STUDIES ON 4β-HYDROXYCHOLESTEROL, A MARKER OF CYP3A ACTIVITY, AND ITS ASSOCIATION WITH 25-HYDROXYVITAMIN D

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Studies on 4β-hydroxycholesterol, a marker of CYP3A activity, and its association with 25-hydroxyvitamin D

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Cholesterol is a vital compound that can undergo cytochrome P450 (CYP) mediated conversion into steroid hormones, bile acids and oxysterols. CYP enzymes are present in all human tissues and mediate the metabolism of several endogenous and exogenous compounds such as steroids and drugs. Vitamin D status has been shown to be important for several biological processes such as drug metabolism, modulation of the immune system and bone health. Enzymes in subfamily CYP3A (CYP3A4, CYP3A5, CYP3A7 and CYP3A43) are present in liver and intestine. They metabolize about 50% of all prescribed drugs. Genetic factors, age, sex, ethnicity and environmental factors influence the activity and expression of CYP3A enzymes. These factors combined cause wide inter-patient variability in CYP3A mediated drug response. There are a number of clinical markers to assess the CYP3A activity, e.g. plasma midazolam clearance, quinine metabolic ratio and 4β-hydroxycholesterol/cholesterol ratio. In the present study the plasma levels of 4β-hydroxycholesterol and the 4β-hydroxycholesterol/cholesterol ratio has been evaluated as markers of CYP3A activity during enzyme induction by a number of drugs (carbamazepine in Papers I and III, rifampicin in Papers IV-V and efavirenz in Paper V) and by pregnancy (Paper III). The association between CYP3A activity and vitamin D status has also been studied (Paper IV-V). In Paper I, carbamazepine treatment in children with epilepsy doubled the plasma levels of 4β-hydroxycholesterol within two weeks of treatment. The increase was 5 to 10-fold within eight weeks treatment. In Paper III, pregnancy increased the 4β-hydroxycholesterol/cholesterol ratio and the plasma levels of cholesterol. Newborn children had the same CYP3A activity as adults as indicated by similar 4β-hydroxycholesterol/cholesterol ratios. Carbamazepine treatment during pregnancy further increased the CYP3A activity in one mother and her child. In Papers IV-V, rifampicin-mediated CYP3A induction did not affect the plasma levels of 25-hydroxyvitamin D in healthy volunteers or in tuberculosis-HIV co-infected patients. In tuberculosis-HIV co-infected patients there was a significant negative correlation between the plasma levels of 25-hydroxyvitamin D and the 4β-hydroxycholesterol/cholesterol ratio already at initiation of treatment (Paper V). Efavirenz treatment caused a transient decrease in the plasma levels of 25-hydroxyvitamin D in HIV-infected patients (Paper V). To summarize, 4β-hydroxycholesterol and the 4β-hydroxycholesterol/cholesterol ratio are useful as markers of CYP3A induction. 4β-Hydroxycholesterol is a non-invasive endogenous clinical marker that is easy to use also in children and vulnerable patient groups. The blood samples can be taken at any time of the day regardless of food intake.
LIST OF SCIENTIFIC PAPERS


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<table>
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<tr>
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<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
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<tr>
<td>ACAT</td>
<td>Acyl-CoA cholesterol acyltransferase</td>
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<td>APOA</td>
<td>Apo lipoprotein A</td>
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<td>ART</td>
<td>Anti-retroviral treatment</td>
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<td>AUC</td>
<td>Area under the curve</td>
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<td>CAR</td>
<td>Constitutive androstane receptor</td>
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<td>CD4</td>
<td>T helper cells</td>
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<td>CYP</td>
<td>Cytochrome P450</td>
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<td>FXR</td>
<td>Farnesoid X receptor</td>
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<td>GR</td>
<td>Glucocorticoid receptor</td>
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<tr>
<td>LCAT</td>
<td>Lecithin-cholesterol acyltransferase</td>
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<td>LXR</td>
<td>Liver X receptor</td>
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<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
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<tr>
<td>PXR</td>
<td>Pregnane X receptor</td>
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<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
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<tr>
<td>SULT</td>
<td>Cytosolic sulfotransferase</td>
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<td>TB</td>
<td>Tuberculosis</td>
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<td>UGT</td>
<td>UDP-glycosyltransferase</td>
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<td>VDR</td>
<td>Vitamin D receptor</td>
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<td>VDRE</td>
<td>Vitamin D responsive elements</td>
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1 INTRODUCTION

1.1 CHOLESTEROL, OXysterOLS AND VITAMIN D

1.1.1 Cholesterol properties, balance and regulation

Cholesterol is a vital component of cellular membranes in all animal cells and lipid bilayers. Some organs and cell types contain large amounts of cholesterol, e.g. the brain and nerve cells. The free form of cholesterol is highly hydrophobic and must be transported in lipoprotein particles for its distribution throughout the body. The brain is an exception as the blood brain barrier prevents lipoprotein-bound cholesterol from passing. The cholesterol in the brain is thus synthesized locally. The origin of the body’s cholesterol pool is from dietary intake and de novo synthesis. All nucleated cells can synthesize cholesterol. The homeostasis of cholesterol is strictly regulated, and excess cholesterol in peripheral cells may be converted to cholesteryl esters by ACAT1, ACAT2 or LCAT (Acyl-CoA cholesterol acyltransferase 1 and 2, Lecithin-cholesterol acyltransferase), or may be transported to the liver by reverse cholesterol transport (RCT) mediated by ApoA1 (Apo lipoprotein A1) containing lipoproteins. The cholesterol homeostasis is in part enzymatically regulated by cytochrome P450 (CYP) enzymes that convert cholesterol into steroid hormones, bile acids and oxysterols. Steroid hormones have important roles as mediators in growth, development and maintenance of cholesterol homeostasis. Bile acids are necessary for the absorption of dietary lipids and fat-soluble vitamins in the intestines. The cholesterol homeostasis and bile acid production is also tightly regulated at a transcriptional and translational level mediated by feedback signaling pathways in which nuclear receptors such as LXRα (liver X receptor α), FXR (farnesoid X receptor) and PPARα (peroxisome proliferator-activated receptor) have vital roles. Oxysterols are oxygenated forms of cholesterol and have important roles as transport forms of cholesterol, intermediates in the bile acid synthesis and co-regulators of several genes.

1.1.2 Oxysterol formation, properties and biological roles

Oxysterols are the mono-oxygenated derivatives of cholesterol, which are formed either spontaneously by cholesterol autoxidation or by enzymatic reactions with cholesterol as substrate. Cholesterol may be oxidized at several different positions leading to a large number of different products. For cholesterol numbering and some products of enzymatic reactions with cholesterol, see Figure 1. The allylic site at carbon 7 is particularly susceptible to autoxidation and 7α-hydroxycholesterol, 7β-hydroxycholesterol and 7-ketocholesterol are common autoxidation products. 7α-Hydroxycholesterol may also be formed by enzymatic
hydroxylation (cytochrome P450 7A1, CYP7A1), and is an important intermediate in the bile acid synthesis.

Plasma oxysterols are present in the nanomolar range. The most abundant oxysterols are 24S- and 27-hydroxycholesterol, at levels around 75 and 150 ng/mL, respectively. The plasma level of 24S-hydroxycholesterol as well as the cerebrospinal fluid (CSF) levels of 24S- and 27-hydroxycholesterol have been proposed as markers of neurodegenerative diseases such as Alzheimer’s disease, Huntington’s disease and multiple sclerosis (MS) [1].

Oxysterols have important roles as co-regulators of several genes regulating the cholesterol efflux, absorption, transport and excretion, mediated by the nuclear receptors LXRα and LXRβ [2]. Oxysterols are less hydrophobic than cholesterol and may serve as transport forms of cholesterol; the above-mentioned 24S-hydroxycholesterol is synthesized in neuronal cells in the brain and is able to cross the blood brain barrier, which contributes to the cholesterol homeostasis in the brain [3]. The oxysterol 25-hydroxycholesterol is formed both by autoxidation and by an enzymatic reaction mediated by cholesterol 25-hydroxylase (CH25H). There are reports on a possible role of 25-hydroxycholesterol in the innate and adaptive immune system [4-6].

![Diagram of CYP enzymatic formation of oxysterols from cholesterol.](image-url)

**Figure 1:** CYP enzymatic formation of oxysterols from cholesterol.
The formation of the oxysterol 4β-hydroxycholesterol is mediated enzymatically by cytochrome P450 3A, CYP3A [7]. The isomer 4α-hydroxycholesterol is formed by cholesterol autoxidation and possibly enzymatically by still unknown enzyme(s). The molecular structures of 4α-hydroxycholesterol and 4β-hydroxycholesterol can be seen in Figure 2.

### 1.1.3 Vitamin D properties and regulation

Vitamin D exists in two main forms in man; Vitamin D₃ and D₂ (Figure 3). Vitamin D₃ is formed naturally in the skin from the precursor 7-dehydrocholesterol upon exposure to sunlight while vitamin D₂ must be ingested as food or supplementation. The bioactive form of vitamin D (D₃ and D₂) is considered to be 1α,25-dihydroxyvitamin D (molecular structure in Figure 3), which is formed from vitamin D in two subsequent enzymatic hydroxylation steps. The 25-hydroxylation take place mainly in liver and other organs and tissues to some extent, mediated by the enzymes CYP2R1 and CYP27A1. The subsequent 1α-hydroxylation takes place in kidney and other organs and tissues to some extent, mainly mediated by the enzyme CYP27B1.

![Figure 2: Formation of 4α-hydroxycholesterol and 4β-hydroxycholesterol from cholesterol.](image-url)
1α,25-dihydroxyvitamin D regulates the transcription of several genes by binding to their vitamin D responsive elements (VDRE) when bound to a RXR-VDR (retinoid X-vitamin D receptor) complex [8]. Such complexes have been shown to induce CYP3A activity and expression at mRNA level in liver and intestinal cells [9]. It is estimated that 200 to 2000 genes have VDREs and the RXR-VDR complexes influence a vast number of additional genes indirectly, possibly by epigenetic mechanisms [10], making the vitamin D status important for numerous biological processes. Under in vitro conditions vitamin D is able to induce enzymes such as CYP3A4, CYP2C9 and CYP2B6 [11, 12].

