism than predicted from *in vitro* studies, especially in the upper part of the therapeutic range, where CYP2C9-mediated phenytoin metabolism may be saturated.
Phenytoin is also an inducer of several CYP enzymes, including CYP3A4, CYP2C9 and CYP2C19. When combining an enzyme inducer with an enzyme inhibitor, the results may be difficult to predict. This is illustrated by the combination of the anti-fungal drug voriconazole (which is primarily metabolised by CYP2C19 and an inhibitor of CYP2B6, CYP3A4, CYP2C9 and CYP2C19 [122]) with phenytoin: Healthy volunteers received either voriconazole (200 mg b.i.d.[*]) and placebo or voriconazole and phenytoin (300 mg q.d.[†]) for 21 days. Those who received phenytoin continued for another week with voriconazole 400 mg b.i.d. The exposure (AUC) to voriconazole on day 21 was decreased by 70% in the subjects receiving phenytoin. This could be compensated for by doubling the voriconazole dose, but this resulted in an 80% increase in the phenytoin exposure [123]. Thus monitoring of plasma concentrations are often mandatory when combining strong inducers and strong inhibitors to avoid underdosing of the inhibitor or overdosing of the inducer.

1.4.5 Noscapine

Noscapine (a.k.a. narcotine) is a naturally occurring opium alkaloid with cough-suppressing effects, but without central opioid effects [124]. It accounts for up to 20% of the alkaloid content in latex from opium poppy (*Papaver somniferum*) varieties [125] and up to 40% of the alkaloid content of poppy seeds [126]. In spite of its, at the most, moderate effect [127] noscapine is a commonly used anti-tussive in Scandinavia with 96% of annual sales sold over-the-counter (OTC) [128]. Noscapine has recently been shown to have anti-tumoural properties and is currently under investigation in two clinical trials in hematological malignancies [129, 130]. The drug interaction potential of noscapine was first investigated in 1967, when it could be shown that noscapine increased the blood-pressure lowering effect of neostigmine in cats [131]. Otherwise the drug interaction potential of noscapine does not seem to have been investigated.

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[*] b.i.d., Latin *bis in diē* (twice daily); t.i.d., *ter in diē* (three times daily), q.i.d., *quattōr in diē* (four times daily)

[†] q.d., Latin *quaque diē* (every day), i.e. once daily

1.4.6 Glucosamine

Glucosamine is an aminosugar that is a component of endogenous glucosaminoglycans, such as heparan sulphate and hyaluronic acid [132, 133]. It was first used in the late 1950's as it was shown to increase the systemic availability of tetracyclines [134]. In recent years, glucosamine has gained interest in treating osteoarthritic pain as it has been shown to stimulate the formation of cartilage *in vitro* and in some *ex vivo* and *in vivo* animal studies [135-137]. However, controlled clinical trials in man have not unequivocally shown a positive effect [138]. Reports from all over the world have raised the suspicion that glucosamine might interact with warfarin to increase the anticoagulant effect. This effect has been seen after several weeks of combined use [132, 139-141]. In a single single case report a decreased anticoagulant effect was observed when acenocoumarol (another oral anticoagulant similar to warfarin) was combined with a glucosamine sulphate preparation [142]. The mechanism for the potential interaction between glucosamine and oral anticoagulants is unknown.

2 AIMS

The overall aim of this thesis was to investigate inter-individual differences in CYP2C dependent drug metabolism and the influence of specific genetic variants and drug interactions, with special emphasis on OTC drugs. The ultimate goal of this work has been to reach a better understanding of the reasons for intra- and inter-individual variations in pharmacokinetics and to improve the safety of pharmacotherapy.

The specific aims of the individual studies were:

Paper I: To investigate whether homozygous carriers of the low-function allele *CYP2C9*3* would accumulate celecoxib (a COX-2 selective non-steroidal anti-inflammatory drug) given repeatedly in a normal dose.

Paper II: To prospectively investigate the influence of the novel *CYP2C19*17* allele on the pharmacokinetics of a single dose of omeprazole and to decide whether the earlier estimates of increased omeprazole metabolism could be verified.

Paper III: To prospectively investigate the influence of the novel *CYP2C19*17* allele on the steady-state pharmacokinetics of *S*-citalopram (escitalopram) and decide whether the preliminary claims of ultrarapid metabolism of omeprazole could be translated to another clinically important CYP2C19 substrate.

Paper IV: To try to answer the question "Does noscapine interact with warfarin?" and to propose a pharmacokinetic mechanism for the suspected interaction based on *in vitro* inhibition assays.

Paper V: To investigate the pharmacokinetic effects of noscapine and glucosamine on *in vivo* CYP enzyme activity measured with different probe drugs. The cocktail of CYP probe drugs was based on the validated "Karolinska cocktail" and the components chosen according to potential relevance for warfarin metabolism.

3 METHODS

3.1 SUBJECTS

Subjects for the studies in Papers **I–III** and **V** were recruited from a database of previously pheno- and/or genotyped individuals that had participated in earlier studies (e.g. [143-145]) at the Clinical Pharmacology Trial Unit (CPTU), Karolinska University Hospital, Huddinge. They were pre-screened by telephone by a research nurse to assure that they were interested in participating in and eligible for the respective study. If interested and eligible, they came to a screening visit were they were given written and oral information and gave written consent before any study-related procedures were undertaken. Subjects were included in the study if physical examination and biochemical screening, including a urinalysis of illicit drug use, were approved. All studies were performed according with the contemporary versions of the WMA Helsinki Declaration and ICH-GCP guidelines and applicable local legislation.
All clinical trials were approved by the Regional Ethics Committee and by the Swedish Medical Products Agency (Läkemedelsverket).

3.2 STUDY DESIGNS

3.2.1 Paper I

This study was an open study of celecoxib 200 mg once daily for seven days given to healthy volunteers stratified according to *CYP2C9* genotype. Plasma sampling for pharmacokinetic analyses was made after the first (pre-dose and at 1, 2, 3, 4, 10 and 24 hours) and after the last dose (at the same time points with extra sampling at 48 hours post-dose).
Inclusion criteria were in brief: written informed consent, *CYP2C9*1/*1*, **1/*3* or **3/*3* genotype, good medical condition, negative urinalysis for drugs of abuse and ECG and biochemical analyses without clinically significant aberrations.
Exclusion criteria included: smoking, body mass index (BMI) >30 kg/m^2, abnormal serum lipids, hypertension, history of cardiovascular disease, concomitant medication (including oral contraceptives, herbal remedies and glucosamine within two months), pregnancy, and lactation.

3.2.2 Papers II–III

This was a two-phase pharmacokinetic study with single dose omeprazole (40 mg) and repeated dose escitalopram (5 mg b.i.d. for 6½ days). Pharmacokinetic plasma sampling was done at 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 10 hours after ingestion of two tablets of Losec® MUPS® 20 mg (AstraZeneca AB, Södertälje, Sweden) in the first phase. In the second phase, plasma samples were drawn on the seventh day before and at 1.5, 3, 4, 5, 8, 10 and 12 hours after the morning dose of Cipralex® 5 mg (H. Lundbeck A/S, Copenhagen, Denmark). Subjects were allowed to participate in the second phase no earlier than one week after participating in the first phase. Informed consent was obtained separately for each study phase.
Inclusion criteria, in brief: written informed consent, *CYP2C19*1/*1* or **17/*17* genotype, good medical condition, negative urinalysis for drugs of abuse and ECG and biochemical analyses without clinically significant abnormalities.

Exclusion criteria included concomitant medication other than paracetamol during the last two weeks. Female subjects were required to abstain from oral contraceptives for at least three weeks and produce a negative pregnancy test before inclusion.

3.2.3 Paper IV

This study was based on the Swedish adverse drug reactions register, a thorough literature search, two different data mining approaches and *in vitro* inhibition tests. These different methodologies are described in more detail below.

3.2.4 Paper V

The study in Paper **V** was an open label two-part study in healthy volunteers. The subjects were phenotyped with a validated combination of CYP activity markers based on the Karolinska cocktail [145]. Since the CYP2D6 probe debrisoquine was no longer available, and CYP2D6 does not play a role in the metabolism of warfarin, we used a cocktail consisting of the four drugs caffeine (for CYP1A2), losartan (for CYP2C9), omeprazole (for CYP2C19) and quinine (for CYP3A4). After initial phenotyping, the subjects were given noscapine 50 mg t.i.d. (Noskapin ACO®, ACO AB, Solna, Sweden) for 7½ days and were again phenotyped on the last day while still on noscapine. The design is outlined in Figure 6. At least three weeks after participating in the noscapine study, subjects were eligible for the glucosamine part. In the glucosamine study, the subjects were again phenotyped before and on the last day of glucosamine 625 mg b.i.d. (Artrox®, Pfizer AB, Sollentuna, Sweden) for 30 days. After two weeks subjects paid a visit to the CPTU for a compliance check and for INR and CRP testing. We included healthy men and women between 18 and 65 years of age that were willing to abstain from caffeine for 16 hours before phenotyping and who tested negative for drugs of abuse, and gave written informed consent.
Main exclusion criteria were: any clinically significant medical condition (either known or discovered during screening), concomitant use of any medication other than adrenergic nasal sprays or paracetamol, and (for female participants) a positive pregnancy test during any part of the study.

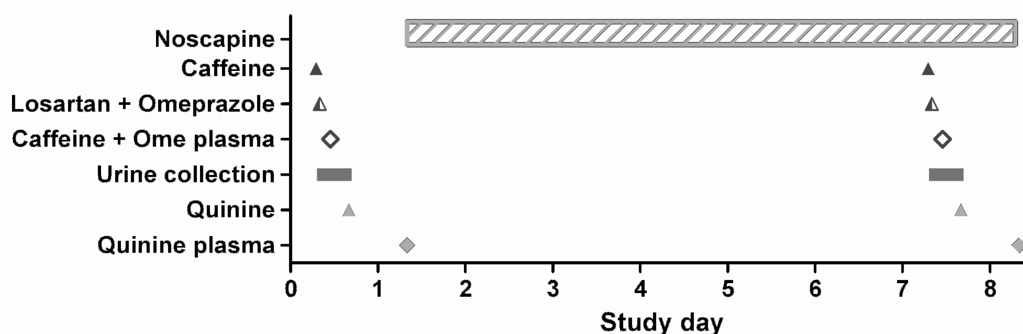

Figure 6 Schematic study design of the noscapine phase in Paper **V**. Baseline phenotyping was done in the morning after 16 hours of caffeine abstinence: 100 mg of caffeine was taken at 7 am, 25 mg of losartan and 20 mg of omeprazole was taken at 8 am. Plasma sampling for caffeine and omeprazole was done at 11 am and urine was collected from 8 am until 4 pm for losartan analysis. 100 mg of quinine was taken at 4 pm and plasma was sampled at 8 the following morning. This procedure was repeated during days 7 and 8 while on concomitant noscapine (at 7 am, 2 and 10 pm).

