Pharmacokinetic variability in patients with obesity and healthy individuals – the role of cytochrome P450

Kine Eide Kvitne

Dissertation for the degree of Philosophiae Doctor (Ph.D)

Section for Pharmacology and Pharmaceutical Biosciences

Department of Pharmacy

Faculty of Mathematics and Natural Sciences

University of Oslo

2022

Series of dissertations submitted to the
Faculty of Mathematics and Natural Sciences, University of Oslo
No. 2550

ISSN 1501-7710

Print production: Graphics Center, University of Oslo.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

The work presented in this thesis has been carried out at the Department of Pharmacy, University of Oslo during the period of 2018 to 2022.

First and foremost, I would like to thank my supervisors, Ida Robertsen, Anders Åsberg, Hege Christensen, and Veronica Krogstad. I am so grateful and privileged to have been part of the pharmacokinetics research group. To my main supervisor, Ida; thank you for your never-ending support, for always making time for me, and for sharing both frustrations and victories over these years. Your positive energy and scientific advice have been especially appreciated. Anders; thank you for your scientific guidance, your swift response to all kinds of questions, and for always being available. I have appreciated your enthusiasm, optimism, and sense of humor. Hege; thank you for your encouragement. Your caring personality and positive energy have been much appreciated. Veronica; thank you for your valuable help and support.

I would like to thank all my co-authors at the Morbid Obesity Center in Tønsberg, AstraZeneca Gothenburg, Uppsala University, and the Center for Psychopharmacology at Diakonhjemmet Hospital for your valuable contribution to this work. A special thanks to Eva for support and help with statistical challenges.

I would also like to thank Hasse; the IntraCYP study would not have been possible without you. A warm thank you to Kristin, Cristell, Merete, May Ellen, and Edmar at the Clinical Research Unit, Oslo University Hospital for your skilled assistance and positive energy during the many and long study days.

To my colleagues at the Department of Pharmacy, thank you for fostering a friendly place to work. Thanks to Eline for your technical assistance and contribution on study days. Thanks to my fellow PhD students Erlend, Marte, Nina, and Abel for making these years so fun. Special thanks to Markus and Ole Martin for being such great colleagues and friends.

I also wish to thank all my friends and family for your cheer and support. Finally, thank you Øystein for your loving support and for always believing in me.

Oslo, June 2022

Kine Eide Kvitne

LIST OF PAPERS

I. **Kvitne KE**, Robertsen I, Skovlund E, Christensen H, Krogstad V, Wegler C, Angeles PC, Wollmann BM, Hole K, Johnson LK, Sandbu R, Artursson P, Karlsson C, Andersson S, Andersson TB, Hjelmesaeth J, Jansson-Löfmark R, Åsberg A. Short- and long-term effects of body weight loss following calorie restriction and gastric bypass on CYP3A-activity - a non-randomized three-armed controlled trial. *Clin Transl Sci.* 2022 Jan;15(1):221-233. doi: 10.1111/cts.13142.

II. **Kvitne KE**, Krogstad V, Wegler C, Johnson LK, Kringen MK, Hovd MH, Hertel JK, Heijer M, Sandbu R, Skovlund E, Artursson P, Karlsson C, Andersson S, Andersson TB, Hjelmesæth J, Åsberg A, Jansson-Löfmark R, Christensen C, Robertsen I. Short- and long-term effects of body weight, calorie restriction and gastric bypass on CYP1A2, CYP2C19 and CYP2C9 activity. *Br J Clin Pharmacol.* 2022 Sep;88(9):4121-4133. doi: 10.1111/bcp.15349.

III. **Kvitne KE**, Åsberg A, Johnson LK, Wegler C, Hertel JK, Artursson P, Karlsson C, Andersson S, Sandbu R, Skovlund E, Christensen H, Jansson-Löfmark R, Hjelmesæth J, Robertsen I. Impact of type 2 diabetes on in vivo activities and protein expressions of cytochrome P450 in patients with obesity. *Clin Transl Sci.* 2022 Aug 29. doi: 10.1111/cts.13394. Online ahead of print.

IV. **Kvitne KE**, Drevland OM, Haugli N, Skadberg, E, Zaré HK, Åsberg A, Robertsen I. Intraindividual variability in absolute bioavailability and clearance of midazolam in healthy individuals. Manuscript submitted to *Br J Clin Pharmacol.*

V. **Kvitne KE**, Hole K, Krogstad V, Wollmann BM, Wegler C, Johnson LK, Hertel JK, Artursson P, Karlsson C, Andersson S, Andersson TB, Sandbu R, Hjelmesæth J, Skovlund E, Christensen H, Jansson-Löfmark R, Åsberg A, Molden E, Robertsen I. Correlations between 4β-hydroxycholesterol and hepatic and intestinal CYP3A4: protein expression, microsomal ex vivo activity, and in vivo activity in patients with a wide body weight range. *Eur J Clin Pharmacol.* 2022 Aug;78(8):1289-1299. doi: 10.1007/s00228-022-03336-9.

ABBREVIATIONS

AUC	Area under the curve
BMI	Body mass index
CAR	Constitutive androstane receptor
CCL	CC-chemokine ligand
CL_{int}	Intrinsic clearance
$CL_{int,u}$	Unbound intrinsic clearance
C_{max}	Maximum concentration
CV	Coefficient of variation
CYP	Cytochrome P450
C/EBP	CCAAT-enhance-binding protein
F	Bioavailability
F_A	The fraction absorbed from the gastrointestinal lumen
F_G	The fraction escaping gut wall metabolism
F_H	The fraction escaping presystemic hepatic metabolism
HbA1c	Hemoglobin A1c
HIV	Human immunodeficiency virus
HIM	Human intestinal microsomes
HLM	Human liver microsomes
hs-CRP	High-sensitivity C-reactive protein
IFN	Interferon
IL	Interleukin
K_a	The absorption rate constant
LCA	Losartan carboxylic acid
LLOQ	Lower limit of quantification
NAFL	Nonalcoholic fatty liver
NAFLD	Nonalcoholic fatty liver disease
NAFLD-LFS	Nonalcoholic fatty liver disease liver fat score
NASH	Nonalcoholic steatohepatitis
NF-κB	Nuclear factor-kappa B
PXR	Pregnane X receptor
QC	Quality control
RYGB	Roux-en-Y gastric bypass

SNP	Single nucleotide polymorphisms
T_{max}	Time to reach maximum concentration
TNF- α	Tumor necrosis factor-α
TNFR	TFN receptor
T2DM	Type 2 diabetes mellitus
UHPLC-MS/MS	Ultra-high performance liquid chromatography tandem mass spectrometry
ULOQ	Upper limit of quantification
4βOHC	4β-hydroxycholesterol
5-OH-omeprazole	5-hydroxyomeprazole

ABSTRACT

Disease-related factors have proven to be an important source of variable drug response among individuals, leading to an increased focus on the impact of various diseases on pharmacokinetics. Due to the rising prevalence of obesity and its comorbidities, and hence the number of bariatric surgeries, clinicians will more often be confronted with how drugs should be dosed in these patients. The overall aim of this thesis was to investigate the impact of obesity, Roux-en-Y gastric bypass (RYGB), weight loss, and type 2 diabetes mellitus (T2DM) on pharmacokinetics, focusing on cytochrome P450 (CYP) 1A2, CYP2C9, CYP2C19, and CYP3A.

This thesis is primarily based on data from the COCKTAIL-study including patients with obesity scheduled for weight loss treatment with RYGB or a strict diet and a non-obese control group scheduled for cholecystectomy. In two of the papers, we showed that metabolism mediated by CYPC219 and CYP3A were decreased in patients with obesity, and, following weight loss induced by RYGB or a strict diet, CYP2C19 activity increased a few weeks later, while CYP3A activity had a longer recovery time. We also showed that body weight, weight loss, and RYGB had a negligible impact on CYP1A2 and CYP2C9 activities. This thesis also included a paper investigating the impact of T2DM in patients with obesity with or without T2DM, where we showed that T2DM downregulated CYP2C19 activity beyond that of obesity, while CYP1A2, CYP2C9, and CYP3A were not influenced.

In the data from the COCKTAIL-study, we observed substantial intraindividual variability in midazolam pharmacokinetics in patients with obesity before and after weight loss over a short period of time. Accordingly, we performed a study in healthy volunteers without obesity (IntraCYP-study) where we showed a low- to moderate intraindividual variability in midazolam absolute bioavailability and systemic clearance on average, indicating a minor variability in CYP3A activity over time. However, a relevant number of the healthy volunteers displayed considerable intraindividual variability that should be considered when using midazolam to assess CYP3A activity. The data in the first paper also indicated that systemic midazolam clearance to a large degree reflected other processes than CYP3A metabolic capacity. Thus, we evaluated 4βOHC as an endogenous biomarker for CYP3A4 activity. In this paper, we showed that 4βOHC appears to reflect hepatic, but not intestinal, CYP3A4 activity, making it a valuable supplement in traditional phenotyping studies using probe drugs such as midazolam.

1 INTRODUCTION

1.1 Variability in drug response

Variability in drug response between individuals is a major clinical challenge, particularly for drugs with a narrow therapeutic range. This is because a standard dose of a given drug may give the desired pharmacological effect in some individuals, while others may experience adverse effects or therapeutic failure. The drug response depends on both pharmacokinetic and pharmacodynamic processes. While pharmacodynamics describes the relationship between drug concentration at the site of action and the resulting effect, pharmacokinetics refers to drug absorption, distribution, metabolism, and elimination processes.[1] The variability in drug response is multifactorial and includes environmental factors, genetic factors, disease-related factors, and drug-drug interactions. Knowledge about factors influencing the drug response and their interplay is essential for predicting interindividual differences and thus optimizing drug therapy.

1.2 Pharmacokinetic processes

The absorption from the intestine following oral dosing is characterized by the rate of absorption (K_a) and the total amount of drug being absorbed. In addition to the physicochemical properties of a drug, several physiological factors may influence these processes, such as gastrointestinal pH, gastric emptying time, intestinal motility, blood flow to the absorption site, drug transporters, and drug-metabolizing enzymes such as the cytochrome P450 (CYP) superfamily.[2, 3] The term bioavailability (F) describes the fraction of a drug reaching the systemic circulation intact and is expressed as the fraction absorbed from the gastrointestinal lumen (F_A), the fraction escaping gut wall metabolism (F_G), and the fraction escaping presystemic hepatic metabolism (F_H) (**Figure 1**). The movement of drug between the blood (the site of measurement) and various tissues of the body is referred to as distribution. The term apparent volume of distribution (V_d) describes the magnitude of distribution of a drug throughout the body relative to the plasma concentration. Various factors influence drug distribution, such as the lipophilicity and ionization of the drug, tissue binding, binding to plasma proteins such as albumin and alpha 1-acid glycoprotein, and body composition.[1] Only the unbound fraction of a drug can exert a pharmacological effect. Besides passive diffusion across cell membranes, carrier-mediated transport is an important mechanism for drug disposition, facilitating the transport of endogenous compounds and xenobiotics into and out of

cells. Drug elimination occurs through excretion of chemically unchanged drug mainly via the kidneys or through metabolism by drug-metabolizing enzymes followed by excretion. The efficacy of drug elimination is described by clearance (CL) which is expressed as the volume of blood cleared for drug per unit of time.

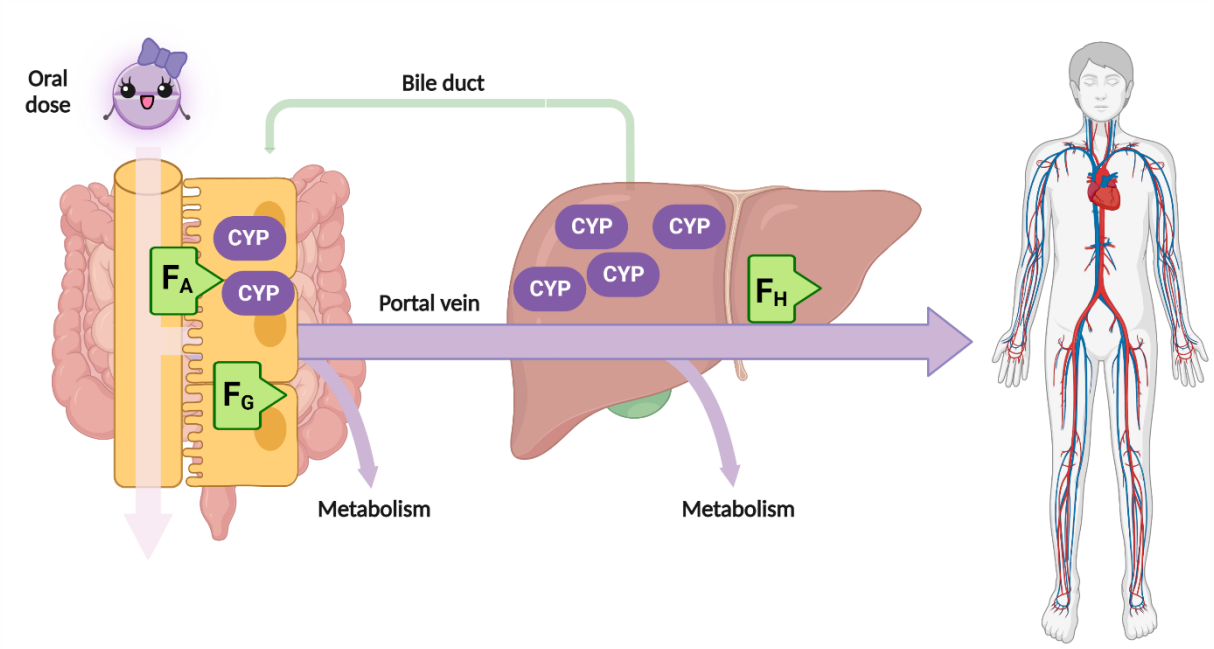

Figure 1. The oral bioavailability (F) of a drug is expressed as $F = F_A \times F_G \times F_H$. The fraction absorbed from the gut lumen to the enterocytes is denoted F_A, F_G denotes the fraction escaping first-pass metabolism in the gut wall, and F_H denotes the fraction escaping first-pass hepatic metabolism. Created with BioRender.com

Abbreviations: CYP, cytochrome P450

1.3 Drug metabolism

Most drugs undergo biotransformation into more hydrophilic compounds to facilitate elimination from the body. Drug metabolizing reactions can be classified into two phases. Primarily, phase I reactions change the chemical structure of a drug through oxidation, reduction, or hydrolysis, resulting in a more hydrophilic substance that is less active than the parent drug or inactive, but phase I reactions also activate prodrugs into metabolic active substances.[1] The phase II reactions involve conjugation of the drug or a phase I metabolite with an endogenous hydrophilic substance such as glucuronide acid, sulfate, or glutathione.

1.3.1 Cytochrome P450 enzymes

The cytochrome P450 (CYP) superfamily is the major enzyme family catalyzing phase I reactions. The superfamily comprises heme-containing enzymes and is essential for the metabolism of xenobiotic compounds and endogenous compounds such as steroids, bile acids, and fatty acids.[4] The enzymes are mainly membrane-bound proteins localized in the endoplasmic reticulum and to a lesser extent in the cell's mitochondria,[5] that catalyze a number of oxidative reactions and some reduction reactions.[6] The highest abundance of CYP enzymes is found in the liver, the primary drug-metabolizing organ, and the small intestine, but the enzymes are also present in other organs, such as the kidneys, lungs, brain, skin, ovary, and testes.[7, 8] The CYP superfamily is grouped into families designated by a number (e.g. CYP2) and subfamilies designated by a capital letter (e.g. CYP2C).[9] For members within the same family and subfamily, the amino acid sequence is >40% and >55% identical, respectively.[9] Isoenzymes within the subfamilies are further specified by numbers (e.g. CYP2C9).[9]

The CYP superfamily contributes to the metabolism of up to approximately 75% of clinically used drugs.[10-12] In humans, the superfamily comprises 57 functional isoforms,[13] but only a small number of these, including the CYP1, CYP2, and CYP3 family, are found to contribute significantly to drug metabolism.[14] Among the isoforms particularly important for drug metabolism are CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/5.[10] The CYP3A subfamily comprises CYP3A4, CYP3A5, CYP3A43, and CYP3A7,[15] and is considered the single most important subfamily of drug-metabolizing enzymes, accounting for around 40% of CYP-mediated drug metabolism.[11] With its large and flexible active site,[16] CYP3A4 is the main contributor to CYP3A-mediated drug metabolism.[10] Due to its high abundance in the small intestine, in addition to the liver,[17] CYP3A4 plays a significant role in the first-pass metabolism of substrate drugs. CYP3A5 has a similar substrate specificity to CYP3A4, with an 84% overlap in the amino acid sequence,[18] but is generally less abundant than CYP3A4.[19, 20] CYP3A7 is the dominant isoform in fetal and newborn liver,[21] but in adults, the contribution from both CYP3A7 and CYP3A43 is considered to be negligible.[22] The CYP2C subfamily includes CYP2C8, CYP2C9, CYP2C18, and CYP2C19,[23] of which CYP2C9 and CYP2C19 are the major isoforms.[10, 11] CYP2C9 and CYP2C19 metabolize a wide range of drugs due to their different substrate specificity, although the isoforms share 91% amino acid sequence homology.[24] CYP1A2 and CYP2D6 are the only isoforms involved in drug metabolism in the CYP1A and CYP2D subfamily, respectively.[12, 25]

CYP expression in the liver and intestine

The content of various CYP isoforms in human liver microsomes (HLMs) is diverse and was already described by Shimada et al. almost thirty years ago.[26] The authors reported that CYP3A and CYP2C had the highest abundance, accounting for approximately 30% and 20% of the total hepatic CYP content, respectively, followed by CYP1A2 (~13%), CYP2E1 (~7%), CYP2A6 (~4%), CYP2D6 (~2%), and CYP2B6 (<1%).[26] A more comprehensive characterization of the hepatic CYP content was described in a meta-analysis by Achour et al. in 2014, summarizing previous studies on the abundance of CYP enzymes in adult Caucasian livers.[19] On average, CYP3A4 accounted for 25% of the hepatic CYP content, CYP3A5 represented 5%, while CYP3A7 and CYP3A43 were less abundant (<3%). However, Kuehl et al. reported that CYP3A5 might represent up to 50% of hepatic CYP3A in individuals expressing at least one functional allele (*CYP3A5*1*) (for genetics, see section 1.4).[27] Even though CYP3A4 is abundantly expressed in the liver, considerable interindividual variability in hepatic CYP3A4 expression has been reported (>100-fold).[12] Achour et al. also described the hepatic CYP2C family more thoroughly, in which CYP2C9 was reported as the most abundant isoform (~16%), followed by CYP2C8 (~6%) and CYP2C19 (~3%).[19] Further, CYP2E1 seems to be more abundantly expressed than first suggested by Shimada et al.[19, 28]

In the intestinal enterocytes, CYP3A4 and CYP2C9 are the most abundant isoforms representing approximately 80% and 15% of the total CYP content in the small intestine, respectively.[17, 20, 28] Other significant drug-metabolizing isoforms expressed at lower levels in the intestine include CYP2C19 and CYP2D6.[17, 20, 28] There is also substantial interindividual variability in intestinal CYP isoforms, especially CYP2D6.[17, 20, 29] Similarly to the liver, the expression of intestinal CYP3A5 seems to be low in homozygote carriers of the *CYP3A5*3* allele (<3%),[20, 28] but the content may reach values observed for the liver in individuals expressing at least one *CYP3A5*1* allele.[17] For the majority of CYP enzymes, the expression is highest in the proximal part of the intestine, i.e. duodenum and jejunum, and lower in the more distal sections.[30] The distribution of CYP3A, however, seems to be more homogenous along the intestine.[20, 28]