1.1.3.1 Vitamin D in health and disease
The best measure of vitamin D status is considered to be the plasma level of 25-hydroxyvitamin D (Figure 3) due to its long half-life (3 weeks). The half-life of the active form 1α,25-dihydroxyvitamin D is only 4 hours. Plasma levels of 25-hydroxyvitamin D < 50 nmol/L are considered deficient, and levels < 72.5 nmol/L insufficient [13].

Figure 3: Molecular structures of vitamin D₃, vitamin D₂, 25-hydroxyvitamin D₃, 25-hydroxyvitamin D₂ and 1,25-dihydroxyvitamin D₃ and 1,25-dihydroxyvitamin D₂.
Hence, vitamin D deficiency is very common in the general population worldwide with a reported incidence ranging between 7-20% in southern Africa and 92% in northern Scandinavia [10]. Vitamin D is involved in several biological processes such as bone health, infection and anti-inflammatory response [10, 14], modulation of the immune system [15-18], reproduction/pregnancy [19] and drug metabolism [11, 12]. Vitamin D deficiency has been reported as a risk factor for some types of cancers [10], tuberculosis [20] and Type 2 diabetes [10]. Vitamin D deficiency has also been reported as a risk factor for a rapid decline in CD4 cell count in HIV patients not on anti-retroviral treatment (ART) [21] and there are reports on a potential benefit on CD4 recovery from vitamin D supplementation during ART [22].

1.2 DRUG METABOLISM

Drug metabolism is a process of enzymatic conversion of drugs into more polar compounds that can be more readily excreted. Drug metabolism takes places mainly in liver, but several tissues and organs can mediate drug metabolism to some extent.

Drug metabolism is usually divided into the following phases [23]:

Phase 0 – hepatic uptake of drugs from systemic circulation. Hepatic clearance of drugs from the portal blood takes place at the basolateral membrane of hepatocytes, mainly mediated by transport proteins such as SLCOs (solute carrier organic anion transporting polypeptides).

Phase 1 – drug hydroxylation by cytochrome P450 (CYP) enzymes and reduction and hydrolysis by other enzymes. There are numerous different CYP enzymes that mediate hydroxylation at regio- and stereospecific sites of a vast amount of different drugs and endogenous substances. The CYP enzymes in families CYP3A and CYP2B mediate the hydroxylation of 80% of prescription drugs.

Phase 2 – conjugation of Phase 1 products with endogenous molecules such as sulfonyl- or glycosyl groups, mediated by SULT (cytosolic sulfotransferase) or UGT (UDP-glycosyltransferase) enzymes.

Phase 3 – hepatic excretion of Phase 2 products into bile. The transport across the membrane is mediated by ATP-binding cassette (ABC) transporter proteins such as P-glycoprotein, that are localized at the canalicular membrane of the hepatocytes.

Nuclear receptors such as PXR, FXR and CAR (constitutive androstane receptor) are ligand-activated transcription factors that regulate the expression of enzymes and membrane transporters participating in Phases 1-3 of drug metabolism [23]. Different drugs activate
different nuclear receptors and affect different drug metabolizing enzymes. In general, the nuclear receptors are localized to the cytoplasm and translocate to the nucleus after activation. The anti-tuberculosis drug rifampicin and the anti-epileptic drug carbamazepine activate enzyme transcription mainly via PXR [24, 25], while the anti-retroviral agent efavirenz activates via CAR [26].

Rifampicin is commonly used as first line anti-tuberculosis treatment in resource-limited settings. Common side effects are rash and gastrointestinal disturbances [27]. Carbamazepine is commonly used to prevent seizures in epilepsy. Common side effects are nausea and drowsiness. Efavirenz is commonly used in combination with two other anti-HIV drugs in resource-limited settings. Common side effects include rash, dizziness, mood changes, depression, anxiety, paranoia, insomnia and changes in body fat composition. Patients with HIV have compromised immune systems and opportunistic infections such as tuberculosis are common. Anti-retroviral and anti-tuberculosis co-treatment is necessary in such cases.

The pharmacological action of a drug is determined by the speed of the Phase 1-3 reactions, which is subject to individual variation due to genetic, environmental and physiological factors.

1.3 CYTOCHROME P450 ENZYMES
Cytochrome P450 enzymes (CYP) are present in all human tissues and are involved in several different biotransformation reactions. There are about 60 different CYP enzymes in human that are classified into different families and subfamilies based on amino acid sequence similarity. The CYP enzymes are localized to the endoplasmatic reticulum or inner membrane of the mitochondria. In general, the enzyme families CYP1, CYP2 and subfamily CYP3A are important in the metabolism of endogenous substances such as steroids, bile acids, fatty acids and vitamins as well as Phase 1 metabolism of drugs. CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 are the major drug metabolizing isoforms [28].

Generally, enzymes participating in drug metabolism have wide substrate specificity and are predominately localized to the liver and intestine, which serve as the main detoxification site and the main barrier against ingested substances, respectively. CYP3A4 has two active sites and wide substrate specificity. Nuclear receptors such as PXR, CAR and VDR are important regulators of CYP enzyme transcription.

The catalytic reaction of CYP enzymes is NADPH-dependent and can be summarized as follows: 
\[
\text{RH} + \text{O}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{NADP}^+ 
\]
One oxygen atom is introduced into the substrate (RH) and the other oxygen atom is reduced to water.

Several factors such as genetic factors, age, sex, ethnicity and environmental factors influence the activity of CYP enzymes. Sex dependent differences in the activity of a number of CYP enzymes, e.g. CYP1A2, CYP2B6, CYP2E1 and CYP3A4 have been demonstrated [29].

Another factor that affects the inter-individual variation in enzyme activity is genetic polymorphism, i.e. variants of any given gene (carried by at least 1% of a certain population). Genetic polymorphism is very common for some drug metabolizing enzymes and the polymorphic trait of CYP2D6, CYP2C19 and CYP2C9 variants are well characterized. Enzyme function may be described as poor, intermediate, extensive or ultra-rapid. There is a 100-fold inter-individual variability in CYP2B6 activity, and women in general have higher activity than men [29]. For certain drugs it may be necessary to monitor drug concentrations during treatment (therapeutic drug monitoring) in order to avoid adverse drug reactions.

Pregnancy increases the activities of the enzymes CYP3A4, CYP2D6 and CYP2C9 [30-33], possibly due to progesterone activation via PXR [30]. Dosages of drugs metabolized by these enzymes may need to be increased during pregnancy in order to avoid loss of efficacy. There are also reports on increased activity of the transporter protein P-glycoprotein during pregnancy, suggesting increased renal clearance of numerous drugs [31].

Pregnancy decreases the activities of the enzymes CYP1A2 and CYP2C19 [30, 32, 34, 35], suggesting that dosage reduction of drugs metabolized by these enzymes may be needed in order to minimize potential toxicity. There are reports on a possible effect of estrogen on the inhibition of CYP2C19 during pregnancy [35].

1.3.1 CYP3A

There are four CYP3A enzymes: CYP3A4, CYP3A5, CYP3A7 and CYP3A43. These have similar amino acid sequences and overlapping, but not identical, substrate specificities. CYP3A enzymes have large active sites that can accommodate several structurally different substrates, possibly simultaneously, and the substrate specificity is thus very broad.

Hepatic CYP3A has large inter-individual variability in expression (≥ 50-fold) and activity (≥ 20-fold), mainly due to genetic factors [36], sex, ethnicity, and physiological and environmental factors [29, 37, 38]. Women have higher CYP3A activity than men [29, 38]. The wide inter-patient variability in CYP3A mediated drug response may be due to the particular complexity of the transcriptional regulation of CYP3A4 mediated by nuclear
receptors such as PXR, CAR, VDR and GR (glucocorticoid receptor) [12, 39-41]. These receptors control the basal and inducible expression of CYP3A. Nuclear receptor polymorphisms may have a role in the large inter-individual variation in CYP3A induction [42].

CYP3A4 is the most abundant CYP3A enzyme and is the dominating enzyme in drug metabolism as it metabolizes over 50% of all prescribed drugs [40]. CYP3A4 is predominantly expressed in liver and intestine, but is present also in other organs and tissues. The allele CYP3A4*20 has been reported to cause loss of function [43].

CYP3A5 has an 84% amino acid sequence similarity with CYP3A4 and is also predominantly expressed in liver and intestines, but is present also in other organs and tissues such as lungs [44, 45]. CYP3A5 is subject to a marked genetic variation (polymorphism) and its expression is abundant in some individuals and non-existent in others. There are a number of common single nucleotide polymorphisms (SNPs) yielding prematurely truncated and thus non-functional enzymes, e.g. CYP3A5*3 and CYP3A5*6 [46]. CYP3A5 is expressed when there is at least one CYP3A5*1 allele present which is the case in 74% of Tanzanians, 33% of Koreans and only 13% of Swedes [38].

CYP3A7 has an 88% amino acid sequence similarity with CYP3A4. CYP3A7 is considered to be the fetal equivalent of CYP3A4 and is expressed in placenta and in liver and intestines in fetus and neonates [47-49]. CYP3A7 accounts for up to 50% of total fetal hepatic CYP enzyme content [48]. There are reports on a transition from expression of CYP3A7 to expression of CYP3A4 during the first years of life [48, 49] and concomitant expression of CYP3A4 and CYP3A7 in neonatal liver [50]. There is a genetic variant of CYP3A7, CYP3A7*1C, with persistent expression in adults [51-53].

CYP3A43 has a 76% amino acid sequence similarity with CYP3A4 [54]. CYP3A43 is expressed at low levels in liver and intestine and has therefore been regarded to be less important in drug clearance. However, the expression of CYP3A43 has shown to be high in the brain and polymorphism of this enzyme may explain inter-individual differences in the clearance of the anti-psychotic drug olanzapine [55, 56], which has a high rate of inefficacy and adverse effects.

Pregnancy increases the activity of CYP3A4 [30-32], possibly due to progesterone activation via PXR or any of the physiological changes that occur, e.g. reduced intestinal movement, altered gastric pH, increased plasma volume, increased hormone levels and/or fetal enzyme activity [30]. The general advice during pregnancy is to avoid drugs, but certain conditions
call for continuous medication, e.g. epilepsy, HIV and tuberculosis. The anti-epileptic drug carbamazepine is known to induce CYP3A and the same is true for the anti-retroviral agent efavirenz and the anti-tuberculosis agent rifampicin. Efavirenz is both substrate and inducer of CYP3A4/5 and CYP2B6 [57]. Dose adjustments may be needed during treatment or pregnancy in order to avoid sub-therapeutic levels in circulation due to altered metabolism. There are reports on altered exposure of the anti-retroviral agent lopinavir in pregnant HIV-infected women [58]. Therapeutic drug monitoring is very useful but may not be available in resource-limited settings.