3.3 ANALYTICAL METHODS

3.3.1 Genotyping

Subjects were re-genotyped for the gene of interest in studies **II–III** and **V**. Genotyping analyses were based on polymerase chain reaction (PCR[*]). The currently applied routine method (mostly allelic discrimination assay on TaqMan; Applied Biosystems, Foster City, CA) at the Clinical Pharmacology Laboratory, Karolinska University Hospital, was used for *CYP2C9*2*, *CYP2C9*3* and *CYP2C19*2*. For *CYP2C19*17* a newly developed method was applied, which is described in more detail in Paper **II**.

3.3.2 Drug analyses

All drug analyses were based on High-Performance Liquid Chromatography with UV (HPLC) or mass spectrometric detection (LC-MS or LC-MS/MS).

3.3.2.1 Celecoxib

A new reversed-phase HPLC method for celecoxib and its two metabolites, hydroxy (OH)-celecoxib and carboxy (COOH)-celecoxib, was elaborated by Mia Sandberg Lundblad and is described in more detail in her thesis [146]. Briefly, plasma (0.5 mL) was mixed with 1.5 mL acetonitrile and centrifuged for 5 minutes at 15,000 × *g*. The supernatant was transferred to new tubes and evaporated using a vacuum centrifuge. Samples were reconstituted in 200 µL of methanol, and 50 µL thereof was injected into the HPLC-system. All samples were run in duplicates. A gradient mobile phase was used, where mobile phase A consisted of 10% acetonitrile and 90% 0.01 M sodium hydrogen phosphate buffer (pH 5.4). Mobile phase B consisted of 80% acetonitrile and 20% 0.01 M sodium hydrogen phosphate buffer (pH 5.4). The gradient elution started with 20% B for 7 minutes, after which it was increased to 50% for 5 minutes. At 12 minutes, mobile phase B was gradually increased to 70% until 19 minutes, when it was decreased to the initial value of 20%. The total run time was 20 minutes, at a constant flow-rate of 1 mL/min. Absorbance was measured at 254 nm. Retention times were approximately 6.8, 11 and 17 min for COOH-celecoxib, OH-celecoxib, and celecoxib, respectively. The range of quantification was 0.025 to 20 µM for each analyte. The lower limit of quantification (LLOQ) was 0.025 µM. The intra-day variability was 7.5%, 10.3% and 6.2% for celecoxib, OH-celecoxib and COOH-celecoxib, respectively, and inter-day variability for the corresponding substances was 9.8%, 9.7% and 8.8%.

[*] Polymerase chain reaction (PCR) is a method to amplify a specific region of DNA by adding region-specific primers, deoxyribonucleotides in excess and thermically stable *Taq* polymerase (a DNA polymerase from the bacterium *Thermus aquaticus* living in the hot springs of Yellowstone National Park) and then repeatedly heating the mixture to around 95ºC to separate the double stranded DNA helix, then cooling to about 51ºC to allow the primers to attach to the DNA strands, then heating to around 72ºC to allow the polymerase to work properly, and then heating to 95ºC again to separate the newly formed DNA strands and so on. In just a few cycles there will be thousands to millions of copies of the DNA region of interest.

3.3.2.2 Omeprazole

Omeprazole and the two metabolites, 5-hydroxyomeprazole and omeprazole sulphone, were analysed by reversed-phase HPLC based on previously published methods and is described in some detail in Paper **II**. Briefly, 100 mL of plasma was extracted with alkalinised methylene chloride:acetonitrile (9:1, v/v). After centrifugation and aspiration of the aqueous phase, the organic phase was evaporated at 60ºC. Samples were reconstituted in methanol and analysed with reversed-phase HPLC. Absorbance was monitored at 302 nm. The range of quantification was of 10–2,500 nmol/L. LLOQ for all three analytes was 10 nmol/L. The intra-day and inter-day variation (CV) for omeprazole and the two major metabolites were <10% and <15%, respectively.

3.3.2.3 Escitalopram

Escitalopram and its metabolites, desmethylcitalopram and didesmethylcitalopram, were analysed according to the contemporarily applied routine method at the Clinical Pharmacology Laboratory at the Karolinska University Hospital and based on the method by Macek *et al.* [147]. Briefly, plasma samples were extracted to diethyl-propylether after alkalinisation followed by extraction into acidic aqueous phase. Escitalopram and its desmethyl and didesmethyl metabolites were subsequently analysed using reversed-phase HPLC. Internal standard was (*S*)-(-)-3-bromo-N-[(1-n-propyl-2-pyrrolidinyl)-methyl]-2,6-dimethoxybenzamide (AstraZeneca AB). The range of quantification was 10–2,000 nmol/L for all analytes. LLOQ was 4 nmol/L for citalopram and 7 nmol/L for desmethyl- and didesmethylcitalopram. Accuracy was 95–100%, and inter-day variability (CV) was approximately 5%.

3.3.2.4 Noscapine

A novel tandem mass spectrometry (LC-MS/MS) method was developed by master student Stella Otto and her supervisors Michèle Masquelier and Jennie Östervall and is described in detail in her master thesis [148].
Noscapine was extracted from plasma using solid phase extraction (SPE). Plasma samples (400 µL) were added 50 µL of internal standard (diphenhydramine hydrochloride) and 400 µL 2% formic acid. SPE columns (Oasis MCX; Waters, Milford, MA) were activated with 1 mL of methanol and washed with 1 mL of water before the addition of the prepared samples. The SPE columns were washed with 1 mL 2% formic acid and 1 mL methanol. Noscapine was then eluted with 1 mL 2% ammonium acetate in methanol. The eluates were concentrated in a vacuum centrifuge for 2×15 min and placed in injection vials for analysis on an Acquity UPLC BEH column (Waters; 2.1 × 50 mm, 1.7 µm) by LC-MS/MS, using a Waters Acquity UPLC system (Waters). Raw data were gathered by MassLynx v4.1 software (Waters). Noscapine was eluted with a mobile phase of 0.1% formic acid and methanol (gradient 45–80% methanol) at a flow rate of 0.4 mL/min. The mass transitions monitored were *m/z* 414–220 for noscapine and 256–167 for the internal standard. Noscapine recovery was 108%. Stability tests showed that noscapine plasma samples could be stored for 5 days at +4 °C. The method was linear in the range 0.35–500 ng/mL (0.85–1,200 nmol/L), with a coefficient of determination (r^2) of 0.9993, and the limit of detection was 0.1 ng/mL. Total imprecision was 5.1% and 8.9% for noscapine concentrations of 75 ng/mL (181 nmol/L) and 3.75 ng/mL (9 nmol/L), respectively.

3.3.2.5 Karolinska cocktail

The cocktail analyses were performed according to the methods described by Christensen *et al.* [145]. That is, the analysis method for omeprazole in Paper **V** differs slightly from the method applied in Papers **II–III**. The most important difference being that they were analysed in different laboratories and on different hardware and that the LLOQ of the method used in Paper **V** was 25 nmol/L (compared to 10 nmol/L in Papers **II–III**). LLOQ for losartan and its carboxy metabolite (E-3174) was 20 and 10 nmol/L, respectively. LLOQ for quinine and 3-OH-quinine was 5 nmol/L, and 0.5 nmol/L for caffeine and paraxanthine.

3.4 STATISTICAL METHODS

3.4.1 General statistics

In all papers we have taken care to apply the relevant statistical methods. Since we have been looking for quite large differences between groups and within individuals under different circumstances, we have applied simple *t*-tests for normally distributed data and tried to transform non-normally distributed data to reasonably normal distribution. When we have succeeded, we have applied *t*-tests as above. When transformation has not been successful, we have applied standard non-parametric statistics (Mann-Whitney U-test for comparison between two groups and Kruskal-Wallis for comparison between more than two groups). Within the limits of this thesis, we have not found a need for more advanced statistical methods as the studies have been limited to healthy volun–teers without co-morbidities.
The one exception was the methods used in Paper **IV**, which will be described next.

3.4.2 Data mining

Adverse drug reactions (ADR) registers are based on spontaneously reported adverse events (AE) during pharmacotherapy that the reporter suspects has a relation to the use of the drug. Not all reported events have a true relation to the use of the suspected drug. Reporting is mandatory for all AEs during the first years after market authorisation, thereafter only for serious ones. The reporting of a certain AE (or combination of AEs) in relation to a certain drug more often than could be expected by chance is in this context referred to as a signal. In other words a signal can be said to occur when a certain drug–AE combination is reported disproportionately often. ADR registers can therefore be said to contain some signals obscured by lots of noise. Different methods have been developed for signal detection (which is virtually based on peak-to-noise enhancement techniques developed for radar and radio communication). When digging into a large database, this process is called data mining, or rather, disproportionality analysis. In the field of pharmacovigilance*, such techniques have been in use since the mid 1990's [149]. In Paper **IV**, we applied two different methods: Proportional reporting ratio (PRR) and Bayesian confidence propagation neuronal network (BCPNN).

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* Pharmacovigilance, from Greek *phármakon* (drug) and French *vigilance* (attentiveness), the science and activities relating to the detection, assessment, understanding and prevention of adverse effects or any other drug-related problem.

3.4.2.1 Proportional reporting ratio

A reporting rate can be calculated by dividing the number of reported adverse events with the number of prescriptions or sales volumes of a certain drug. This ratio is biased however, by delays in sales statistics and more frequent reporting of AEs for new drugs. Another approach is to take the proportion of the number of reports of an AE of interest for a certain drug divided by the total number of reports for that drug. This proportion can further be enhanced by dividing it with the proportion of all reports of the AE of interest for every other drug to all other reports of any other AE in the database. This ratio is called the proportional reporting ratio (PRR) [149].
The mathematics behind the PRR are explained in Table 3. Accordingly, the PRR will be 1 if there is no association between the drug and the reaction of interest. Evans and co-workers suggested that a signal is worth investigating when the PRR is greater than 2, χ^2 is greater than 4, and the number of reports of the drug–AE combination of interest (a) is greater than 3 [149]. For a two-by-two table (with 1 *df*[*]), the critical value of χ^2 is actually 3.84 for a significance level of $\alpha = 0.05$ [150], so the criteria by Evans *et al.* implies slightly stricter criteria than are generally considered statistically significant.

	Drug of interest	All other drugs
Reaction(s) of interest	a	b
All other reactions	c	d

$$PRR = \frac{a/(a+c)}{b/(b+d)}$$

$$\chi^2 = \sum_{i=1}^{r}\sum_{j=1}^{c}\frac{(O_{ij}-E_{ij})^2}{E_{ij}}, \textit{ where}$$

$$E_{ij} = \frac{\textit{row total} \times \textit{column total}}{\textit{total sample size}}$$

Table 3 Calculation of proportional reporting ratios according to Evans *et al.* Pharmacoepidemiol Drug Saf, 2001;10(6):483-6. The formula for chi-squared (χ^2) is redrawn from Machin *et al.* [150]. Explanations: r is the number of rows, c is the number of columns, O_{ij} is the observed count in cell ij, and E_{ij} is the expected count in cell ij under the assumption of chance distribution.

