4β-Hydroxycholesterol as biomarker for variation in CYP3A activity

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LIST OF PUBLICATIONS

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ABBREVIATIONS

4βOHC	4β-hydroxycholesterol
4βOHC/C	4β-hydroxycholesterol/cholesterol
AUC	Area under the concentration versus time curve
BMI	Body mass index
CYP	Cytochrome P450
EIAED	Enzyme-inducing antiepileptic drug
GFR	Glomerular filtration rate
IL	Interleukin
P-gp	P-glycoprotein
POR	Cytochrome P450 oxidoreductase
TDM	Therapeutic drug monitoring

ABSTRACT

Individual differences in drug metabolism can cause extensive variability in drug exposure and efficacy. Better characterization of the different factors influencing drug metabolism can contribute to individualizing and optimizing drug therapy. The overall objective of this thesis was to investigate the suitability of 4β-hydroxycholesterol (4βOHC) as biomarker for the variability in the activity of CYP3A-mediated drug metabolism.

In a large naturalistic study of psychiatric patients, we reported 120-fold variation of 4βOHC levels. Impact of enzyme-inducing drugs was the factor with greatest quantitative effect on 4βOHC levels, and the findings support the suitability of 4βOHC as a biomarker for hepatic CYP3A induction. 4βOHC was also responsive to long-term treatment with moderate CYP3A inhibitors, but its suitability as a biomarker for CYP3A inhibition is yet to be established.

Genetic polymorphism is known to be important for CYP3A5 activity, and to contribute to the total CYP3A activity. The contribution of CYP3A5 to *in vivo* formation of 4βOHC was, however, inconclusive in the present studies. Impact of other genetic polymorphisms on 4βOHC levels was limited.

End-stage renal disease is associated with reduced 4βOHC levels, and recovery of renal function after kidney transplantation was associated with recovery of CYP3A activity. The clinical impact of this is yet to be established, but might indicate higher dose requirements of CYP3A-metabolized drugs after vs. before kidney transplantation.

Body weight and body mass index (BMI) impacted CYP3A activity both in kidney transplant and anorexic patients, with increasing level of 4βOHC levels with decreasing body weight and BMI. This suggests that clearance of CYP3A substrates increases with decreasing body weight/BMI.

The present thesis supports the suitability of 4βOHC as a hepatic CYP3A biomarker since the factors which in these studies describe variation in 4βOHC levels coincide with previous findings on variation in CYP3A phenotype. Further studies should investigate 4βOHC as a potential biomarker for personalized dosing of CYP3A substrates.

1 INTRODUCTION

1.1 Variability in drug response

A major problem during drug therapy is variability in drug response. Standard doses of a given drug can lead to the desired effect in some patients, while others experience subtherapeutic response or side effects. Personalized dosing can therefore improve the outcome of drug treatment by reducing the occurrence of adverse effects and lack of effect. However, personalized dosing is not a straightforward procedure. The effect a drug dose will produce in a given patient is influenced by pharmacokinetic and pharmacodynamic processes, which describe the interaction between the body and the drug. Pharmacodynamics is the study of the effects of drugs on the body, and variability can be caused by e.g. genetic differences in target receptors, drug-drug interactions at the target site or disease status. Pharmacokinetic variability is one of the main sources for variation in drug response. Pharmacokinetic variability comprises variation in drug exposure due to differences in e.g. absorption, distribution, metabolism and excretion of a given drug. These processes influence drug concentration at the target site, and therefore lead to variations in drug response. Several factors are known to impact pharmacokinetic processes, such as age, sex, body weight, disease status, kidney function, genetics and drug-drug interactions. Better characterization of the different factors influencing pharmacokinetic variability can contribute to individualizing and optimizing drug therapy.

1.2 Drug metabolism

Drug metabolism is an important pharmacokinetic process. To facilitate transfer across human cell membranes, most drugs are lipophilic compounds. However, drugs are often converted *in vivo* to more water-soluble compounds before hepatic or renal excretion. This conversion can be divided into phase I and phase II metabolism. In phase I metabolism, drugs undergo simple modifications such as oxidation, reduction or hydrolysis. In phase II metabolism, drugs are conjugated to hydrophilic molecules such as glucuronic acid or glutathione. Several enzymes catalyze phase I and phase II metabolism. Interindividual differences in protein expression and activity of metabolizing enzymes contribute substantially to pharmacokinetic variability.

1.2.1 CYP enzymes

Cytochrome P450 (CYP) is the superfamily of enzymes which is considered the most important group of phase I metabolizing enzymes. CYP enzymes contain the iron-incorporated cofactor heme, which enables them to carry out electron transfer reactions. Human CYP enzymes are for the most part membrane-bound proteins located intracellularly in the endoplasmic reticulum, the protein production center of the cells; and the mitochondria, the energy center of the cells. The majority of drug metabolizing CYP enzymes are found in the liver and small intestine. Intestinal CYP is located in epithelial cells along the mucosal lining, and contribute to metabolism before the drug reaches systemic blood flow: first-pass metabolism (Figure 1).[1] After the drug is absorbed from the intestines, it passes through the portal vein to the liver, and then into the systemic blood flow. The liver is therefore involved both in first-pass metabolism and systemic metabolism of orally administered drugs. Although CYP enzymes are found in other organs – such as the kidneys, the lungs and the brain – the contribution to drug metabolism from these tissues is considered low, and is usually disregarded.

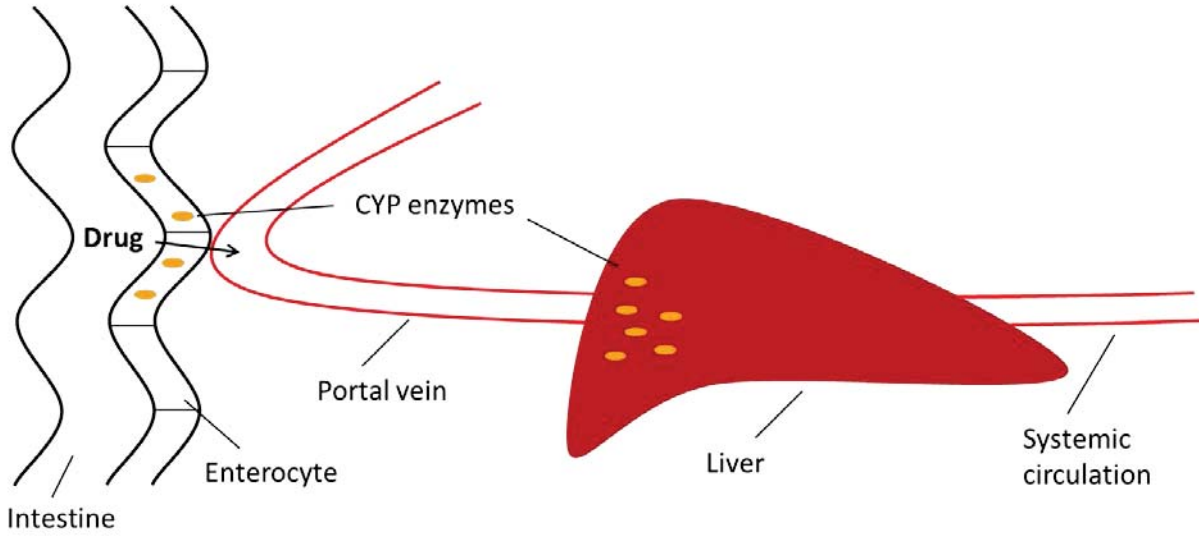

Figure 1. Illustration of the presystemic drug metabolism following oral dosing, mediated by intestinal and hepatic CYP enzymes.