The risk of prostate and breast cancer has been associated with certain genetic variants of CYP3A4, CYP3A5 and CYP3A43 [59-62].

CYP3A enzymes mediate the formation of 4β-hydroxycholesterol from cholesterol both in vivo (CYP3A4 and CYP3A5 [7, 38, 63]) and in vitro (CYP3A7 [64]). The 4-hydroxylase activity of CYP3A43 remains to be determined. The isomer 4α-hydroxycholesterol is formed by cholesterol autoxidation and possibly enzymatically by still unknown enzyme(s) (Figure 2, page 7).

1.3.1.1 Markers of CYP3A activity

Fifty percent of all prescribed drugs are metabolized by CYP3A [40] and the pharmaceutical industry need to study the effect of candidate drugs on the CYP3A activity in order to evaluate possible drug-drug interactions. There are a number of clinical markers for this, e.g. plasma midazolam clearance [65], erythromycin breath test [66], 6β-hydroxycortisol/cortisol ratio in urine [67], quinine metabolic ratio [68] and the plasma levels of 4β-hydroxycholesterol [69].

In plasma midazolam clearance the concentration of midazolam is measured repeatedly after oral or intravenous administration of the drug midazolam and the clearance is calculated from the AUC (area under the curve).

In the erythromycin breath test radiolabeled N-methyl-erythromycin is given intravenously and the amount of exhaled radiolabeled carbon dioxide is measured.

The 6β-hydroxycortisol/cortisol ratio in urine is an endogenous marker, but the diurnal variation of cortisol calls for at least 4-hour and preferably 24-hour collections of urine.
To determine the quinine metabolic ratio (quinine/3-hydroxyquinine) quinine is given as an oral dose and the plasma concentrations of quinine and the metabolite 3-hydroxyquinine is determined in a sample drawn 16-hour post-dose.

The plasma levels of 4β-hydroxycholesterol have been shown to be related to the degree of induction or inhibition of the CYP3A enzyme activity. The plasma levels of 4β-hydroxycholesterol were markedly increased in patients treated with any of the CYP3A inducers carbamazepine, phenytoin, phenobarbital (anti-epileptics [7]), rifampicin (anti-tuberculosis [70]) or ursodeoxycholic acid (anti gallstone [7]). The 4β-hydroxycholesterol/cholesterol ratio may be used as a marker of CYP3A activity in studies where the cholesterol levels are expected to change during disease progression or treatment [71, 72]. The plasma level of 4β-hydroxycholesterol and the 4β-hydroxycholesterol/cholesterol ratio have been shown to be correlated to the number of functional CYP3A5 alleles (CYP3A5*1) in four populations (Swedes, Tanzanians, Koreans and Ethiopians) [38, 63]. Both 4β-hydroxycholesterol and cholesterol have half-lives of several days [69, 73] which is useful in a clinical setting as the blood samples can be taken at any time of the day. The long half-life of 4β-hydroxycholesterol is an advantage in situations where long time changes are studied, e.g. how long-term treatment with an inducer or an inhibitor affects the enzyme activity during the course of weeks or months.

Of the above-mentioned markers 4β-hydroxycholesterol is the easiest to use in children, elderly, certain vulnerable patient groups and pregnant women due to technical and/or ethical issues. Many drugs that are given to children are not licensed for pediatric use and/or are given by off-label prescription. Ethical aspects make it difficult to define pediatric pharmacokinetics in clinical trials. Children and neonates are often prescribed weight-adjusted dosing of drugs based on the pharmacokinetics in adults, but precaution must be taken since the physiology of neonates is different compared to the physiology of adults and older children. As an example, neonates have higher gastric pH, lower gastrointestinal motility, lower serum proteins levels and larger extra cellular water volume than older children and adults, which may have an effect on the bioavailability of the drug [74, 75]. For some drugs, a lower than the weight-adjusted dose may be necessary in order to avoid toxic effects. For some drugs a higher dose than the weight-adjusted dose may be needed in order to reach therapeutic concentrations.
2 AIMS OF THE STUDY

The specific aims of the present study were the following:

- To develop an optimized sample preparation procedure for the analysis of 4β-hydroxycholesterol in plasma (Paper II).
- To develop a sample preparation procedure and a LC-MS/MS method for the analysis of 25-hydroxyvitamin D in plasma (Paper IV).
- To evaluate 4β-hydroxycholesterol as a marker CYP3A activity:
  - In children with epilepsy after initiation of carbamazepine treatment (Paper I).
  - In mothers and neonates at birth (Paper III).
  - In tuberculosis-HIV co-infected or HIV-infected patients after initiation of anti-tuberculosis and/or anti-retroviral treatment (Paper V).
- To investigate the possible relation between CYP3A activity and vitamin D status.
  - In healthy volunteers (Paper IV).
  - In tuberculosis-HIV co-infected or HIV-infected patients after initiation of anti-tuberculosis and/or anti-retroviral treatment (Paper V).
3 METHODOLOGY

3.1 CHEMICALS

4β-Hydroxycholesterol was purchased from Steraloids (Newport, RI, USA) or synthesized along with $^2$H$_6$-4β-hydroxycholesterol according to Breuer [76] (Papers I-V). 25-Hydroxyvitamin D2/D3 Control (level 1) and Calibration Standard were purchased from Chromsystems (Munich, Germany) (Papers IV-V). $^2$H$_6$-25-Hydroxyvitamin D$_3$ was purchased from Synthetica (Oslo, Norway) (Papers IV-V).

3.2 MATERIALS

SI solid phase extraction columns were purchased from International Sorbent Technology (Mid Glanorgan, UK) (Papers I and III). Hybrid SPE precipitation 96-well plates (50 mg/well) were purchased from Supelco (Sigma Aldrich, Saint Louis, MO, USA) (Papers IV-V). Strata-X 30 mg/1mL SPE columns were purchased from Phenomenex (Skandinaviska Genetech AB, Västra Frölunda, Sweden) (Papers II, IV-V).

3.3 SAMPLE HANDLING AND PREPARATION

Venous blood samples were drawn in EDTA-containing tubes. After centrifugation the plasma was stored at -70°C until analysis. Aliquots of whole blood were transferred to plastic tubes and stored at -70°C until genotyping for common variant alleles in CYP3A5, CYP2B6, UGT2B7, ABCB1, SLCO1B1 and VDR: CYP3A5*3 in Papers I, III and V. CYP3A5*6 in Paper V. CYP2B6*6, UGT2B7*2, ABCB1 c.3435C/T, ABCB1 c.4036A/G (rs3842), SLCO1B1*1b and SLCO1B1*5 in Paper V. VDR rs1544410, VDR rs11568820 and VDR rs4516045 in Paper IV.

In Paper I venous blood samples were drawn from children (n=8) with newly diagnosed epilepsy before and after 1, 2, 4, 8, 16 and 23 weeks treatment with the anti-epileptic drug carbamazepine in doses ranging 5-30 mg/kg bodyweight/day.

In Paper II venous blood samples (n=90) were drawn from healthy volunteers.

In Paper III venous blood samples were drawn from untreated mothers at the neonatal screening (vaginal delivery, n=14) or prior to elective caesarean section (CS, n=7). Venous blood samples were drawn from children born by vaginal delivery at the neonatal screening (n=14) or from the umbilical cord immediately after CS (n=8). Venous blood samples were drawn from both mothers and children at the four-month follow up (Mothers: vaginal
delivery n=5, CS n=5. Children: Vaginal delivery n=5, CS n=6). Samples were also drawn from one carbamazepine-treated mother and her child at time of CS delivery.

In Paper IV samples from population studies in Swedes (n=65) and Koreans (n=67) were drawn before and after administration of the “Karolinska cocktail” (consisting of five drugs to determine the phenotype of the CYP enzymes 1A2, 2C9, 2C19, 2D6 and 3A4 [77]). Samples from another study (healthy volunteers, n=24) were taken before and after two weeks treatment with rifampicin in three different doses (10, 20 or 100 mg/daily).

In Paper V samples were drawn at initiation of efavirenz-based ART (week 0) and at weeks 4, 16 and 48 of treatment (HIV only patients, n=94). For tuberculosis-HIV co-infected patients (n=102) samples were also drawn at the initiation of anti-tuberculosis treatment (rifampicin), 4 or 8 weeks prior to ART (week -8, n=33 and week -4, n=69, respectively) The time scheme for anti-tuberculosis treatment and ART is shown in Figure 4. The efavirenz-based anti-retroviral treatment was 600 mg efavirenz/lamivudine/zidovudine, stavudine or tenofovir). The anti-tuberculosis treatment was rifampicin/isoniazid/pyrazineamide/ethambutol for the first two months and rifampicin/isoniazid for four months.

3.3.1 Sample preparation of plasma 4α- and 4β-hydroxycholesterol

3.3.1.1 Original method (Papers I and III)

Many oxysterols, including 4α-hydroxycholesterol and 4β-hydroxycholesterol, are present as long chain fatty acid esters in plasma. Hydrolysis is often used to get the oxysterols into free form. For sample preparation of 4α-hydroxycholesterol and 4β-hydroxycholesterol in plasma with the original method, hydrolysis was performed by adding 0.35 M potassium hydroxide in ethanol (KOH) (10 mL) to a screw-capped vial with Teflon-lined septum containing 1 mL plasma, 200 µg EDTA (10 mg/mL water), 50 µg BHT (5 mg/mL water) and 100 ng internal standard (2H6-4β-hydroxycholesterol).

![Figure 4](Image)

**Figure 4**: Time scheme of rifampicin and efavirenz-based anti-retroviral treatment in tuberculosis-HIV and/or HIV only patients.
The screw-capped vials were left at room temperature under argon with continuous stirring for two hours. Liquid-liquid extraction was done after transferring the hydrolysis solution along with chloroform (18 mL) and sodium chloride (0.15 M) (6 mL) to separatory funnels (pH adjusted to 7 with phosphoric acid). The separatory funnels were shaken vigorously and left to phase separate for 20 minutes. Chloroform was rotary evaporated at room temperature. The residues were dissolved in toluene (1 mL) and put on preconditioned (hexane, 2 mL) SI solid phase extraction columns. The SI columns were washed with hexane (1 mL). Cholesterol was eluted using 2-propanol in hexane (0.5%, 8 mL). Oxysterols were subsequently eluted using 2-propanol in hexane (30%, 5 mL). The eluates were evaporated to dryness under argon and derivatized using t-butyldimethylsilylimidazole dimethylformamide (100 µL, 60°C) for four hours. Liquid-liquid extraction of the derivatized oxysterols was done using isooctane (2 x 2 mL) and water (1 mL). Finally, the isooctane phase was evaporated and the residues dissolved in hexane (70 µL) and transferred to 2 mL vials prior to analysis by GC-MS [7, 78].