3.4.2.2 Bayesian confidence propagation neuronal network

Bayesian statistics are named after Reverend Thomas Bayes (1702-1761), who realised that the probability for event A (lets say an afternoon rain shower) is different when you know condition B (you can see heavy clouds at the horizon coming closer), than if you do not know about B. He stipulated Bayes' theorem:

$$p(A|B) = \frac{p(B|A)\,p(A)}{p(B)}$$, where

$p(A)$ is the probability of A, called prior probability as it does not take account to B,
$p(A|B)$ is the conditional probability of A, given B, also called the posterior probability
$p(B|A)$ is the conditional probability of B, given A, also called the likelihood, and
$p(B)$ is the prior probability of B.

[*] *df*, degree(s) of freedom, is a statistical/mathematical concept that is not easily explained within the constraints of a footnote, but the interested reader can find the most elegant explanation in Wikipedia (www.wikipedia.org).

Now, back to the ADR register: if $p(x)$ is the probability of a specific drug being listed on a case report; $p(y)$ is the probability of a specific ADR being listed on a case report; and $p(x,y)$ is the probability of both the drug and the ADR being listed on a case report, then we can construct a measure of disproportionality called the information component (IC):

$$IC = \log_2 \frac{p(x,y)}{p(x)\,p(y)} = \log_2 \frac{p(x|y)}{p(y)}$$

The IC will be close to zero if there is no association between the drug and the ADR, it will be positive if there is a positive relation, and negative if the drug is less associated with the ADR than expected.
Using a computerised neuronal network, the IC can be calculated as a point estimate with a confidence interval or a probability distribution. The Bayesian approach allows for the applicability of low (and zero) counter values, calculation with missing data (confidence interval will be wide), analysis of other and multiple variables, and there is an intuitive relationship between the IC estimate and its confidence interval. The neuronal network also allows for automatic signal detection [151].

3.5 *IN VITRO* EXPERIMENTS

In Paper **IV** we applied commercially available kits (Vivid® CYP450 Screening Kit, Invitrogen Corp, Madison, WI) for *in vitro* inhibition screening of CYP2C9 and CYP3A4, the main enzymes involved in the metabolism of *S*- and *R*-warfarin, respectively. The Vivid® screening kit contains microsomes called Baculosomes® prepared from insect cells transfected[*] with baculoviruses and expressing recombinant human CYP enzymes. The kit also contains all reagents needed including a fluorescent dye linked to a blocker that is a substrate for the specific CYP enzyme. When the blocker is cleaved, the dye is released and becomes fluorescent. If the enzyme is inhibited, less dye is released and thus the sample becomes less fluorescent [152]. The kit comes in different colours. We used the green kit for both CYP2C9 and CYP3A4.

3.6 SOFTWARE

3.6.1 Statistical software

For statistical analyses we used Statistica (Statsoft Corp, Tulsa, OK), version 6.1 in Paper **I** and version 8.0 in Paper **V**. In Paper **II** statistical analyses were made with GraphPad Prism 5.0 (GraphPad Software Inc, La Jolla, CA) and Microsoft Excel (Microsoft Corp, Redmond, WA). StatsDirect version 2.6.5 (StatsDirect Ltd, Altrincham, UK) was used in Paper **III**.

3.6.2 Pharmacokinetic analyses

For pharmacokinetic analyses, we used WinNonLin (Pharsight Corp, Mountain View, CA). Version 4.1 was used in Paper **I** and version 5.1 in Papers **II–III**. GraphPad Prism 5.03 (GraphPad Software Inc, La Jolla, CA) was used for curve-fitting purposes for

[*] transfection, refers to the transfer of a gene construct into a cell by infecting it with a virus containing the gene construct in question. Thus, human genes can be transfected to and expressed in e.g. insect, yeast or bacterial cells.

figures in this thesis together with an equation from Gabrielsson & Weiner [3] for a one-compartment oral pharmacokinetics model.

3.6.3 Graphs

Graphs were drawn with GraphPad Prism 5.0–5.03 (GraphPad Software Inc, La Jolla, CA) or Microsoft Excel (Microsoft Corp, Redmond, WA).

4 RESULTS

4.1 PAPER I

Initially we planned to include four to five subjects in all genotype groups. Due to the low allele frequency of *CYP2C9*3* in a Swedish population (6–9% with <1% homozygous individuals) [47] and the limited number of subjects in the database (about 200 individuals), we were unable to recruit more than three subjects in the **3/*3* and **1/*3* groups. This was somewhat compensated for by including seven subjects in the wild-type group.
Arithmetic means are sensitive to extremes, which is why we chose to analyse differences in median AUC between groups with the Kruskal-Wallis test.

4.1.1 Single dose data

Median $AUC_{0\text{-}24\,h}$ differed 3.5-fold between the homozygous *CYP2C9*3* carriers compared to heterozygous and wild-type individuals (Figure 7).

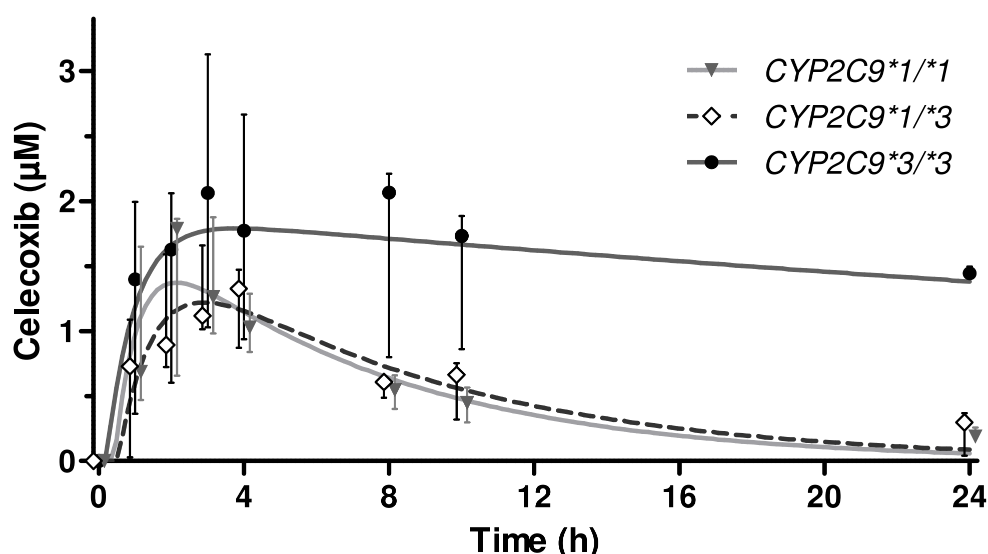

Figure 7 Single dose data after the first dose of 200 mg celecoxib to healthy volunteers with different *CYP2C9* genotypes (7 subjects in the **1/*1* group and 3 subjects each in the **1/*3* and **3/*3* groups). Symbols and whiskers represent medians and interquartile ranges, respectively. The curves are fitted to a one-compartment oral model using all individual data. Symbols in the **1/*1* and **1/*3* groups are nudged to prevent overlap.

4.1.2 Repeated dose data

On day seven, the median $AUC_{0\text{-}24\,h}$ was seven-fold higher in individuals homozygous for *CYP2C9*3* compared to heterozygous and wild-type subjects. This is illustrated in the figure in Paper **I**. Figure 8 shows the metabolite levels of hydroxy- and carboxy-celecoxib on day 7. Metabolites reached a lower maximal concentration in subjects genotyped as *CYP2C9*3/*3* compared to the other genotype groups due to a lower formation rate. However, metabolite levels stayed higher for a longer time in homozygous *CYP2C9*3* carriers due to accumulation of the parent compound.

Pharmacokinetic parameters for celecoxib after single and repeated doses are given in Table 4.

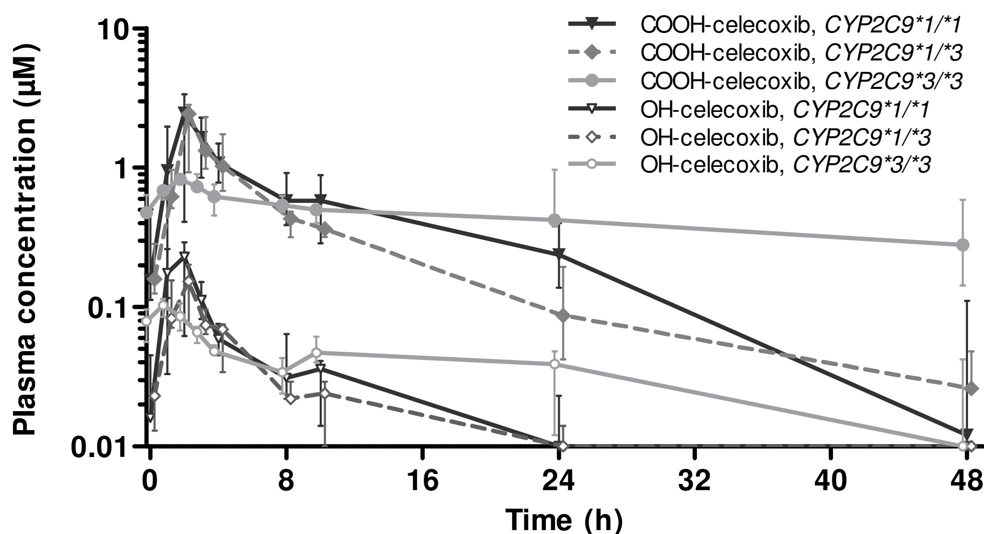

Figure 8 Plasma levels of the celecoxib metabolites, hydroxycelecoxib and carboxycelecoxib, on the seventh day of repeated dosing of celecoxib 200 mg once daily in healthy subjects with different *CYP2C9* genotypes. Symbols and whiskers represent medians and interquartile ranges, respectively. Symbols in the **1/*1* and **1/*3* groups are nudged to prevent overlap. Note the logarithmic scale on the y-axis.