CYP enzymes metabolize a large variety of substrates: both endogenous compounds, such as steroids and fatty acids; and exogenous chemicals, such as drugs and herbal compounds. The human genome has 57 functional CYP genes.[2] The nomenclature of CYPs organizes them into families designated by numbers (CYP2, CYP3), subfamilies designated by capital letters (CYP2D, CYP3A), and isoenzymes designated by numbers (CYP2D6, CYP3A4). The gene

encoding the enzyme is referred to in italics (*CYP3A4*), while the protein is referred to in non-italics (CYP3A4). CYP enzymes within the same family and subfamily usually have >40% and >55% amino acid sequence homology, respectively.[3, 4] The enzymes belonging to the 1, 2 and 3 CYP families are the most important in drug metabolism. Three-fourths of the human drug metabolizing CYP reactions can be accounted for by five isoenzymes: CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4.[5]

1.3 CYP3A metabolism

1.3.1 CYP3A enzymes

The CYP3A subfamily metabolizes the largest fraction of CYP reactions in humans, and consists of four different isoenzymes: CYP3A4, CYP3A5, CYP3A7 and CYP3A43.[2, 5] CYP3A enzymes account for ~80% of total CYP content in the intestines, and ~30% of total CYP content in the liver.[6, 7]

In most human livers, CYP3A4 is the major contributor to CYP3A-mediated metabolism. Due to a large and flexible active site,[8] CYP3A4 can metabolize a large range of substrates, both small molecules and larger compounds. A recent review accounted that CYP3A4 participate in the metabolism of 27% of all drugs: e.g. immunosuppressants, benzodiazepines and statins.[5]

CYP3A5 has 84% amino acid sequence homology with CYP3A4,[9] and the substrate specificity of the enzymes overlaps to a large degree. However, some substrates exhibit different enzyme preferences.[10] Many individuals do not express functional CYP3A5 enzymes, but for those who do, the isoenzyme contributes on average 50% and 27% of total CYP3A protein content in liver and intestines, respectively.[6, 11]

The remaining CYP3A isoenzymes – CYP3A7 and CYP3A43 – are considered to be less important for the overall CYP3A activity. CYP3A7 is primarily expressed in fetal livers, but is also found in adult liver and intestines.[12] However, only 10% of adult livers contain quantifiable amounts of CYP3A7 protein.[13] CYP3A7 substrate specificity overlaps with the other CYP3A enzymes – as it expresses 88% amino acid sequence homology with CYP3A4 – but the clinical significance of CYP3A7 in adults has not been well studied.[14] CYP3A43 is mainly expressed in the prostate.[15, 16] The CYP3A43 isoenzyme displays 76% homology with CYP3A4,[17] but the expression of CYP3A43 is only 0.2% of CYP3A4 expression in an

average human liver.[15] The contribution of CYP3A43 to the overall CYP3A metabolism is therefore considered negligible.

1.3.2 CYP3A variability

Substantial interindividual variability in CYP3A expression and activity has been reported. The distribution of CYP3A variability is unimodal, with no clear distinction between poor and rapid metabolizers. A combination of environmental and physiological factors causes the variability.

Exogenous factors

A number of compounds interact with CYP enzymes, causing substantial variation in enzyme activity by induction or inhibition. Up to 400-fold difference in *in vivo* CYP3A activity has been reported in subjects switching from treatment with a CYP3A inhibitor to an inducer.[18]

CYP induction involves upregulation of enzyme synthesis, and hence increased protein number. When starting treatment with an enzyme inducer, it takes time for the induction to become apparent and for enzyme levels to stabilize at a new steady state. When ceasing treatment with an enzyme inducer, it also takes time for the enzymes to degrade and enzyme levels to normalize, as the half-life of hepatic CYP3A4 enzymes is expected to be 3-6 days.[19] Potent inducers of CYP3A include the antiepileptics carbamazepine and phenytoin; the antibiotic rifampicin; and the HIV antivirals efavirenz and nevirapine.[20]

Enzyme inhibition can occur by two main mechanisms: reversible inhibition and mechanism-based inhibition. Reversible inhibition is divided into competitive, noncompetitive and uncompetitive inhibition. It involves rapid association and dissociation between substrates and enzymes, and is concentration dependent. Mechanism-based inhibition can be irreversible or quasi-irreversible, and involves inactivation of the enzyme. This generates longer-lasting effects than reversible inhibition, since enzyme activity needs to be restored by protein synthesis. Strong CYP3A inhibitors include the antibiotics erythromycin and clarithromycin; the antifungals itraconazole and ketoconazole; and the HIV antivirals indinavir, nelfinavir and ritonavir.[20]

Genetic polymorphism

Genetic polymorphism is the occurrence of common DNA sequence variations. For CYP2D6 and CYP2C19, the impact of genetic polymorphism on enzyme activity is well established. Depending on the genotype, enzyme function is described as poor, intermediate, extensive or ultra-rapid. Early twin studies indicated strong heritability also for CYP3A4 metabolism,[21] but the reported impact of discovered genetic polymorphisms is limited. Multiple *CYP3A4* polymorphisms have been found, but few predict clinically relevant alterations in enzyme activity. Only one report exists of a patient completely lacking CYP3A4 activity, and homozygous expression of a premature stop codon was found in the patient.[22] The *CYP3A4*22* (rs35599367) allele was identified in 2011, and has been linked to reduced CYP3A4 mRNA expression, enzyme activity and protein levels in human livers.[23, 24] The allele frequency is reported to be 4–9% among Europeans.[25]

Variability in CYP3A5 activity is largely explained by genetic polymorphism. The most studied gene variant for *CYP3A5* is the *CYP3A5*3* (rs776746) allele, which alters mRNA splicing and introduces a premature stop codon. This produces a truncated and nonfunctional enzyme. Functional CYP3A5 enzyme is displayed in subjects who have at least one *CYP3A5*1* allele. The allele frequency of *CYP3A5*3* displays major interethnic differences, and is approximately 95% in Europeans, 70% in Asians and 20% in Africans.[25] It has been estimated that in carriers of *CYP3A5*1*, up to 50% of the hepatic CYP3A content is CYP3A5.[26]

CYP3A activity may be influenced by genetic polymorphisms outside the *CYP3A* loci. CYP oxidoreductase (POR) is responsible for electron transfer to CYP enzymes. The *POR*28* (rs1057868) allele is a common polymorphism characterized by an amino acid substitution in the electron binding domain, and the allele frequency is 30% in European populations.[25] The *POR*28* allele has been associated with both increased and decreased drug metabolism, depending on the CYP enzyme and substrate investigated.[27-30]

Non-genetic endogenous factors

Several intrinsic factors influence CYP3A variability. At birth, CYP3A7 is the predominant CYP3A enzyme in humans. This shifts during the first years of life, when CYP3A4 and CYP3A5 activity reaches adult levels while CYP3A7 decreases.[31, 32] At the other end of life, the elderly are reported to have decreased drug clearance. However, this is not necessarily

because of reduced enzyme capacity, but rather due to factors such as impaired renal function and decreased liver blood flow. The reported effect of age on CYP3A metabolism in adults is inconclusive, although an increase in CYP3A activity with advancing age has been reported.[33]

Sex may alter CYP3A activity, with reported higher enzyme activity and expression in females compared to males.[34] The mechanism behind this is not elucidated, but may involve differences in growth hormone.[35] Obesity has also been linked to alterations in drug metabolism, with reported lower CYP3A4 protein expression and activity with increasing body weight or body mass index (BMI).[36, 37]

Disease states can impact drug metabolism, not only via direct impact on renal or hepatic function, but via inflammation status. Cytokines are produced and released as part of the immunological response to various conditions like autoimmune diseases, infections and cancer. Cytokines regulate the expression and activity of drug metabolizing enzymes, and may therefore alter drug pharmacokinetics. E.g., CYP3A4 activity is reduced in response to interleukin(IL)-1β, IL-2, IL-4, IL-6 and tumor necrosis factor-α.[38, 39] Furthermore, decreased CYP3A activity is associated with several disease states, such as end-stage renal disease and obesity.[37, 40]

1.4 CYP3A biomarkers

Quantifying the CYP3A activity of an individual is useful in several settings. During the development of new drugs, it is necessary to determine whether the drugs have potential for drug-drug interactions. Measurement of *in vivo* CYP3A activity before and after dosing with a new drug can indicate whether the drug is an inducer or inhibitor of CYP3A. In a clinical setting, quantifying the CYP3A activity of a patient could help predict the best dosing strategy, and hence improve therapeutic efficacy. This dosing strategy is used for several renally eliminated drugs, where measurement of the glomerular filtration rate (GFR) contributes to individualized dosing.[41] However, GFR estimates elimination via one organ, while CYP3A expression is extensive in two organs; liver and intestines. An ideal CYP3A biomarker would therefore reflect the combined presystemic and systemic elimination via CYP3A enzymes.