3.3.1.2 Optimized sample preparation method (Papers II, IV-V)

In the original method several precautions were taken to protect the most sensitive oxysterols from autoxidation and/or decomposition. For analysis of 4β-hydroxycholesterol a number of modifications were made in order to tailor the preparation. The steps chosen for modification was the sample volume and the conditions during hydrolysis and extraction.

Hydrolysis was performed by adding 0.7 M KOH in ethanol (1 mL) to a glass centrifuge tube containing 250 µL plasma, 200 µg EDTA (10 mg/mL water), 50 µg BHT (5 mg/mL water) and 100 ng internal standard (2H6-4β-hydroxycholesterol). After vortex mixing (30 seconds) the samples were kept at room temperature for 30 minutes without stirring. Before centrifugation (10 min, 1000g) the pH was adjusted to 7 with phosphoric acid. For extraction the samples were put on preconditioned (methanol, 1 mL) and equilibrated (deionized water, 1 mL) Strata-X 30 mg/1mL SPE columns. The columns were washed with methanol in water (10%, v/v). The samples were eluted with acetonitrile in water (85%, v/v, 1 mL). The eluates were evaporated to dryness under argon before derivatization and analysis by GC-MS as described previously [7, 78].

3.3.2 Sample preparation of plasma/serum 25-hydroxyvitamin D

In Papers IV-V, plasma/serum (100 µL) was added to a Hybrid SPE precipitation 96-well plate (50 mg/well) followed by 2-propanol in acetonitrile (12% v/v, 1% formic acid, 300 µL)
containing $^2$H$_6$-25-hydroxyvitamin D$_3$. The 96-well plate was vortexed prior to elution into a 96-well polypropylene plate. The plate was sealed and stored at 8°C until analysis.

3.4 SAMPLE ANALYSES

3.4.1 GC-MS analysis of plasma 4α- and 4β-hydroxycholesterol

In Papers I-V, the t-butyldimethylsilyl ethers of 4α-hydroxycholesterol, 4β-hydroxycholesterol and $^2$H$_6$-4β-hydroxycholesterol were analyzed on a GC-MS (Hewlett Packard 6890 GC-5973 MS) equipped with a HP5-MS capillary column (30 m x 0.25 mm i.d. x 0.25 µm phase thickness) using single ion monitoring (SIM) (m/z 573 for 4α-hydroxycholesterol and 4β-hydroxycholesterol and m/z 579 for $^2$H$_6$-4β-hydroxycholesterol) [7, 78]. The CVs for 4β-hydroxycholesterol with the original and modified sample preparation methods were 7.7% (n=78) and 8.2% (n=59), respectively at level 25 ng/mL.

3.4.2 LC-MS/MS analysis of 25-hydroxyvitamin D

In Papers IV-V, 25-hydroxyvitamin D$_3$ and 25-hydroxyvitamin D$_2$ in plasma/serum were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) with $^2$H$_6$-25-hydroxyvitamin D$_3$ as internal standard. The samples were analyzed on a Waters Acquity Quattro Premier LC-MS/MS equipped with a Waters UPLC BEH C18 column (1.7 µm i.d., 2.1 x 50 mm) set at 55°C. The mobile phase was a 4-minute gradient of 50-95% acetonitrile in water (formic acid, 0.1%). The flow rate was 0.3 mL/min and the total run time 5 minutes.

The mass spectrometer was operated in positive electrospray mode and the following transitions were followed: $^2$H$_6$-25-hydroxyvitamin D$_3$: 407 → 158, 25-hydroxyvitamin D$_3$: 401 → 158, 25-hydroxyvitamin D$_2$: 413 → 82.

3.4.3 Other analyses

Carbamazepine and carbamazepine-epoxide were analyzed in plasma using HPLC-UV as described previously [79] (Paper I).

Plasma cholesterol was analyzed by a commercial enzymatic method, Chod-PAPP (Roche Diagnostics, Mannheim, Germany) (Papers I-V).

Efavirenz was analyzed using HPLC-MS/MS as described previously [57, 80, 81] (Paper V).

Quinine and 3-hydroxyquinine were analyzed using HPLC-fluorescence as described previously [82] (Paper IV).
Genotyping for common variant alleles in CYP3A5, CYP2B6, UGT2B7, ABCB1 and VDR were performed using QIAmp DNA Mini Blood kit (Qiagen, Crawley, UK) and TaqMan genotyping assays (Applied Biosystem, Foster City, USA) [81, 83]. Genotyping for SLCO1B1 was performed using LightCycler 480-based methods as described previously [84].

3.5 STATISTICAL ANALYSES

Statistical calculations were done using Statistica version 7.1 (Paper I), version 8 (Paper III), version 12 (Paper V) (StatSoft Inc., Tulsa, OK, USA), GraphPad Prism version 5.0 (Paper V), version 5.03 (Paper IV) (San Diego, CA, USA), R version 2.15.0 (Paper IV) (R Development Core Team, Vienna, Austria) or SPSS Statistics version 23.0 (Paper V) (IBM Corporation, Somers, NY).

3.6 BRIEF OUTLINE OF PAPERS I-V

3.6.1 Paper I

Paper I describes the effect of treatment with the anti-epileptic drug carbamazepine on the plasma levels of 4β-hydroxycholesterol, 4α-hydroxycholesterol, cholesterol and the 4β-hydroxycholesterol/cholesterol ratio in pediatric patients (n=8). The patients had newly diagnosed epilepsy and were monitored during the first 0-23 weeks of treatment. The treatment at least doubled the plasma levels of 4β-hydroxycholesterol within two weeks treatment.

3.6.2 Paper II

Paper II describes a sample preparation method optimized for the subsequent GC-MS analysis of 4β-hydroxycholesterol in plasma. The optimized method is a refinement of a previously published method that is still widely used [7, 78]. The optimized sample preparation method allowed higher sample throughput and reduced usage of organic solvents.

3.6.3 Paper III

Paper III describes the plasma levels of 4α-hydroxycholesterol, 4β-hydroxycholesterol, cholesterol and the 4α- and 4β-hydroxycholesterol/cholesterol ratios in mothers (n=21) and their children (n=22) at time of delivery/birth and four months later. Mothers had increased CYP3A activity at time of delivery and children had the same CYP3A activity as adults already at time of birth.
3.6.4 Paper IV

Paper IV describes the plasma levels of 25-hydroxyvitamin D, VDR polymorphisms and CYP3A activity (measured as 4β-hydroxycholesterol/cholesterol ratio, quinine metabolic ratio, midazolam clearance and 6β-hydroxycortisol/cortisol ratio) in Koreans (n=67) and Swedes (n=65). A method for analysis of 25-hydroxyvitamin D in plasma was developed and used to determine vitamin D status. All Koreans had deficient or insufficient vitamin D status. Swedish women using oral contraceptives (OCs) had increased plasma levels of 25-hydroxyvitamin D compared to Swedish women not using OCs. The impact of rifampicin treatment on vitamin D status was also investigated (healthy volunteers, n=24).

3.6.5 Paper V

Paper V describes the initiation effect of anti-tuberculosis and anti-retroviral treatment on the CYP3A activity (4β-hydroxycholesterol/cholesterol ratio) and vitamin D status (25-hydroxyvitamin D). Patients with tuberculosis-HIV co-infection (n=102) and HIV-infection (n=94) were monitored during treatment. The influence on the plasma levels of 25-hydroxyvitamin D of common genotypes in CYP3A5, CYP2B6, UGT2B7, ABCB1 and SLCO1B1 were investigated. All patients had insufficient or deficient vitamin D status measured as plasma levels of 25-hydroxyvitamin D.
4 RESULTS AND DISCUSSION

4.1 EFFECT OF CARBAMAZEPINE TREATMENT ON CYP3A ACTIVITY

In paper I the kinetics of formation of plasma 4β-hydroxycholesterol after CYP3A induction with the anti-epileptic drug carbamazepine was monitored. Ten pediatric patients with newly diagnosed epilepsy were enrolled and eight of them completed the study (patients A to H). Blood samples were drawn before and after 1, 2, 4, 8, 16 and 23 weeks of treatment with carbamazepine. The plasma levels of 4β-hydroxycholesterol, 4α-hydroxycholesterol, cholesterol, carbamazepine and carbamazepine-epoxide were monitored. The 4β-hydroxycholesterol/cholesterol ratio was also calculated.

The carbamazepine treatment (ranging between 5-30 mg/kg body weight/day) at least doubled the 4β-hydroxycholesterol/cholesterol ratio within two weeks treatment in all patients (Figure 5). After 7-23 weeks of treatment the increase in the 4β-hydroxycholesterol/cholesterol ratio was 4 to 11-fold in all patients. The plasma levels of 4β-hydroxycholesterol in each patient at least doubled within two weeks of treatment (Figures 6a and 6b, Table 1). As treatment continued, the plasma levels of 4β-hydroxycholesterol further increased and were 5 to 10-fold higher at week 8 compared to the pre-treatment levels.

Table 1: Levels of 4β-hydroxycholesterol (4b-OHC), 4α-hydroxycholesterol (4a-OHC) and cholesterol in children before, after 7-9 weeks and after 15-23 weeks of carbamazepine treatment (mean±sd).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Before (n=8)</th>
<th>7-9 weeks (n=8)</th>
<th>15-23 weeks (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4b-OHC (ng/mL)</td>
<td>43 ± 25</td>
<td>296 ± 104 **</td>
<td>321 ± 125 **</td>
</tr>
<tr>
<td>4a-OHC (ng/mL)</td>
<td>5.4 ± 2.1</td>
<td>7.4 ± 2.0 **</td>
<td>7.0 ± 1.9 a</td>
</tr>
<tr>
<td>cholesterol (mmol/L)</td>
<td>3.7 ± 0.8</td>
<td>4.4 ± 0.5  *</td>
<td>4.2 ± 0.8 a</td>
</tr>
<tr>
<td>carbamazepine-epoxide/</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>carbamazepine</td>
<td>0.14 ± 0.06 b</td>
<td>0.14 ± 0.04 a</td>
<td>0.17 ± 0.08 a</td>
</tr>
</tbody>
</table>

p-values from student’s T-test for dependent samples. Reported by * (p<0.05), ** (p<0.01), a (p>0.05). b = ratio given for 1 week of treatment.
The ratio of the plasma levels of carbamazepine-epoxide/carbamazepine reached steady state within one week of carbamazepine treatment possibly indicating complete induction of CYP3A already within this short time period (Table 1). The continued increase of 4β-hydroxycholesterol levels in circulation may be due to slow equilibriums between different compartments, induction of transporter proteins or of activity of other drug metabolizing enzymes [25]. In vitro experiments with CYP1A2, CYP2C9, CYP2D6 and CYP2B6 failed to show conversion of cholesterol to 4β-hydroxycholesterol [7].