	***CYP2C9* genotype**	**n**	**$T_{1/2}$ (h)**	**C_{max} (µM)**	**$AUC_{0\text{-}24h}$ (h*µmol/L)**	**Cl/F (L/h)**
Day 1	**1/*1*	7	13 (6.4–15)	1.8 (1.0–1.9)	12 (10–24)	n.d.
	**1/*3*	3	7.5 (4.6–20)	1.6 (0.97–1.7)	12 (11–17)	n.d
	**3/*3*	3	30 (25–37)*†	2.1 (1.4–3.1)	41 (24–44)*†	n.d.
Day 7	**1/*1*	7	14 (6.7–24)	2.0 (0.92–3.5)	18 (12–24)	29 (21–45)
	**1/*3*	3	10 (7.5–13)	1.9 (1.5–3.6)	17 (14–23)	32 (22–38)
	**3/*3*	3	52 (24–60)*†	7.7 (7.3–11)*†	125 (90–154)*†	4.2 (3.4–5.8)*†

Table 4 Pharmacokinetic parameters for celecoxib after the first and seventh daily dose in subjects with different *CYP2C9* genotypes. Cl/F: oral clearance, *: $p < 0.05$ compared to *CYP2C9*1/*1* subjects, †: $p < 0.05$ compared to *CYP2C9*1/*3* subjects, n.d.: not determined. Oral clearance of celecoxib could not be determined after the first dose as the extrapolated AUC beyond 24 hours was too large.

4.2 PAPER II

Eighteen subjects were screened, one was excluded because confirmatory genotyping proved him to be heterozygous *CYP2C19*17* carrier. Five subjects genotyped as *CYP2C19*17/*17* and twelve homozygous *CYP2C19* wild-type carriers were included. One of the subjects genotyped as *CYP2C19*1/*1* displayed markedly aberrant ome-

prazole pharmacokinetics with extremely delayed absorption. This made the extrapolated $AUC_{10-\infty}$ very large and thus unusable. The data analysis was thus based on five **17/*17* and eleven **1/*1* individuals.

Several of the included subjects displayed bimodal omeprazole pharmacokinetics indicative of delayed absorption of one of the two Losec® MUPS® tablets.

The mean $AUC_{0-\infty}$ for omeprazole was 52% lower in *CYP2C19*17/*17* subjects than in wild-type subjects ($p = 0.04$). There was no significant difference in the $AUC_{0-\infty}$ of 5-hydroxyomeprazole between genotype groups. However, the exposure of omeprazole sulphone, was 68% lower in the **17* carriers ($p = 0.03$). The corresponding difference in oral clearance of omeprazole was 48% higher in the **17/*17* group, which did not reach statistical significance ($p = 0.37$).

One subject became pregnant after the follow-up visit and later gave birth to a healthy daughter.

4.3 PAPER III

The subject that was excluded from the data analysis in Paper **II**, participated in the escitalopram study, but the pregnant subject did not participate in the second part of the study. The escitalopram study thus comprised the same number of individuals in each genotype group as the omeprazole study, but only ten individuals genotyped as *CYP2C19*1/*1* participated in both study parts. This explains the small differences in demographic characteristics between Paper **II** and Paper **III**.

The mean difference in escitalopram $AUC_{0-\tau}$ was 21% but this difference did not meet predefined criteria for statistical significance ($p = 0.08$).

Eight subjects reported mild to moderate adverse events which were judged to have a possible relation to the study drug. No unexpected adverse events were reported.

There was a significant correlation between the exposures of escitalopram at steady-state and omeprazole after a single dose (Spearman $r^2 = 0.67$, $p = 0.006$). There was a tendency for a curvilinear relationship, so that the subjects with the highest escitalopram exposure tended to have an even higher omeprazole exposure (see Figure 2 in Paper **III**). This will be discussed more in detail in the Discussion section.

4.4 PAPER IV

4.4.1 Literature searches

When searching the literature (PubMed, Embase and standard text books on drug interactions), we found nothing about a documented interaction between noscapine and warfarin. What we did find, however, was an animal study, that showed a decreased CYP content in rat liver after treating rats with very high noscapine doses (200 mg/kg) for five days [153]. We thought it difficult to interpret this finding in a clinically meaningful way.

4.4.2 The Swedish ADR register

In the Swedish ADR register (SWEDIS), there were five previous ADR reports concerning the combined use of warfarin and noscapine. The oldest report was from 1978 and the reported event was a haematoma. All other reports were about change in INR. However, the term in the database for increased anticoagulant effect is “prothrombin complex decreased” and one of the reports had been classified as “prothrombin com-

plex increased”. When reading the full reports it was clear, however, that all reports were about increased anticoagulation* (i.e. “prothrombin complex decreased”). The classification of the misclassified report has been corrected in the register. Another two cases were reported during the summer of 2006 and are described in Paper **IV**, although the statistics were based on the first six cases.

4.4.3 Data mining

Table 5 shows the number of reports in SWEDIS as of June 2006 arranged according to specified properties.

	Noscapine + warfarin	All reports with ≥ 2 drugs (not warfarin + noscapine)
INR increased and/or haemorrhage	6	2,241
All other reactions	2	33,350

Table 5 Number of reports in SWEDIS as of June 2006 arranged in accordance with **Table 3**.

The proportional reporting ratio (PRR) could be calculated to 11.9 and χ^2 to 63.8, which was highly significant ($p < 0.001$). The PRR could also be expressed as a 95% confidence interval (4.1–34.4).
The information component (IC) value could be calculated to 2.16 with a 95% confidence interval of 0.73–3.59, i.e. also highly statistically significant.

4.4.4 *In vitro* inhibition tests

The *in vitro* inhibition tests showed that noscapine potently inhibits both CYP2C9 and CYP3A4 with IC_{50} values of 0.90 (95% CI 0.54-1.50) and 1.06 (95% CI 0.87-1.31), respectively (Figure 9).

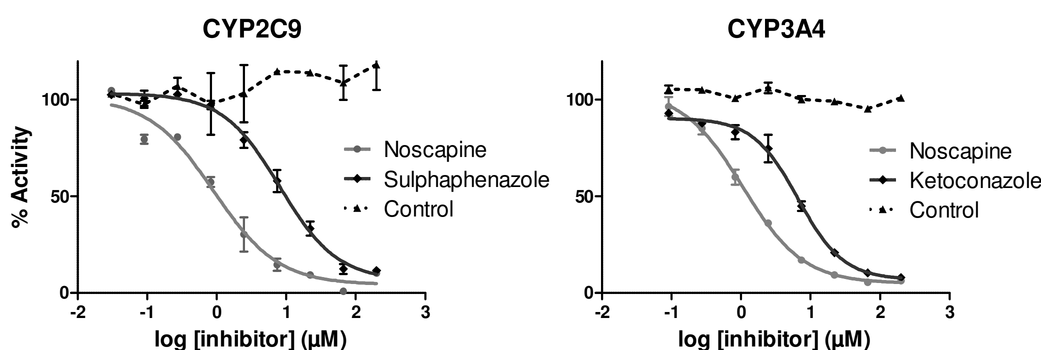

Figure 9 *In vitro* inhibition tests using the Vivid® CYP450 Screening Kits for CYP2C9 and CYP3A4. Symbols and whiskers represent geometric means and standard deviations, respectively

* I can gladly recommend the interested reader who wants an in-depth explanation about the relation between levels of “prothrombin complex” and INR to read pages 11-12 in the thesis by Jonatan Lindh [86].

4.5 PAPER V

4.5.1 Subjects

A total of fourteen subjects were included. Subjects gave separate informed consents for each study part. Eleven of the subjects participated in both study parts, and two subjects participated in only one of the study parts, either the noscapine part or the glucosamine part. Thus twelve subjects participated in the noscapine study and twelve in the glucosamine study. In both studies six subjects of each *CYP2C9* genotype were included. Compliance with the study medication was over 96% in the noscapine part and about 80% in the glucosamine part. There were two screening failures; one subject had spontaneously and repeatedly elevated INR (about 1.5) and was referred to the Unit of coagulation, where she was diagnosed with von Willebrand disorder. Another subject was dismissed due to a positive urine test for illicit drug use.

4.5.2 Phenotyping results

4.5.2.1 Noscapine

Baseline phenotyping results were in accordance with earlier findings from our department [143-145]. In the noscapine part, all subjects increased in losartan metabolic ratio[*], on average 4.9-fold (95% CI 2.8–8.4, p < 0.001). One of the subjects had a very high metabolic ratio of losartan during noscapine treatment due to very low levels of the losartan carboxy-metabolite, E3174 (Figure 10). After exclusion of this subject, the mean increase in losartan metabolic ratio was still 3.9-fold (95% CI 3.0–5.0, p < 0.001). The degree of inhibition was similar in individuals genotyped as *CYP2C9*1/*1* compared to *CYP2C9*1/*3* (see Figure 11).

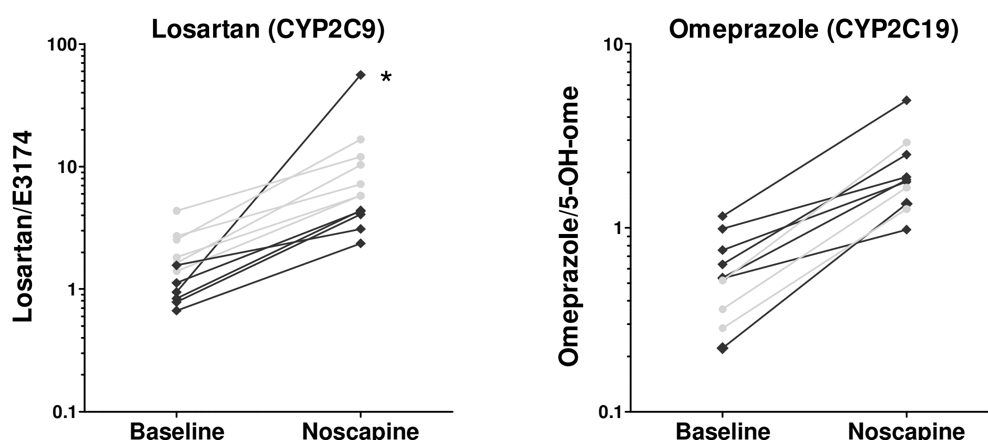

Figure 10 Metabolic ratios of losartan and omeprazole at baseline and during noscapine, respectively. The outlier with almost 60-fold increase in the metabolic ratio of losartan is marked with an asterisk. Homozygous wild-type carriers are shown in black, while grey indicates subjects genotyped as *CYP2C9*1/*3* in the losartan panel and *CYP2C19*1/*17* in the omeprazole panel.

[*] Metabolic ratio (MR) is the ratio between parent substance and metabolite. By convention, the ratio of paraxanthine/caffeine is used as a phenotypic marker for CYP1A2, and this is not a metabolic ratio, which is why we chose to use the term phenotypic index (PI) in Paper **V**.

One of the subjects either forgot to take her omeprazole on the morning of the second phenotyping or had delayed absorption, as she had no detectable levels of either omeprazole or metabolites in her plasma. The plasma from another subject gave uninterpretable chromatograms due to interference and quantification could not be done. Thus, omeprazole data were assessable from ten subjects. All these subjects demonstrated an increased metabolic ratio of omeprazole on noscapine compared to baseline. The mean increase was 3.6-fold (95% CI 2.6–4.8, $p < 0.001$). There was a trend ($p = 0.16$) towards *CYP2C19*17* carriers (three subjects) being inhibited to a higher degree than subjects genotyped as *CYP2C19*1/*1* (Figure 11).