Measurement of enzyme activity is referred to as phenotyping. There are several probe drugs available to measure CYP3A phenotype. They vary in specificity for CYP3A enzymes, reflection of hepatic and intestinal metabolism, and validity. The short-acting benzodiazepine

midazolam is the most used and acknowledged CYP3A phenotype marker. It is almost exclusively converted by CYP3A isoenzymes to the main metabolite α-OH-midazolam. The best measure of individual CYP3A activity is to measure midazolam concentration at multiple time points and calculate the area under the concentration-time curve (AUC). However, this is invasive and time-consuming, and newer methods measuring a single time point midazolam concentration as a predictor of the AUC are prevailing.[42]

The erythromycin breath test has been popular due to a fast and noninvasive sampling procedure. Radiolabeled erythromycin is injected in the subject, and after a given amount of time a breath sample is collected. Radioactive carbon dioxide in the breath sample reflects erythromycin demethylation, a measure of CYP3A phenotype. A major limitation of this probe is that P-glycoprotein (P-gp) contributes to the exposure of erythromycin, therefore the breath test does not exclusively reflect CYP3A activity.[43]

CYP3A metabolizes endogenous cortisol to 6β-hydroxycortisol, and the ratio between the metabolite and mother compound measured in urine can be used as a phenotype marker. Another endogenous biomarker is the cholesterol metabolite 4β-hydroxycholesterol (4βOHC). Endogenous CYP3A biomarkers have the advantage of not needing probe drug administration, and for 4βOHC, a single blood sample can predict individual CYP3A phenotype.

1.4.1 4β-hydroxycholesterol

Formation of 4β-hydroxycholesterol

Cholesterol oxidation products (oxysterols) can be formed either by autoxidation, by cholesterol-metabolizing enzymes, or by a combination of these two mechanisms. Compared to other oxysterols, 4βOHC is present in the human circulation in relatively high concentrations (nanomolar range). It was first suggested as a CYP3A biomarker by Bodin *et al.* in 2001.[44] Very little was formerly known of the formation and metabolism of 4βOHC, except that its formation by cholesterol autoxidation is minimal, unlike its stereoisomer 4αOHC.[45] After finding highly elevated plasma 4βOHC levels in patients treated with strong CYP3A inducers, Bodin *et al.* conducted *in vitro* studies showing that recombinant CYP3A4 converted cholesterol to 4βOHC (Figure 2).[44] Recombinant CYP1A2, CYP2B6 or CYP2C9 did not convert cholesterol to 4βOHC, nor did control microsomes without CYP enzymes.[44] Further *in vitro* studies showed that CYP3A5 and CYP3A7 also converted cholesterol to

4βOHC, but with a relative conversion rate which was only 6% and 3% of CYP3A4, respectively.[46] Recently, Nitta *et al.* confirmed *in vitro* formation of 4βOHC from cholesterol by CYP3A4 and CYP3A5. Furthermore, only negligible 4βOHC formation was reported by seven other CYP isoforms: CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP2E1.[47] This strengthens the position of 4βOHC as a distinctive CYP3A phenotype marker. Nitta *et al.* also reported strong correlation between hepatic CYP3A protein levels and hepatic and plasma 4βOHC in a humanized mouse model ($r \geq 0.6$), indicating that plasma 4βOHC is an appropriate biomarker for hepatic CYP3A.[47] Gjestad *et al.* suggested that intestinal CYP3A is involved in the formation of 4βOHC, since carbamazepine daily dose but not concentration correlated well with serum 4βOHC.[48] It is not yet clear, however, to which degree 4βOHC is produced by intestinal CYP3A enzymes. Arterial blood flow to the mucosal villi where CYP3A is expressed is not substantial, therefore it is not expected that intestinal CYP3A contributes a great deal to 4βOHC formation.[49]

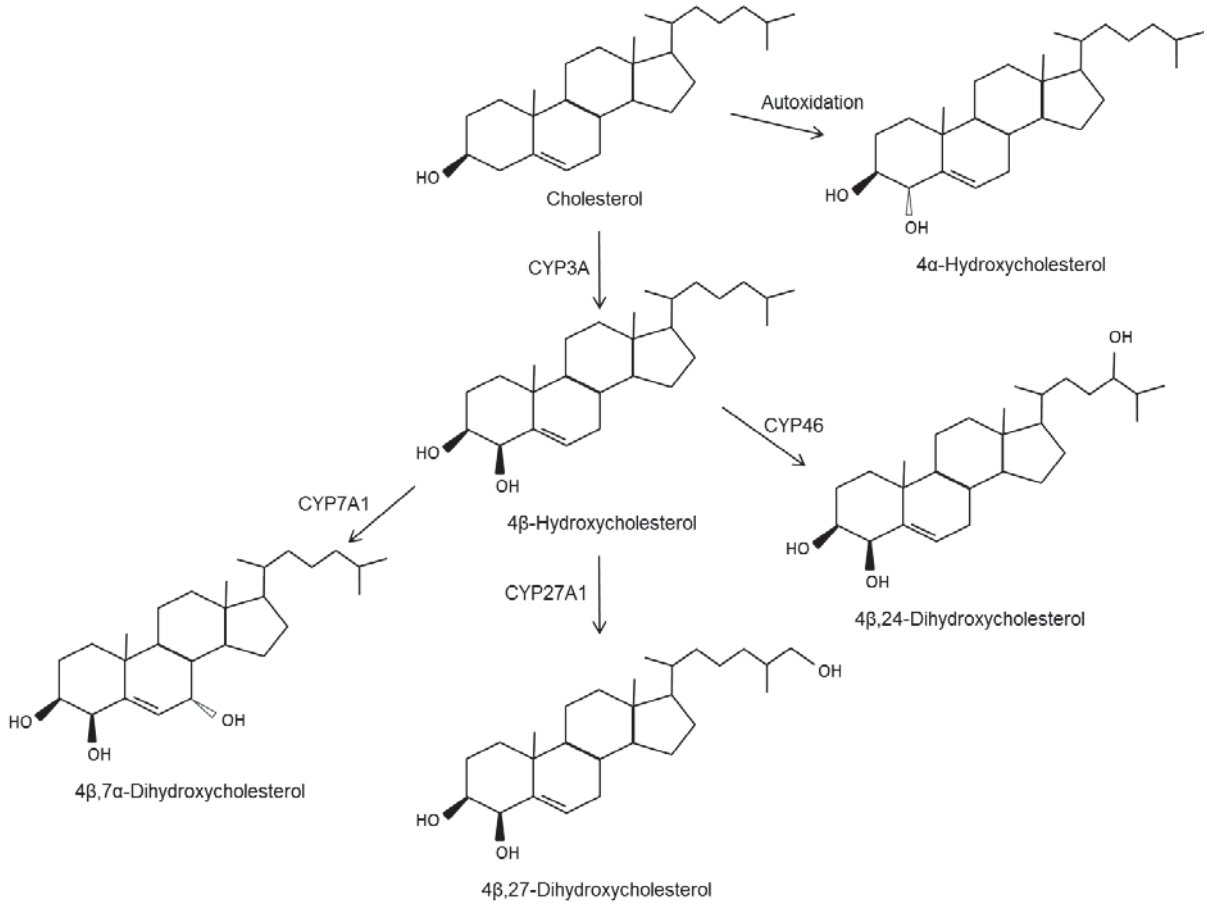

Figure 2. Formation and metabolism of 4β-hydroxycholesterol

Metabolism of 4β-hydroxycholesterol

In general, oxysterols are degraded to bile acids, with 7α-hydroxylation as the rate-limiting step. Incubation of 4βOHC with human recombinant CYP enzymes showed that CYP7A1 7α-hydroxylated 4βOHC and CYP27A1 27α-hydroxylated 4βOHC (Figure 2). The turnover rates were 0.087 and 0.41 nmol/min × nmol CYP for CYP7A1 and CYP27A1, respectively.[46] Both enzymes are important in bile acid synthesis. CYP46 also contributed to the metabolism of 4βOHC, but since CYP46 only is expressed in the brain, it is likely negligible in the systemic elimination of 4βOHC.[46]