Accumulation of 4β-hydroxycholesterol in circulation due to carbamazepine inhibition of its further metabolism may also be possible. However, the half-life of elimination of 4β-hydroxycholesterol from circulation has been reported to be approximately the same in controls (n=2) as in one carbamazepine treated patient [64]. Only one patient (G) expressed functional CYP3A5 (CYP3A5*1/*3) and therefore no statistical calculations could be done. However, it is noteworthy that patient G reached the highest 4β-hydroxycholesterol/cholesterol ratio (Figure 5), indicating the greatest CYP3A induction.

**Figure 5:** 4β-Hydroxycholesterol/cholesterol ratio (4bOHcholesterol/cholesterol ratio) in patients A-H as a time function of carbamazepine treatment.
To summarize, the plasma levels of $\beta$-hydroxycholesterol and the $\beta$-hydroxycholesterol/cholesterol ratio was monitored and used as markers of CYP3A induction during carbamazepine treatment in children with epilepsy. The carbamazepine treatment increased the plasma levels of $\beta$-hydroxycholesterol 5 to 10-fold and the $\beta$-hydroxycholesterol/cholesterol ratio 4 to 11-fold within eight weeks of treatment. The carbamazepine treatment at least doubled the plasma levels of $\beta$-hydroxycholesterol and the $\beta$-hydroxycholesterol/cholesterol ratio within the first two weeks of treatment.
Figure 6b: Plasma levels of 4β-hydroxycholesterol, carbamazepine and carbamazepine-epoxide and daily dose of carbamazepine at week 0-25 of treatment (patients E-H). Upper panels: 4β-hydroxycholesterol; 4b-OH, filled diamonds, dashed line. Carbamazepine; CBZ, filled triangles, solid line. Carbamazepine-epoxide; CBZ-E, filled squares, solid line. Lower panels: Daily dose (mg/mL) of carbamazepine.

4.2 OPTIMIZED SAMPLE PREPARATION FOR 4β-HYDROXYCHOLESTEROL

In Paper II an optimized sample preparation method for the subsequent GC-MS analysis of 4β-hydroxycholesterol in plasma was developed.

The original method is very labor intensive but still widely used for the simultaneous analysis of a number of oxysterols (7α-hydroxycholesterol, 7β-hydroxycholesterol, 24S-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, 7-ketocholesterol, cholesterol-5,6-epoxide and 5α-cholestane-3β,5α,6β-triol) [78]. These oxysterols have different chemical properties and varied susceptibility towards autoxidation and decomposition. Several compromises were necessary in order to be able to analyze the
oxysterols accurately simultaneously. If there is no particular need to analyze all oxysterols at the same time, the sample preparation procedure can be modified to tailor it for the oxysterol/s of interest. In the case of 4β-hydroxycholesterol, the sample volume, hydrolysis and extraction steps were optimized for that purpose.

In the original method a large sample volume was needed (1 mL) in order to comply with the mass spectrometric detection limit for low abundance oxysterols, i.e. 25-hydroxycholesterol and 7β-hydroxycholesterol (~2 and ~3 ng/mL, respectively). Since 4β-hydroxycholesterol normally is present at levels about 30 ng/mL in plasma, the sample volume was reduced from 1 mL to 250 μL and the hydrolysis solution volume could be reduced from 10 mL to 1 mL.

KOH in ethanol was used for hydrolysis at two different concentrations and three different temperatures; 0.7 M and 0.35 M at room temperature, 37°C and 54°C. With the original method hydrolysis was performed under argon with 0.35 M KOH in ethanol during two hours at room temperature to prevent decomposition of 7-ketocholesterol.

The optimizations of the hydrolysis conditions were done stepwise. First, hydrolysis with 0.35 M KOH in ethanol during 30 minutes, 1 hour or 2 hours was performed at room temperature, 37°C and 54°C. Samples were then processed according to the original method. The changes in hydrolysis conditions did not cause any differences in the 4β-hydroxycholesterol concentrations; neither did hydrolysis with 0.35 M or 0.7 M KOH in ethanol during 30 minutes at room temperature. 0.7 M KOH in ethanol during 30 minutes at room temperature was chosen for hydrolysis, hence reducing the hydrolysis time by 90 minutes while maintaining the efficiency. Shorter hydrolysis times were also tested (5, 10 and 20 minutes) without major changes in the analyzed 4β-hydroxycholesterol concentrations. However, a shorter hydrolysis time may have a negative impact on the method variability (CV) and the hydrolysis time was set to 30 minutes.

In the original method, oxysterols were extracted from the 10 mL hydrolysis solution by liquid-liquid extraction with 18 mL chloroform. This large volume was rotary evaporated, which was time consuming and posed a bottle neck in the sample preparation procedure as the number of samples that could be processed simultaneously was limited to four (the number of available rotary evaporators). The reduced hydrolysis volume in the optimized method allowed for faster extraction as solid phase extraction could be used. Two solid phase extraction columns were investigated: Strata-X and Extrelut (Merck, Darmstadt, Germany). The SI column used in the original method was used as a reference. The percent recovery of \(^{2}\)H\(_{6}\)-4β-hydroxycholesterol with the three columns was highest with the SI column and lowest
with the Extrelut column (15-fold lower). Therefore, the Extrelut column was omitted from further experiments.

A number of different elution conditions were investigated for the Strata-X column: 70, 80, 85 and 90% (v/v) acetonitrile in water. Acetonitrile in water (85%, v/v) showed acceptable recovery of 4β-hydroxycholesterol and $^2$H$_6$-4β-hydroxycholesterol. The Strata-X column was washed with methanol in water (10%, v/v) prior to elution. The differences between the sample preparation procedures are given in Table 2.

Samples previously analyzed with the original method were prepared using the optimized sample preparation procedure and then analyzed by GC-MS. The correlation between 4β-hydroxycholesterol results with the two methods was high ($y=1.0x-2.1$, $r^2 = 0.99$, $y$=new method, $n=90$). The CVs for 4β-hydroxycholesterol were 8.2% ($n=59$) and 7.7% ($n=78$) for the new and the original method, respectively at level 25 ng/mL.

### Table 2: Difference between the original and optimized sample preparation methods.

<table>
<thead>
<tr>
<th></th>
<th>Optimized sample preparation</th>
<th>Original sample preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>250 µL</td>
<td>1 mL</td>
</tr>
<tr>
<td>Hydrolysis</td>
<td>0.7 M KOH (1 mL)</td>
<td>0.35 M KOH (10 mL)</td>
</tr>
<tr>
<td></td>
<td>30 min, no stirring</td>
<td>2 hours, continuous stirring</td>
</tr>
<tr>
<td>Extraction 1</td>
<td>Solid phase extraction (Strata-X)</td>
<td>Liquid-liquid extraction</td>
</tr>
<tr>
<td></td>
<td>To evaporate under argon: acetonitrile in water (85%), 1 mL</td>
<td>Separatory funnel</td>
</tr>
<tr>
<td></td>
<td></td>
<td>To evaporate in rotary evaporator: chloroform, 18 mL</td>
</tr>
<tr>
<td>Extraction 2</td>
<td></td>
<td>Solid phase extraction (SI)</td>
</tr>
<tr>
<td></td>
<td>To evaporate under argon: 2-propanol in hexane (30%), 5 mL</td>
<td></td>
</tr>
</tbody>
</table>
The optimized method has several advantages over the original method when it comes to sample preparation for subsequent GC-MS analysis of 4β-hydroxycholesterol:

- It is faster and less labor intensive. The sample throughput is approximately three times higher compared to the original method.
- The use of disposable glassware makes the new method ideal for analysis of infectious samples.
- The use of less organic solvents is beneficial from an environmental and risk assessment point of view.

### 4.3 CYP3A ACTIVITY IN MOTHERS AND NEWBORN CHILDREN

The initial aim in Paper III was to define the effect of carbamazepine treatment during pregnancy on the CYP3A activity as measured by the plasma levels of 4β-hydroxycholesterol and 4β-hydroxycholesterol/cholesterol ratio in mothers and their newborn children. CYP3A is induced by carbamazepine and the effect from maternal anti-epileptic treatment on fetal hepatic drug metabolism was to be studied. However, recruitment of carbamazepine treated women was more difficult than anticipated and the initial project was cancelled. Instead focus was switched to the control group (healthy women and their newborn children). Mothers were recruited at the delivery ward at Karolinska University Hospital and the plasma levels of 4β-hydroxycholesterol, 4α-hydroxycholesterol, cholesterol and the 4α- and 4β-hydroxycholesterol/cholesterol ratios were monitored in the women (n=21) and their newborns (n=22) at time of delivery/birth and four months later.

In the mothers the CYP3A activity was significantly increased at time of delivery, with higher 4β-hydroxycholesterol/cholesterol ratios compared to the ratios in a cohort of healthy non-pregnant women (Table 3). Four months post partum there were no longer any difference in plasma levels of 4β-hydroxycholesterol, cholesterol or 4β-hydroxycholesterol/cholesterol ratios between mothers and the cohort of non-pregnant women. These results are in line with previously published results indicating that both the CYP3A activity and the plasma levels of cholesterol in circulation are increased during pregnancy [30-32, 85, 86].

The formation of the isomer 4α-hydroxycholesterol is not mediated by CYP3A but may be formed by cholesterol autoxidation, possibly during oxidative stress. Mothers had significantly higher plasma levels of 4α-hydroxycholesterol both at time of delivery and four months post partum compared to the cohort of non-pregnant women.
Table 3: Median plasma levels of 4β-hydroxycholesterol (4b-OHC), 4α-hydroxycholesterol (4a-OHC), cholesterol and ratios (4b-OHC/chol, 4a-OHC/chol) in mothers and non-pregnant healthy women.