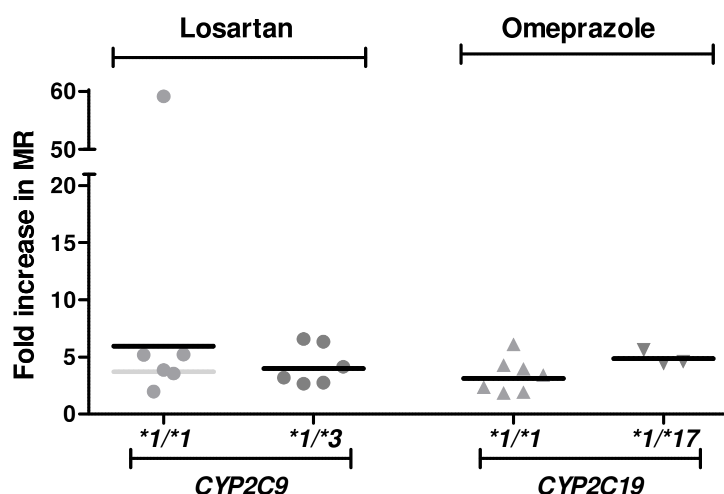

Figure 11 Fold increase in the metabolic ratios (MRs) during noscapine compared to baseline. MRs of losartan and omeprazole are shown in relation to *CYP2C9* and *CYP2C19* genotypes, respectively. Horizontal bars represent geometric means. The grey bar in the *CYP2C9*1/*1* group represents the geometric mean without the outlier.

There were no significant differences or trends in the phenotypic indices of caffeine or quinine during noscapine treatment compared to baseline (see Figure 1 in Paper **V**).

4.5.2.2 Glucosamine

There were no significant differences or any trends in phenotypic indices for any of the phenotyping drugs during concomitant glucosamine. Neither was there any change in C-reactive protein (CRP) levels or INR.

4.5.3 Noscapine analyses

The possibility to analyse noscapine emerged after all the paperwork with the authorities was done. Therefore no extra pharmacokinetic sampling was performed, but noscapine was analysed in the back-up samples for caffeine (4 hours post-dose on day 7) and quinine (one hour after dose on day 8).

Since noscapine is rapidly absorbed, reaching peak levels in less than an hour and has a relatively short half-life (4.5 hours) [124], we concluded that the 4-hour plasma level would better predict noscapine exposure than the 1-hour level.

There was a correlation between 4-hour noscapine levels and multiples of increase in losartan metabolic ratio. However, the subject that had a metabolic ratio of nearly 60 during concomitant noscapine treatment was also an outlier in this correlation. Interestingly, she had two to three-fold higher plasma levels of noscapine at one hour after intake than the other subjects. Excluding this subject from the regression analysis, there was a log-linear relation ($r^2 = 0.79$, $p < 0.001$) between noscapine levels and CYP2C9 inhibition expressed as multiples in increase of the losartan metabolic ratio (See Figure 2 in Paper **V**).

4.5.4 Adverse events

In total, nine adverse events were reported during the noscapine study: headaches and stomach discomfort being most common. In the glucosamine study a total of 22 adverse events were noted. The most frequent of these were: 3 cases each of nausea, frequent and/or loose stools and common cold; 2 events each of headache and abdominal pain. The most unexpected AE was one case of rank odour of the urine without any evidence of infection or renal impairment. Increased bilirubin was noted in one subject shown to carry the *UGT1A1*28* allele associated with Gilbert's syndrome[*]. No serious adverse events were noted.

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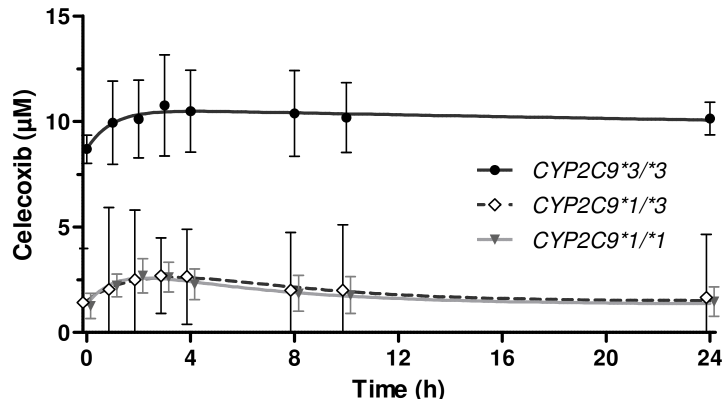
[*] Gilbert's syndrome is named after the French gastroenterologist Augustin Nicolas Gilbert (1858-1927) who described a hereditary form of jaundice which was later shown to be caused by decreased capacity to conjugate bilirubin to glucuronic acid due to defective uridine diphosphate-glucuronosyl transferase 1A1 (UGT1A1). Gilbert's syndrome affects some 5% of a Caucasian population and is normally without other clinical significance than transient jaundice during febrile illness. However, it has been associated with increased risk of toxicity in patients treated with the anticancer drug irinotecan and some antiviral drugs [154].

5 DISCUSSION

5.1 PAPER I

Celecoxib is metabolised by CYP2C9 to hydroxycelecoxib (OH-celecoxib), which is further metabolised by alcohol dehydrogenase to carboxycelecoxib (COOH-celecoxib). Microsomes prepared from human livers genotyped as *CYP2C9*3/*3* have been shown to metabolise celecoxib considerably slower than other human liver microsomes [155]. Our data on single-dose kinetics are in agreement with previous studies, that have reported two to three-fold higher $AUC_{0-\infty}$ of celecoxib in individuals genotyped as *CYP2C9*3/*3* compared to *CYP2C9*1/*1* [156, 157].
However, the steady-state data showing a seven-fold higher median $AUC_{0-\infty}$ of celecoxib in individuals homozygous for *CYP2C9*3* compared to heterozygotes and homozygous wild-type subjects, contradict a previous study of celecoxib 200 mg b.i.d. for 15 days that could not demonstrate any difference in pharmacokinetics in a single elderly participant homozygous for *CYP2C9*3/*3* compared to the genotype groups of the other 23 subjects [158].
At first glance, we were surprised at the marked accumulation of celecoxib at steady-state in individuals homozygous for *CYP2C9*3*. However, the accumulation at steady state in homozygous *CYP2C9*3* carriers can be simulated by repeatedly superimposing the single-dose data (Figure 12). This gives an approximate 6-fold difference in mean $AUC_{0\text{-}24\,h}$ between the *CYP2C9*1/*1* and *CYP2C9*3/*3* groups. The original data from day 7 are depicted in Figure 1 in Paper **I**.

Figure 12 Simulation of celecoxib pharmacokinetics on day 7 based on repeatedly superimposing single-dose data. Simulated means and 95% confidence intervals are shown.



It has been proposed that celecoxib may induce CYP3A4 [159] and that this mechanism may explain the discrepancy between our findings and the 15-day study by Brenner *et al.* [158]. CYP3A4 has been shown to metabolise celecoxib *in vitro* in CYP2C9 deficient human liver microsomes [156] and in primary hepatocyte cultures treated with sulphaphenazole [159].
On September 30, 2004, rofecoxib (Vioxx®) was withdrawn from the market due to an increased risk of adverse cardiovascular events in a trial of rofecoxib for the prevention of colorectal polyps (the APPROVe study). This prompted the data and safety monitoring board of the similar Adenoma Prevention with Celecoxib (APC) trial to perform an assessment of cardiovascular safety with celecoxib. This review showing a

dose-dependent increase in adverse cardiovascular events (hazard ratio* 2.3 in the 200 mg b.i.d. arm and 3.4 in the 400 mg b.i.d. arm) with celecoxib [160], was long the only data published from the APC trial. Recently, new data have been published from the APC trial, which included 2,035 patients with a history of colorectal polyps and randomised to either of three arms; placebo, celecoxib 200 mg b.i.d., or celecoxib 400 mg b.i.d. These new data include *CYP2C9* genotyping data. 1,660 patients were successfully genotyped for *CYP2C9*2* and **3*. When analysing all patients regardless of genotype, there was a dose-dependently reduced risk for recurrent adenomas with celecoxib compared to placebo. After stratification by *CYP2C9* genotype, the dose-dependency of the reduced risk was limited to the *CYP2C9*3* carriers. Celecoxib also increased the risk of adverse cardiovascular events in a dose-dependent way, which seems to be largely driven by the increased risk in *CYP2C9*3* carriers given the high dose [161] (although this might be jumping to conclusions as the number of events was too small to reach statistical significance in any of the dose/genotype strata).
When submitting the original full-length manuscript of Paper **I**, we tried to suggest that the pharmacokinetic differences between the genotype groups might have implications for cardiovascular and other adverse effects, but this speculation was disliked by the reviewers. The data from the APC trial show at least a tendency that we were right.
The mechanism for how COX-2 selective NSAIDs pose a cardiovascular hazard is not fully understood, but one proposed mechanism is that it disturbs the balance between thromboxane A_2 (which is primarily produced by COX-1 in platelets and stimulates platelet aggregation and vasoconstriction) and prostacyclin (which is produced primarily by endothelial† cells expressing COX-2, and inhibits platelet aggregation and dilates vessels). The mechanism may also involve interference with other arachidonic acid metabolites, many of which are formed and/or metabolised by CYP2C8 and CYP2C9 (see Table 1).

5.2 PAPERS II AND III

5.2.1 Our *CYP2C19*17* study

The *CYP2C19*17* allele was discovered by sequencing the *CYP2C19* genes of individuals found to be extensive metabolisers in earlier studies with omeprazole or mephenytoin as probe drugs. Based on retrospectively re-genotyping the subjects and re-analysing their phenotyping data, an approximate 40% mean difference in omeprazole exposure was estimated between genotype groups [56]. We designed the study behind Papers **II** and **III** to be able to detect such a large difference. This succeeded in the case of omeprazole. However, in the case of escitalopram, the mean difference in AUC was considerably smaller than estimated and this difference did not reach statistical significance ($p = 0.08$). We considered recruiting more subjects to reach statistical significance, but this course of action would only result in chasing p-values. We had recruited almost all homozygous *CYP2C19*17* subjects in our database and their data

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* Hazard ratio (HR) refers in medical statistics to the odds ratio (OR) of an adverse event. Odds is the ratio of the probability of an event divided by the probability of a non-event, thus the hazard ratio between groups A and B is $HR = \frac{p_A/(1-p_A)}{p_B/(1-p_B)}$, while the relative risk (RR) is p_A/p_B.