1.4 Factors influencing CYP-mediated metabolism

1.4.1 Genetics

The genetic influence on CYP enzymes has been studied in great detail and is recognized as an important source of interindividual variability in CYP-mediated metabolism. Genetic polymorphism can cause both decreased and increased enzyme function, and based on genotype-predicted-phenotype, individuals may be categorized as extensive (normal), poor, intermediate, or rapid/ultrarapid metabolizers. Genetic polymorphism is one of the major causes of interindividual variability in CYP2C9, CYP2C19, and CYP2D6 phenotypes. So far, more than 60 CYP2C9 variant alleles have been identified,[31] of which the reduced function alleles *CYP2C9*2* (*rs1799853*) and *CYP2C9*3* (*rs1057910*) are the most frequently expressed with allele frequencies of 11.7% and 5.6% in Europeans, respectively.[32] *CYP2C9*2* is characterized by a 430C>T exchange in exon 3, causing an Arg144Cys amino acid substitution, whereas *CYP2C9*3* is characterized by a 1075A>C exchange in exon 7, resulting in an Ile359Leu amino acid substitution.[33] Both mutations result in decreased enzyme activity. Hence, individuals expressing these variant alleles may require lower doses of CYP2C9 substrate drugs such as warfarin.[34] The other CYP2C9 variant alleles are rare in the Caucasian population, and *CYP2C9*5, *6, *8, *9,* and **11* are almost exclusively expressed in the African population.[32] The most common CYP2C19 variant alleles include *CYP2C19*2 (rs4244285), CYP2C19*3 (rs4986893),* and *CYP2C19*17 (rs12248560).*[32] The *CYP2C19*2* and **3* alleles carry polymorphism at 681G>A in exon 5, causing a splicing defect, and at 636G>A in exon 4, producing a premature stop codon, respectively, both resulting in a non-functional enzyme.[35, 36] While *CYP2C19*3* is almost exclusively restricted to East Asians and native Oceanians, *CYP2C19*2* is prevalent in all populations (approximately 15% of Europeans).[32, 37] *CYP2C19*17* however, which is characterized by two single nucleotide polymorphisms (SNP) in the 5'-flanking region of the gene (-806C>T and -3402C>T) that mediates increased gene transcription, is associated with enhanced CYP2C19 enzyme activity[38] and more than 20% of Europeans express this allele.[32, 37] Recently, a novel CYP2C haplotype (*CYP2C:TG*) associated with increased clearance of the CYP2C19 substrate escitalopram was discovered.[39] CYP2D6 phenotype depends largely on CYP2D6 genotype,[40-42] and a wide spectrum of variant alleles have been described ranging from non-functional alleles to alleles with increased function.[42] Among the most important CYP2D6 alleles are the normal functioning **2,* the non-functional **4, *5, *10,* and the reduced functioning **17* and **41*.[42] The most frequent non-functional allele

in Europeans is *CYP2D6*4 (*1846G>A; *rs3892097).*[32] Studies have shown that CYP2D6 poor metabolizers are at greater risk for adverse events during treatment with CYP2D6 substrate drugs such as metoprolol, aripiprazole, or risperidone.[43-45]

In contrast, genetic factors seem to be of limited importance for the substantial variability in CYP3A4 phenotype.[46, 47] Even though the SNP in intron 6 of CYP3A4 (c.522-191C>T; *rs35599367; CYP3A4*22*) is associated with a lower expression and activity of CYP3A4,[48-53] only approximately 5% of Europeans express this allele.[32] Nevertheless, individuals expressing the *CYP3A4*22* allele may require dose adjustments of selected drugs.[54] Variability in CYP3A5-mediated metabolism, however, is to a large extent explained by genetic polymorphism. The non-functional allele *CYP3A5*3* (6986A>G; *rs776746*) is the most common allele in Europeans (~94%), admixed Americans (~80%), and Asians (~70%).[32] In this allele, a SNP in the third intron causes an alternatively spliced mRNA variant, and protein truncation results in a non-functional enzyme.[27] With respect to *CYP3A5**1, individuals with at least one functional allele may require higher doses of drugs metabolized by CYP3A, such as tacrolimus, compared with CYP3A5 non-expressers.[55, 56]

Genetic factors are considered to be of limited importance for variability in CYP1A2-mediated metabolism. Nevertheless, upon exposure to specific inducers such as smoking, *CYP1A2*1F* (*rs762551*) represents an inducible allele associated with increased CYP1A2 activity.[57] More than 60% of Caucasians express *CYP1A2*1F*,[32] which is characterized by the -163C>A substitution in intron 1.[57]

1.4.2 Disease state

The current literature indicates that CYP3A-mediated metabolism is decreased in chronic conditions such as obesity, type 2 diabetes mellitus (T2DM), human immunodeficiency virus (HIV), cancer, nonalcoholic fatty liver disease (NAFLD), and rheumatoid arthritis.[58-64] Previous studies have also observed a downregulation in CYP2C19-mediated metabolism in patients with cancer,[65, 66] and CYP2D6-mediated metabolism in patients with HIV.[67, 68] Metabolism mediated by various CYP enzymes also appears to be influenced by acute inflammation.[69-71] Recently, Lenoir et al. reported that CYP1A2, CYP2C19, and CYP3A activities decreased by ~52%, ~75%, and ~23%, respectively, during severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, whereas CYP2B6 and CYP2C9 activities

increased by ~101% and ~56%, respectively.[70] In later years, inflammation has been recognized as a major factor for altered activity and expression of several CYP enzymes.[72, 73]

Inflammation is an essential immune response that is defined as the presence of pain, redness, and swelling resulting from a response to stimuli, such as pathogens, damaged cells, or irritants.[73] During this complex process, intracellular signaling pathways are activated, and inflammatory mediators such as cytokines and chemokines are produced and released.[73] Interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) are considered key pro-inflammatory cytokines that mediate inflammation through activation of Toll-like receptors, IL-6 receptor, and TFN receptors (TNFR), respectively.[74] This triggers intracellular signaling pathways such as the nuclear factor-kappa B (NF-κB), Janus kinase (JAK)/signal transducer and activator of transcription protein-3 (STAT3), and mitogen-activated protein kinase (MAPK), causing transcription of genes encoding the majority of inflammatory cytokines.[74] Several *in vitro* studies have reported an altered activity and/or expression of CYP enzymes in response to inflammatory cytokines. Aitken et al. showed that IL-1β, IL-6, and TNF-α downregulated CYP3A4 mRNA expression in human hepatocytes, whereas only IL-6 downregulated CYP2C9 and CYP2C19.[75] In fact, several studies have reported a decreased CYP3A4 mRNA expression and activity in human hepatocytes or HepaRG cells upon IL-6, IL-1β, or TNF-α exposure.[76-78] These studies also indicated decreased CYP1A2, CYP2C9, and CYP2C19 mRNA expressions. However, it appears that higher levels of inflammatory cytokines are required to downregulate the expression of these isoforms compared with CYP3A.[79]

Suggested mechanistic pathways for CYP downregulation

One of the principal mechanisms in which inflammation modulates CYP enzymes appears to be altered gene transcription mediated by specific cytokines (**Figure 2**).[79] This may occur through transcriptional downregulation of nuclear receptors such as the PXR or the constitutive androstane receptor (CAR), which are identified as key transcriptional regulators of CYP2C9, CYP2C19, and CYP3A expression.[79] This has been suggested because IL-6 and IL-1β have been observed to decrease the expression of PXR and CAR mRNA in human hepatocytes.[80, 81] It has also been hypothesized that activation of the NF-κB pathway may alter the activity of PXR or CAR through changes in dimerization or translocation.[79] The transcriptional activity of the aryl hydrocarbon receptor, which is the main regulator of CYP1A2 expression, has also been shown to be inhibited by NF-κB.[82] Other suggested mechanisms include a direct

modulation by NF-κB through interaction with the CYP promotor region, altered liver-enriched CCAAT-enhance-binding protein (C/EBP) signaling, and post-transcriptional mechanisms in which microRNA activity regulates CYP mRNA and protein concentration.[79]

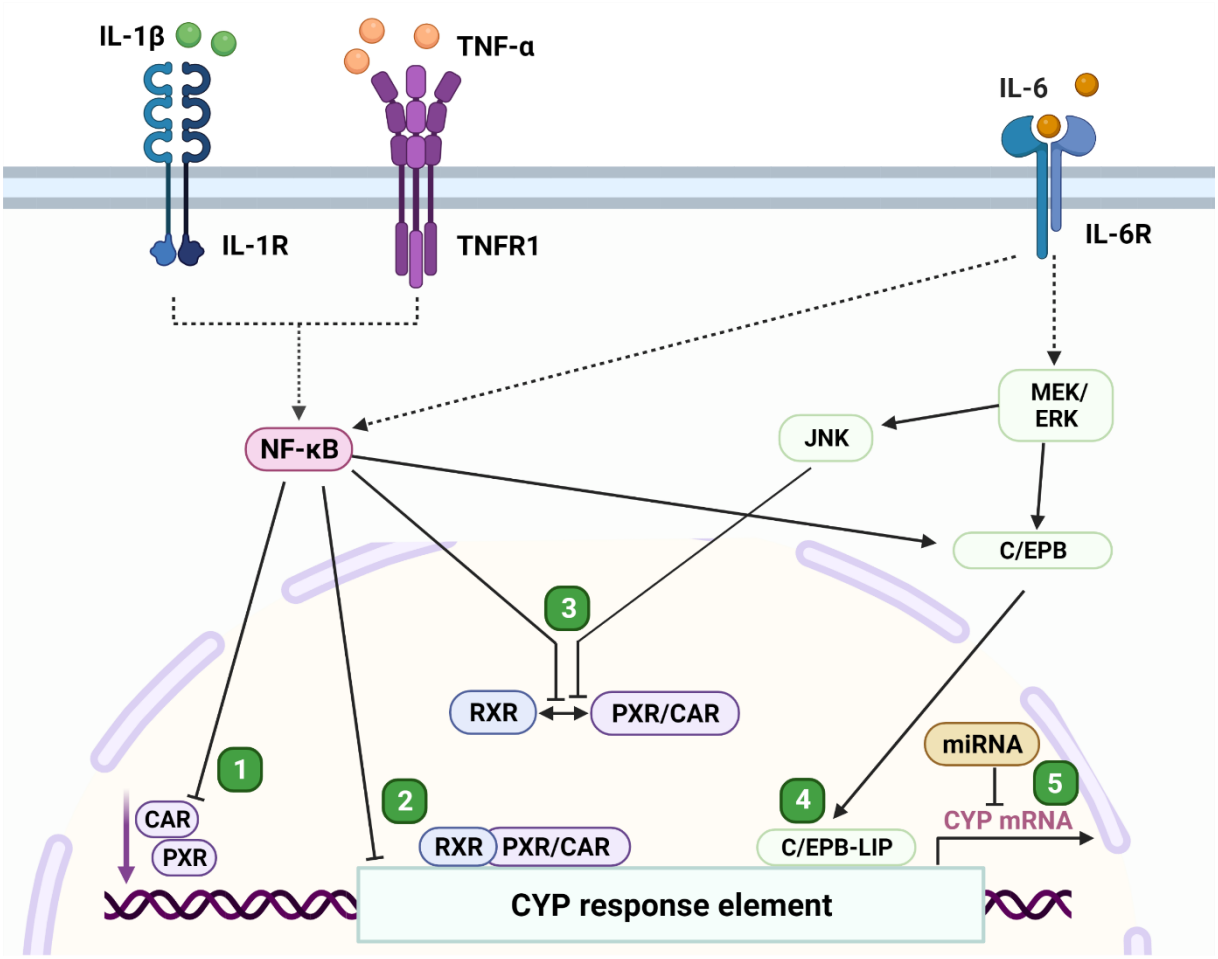

Figure 2. Suggested mechanistic pathways for downregulation of CYP activity and expression during inflammation. 1) Transcriptional downregulation of nuclear receptors such as PXR and CAR; 2) direct modulation through interaction with the CYP promotor region, 3) altered activity of nuclear receptors through changes in dimerization or translocation, 4) altered signaling of liver-enriched C/EBP, or 5) post-transcriptional mechanisms. Modified from de Jong et al.[79] Created with BioRender.com

Abbreviations: CAR, constitutive androstane receptor; C/EPB-LIP, CCAAT-enhance-binding protein-liver inhibiting protein; CYP, cytochrome P450; ERK, extracellular signal-regulated kinase; IL, interleukin; JNK, Jun-N-terminal kinase; MEK, mitogen-activated protein kinase kinase; miRNA, MicroRNA; NF-κB, nuclear factor-kappa-B; PXR, pregnane X receptor; RXR, retinoid X receptor; TNF-α, tumor necrosis factor-α, TNFR1; tumor necrosis factor receptor 1.

1.4.3 Environmental factors

Many environmental factors, including drug-drug interactions, have been shown to induce or inhibit CYP-mediated metabolism, although the clinical significance varies. In a study by Fichtenbaum et al., the systemic exposure of the CYP3A substrate simvastatin increased 30-fold during concomitant treatment with the protease inhibitors ritonavir and saquinavir,[83] illustrating that drug-drug interactions may lead to considerable changes in systemic exposure of drugs. Grapefruit juice is another potent inhibitor, reducing the first-pass metabolism of substrate drugs through inhibition of intestinal CYP3A4.[84] The herbal medicine St. John's wort, is a potent CYP3A4 inducer that activates pregnane X receptor (PXR) and thus transcription of CYP3A4.[85] In a case report by Ruschitzka et al., the authors reported that St. John's wort caused acute transplant rejection due to a decrease in the systemic exposure of the CYP3A substrate cyclosporine.[86] Smoking induces CYP1A2 through binding of the polycyclic aromatic hydrocarbons from cigarette smoke to the aryl hydrocarbon receptor causing transcriptional activation of the *CYP1A2* gene.[87, 88]

1.4.4 Age and sex

Generally, the existing literature on the impact of age and sex on various CYP enzymes is ambiguous. In a review article by Schwartz, it was concluded that CYP2D6-mediated metabolism was decreased in older patients compared with those of younger age, and in females compared with males.[89] In contrast, studies have reported an increased CYP3A-mediated metabolism in females compared with males.[90, 91] However, other studies have not found any significant sex-related differences in CYP3A.[92, 93] The literature is also inconsistent with respect to the impact of age on CYP3A, with reports of both decreased and unaltered CYP3A-mediated metabolism in older patients.[89, 94] CYP1A2-mediated metabolism has been found to be higher in males than in females[95, 96] and similar between the genders.[97] The impact of age and sex on CYP2C9- and CYP2C19-mediated metabolism are unclear.[89, 96]

1.5 Metrics to determine CYP activities

Phenotyping is used to determine the individual activity of a target CYP isoform. This is of high interest from a clinical perspective to evaluate sources of interindividual variability, such as changes in enzyme activity during different disease states or following an intervention (e.g. bariatric surgery), but the approach is also useful to quantify drug-drug interactions during the development of new drugs. Individual phenotype is most often assessed using probe drugs, but specific endogenous substances can also be used. An advantage of probe drugs is that they may be administered in combination, often referred to as the cocktail approach, allowing for simultaneous assessment of multiple CYP activities.[98] Ideally, probe drugs should exclusively be metabolized by the target CYP isoform, so that clearance of the probe drug corresponds directly to the metabolic capacity, i.e. intrinsic clearance (CL_{int}) of that enzyme. To date, however, no ideal probe drug exists and usually, the pharmacokinetics of currently available probe drugs also depends on other pharmacokinetic processes.

CYP3A metrics

There are several metrics available to assess CYP3A phenotype, in which midazolam, a short-acting benzodiazepine, is currently recognized as the gold standard method for assessing CYP3A phenotype *in vivo*.[91, 99, 100] Midazolam is almost exclusively metabolized by CYP3A to the major metabolite 1'-hydroxymidazolam, and to a minor extent, to 4-hydroxymidazolam.[101, 102] Although midazolam is usually considered a probe drug for the combined CYP3A4 and CYP3A5 activities, there have been studies suggesting a minor contribution of CYP3A5 to the hydroxylation of midazolam *in vivo*.[103-105] There are also some controversies to which extent midazolam clearance reflects hepatic CYP3A activity in all patient populations, given its highly variable extraction ratio, ranging from 0.32 to 0.96 (mean of 0.55) in the literature.[106-108] Another CYP3A metric that was first suggested by Bodin et al. in 2001 is the endogenous biomarker 4β-hydroxycholesterol (4βOHC).[109] CYP3A4 accounts for most of the cholesterol formation to 4βOHC, whereas data on the contribution from CYP3A5 is more conflicting.[47, 110-113] The current literature indicates that 4βOHC concentrations reflect interindividual variability in CYP3A activity, and it also appears that 4βOHC is a useful biomarker to identify CYP3A induction.[114, 115] However, there are some challenges associated with phenotyping using 4βOHC, such as that it is not yet known which role intestinal CYP3A has in the formation of 4βOHC,[115, 116] and the long elimination half-life of ~17 days may limit the ability to detect acute changes in CYP3A activity.[117]

Probe drugs for CYP1A2, CYP2C9, and CYP2C19 phenotyping

Caffeine is considered the preferred probe to study CYP1A2 phenotype.[98, 100, 118] The metabolism of caffeine involves several enzymes, but the main route is through N-3 demethylation to paraxanthine mediated by CYP1A2.[119]An advantage of caffeine is that it is safe and easily available. However, because it is found in several foods and beverages, caffeine intake should be avoided for 36 hours before and during phenotyping studies.[118] Several probe drugs have been suggested for CYP2C9 phenotyping, including tolbutamide, flurbiprofen, warfarin, and losartan.[99, 120] Tolbutamide is the best validated probe drug for CYP2C9 phenotyping, but there have been concerns about hypoglycemia and limited availability.[120, 121] A safer alternative that has been included in validated cocktail approaches is losartan.[122, 123] Losartan is primarily metabolized by CYP2C9, and partly by CYP3A4, to the active metabolite losartan carboxylic acid (LCA; E-3174), with a transformation rate around 3-4 times greater for CYP2C9 than CYP3A4.[124] Traditionally, mephenytoin has been the standard probe drug to assess CYP2C19 activity, but due to concerns about safety and sample stability, omeprazole has emerged as the recommended probe drug.[98, 125-127] Omeprazole is mainly metabolized to 5-hydroxyomeprazole (5-OH-omeprazole) by CYP2C19, but also partly to omeprazole sulphone by CYP3A4.[128] However, the affinity for CYP3A4 is around 10 times lower than for CYP2C19.[128] There are concerns about how reliable omeprazole is for exact quantification of CYP2C19 activity, particularly because of variable absorption.[127] Due to the enteric-coated formulation of oral omeprazole, the absorption may have considerable and unpredictable lag time. Other limitations include that an unknown fraction of omeprazole is subjected to first-pass metabolism in the gut wall and that omeprazole is a substrate for the efflux transporter P-glycoprotein.[127] Nevertheless, to date it does not appear to be any significantly better alternatives for CYP2C19 phenotyping.

1.6 Pharmacokinetic variability in selected patient populations

1.6.1 Obesity

Obesity, defined as abnormal or excessive accumulation of adipose tissue that may impair health,[129] is a global epidemic.[130] Individuals with a body mass index (BMI) greater than or equal to 30 kg/m^2 are classified as obese, whereas severe obesity is classified as BMI ≥40 kg/m^2 or BMI ≥35 kg/m^2 when in combination with at least one obesity-related comorbidity.[131] In Norway, the prevalence of obesity in the adult population is approximately 25%.[132, 133] The

main cause of obesity is long-term, positive energy balance, however, the pathogenesis of obesity is multifactorial and involves a complex interplay between environmental, physiological, socioeconomic, and genetic factors.[131] The pathophysiology is characterized by low-grade, chronic inflammation of the adipose tissue and elevated circulating levels of proinflammatory cytokines such as IL-6, TNF-α, IL-1β, and interferon (IFN)-γ, and adipokines such as leptin.[134-136] The altered production and release of cytokines are related to an accumulation of pro-inflammatory M1-polarized macrophages in the adipose tissue.[134] Obesity is a major risk factor for T2DM and coronary heart disease,[137-139] but is also associated with a number of other diseases such as certain types of cancer, osteoarthritis, and NAFLD[140-143] leaving patients with obesity often in need of pharmacological treatment.