<table>
<thead>
<tr>
<th></th>
<th>mothers</th>
<th></th>
<th></th>
<th>women, non-pregnant</th>
<th></th>
<th></th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>median</td>
<td>QR</td>
<td>n</td>
<td>median</td>
<td>QR</td>
<td>n</td>
<td></td>
</tr>
<tr>
<td>4b-OHC (ng/mL)</td>
<td>delivery</td>
<td>50.0</td>
<td>10.7</td>
<td>21</td>
<td>28.9</td>
<td>14.4</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td></td>
<td>4 months</td>
<td>32.6 **</td>
<td>11.9</td>
<td>10</td>
<td>n.s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4a-OHC (ng/mL)</td>
<td>delivery</td>
<td>13.9</td>
<td>3.1</td>
<td>21</td>
<td>6.1</td>
<td>2.05</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td></td>
<td>4 months</td>
<td>10.8 **</td>
<td>3.9</td>
<td>10</td>
<td>n.s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4b-OHC/chol</td>
<td>delivery</td>
<td>0.19</td>
<td>0.06</td>
<td>21</td>
<td>0.15</td>
<td>0.07</td>
<td>0.0009</td>
</tr>
<tr>
<td></td>
<td>4 months</td>
<td>0.15 a</td>
<td>0.09</td>
<td>10</td>
<td>n. s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4a-OHC/chol</td>
<td>delivery</td>
<td>0.06</td>
<td>0.01</td>
<td>21</td>
<td>0.03</td>
<td>0.01</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td></td>
<td>4 months</td>
<td>0.05 *</td>
<td>0.01</td>
<td>10</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cholesterol (mmol/L)</td>
<td>delivery</td>
<td>6.2</td>
<td>1.7</td>
<td>21</td>
<td>4.6</td>
<td>0.9</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td></td>
<td>4 months</td>
<td>5.2 *</td>
<td>2.5</td>
<td>10</td>
<td>n. s.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

QR = quartile range. p-values from Mann Whitney U test. The change in 4b- and 4a-OHC/cholesterol ratios and cholesterol from delivery to four months later were statistically tested by Wilcoxon Matched Pairs. p-values are reported by * (p<0.05), ** (p<0.01) and a (p>0.05). Ratios for 4b-OHC/chol and 4a-OHC/chol are multiplied by 10^4.

Also the 4α-hydroxycholesterol/cholesterol ratio in mothers was higher both at delivery and four months post partum when compared to the ratios in the cohort of non-pregnant women. At time of birth, children born by elective CS had 4β-hydroxycholesterol/cholesterol ratios comparable to the ratios in a cohort of healthy adults (Table 4), indicating that neonates had the same total CYP3A activity as adults. No ratios were calculated for children born by vaginal delivery due to limited sample material. The newborn children had lower plasma levels of 4β-hydroxycholesterol and cholesterol at birth compared to the cohort of healthy adults, but similar levels four months post partum. CYP3A7 is abundant in fetal and neonatal...
liver [47-49] and a postnatal decrease in CYP3A7 and a concomitant increase in CYP3A4 may result in unchanged total CYP3A activity. CYP3A7 catalyzes the conversion of cholesterol to 4β-hydroxycholesterol to a small extent in vitro [64], but it is not known if this conversion takes place also in vivo. Both at time of birth and at four months of age children had higher 4α-hydroxycholesterol/cholesterol ratio than the cohort of adults (Table 4). Children born by vaginal delivery had twice the plasma levels of 4α-hydroxycholesterol at the time of birth compared to children born by elective CS (Table 5).

Table 4: Median plasma levels of 4β-hydroxycholesterol (4b-OHC), 4α-hydroxycholesterol (4a-OHC), cholesterol and ratios (4b-OHC/chol, 4a-OHC/chol) in children and adults.

<table>
<thead>
<tr>
<th></th>
<th>children</th>
<th></th>
<th></th>
<th></th>
<th>adults</th>
<th></th>
<th></th>
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<tr>
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<td>QR</td>
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<td>QR</td>
<td>n</td>
<td>median</td>
<td>QR</td>
<td>n</td>
</tr>
<tr>
<td>4b-OHC (ng/mL)</td>
<td>delivery</td>
<td>18.8</td>
<td>8.6</td>
<td>22</td>
<td>27.2</td>
<td>13.8</td>
<td>125</td>
<td>&lt;0.00001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 months</td>
<td>39.6**</td>
<td>21.2</td>
<td>11</td>
<td>0.03</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>4a-OHC (ng/mL)</td>
<td>delivery</td>
<td>26.2</td>
<td>21.1</td>
<td>22</td>
<td>6.0</td>
<td>2.8</td>
<td>125</td>
<td>&lt;0.00001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 months</td>
<td>15.2a</td>
<td>5.1</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.00001</td>
<td></td>
</tr>
<tr>
<td>4b-OHC/chol</td>
<td>delivery</td>
<td>0.19</td>
<td>0.07</td>
<td>8</td>
<td>0.15</td>
<td>0.07</td>
<td>125</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 months</td>
<td>0.20a</td>
<td>0.11</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td>0.005</td>
<td></td>
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<tr>
<td>4a-OHC/chol</td>
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<td>0.20</td>
<td>0.04</td>
<td>8</td>
<td>0.03</td>
<td>0.01</td>
<td>125</td>
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<tr>
<td></td>
<td>4 months</td>
<td>0.09a</td>
<td>0.02</td>
<td>11</td>
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<tr>
<td>cholesterol</td>
<td>delivery</td>
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<td>0.8</td>
<td>8</td>
<td>4.5</td>
<td>0.9</td>
<td>125</td>
<td>&lt;0.00001</td>
<td></td>
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<tr>
<td>(mmol/L)</td>
<td>4 months</td>
<td>4.2*</td>
<td>0.8</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td>n.s.</td>
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QR = quartile range. p-values from Mann Whitney U test. The change in 4b- and 4a-OHC/cholesterol ratios and cholesterol from delivery to four months later were statistically tested by Wilcoxon Matched Pairs. p-values are reported by * (p<0.05), **(p<0.01) and * (p>0.05). Ratios for 4b-OHC/chol and 4a-OHC/chol are multiplied by 10^4.
Interestingly, all children born by vaginal delivery had higher $4\alpha$-hydroxycholesterol than their mother, while all children born by elective CS had lower $4\alpha$-hydroxycholesterol than their mother (Figure 7). Oxidative stress and other factors during vaginal delivery may explain the increased levels of $4\alpha$-hydroxycholesterol in children born by vaginal delivery compared to children born by elective CS. There are reports on the oxidative effect of vaginal delivery in both mothers and children [87, 88]. The time difference in drawing of samples (at birth and two days later for children born by elective CS and vaginally, respectively) may have an impact on the results. However, rapid changes in the plasma levels of $4\alpha$-hydroxycholesterol was not anticipated based on the characteristics of $4\beta$-hydroxycholesterol. The $4\alpha$-hydroxycholesterol/cholesterol ratio decreased significantly between birth and four months post partum in children born by elective CS. However, the ratio was still three times higher than in the cohort of healthy adults. No ratios were calculated for children born by vaginal delivery due to limited sample material (Table 5).

**Figure 7**: Plasma levels of $4\beta$-hydroxycholesterol (upper panel) and $4\alpha$-hydroxycholesterol (lower panel) in mothers and neonates. Open squares = elective CS. Filled diamonds = vaginal delivery.
Table 5: Median plasma levels of 4β-hydroxycholesterol (4b-OHC), 4α-hydroxycholesterol (4a-OHC) and 4α- and ratios (4b-OHC/chol, 4a-OHC/chol) in mothers and children, divided by mode of delivery (elective CS and vaginal delivery).

<table>
<thead>
<tr>
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<th>Elective CS</th>
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<tr>
<td></td>
<td>median QR n</td>
<td>median QR n</td>
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<tr>
<td><strong>Mothers</strong></td>
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<tr>
<td>4b-OHC (ng/mL)</td>
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<tr>
<td>delivery</td>
<td>53.2 32.5 7</td>
<td>48.0 10.6 14</td>
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</tr>
<tr>
<td>4 months</td>
<td>28.6* 3.7 5</td>
<td>40.5* 7.8 5</td>
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<tr>
<td>4a-OHC (ng/mL)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>delivery</td>
<td>16.8 7.3 7</td>
<td>13.5 2.6 14</td>
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</tr>
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<td>11.5* 3.7 5</td>
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<tr>
<td>delivery</td>
<td>0.19 0.06 7</td>
<td>0.20 0.08 14</td>
<td>n.s.</td>
</tr>
<tr>
<td>4 months</td>
<td>0.12** 0.01</td>
<td>0.21** 0.07 5</td>
<td>n.s.</td>
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<tr>
<td>delivery</td>
<td>0.06 0.01 7</td>
<td>0.05 0.02 14</td>
<td>n.s.</td>
</tr>
<tr>
<td>4 months</td>
<td>0.05** 0.01</td>
<td>0.05** 0.002 5</td>
<td>n.s.</td>
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</tr>
<tr>
<td>birth</td>
<td>12.0 5.4 8</td>
<td>20.2 4.7 14</td>
<td>0.003</td>
</tr>
<tr>
<td>4 months</td>
<td>43.0* 16.0</td>
<td>25.3* 16.0 5</td>
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<tr>
<td>birth</td>
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<td>30.2 9.9 14</td>
<td>0.00001</td>
</tr>
<tr>
<td>4 months</td>
<td>16.4* 6.4 6</td>
<td>15.1* 1.6 5</td>
<td>n.s.</td>
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<td>4b-OHC/chol</td>
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<tr>
<td>birth</td>
<td>0.19 0.07 8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4 months</td>
<td>0.24** 0.10</td>
<td>0.18 0.11 5</td>
<td>n.s.</td>
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<td>4a-OHC/chol</td>
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<tr>
<td>birth</td>
<td>0.20 0.04 8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4 months</td>
<td>0.09* 0.04 6</td>
<td>0.10 0.01 5</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

QR = quartile range. Vaginal delivery: Blood samples were taken at the neonatal screening two-three days after birth. p-values from Mann Whitney U test. The change in concentrations of 4a-OHC and 4b-OHC from birth to four months later were statistically tested by Wilcoxon Matched Pairs. p-values are reported by * (p<0.05), ** (p>0.05). Ratios for 4b-OHC/chol and 4a-OHC/chol are multiplied by 10^4.
Only 3 mothers and 3 neonates expressed functional CYP3A5 (CYP3A5*1/*3) out of the 10 mothers and 11 neonates that were genotyped (scheduled at the four month follow up). No statistical calculations were done due to the size of the data set.