† Endothelial, from Greek *éndon* (within) and *thēlē* (teat, nipple), refers to the endothelium, the inner cell lining of blood vessels and heart chambers, confer the footnote on page 2 (epithelial).

were tightly grouped. Recruiting more *CYP2C19* wild-type individuals would have yielded a $p < 0.05$, but it would have been unlikely to get a larger mean difference in escitalopram AUC. In order to get a larger effect in an extended study, we would have had to recruit subjects genotyped as *CYP2C19*1/*1* with a lower than average CYP2C19 activity. We thus concluded that the *CYP2C19*17* allele did not have a clinically significant effect on escitalopram pharmacokinetics.
The primary metabolism of omeprazole is to a great extent catalysed by CYP2C19 to yield 5-hydroxyomeprazole (5-OH-omeprazole), whereas sulphoxidation of omeprazole to omeprazole sulphone is catalysed by CYP3A4. The *R*-enantiomer is hydroxylated to a greater extent than *S*-omeprazole [162, 163], while both enantiomers have equal pharmacological activity [164]. In the omeprazole study, it was interesting to note that there was a similar difference in the exposure to omeprazole sulphone (Paper **II**). This is probably due to CYP2C19 being responsible for a rate limiting step in the clearance of omeprazole sulphone to the secondary metabolite 5-hydroxyome-prazole sulphone [165]. The correlation between the pharmacokinetics of omeprazole and omeprazole sulphone has also been shown in a study where healthy volunteers were first given omeprazole 40 mg once daily for seven days and in another study period 60 mg twice daily for seven days. This study showed reduced CYP2C19-dependent clearance of omeprazole (2.3-fold) and sulphone (2.2-fold) when increasing the omeprazole dose. Simultaneously, the formation of 5-OH-omeprazole was delayed while its CYP3A4-dependent clearance was increased by 20% during high dose omeprazole treatment [103]. We can thus conclude that omeprazole is eliminated according to the scheme in Figure 13.

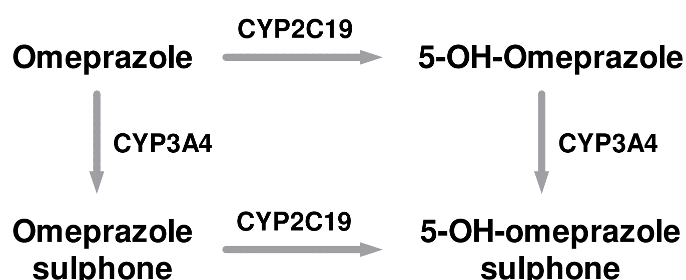

Figure 13 Schematic representation of omeprazole metabolism, where CYP2C19 mediates 5-hydroxylation of both omeprazole and omeprazole sulphone and CYP3A4 catalyzes sulphoxidation of both omeprazole and 5-OH-omeprazole.

The apparent curvi-linear relationship between escitalopram and omeprazole exposures described in Figure 2 in Paper **III** merits some discussion, although it is not excluded that it is a chance finding. The ability of omeprazole to inhibit its own metabolism at high exposures may be an explanation. Another possibility is saturation of omeprazole metabolism in those EMs that have the lowest metabolic capacity.

5.2.2 *CYP2C19*17* and PPIs

The successful healing of peptic ulcers has been associated with *CYP2C19* loss of function alleles using a relatively low dose of omeprazole (20 mg daily) in Asian populations with a high frequency of mutant alleles [166]. In Europe, where there is a lower prevalence of defective *CYP2C19* alleles [167], doses of proton pump inhibitors are generally higher, but still *CYP2C19*2* carriers have better ulcer healing according to two Polish studies of 125 and 139 patients, respectively [168, 169]. It is unclear whether the second study includes the same patients as the first study, but the results are still interesting. These studies also show that neither heterozygous, nor homozygous

*CYP2C19*17* carriers differ in ulcer healing rates from wild-type subjects, although plasma levels of pantoprazole were lower in the former group [169].

5.2.3 *CYP2C19*17* and antidepressants

5.2.3.1 Citalopram and escitalopram

The results of our prospective trial seemingly contradicts the findings by Rudberg *et al.*, who found a 42% difference in geometric mean plasma concentrations of escitalopram between *CYP2C19*17/*17* and **1/*1* genotype groups in a therapeutic drug monitoring (TDM) material [170]. However, a TDM material may be biased in the sense that patients with lack of effect or side-effects may be more likely to be sampled, which may exaggerate differences in metabolic capacity. The wide sampling window (samples were taken 10–30 hours after intake) might also have introduced bias.
It is interesting to note that individuals homozygous for *CYP2C19*17* consistently have a relatively small interindividual variability in the metabolic capacity of CYP2C19 substrates, whereas homozygous wild-type carriers are dispersed over a wide range of metabolic capacity with some individuals having a more extensive metabolism than any of the **17* homozygotes [35, 56, 170, 171] (Figure 14, and Papers **II**–**III**). It remains to be investigated whether these differences depend on hitherto undiscovered genetic variants or on environmental factors affecting *CYP2C19* gene expression.

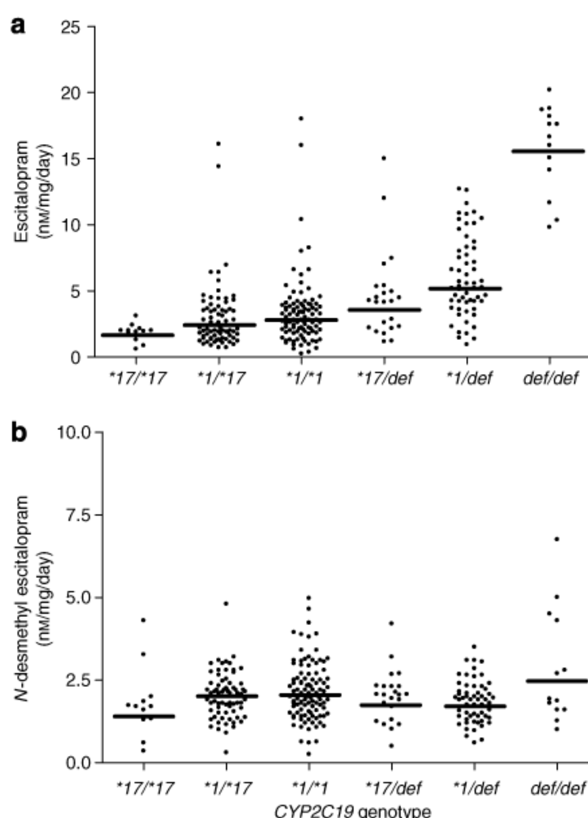

Figure 14 Serum concentrations of (**a**) escitalopram and (**b**) *N*-desmethylescitalopram in relation to CYP2C19 genotype. Lines indicate geometric means. Note the relatively small spread within the **17/*17* group and the considerable overlap between wild-type and *CYP2C19*17* carriers.

Reproduced from Rudberg *et al.* Clin Pharmacol Ther, 2007; 83(2):322-7.

(*S*-)-Citalopram is metabolised to desmethylcitalopram by CYP2C19 and further to didesmethylcitalopram by CYP2D6 [172]. Interestingly, adjusting for *CYP2D6* genotype did not diminish interindividual variability in escitalopram plasma concentrations in the functional *CYP2C19* genotype groups [173]. This might partly be explained by a novel CYP2C19-catalysed metabolic pathway to citalopram propionic acid [174].

5.2.3.2 Sertraline

Sertraline is an SSRI that is primarily metabolised by CYP3A4, but also by several other CYPs, including CYP2C19 [175]. Carriage of defective *CYP2C19* alleles have been associated with higher exposure of sertraline [176, 177], while no effect could be demonstrated for *CYP2C19*17* in a TDM study in 121 patients [177].

5.2.3.3 Imipramine

Recently, the impact of *CYP2C19*17* on the pharmacokinetics on imipramine was reported. Imipramine is a tricyclic antidepressant that is metabolised by CYP2C19 to the active metabolite desipramine. Patients were prospectively prescribed imipramine in doses from 25 to 900 mg daily and were sampled at steady-state aiming for trough[*] concentrations of the sum of imipramine+desipramine between 200 and 300 µg/L (about 700–1100 nmol/L). It was found that patients homozygous for *CYP2C19*17* had 30% lower imipramine levels than patients classified as *CYP2C19*1/*1*, but the concentrations of the active moiety (imipramine+desipramine) did not differ between *CYP2C19* genotype groups [171].

5.2.4 *CYP2C19*17* and other drugs

5.2.4.1 Clopidogrel

The platelet aggregation inhibitor clopidogrel is a prodrug, i.e. it needs to be activated by metabolism in order to be pharmacologically active. This bioactivation is mediated by several CYPs *in vitro* [178]. However, *in vivo*, *CYP2C19* genotype has been shown to influence pharmacodynamics [95, 179-181], pharmacokinetics of the active metabolite, as well as clinical outcome in clopidogrel treated patients [35, 180]. This speaks in favour of CYP2C19 being the most important enzyme for clopidogrel bioactivation *in vivo*. The *CYP2C19*17* allele has been associated with enhanced clinical effect [35, 180, 182], but also increased risk of bleeding in patients treated with clopidogrel [180]. Just recently, three more studies on the clopidogrel–*CYP2C19* genotype theme have been published almost simultaneously: one is a re-analysis of the 10,285 patients from the PLATO trial comparing clopidogrel to a reversible platelet aggregation inhibitor, ticagrelor, that does not need bioactivation [183]; the other is a genetic substudy of 2,932 participants in the TRITON-TIMI 38 trial comparing clopidogrel with a similar compound, prasugrel that is less dependent on CYP2C19 for bioactivation [178, 184]; the third is a genotype analysis of 5,059 participants from the pivotal clopidogrel study (CURE) [185]. The first study (funded by the manufacturer of ticagrelor) concludes that ticagrelor is superior to clopidogrel irrespective of *CYP2C19* genotype, and that clopidogrel is associated with a higher risk of major bleedings in *CYP2C19*17* carriers [183]. The second study (funded by the manufacturers of prasugrel) concludes that nearly half of the population is at risk of unfavourable response to clopidogrel (and

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[*] Trough does not, in this context, refer to the feeding of farm animals, but to the to the lowlands of the concentration–time curve after each dose, i.e. the end of the dosing interval, before the next dose; as opposed to the peak, the timing of which depends not only on the rate of elimination, but also on the rate of absorption. Trough concentrations are easier to sample and are more reproducible than peak concentrations that are very sensitive to the timing of the sampling. See also Figure 1 on page 1.

should be treated with prasugrel) [184]. Finally, the third study (funded by the manufacturers of clopidogrel) fails to see a relation between *CYP2C19* genotype and neither lack of response, nor major bleeding in the first study attempting a comparison of clopidogrel to placebo after stratification by *CYP2C19* genotype [185]. However, it is striking how the favourable clopidogrel effect in the CURE trial seems to have been driven by clopidogrel treated *CYP2C19*17* carriers, as neither of the other genotype groups had an effect that significantly differed from placebo. The strict definition of major bleeding (haemorrhage requiring transfusion of at least 2 units of blood) may have contributed to the negative correlation between genotype and bleeding events. Another interesting finding of this latter study, was that clopidogrel seemed to have some beneficial effect in preventing major coronary events regardless of *CYP2C19* genotype, as there was a consistent trend for the clopidogrel treated patients to do better than the placebo treated patients, although this was significant only for **17* carriers [185].