Half-life of 4β-hydroxycholesterol

Only a few small studies have investigated the half-life of 4βOHC, which seems to be unusually long. In one study, deuterated 4βOHC was administered intravenously in two healthy volunteers, and changes in plasma concentration of deuterated 4βOHC were monitored. The apparent half-life of 4βOHC was then determined to be 60–64 h, as calculated from the elimination phase.[46] However, this study does not account for the distribution that would have taken place after administering exogenous 4βOHC, and the half-life is likely underestimated. A population pharmacokinetic model including data from 21 subjects have estimated a half-life of 5 days.[50] The model was, however, based on 4βOHC levels during CYP3A induction, and may also have underestimated the half-life. Diczfalusy *et al.* performed a new half-life study, where they measured 4βOHC 4 and 8 weeks after termination of rifampicin treatment; a CYP3A4 inducer which elevates 4βOHC levels. The half-life was estimated to be approximately 17 days, and was based on data from two subjects.[51] This is the estimation which is most accepted in the literature, and is supported by a pharmacokinetic model by Yang and Rodrigues.[52] Neuhoff and Tucker, however, suggest that since rifampicin may decrease CYP7A1 levels, 4βOHC half-life may have been over-estimated in this study.[53, 54]

Distribution of 4β-hydroxycholesterol

Some 4βOHC is present in plasma in free form, but about 85% of plasma 4βOHC is esterified to long-chain fatty acids.[44] The distribution of 4βOHC in plasma lipoproteins parallels the cholesterol distribution, with 71% in low density lipoproteins, 27% in high density lipoproteins and 2% in very low density lipoproteins. Autopsy material has shown that

4βOHC is present in multiple tissues; such as liver, kidneys, duodenum and adipose tissue, and high levels were found in brain tissue.[44]

2 AIM OF THE THESIS

The overall objective of the thesis was to investigate the suitability of the biomarker 4β-hydroxycholesterol to describe variations in CYP3A activity.

Specific aims were to investigate the following points:

- 4βOHC as biomarker for CYP3A induction and inhibition (paper I & III)
- the impact of relevant polymorphisms on 4βOHC levels (paper I & II)
- the impact of end-stage renal disease and renal transplantation on 4βOHC levels (paper II)
- the association between body weight/BMI and 4βOHC levels (paper II & IV)

3 SUMMARY OF RESULTS

Paper I

Impact of genetic and nongenetic factors on interindividual variability in 4β-hydroxycholesterol concentration

The aim of this study was to evaluate the explanatory power of candidate genetic variants and key nongenetic factors on interindividual variability in 4βOHC levels in a large naturalistic patient population. We measured 4βOHC concentration in serum samples from 655 patients, and used multiple linear regression analysis to estimate the quantitative effects of *CYP3A4*22*, *CYP3A5*3* and *POR*28* variant alleles; comedication with CYP3A inducers, inhibitors and substrates; sex, and age on individual 4βOHC levels. 4βOHC concentration ranged 120-fold in the population and the multiple linear regression model explained about one fourth of the variability (r^2 = 0.23). Only comedication with inducers or inhibitors, sex and *POR* genotype were significantly associated with individual variability in 4βOHC level. The estimated quantitative effects on 4βOHC levels were greatest for inducer comedication (+>313%, $p < 0.001$), inhibitor comedication (–34%, $p = 0.021$), and female sex (+30%, $p < 0.001$), while only a modestly elevated 4βOHC level was observed in carriers vs. non-carriers of *POR*28* (+11%, $p = 0.023$). The current findings suggest that the *CYP3A4*22*, *CYP3A5*3* and *POR*28* variant alleles are of limited importance for overall individual variability in 4βOHC levels compared to nongenetic factors.

Paper II

Recovery of CYP3A phenotype after kidney transplantation

The aim of this study was to evaluate the change in CYP3A activity after kidney transplantation using 4βOHC as biomarker. In total, data from 58 renal transplant recipients with 550 prospective 4βOHC measurements were included in the study. One sample per patient was collected before transplantation, and 2-12 samples per patient were collected 1-82 days after transplantation. The measured pre-transplant 4βOHC concentrations ranged 7-fold with a median value of 22.8 ng/mL. Linear mixed model analysis identified a 0.16 ng/mL increase in 4βOHC concentration per day after transplantation ($p<0.001$), indicating a regain in CYP3A activity. Increasing estimated glomerular filtration rate after transplantation was associated with increasing 4βOHC concentration ($p<0.001$), supporting that CYP3A activity increases with recovering uremia. In conclusion, this study indicates that CYP3A activity is regained subsequent to kidney transplantation.

Paper III

Comparison of CYP3A4-inducing capacity of enzyme-inducing antiepileptic drugs using 4β-hydroxycholesterol as biomarker

The aim of this study was to estimate CYP3A4-inductive potency of enzyme-inducing antiepileptic drugs (EIAEDs) by comparing CYP3A4 activity in patients treated with carbamazepine, phenobarbital, or phenytoin, or two of the inducers simultaneously. Residual serum samples from patients treated with EIAEDs or levetiracetam were collected from a therapeutic drug monitoring service for analysis of 4βOHC. Concentration of 4βOHC, EIAEDs and levetiracetam was measured by ultra-performance liquid chromatography tandem mass spectrometry. Kruskal-Wallis and Mann-Whitney tests were used for comparison of 4βOHC levels between the subgroups. In total, 4βOHC measurements for 343 and 339 patients treated with EIAEDs and levetiracetam, respectively, were included in the study. Compared to levetiracetam-treated patients, the median 4βOHC concentration was 3-fold, 6-fold, 7-fold, and 9-fold higher in patients using phenobarbital, phenytoin, carbamazepine, or two inducers simultaneously, respectively ($p<0.0001$). Phenytoin users (n=65) and carbamazepine users (n=225) had 2-fold higher median 4βOHC concentration than phenobarbital users (n=28), respectively ($p\leq0.0001$). This study indicates that phenytoin and carbamazepine have approximately twice the CYP3A4-inducing potency of phenobarbital. The clinical implications may be that dose requirements of CYP3A4-metabolized drugs are generally higher during concurrent treatment with phenytoin or carbamazepine compared with phenobarbital.

Paper IV

Elevated 4β-hydroxycholesterol/cholesterol ratio in anorexia nervosa patients

The aim of this study was to examine potential differences in CYP3A activity between underweight patients with anorexia nervosa and normal-weight volunteers by measuring plasma 4βOHC/C ratio. Furthermore, we wished to describe any association between BMI and 4βOHC/C ratio in underweight patients. A total of 20 underweight patients and 16 normal-weight volunteers were included in the study, all females. Underweight patients had a median 4*β*OHC/C ratio (molar ratio × 10^{-5}) of 2.52 (range, 0.90–11.3) compared to 1.29 (0.56–2.09) in normal-weight subjects (Mann-Whitney $P = 0.0005$). 4βOHC/C ratio was negatively correlated with BMI in underweight patients ($r = –0.56$, $P = 0.011$), and in the whole study population ($r = –0.67$, $P < 0.0001$). This suggests that the negative correlation between 4βOHC/C and BMI, which has previously been reported between 4βOHC/C and body weight in healthy volunteers, extends to underweight patients. The findings indicate that CYP3A activity increases with decreasing BMI, and that underweight patients have higher CYP3A activity than normal-weight subjects. The potential clinical relevance needs to be studied further by comparing pharmacokinetics of drugs subjected to CYP3A-mediated metabolism in underweight vs. normal-weight individuals.

4 DISCUSSION

4.1 4β-hydroxycholesterol as biomarker for CYP3A variability

In this thesis, we have used 4βOHC and 4βOHC/C ratio as biomarkers for *in vivo* CYP3A activity. CYP3A activity varies greatly between individuals, and 4βOHC is an emerging endogenous biomarker to describe these variations.