One mother treated with carbamazepine during the entire pregnancy had high plasma level of 4β-hydroxycholesterol two days after an elective CS (392 ng/mL) when compared to untreated mothers (elective CS; 53.2 ng/mL). The 4β-hydroxycholesterol/cholesterol ratio was about nine-fold higher than in untreated mothers (1.8·10^{-4} and 0.19·10^{-4}, respectively). The newborn child had a close to five-fold higher 4β-hydroxycholesterol/cholesterol ratio as compared to children of untreated mothers (0.99·10^{-4} and 0.19·10^{-4}, respectively). The level of 4β-hydroxycholesterol in cord blood plasma of the child was higher compared to the levels in cord blood from children born by elective CS of untreated mothers (76 ng/mL and 12.0 ng/mL, respectively).

To summarize, 4β-hydroxycholesterol and the 4β-hydroxycholesterol/cholesterol ratio was used to evaluate CYP3A activity in mothers and newborn children. Mothers had higher CYP3A activity compared to non-pregnant women. Newborn children had the same CYP3A activity as adults, possibly due to neonatal CYP3A7 activity. Carbamazepine treatment during pregnancy caused increased levels of 4β-hydroxycholesterol in one mother and her child, indicating carbamazepine induction of the CYP3A activity.

4.4 CYP3A ACTIVITY AND VITAMIN D STATUS IN HEALTHY VOLUNTEERS

Paper IV describes the plasma levels of 25-hydroxyvitamin D, VDR polymorphisms and CYP3A activity (4β-hydroxycholesterol/cholesterol ratio, quinine metabolic ratio, midazolam clearance and 6β-hydroxycortisol/cortisol ratio) in Koreans (n=67) and Swedes (n=65). The impact of rifampicin treatment on vitamin D status was also investigated in Swedes (healthy volunteers, n=24). Plasma samples were retrieved from a biobank and analyzed for 25-hydroxyvitamin D. Cholesterol and CYP3A markers had been analyzed previously.

All Koreans (n=67) had poor vitamin D status with deficient or insufficient plasma levels of 25-hydroxyvitamin D. Swedes (n=65) had significantly higher plasma levels of 25-hydroxyvitamin D when compared to Koreans (n=67), 75 and 31 nmol/L, respectively (p<0.001). This is in line with earlier reports on exceptionally low levels of 25-hydroxyvitamin D in Asians [89, 90]. Sub-group analysis based on ethnicity, sex and use of oral contraceptives (OCs) indicated that Swedish women not on OCs (n=21) had higher plasma levels of 25-hydroxyvitamin D when compared to Korean women not on OCs (n=34)
Swedish women on OCs (n=19) had higher plasma levels of 25-hydroxyvitamin D compared to Swedish women not on OCs (n=21) (p=0.02), 89 and 72 nmol/L, respectively. This is in line with previous reports [91, 92]. Only one Korean woman used OCs and no statistical calculations were done. Swedish men (n=25) had higher plasma levels of 25-hydroxyvitamin D compared to Korean men (n=32) (p<0.001). The plasma levels of 25-hydroxyvitamin D in Swedish and Korean men and women are shown in Figure 8.

There were no significant associations between the plasma levels of 25-hydroxyvitamin D and any of the markers of CYP3A activity investigated, i.e. quinine metabolic ratio, 4β-hydroxycholesterol/cholesterol ratio, midazolam clearance and 6β-hydroxycortisol/cortisol ratio (data not shown). Rifampicin induction of CYP3A activity in healthy volunteers (n=24) during two weeks did not influence the plasma levels of 25-hydroxyvitamin D either (data not shown).

The influence of the plasma levels of 25-hydroxyvitamin D and three common VDR polymorphisms (known to influence the CYP3A activity in intestine [93]) on the CYP3A activity was investigated in Swedes (n=53). For one of the VDR polymorphisms, rs4516035, there was a significant association between the plasma levels of 25-hydroxyvitamin D and the CYP3A activity as measured by the quinine metabolic ratio (in a multivariate regression model) (p=0.0044, n=52).

**Figure 8**: Plasma levels of 25-hydroxyvitamin D₃ (25-OH Vit D₃) in Swedes and Koreans. Statistics were done using unpaired t-test. Mean concentrations marked with lines. -OC= without oral contraceptives. +OC= with oral contraceptives.
The increased plasma levels of 25-hydroxyvitamin D were associated with lower quinine metabolic ratio, i.e. increased CYP3A activity. There was also a significant association between the rs4516035 genotype itself and the CYP3A activity (p=0.0014).

There were no significant associations between the plasma levels of 25-hydroxyvitamin D, the two other polymorphisms (rs1544410 and rs11568820) or any of the other markers of CYP3A activity (data not shown). This may be due to the intrinsic differences between the markers and the fact that they partly reflect different things; Midazolam clearance and the quinine metabolic ratio reflect both hepatic and intestinal CYP3A activity while the 4β-hydroxycholesterol/cholesterol ratio reflects the hepatic CYP3A activity. It is not known if it also reflects the intestinal CYP3A activity. The impact of VDR polymorphism is likely to have larger effect on markers measuring the impact in intestines, as the expression of VDR is higher in intestines compared to the liver. The overall influence of 25-hydroxyvitamin D on the CYP3A activity may be of importance to some extent for carriers of certain genetic variants of the VDR.

The samples were analyzed for the plasma levels of 25-hydroxyvitamin D using an LC-MS/MS method and sample preparation procedure developed for this purpose.

The sample preparation was done using a Hybrid SPE precipitation in order to eliminate phospholipids and to precipitate proteins. The use of 2-propanol in acetonitrile (12%, 1% formic acid) increased the recoveries of 25-hydroxyvitamin D$_3$ and 25-hydroxyvitamin D$_2$, respectively as compared to the generic method (acetonitrile, 1% formic acid). The recoveries after Hybrid SPE was 90.6% and 80.1% for 25-hydroxyvitamin D$_3$ and 25-hydroxyvitamin D$_2$, respectively. The plasma levels of 25-hydroxyvitamin D were not affected by multiple freeze-thaw cycles nor long-time storage (1 year) at -20°C (data not shown). 25-hydroxyvitamin D in plasma has been reported to have over 10-year stability [94]. However, 25-hydroxyvitamin D was proven not stable in solution and initially there were difficulties with reproducibility due to this matter, but this was diminished with the introduction of lyophilized calibrator. The lower limit of detection (LOD) was 2.5 nmol/L for both 25-hydroxyvitamin D$_3$ and 25-hydroxyvitamin D$_2$. However, the lower limit of quantification was set at 3x LOD for 25-hydroxyvitamin D$_3$ (7 nmol/L) and 5x LOD 25-hydroxyvitamin D$_2$ (12 nmol/L) due to a noisier baseline for 25-hydroxyvitamin D$_2$. The linear ranges were 2.5-625 nmol/L and 2.5-605 nmol/L for 25-hydroxyvitamin D$_3$ and 25-hydroxyvitamin D$_2$, respectively. The CVs for 25-hydroxyvitamin D$_3$ were 12% and 10% at levels 22.3 and 84.1 nmol/L (n=58 and 60, respectively) (mean ± sd). The CVs for 25-hydroxyvitamin D$_2$ were 15% at levels 26.1 and 44.6 nmol/L (n=58).
To summarize, an analysis and sample preparation method was developed and used to analyze the plasma levels of 25-hydroxyvitamin D in samples from Koreans and Swedes. All Korean men and women had insufficient or deficient vitamin D status as measured by 25-hydroxyvitamin D. In Swedish women, users of oral contraceptives (OCs) had higher plasma levels of 25-hydroxyvitamin D compared to non-users of OCs. There was an association between one of the VDR polymorphisms investigated, suggesting that plasma levels of 25-hydroxyvitamin D may have an impact on the CYP3A activity for carriers of certain genetic variants of VDR.

4.5 CYP3A ACTIVITY, VITAMIN D STATUS AND CHOLESTEROL LEVELS IN PATIENTS WITH TUBERCULOSIS AND/OR HIV

In Paper V the influence of efavirenz-based ART and rifampicin-based anti-tuberculosis treatment on vitamin D status in HIV only (n=94) and in tuberculosis-HIV co-infected patients (n=102) from Ethiopia were investigated. Plasma samples were retrieved from a biobank and analyzed for 25-hydroxyvitamin D. 4β-Hydroxycholesterol, cholesterol and efavirenz had been analyzed previously.

The samples were taken at the initiation of ART (week 0) and at weeks 4, 16 and 48 of treatment. For the tuberculosis-HIV co-infected patients samples were also taken at the initiation of anti-tuberculosis treatment, four or eight weeks prior to initiation of ART (baseline, week -8 or -4) (Figure 4, page 17).

All patients had poor vitamin D status with deficient or insufficient plasma levels of 25-hydroxyvitamin D at all time points investigated. The median plasma levels of 25-hydroxyvitamin D at baseline and at weeks 0, 4, 16 and 48 of treatment as well as percent change in each treatment group is presented in Table 6. The median plasma levels of 25-hydroxyvitamin D were lower in tuberculosis-HIV co-infected patients than HIV only patients at initiation treatment, 23.2 nmol/L and 32.5 nmol/L, respectively (p<0.0001).

Treatment group analysis using repeated measure ANOVA in HIV only patients indicated that ART reduced the plasma levels of 25-hydroxyvitamin D. The levels were lower at weeks 4 and 16 compared with week 0 (both p<0.0001, Bonferroni’s Multiple Comparison Test). At the week 48 follow-up the levels were restored to the pre-treatment levels and there was no longer any significant difference. This may be due to an overall improved health status.

Treatment group analysis using repeated measure ANOVA in the tuberculosis-HIV co-infected patients indicated that initiation of ART decreased the plasma levels 25-hydroxyvitamin D. The levels were lower at weeks 4 and 16 compared to week 0 (p=0.013
and \( p=0.0002 \), respectively). Initiation of anti-tuberculosis treatment at week -8 or -4 had no significant effect on the vitamin D status. The time profile for plasma levels of 25-hydroxyvitamin D during treatment is presented in Figure 9 (page 40).