5.2.4.2 Voriconazole

The broad spectrum antifungal agent voriconazole is primarily metabolised by CYP 2C19 with some contribution from CYP3A4 [31]. Wang *et al.* genotyped 315 subjects to recruit 20 healthy volunteers with genotypes *CYP2C19*1/*1* (n = 8), **2/*2* (n = 8) and **1/*17* (n = 4). Due to the scarcity of the **17* allele in Asian populations, no homozygous individuals were found. The results showed 48% lower $AUC_{0-\infty}$ in *CYP2C19 *17* carriers compared to wild-type subjects [186]. These data might not be directly applicable to Caucasian populations, as Asian extensive metabolisers of CYP2C19 substrates generally have a lower metabolic capacity than Caucasian EMs [167], even after stratification for genotype [187], and the **17* allele might therefore have a larger impact in Asians.

5.2.4.3 Tamoxifen

Tamoxifen is an oestrogen receptor antagonist that needs to be bioactivated for full efficacy. It is used as adjunctive therapy in breast cancer when the tumour expresses the oestrogen receptor [188, 189]. Tamoxifen is primarily metabolised by CYP2D6 to yield the 100-fold more potent metabolites 4-hydroxytamoxifen and 4-hydroxy-*N*-desmethyltamoxifen (endoxifen), but CYP2C19 also contributes [190, 191].
In a retrospective analysis of 206 tamoxifen-treated and 280 non-tamoxifen treated patients, *CYP2D6* genotype was strongly associated with relapse-free survival. It was also found that *CYP2C19*17* carriers had a better relapse-free survival than non-carriers, especially if they were carriers of fully functional *CYP2D6* alleles [188]. This finding could not be reproduced in a smaller case–control study (47 breast cancer patients and 135 matched controls), where the participants were selected from a trial of tamoxifen prevention in 5,408 women of average breast cancer risk. However, there was still a trend for a beneficial effect of *CYP2C19*17* carrier status (OR[*] 3.50, 95% CI 0.46–26.6) [189].
CYP2C19 is also involved in the metabolism of oestrogens [192, 193] and if *CYP2C19 *17* proves to be a marker of favourable response to tamoxifen, it remains to clarify

[*] OR (odds ratio), the ratio between the odds for the cases divided by the odds for the controls, cf footnote on page 34.

whether this is due to an increased bioactivation of tamoxifen, increased catabolism of oestrogens, or other factors coupled to the **17* allele. Indeed, *CYP2C19*17* has been associated with decreased risk of developing breast cancer (OR 0.64, 95% CI 0.44–0.94) [194].

5.2.5 The clinical impact of *CYP2C19*17*

As written above, the *CYP2C19*17* allele is associated with a uniform and higher than average expression and metabolic activity. However, not even homozygous **17* carriers have a more rapid metabolism than the most extensive metabolisers genotyped as **1/*1*. Thus, it is inappropriate to attribute *CYP2C19*17* carriers an ultrarapid metaboliser pheno-type. It may be warranted to compare with the distribution of CYP2D6 phenotype, where the UMs form a tail at the extreme of extensive metabolic capacity (see Figure 3). Therefore, even if *CYP2C19*17* proves to be clinically significant on a population level, it will hardly prove to be clinically significant on an individual level. In selected cases, however, retrospectively genotyping for *CYP2C19*17* may provide an explanation for treatment failures or lower than expected plasma levels of therapeutic drugs. We have not yet reached the end of the CYP2C19–clopidogrel story, but pre-emptive *CYP2C19* genotyping (at least for the loss of function alleles **2* and **3*) may prove to be cost-effective, especially as clopidogrel has recently come off patent and is likely to become considerably cheaper than its alter-natives. The more expensive alternatives could thus be reserved for those unlikely to benefit from clopidogrel therapy.

5.3 PAPER IV

5.3.1 Warfarin metabolism

Warfarin is the drug that causes the greatest number of serious adverse drug reactions in Sweden [195], the only exception being 2009, when the pandemic flu vaccine had more serious ADR reports [196]. This was probably more a result of the high reporting rate for the new flu vaccine than it being extraordinarily harmful. Warfarin is a race-mate where *S*-warfarin is a more potent anticoagulant than *R*-warfarin. *S*-warfarin is metabolised by CYP2C9, whereas *R*-warfarin is mainly metabolised by CYP3A4 and CYP1A2 with some contribution from CYP2C19 [88].

5.3.2 ADR reports and disproportionality analyses

Since first reporting our findings of an association between noscapine use and increased effect of warfarin at the annual meeting (Riksstämman) of the Swedish Society of Medicine in late 2006, two more case series have been published. Scordo *et al.* reported of four cases of increased INR during concomitant noscapine and warfarin treatment and on the return to therapeutic levels after withdrawal of noscapine [197]. Myhr reported the same finding in four cases from Norway [198]. Until October 2010, there have been 20 reports in Sweden, 7 reports in Norway, and 1 report in the Netherlands of this suspected interaction [199, 200].

Although the disproportionality analyses do not prove causality, the association is strengthened by the fact that the signal is significant with two different methods. We have not recalculated the PRR and IC after the last reports, but they would be higher, since there are more reports showing an association and no reports that there is not.

5.3.3 *In vitro* findings

Our suspicion of a pharmacokinetic interaction between noscapine and warfarin was supported by the *in vitro* findings. Yet, we only used one test system and it couldn't be completely ruled out that noscapine may disrupt the test system in some other way than enzyme inhibition. Recently, Fang *et al.* have reproduced and elaborated our initial *in vitro* findings with proper enzyme inhibition kinetics using human liver microsomes (HLMs). First they tested the ability of 100 µM of noscapine to inhibit the metabolism of seven different CYP probe substrates (phenacetin for CYP1A2, coumarin for CYP 2A6, paclitaxel for CYP2C8, diclofenac for CYP2C9, dextromethorphan for CYP2D6, chlorzoxazone for CYP2E1, and testosterone for CYP3A4). In the presence of noscapine, the catalytic capacity was reduced with more than 80% for diclofenac 4'-hydroxylation (CYP2C9) and testosterone-6β-hydroxylation (CYP3A4). Further studies were therefore performed to elucidate the inhibition kinetics of noscapine towards CYP2C9 and CYP3A4. Mixed HLMs were preincubated with different concentrations of noscapine (0–50 µM) for various times (0–20 min) before 10-fold dilution and testing of residual catalytic capacity. The results showed a time-dependent inhibition of both CYP2C9 and CYP3A4 activities as shown in Figure 15.

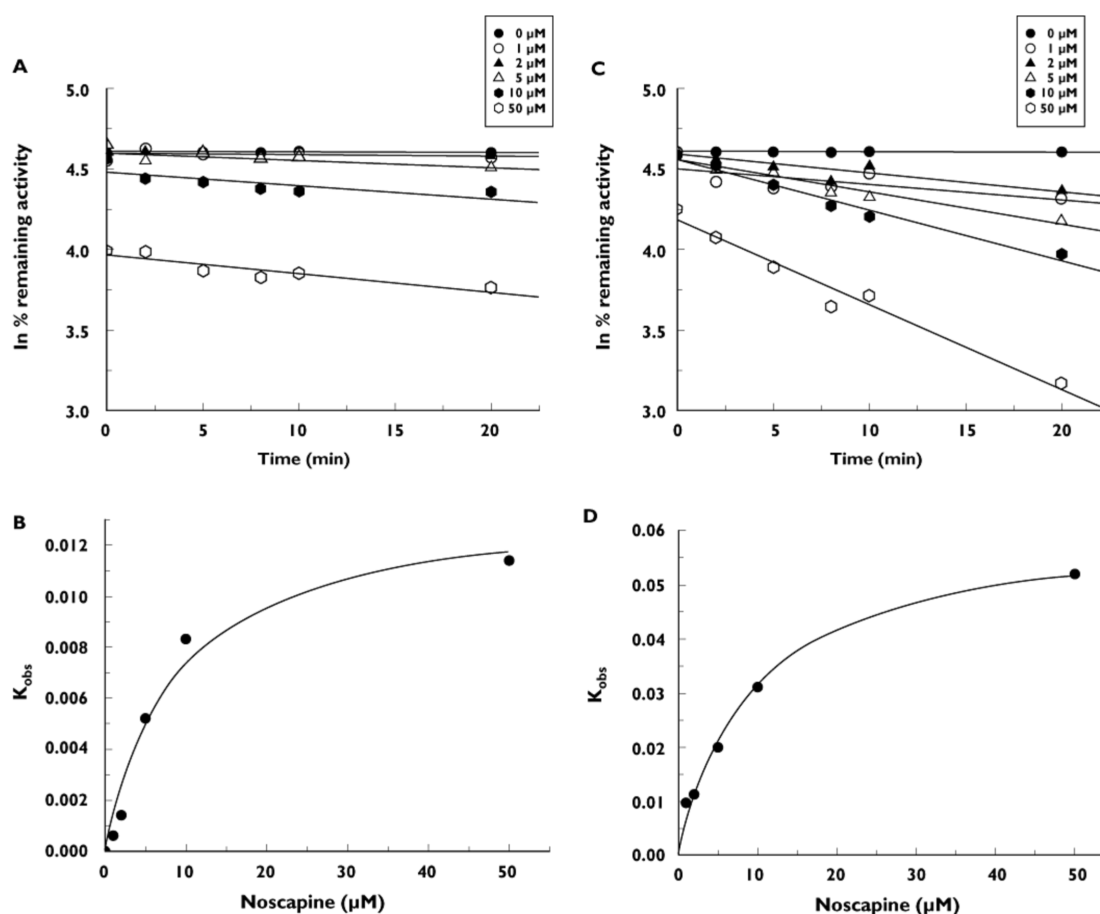

Figure 15 Time-dependent inhibition of CYP3A4 and CYP2C9 by noscapine. (A) Time- and concentration dependent inhibition of CYP2C9 by noscapine. (B) The hyperbolic plot of k_{obs} (the observed inactivation rate) of CYP2C9 vs. noscapine concentrations. (C) Time- and concentration-dependent inhibition of CYP3A4 by noscapine. (D) The hyperbolic plot of k_{obs} of CYP3A4 vs. noscapine concentrations. Reproduced from Fang *et al.*, Br J Clin Pharmacol., 2010; 69(2):193-9.

The authors predicted a 42% and 33% increase in *S*- and *R*-warfarin, respectively, at the maximal plasma concentration reached after a single 50 mg dose of noscapine [201]. However, noscapine does not have a linear dose–concentration relationship, as the bioavailability increases with increasing or repeated doses [124]. Pharmacokinetic studies of noscapine with more than two consecutive doses have not been published. This contributes to the uncertainty of extrapolating *in vitro* findings to *in vivo* conditions.