4.1.1 CYP3A induction and inhibition

In paper I, we found 120-fold variability of 4βOHC concentration in a naturalistic patient population including users of both CYP3A inhibitors and inducers. Induction and inhibition of CYP3A enzymes are known to have substantial impact on phenotype, with up to 400-fold difference in oral midazolam AUC reported in patients switching from an inhibitor to an inducer.[18] The 120-fold range of variability we reported in 4βOHC levels was smaller than for oral midazolam exposure, and coincides with previous reports that the magnitude of induction measured by oral midazolam exposure is greater than for 4βOHC. After 10-15 days of 600 mg rifampicin treatment, oral midazolam clearance increased 13-fold, while 4βOHC levels and intravenous midazolam clearance increased 2- to 3-fold.[55-57] This suggests that 4βOHC dynamics are comparable to intravenous midazolam clearance when quantifying potency of CYP3A inducers. However, these studies did not last not long enough for 4βOHC levels to reach steady state, and the dynamic impact of induction on 4βOHC is therefore likely to be slightly larger than intravenous midazolam clearance when studies are designed to account for the long half-life of 4βOHC. A study with population pharmacokinetic modelling concluded that after exposure to a CYP3A inducer, the maximum 4βOHC level was achieved after >43 days. The model indicated that 4βOHC levels increased rapidly early after start-up with a CYP3A inducer, to slow down and reach steady-state at a later stage.[50] The slow plateauing of 4βOHC levels after CYP3A induction is supported by another study, where 4βOHC levels of pediatric patients continued to increase until at least 8 weeks after initiation with carbamazepine treatment.[58] However, an increase in 4βOHC is detectable already 3 days after initiation with inducer treatment,[55] and 4βOHC levels are doubled after a week of induction therapy.[58, 59] These reports indicate that 4βOHC rapidly responds to increase in enzyme activity, although it may take weeks to reach steady-state, where one can estimate the relative change in enzyme activity. Therefore, it is not expected that longer-lasting induction studies than described above would reveal dramatically larger increase in 4βOHC levels. While oral

midazolam clearance is impacted by both intestinal and hepatic CYP3A, 4βOHC seems to be a marker which predominantly reflects hepatic CYP3A metabolism, much like intravenous midazolam. The contribution of intestinal CYP3A to 4βOHC levels is yet to be determined.

In paper I, we found that use of CYP3A inducers was associated with >4-fold increase in 4βOHC concentration, and it was the factor with largest quantitative impact on 4βOHC levels. In paper III, use of the enzyme-inducing antiepileptic drugs phenobarbital, phenytoin and carbamazepine was associated with 3-fold, 6-fold and 7-fold higher median 4βOHC levels than in non-induced patients. This suggests that 4βOHC has the dynamic range to at least distinguish between moderate and strong CYP3A inducers, as the Food and Drug Administration defines weak, moderate and strong inducers to result in 1.25- to 2-fold, 2- to 5-fold and ≥5-fold decrease in AUC for the victim drug, respectively.[60] In a simulation study by Yang & Rodriguez, 4βOHC is expected to distinguish between none, weak, moderate and strong induction after 2 weeks of dosing.[52]

The dynamic range of 4βOHC seems to be smaller for CYP3A inhibition than for induction, which may contribute to explain our findings of 120-fold variability in 4βOHC levels compared to 400-fold variability in oral midazolam exposure. While 14 days of ketoconazole treatment produced 4-fold decrease in intravenous midazolam clearance and 11-fold decrease in oral midazolam clearance, 4βOHC levels were only decreased by 23%.[55] In paper I, we reported 34% lower 4βOHC levels in users of weak to moderate CYP3A inhibitors compared with patients not using CYP3A-interacting drugs. The patients included in this study were likely long-time users of inhibitors, so that steady state of both inhibitor concentration and 4βOHC levels were assumed. This seems particularly important for measuring inhibition by 4βOHC. A pharmacokinetic model by Yang & Rodrigues predicted that inhibitors needed to be administered for at least 1 month for 4βOHC to fully differentiate between weak, moderate and potent inhibitors.[52]

The elimination pathway of 4βOHC is not yet fully elucidated, but elimination by CYP7A1 and CYP27A1 is reported *in vitro*.[46] Since rifampicin is reported to inhibit CYP7A1 *in vitro*,[53] it has been suggested that elevated 4βOHC levels by CYP3A induction may be enhanced by reduced elimination via CYP7A1.[54] However, this theory is yet to be substantiated *in vivo*.

In conclusion, 4βOHC is an appropriate biomarker for measuring hepatic CYP3A induction, and its dynamic range is comparable to that of intravenous midazolam. It is important to take

into account the long half-life of 4βOHC, however, as it may take weeks before steady-state is reached after initiation with induction therapy. The dynamics of 4βOHC for measuring CYP3A inhibition are more disputed. 4βOHC may be useful in this area too, but further studies are needed to establish the dynamic range of 4βOHC as an inhibition marker, and such studies should last at least 1 month for 4βOHC levels to adjust to the inhibition therapy.

4.1.2 Genetic polymorphism

Polymorphism of *CYP3A5* is the main reason for variability in CYP3A5 activity, and it contributes substantially to variability in the overall CYP3A activity. *In vitro* data have suggested that CYP3A4 is 20-fold more effective than CYP3A5 in catalyzing 4βOHC formation,[46] but there are conflicting *in vivo* reports regarding impact of *CYP3A5* polymorphism on 4βOHC levels. When compared to subjects who are homozygote null allele carriers, carriers of one and two *CYP3A5*1* alleles are reported to have 10-20% and 40-65% higher mean 4βOHC levels, respectively.[61, 62] In paper I, we showed that the *CYP3A4*22* and *CYP3A5*3* alleles had no impact on 4βOHC levels in a large, naturalistic population. In paper II, however, patients who carried the *CYP3A5*1* allele had 50% higher 4βOHC concentration than homozygote carriers of *CYP3A5*3* before renal transplantation. A possible explanation of these discrepancies could be that CYP3A4 is more susceptible to inhibition by impaired renal function than CYP3A5, but this does not coincide with findings by Suzuki *et al.*, who reported no difference in 4βOHC levels between *CYP3A5* genotypes before renal transplantation.[63] The patient population in paper II was smaller and more homogenous than in paper I, which may contribute to explain the different results. Since we did not have complete medicinal lists for patients in paper I, CYP3A interacting drugs may have been used by some of the patients. Furthermore, the retrospective study design limits availability of patient characteristics which could influence 4βOHC levels, such as comorbidity, organ function, and inflammation status. These factors are likely to add some 'noise' to the variability in 4βOHC concentrations, and could make it harder to detect small variations in 4βOHC levels. Another possible explanation for the lack of increased 4βOHC levels in *CYP3A5*1* carriers is other polymorphisms not accounted for in the study. Vanhove *et al.* reported higher 4βOHC/C ratio in patients carrying the *CYP3A5*1* allele in univariate analysis, but when doing multivariate analysis, it was the *CYP3A4*1B* allele which predicted higher 4βOHC/C ratio, and not *CYP3A5*1*.[64] Since *CYP3A4*1B* is in linkage disequilibrium with *CYP3A5*1*, it is possible that *CYP3A4*1B* is the variant allele which actually impacts 4βOHC levels, and not *CYP3A5*1*. However, *CYP3A4*1B* has generally not been considered

a clinically relevant polymorphism, and further studies are needed to clarify its impact on CYP3A activity. E.g., two recent meta-analyses have emerged regarding the clinical impact of *CYP3A4*1B*: one concluding that renal transplant recipients need higher tacrolimus doses if carrying *CYP3A4*1B,* the other concluding that the same patient group needs lower cyclosporine doses if carrying *CYP3A4*1B.*[65, 66] Further studies are needed to elucidate the differential impact of *CYP3A4*1B* and *CYP3A5*1* on 4βOHC levels.

Conflicting findings are reported also for the impact of other polymorphisms on 4βOHC levels. Vanhove *et al.* reported that *CYP3A4*22* explained 5% of the variation in 4βOHC/C ratio in a multivariate analysis, while Woolsey *et al.* found no impact of *CYP3A4*22, CYP3A5*3* or *POR*28* on 4βOHC/C ratio.[64, 67] The latter paper only genotyped 46 patients, however. In paper I, we found that being carrier of the *POR*28* allele was associated with an 11% higher 4βOHC concentration than being *POR*1/*1* homozygote, indicating that *POR*28* contributes to a slightly increased CYP3A metabolic capacity. Polymorphisms in *CYP7A1* and *CYP27A1* may also impact the elimination of 4βOHC, but this is yet to be investigated.