Pharmacokinetics in patients with obesity

The pharmacokinetics of drugs may be altered in patients with obesity due to a wide range of physiological alterations. In general, it appears that CYP-mediated metabolism is altered in an isospecific manner,[144, 145] possibly due to elevated levels of inflammatory cytokines.[79] A number of studies have investigated *in vivo* CYP3A activity in patients with obesity using various CYP3A probe drugs, including alprazolam, triazolam, midazolam, carbamazepine, dextrorphan, and cyclosporine. In these studies, patients with obesity had lower oral or systemic clearance than non-obese, suggesting a decreased CYP3A activity in obesity, although the difference did not reach statistical significance in all studies.[146-150] Additionally, *in vitro* and *in vivo* studies have shown an inverse correlation between BMI or body weight and CYP3A activity.[151-154] A few years ago, Brill et al. determined absolute bioavailability and systemic clearance of midazolam following semi-simultaneous oral and intravenous administration and reported that patients with obesity had significantly higher absolute bioavailability than normal-weight individuals, while systemic clearance was similar.[62] The authors speculated that a higher liver volume in patients with obesity may have compensated for a decreased hepatic CYP3A activity, thus resulting in a similar systemic clearance in the two groups. Later, the authors used a semiphysiologically based pharmacokinetic model to estimate hepatic CL_{int} and found that it was lower in the patients with obesity compared with normal-weight individuals.[155] Obesity is characterized by increased blood volume and cardiac output, and probably also increased blood flow to perfuse the gut and liver.[144] Thus, patients with obesity may have an increased clearance of drugs with an intermediate or high extraction ratio, as such drugs are partly or primarily dependent on hepatic blood flow. In fact, studies have shown an increased clearance of drugs with a high extraction ratio such as fentanyl and propofol in patients with obesity.[156-158] Given

that midazolam clearance is partly dependent on hepatic blood flow,[107, 155] a reduced hepatic CYP3A activity in patients with obesity in the study by Brill et al.[62] may have been compensated by an increased hepatic blood flow.[144] With respect to CYP1A2, CYP2C9, CYP2C19, and CYP2D6, these CYP activities appear to be similar or slightly increased in patients with obesity compared with patients without obesity,[145, 148, 159] but the existing literature is sparse. In contrast, CYP2E1 activity is found to be increased in patients with obesity.[160, 161] Besides altered CYP-mediated metabolism, the overall oral drug absorption may also be influenced by an increased gut wall permeability and accelerated gastric emptying.[144, 162] The apparent volume of distribution may also change depending on the drug's physicochemical properties, but these changes appear to be difficult to predict upfront.[144] Plasma proteins such as albumin do not seem to be significantly altered in patients with obesity,[144] although levels of alpha 1-acid glycoprotein may be increased.[163]

1.6.2 Nonalcoholic fatty liver disease

NAFLD is defined by the presence of hepatic steatosis either by histology or imaging after the exclusion of secondary causes of hepatic fat accumulation such as significant alcohol consumption, hereditary disorders, or long-term use of drugs that can cause hepatic steatosis.[164] It is a spectrum of liver disease that can be categorized histologically into nonalcoholic fatty liver (NAFL) or nonalcoholic steatohepatitis (NASH). NAFL is characterized by the presence of ≥5% hepatic steatosis without evidence of hepatocellular injury, whereas in NASH inflammation with hepatocyte injury, with or without fibrosis, is also present in addition to ≥5% hepatic steatosis.[164] NAFLD is recognized as the most frequent liver disease worldwide, and the global burden is rapidly increasing with a prevalence approaching 30%.[165, 166] The prevalence is even higher in patients with obesity, and Machado et al. reported that steatosis was present in approximately 90% of patients with severe obesity submitted to bariatric surgery.[167] NAFLD often coexists with T2DM, and the relationship seems to be bidirectional.[168] T2DM is recognized as a risk factor for the faster progression of NAFLD. However, NAFLD is also associated with an increased risk of developing T2DM.[168]

Pharmacokinetics in patients with NAFLD

Even though the liver is the major site for drug metabolism, the current knowledge about the effect of NAFLD on CYP-mediated metabolism *in vivo* is limited.[169] Data from *in vitro* studies indicate that NAFLD is associated with decreased hepatic expression and activity of CYP1A2,

CYP2C19, CYP3A4/5, and CYP2D6, although not all findings were statistically significant.[61, 170-174] The current literature is inconsistent with respect to CYP2C9 and CYP2E1, with reports of both increased and decreased expressions and activities in NAFLD.[170, 171, 173, 174] In the study by Jamwal et al., CL_{int} values for CYP3A4 were 2.7-fold and 4.1-fold lower in livers from donors with NAFL and NASH, respectively, compared with normal donors.[61] Woolsey et al. were the first to report that *in vivo* hepatic CYP3A activity is decreased in patients with NAFLD compared with healthy controls.[60] The authors hypothesized that the effects of inflammation and elevated cytokine levels on hepatic drug metabolism gene expression were involved in the downregulation of CYP3A4 activity in patients with NAFLD.[60] Another factor that may influence the pharmacokinetics of drugs dependent on blood flow is that hepatic blood flow may be decreased over time, particularly in patients with NASH or liver cirrhosis.[175]

1.6.3 Type 2 diabetes mellitus

T2DM is a chronic metabolic disease characterized by insulin deficiency due to insulin resistance and impaired β-cell function,[134, 176] that is also rapidly increasing worldwide.[177, 178] Obesity is considered the main preventable cause of T2DM,[134, 179] and 60% of patients with T2DM are patients with obesity.[176] The prevalence of NAFLD in patients with T2DM is also very high, and Younossi et al. recently reported a global prevalence of almost 60%, with the highest prevalence in Europe (~68%).[180] In later years, inflammation has been recognized to have an important role in the pathophysiology of T2DM.[181] The mechanisms for insulin resistance and β-cell dysfunction are complex, however, excessive levels of glucose and free fatty acids appear to promote the production and release of several cytokines and chemokines such as IL-1β, TNF-α, CC-chemokine ligand (CCL) 2, and CCL3.[182]

Pharmacokinetics in patients with T2DM

It seems that patients with T2DM often have a variable and different drug response than other patient populations, possibly due to changes in the expressions and activities of CYP enzymes. In an *in vitro* study by Dostalek et al., hepatic CYP3A4 activity and expression were significantly lower in diabetic HLMs compared with non-diabetic HLMs.[183] Furthermore, Jamwal et al. found that microsomal CYP3A4 activity was lower in HLMs from donors with NAFLD and diabetes compared with HLMs from donors with NAFLD only.[61] *In vivo* studies have also suggested a decreased CYP3A activity in various patient populations with diabetes.[184-186] However, it is difficult to draw conclusions from these studies, as they were not

designed to show such an effect and did not use validated probe drugs to study CYP3A phenotype. The literature also indicates that CYP2C19 activity may be decreased in patients with T2DM, given that patients with T2DM appear to have an inadequate effect of clopidogrel, a prodrug which to a large extent is activated by CYP2C19.[187-189] Recently, Gravel et al. investigated the impact of T2DM on different CYP activities *in vivo* using oral probe drugs in patients with and without T2DM.[59] The authors reported that low-grade inflammation associated with T2DM altered CYP activities in an isoform-specific manner and that CYP2B6, CYP2C19, and CYP3A activities were 45%, 46%, and 38% lower in patients with T2DM. CYP1A2 and CYP2C9 appeared to be slightly increased in patients with T2DM but did not reach statistical significance, while CYP2D6 and CYP2E1 were similar in the two groups. Urry et al. also reported increased CYP1A2 activity in patients with T2DM compared with patients without T2DM.[190] Elevated levels of circulating cytokines have been hypothesized to play a role in the modulation of CYP enzymes in patients with T2DM.[59, 191] Nevertheless, a major limitation of the existing literature is that other factors closely linked to T2DM such as obesity and NAFLD have not been accounted for when the impact of T2DM on different CYP activities has been investigated, thereby complicating the interpretation of previous findings.

1.6.4 Bariatric surgery

Bariatric surgery has emerged as the principal treatment option for the majority of patients with severe obesity and a high risk for obesity-related comorbidity and mortality. In terms of achieving long-lasting weight loss and improvement of comorbidities, bariatric surgery is superior compared with nonsurgical treatment.[192-194] Recent studies have also suggested that bariatric surgery is an effective treatment for T2DM that should be considered in the management of patients with T2DM and obesity.[179, 195, 196] This practice is commonly referred to as metabolic surgery.[197] Among the most performed bariatric procedures are sleeve gastrectomy followed by Roux-en-Y gastric bypass (RYGB).[198, 199] During RYGB, a small gastric pouch (~20–30 mL) from the proximal stomach is anastomosed with the mid-jejunum, creating the 75 to 150 cm Roux limb.[200, 201] Thereby, ingested nutrients bypass the majority of the stomach, duodenum, and proximal jejunum.[200] The biliopancreatic limb, which allows for drainage of bile acids and pancreatic secretions, is reconnected to the distal jejunum to create a common channel of the remaining small intestine.

Pharmacokinetics after bariatric surgery

RYGB leads to anatomical and physiological alterations in the gastrointestinal tract that may change various factors influencing pharmacokinetics.[202] It has been hypothesized that first-pass metabolism of drugs may decrease following surgery, given that the proximal small intestine rich in CYP enzymes is bypassed, placing drugs directly into the more distal part of the intestine with less metabolic capacity.[202] Thus, oral bioavailability may increase after RYGB. This is particularly relevant for CYP3A substrate drugs due to the high abundance of CYP3A enzymes in the duodenum and proximal jejunum.[20, 28] Depending on the physicochemical properties of a drug, other factors such as increased gastric pH, altered gastric emptying time, altered gastrointestinal transit time, or the removal of a large part of the total surface area available for drug absorption may also influence the fraction absorbed.[203] Several studies have reported higher maximum concentration (C_{max}) and shorter time to reach C_{max} (T_{max}) for various substrates post-RYGB,[159, 204-206] suggesting a faster absorption of orally administered drugs in patients subjected to gastric bypass. However, these findings are not consistent[207-210] suggesting that the rate and extent of drug absorption following RYGB is substrate-dependent.

Besides the gastrointestinal alterations, the subsequent weight loss and improvement of comorbidities such as T2DM, NAFLD, and inflammation status after surgery may also influence pharmacokinetics.[192, 211-214] Brill et al. observed an increased systemic midazolam clearance one year after bariatric surgery, and suggested that hepatic CYP3A activity had recovered due to decreased inflammation status.[206] The authors found no change in the absolute bioavailability of midazolam. However, they hypothesized that this might be explained by the fact that F_G increased due to the bypass and that the extent was similar to the decrease in $F_{H,}$ thus resulting in a similar bioavailability as before surgery. Later, using a semiphysiologically based pharmacokinetic model, the same authors estimated that hepatic CL_{int} for CYP3A was 1.5 times higher post-bariatric surgery.[155] The findings by Rodríguez-Morató et al. and Puris et al. support an increased CYP3A activity after RYGB or sleeve gastrectomy.[148, 207] The current literature also suggests that CYP2C19 activity increases after RYGB,[204, 215] although Puris et al. found no difference in CYP2C19 activity before and after RYGB.[207] CYP1A2 activity appears to temporarily decrease in the early period after RYGB,[148] and then recover in the long-term perspective.[148, 207] Bariatric surgery does not seem to influence CYP2C9 and CYP2D6 activities.[148, 207]

There is growing evidence on the impact of bariatric surgery on pharmacokinetics. However, general dosing recommendations are still lacking, and it remains uncertain whether pharmacokinetic changes after RYGB are due to the surgery *per se* or the subsequent weight loss. Given that the number of patients subjected to bariatric surgery is increasing and that clinicians will increasingly be confronted with how to dose these patients, more knowledge is warranted. Pharmacokinetic changes focusing on CYP enzymes in obesity, NAFLD, T2DM, RYGB, and weight loss based on the current literature are summarized in **Figure 3**.

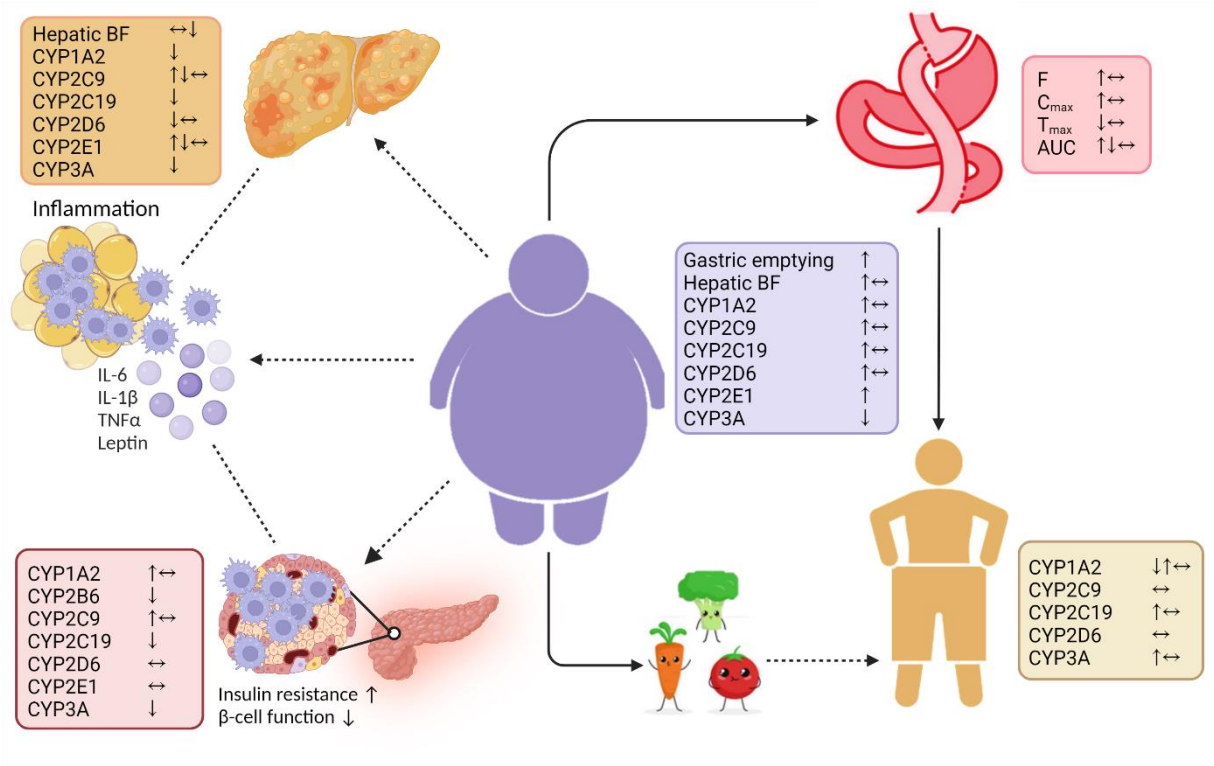

Figure 3. Summary of pharmacokinetic changes focusing on CYP enzymes in obesity, nonalcoholic fatty liver disease, type 2 diabetes mellitus, Roux-en-Y gastric bypass, and weight loss based on the current literature. The arrows indicate an increase (↑), decrease (↓), or no change (↔) in the activity and/or expression of various CYP enzymes. Created with BioRender.com

Abbreviations: AAG, alpha 1-acid glycoprotein; AUC, area under the curve; BF, blood flow; C_{max}, maximum plasma concentration after oral dosing; CYP, cytochrome P450; F, oral bioavailability; IL, interleukin; T_{max}, time to reach C_{max}; TNF-α, tumor necrosis factor-α

2 AIMS OF THE PRESENT STUDIES

The overall aim of this thesis was to investigate pharmacokinetic variability in selected patient populations. The focus was on CYP1A2-, CYP2C9-, CYP2C19-, and CYP3A-mediated metabolism in patients with obesity subjected to RYGB or non-surgical calorie restriction (**Papers I and II**) and in patients with obesity with and without T2DM (**Paper III**). Due to substantial intraindividual variability in the absolute bioavailability and systemic clearance of midazolam over a short period of time in the patients with obesity following weight loss in **Paper I**, we additionally investigated intraindividual variability in absolute bioavailability and systemic clearance of midazolam in healthy volunteers (**Paper IV**). The endogenous biomarker 4βOHC was also evaluated against hepatic and intestinal CYP3A4 protein expression, *ex vivo* microsomal CYP3A4 activity in paired liver and jejunum samples, and *in vivo* midazolam absolute bioavailability and systemic clearance (**Paper V**).

The main objectives were as follows:

- Investigate the effect of body weight, weight loss, RYGB, and T2DM on CYP1A2, CYP2C9, CYP2C19, and CYP3A phenotypes (**Papers I, II,** and **III**)

- Assess intraindividual variability in absolute bioavailability and systemic clearance of midazolam in healthy individuals over a 2-month period (**Paper IV**)

- Evaluate 4βOHC as a metric to assess CYP3A phenotype (**Papers V**)

3 METHODS

3.1 Study design, patients, and investigations

COCKTAIL-study

Papers I, II, III, and V were part of the extensive COCKTAIL-study (NCT02386917) and included investigations of CYP1A2, CYP2C9, CYP2C19, and CYP3A phenotypes using a cocktail of probe drugs (caffeine, losartan, omeprazole, and midazolam, respectively). The COCKTAIL-study was an open-label, non-randomized, three-armed, single-center controlled study performed at Vestfold Hospital Trust (Norway) between April 2015 and June 2019. Patients with severe obesity scheduled for weight loss treatment with RYGB (labelled RYGB group) or non-surgical calorie restriction (labelled diet group) based on clinical indications were included and followed prospectively for two years. Additionally, a cross-sectional control group of normal- to overweight individuals (BMI <30 kg/m^2) was included (labelled controls). The study design also included a stratification for diabetic status in the patients with obesity. Patients aged 18 years or above, with BMI ≥18.5 kg/m^2 and stable body weight (<5 kg weight change) over the last three months were eligible for inclusion. Key exclusion criteria included previous bariatric or upper gastrointestinal surgery, estimated glomerular filtration rate (GFR) <30 mL/min/1.73 m^2, and pregnancy or breastfeeding mothers.

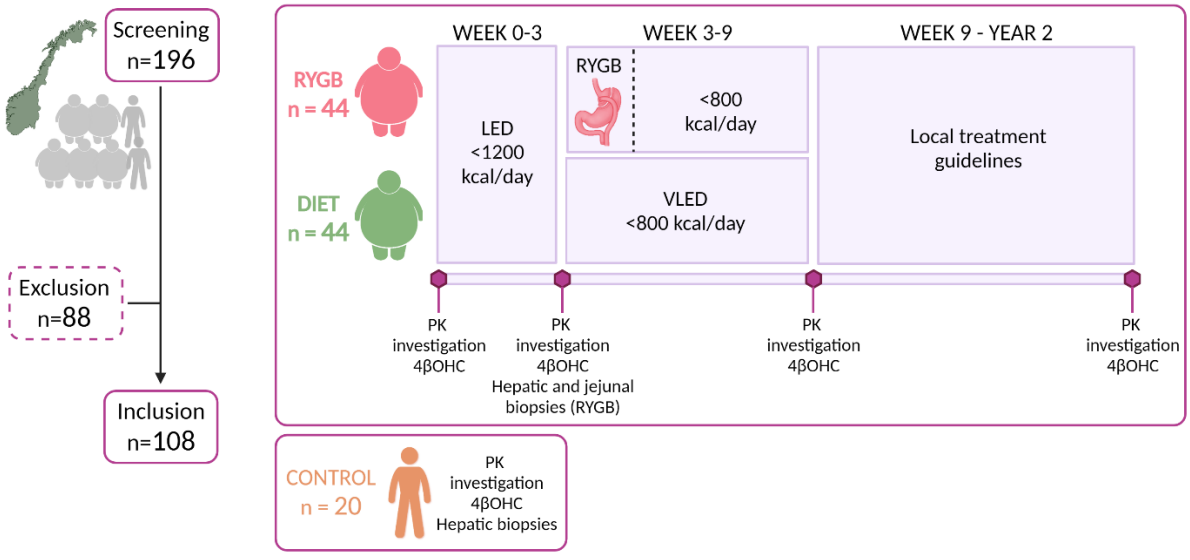

Figure 4. Study design of the COCKTAIL-study.