5.3.4 Causality and confounding

The association between increased anticoagulant effect of warfarin and concomitant use of noscapine could possibly be explained by confounding by indication, i.e. the respiratory infection that caused the cough might have reduced the clearance of warfarin by inflammatory downregulation of *CYP* gene expression. Inflammation has been shown to lower the expression of several CYPs [202] and decreased drug clearance has been noted during respiratory infection (influenza B) [203].
However, we did not find any reports of an interaction between warfarin and any other opioid cough syrups, neither in the literature, nor in the ADR registers. Recently, however, there was a report about increased INR in three maintenance warfarin treated patients concomitantly using a cough syrup containing ethylmorphine and *Senega* extract (Cocillana-etyfin®) [204]. This report warrants further investigation.

The results of Paper **IV** prompted the Swedish Medical Products Agency (MPA) to ask the manufacturer of warfarin to add a warning for concomitant use of noscapine to the warfarin Summary of Product Characteristics (SPC). It also made the board of the MPA willing to finance the study that resulted in Paper **V**.

5.4 PAPER V

5.4.1 Noscapine

We could clearly show that noscapine inhibits CYP2C9 dependent losartan metabolism and CYP2C19 dependent omeprazole metabolism. The concept of metabolic ratios is a useful measure of the activity of different CYP enzymes. However, it is not readily interpreted from a clinical point of view. In an attempt to evaluate the possible clinical impact of the degree of CYP2C9 inhibition seen in the study, we compared our data with the geno–phenotyping data of Yasar *et al.* [144]. The results of this comparison is illustrated in Figure 16 and described numerically in Paper **V**.
The basal metabolic ratio of losartan ($MR_{losartan}$) of the *CYP2C9*1/*1* subjects in our study was very similar to that of the previous study. On concomitant noscapine, the $MR_{losartan}$ increased to numbers comparable to the *CYP2C9*2/*3* group in Yasar's study. A recent metaanalysis of the impact of warfarin dose in relation to *CYP2C9* genotype showed that *CYP2C9*2/*3* carriers on average have less than 50% of the dose of *CYP2C9* wild-type individuals [46]. This comparison justified the declaration that the observed inhibition of CYP2C9 by noscapine was clinically significant.

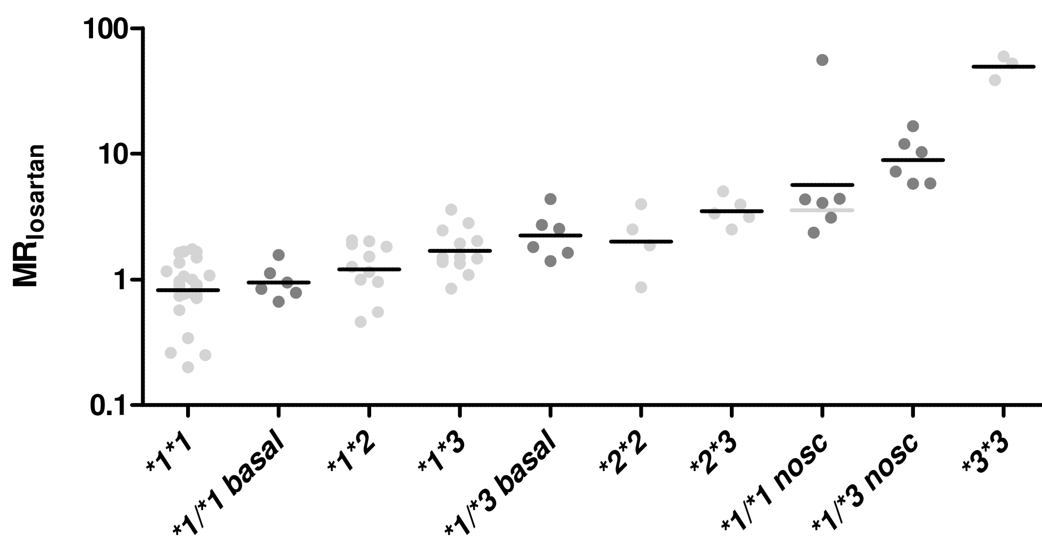

Figure 16 Comparison of losartan phenotyping data from the geno–phenotype study by Yasar *et al.* (light grey symbols) and our noscapine interaction study (dark grey symbols). Bars represent geometric means. The light grey bar in the **1/*1* group on noscapine represents the geometric mean when the outlier is excluded.

When it comes to a similar comparison regarding the omeprazole data, it is more difficult to draw any clearcut conclusions. Comparing our data with the *CYP2C19* geno–phenotyping study by Chang *et al.* [143], showed that the inhibition of omeprazole metabolism was comparable to converting an extensive metaboliser to intermediate metaboliser phenotype. This may still be of clinical importance for drugs with narrow therapeutic intervals. The bioactivation of clopidogrel is one example. Several drugs are dependent on both CYP2C9 and CYP2C19 for bioactivation, e.g. the cytostatic drugs cyclophosphamide and ifosfamide [205], or elimination, e.g. phenytoin [115].

It is interesting to note that we did not observe any *in vivo* inhibition of CYP3A4 activity, neither measured with the quinine/3hydroxyquinine ratio, nor with the omeprazole/omeprazole sulphone ratio (data not shown). The omeprazole/omeprazole sulphone ratio has been proven useful for CYP3A4 phenotyping under both inhibition and induction conditions [206]. The quinine/3-hydroxyquinine ratio has been shown sensitive for induction [207], but has not previously been used for inhibition studies. The lack of demonstrable *in vivo* CYP3A4 inhibition is in contrast to both our preliminary *in vitro* data presented in Paper **IV** and above and to the data of Fang *et al.* [201]. Since CYP3A4 is highly expressed in the gut [208], one highly speculative explanation might be that noscapine concentrations are high enough to inhibit enteric* CYP3A4, thus reducing first-pass metabolism, while leaving enough hepatic† CYP3A4 activity for unaffected systemic quinine metabolism. Our data might support this idea, as quinine concentrations were higher after noscapine than on basal phenotyping ($p \approx 0.03$), while the quinine/3-hydroxyquinine ratio was unaffected. This may well be a chance finding and would need a complete pharmacokinetic profile to verify. Another

* Enteric, from Greek *énteron* (intestine, bowel), concerning the bowels

† Hepatic, from Greek *hêpar* (liver), concerning the liver

possible, and maybe less speculative, explanation might be that noscapine is a mechanism-based (i.e. irreversible) inhibitor of CYP2C9, while being a reversible, yet time-dependent, inhibitor of CYP3A4. Then CYP3A4 activity would possibly regain quickly enough for leaving the metabolism of CYP3A4 substrates unimpaired. A third explanation for the discrepancy between *in vitro* and *in vivo* data is the high plasma protein binding of noscapine. Fang *et al.* assumed a protein binding of 65% [201], whereas other investigators have shown that noscapine is protein bound to a considerably greater extent (85-93%) [209].
Fact remains: *in vitro* findings are not always readily applicable for *in vivo* conditions!

5.4.2 Glucosamine

We could also clearly show, that glucosamine does not affect the activity of either of the probed cytochrome P450s. Thus a metabolic interaction between glucosamine and warfarin can be ruled out. However, several case reports of high quality suggest that there is some kind of drug-drug interaction between these two substances. This is probably pharmacodynamic. We did not notice any direct anticoagulant effect of glucosamine in our study, as INR values were unchanged in our subjects.
A pharmacodynamic interaction between glucosamine and warfarin may occur at any of several steps in the complex chain of events leading from inactive precursors to active vitamin-K dependent coagulation factors [139].
A potential pharmacokinetic interaction between glucosamine and warfarin that we have not addressed in our study is enhanced uptake. Glucosamine has been shown to enhance the bioavailability of tetracycline and during the late 1950's and early 1960's there was a combined preparation available called Cosa-Tetracyn® [134, 210]. The mechanism seems not to have been extensively elucidated, but since warfarin and tetracyclines are both weak acids [211], absorption may be enhanced by a weak base such as glucosamine. However, since warfarin is readily absorbed, whereas tetracycline has a lower and varying bioavailability of 60–80% [212], this mechanism is unlikely for the warfarin–glucosamine interaction.

5.5 THE ANSWER

The question in paragraph 1.1 cannot be answered with absolute certainty as we have not, yet, done a direct interaction study with noscapine and warfarin, but the evidence for an interaction is very strong: an increasing number of case reports, *in vitro* and *in vivo* findings that noscapine inhibits the key enzyme in warfarin metabolism. The *in vitro* findings have also been reproduced by an independent group and in a different test system than we used in Paper **IV**.
The Swedish Medical Products Agency has taken regulatory action on the results in Paper **V**: noscapine is still available OTC, but it cannot be sold outside of pharmacies [213].

6 FUTURE PERSPECTIVES

6.1 *CYP2C19*17*

There are preliminary data suggesting that oestrogens do not inhibit the expression of the *CYP2C19*17* allele and *CYP2C19*17* carriers may therefore not be inhibited by oral contraceptives as are *CYP2C19* wild-type carriers. This would be interesting to confirm in a clinical trial.

In the near future, we will also hear more about the role of CYP2C19 activity in clopidogrel resistance (both from the pharmacogenomics and the drug interaction perspective).

The most important pharmacokinetic feature of *CYP2C19*17* carriers may be their high and uniform CYP2C19 activity. It remains to determine the causes of the wide range of metabolic activity seen in individuals with *CYP2C19* wild-type genotype.

6.2 NOSCAPINE

We are currently performing a direct interaction study with noscapine in stable maintenance warfarin treated patients. We give noscapine 50 mg t.i.d. for a maximum of ten days. The patients are sampled every day and noscapine is withdrawn if the INR reaches a prespecified level.

It would also be interesting to do a study of how the pharmacokinetics and pharmacodynamics of oral antidiabetics are affected when co-administered with noscapine. Such a study could be done in a fashion similar to the cocktail study of Paper **V**. Pharmacodynamics could be monitored by the glucose–insulin clamp technique or, simpler, by measuring plasma glucose and insulin during an oral glucose tolerance test. It could also be done under steady-state conditions in stable patients with diabetes type 2.

The impact (or lack of impact) of noscapine on *in vivo* CYP3A4 activity could also be worth further investigation with other measures of CYP3A4 activity.

6.3 GLUCOSAMINE

It would be interesting to design and perform a clinical trial to try to elucidate the mechanism of the potential drug–drug interaction between glucosamine and warfarin. Such a trial would probably need to be performed in patients on stable maintenance warfarin, as warfarin pharmacodynamics may be difficult to study after single doses in healthy volunteers.

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[*] Senior Scientific Advisor is a near verbatim translation of the Swedish appellation *klok gubbe*.

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