In conclusion, the impact of genetic polymorphisms on 4βOHC levels is still unclear, as conflicting reports have emerged for *CYP3A5*3, CYP3A4*22* and *POR*28* variant alleles. Nongenetic factors, such as enzyme induction, are of far greater importance for 4βOHC levels than genetic polymorphisms. The *in vivo* contribution of CYP3A5 on 4βOHC concentration is still not completely established.

4.1.3 Renal function and CYP3A activity

End-stage renal disease impairs CYP metabolism, perhaps by uremic toxins causing direct inhibition or reduced protein expression.[68, 69] In paper II, we found that median 4βOHC concentration before kidney transplantation in patients with combined *CYP3A4*1/*1* and *CYP3A5*3/*3* genotypes was 22.3 ng/mL, which is 10% lower than in psychiatric patients with the same genotype combination (paper I). Previously, end-stage renal disease has been reported to produce ~30% decrease in CYP3A activity measured by the erythromycin breath test,[40] and acute kidney injury results in reduced hepatic midazolam metabolism.[70] The unusually long half-life of 4βOHC makes it a suitable biomarker for baseline CYP3A activity, as the intraindividual variability in 4βOHC levels over time is low. Over a 3 month period, an average intraindividual variation of 7% was measured in healthy volunteers.[51]

From before to 3 months after renal transplantation, we reported a gradual increase in 4βOHC concentration of ~50% (paper II). This coincides with findings from Suzuki *et al.*, who reported 30-50% increase in 4βOHC levels 3-6 months after renal transplantation.[63, 71] Tacrolimus clearance is, however, reported to decrease with time after kidney transplantation, and 4βOHC measurements do not contribute to explain this.[72] Since tacrolimus exposure is influenced by multiple factors, such as perioperative changes in gastrointestinal function, hematocrit levels, albumin levels and P-gp, the failure of 4βOHC to describe early tacrolimus exposure is not too surprising. Further studies involving other CYP3A substrates than tacrolimus are needed to investigate whether these findings are clinically relevant.

4.1.4 Body weight and body mass index

In paper IV, we reported the novel association between low BMI and high 4βOHC levels. An association between body weight and 4βOHC levels was reported in renal transplant patients in paper II, but this association has not been reported in severely underweight patients before. Females with anorexia nervosa had twice the 4βOHC levels and 4βOHC/C ratio compared to normal-weight females, and BMI was negatively correlated with 4βOHC/C. This indicates increasing CYP3A activity with decreasing BMI. In obese patients, negative correlations between BMI and CYP3A4 protein expression in both liver (r=–0.77) and intestines (r=–0.56) have been reported, as well as negative correlation between BMI and atorvastatin clearance (r=–59).[36] It is therefore likely that the association we found between 4βOHC/C and BMI in anorexic patients reflects elevated CYP3A activity.

The clinical implications of elevated CYP3A activity in underweight patients are unclear, as drug exposure is not only dependent on metabolic capacity, but also factors such as distribution volume, protein binding and hepatic extraction ratio. For obese patients, however, studies on the clearance of a number of CYP3A substrates suggest that CYP3A activity is reduced by 10-35%.[37] Therefore, based on our results, it is likely that severely underweight patients have increased clearance of CYP3A substrates. Further studies should be performed investigating the association between low BMI and clearance of other agents metabolized by CYP3A, preferably clinically used drugs.

4.1.5 Future perspectives

Although there is increasing number of reports using 4βOHC as a CYP3A biomarker, it is yet to be brought into clinical use. To bring 4βOHC to the next level, we need to know if it can contribute to personalizing treatment with CYP3A substrates.

Conflicting reports exist regarding correlation between oral midazolam parameters and 4βOHC data, with everything from no correlation to weak or moderate correlation (r=0.35-0.54).[67, 73, 74] The correlation with intravenous midazolam clearance is moderate (r=0.27-0.62).[56, 74] Phenotyping data obtained with one CYP3A biomarker does not necessarily correlate well with data from another biomarker. E.g., data from the erythromycin breath test does not correlate with midazolam clearance.[43] The large active sites of the CYP3A isoenzymes contain overlapping substrate binding regions, and one CYP3A substrate does not always have the same metabolizing rate as another substrate. Different substrates will also have different isoenzyme preferences, and some CYP3A probes are substrates of P-gp, which contributes to pharmacokinetic variability. Therefore, to clinically implement a CYP3A marker for dose predictions, it should be validated for each drug subjected to CYP3A metabolism. So far, 4βOHC is reported to correlate with clearance of several CYP3A substrates; such as atorvastatin, carbamazepine, midostaurin, quetiapine and with tacrolimus in stable kidney transplant recipients,[48, 64, 75-78] but not with taxanes or with tacrolimus early after kidney transplantation.[72, 79, 80] These reports support the potential suitability of 4βOHC as a CYP3A phenotype marker, but additional factors should probably be accounted for if using 4βOHC as a metric for individual dose recommendations. Further prospective studies, including multiple factors of potential importance, should be performed to provide 4βOHC-based algorithms for personalized dosing of CYP3A substrates.

4.2 Methodological considerations

Paper I and III had naturalistic designs, and included material from a therapeutic drug monitoring (TDM) service at Diakonhjemmet Hospital, Oslo. Serum samples were collected and analyzed for 4βOHC after the requested TDM analyses were completed. In paper I, whole blood from all included patients was also collected from the TDM biobank and analyzed for relevant genetic polymorphisms. Strengths of this design are e.g. the opportunity to include a large number of real-life patients who are likely to have reached steady-state conditions on their drug treatment. The retrospective study design, however, limited the information we were able to collect about each patient. Requisition forms for all included samples were

reviewed to identify use of interacting drugs or herbal agents, but since requisition forms usually do not contain complete medicinal lists, some interacting drugs may have been omitted. Also, the studies would have benefitted from knowing more patient characteristics that could influence CYP3A4 phenotype, such as somatic conditions, ethnicity and inflammation status.

Genotyping of *CYP3A4* and *CYP3A5* in paper I and II involved determination of the relatively common variant alleles *CYP3A4*22* and *CYP3A5*3*, and absence of these alleles was interpreted as presence of the **1* alleles. However, presence of the *CYP3A4*1B* (rs2740574) allele was not accounted for. *CYP3A4*1B* has an allele frequency of 3% in European populations, and is in linkage disequilibrium with the *CYP3A5*1* allele, which means that these polymorphisms are non-randomly associated.[25] Therefore, findings associated with the impact of *CYP3A5*1* on 4βOHC concentration may be biased by the *CYP3A4*1B* polymorphism.

Cholesterol is reported to explain ~10% of the variation in 4βOHC levels, and 4βOHC/C ratio is regarded a more exact measure of CYP3A activity than 4βOHC concentration alone.[81] Due to limited serum and plasma volumes in paper I-III, we were not able to analyze cholesterol levels in the included samples. However, our group has previously reported strong correlation between 4βOHC/C ratio and 4βOHC levels ($r \geq 0.9$).[72, 78] Furthermore, serum cholesterol levels are in the millimolar range and always in surplus compared to serum 4βOHC levels, which are in the nanomolar range. Therefore, we do not suspect that using the 4βOHC/C ratio would have led to different results in the current studies.

5 CONCLUSIONS

Overall, this thesis supports the suitability of 4βOHC as a hepatic CYP3A biomarker, since the factors which in the present studies describe variation in 4βOHC levels coincide with previous reports on variation in hepatic CYP3A phenotype. We found 120-fold variation in 4βOHC levels in a naturalistic patient population, and the factor with greatest quantitative effect on CYP3A activity was enzyme-inducing drugs. 4βOHC was also shown to be responsive to long-term treatment with moderate CYP3A inhibitors, but its suitability as a biomarker for CYP3A inhibition is yet to be established. The contribution of CYP3A5 to *in vivo* formation of 4βOHC was inconclusive in the present studies. Impact of other genetic polymorphisms on 4βOHC levels was limited. Furthermore, it was shown that end-stage renal disease was associated with reduced 4βOHC levels, and accordingly that improved renal function after kidney transplantation was associated with recovery of CYP3A activity. This may indicate higher dose requirements of CYP3A substrates after vs. before kidney transplantation, but the clinical impact of these findings should be further elucidated. Body weight and BMI impacted *in vivo* CYP3A activity, with increasing level of 4βOHC levels with decreasing body weight and BMI. This suggests that clearance of CYP3A substrates increases with decreasing body weight/BMI.