Abbreviations: LED, low-energy diet; PK, pharmacokinetic; RYGB, Roux-en-Y gastric bypass; VLED, very-low-energy diet; 4βOHC, 4β-hydroxycholesterol

A total of four pharmacokinetic investigations were performed in the intervention groups (RYGB and diet), at weeks 0, 3, and 9, and at year 2 (**Figure 4**). The RYGB group and diet group started a 3-week low-energy diet (<1200 kcal/day) immediately after the investigation at baseline, followed by 6 weeks of a very-low-energy diet or RYGB (both <800 kcal/day). After that, patients were instructed to follow local treatment guidelines until the final study visit (year 2). The controls only underwent one pharmacokinetic investigation the day before the cholecystectomy. Hepatic and jejunal (only RYGB) biopsies were obtained from the patients subjected to RYGB or cholecystectomy (controls) at the time of surgery.

The patients fasted (food, drink, and drugs) from 10:00 p.m. the day before the investigation, except for water. Medications known to alter the pharmacokinetics of the probe drugs were discontinued at least seven half-lives before the investigational days. On the study days, patients first met for blood sampling at 07.30 a.m. before they received 100 mg oral caffeine, followed by 1.5 mg oral midazolam syrup, 25 mg oral losartan, and 20 mg oral omeprazole one hour later. Intravenous midazolam (1.0 mg) was administered four hours after the oral midazolam syrup. Blood samples for analysis of omeprazole and caffeine were collected three and four hours after administration of respective drug. For midazolam the blood samples were collected at times: 0.25, 0.5, 1, 1.5, 2, 3, 4, 4.25, 4.5, 5, 5.5, 6, 8, 10, 12, 23, and 24-hrs after the administration of oral midazolam syrup. Urine was collected in a container for eight hours after the administration of losartan, and the total volume was recorded.

IntraCYP-study

The study in **Paper IV** was a single-armed, open-label, single-center study (IntraCYP-study) performed at the Oslo University Hospital, Rikshospitalet (Norway), between June and December 2021. Healthy individuals with no underlying disease, aged 18 years or above, and with BMI <30 kg/m^2 were eligible for inclusion in the study. Individuals expressing functional CYP3A5 (*CYP3A5*1/*3* or *CYP3A5*1/*1*) or pregnant or nursing mothers were not eligible to participate in the study. Exclusion criteria also included conditions anticipated to interfere with gastrointestinal or hepatic drug disposition, and treatment with substances that may influence midazolam pharmacokinetics. A total of four 6-hrs midazolam pharmacokinetic investigations were performed, at weeks 0, 2, 4, and 8.

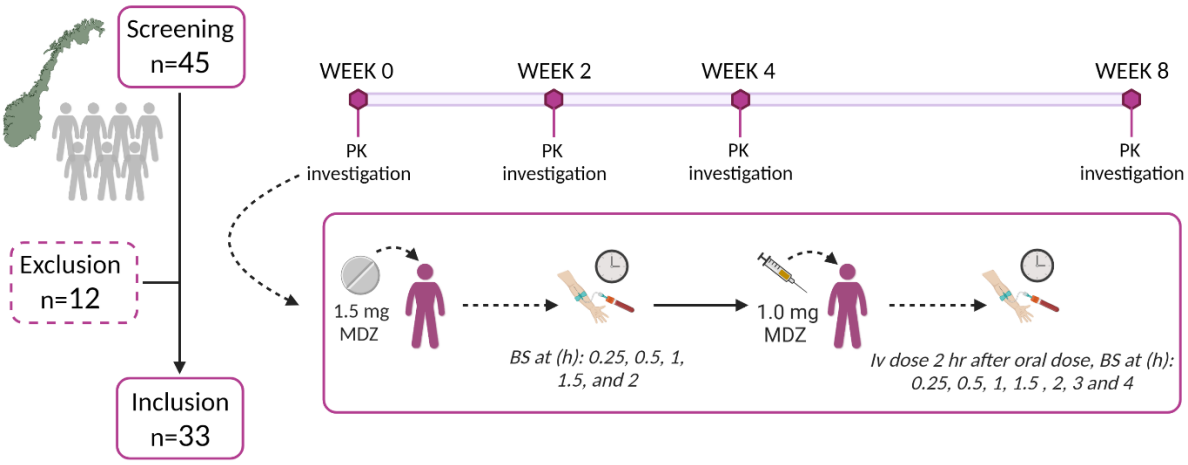

Figure 5. Study design of the IntraCYP-study.

Abbreviations: BS, blood sample; iv, intravenous; MDZ, midazolam; PK, pharmacokinetic

The participants fasted (food, drinks, and drugs) two hours prior to the pharmacokinetic investigations except for water. On the study days, 1.5 mg oral midazolam syrup was administered, followed by 1.0 mg intravenous midazolam 2 hours later (**Figure 5**). A total of 13 venous blood samples were obtained at times: 0, 0.25, 0.5, 1, 1.5, 2, 2.25, 2.5, 3, 3.5, 4, 5, and 6 hours after oral midazolam administration.

3.2 Ethical aspects

All studies were performed in accordance with Good Clinical Practice and the Declaration of Helsinki and approved by the Regional Committee for Medical and Health Research Ethics. All participants gave written informed consent before study participation and had the opportunity to withdraw from the studies at any time. The patients in the COCKTAIL-study were scheduled for weight loss treatment with RYGB or a strict diet based on clinical indications. Generally, pharmacokinetic studies are time-consuming and require a large number of blood samples. Using the cocktail approach, as we did in the COCKTAIL-study, it was possible to assess the activities of multiple CYP isoforms simultaneously, thus reducing the number of study investigations. The probe drugs were carefully selected and administered in low doses to minimize the risk of side effects (**Papers I-V**). To limit the discomfort of multiple blood sampling, all blood samples were obtained from a peripheral venous catheter (**Papers I-V**). The volume of blood drawn was not considered to be clinically harmful. Jejunal and hepatic biopsies were only taken from tissues already exposed as part of the surgical procedure (RYGB or cholecystectomy) (**Papers I-III** and **V**).

3.3 Bioanalytical methods

Midazolam

Plasma concentrations of midazolam were determined using a previously validated ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) methods (**Papers I, III-V**).[216] The sample preparation was carried out by two different extraction techniques, and the calibration curve range differed between the two methods applied. In **Papers I, III, and V**, midazolam was extracted from the plasma samples by liquid-liquid extraction, while the samples in **Paper IV** were prepared by protein precipitation. Due to limited capacity, the UHPLC-MS/MS method in **Papers I, III,** and **V** were carried out in two different labs; the Department of Pharmacy, University of Oslo, and at the Center for Psychopharmacology, Diakonhjemmet Hospital. Because of different instrumentation, inter-laboratory cross-validation was performed using clinical study samples, and the percentage bias was within ±12.5%. In **Paper IV**, the UHPLC-MS/MS method was only carried out at the Department of Pharmacy, University of Oslo.

Calibrators and quality control (QC) samples were prepared in blank plasma and analyzed in each analytical run. For the bioanalytical method used in **Papers I, III,** and **V**, 8 calibrators in the range 0.1 to 20 ng/mL were applied, and back-calculated values of calibrators within 80 to 120% were accepted. The lower limit of quantification (LLOQ) was 0.1 ng/mL, and the upper limit of quantification (ULOQ) was 20 ng/mL. Samples with midazolam concentrations above ULOQ were diluted in blank plasma and reanalyzed. Dilution integrity with dilution factors of 1/2, 1/5, 1/10, 1/20, and 1/50 was established; mean accuracy ranged from 88.9% to 103.8%, and the imprecision was <4.5%. Within-series and between-series performance were assessed with a resulting coefficient of variation (CV) <12.3%, and the mean accuracy ranged from 99.3 to 104.3%. For the method used in **Paper IV**, 10 calibrators in the range 0.25 to 100 ng/mL were applied. LLOQ was 0.25 ng/mL, and ULOQ was 100 ng/mL. All study samples from each individual were analyzed in the same analytical run to minimize the bioanalytical variation. Within-series and between-series performance were assessed with a CV of <4.3% and <10.4%, and the mean accuracy ranged from 93 to 101% and 93 to 102 %, respectively.

Caffeine, losartan, omeprazole

Plasma concentrations of caffeine, paraxanthine, omeprazole, and 5-OH-omeprazole, and urinary concentration of losartan and LCA were determined using validated LC-MS/MS methods at Covance Laboratories, Madison (Wisconsin, USA) (**Papers II** and **III**).

4β-hydroxycholesterol

Plasma concentrations of 4βOHC were determined using UHPLC-MS/MS at the Center for Psychopharmacology, Diakonhjemmet Hospital (**Papers I, III,** and **V**).[116, 217] Liquid-liquid extraction with an added filtration step to remove lipid precipitations was used as sample preparation. In brief, 4βOHC was de-esterified from fatty acids by ethanolic sodium methoxide and isolated from plasma by liquid-liquid extraction with hexane. Extracts were evaporated by nitrogen and reconstituted in methanol before filtration. Within- and between-series imprecision and inaccuracy were <15% at 10 ng/mL and <4% at 644 ng/mL (n = 6).

3.4 Liver and jejunal biopsies

Protein quantification

Proteomics analysis was performed with LC-MS/MS-based global proteomics at the Max Planck Institute of Biochemistry, Martinsried (Germany) (**Papers I-III and V**).[29] CYP protein concentrations were quantified using the Total Protein Approach.[218] Proteins were extracted from jejunum and liver biopsies in an SDS-containing (2% w/v) lysis buffer. Samples were prepared for proteomics analysis using the multi-enzyme digestion filter-aided sample preparation protocol, using LysC and trypsin.[219]

Preparation of microsomes and ex vivo activity assay

Ex vivo activity of CYP3A4 was determined in individual HLMs and human intestinal microsomes (HIMs) by incubation with midazolam, followed by LC-MS quantification of metabolite formation (1'-hydroxymidazolam) in 20 patients in the RYGB group at AstraZeneca, Gothenburg (Sweden) (**Paper V**).[220] Enzyme kinetic parameters were determined using untransformed data and GraphPad prism 7 by fitting the reaction velocity versus the substrate concentration data to the Michaelis-Menten model or the substrate inhibition model.

The unbound CL_{int} ($CL_{int,u}$) was calculated from the ratio of maximum velocity (V_{max}) to the unbound Michaelis constant (K_m).

3.5 Genotype analyses

Taqman-based real-time polymerase chain reaction (PCR) assays at the Center for Psychopharmacology, Diakonhjemmet Hospital (**Papers II, III, and V**) or at Oslo University Hospital, Rikshospitalet (**Paper IV**) were used to analyze the following variant alleles: *CYP1A2*; the increased induction allele **1F* (rs762551), *CYP2C9*; the reduced-function alleles **2* (rs1799853) and **3* (rs1057910), *CYP2C19*; the null alleles **2* (rs4244285), **3* (rs4986893), and **4* (rs28399504), the gain-of-function allele **17* (rs12248560), *CYP3A4*; the reduced function allele **22* (rs35599367), and *CYP3A5*; the null allele **3* (rs776746).

3.6 Pharmacokinetic analyses

In **Papers I** and **III-V,** CYP3A activity was assessed by absolute bioavailability and systemic clearance of midazolam, as well as plasma concentrations of 4βOHC (only **Papers I, III,** and **V**). A population pharmacokinetic model was developed and validated for description of individual pharmacokinetic profiles in order to retrieve individual predictions of midazolam absolute bioavailability and area under the curve (AUC) (**Paper I**). The midazolam pharmacokinetic parameters determined in **Paper I** was also used in **Papers III** and **V**. The model from **Paper I** was further used as Bayesian prior to predict midazolam absolute bioavailability and AUC in **Paper IV** (see below). CYP1A2 activity was assessed by the 4-hour plasma paraxanthine/caffeine ratio, CYP2C19 activity by the 3-hour plasma 5-OH-omeprazole/omeprazole ratio, and CYP2C9 activity by the 8-hour urine losartan/LCA ratio (**Papers II** and **III**).

Population pharmacokinetic modeling

In **Papers I** and **V**, population pharmacokinetic modeling using the nonparametric adaptive grid (NPAG) approach implemented in Pmetrics® (version 1.5.2) for R was performed (version 3.6.2 or later versions). In short, the model was a catenary three-compartment model with absorption lag-time and first-order elimination from the central compartment, with no covariates. The model was parameterized to determine absolute bioavailability with data from semi-simultaneous oral and intravenous midazolam administration. A total of 5,414 midazolam concentrations corresponding to 306 unique 24-hour pharmacokinetic profiles were available

from 98 patients. In **Paper IV**, the previously developed and validated midazolam model in **Paper I** was used as Bayesian prior for predictions of absolute bioavailability and AUC of midazolam. In **Paper IV**, a total of 1,737 midazolam concentrations corresponding to 134 unique pharmacokinetic profiles were available from a total of 38 individuals.

3.7 Calculations

Pharmacokinetic calculations

Absolute bioavailability, AUC, C_{max}, and T_{max} after oral midazolam administration were obtained directly from the model predictions, while systemic clearance was calculated using dose and model-derived AUC from zero to infinity. Systemic clearance was estimated in that way to minimize any potential correlation biases in the transfer rate constants.

$$\text{Clearance (L/h)} = \frac{\text{Absolute bioavailability (F)} \times 1.5\ \text{(mg)} + 1.0\ \text{(mg)}}{AUC_{0-Inf}\ (\text{mg} \times \text{h/L})}$$

Prediction of NAFLD

The equation by Kotronen et al. was used to calculate the NAFLD liver fat score (NAFLD-LFS), in which values greater than -0.640 were indicative of NAFLD (**Papers II** and **III**).[221]

$$\begin{aligned}\text{NAFLD liver fat score} = {} & -2.89 + 1.18 \times \text{metabolic syndrome (yes} = 1, \text{no} = 0) + \\ & 0.45 \times \text{T2DM (yes} = 2, \text{no} = 0) + 0.15 \times \text{fS - insulin (mU/L)} + \\ & 0.04 \times \text{fS - AST (U/L))} - 0.94 \times \text{AST/ALT}\end{aligned}$$

3.8 Statistics

Normality of data was assessed by visual inspection of plots and the Shapiro-Wilk test (**Papers I-V**). In **Papers I** and **II**, Student's t-test for normally or log-transformed normally distributed variables was used in the cross-sectional comparison between patients with obesity and controls at baseline, while linear mixed-effects models were used in the longitudinal analysis to estimate within-group changes and between-group differences (RYGB versus diet). The midazolam pharmacokinetic parameters (**Paper I**) and metabolic ratios (**Paper II**) were treated as dependent variables, while visit (time), group (RYGB and diet), and their interaction (visit × group) were treated as fixed effects. To account for individual variability, the unique patient id was used as a random effect (individual intercepts). To adjust for comparison between multiple study visits in the mixed-effects model, confidence intervals (CI) were adjusted with the Tukey

method. Contrast analyses were performed for parameters of interest. In **Paper III**, Wilcoxon rank-sum test for non-normally distributed variables was used to compare differences between 1) patients with T2DM and obesity and patients with obesity only (without T2DM), and 2) patients with obesity (without T2DM) and controls. To describe the relationship between variables, the Pearson's correlation coefficient (**Paper I**) or the Spearman's rank order correlation test (**Papers II** and **V**) were performed depending on the normality of the data. In **Paper IV**, intraindividual variability was determined by calculating CV% of absolute bioavailability and systemic clearance of midazolam. All statistical analyses were performed using R for Windows (version 3.6.2 or 4.1.2), and a P value <0.05 was considered statistically significant.

4 SUMMARY OF PAPERS

Paper I

Short- and long-term effects of body weight loss following calorie restriction and gastric bypass on CYP3A-activity – a non-randomized three-armed controlled trial

In this study, we aimed to compare the effects of RYGB (n=41) and non-surgical calorie restriction (n=41) on CYP3A activity, both in the short-term and the long-term perspective. Midazolam, which was administered semi-simultaneously, was used as a probe drug, and absolute bioavailability and systemic clearance were considered the main CYP3A metrics, although plasma 4βOHC concentrations and intestinal- and hepatic CYP3A4 protein concentrations also were determined. Both absolute bioavailability and systemic clearance of midazolam were unaltered in the RYGB group and diet group at week 9 (6 weeks post-RYGB) despite a significant weight loss of 13±2.4% and 11±3.6% from baseline, respectively. At year 2, mean weight loss from baseline in the RYGB group (-30±7.0%) and diet group (-3.1±6.3%) differed substantially. Nevertheless, mean absolute bioavailability was decreased in both groups (RYGB group: 40±7.5%, diet group: 32±6.1%) at year 2, while systemic clearance was unaltered. Also, 4βOHC concentrations were increased in both the RYGB group (60±22%) and the diet group (23±6.8%). The results suggest that neither weight loss induced by RYGB or non-surgical calorie restriction, nor RYGB *per se*, impact absolute bioavailability and systemic clearance of midazolam in the short-term perspective (week 9). The decreased absolute bioavailability and increased 4βOHC concentrations in both groups at year 2 indicate that the recovered CYP3A activity is not only dependent on weight loss. A secondary objective of the study was to investigate the effect of body weight by comparing CYP3A activity in patients with severe obesity (n=78) and mainly normal-weight controls (n=18). Absolute bioavailability and systemic midazolam clearance were significantly higher in the patients with obesity compared with the controls (153% and 46%, respectively), whereas 4βOHC concentrations were lower (44%). Altogether, the results in this study indicate that CYP3A activity is decreased in patients with obesity compared with primarily normal-weight individuals.

Paper II

Short- and long-term effects of body weight, calorie restriction, and gastric bypass on CYP1A2, CYP2C19, and CYP2C9 activity

The objective of this study was to compare short-term and long-term effects of RYGB (n=40) and non-surgical calorie restriction (n=41) on CYP1A2, CYP2C9, and CYP2C19 activities using caffeine, losartan, and omeprazole as probe drugs. Secondary, we also aimed to compare these CYP activities in patients with obesity (n=81) with mainly normal-weight controls (n=18). Given that this study was based on the same patient population as in **Paper I**, the weight loss during the study period was also very similar (13±2.4% in the RYGB group and 11±3.9% in the diet group at week 9). CYP2C19 activity increased similarly in the RYGB group (43% [95% CI: 16, 55]) and diet group (48% [95% CI: 22, 60]) from week 0 to week 3. Six weeks post-RYGB, however, CYP2C19 activity had increased by an additional 30% [95% CI: 2.6, 43], while no change was observed in the diet group (week 3-9). At year 2, mean body weight had decreased 19 ± 8.9% in the RYGB group and increased 9.0 ± 8.0% in the diet group from week 9, leaving CYP2C19 activity elevated in the patients subjected to surgery but not in the patients subjected to non-surgical calorie restriction. Only minor, not clinically significant changes were observed for CYP1A2 and CYP2C9. With respect to the secondary objective, controls had 2.7-fold higher CYP2C19 activity compared with patients with obesity, while CYP1A2 and CYP2C9 activity were similar. This study showed that CYP2C19 activity is lower in patients with obesity compared with normal-weight individuals and increases following weight loss, which may be clinically relevant for dosing of CYP2C19 substrate drugs.