The lower pre-treatment levels of 25-hydroxyvitamin D in tuberculosis-HIV co-infected patients may be due to an added effect of the two conditions. Studies have shown that patients with HIV or active tuberculosis have lower levels of 25-hydroxyvitamin D when compared to healthy controls and patients with latent tuberculosis, respectively [95, 96]. There are several reports on vitamin D deficiency in HIV patients on non-nucleoside reverse transcriptase inhibitors (NNRTIs), such as efavirenz [95, 97-99]. This may be due to efavirenz-mediated inhibition of CYP2R1 expression [100] and a concomitant efavirenz-mediated induction of CYP24 expression [101]. CYP2R1 is involved in the 25-hydroxylation of vitamin D while CYP24 is involved in the formation of di- and tri-hydroxylated analogs of vitamin D that have potential to further induce CYP24 expression [102] or inhibit CYP2R1 expression (1\(\alpha\),25-dihydroxyvitamin D) [100]. This may lead to reduced formation of 25-hydroxyvitamin D and concomitant increase in 25-hydroxyvitamin D turn-over and reduced levels in circulation. Other types of ART, such as protease inhibitors do not have the same reducing effect on the plasma levels of 25-hydroxyvitamin D [103] and discontinuation of efavirenz treatment has been shown to improve vitamin D status [104]. Studies in mice have shown that rifampicin and rifampicin/isoniazid co-treatment induce CYP2R1 and/or CYP27A1 expression [105]. Both enzymes are hepatic 25-hydroxylases. Rifampicin/isoniazid co-treatment was also shown to inhibit the expression of CYP27B1 (1\(\alpha\)-hydroxylase in kidney) [105]. This would lead to increased plasma levels of 25-hydroxyvitamin D following rifampicin treatment. However, this is not the case in the present study where there is no effect on the plasma levels of 25-hydroxyvitamin D from initiation of rifampicin treatment. After longer treatment, there is even a decrease in the plasma levels of 25-hydroxyvitamin D, possibly due to rifampicin-mediated induction of CYP3A which leads to increased formation of 4-hydroxylated 25-hydroxyvitamin D metabolites (4\(\beta\),25-hydroxyvitamin D) [106]. The initiation of efavirenz treatment further induces CYP3A, which may have an effect on the plasma levels of 25-hydroxyvitamin D.

The median plasma levels of cholesterol at baseline and at weeks 0, 4, 16 and 48 of treatment as well as percent change in each treatment group is presented in Table 7. The median plasma levels of cholesterol were lower in tuberculosis-HIV co-infected patients than in HIV only patients at initiation of the respective treatment, 2.7 and 3.1 mmol/L, respectively (\( p=0.007 \)).
This may be an effect of mycobacterial catabolism of host cholesterol esters and oxysterol esters, mediated by CYP142 [107] and CYP125 [108] to provide carbon for bacterial growth.

While initiation of efavirenz-based ART reduced the mean plasma levels of cholesterol until week 16 (p=0.0001) in the HIV only patients (repeated measure ANOVA), rifampicin anti-tuberculosis treatment initially increased the plasma levels of cholesterol in the tuberculosis-HIV patients. This is possibly due to the combined effect of the discontinuation of mycobacterial use of host cholesterol [107] and rifampicin-PXR inhibition of CYP7A1 transcription, which blocks the bile acid synthesis [109] and causes accumulation of cholesterol.

Initiation of efavirenz-based ART in tuberculosis-HIV patients (week 0) lead to decreased plasma cholesterol levels in these patients. By week 16 there was no difference in the plasma levels of cholesterol between the patient groups (repeated measure ANOVA). The time profile for plasma levels of cholesterol during treatment is presented in Figure 9 (page 40). The efavirenz-mediated decrease in cholesterol levels was transient in the present study and there are several reports on elevated markers of dyslipidemia and lipodystrophy in long-term efavirenz users [110-113].

In tuberculosis-HIV patients there was a significant negative correlation between the plasma level of 25-hydroxyvitamin D and the 4β-hydroxycholesterol/cholesterol ratio already at initiation of rifampicin treatment (baseline, week -4 or -8, p=0.003, r²=0.18). The significance remained until four weeks of anti-tuberculosis/ART co-treatment (week 4, p=0.0007, r²=0.16). No such correlation could be seen in HIV only patients at any time point. The correlation is such that low levels of 25-hydroxyvitamin D are associated with high CYP3A activity, which may be explained by rifampicin-mediated induction of CYP3A activity as the treatment progresses. The correlation already at baseline, before initiation of treatment may be due to mycobacterial effects on the host’s cholesterol pool [107], causing a pseudo-increase in the 4β-hydroxycholesterol/cholesterol ratio.

There was no influence of any of the genotypes investigated on within or between group differences in plasma 25-hydroxyvitamin D levels (data not shown).

To summarize, the plasma levels of 25-hydroxyvitamin D were monitored in tuberculosis-HIV co-infected or HIV-infected patients during treatment with rifampicin and/or efavirenz-based ART. There was no effect of initiation of rifampicin treatment on the plasma levels of 25-hydroxyvitamin D, while initiation of efavirenz treatment caused a transient decrease.
There was a negative correlation between the plasma levels of 25-hydroxyvitamin D and the 4β-hydroxycholesterol/cholesterol ratio already at initiation of rifampicin treatment, which may be due to mycobacterial effects on the host’s cholesterol pool.

**Table 6**: Time effect on median plasma levels of 25-hydroxyvitamin D levels in tuberculosis-HIV and HIV only patients. Median percent change is calculated between baseline and the respective week using Mann Whitney U test. Baseline in the TB-HIV patients was at initiation of rifampicin treatment (week -4 or -8). Baseline in the HIV only patients was at initiation of efavirenz treatment (week 0).

<table>
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<th>Tuberculosis-HIV</th>
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</thead>
<tbody>
<tr>
<td><strong>25-hydroxyvitamin D (nmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline (week -8 or -4)</td>
<td>102 23.2</td>
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<td>&lt;0.0001</td>
</tr>
<tr>
<td>Baseline (week 0)</td>
<td></td>
<td>89 32.5</td>
<td></td>
</tr>
<tr>
<td>Week 0</td>
<td>76 23.9 -1%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 4</td>
<td>102 19.3 -17%</td>
<td>88 20.0 -40%</td>
<td>0.7</td>
</tr>
<tr>
<td>Week 16</td>
<td>94 16.9 -21%</td>
<td>85 15.2 -50%</td>
<td>0.55</td>
</tr>
<tr>
<td>Week 48</td>
<td></td>
<td>40 29.2 -14%</td>
<td></td>
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</tbody>
</table>

**Table 7**: Time effect on median plasma levels of cholesterol in tuberculosis-HIV and HIV only patients. Median percent change is calculated between baseline and the respective week using Mann Whitney U test. Baseline in the TB-HIV patients was at initiation of rifampicin treatment (week -4 or -8). Baseline in the HIV only patients was at initiation of efavirenz treatment (week 0).

<table>
<thead>
<tr>
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<th>Tuberculosis-HIV</th>
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<th>p</th>
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<tr>
<td><strong>Cholesterol (mmol/L)</strong></td>
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<td></td>
</tr>
<tr>
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<td></td>
<td>0.007</td>
</tr>
<tr>
<td>Baseline (week 0)</td>
<td></td>
<td>94 3.1</td>
<td></td>
</tr>
<tr>
<td>Week 0</td>
<td>76 3.4 21%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 4</td>
<td>102 3.1 21%</td>
<td>94 2.2  -33%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Week 16</td>
<td>94 2.3 -11%</td>
<td>93 2.3  -25%</td>
<td>0.91</td>
</tr>
<tr>
<td>Week 48</td>
<td></td>
<td>39 4.7  33%</td>
<td></td>
</tr>
</tbody>
</table>
Figure 9: Time profile of cholesterol (upper panel) and 25-hydroxyvitamin D (lower panel) levels (log) during rifampicin anti-tuberculosis and/or efavirenz-based anti-retroviral treatment.
4.6 GENERAL SUMMARY

In the present study the use of 4β-hydroxycholesterol and the 4β-hydroxycholesterol/cholesterol ratio was evaluated as markers of CYP3A induction by a number of drugs and by pregnancy:

- Carbamazepine treatment in children with epilepsy doubled the plasma levels of 4β-hydroxycholesterol and the 4β-hydroxycholesterol/cholesterol ratio within two weeks of treatment. The increase was 5 to 10-fold and 4 to 11-fold, respectively within eight weeks treatment.
- Pregnancy increased the 4β-hydroxycholesterol/cholesterol ratio and the plasma levels of cholesterol in women. Newborn children had the same CYP3A activity as adults as indicated by similar 4β-hydroxycholesterol/cholesterol ratio.
- Rifampicin mediated CYP3A induction did not affect the plasma levels of 25-hydroxyvitamin D in healthy volunteers or in tuberculosis-HIV co-infected patients. However, in tuberculosis-HIV co-infected patients there was a significant negative correlation between the plasma levels of 25-hydroxyvitamin D and the 4β-hydroxycholesterol/cholesterol ratio already at initiation of treatment.
- Efavirenz treatment caused a transient decrease in the plasma levels of 25-hydroxyvitamin D in HIV infected patients.

To summarize, 4β-hydroxycholesterol and the 4β-hydroxycholesterol/cholesterol ratio appear to be useful as markers of CYP3A induction. 4β-Hydroxycholesterol is a non-invasive marker that is easy to use in children and vulnerable patient groups where urine collections are difficult to perform or probe drugs are unethical to administer. Blood samples can be taken at any time of the day regardless of food intake.
5 FUTURE PERSPECTIVES

Several studies have been done on the usefulness of $4\beta$-hydroxycholesterol and the $4\beta$-hydroxycholesterol/cholesterol ratio as markers of CYP3A activity. Some of the studies that remain are the following:

- To investigate the possible 4-hydroxylase activity of CYP3A7 and CYP3A43.
- To investigate the effect on the CYP3A activity of various drug treatments during pregnancy.

Further characterization of the properties of $4\alpha$-hydroxycholesterol:

- To investigate the formation and tissue distribution of $4\alpha$-hydroxycholesterol.
  - In newborns during the first days of life.
  - In healthy volunteers.

Further studies to elucidate the possible association between vitamin D status, CYP activity and immune response in CYP3A enzymes and other CYP enzymes.
6 ACKNOWLEDGEMENTS

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