The studies of this thesis highlight the potential usefulness of 4βOHC as a dosing biomarker of CYP3A substrates, but this needs further investigation in prospective studies to provide 4βOHC-based algorithms for personalized dosing of CYP3A-metabolized drugs.

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IV

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ORIGINAL ARTICLE

Elevated 4β-hydroxycholesterol/cholesterol ratio in anorexia nervosa patients

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Abstract

Recent studies have shown that the cytochrome P450 (CYP) 3A phenotype marker 4β-hydroxycholesterol/cholesterol (4βOHC/C) ratio is negatively correlated with body weight in healthy volunteers, and that obese patients have lower 4βOHC levels than healthy controls. However, 4βOHC/C ratio in underweight patients has yet to be reported. The aim of this study was to examine potential differences in CYP3A activity between underweight patients with anorexia nervosa and normal-weight volunteers by measuring plasma 4βOHC/C ratio. Furthermore, we wished to describe any association between body mass index (BMI) and 4βOHC/C ratio in underweight patients. A total of 20 underweight patients and 16 normal-weight volunteers were included in the study, all females. Underweight patients had a median 4βOHC/C ratio (molar ratio $\times 10^{-5}$) of 2.52 (range, 0.90–11.3) compared to 1.29 (0.56–2.09) in normal-weight subjects (Mann-Whitney $P = 0.0005$). 4βOHC/C ratio was negatively correlated with BMI in underweight patients ($r = -0.56$, $P = 0.011$), and in the whole study population ($r = -0.67$, $P < 0.0001$). This suggests that the negative correlation between 4βOHC/C and BMI, which has previously been reported between 4βOHC/C and body weight in healthy volunteers, extends to underweight patients. The findings indicate that CYP3A activity increases with decreasing BMI, resulting in higher CYP3A activity in underweight patients compared to normal-weight subjects. The potential clinical relevance of this needs to be studied further by comparing pharmacokinetics of drugs subjected to CYP3A-mediated metabolism in underweight vs. normal-weight individuals.

KEYWORDS
4β-hydroxycholesterol, anorexia nervosa, BMI, CYP3A

1 | INTRODUCTION

Cytochrome P450 (CYP) 3A enzymes play a major role in the metabolism of about 30% of clinically used drugs.[1] Substantial

Abbreviations: 4βOHC/C, 4β-hydroxycholesterol/cholesterol; BMI, body mass index; CYP, cytochrome P450.

inter-individual variability in CYP3A activity exists due to a combination of genetic, environmental, and endogenous factors.[2] CYP3A phenotype can be measured by the endogenous biomarker 4β-hydroxycholesterol (4βOHC), which is metabolized from cholesterol by CYP3A4 and CYP3A5, the two most important CYP3A enzymes in humans.[3,4] Variations in cholesterol concentration have been found to explain about 10% of 4βOHC variation,[5] and 4βOHC/cholesterol (4βOHC/C) ratio is preferable to 4βOHC as CYP3A biomarker in patient groups where cholesterol levels are abnormal.[6]

Recent studies have shown that body weight is negatively correlated with 4βOHC/C ratio in healthy volunteers,[7] and that obese patients have lower 4βOHC levels than healthy controls.[8] Studies on the clearance of a number of other CYP3A substrates suggest that CYP3A activity is reduced by 10-35% in obese patients.[9] Furthermore, Ulvestad et al. reported strong negative correlation between body mass index (BMI) and CYP3A protein expression in liver and intestines.[10] Altogether, this indicates that CYP3A activity decreases with increasing body weight. However, 4βOHC/C ratio in underweight patients is yet to be reported, and it is not known whether the correlation between BMI and CYP3A activity extends to underweight patients.

The aim of our study was to examine potential differences in CYP3A activity between underweight patients with anorexia nervosa and normal-weight volunteers by measuring plasma 4βOHC/C ratio. Furthermore, we wished to describe any association between BMI and 4βOHC/C ratio in underweight patients.

2 | MATERIALS AND METHODS

2.1 | Subjects

Patients with severe anorexia nervosa (n = 20) were included from an inpatient unit at the Regional Department for Eating Disorders, Division of Mental Health and Addiction, Oslo University Hospital, Norway, from May 2012 to September 2013. Inclusion criteria were (i) anorexia nervosa diagnosis according to DSM-IV; (ii) female sex; and (iii) BMI < 18.5. Normal-weight control subjects (n = 16) were recruited from School of Pharmacy, University of Oslo, Norway in May 2016. Inclusion criteria for normal-weight volunteers were (i) female sex; and (ii) BMI ≥ 18.5.

All subjects gave written, informed consent. The study was approved by the Regional Committee for Medical and Health Research Ethics South-East.

2.2 | 4βOHC analyses

Plasma concentration of 4βOHC was determined by a previously described UPLC-MS/MS assay.[11,12] The lower limit of quantification was 10 ng/mL. Intra- and interday imprecision and inaccuracy for the method were <15% at 10 ng/mL and <4% at 644 ng/mL (n = 6).[11] All samples were stored at −80°C between sampling and analysis. For the underweight patient samples, stability of 4βOHC during storage was ensured by controlling that 4αOHC concentration was lower than 4βOHC concentration.[13] This was not done for normal weight samples, since they were analyzed only 5 months after sampling; within the time frame that 4βOHC is known to maintain stability.[14]

2.3 | Endpoints and statistical analyses

BMI was calculated as body weight divided by height squared (kg/m^2). 4βOHC/C ratio was calculated as 4βOHC concentration (nmol/L) divided by total cholesterol concentration (mmol × 10^6/L). Mann-Whitney *U* tests were used for comparisons of 4βOHC/C ratio and other characteristics between anorexia nervosa patients and normal-weight subjects. Spearman correlation was used to evaluate association between BMI and 4βOHC/C ratio and between 4βOHC and cholesterol. Statistical significance was considered as $P < 0.05$. GraphPad Prism for Windows, version 6.01 (GraphPad Software, La Jolla, CA), was used for statistical analyses and graphical presentations.

3 | RESULTS

Clinical and demographic characteristics of included subjects are listed in Table 1. All included subjects were female, and there were no differences in age or total cholesterol between the two groups ($P > 0.1$). None of the included subjects used CYP3A inducers according to the Flockhart CYP Drug Interaction Table.[15] One of the anorexia nervosa patients used fluoxetine, a CYP3A inhibitor.[15] The patient was not excluded since results showed higher 4βOHC concentration in underweight patients compared to normal-weight subjects. All drugs used by included subjects are listed in Table 2.

The association between 4βOHC and cholesterol concentration in the whole study population was significant ($r = 0.41$, $P = 0.013$). The median 4βOHC concentration in underweight patients was 49.0 ng/mL (range, 18.5–129 ng/mL) compared to 22.0 ng/mL (10.8–41.9 ng/mL) in normal-weight subjects ($P < 0.0001$) (Figure 1A). Underweight patients had a median 4βOHC/C ratio (molar ratio × 10^{-5}) of 2.52 (0.90–11.3) compared to 1.29 (0.56–2.09) in normal-weight subjects ($P = .0005$) (Figure 1B). 4βOHC/C ratio was

TABLE 1 Clinical and demographic characteristics

Variables	Anorexia nervosa (n = 20)	Normal-weight (n = 16)	*P* value
Age, years	24 (15-47)	23 (19-48)	0.81
Body weight, kg	43 (29-53)	61 (43-77)	<0.0001
Body mass index, kg/m^2	14.9 (10.1-18.0)	21.5 (19.4-25.2)	<0.0001
Total cholesterol, mmol/L	5.07 (2.83-6.88)	4.54 (3.44-5.80)	0.12

Data are expressed as median (range), and *P* values are derived from Mann-Whitney *U* tests.