Paper III

Impact of type 2 diabetes on in vivo activities and protein expressions of cytochrome P450 in patients with obesity

In this study, the aim was to disentangle the effect of T2DM and obesity on *in vivo* activities and intestinal and hepatic protein expressions of CYP1A2, CYP2C9, CYP2C19, and CYP3A in a cross-sectional analysis (week 3). Patients from the COCKTAIL-study were separated into three groups based on diabetes status and BMI: (1) patients with T2DM and obesity (BMI ≥30 kg/m^2) (n=29), 2) patients with obesity without T2DM (n=53), and 3) controls without T2DM and obesity (BMI <30 kg/m^2) (n=17). The patients with T2DM and obesity had 63% lower CYP2C19 activity and 40% lower jejunal CYP2C19 concentrations than patients with obesity

only. By contrast, there was no difference in *in vivo* activities and jejunal and hepatic protein concentrations of CYP1A2, CYP2C9, and CYP3A between patients with T2DM and obesity and patients with obesity only (without T2DM). However, altogether, the patients with obesity (without T2DM) had lower CYP3A activity than controls. This study suggests that T2DM downregulates CYP2C19 activity and jejunal CYP2C19 protein concentration in patients with obesity, but not the activities and protein concentrations of CYP1A2, CYP2C9, and CYP3A. Accordingly, the effect of T2DM appears to be isoform-specific.

Paper IV

Intraindividual variability in absolute bioavailability and clearance of midazolam in healthy individuals

This study aimed to assess intraindividual variability in midazolam absolute bioavailability and systemic clearance in healthy individuals without obesity during a 2-month period including four pharmacokinetic investigations (weeks 0, 2, 4, and 8). A total of 33 individuals were included (28 ± 8 years, 21% males, BMI 23 ± 2.5 kg/m^2), after exclusion of 5 heterozygous carriers of the *CYP3A5*1* allele. Mean absolute bioavailability and systemic clearance of midazolam at baseline (week 0) were 46 ± 18% and 31 ± 10 L/h, respectively. For absolute bioavailability, mean coefficient of variation (CV) % was 26 ± 15%, while it was lower for systemic clearance (20 ± 10%). Approximately 30% and 13% had a CV >30% for absolute bioavailability and systemic clearance, respectively. The intraindividual variability was low to moderate on average, however, a relevant number of the individuals had a significant intraindividual variability. Hence, midazolam may not be suitable as a probe drug for CYP3A activity in certain studies.

Paper V

Correlations between 4β-hydroxycholesterol and hepatic and intestinal CYP3A4: protein expression, microsomal ex vivo activity, and in vivo activity in patients with a wide body weight range

In this study, the objective was to evaluate 4βOHC as an endogenous biomarker for CYP3A4 phenotype in 96 patients from the COCKTAIL-study (78 patients with obesity and 18 mainly normal-weight individuals). Spearman's rank order test was used to describe correlations between 4βOHC concentrations and three other CYP3A metrics: 1) CYP3A4 expression and

2) ex vivo microsomal CYP3A4 activity in paired jejunum and hepatic biopsies and 3) midazolam absolute bioavailability, apparent oral clearance, and systemic clearance. A positive correlation was observed between plasma 4βOHC concentrations and hepatic microsomal CYP3A4 activity (ρ=0.53, P <0.001) and hepatic CYP3A4 concentrations (ρ=0.30, P <0.027). However, 4βOHC concentrations were not correlated with intestinal CYP3A4 concentrations and microsomal CYP3A4 activity. A weak correlation was observed between 4βOHC concentrations and midazolam absolute bioavailability (ρ=-0.23, P <0.027) and apparent oral clearance (ρ=0.28, P <0.008), whereas no correlation was observed with systemic clearance. This study shows that 4βOHC seems to be a valuable CYP3A4 metric for hepatic, but not intestinal, CYP3A4 activity.

5 DISCUSSION

In later years, there has been an increased focus on the impact of obesity and RYGB on pharmacokinetics in general and *in vivo* CYP activities in particular. Obesity-related comorbidities such as T2DM and NAFLD and their impact on pharmacokinetics have also gained more attention recently.[73] This, because variable activity of specific CYP enzymes due to disease-related factors appears to be an important source of interindividual variability in drug response.[72] Due to the rapidly increasing prevalence of obesity and its comorbidities,[130, 165, 177] and accordingly the number of patients subjected to bariatric procedures,[199] clinicians are likely to be more frequently confronted with how drugs should be dosed in these patients. The currently available data, however, are not sufficient enough to give general dosing recommendations for these patients. The prediction of pharmacokinetic alterations after RYGB is further complicated by the fact that weight loss itself may alter drug disposition, and previous studies have not been able to disentangle this effect from the surgery effect *per se*.[222, 223] In **Papers I**-**III**, we investigated the effect of body weight, T2DM, RYGB, and weight loss on CYP1A2-, CYP2C9-, CYP2C19-, and CYP3A-mediated metabolism in a patient population consisting of patients with severe obesity with and without T2DM and controls without T2DM and obesity (mainly normal-weight individuals).

5.1 Impact of obesity and T2DM on specific CYPs

CYP3A

Several CYP3A metrics were assessed in **Paper I**; *in vivo* midazolam absolute bioavailability and systemic clearance, plasma 4βOHC concentrations, and CYP3A4 protein expression. Based on data from previous animal studies[224, 225] and *in vitro* studies,[151, 152] as well as *in* vivo studies,[146-148, 150] we expected a lower CYP3A activity in patients with obesity. In our study, the patients with obesity had higher systemic midazolam clearance (~50%), indicating a higher hepatic CYP3A activity, compared with mainly normal-weight controls (**Paper I**). However, we also showed that patients with obesity had lower 4βOHC concentrations than the controls. According to the finding in **Paper V**, in which we showed that 4βOHC seems to be a valuable biomarker for hepatic CYP3A4, the lower 4βOHC concentrations contradict the higher systemic clearance and suggest that hepatic CYP3A4 activity actually is lower in patients with obesity. The reason for the discrepancy between systemic midazolam clearance and 4βOHC concentrations may be attributed to the fact that midazolam is a medium to high extraction ratio

drug, and thus clearance is not only dependent on hepatic CYP3A activity but also on hepatic blood flow.[107, 144] Also, the extraction ratio of midazolam is reported to be highly variable,[107] and it may differ in specific populations.[226] Patients with obesity are considered to have an increased hepatic blood flow due to increased blood volume and cardiac output.[144] Thus, it may be that systemic clearance in patients with obesity is more dependent on hepatic blood flow than CYP3A activity, leaving them with higher clearance values than the non-obese controls despite that hepatic CYP3A activity might be lower. The low bioavailability in the present study supports that midazolam behaves like a high extraction ratio drug in this patient population. Previous studies have also suggested that midazolam clearance is influenced by a higher hepatic blood flow in patients with obesity.[155, 227] The findings in **Paper V** support that systemic midazolam clearance seems to poorly reflect *in vivo* CYP3A activity in patients with obesity. In **Paper V**, we show that there was no correlation between hepatic microsomal CYP3A4 activity, i.e. $CL_{int,u}$, and *in vivo* systemic midazolam clearance in the same patient population. However, a moderate correlation was observed between 4βOHC concentrations and hepatic $CL_{int,u}$, suggesting that 4βOHC concentrations reflect individual hepatic CYP3A4 phenotype. In agreement, hepatic CYP3A4 concentrations were significantly correlated with 4βOHC concentrations (**Paper V**), but not with systemic midazolam clearance (**Paper I**). Midazolam is highly protein bound, mainly to albumin, which means that clearance of midazolam is expected to increase if the fraction of unbound drug increases.[228, 229] However, there was no difference in albumin concentrations between the patients with obesity and the controls, thus we do not believe that alterations in protein binding have influenced systemic midazolam clearance in this study (**Paper I**). The patients with obesity also had higher absolute bioavailability (3-fold) of midazolam than the controls (**Paper I**). It is not expected that this is due to any difference in F_A, given that midazolam is a highly permeable drug that is anticipated to be almost completely absorbed in the gut (F_A approximately 1).[3] Hence, the higher absolute bioavailability may be attributed to an increase in both F_G and F_H, assuming that hepatic CYP3A activity actually is lower in patients with obesity. CYP3A4 expressions in the small intestine and liver have been reported to be lower in patients with obesity.[151] In line with this, we also observed a negative correlation between hepatic CYP3A4 concentrations and body weight in **Paper I**. Intestinal blood flow alterations can possibly also have contributed to the higher absolute bioavailability in patients with obesity. In case of an increased blood flow to the gut, midazolam may have decreased contact with CYP3A in the enterocytes, thus causing an increase in F_G.[230] Some years ago, Brill et al. investigated CYP3A activity in patients with severe obesity and healthy volunteers using a similar approach as we did in **Paper I** with semi-

simultaneous administration of midazolam.[62] They reported a higher absolute bioavailability in the patients with severe obesity compared with healthy volunteers, which is in agreement with the findings in our study, but no difference in systemic clearance. To speculate, this discrepancy in midazolam clearance may be explained by an age difference. In our study, the patients with obesity and controls had very similar ages, while in the study by Brill et al. the healthy volunteers had a considerably younger age than the patients with obesity (mean of 22 years versus 44 years). Younger individuals will typically have higher hepatic blood flow than older individuals, resulting in a higher systemic midazolam clearance,[227] and may explain why Brill and co-authors found a similar clearance between patients with obesity and healthy volunteers in their study. Altogether, the results from **Paper I** supports previous literature indicating that patients with obesity have decreased CYP3A activity.

In later years, inflammation and associated cytokines have been suggested as one of the major mechanisms for the downregulation of CYP3A activity in obesity,[62, 206] but also in other diseases characterized by inflammation such as T2DM.[59] Recently, Gravel et al. reported a lower CYP3A activity in patients with T2DM than in patients without T2DM.[59] By contrast, we did not observe any difference in CYP3A activity or jejunal- or hepatic CYP3A4 protein concentrations when we compared patients with T2DM and obesity with patients with obesity only (without T2DM) (**Paper III**). However, the patients with obesity (without T2DM) had lower CYP3A activity compared with controls (**Paper III**). According to these findings, it appears that obesity, but not T2DM, plays a role in the downregulation of CYP3A activity in patients with obesity. This discrepancy between our study and the study by Gravel et al. may be explained by the fact that Gravel and co-authors compared two groups with significantly different BMI (mean of 29.1 kg/m^2 in patients with T2DM versus 25.7 kg/m^2). Thus, it is possible that higher BMI rather than T2DM *per se* is the reason for the lower CYP3A activity in patients with T2DM in their study. This is supported by the finding in **Paper V**, in which we report an inverse correlation between BMI and 4βOHC concentrations (e.g. hepatic CYP3A4 activity), and also by previous studies showing a negative correlation between BMI or body weight and various CYP3A metrics.[151-154] Gravel et al. later investigated differences in CYP3A mRNA expression in duodenal biopsies from individuals with T2DM and without T2DM and found no difference in CYP3A expression or activity.[231] However, they hypothesized that their previous findings may be due to a tissue-specific modulation of hepatic CYP3A. The hepatic CYP3A4 protein concentrations in our study do not support this hypothesis (**Paper III**). Nevertheless, we cannot rule out that CYP3A activity is decreased in patients with T2DM

without obesity, but T2DM does not appear to cause any additional downregulation of CYP3A activity when obesity already is present.

CYP2C19

One of the main findings in **Paper II** was that CYP2C19 activity was decreased in patients with obesity (with and without T2DM). Although previous literature is sparse, there have been indications of an unaltered or increased CYP2C19 activity in patients with obesity than in individuals without obesity.[232, 233] However, these studies did not use validated CYP2C19 probe drugs. In **Paper III**, we also show that T2DM appears to downregulate CYP2C19 activity beyond that of obesity, given that CYP2C19 activity was lower in patients with T2DM and obesity than in patients with obesity only (~60%), and also lower in patients with obesity (without T2DM) than in mainly normal-weight controls (~55%). The patients with T2DM and obesity also had lower concentrations of jejunal CYP2C19 but not hepatic CYP2C19. A possible explanation for this discrepancy may be different exposure to endogenous and environmental factors or that different expression patterns regulate transcription factors in the intestine and liver.[8] In agreement with our finding, Gravel et al. also found a similar decrease in CYP2C19 activity (~50%) in patients with T2DM compared with individuals without T2DM.[59] The diabetic patients in our study and the study by Gravel et al. had T2DM that was relatively well-controlled, with average hemoglobin A1c (HbA1c) of 50 mmol/mol (6.7%) and 54 mmol/mol (7.1%), respectively. Thus, the effect of T2DM may be even larger in patients with poorly controlled diabetes. The studies showing poor effect of clopidogrel at studied doses also support that patients with T2DM have decreased CYP2C19 activity.[187, 188] In **Paper II**, we also showed that patients with a NAFLD-LFS indicative of NAFLD had lower CYP2C19 activity than patients without, suggesting that NAFLD may be an important contributor to the downregulation of CYP2C19 in patients with obesity. The proportion with NAFLD was higher in the patients with T2DM and obesity than in the patients with obesity only (100% versus 62%, respectively) (**Paper III**), indicating that NAFLD may also play a major role in the downregulation of CYP2C19 in T2DM. However, the impact of NAFLD on CYP activities warrants further investigations.

CYP1A2 and CYP2C9

CYP1A2 and CYP2C9 activities were not influenced by obesity or T2DM (**Papers II** and **III**), suggesting that these isoforms are less susceptible to alterations than CYP2C19 and CYP3A in patients with metabolic conditions. This is in agreement with the majority of the current literature,[148, 159, 207, 234, 235] although a few studies have indicated that the activity of these CYP isoforms may be increased in T2DM.[59, 190] However, it may be that the significantly larger proportion of males in the groups with T2DM than in the non-diabetic groups in these studies have influenced the results, given that there have been indications of increased CYP1A2 activity in males.[95, 96]

5.2 CYP activities following weight loss induced by RYGB or strict diet

Before the initiation of the COCKTAIL-study it remained uncertain whether pharmacokinetic changes following RYGB are due to the surgery *per se* or weight loss. Thus, in **Papers I** and **II**, we assessed changes in CYP1A2, CYP2C9, CYP2C19, and CYP3A activities in patients with obesity scheduled for RYGB or non-surgical calorie restriction. The two groups had a comparable weight loss until week 9, which enabled us to separate the surgery effect from the weight loss effect in the early period after RYGB. Thereafter, the RYGB group continued to lose weight, whereas the diet group regained weight as several patients tended to return to their baseline body weight.

CYP3A

It has been hypothesized that RYGB will increase the oral bioavailability of CYP3A substrates due to its high abundance in the duodenum and proximal jejunum.[202] We were therefore surprised to see that the considerable rearrangement of the gastrointestinal tract after RYGB did not lead to any changes in the absolute bioavailability of midazolam early after surgery (6 weeks post-RYGB) (**Paper I**). The previously discussed findings of downregulated CYP3A activity in patients with obesity also suggest that the activity of CYP3A may recover upon weight loss. Nevertheless, neither of the CYP3A metrics investigated changed during the first 9 weeks of the study period, despite a considerable weight loss, indicating that this short period of time is not enough for CYP3A activity to recover. However, and as discussed previously, systemic midazolam clearance appears to reflect other processes than solely hepatic CYP3A activity. Also, 9 weeks may not have been long enough time to detect a change in 4βOHC given its long elimination half-life (~17 days).[117] Hence, we cannot rule out that these factors have

limited a true understanding of alterations in CYP3A activity, particularly short-term. Nevertheless, the increased 4βOHC concentrations in the RYGB group at year 2 indicate that hepatic CYP3A activity increases with weight loss given enough time, which is in agreement with the existing literature.[148, 206, 207] Thus, the lower absolute bioavailability in RYGB patients at year 2 may be explained by a decrease in F_H, or possibly both F_G and F_H if intestinal CYP3A also recovers. However, it seems more likely that F_G increases rather than decreases considering that the most CYP3A-rich part of the intestine is bypassed. Others have also suggested that F_G increases after RYGB.[206] Surprisingly, although to a lesser extent, similar changes in 4βOHC and absolute bioavailability were also present in the diet group which had regained body weight. We have no explanation for these findings, but overall it suggests that recovery of CYP3A activity is not only dependent on weight loss.

CYP2C19

One of the main findings in **Paper II** was that CYP2C19 activity increased after weight loss induced by RYGB or a strict diet, suggesting a recovery of CYP2C19 activity with weight loss. However, as we show in **Paper III**, the increase in CYP2C19 activity between weeks 0 and 3 was significantly lower in patients with T2DM and obesity compared with patients with obesity only. Accordingly, weight loss appears to have a minor impact on CYP2C19 activity in patients with obesity when T2DM also is present. Bariatric surgery has evolved as an effective treatment for T2DM in patients with obesity, and up to 75% of patients with T2DM treated with RYGB experience diabetes remission 1-year post-RYGB.[236, 237] To which degree CYP2C19 activity recovers in these patients is yet to be investigated. In **Paper II**, we also show that in case of regained body weight, such as in the diet group at year 2, the activity of CYP2C19 also reverts. Interestingly, there was a tendency for an additional effect on CYP2C19 activity in the RYGB group six weeks after surgery. However, it may be that the single time point metabolic ratio has been influenced by alterations in the absorption post-surgery, as a result of the anatomical changes in the gastrointestinal tract (described more thoroughly in section 5.6.1). Nevertheless, others have also indicated an increased CYP2C19 activity after RYGB.[204, 215] In these studies, a lower systemic exposure of omeprazole was observed 1 and 6 months, and approximately 2 months after RYGB, respectively. No change in CYP2C19 activity after bariatric surgery has also been reported,[207, 238] however, these studies did not include a proper control group and had a lower number of participants.

CYP1A2 and CYP2C9

Overall, only minor, not clinically significant changes were observed for CYP1A2 and CYP2C9 activities after weight loss induced by RYGB or a strict diet (**Paper II**). Interestingly, CYP1A2 activity increased in the diet group short-term and remained elevated until the 2-year study visit, even though clearance mediated by CYP1A2 appears to be similar in individuals with different body weights. There was a weak negative association between body weight and hepatic CYP1A2 concentrations. To speculate, it may be that the increased CYP1A2 activity following weight loss is explained by an increase in CYP1A2 expression. In contrast to the findings in the diet group, we did not observe any increase in CYP1A2 activity in the RYGB group short-term. However, it was increased at year 2 which is in line with the findings by Puris et al.[207] Furthermore, Rodríguez-Morató et al. described a lower CYP1A2 activity 4 weeks after RYGB or sleeve gastrectomy, which then was recovered after 6 months.[148] Hence, the lack of changes in the RYGB group short-term may be explained by effects from the surgery counteracting the effect of weight loss. With respect to CYP2C9, the RYGB group had higher CYP2C9 activity than the diet group at baseline (**Paper II**). We have no explanation for this, however, the difference diminished over time as CYP2C9 activity in the RYGB group decreased following three weeks of low-energy diet. Taken together, CYP2C9 activity does not seem to be influenced by weight loss or RYGB, which also is in agreement with other studies.[148, 207]

5.3 Mechanisms for regulation of CYPs

The underlying mechanisms for the isoform-specific modulation of CYP enzymes in **Papers I-III** are not fully known. In **Papers I** and **II**, we observed that patients with obesity had elevated concentrations of the inflammatory marker high-sensitivity C-reactive protein (hs-CRP) compared with controls. Patients with obesity (without T2DM) also had higher levels of TNF-α and IL-6 than controls without T2DM and obesity, while there was no difference between patients with T2DM and obesity and patients with obesity only (**Paper III**). Correlation analyses revealed a weak negative association between hs-CRP and hepatic CYP3A4 concentration (**Paper I**), and 4βOHC concentrations (**Paper V**), suggesting that inflammation is involved in the downregulation of hepatic CYP3A expression and activity in patients with obesity. There was also a weak negative association between hs-CRP and hepatic CYP2C19 concentration (ρ=-0.28, P = 0.04) but not CYP2C19 activity as assessed by the 5-OH-omeprazole/omeprazole ratio (ρ=-0.15, P = 0.15) (using Spearman's rank order correlation test, data have not been shown previously). In a recent review by Lenoir et al. it was concluded that

inflammation is a major contributing factor to the downregulation of CYP3A and CYP2C19.[72] Nevertheless, *in vitro* hepatocyte models indicate that CYP3A4 is more susceptible to downregulation by various cytokines such as IL-6 and TNF-α than CYP2C19.[79] Hence, it may be speculated that the low-grade inflammation in obesity and associated cytokine concentrations are not high enough to downregulate CYP2C19 activity, and that other (unknown) mechanisms are more important. None of the inflammatory markers investigated in **Paper III**, including IL-1β, IL-6, TNF-α, could explain the additional effect of T2DM on CYP2C19 activity. Altogether, this suggests that elevated cytokine levels may not be the major mechanism for the downregulation of CYP2C19 in patients with T2DM and obesity. Another factor that may contribute to the downregulation of CYP2C19 and CYP3A in obesity and T2DM (only CYP2C19) is NAFLD (**Papers I-III**). Using the NAFLD-LFS, data from **Papers I** and **II** indicate that metabolism mediated by CYP2C19 and CYP3A are lower in patients with NAFLD than in patients without. Similarly, Woolsey et al. reported lower CYP3A activity assessed with midazolam and 4βOHC in individuals with biopsy-proven NAFLD.[60] Several *in vitro* studies have also indicated that CYP2C19 and CYP3A protein expressions and activities are decreased in NAFLD,[60, 61, 170-173] and that the downregulation increases with NAFLD severity.[171] TNFR1, which has been associated with the severity of NAFLD,[239, 240] was higher in the patients with T2DM and obesity than the patients with obesity only (**Paper III**). Hence, it may be speculated that the additional effect of T2DM on CYP2C19 activity is because NAFLD may have progressed to more severe stages in the patients with T2DM and obesity than in those with obesity only. This may also explain why a short-term weight loss has less impact on CYP2C19 activity in patients with T2DM and obesity than patients with obesity only, as we show in **Paper III**, given that three weeks probably is not enough time for a significant NAFLD improvement. Even though *in vitro* studies have indicated a decreased CYP3A4 expression and activity in HLMs from diabetic donors or diabetic NAFLD donors,[61, 183] we showed in **Paper III** that T2DM has no impact on CYP3A activity *in vivo* when obesity already is present.

Interestingly, CYP2C19 activity increased already during the three first weeks of the study in both intervention groups (**Paper II**), whereas CYP3A activity did not change in the short-term perspective (weeks 3 and 9) (**Paper I**), suggesting that it takes a longer time for CYP3A to recover. To speculate, this may partly be attributed to a difference in turn-over half-life between the two isoforms (~53 hours for CYP2C19 versus 70-140 hours for CYP3A).[241, 242] Another possible explanation may be that since CYP3A4 appears to be more sensitive to the effects of

inflammation[79], circulating levels of inflammatory cytokines may still be too high for CYP3A to recover. Chronic, low-grade inflammatory conditions such as obesity or T2DM do not appear to downregulate CYP2C9 activity *in vivo*, even though CYP2C9 expression also is regulated by PXR and CAR. It may be speculated that the inflammatory effects are counteracted by NAFLD, given that Fisher et al. reported increased CYP2C9 activity with progressive states of NAFLD, possibly due to hypoxia.[171] Furthermore, CYP1A2 activity did not appear to be downregulated in obesity or T2DM (**Papers II** and **III**), even though studies have shown that IL-6 decreases the expression of CYP1A2.[77, 243] However, as we expect the inflammatory state with associated cytokines such as IL-6 to improve following weight loss, this may explain the minor, but significant increase in CYP1A2 activity after RYGB and strict diet. Nevertheless, the effect of inflammation on CYP1A2 and CYP2C9 expressions and activities remains unclear.[72]

5.4 Impact of genotypes

CYP3A

CYP3A5 may contribute significantly to the drug metabolism of CYP3A substrates in individuals that express functional enzyme.[55, 56] Hence, it should be addressed that we did not distinguish between CYP3A5 nonexpressers (*CYP3A5*3/*3*) and heterozygous *CYP3A5*1* carriers when we investigated the effect of body weight, weight loss, and RYGB (**Paper I**), and T2DM (**Paper III**) on CYP3A activity *in vivo*, mainly due to the low (~10%) proportion expressing functional CYP3A5 in our study population. Theoretically, patients expressing functional CYP3A5 may display an increased systemic clearance of midazolam. However, results from previous studies have indicated that midazolam primarily reflects CYP3A4 activity *in vivo* and that the contribution from CYP3A5 is limited.[90, 103-105] Also, the individuals that expressed functional enzyme were distributed relatively evenly between the groups, and none were homozygous carriers of *CYP3A5*1*. Hence, we do not believe that CYP3A5 genotype has influenced the results. In **Paper IV**, we also observed that heterozygous carriers of the *CYP3A5*1* allele had similar absolute bioavailability and systemic clearance as homozygous carriers of the nonfunctional *CYP3A5*3* allele, supporting a minor contribution from CYP3A5 to the metabolism of midazolam. However, it is not possible to draw any conclusions considering the low number of individuals expressing functional CYP3A5 in this study. With respect to 4βOHC, the current literature on the contribution from CYP3A5 to the formation of 4βOHC is conflicting.[110-113] We did not observe any significant difference in 4βOHC

concentrations in heterozygous *CYP3A5*1* carriers and nonexpressers (*CYP3A5*3/*3*), as shown in **Paper V**. In agreement, Hole et al. reported in a recently performed study that CYP3A5 seems to have a limited role in the formation of 4βOHC *in vivo* in a similar study population to ours.[47] Although the low proportion of heterozygous *CYP3A5*1* carriers in our study makes it challenging to draw any conclusion on the effect of CYP3A5 on 4βOHC concentrations, we do not believe that these individuals have influenced the interpretation of the results to any significant degree.

CYP2C9 and CYP2C19

We also investigated whether alterations in metabolism mediated by CYP1A2, CYP2C9, and CYP2C19 following RYGB and/or weight loss were dependent on genotype (**Paper II**). This is particularly relevant for CYP2C9 and CYP2C19, given that genetic polymorphism contributes significantly to the interindividual variability in metabolism mediated by these isoforms.[12] Changes in CYP2C19 activity appeared to be partly dependent on genotype. As could be expected, CYP2C9 and CYP2C19 activities did not change in individuals genotypic categorized as poor metabolizers (*CYP2C9*3/*3, CYP2C19*2/*2* or **2/*4*). Interestingly, CYP2C19 activity increased primarily in individuals categorized as rapid or ultrarapid metabolizers based on genotype, suggesting that the *CYP2C19*17* allele may be more susceptible to modulation with fluctuations in body weight than the *CYP2C19*1* allele. In **Paper III**, the proportion of genotypic rapid- or ultrarapid metabolizers was lower (but the difference was not statistically significant) in the patients with T2DM and obesity than in patients with obesity only. However, there was no significant difference in CYP2C19 activity between the various genotype-predicted-phenotype categories, and, as such, we do not believe that this has influenced the results. Given the previous finding suggesting a partly genotype-dependent alteration of CYP2C19 activity with fluctuations in body weight, it may be speculated that the minor increase in CYP2C19 activity upon weight loss in patients with T2DM and obesity may actually be due to fewer genotypic rapid- or ultrarapid metabolizers in this group compared to the patients with obesity only. Nevertheless, the genotype analyses in these studies are challenged by the low number of individuals in each category, and, as such, it is not possible to draw any certain conclusions with regard to the impact of genotype.

5.5 Midazolam as a CYP3A probe drug

5.5.1 Absolute bioavailability

We observed large interindividual variability in various CYP3A metrics in patients within a wide body weight range (**Paper I**) and in healthy volunteers (**Paper IV**). In **Papers I** and **IV**, a similar semi-simultaneous approach with the same doses was used to assess the absolute bioavailability and systemic clearance of midazolam. Thus, we were surprised to see that the healthy volunteers in **Paper IV** had considerably higher mean absolute bioavailability (~50%) than the patients with obesity (~25%) and the controls (~10%) in **Paper I**. The reasons for this large difference in absolute bioavailability are unknown. It may be speculated that food intake has influenced the results as the fasting period was significantly different in the two studies. In **Paper IV**, the participants were instructed to fast for at least 2 hours before the study investigation compared with ~9 hours in **Paper I**. Thus, many of the participants in **Paper IV** may have eaten a large meal ~2 hours before the study investigation. Food may influence the absorption of drugs through delayed gastric emptying, altered gastrointestinal pH, physical and chemical drug modification, increased splanchnic blood flow, and altered intestinal drug metabolism.[3] Because splanchnic blood flow increases significantly in response to a meal,[244] the bioavailability of drugs that undergo substantial first-pass metabolism, such as propranolol, may also increase.[245, 246] Previous studies with midazolam have indicated that F_G may be influenced by variability in intestinal blood flow.[230, 247] Furthermore, Bornemann et al. showed a delayed and decreased rate of absorption when 15 mg oral midazolam was administered one hour after a meal, although it was minor.[248] Altogether, we cannot say for sure that the short fasting period not has influenced the absolute bioavailability in **Paper IV**. Nevertheless, midazolam absolute bioavailability estimates reported in the literature also vary substantially, ranging from 20 to 70% in different populations and a range of doses, although it tends to be around 20-30% in healthy volunteers. [62, 216, 249-252]

5.5.2 Systemic clearance

The healthy individuals in **Paper IV** also had higher systemic clearance (~30 L/h) than the patients with obesity (~25 L/h) and the controls (~17 L/h) in **Paper I**. We have previously suggested that midazolam appears to be more dependent on hepatic blood in patients with obesity than previously assumed and that higher hepatic blood flow in these patients has resulted in higher systemic midazolam clearance compared with the non-obese controls.

Nevertheless, we had expected the clearance estimates for the healthy individuals in **Paper IV** to be similar to those for the control group in **Paper I**. To speculate, this discrepancy may be explained by a difference in age (mean of 28 years versus ~45 years, respectively) and hence different hepatic blood flow, leaving the healthy volunteers with a higher systemic clearance. Van Rongen et al. recently observed that obese adolescents had considerably higher systemic midazolam clearance than morbidly obese adults (~42 L/h versus ~26 L/h, respectively), and hypothesized that this was partly due to an increased hepatic blood flow in the obese adolescents.[227] Another possible explanation may be that the controls (normal- to overweight individuals scheduled for cholecystectomy) in **Paper I** are not as similar to healthy volunteers as we first had assumed. Klein et al. have reported a decreased CYP1A2 protein expression and activity in liver tissue from patients with cholestasis,[253] and cholestasis or bile duct obstruction have also been reported to alter hepatic CYP activities in animal studies.[254, 255] Thus, we cannot rule out that CYP3A activity has been altered in these patients. However, this seems unlikely given the low absolute bioavailability and that the controls had similar values of 4βOHC as reported for corresponding non-obese Caucasians in other studies.[111, 153, 256, 257] It would have been interesting to determine 4βOHC concentrations in the healthy individuals included in **Paper IV** and compare these concentrations with the control group in **Paper I**. Unfortunately, these data are not yet available due to limited analytical capacity. Nevertheless, the systemic clearance estimates in **Papers I** and **IV** are similar to clearance values reported in the literature, which on average appear to be around 20 L/h.[62, 249, 252]

5.5.3 Intraindividual variability

We were surprised by the substantial intraindividual variability in absolute bioavailability and systemic clearance of midazolam in patients with obesity before and after weight loss induced by RYGB or a strict diet over a 9-week period (**Paper I**). For absolute bioavailability, more than 60% of the patients had an intraindividual CV >30%, whereas almost half of the patients also displayed a CV >30% for systemic clearance. However, due to the study design, it is challenging to determine if the large intraindividual variability that we observed in **Paper I** is attributed to an actual change in CYP3A activity or if it is midazolam pharmacokinetics that varies due to other factors such as changes in blood flow and/or protein binding. Nevertheless, a high intraindividual variability challenges the use of midazolam for CYP3A phenotyping as low intraindividual variability is a prerequisite for phenotyping metrics used to study changes in CYP activities over time. Therefore, in view of the high within-subject variability in **Paper**

I, we assessed intraindividual variability in absolute bioavailability and systemic midazolam clearance in healthy volunteers over a 2-month period (**Paper IV**). In this study, we report a mean CV% of 26% and 20% for absolute bioavailability and systemic clearance, respectively, suggesting that CYP3A activity is relatively constant over time in most healthy individuals. Others have also reported a low intraindividual variability in midazolam AUC or systemic clearance.[93, 258-260] However, ~30% of the participants had a CV >30% for absolute bioavailability, and the CV values ranged from 6 to 69% suggesting that a proportion of individuals display considerable within-subject variability. Overall, the findings in this study are in contrast to what we observed in **Paper I**. Altogether, it appears that the considerable intraindividual variability observed in the patients with obesity was attributed to intraindividual variability in absolute bioavailability and systemic clearance of midazolam, possibly due to major physiological alterations following the interventions, rather than an actual intraindividual variability in CYP3A activity.

5.5.4 *In vivo* versus *ex vivo*

The majority of probe drugs are validated in healthy individuals, and even though they ideally should, they may not always reflect the same processes in patient populations which was illustrated in **Papers I** and **V**. In **Paper V**, we reported a lack of correlation between *in vivo* systemic midazolam clearance and hepatic microsomal CYP3A4 $CL_{int,u}$. This may be due to limitations with the $CL_{int,u}$ estimate, such as that the small hepatic tissue sample may not be representative of the CYP3A4 activity across the whole liver.[261] However, we believe that the main reason for the poor correlation between *in vivo* and *ex vivo* activity is due to the fact that systemic midazolam clearance reflects other processes than metabolic CYP3A capacity. This was also supported by the lack of correlation between hepatic CYP3A4 concentrations and systemic clearance (**Paper I**). With regards to absolute bioavailability, there was a weak inverse association between jejunal CYP3A4 concentrations and absolute bioavailability, supporting that intestinal CYP3A4 plays an important role in the first-pass metabolism of midazolam. Altogether, when using midazolam as a probe drug to assess CYP3A activity it should be taken into account that physiological changes may influence the pharmacokinetics of midazolam more than previously assumed in specific patient populations. Nevertheless, to date, no ideal probe drug or another phenotyping method exists.

5.6 Methodological considerations

5.6.1 Strengths and limitations of the study design

The COCKTAIL-study, which **Papers I-III** and **V** were based on, had a large study population, included multiple study visits (short-term and long-term), and had rich pharmacokinetic data for midazolam. Also, the matched short-term weight loss induced by RYGB or a strict diet made it possible to separate the effect of surgery from the weight loss effect (**Papers I** and **II**). Another major strength was that the interpretation of *in vivo* CYP activities in the various studies was supported by *ex vivo* CYP activities (only **Paper V**) and proteomics data. However, there are also several limitations that should be addressed. The administration of multiple probe drugs during a short period of time, e.g., the cocktail approach, increases the risk for drug-drug interactions. Even though parts of the cocktail were based on the validated Karolinska cocktail,[122] midazolam, rosuvastatin, and digoxin (the latter two are beyond the scope of this thesis) were also included. Hence, the cocktail has not been validated. However, the drug-drug interaction potentials between the drugs included in the cocktail were extensively investigated during the planning of the study. Another challenge with probe drugs for phenotyping is that other enzymes may also be involved in the metabolism. Omeprazole, for example, is also partly metabolized by CYP3A4 in addition to CYP2C19.[128] However, Fuhr et al. have suggested that limitations associated with specificity appears to be of minor relevance.[127] To prevent caffeine in food and drinks to interfere with the phenotyping, caffeine was not allowed from 36 hours before and through 4 hours after the caffeine administration at the investigational day.

In **Paper IV**, we aimed to perform the investigations at the same time of day (~02:00 p.m.) for each study visit to minimize any influence by circadian variations. However, 15 individuals had one or two study investigations that started in the morning (~09:00 a.m.) which is a limitation of the study. While the diurnal variation in systemic clearance appears to be small,[252, 262] Van Rongen et al. showed that oral bioavailability of midazolam displayed 24-hour variation. The relative difference between peak and trough levels was ~30%, with the largest difference between day- and night time.[252] However, in a mixed-effects model analysis, time of the day had no significant impact on absolute bioavailability and systemic clearance of midazolam in our study. Also, approximately half of the healthy individuals with large intraindividual variability had all their study visits at the same time of the day. Accordingly, we do not suspect that diurnal variation has influenced the results significantly.

In **Papers III** and **V**, the patients with obesity had been subjected to a three-week low-energy diet before the study investigation, and hence we cannot rule out that this has influenced the results. However, 4βOHC concentrations, absolute bioavailability, and systemic clearance of midazolam did not change from before to after the diet. In **Paper III**, we performed an additional analysis to investigate if the observed differences at week 3 (after the low-energy diet) also were present before the low-energy diet given that CYP2C19 activity increased in this period (as shown in **Paper II**). Thus, we do not believe that the initial three-week diet intervention has influenced the result in **Paper III** and **V** to any significant degree.

Semi-simultaneous administration of midazolam

The semi-simultaneous administration of midazolam that was used in **Paper I** and **IV** has proven to be suitable to assess intestinal and hepatic CYP3A activity in healthy volunteers as it is more time-efficient, cost-effective, and minimizes the influence of intraindividual variability compared to traditional methods.[251] The method has also been used in patients with obesity before and after bariatric surgery previously.[62, 206] Traditionally, using non-compartmental methods, it is critical that the absorption of the oral dose is almost complete before administering the intravenous dose to separate the contribution from the oral versus the intravenous dose. However, it may be challenging to predict when the absorption phase is complete, especially in specific patient populations with an altered absorption or in cases of considerable lag time for absorption. Population pharmacokinetic methods use mathematical and statistical models to describe the dynamic relationship between drug dose and drug concentration in the body over time.[263] By describing drug transport into, between, and out of theoretical body compartments, the models are a valuable tool for understanding and predicting the behavior of a drug in the body.[264] Population pharmacokinetic models are more robust than non-compartmental methods, and yield accurate estimates of pharmacokinetic parameters, especially absolute bioavailability, even though the absorption phase is not virtually complete. Hence, we used a population pharmacokinetic model for the estimation of absolute bioavailability and AUC. Prior to the study start, we performed simulations to assess the appropriateness of separating the oral and intravenous administration by 4 hours with regards to accurate determination of absolute bioavailability (**Paper I**). Similarly, we investigated the predictive performance for the determination of absolute bioavailability with 2 hours separation between the oral and intravenous dose as we used in **Paper IV**, and the predictions were considered to be adequate for its purpose.