TABLE 2 Overview of drugs used by included subjects

Anorexia nervosa patients		Normal-weight subjects	
Drugs	Number of patients	Drugs	Number of subjects
Alimemazine	1	Cetirizine	2
Chlorprothixene	1	Combination contraceptives	5
Desloratadine	1	Desloratadine	2
Fluoxetine	1	Levothyroxine	2
Levothyroxine	1	Naproxen	1
Melatonin	1	Paracetamol	1
Metoprolol	1	Progesterone only contraceptives	6
Promethazine	1	Valerian root	1
Quetiapine	1		

negatively correlated with BMI in underweight patients ($r = -0.56$, $P = .011$) (Figure 2A), and in the whole study population ($r = -0.67$, $P < 0.0001$) (Figure 2B).

4 | DISCUSSION

We found that the underweight patients had twice the plasma 4βOHC/C ratio of normal-weight volunteers. Furthermore, 4βOHC/C ratio was strongly correlated with BMI in the whole population regardless of the participants' status as underweight or normal-weight. This suggests that the negative correlation between 4βOHC/C and BMI, which has previously been reported between 4βOHC/C and body weight in healthy volunteers, extends to underweight patients. The findings indicate that CYP3A activity increases with decreasing BMI, resulting in higher CYP3A activity in underweight patients compared to normal-weight subjects. However, this needs to be studied further with other CYP3A substrates in underweight vs. normal-weight individuals.

Studies evaluating CYP3A metabolism in anorexia nervosa patients are scarce. Boyar et al. reported reduced clearance of cortisol, a partial CYP3A substrate, in anorexia nervosa patients compared to healthy subjects.[16] Cachectic patients have been reported to have both increased and decreased CYP3A metabolism,[17,18] but not to have altered liver content of CYP3A proteins.[19] Altogether, conflicting reports make it difficult to conclude whether CYP3A metabolism in underweight patients diverges from normal-weight subjects.

In this study, we report that anorexia nervosa patients have higher 4βOHC concentration and 4βOHC/C ratio compared to normal-weight volunteers, and hypothesize that this reflects elevated CYP3A activity in underweight patients. Anorexia nervosa patients often have hypercholesterolemia.[20] Hyperadiponectinemia is known to occur in anorexic patients,[21] and may be related to increased cholesterol synthesis.[22] To account for impact of altered cholesterol levels on 4βOHC concentration, we consider 4βOHC/C ratio to be a more appropriate CYP3A biomarker in this study population. However, whether using 4βOHC concentration or 4βOHC/C ratio, we report approximately twice the biomarker level in underweight patients compared to normal-weight volunteers. The normal-weight volunteers had 4βOHC levels and 4βOHC/C ratio within the normal range.[23] Obesity has been associated with reduced plasma 4βOHC levels,[8] and hence emaciation could lead to increased 4βOHC levels. However, distorted 4βOHC levels could be caused by changes both in production and elimination, and 4βOHC is further metabolized via CYP7A1 and CYP27A1.[3] Reduced 4βOHC and elevated plasma 27OHC levels have been reported in an obese mouse model, which could indicate that obesity is associated with increased CYP27A1 activity and therefore increased elimination of 4βOHC.[24] However, Ulvestad et al. reported strong negative correlation between BMI and both hepatic and small-intestinal CYP3A protein expression in obese patients,[10] supporting our findings that CYP3A activity is correlated with BMI.

Theoretically, reduced tissue distribution of 4βOHC and/or increased lipolysis may result in increased 4βOHC/C ratio in anorexia nervosa patients. Thus, it cannot be ruled out that increased 4βOHC/C ratio might be caused by other factors than increased CYP3A activity in this underweight population. However, due to the strong correlation between BMI and 4βOHC/C ratio in the whole study population, including both underweight and normal-weight subjects, we find it likely that the increased 4βOHC/C ratio reflects an increase of CYP3A activity.

A limitation of the present study is that only one CYP3A biomarker was tested. Unfortunately, no probe drug such as midazolam was given to the participants at the time of the study. Thus, additional studies with other CYP3A substrates are necessary to confirm our results. Genotyping of CYP3A4 and CYP3A5 would also have been of interest. Expression of the *CYP3A5*1* allele has been

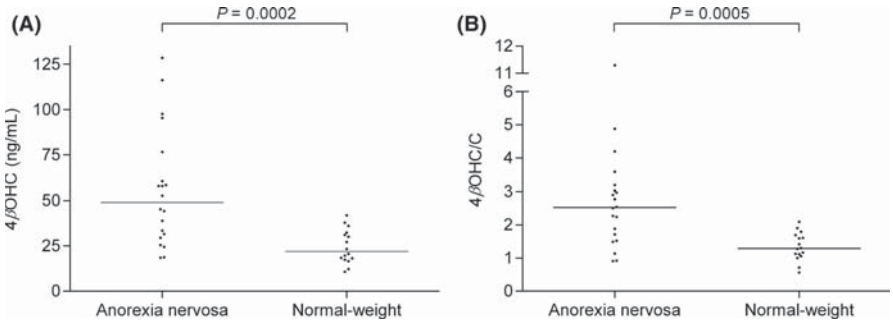

FIGURE 1 (A) 4β-hydroxycholesterol (4βOHC) concentration and (B) 4β-hydroxycholesterol/cholesterol (4βOHC/C) ratio in anorexia nervosa patients (n = 20) and normal-weight subjects (n = 16). 4βOHC/C is expressed as molar ratio × 10^{-5}. *P* values are derived from Mann-Whitney *U* tests, and medians are expressed as solid lines

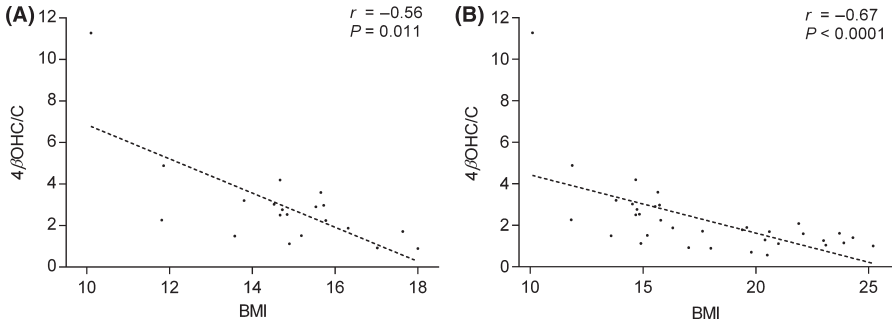

FIGURE 2 Correlations between 4β-hydroxycholesterol/cholesterol (4βOHC/C) ratio and body mass index (BMI) in (A) anorexia nervosa patients (n = 20) and in (B) the whole study population (n = 36). 4βOHC/C is expressed as molar ratio × 10^{-5}. *P* and *r* values are derived from Spearman correlations, and linear trend lines are added for visual purposes

associated with elevated 4βOHC levels,[5,25] although a larger study did not find any association between 4βOHC levels and *CYP3A5*3* or *CYP3A4*22* polymorphisms.[2] Other potential sources of inter-individual variation in 4βOHC levels are ethnicity and inflammation.[5,26]

An advantage with 4βOHC as biomarker is its selectivity; CYP3A4 and CYP3A5 convert cholesterol to 4βOHC, while only negligible amounts of 4βOHC were produced by seven other CYP enzymes.[3,4] Furthermore, 4βOHC has an unusually long half-life of up to 17 days, which leads to low intra-individual variability.[27] Since 4βOHC is sensitive to CYP3A induction,[27] we consider it a suitable biomarker for the present study where anorexic patients displayed elevated 4βOHC levels.

In conclusion, we report that anorexia nervosa patients have twice the plasma 4βOHC/C ratio of normal-weight volunteers, and that 4βOHC/C ratio has a strong negative correlation with BMI. The findings indicate that CYP3A activity increases with decreasing BMI, resulting in higher CYP3A activity in underweight patients compared to normal-weight subjects. However, the potential clinical relevance needs to be studied further by comparing pharmacokinetics of drugs subjected to CYP3A-mediated metabolism in underweight vs. normal-weight individuals.

AUTHOR CONTRIBUTIONS

Participated in research design: Hole, Heiberg, Molden. Contributed to acquisition of data: Hole, Heiberg, Gjestad, Mehus, Rø, Molden. Performed data analysis: Hole. Wrote or contributed to the writing of the manuscript: Hole, Heiberg, Gjestad, Mehus, Rø, Molden.

DISCLOSURE

None declared.

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