Single time-point metabolic ratios to assess CYP activities

CYP1A2, CYP2C9, and CYP2C19 activities were determined using a single time-point metabolic ratio (**Papers II** and **III**). The time-points (paraxanthine/caffeine ratio, 4 hours; 5-OH-omeprazole/omeprazole ratio, 3 hours; and losartan/LCA ratio; 8 hours) were carefully selected and had been validated in previous studies.[122, 265] This approach is frequently used, although it has not been validated in the population investigated. An important limitation is that single-time point ratios do not provide detailed information about the pharmacokinetic processes of the probe drugs. Thus, we cannot rule out that differences in the absorption rate or extent may have influenced the metabolic ratios. Patients with obesity may have altered absorption compared to normal-weight individuals,[144] and following RYGB, the absorption rate and extent have been shown to be both increased and decreased.[159, 204-206, 208, 238, 266] This is particularly relevant for omeprazole, given that the absorption may have considerable lag-time.[127] To investigate any potential impact of a changed absorption on the 3-hour 5-OH-omeprazole/omeprazole ratio, we performed simulations using literature data.[267, 268] However, sparse data in the literature made it challenging to perform a proper evaluation. Nevertheless, the simulations indicated that a 30% change in K_a in both directions did not influence average or low metabolic ratios to any significant degree. However, for patients with a high metabolic ratio, alterations in K_a appeared to have a potential impact. Also, patients with T2DM may have delayed absorption.[269] However, our data on oral midazolam did not indicate any difference in absorption rate or lag-time between patients with T2DM and obesity and patients with obesity only. The protein concentrations of CYP1A2, CYP2C9, and CYP2C19 also support that the single time-point metabolic ratios to a large extent reflects CYP1A2, CYP2C9, and CYP2C19 activities, presuming that the expression also reflects the activity. Ideally, we should have collected multiple blood samples to provide more information about the pharmacokinetic processes of the probe drugs, e.g., absorption phase and elimination phase. Unfortunately, this was not possible due to high costs associated with the analysis of the samples. Also, the study personnel considered that it was not possible to collect more blood samples due to ethical reasons.

5.6.2 Calculations and statistical considerations

NAFLD liver fat score

NAFLD-LFS was used to predict NAFLD in the study participants (**Papers I, II, III, and IV**). A major advantage of this score is that it is cost-effective and allows for prediction of NAFLD based on routinely available data.[221] With a cut-off value of -0.640 as we used, the score has been shown to have a sensitivity of 86% and a specificity of 71%,[221] and it is therefore a risk that a proportion of the individuals have been misdiagnosed. An important limitation of the NAFLD-LFS is that it covers a spectrum of liver diseases and does not allow for any determination of the severity of NAFLD. As such, it may have been more correct to apply the cut-off value in **Paper I**, rather than a continuous score as we used.

Systemic clearance

The midazolam population pharmacokinetic model developed in **Paper I** was not parameterized by systemic clearance. Thus, individual model parameter estimates of the volume of distribution and elimination rate constant were required to derive systemic clearance estimates from the model. However, a limitation of the developed model was that the initial distribution volume was very low for a small proportion of the patients, probably because these patients showed extremely high midazolam plasma concentrations immediately after the intravenous dose, thus resulting in the low volume parameter estimates in the model. With this in consideration, we determined to estimate systemic clearance from the model predicted area under the curve (equation shown in section 3.7) instead of the derived clearance from the individual model parameter estimates of elimination rate constant and volume of distribution. Nevertheless, on average, the clearance estimate from the model predicted volume parameter and elimination rate constant was very similar to the clearance estimated from the model predicted AUC. As such, we do not believe that this has influenced the results to any significant degree.

Sample size

The designers of the COCKTAIL-study performed a sample size calculation based on midazolam oral bioavailability in the intervention groups. The sample size was calculated to find an anticipated change of 40% between the time of surgery and six weeks post-RYGB with an 80% power and a 5% significance level. No strict sample size calculation was performed in **Papers II-V**, due to the exploratory and/or descriptive nature of the research questions in these

studies. Compared to previous studies with similar objectives, the sample size in our study was large. When performing multiple statistical tests there is an increased risk of type 1 errors. The confidence intervals in the longitudinal analyses were adjusted using the Tukey method, but an important limitation with the statistical analyses in **Papers III** and **V** is that we did not adjust for multiple testing. We considered it most correct to not adjust for multiple testing given that the likelihood of type II errors, i.e. failing to demonstrate an effect when there is one, increases if the study is not sufficiently powered which might have been the case in these studies. In **Papers III** and **V**, we used a nonparametric approach which may be overly conservative given the sample size. However, a parametric approach yielded almost exactly the same results. Thus, we considered non-parametric tests more appropriate to avoid the problems following with back-transformed estimates.

6 CONCLUSION

The overall aim of the thesis was to investigate pharmacokinetic variability in selected patient populations, focusing on CYP1A2, CYP2C9, CYP2C19, and CYP3A. Based on the papers in this thesis, the following conclusions have been made:

- CYP2C19 and CYP3A activities are lower in patients with obesity, while CYP1A2 and CYP2C9 activities are unaltered. T2DM appears to downregulate CYP2C19 activity beyond that of obesity, but has no impact on CYP1A2, CYP2C9, and CYP3A when obesity already is present. CYP2C19 activity increases rapidly following weight loss induced by RYGB or a strict diet, and reverts in case of regained body weight. CYP3A activity is not influenced in the early period following RYGB or diet-induced weight loss, but hepatic CYP3A activity seems to recover given enough time. RYGB and/or weight loss seems to have minor effects on CYP1A2 and CYP2C9.

- On average, there is a low- to moderate intraindividual variability in absolute bioavailability and systemic clearance of midazolam in healthy individuals without obesity, indicating a minor variability in CYP3A activity over time. However, a relevant proportion may have considerable intraindividual variability that may limit the use of midazolam in certain studies.

- The endogenous biomarker 4βOHC reflected hepatic, but not intestinal, CYP3A4 activity. Thus, 4βOHC may be a valuable supplement to traditional phenotyping using probe drugs to assess CYP3A activity.

7 CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

The findings in **Papers I-III** suggest that clinicians should be observant of adverse effects, or treatment failure in the case of prodrugs, when prescribing drugs primarily dependent on metabolism mediated by CYP2C19 and CYP3A in patients with obesity, and particularly when prescribing CYP2C19 substrates in patients with T2DM and obesity. The findings in **Papers I** and **II** also indicate that RYGB has less clinical relevance for early post-surgery drug dosing of CYP1A2, CYP2C9, CYP2C19, and CYP3A substrates than previously assumed. However, differences in such as substrate specificity, extraction ratio, and physicochemical properties make it challenging to extrapolate the results in this thesis to other drugs, also those that are primarily metabolized by the same enzymes. The data and findings in **Papers I-III** will potentially be relevant in the development of advanced physiologically based pharmacokinetic (PBPK) models that may improve our mechanistic understanding of how obesity, T2DM, RYGB, and weight loss changes drug disposition.

In **Paper IV** we showed that a relevant proportion of the healthy volunteers displayed significant intraindividual variability in midazolam absolute bioavailability and systemic clearance, which may limit the use of midazolam as a sole metric to assess CYP3A activity in certain studies. The findings in **Paper V**, suggest that 4βOHC reflects hepatic CYP3A4 activity sufficiently. Future studies should therefore consider to implement 4βOHC in clinical trials using probe drugs to assess CYP3A activity, as it may give complementary information in cases where other factors influence the pharmacokinetics of probe drugs.

REFERENCES

1. Rowland, M., Tozer, T.N., Clinical Pharmacokinetics and Pharmacodynamics - Concepts and applications. 4th. (Lippincott Williams & Wilkins, a Wolters Kluwer business: Philadelphia PA, 2011).
2. Abuhelwa, A.Y., et al., Food, gastrointestinal pH, and models of oral drug absorption. *Eur J Pharm Biopharm*, 2017. **112**: p. 234-248.
3. Jamei, M., et al., Population-based mechanistic prediction of oral drug absorption. *Aaps j*, 2009. **11**(2): p. 225-37.
4. Chang, G.W. and P.C. Kam, The physiological and pharmacological roles of cytochrome P450 isoenzymes. *Anaesthesia*, 1999. **54**(1): p. 42-50.
5. Neve, E.P. and M. Ingelman-Sundberg, Intracellular transport and localization of microsomal cytochrome P450. *Anal Bioanal Chem*, 2008. **392**(6): p. 1075-84.
6. Guengerich, F.P., Mechanisms of cytochrome P450 substrate oxidation: MiniReview. *J Biochem Mol Toxicol*, 2007. **21**(4): p. 163-8.
7. Lin, J.H. and A.Y. Lu, Interindividual variability in inhibition and induction of cytochrome P450 enzymes. *Annu Rev Pharmacol Toxicol*, 2001. **41**: p. 535-67.
8. Pavek, P. and Z. Dvorak, Xenobiotic-induced transcriptional regulation of xenobiotic metabolizing enzymes of the cytochrome P450 superfamily in human extrahepatic tissues. *Curr Drug Metab*, 2008. **9**(2): p. 129-43.
9. Nelson, D.R., et al., P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics*, 1996. **6**(1): p. 1-42.
10. Rendic, S. and F.P. Guengerich, Survey of Human Oxidoreductases and Cytochrome P450 Enzymes Involved in the Metabolism of Xenobiotic and Natural Chemicals. *Chem Res Toxicol*, 2015. **28**(1): p. 38-42.
11. Saravanakumar, A., et al., Physicochemical Properties, Biotransformation, and Transport Pathways of Established and Newly Approved Medications: A Systematic Review of the Top 200 Most Prescribed Drugs vs. the FDA-Approved Drugs Between 2005 and 2016. *Clin Pharmacokinet*, 2019. **58**(10): p. 1281-1294.
12. Zanger, U.M. and M. Schwab, Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther*, 2013. **138**(1): p. 103-41.
13. Nelson, D.R., et al., Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. *Pharmacogenetics*, 2004. **14**(1): p. 1-18.
14. Wilkinson, G.R., Drug metabolism and variability among patients in drug response. *N Engl J Med*, 2005. **352**(21): p. 2211-21.
15. Daly, A.K., Significance of the minor cytochrome P450 3A isoforms. *Clin Pharmacokinet*, 2006. **45**(1): p. 13-31.
16. Scott, E.E. and J.R. Halpert, Structures of cytochrome P450 3A4. *Trends Biochem Sci*, 2005. **30**(1): p. 5-7.
17. Paine, M.F., et al., The human intestinal cytochrome P450 "pie". *Drug Metab Dispos*, 2006. **34**(5): p. 880-6.
18. Aoyama, T., et al., Cytochrome P-450 hPCN3, a novel cytochrome P-450 IIIA gene product that is differentially expressed in adult human liver. cDNA and deduced amino acid sequence and distinct specificities of cDNA-expressed hPCN1 and hPCN3 for the metabolism of steroid hormones and cyclosporine. *J Biol Chem*, 1989. **264**(18): p. 10388-95.

19. Achour, B., J. Barber, and A. Rostami-Hodjegan, Expression of hepatic drug-metabolizing cytochrome p450 enzymes and their intercorrelations: a meta-analysis. *Drug Metab Dispos*, 2014. **42**(8): p. 1349-56.
20. Grangeon, A., et al., Determination of CYP450 Expression Levels in the Human Small Intestine by Mass Spectrometry-Based Targeted Proteomics. *Int J Mol Sci*, 2021. **22**(23).
21. de Wildt, S.N., et al., Cytochrome P450 3A: ontogeny and drug disposition. *Clin Pharmacokinet*, 1999. **37**(6): p. 485-505.
22. Lamba, J.K., et al., Genetic contribution to variable human CYP3A-mediated metabolism. *Adv Drug Deliv Rev*, 2002. **54**(10): p. 1271-94.
23. Goldstein, J.A., Clinical relevance of genetic polymorphisms in the human CYP2C subfamily. *Br J Clin Pharmacol*, 2001. **52**(4): p. 349-55.
24. Isvoran, A., et al., Pharmacogenomics of the cytochrome P450 2C family: impacts of amino acid variations on drug metabolism. *Drug Discov Today*, 2017. **22**(2): p. 366-376.
25. Zhou, S.F., et al., Structure, function, regulation and polymorphism and the clinical significance of human cytochrome P450 1A2. *Drug Metab Rev*, 2010. **42**(2): p. 268-354.
26. Shimada, T., et al., Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J Pharmacol Exp Ther*, 1994. **270**(1): p. 414-23.
27. Kuehl, P., et al., Sequence diversity in CYP3A promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. *Nat Genet*, 2001. **27**(4): p. 383-91.
28. Drozdzik, M., et al., Protein Abundance of Clinically Relevant Drug-Metabolizing Enzymes in the Human Liver and Intestine: A Comparative Analysis in Paired Tissue Specimens. *Clin Pharmacol Ther*, 2018. **104**(3): p. 515-524.
29. Wegler, C., et al., Drug Disposition Protein Quantification in Matched Human Jejunum and Liver From Donors With Obesity. *Clin Pharmacol Ther*, 2022.
30. Fritz, A., et al., Expression of clinically relevant drug-metabolizing enzymes along the human intestine and their correlation to drug transporters and nuclear receptors: An intra-subject analysis. *Basic Clin Pharmacol Toxicol*, 2019. **124**(3): p. 245-255.
31. PharmVar. *CYP2C9*. [cited 2022 April 26]; Available from: https://www.pharmvar.org/gene/CYP2C9.
32. Zhou, Y., M. Ingelman-Sundberg, and V.M. Lauschke, Worldwide Distribution of Cytochrome P450 Alleles: A Meta-analysis of Population-scale Sequencing Projects. *Clin Pharmacol Ther*, 2017. **102**(4): p. 688-700.
33. Van Booven, D., et al., Cytochrome P450 2C9-CYP2C9. *Pharmacogenet Genomics*, 2010. **20**(4): p. 277-81.
34. Aithal, G.P., et al., Association of polymorphisms in the cytochrome P450 CYP2C9 with warfarin dose requirement and risk of bleeding complications. *Lancet*, 1999. **353**(9154): p. 717-9.
35. de Morais, S.M., et al., The major genetic defect responsible for the polymorphism of S-mephenytoin metabolism in humans. *J Biol Chem*, 1994. **269**(22): p. 15419-22.
36. De Morais, S.M., et al., Identification of a new genetic defect responsible for the polymorphism of (S)-mephenytoin metabolism in Japanese. *Mol Pharmacol*, 1994. **46**(4): p. 594-8.
37. Ionova, Y., et al., CYP2C19 Allele Frequencies in Over 2.2 Million Direct-to-Consumer Genetics Research Participants and the Potential Implication for Prescriptions in a Large Health System. *Clin Transl Sci*, 2020. **13**(6): p. 1298-1306.

38. Sim, S.C., et al., A common novel CYP2C19 gene variant causes ultrarapid drug metabolism relevant for the drug response to proton pump inhibitors and antidepressants. *Clin Pharmacol Ther*, 2006. **79**(1): p. 103-13.
39. Bråten, L.S., et al., A Novel CYP2C-Haplotype Associated With Ultrarapid Metabolism of Escitalopram. *Clin Pharmacol Ther*, 2021. **110**(3): p. 786-793.
40. Caudle, K.E., et al., Standardizing CYP2D6 Genotype to Phenotype Translation: Consensus Recommendations from the Clinical Pharmacogenetics Implementation Consortium and Dutch Pharmacogenetics Working Group. *Clin Transl Sci*, 2020. **13**(1): p. 116-124.
41. Gaedigk, A., et al., The CYP2D6 activity score: translating genotype information into a qualitative measure of phenotype. *Clin Pharmacol Ther*, 2008. **83**(2): p. 234-42.
42. Ingelman-Sundberg, M., Genetic polymorphisms of cytochrome P450 2D6 (CYP2D6): clinical consequences, evolutionary aspects and functional diversity. *Pharmacogenomics J*, 2005. **5**(1): p. 6-13.
43. Oshikoya, K.A., et al., CYP2D6 genotype and adverse events to risperidone in children and adolescents. *Pediatr Res*, 2019. **85**(5): p. 602-606.
44. Meloche, M., et al., CYP2D6 polymorphism and its impact on the clinical response to metoprolol: A systematic review and meta-analysis. *Br J Clin Pharmacol*, 2020. **86**(6): p. 1015-1033.
45. Jukic, M.M., et al., Effect of CYP2D6 genotype on exposure and efficacy of risperidone and aripiprazole: a retrospective, cohort study. *Lancet Psychiatry*, 2019. **6**(5): p. 418-426.
46. Klein, K. and U.M. Zanger, Pharmacogenomics of Cytochrome P450 3A4: Recent Progress Toward the "Missing Heritability" Problem. *Front Genet*, 2013. **4**: p. 12.
47. Hole, K., et al., Impact of genetic and nongenetic factors on interindividual variability in 4β-hydroxycholesterol concentration. *Eur J Clin Pharmacol*, 2017. **73**(3): p. 317-324.
48. Wang, D., et al., Intronic polymorphism in CYP3A4 affects hepatic expression and response to statin drugs. *Pharmacogenomics J*, 2011. **11**(4): p. 274-86.
49. Klein, K., et al., PPARA: a novel genetic determinant of CYP3A4 in vitro and in vivo. *Clin Pharmacol Ther*, 2012. **91**(6): p. 1044-52.
50. Okubo, M., et al., CYP3A4 intron 6 C>T polymorphism (CYP3A4*22) is associated with reduced CYP3A4 protein level and function in human liver microsomes. *J Toxicol Sci*, 2013. **38**(3): p. 349-54.
51. Elens, L., et al., CYP3A4 intron 6 C>T SNP (CYP3A4*22) encodes lower CYP3A4 activity in cancer patients, as measured with probes midazolam and erythromycin. *Pharmacogenomics*, 2013. **14**(2): p. 137-49.
52. Holmberg, M.T., et al., CYP3A4*22 Impairs the Elimination of Ticagrelor, But Has No Significant Effect on the Bioactivation of Clopidogrel or Prasugrel. *Clin Pharmacol Ther*, 2019. **105**(2): p. 448-457.
53. Olagunju, A., et al., CYP3A4*22 (c.522-191 C>T; rs35599367) is associated with lopinavir pharmacokinetics in HIV-positive adults. *Pharmacogenet Genomics*, 2014. **24**(9): p. 459-63.
54. Mulder, T.A.M., et al., CYP3A4(∗)22 Genotyping in Clinical Practice: Ready for Implementation? *Front Genet*, 2021. **12**: p. 711943.
55. Kniepeiss, D., et al., The role of CYP3A5 genotypes in dose requirements of tacrolimus and everolimus after heart transplantation. *Clin Transplant*, 2011. **25**(1): p. 146-50.

56. Quteineh, L., et al., Influence of CYP3A5 genetic polymorphism on tacrolimus daily dose requirements and acute rejection in renal graft recipients. *Basic Clin Pharmacol Toxicol*, 2008. **103**(6): p. 546-52.
57. Koonrungsesomboon, N., et al., The impact of genetic polymorphisms on CYP1A2 activity in humans: a systematic review and meta-analysis. *Pharmacogenomics J*, 2018. **18**(6): p. 760-768.
58. Jetter, A., et al., Do activities of cytochrome P450 (CYP)3A, CYP2D6 and P-glycoprotein differ between healthy volunteers and HIV-infected patients? *Antivir Ther*, 2010. **15**(7): p. 975-83.
59. Gravel, S., et al., Modulation of CYP450 Activities in Patients With Type 2 Diabetes. *Clin Pharmacol Ther*, 2019. **106**(6): p. 1280-1289.
60. Woolsey, S.J., et al., CYP3A Activity and Expression in Nonalcoholic Fatty Liver Disease. *Drug Metab Dispos*, 2015. **43**(10): p. 1484-90.
61. Jamwal, R., et al., Nonalcoholic Fatty Liver Disease and Diabetes Are Associated with Decreased CYP3A4 Protein Expression and Activity in Human Liver. *Mol Pharm*, 2018. **15**(7): p. 2621-2632.
62. Brill, M.J., et al., Midazolam pharmacokinetics in morbidly obese patients following semi-simultaneous oral and intravenous administration: a comparison with healthy volunteers. *Clin Pharmacokinet*, 2014. **53**(10): p. 931-41.
63. Rivory, L.P., K.A. Slaviero, and S.J. Clarke, Hepatic cytochrome P450 3A drug metabolism is reduced in cancer patients who have an acute-phase response. *Br J Cancer*, 2002. **87**(3): p. 277-80.
64. Wollmann, B.M., et al., 4β-Hydroxycholesterol Level in Patients With Rheumatoid Arthritis Before vs. After Initiation of bDMARDs and Correlation With Inflammatory State. *Clin Transl Sci*, 2017. **10**(1): p. 42-49.
65. Burns, K.E., et al., CYP2C19 genotype-phenotype discordance in patients with multiple myeloma leads to an acquired loss of drug-metabolising activity. *Cancer Chemother Pharmacol*, 2014. **73**(3): p. 651-5.
66. Helsby, N.A., et al., CYP2C19 pharmacogenetics in advanced cancer: compromised function independent of genotype. *Br J Cancer*, 2008. **99**(8): p. 1251-5.
67. Jones, A.E., et al., Variability in drug metabolizing enzyme activity in HIV-infected patients. *Eur J Clin Pharmacol*, 2010. **66**(5): p. 475-85.
68. O'Neil, W.M., et al., Genotype and phenotype of cytochrome P450 2D6 in human immunodeficiency virus-positive patients and patients with acquired immunodeficiency syndrome. *Eur J Clin Pharmacol*, 2000. **56**(3): p. 231-40.
69. Cojutti, P.G., et al., Comparative Population Pharmacokinetics of Darunavir in SARS-CoV-2 Patients vs. HIV Patients: The Role of Interleukin-6. *Clin Pharmacokinet*, 2020. **59**(10): p. 1251-1260.
70. Lenoir, C., et al., Impact of SARS-CoV-2 Infection (COVID-19) on Cytochromes P450 Activity Assessed by the Geneva Cocktail. *Clin Pharmacol Ther*, 2021. **110**(5): p. 1358-1367.
71. Lenoir, C., et al., Impact of Acute Inflammation on Cytochromes P450 Activity Assessed by the Geneva Cocktail. *Clin Pharmacol Ther*, 2021. **109**(6): p. 1668-1676.
72. Lenoir, C., et al., Influence of Inflammation on Cytochromes P450 Activity in Adults: A Systematic Review of the Literature. *Front Pharmacol*, 2021. **12**: p. 733935.
73. Stanke-Labesque, F., et al., Inflammation is a major regulator of drug metabolizing enzymes and transporters: Consequences for the personalization of drug treatment. *Pharmacol Ther*, 2020. **215**: p. 107627.
74. Turner, M.D., et al., Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. *Biochim Biophys Acta*, 2014. **1843**(11): p. 2563-2582.

75. Aitken, A.E. and E.T. Morgan, Gene-specific effects of inflammatory cytokines on cytochrome P450 2C, 2B6 and 3A4 mRNA levels in human hepatocytes. *Drug Metab Dispos*, 2007. **35**(9): p. 1687-93.
76. Rubin, K., et al., HepaRG cells as human-relevant in vitro model to study the effects of inflammatory stimuli on cytochrome P450 isoenzymes. *Drug Metab Dispos*, 2015. **43**(1): p. 119-25.
77. Klein, M., et al., A systematic comparison of the impact of inflammatory signaling on absorption, distribution, metabolism, and excretion gene expression and activity in primary human hepatocytes and HepaRG cells. *Drug Metab Dispos*, 2015. **43**(2): p. 273-83.
78. Dickmann, L.J., et al., Effects of interleukin 1β (IL-1β) and IL-1β/interleukin 6 (IL-6) combinations on drug metabolizing enzymes in human hepatocyte culture. *Curr Drug Metab*, 2012. **13**(7): p. 930-7.
79. de Jong, L.M., et al., Distinct Effects of Inflammation on Cytochrome P450 Regulation and Drug Metabolism: Lessons from Experimental Models and a Potential Role for Pharmacogenetics. *Genes (Basel)*, 2020. **11**(12).
80. Pascussi, J.M., et al., Interleukin-6 negatively regulates the expression of pregnane X receptor and constitutively activated receptor in primary human hepatocytes. *Biochem Biophys Res Commun*, 2000. **274**(3): p. 707-13.
81. Assenat, E., et al., Interleukin 1beta inhibits CAR-induced expression of hepatic genes involved in drug and bilirubin clearance. *Hepatology*, 2004. **40**(4): p. 951-60.
82. Ke, S., et al., Mechanism of suppression of cytochrome P-450 1A1 expression by tumor necrosis factor-alpha and lipopolysaccharide. *J Biol Chem*, 2001. **276**(43): p. 39638-44.
83. Fichtenbaum, C.J., et al., Pharmacokinetic interactions between protease inhibitors and statins in HIV seronegative volunteers: ACTG Study A5047. *Aids*, 2002. **16**(4): p. 569-77.
84. Lown, K.S., et al., Grapefruit juice increases felodipine oral availability in humans by decreasing intestinal CYP3A protein expression. *J Clin Invest*, 1997. **99**(10): p. 2545-53.
85. Moore, L.B., et al., St. John's wort induces hepatic drug metabolism through activation of the pregnane X receptor. *Proc Natl Acad Sci U S A*, 2000. **97**(13): p. 7500-2.
86. Ruschitzka, F., et al., Acute heart transplant rejection due to Saint John's wort. *Lancet*, 2000. **355**(9203): p. 548-9.
87. Dobrinas, M., et al., Impact of smoking, smoking cessation, and genetic polymorphisms on CYP1A2 activity and inducibility. *Clin Pharmacol Ther*, 2011. **90**(1): p. 117-25.
88. Hukkanen, J., et al., Effect of nicotine on cytochrome P450 1A2 activity. *Br J Clin Pharmacol*, 2011. **72**(5): p. 836-8.
89. Schwartz, J.B., The current state of knowledge on age, sex, and their interactions on clinical pharmacology. *Clin Pharmacol Ther*, 2007. **82**(1): p. 87-96.
90. Kharasch, E.D., et al., Influence of CYP3A5 genotype on the pharmacokinetics and pharmacodynamics of the cytochrome P4503A probes alfentanil and midazolam. *Clin Pharmacol Ther*, 2007. **82**(4): p. 410-26.
91. Chung, E., et al., Comparison of midazolam and simvastatin as cytochrome P450 3A probes. *Clin Pharmacol Ther*, 2006. **79**(4): p. 350-61.
92. Thummel, K.E., et al., Oral first-pass elimination of midazolam involves both gastrointestinal and hepatic CYP3A-mediated metabolism. *Clin Pharmacol Ther*, 1996. **59**(5): p. 491-502.

93. Kashuba, A.D., et al., Quantification of 3-month intraindividual variability and the influence of sex and menstrual cycle phase on CYP3A activity as measured by phenotyping with intravenous midazolam. *Clin Pharmacol Ther*, 1998. **64**(3): p. 269-77.
94. Cotreau, M.M., L.L. von Moltke, and D.J. Greenblatt, The influence of age and sex on the clearance of cytochrome P450 3A substrates. *Clin Pharmacokinet*, 2005. **44**(1): p. 33-60.
95. Relling, M.V., et al., Racial and gender differences in N-acetyltransferase, xanthine oxidase, and CYP1A2 activities. *Clin Pharmacol Ther*, 1992. **52**(6): p. 643-58.
96. Scandlyn, M.J., E.C. Stuart, and R.J. Rosengren, Sex-specific differences in CYP450 isoforms in humans. *Expert Opin Drug Metab Toxicol*, 2008. **4**(4): p. 413-24.
97. Ghotbi, R., et al., Comparisons of CYP1A2 genetic polymorphisms, enzyme activity and the genotype-phenotype relationship in Swedes and Koreans. *Eur J Clin Pharmacol*, 2007. **63**(6): p. 537-46.
98. Tanaka, E., N. Kurata, and H. Yasuhara, How useful is the "cocktail approach" for evaluating human hepatic drug metabolizing capacity using cytochrome P450 phenotyping probes in vivo? *J Clin Pharm Ther*, 2003. **28**(3): p. 157-65.
99. Fuhr, U., A. Jetter, and J. Kirchheiner, Appropriate phenotyping procedures for drug metabolizing enzymes and transporters in humans and their simultaneous use in the "cocktail" approach. *Clin Pharmacol Ther*, 2007. **81**(2): p. 270-83.
100. Keller, G.A., et al., In vivo Phenotyping Methods: Cytochrome P450 Probes with Emphasis on the Cocktail Approach. *Curr Pharm Des*, 2017. **23**(14): p. 2035-2049.
101. Floyd, M.D., et al., Genotype-phenotype associations for common CYP3A4 and CYP3A5 variants in the basal and induced metabolism of midazolam in European- and African-American men and women. *Pharmacogenetics*, 2003. **13**(10): p. 595-606.
102. Vossen, M., et al., Dynamically simulating the interaction of midazolam and the CYP3A4 inhibitor itraconazole using individual coupled whole-body physiologically-based pharmacokinetic (WB-PBPK) models. *Theor Biol Med Model*, 2007. **4**: p. 13.
103. Yu, K.S., et al., Effect of the CYP3A5 genotype on the pharmacokinetics of intravenous midazolam during inhibited and induced metabolic states. *Clin Pharmacol Ther*, 2004. **76**(2): p. 104-12.
104. de Jonge, H., et al., Impact of CYP3A5 genotype on tacrolimus versus midazolam clearance in renal transplant recipients: new insights in CYP3A5-mediated drug metabolism. *Pharmacogenomics*, 2013. **14**(12): p. 1467-80.
105. He, P., et al., Genotype-phenotype associations of cytochrome P450 3A4 and 3A5 polymorphism with midazolam clearance in vivo. *Clin Pharmacol Ther*, 2005. **77**(5): p. 373-87.
106. Klotz, U. and G. Ziegler, Physiologic and temporal variation in hepatic elimination of midazolam. *Clin Pharmacol Ther*, 1982. **32**(1): p. 107-12.
107. Rogers, J.F., et al., An evaluation of the suitability of intravenous midazolam as an in vivo marker for hepatic cytochrome P4503A activity. *Clin Pharmacol Ther*, 2003. **73**(3): p. 153-8.
108. Kirwan, C., I. MacPhee, and B. Philips, Using drug probes to monitor hepatic drug metabolism in critically ill patients: midazolam, a flawed but useful tool for clinical investigation of CYP3A activity? *Expert Opin Drug Metab Toxicol*, 2010. **6**(6): p. 761-71.
109. Bodin, K., et al., Antiepileptic drugs increase plasma levels of 4beta-hydroxycholesterol in humans: evidence for involvement of cytochrome p450 3A4. *J Biol Chem*, 2001. **276**(42): p. 38685-9.

110. Bodin, K., et al., Metabolism of 4 beta -hydroxycholesterol in humans. *J Biol Chem*, 2002. **277**(35): p. 31534-40.
111. Diczfalusy, U., et al., 4Beta-hydroxycholesterol is a new endogenous CYP3A marker: relationship to CYP3A5 genotype, quinine 3-hydroxylation and sex in Koreans, Swedes and Tanzanians. *Pharmacogenet Genomics*, 2008. **18**(3): p. 201-8.
112. Gebeyehu, E., et al., Sex and CYP3A5 genotype influence total CYP3A activity: high CYP3A activity and a unique distribution of CYP3A5 variant alleles in Ethiopians. *Pharmacogenomics J*, 2011. **11**(2): p. 130-7.
113. Nitta, S.I., et al., Evaluation of 4β-Hydroxycholesterol and 25-Hydroxycholesterol as Endogenous Biomarkers of CYP3A4: Study with CYP3A-Humanized Mice. *Aaps j*, 2018. **20**(3): p. 61.
114. Mao, J., et al., Perspective: 4β-hydroxycholesterol as an emerging endogenous biomarker of hepatic CYP3A. *Drug Metab Rev*, 2017. **49**(1): p. 18-34.
115. Penzak, S.R. and C. Rojas-Fernandez, 4β-Hydroxycholesterol as an Endogenous Biomarker for CYP3A Activity: Literature Review and Critical Evaluation. *J Clin Pharmacol*, 2019. **59**(5): p. 611-624.
116. Gjestad, C., et al., 4β-hydroxycholesterol correlates with dose but not steady-state concentration of carbamazepine: indication of intestinal CYP3A in biomarker formation? *Br J Clin Pharmacol*, 2016. **81**(2): p. 269-76.
117. Diczfalusy, U., et al., 4beta-hydroxycholesterol as an endogenous marker for CYP3A4/5 activity. Stability and half-life of elimination after induction with rifampicin. *Br J Clin Pharmacol*, 2009. **67**(1): p. 38-43.
118. Faber, M.S., A. Jetter, and U. Fuhr, Assessment of CYP1A2 activity in clinical practice: why, how, and when? *Basic Clin Pharmacol Toxicol*, 2005. **97**(3): p. 125-34.
119. Grzegorzewski, J., et al., Pharmacokinetics of Caffeine: A Systematic Analysis of Reported Data for Application in Metabolic Phenotyping and Liver Function Testing. *Front Pharmacol*, 2021. **12**: p. 752826.
120. Lee, C.R., et al., Tolbutamide, flurbiprofen, and losartan as probes of CYP2C9 activity in humans. *J Clin Pharmacol*, 2003. **43**(1): p. 84-91.
121. Daly, A.K., et al., Pharmacogenomics of CYP2C9: Functional and Clinical Considerations. *J Pers Med*, 2017. **8**(1).
122. Christensen, M., et al., The Karolinska cocktail for phenotyping of five human cytochrome P450 enzymes. *Clin Pharmacol Ther*, 2003. **73**(6): p. 517-28.
123. Ryu, J.Y., et al., Development of the "Inje cocktail" for high-throughput evaluation of five human cytochrome P450 isoforms in vivo. *Clin Pharmacol Ther*, 2007. **82**(5): p. 531-40.
124. Stearns, R.A., et al., Biotransformation of losartan to its active carboxylic acid metabolite in human liver microsomes. Role of cytochrome P4502C and 3A subfamily members. *Drug Metab Dispos*, 1995. **23**(2): p. 207-15.
125. Mahmoudi, M., et al., Application of Microdosed Intravenous Omeprazole to Determine Hepatic CYP2C19 Activity. *J Clin Pharmacol*, 2021. **61**(6): p. 789-798.
126. Streetman, D.S., J.S. Bertino, Jr., and A.N. Nafziger, Phenotyping of drug-metabolizing enzymes in adults: a review of in-vivo cytochrome P450 phenotyping probes. *Pharmacogenetics*, 2000. **10**(3): p. 187-216.
127. Fuhr, U., et al., Assessment of Pharmacokinetic Drug-Drug Interactions in Humans: In Vivo Probe Substrates for Drug Metabolism and Drug Transport Revisited. *Annu Rev Pharmacol Toxicol*, 2019. **59**: p. 507-536.
128. Ishizaki, T. and Y. Horai, Review article: cytochrome P450 and the metabolism of proton pump inhibitors--emphasis on rabeprazole. *Aliment Pharmacol Ther*, 1999. **13 Suppl 3**: p. 27-36.

129. World Health Organization. *Obesity and overweight*. [cited 2022 June 04]; Available from: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight.
130. NCD Risk Factor Collaboration, Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19·2 million participants. *Lancet*, 2016. **387**(10026): p. 1377-1396.
131. González-Muniesa, P., et al., Obesity. *Nat Rev Dis Primers*, 2017. **3**: p. 17034.
132. Midthjell, K., et al., Trends in overweight and obesity over 22 years in a large adult population: the HUNT Study, Norway. *Clin Obes*, 2013. **3**(1-2): p. 12-20.
133. Løvsletten, O., et al., Prevalence of general and abdominal obesity in 2015-2016 and 8-year longitudinal weight and waist circumference changes in adults and elderly: the Tromsø Study. *BMJ Open*, 2020. **10**(11): p. e038465.
134. Zatterale, F., et al., Chronic Adipose Tissue Inflammation Linking Obesity to Insulin Resistance and Type 2 Diabetes. *Front Physiol*, 2019. **10**: p. 1607.
135. Zorena, K., et al., Adipokines and Obesity. Potential Link to Metabolic Disorders and Chronic Complications. *Int J Mol Sci*, 2020. **21**(10).
136. Rohm, T.V., et al., Inflammation in obesity, diabetes, and related disorders. *Immunity*, 2022. **55**(1): p. 31-55.
137. Katta, N., et al., Obesity and Coronary Heart Disease: Epidemiology, Pathology, and Coronary Artery Imaging. *Curr Probl Cardiol*, 2021. **46**(3): p. 100655.
138. Schnurr, T.M., et al., Obesity, unfavourable lifestyle and genetic risk of type 2 diabetes: a case-cohort study. *Diabetologia*, 2020. **63**(7): p. 1324-1332.
139. Guh, D.P., et al., The incidence of co-morbidities related to obesity and overweight: a systematic review and meta-analysis. *BMC Public Health*, 2009. **9**: p. 88.
140. De Pergola, G. and F. Silvestris, Obesity as a major risk factor for cancer. *J Obes*, 2013. **2013**: p. 291546.
141. Reyes, C., et al., Association Between Overweight and Obesity and Risk of Clinically Diagnosed Knee, Hip, and Hand Osteoarthritis: A Population-Based Cohort Study. *Arthritis Rheumatol*, 2016. **68**(8): p. 1869-75.
142. Li, L., et al., Obesity is an independent risk factor for non-alcoholic fatty liver disease: evidence from a meta-analysis of 21 cohort studies. *Obes Rev*, 2016. **17**(6): p. 510-9.
143. Younossi, Z.M., Non-alcoholic fatty liver disease - A global public health perspective. *J Hepatol*, 2019. **70**(3): p. 531-544.
144. Smit, C., et al., Obesity and drug pharmacology: a review of the influence of obesity on pharmacokinetic and pharmacodynamic parameters. *Expert Opin Drug Metab Toxicol*, 2018. **14**(3): p. 275-285.
145. Brill, M.J., et al., Impact of obesity on drug metabolism and elimination in adults and children. *Clin Pharmacokinet*, 2012. **51**(5): p. 277-304.
146. Abernethy, D.R., et al., The influence of obesity on the pharmacokinetics of oral alprazolam and triazolam. *Clin Pharmacokinet*, 1984. **9**(2): p. 177-83.
147. Caraco, Y., et al., Carbamazepine pharmacokinetics in obese and lean subjects. *Ann Pharmacother*, 1995. **29**(9): p. 843-7.
148. Rodríguez-Morató, J., et al., Short- and medium-term impact of bariatric surgery on the activities of CYP2D6, CYP3A4, CYP2C9, and CYP1A2 in morbid obesity. *Sci Rep*, 2019. **9**(1): p. 20405.
149. Greenblatt, D.J., et al., Effect of age, gender, and obesity on midazolam kinetics. *Anesthesiology*, 1984. **61**(1): p. 27-35.
150. Flechner, S.M., et al., The impact of body weight on cyclosporine pharmacokinetics in renal transplant recipients. *Transplantation*, 1989. **47**(5): p. 806-10.

151. Ulvestad, M., et al., Impact of OATP1B1, MDR1, and CYP3A4 expression in liver and intestine on interpatient pharmacokinetic variability of atorvastatin in obese subjects. *Clin Pharmacol Ther*, 2013. **93**(3): p. 275-82.
152. Krogstad, V., et al., Correlation of Body Weight and Composition With Hepatic Activities of Cytochrome P450 Enzymes. *J Pharm Sci*, 2021. **110**(1): p. 432-437.
153. Hole, K., et al., Elevated 4β-hydroxycholesterol/cholesterol ratio in anorexia nervosa patients. *Pharmacol Res Perspect*, 2018. **6**(5): p. e00430.
154. Woolsey, S.J., et al., Relationships between Endogenous Plasma Biomarkers of Constitutive Cytochrome P450 3A Activity and Single-Time-Point Oral Midazolam Microdose Phenotype in Healthy Subjects. *Basic Clin Pharmacol Toxicol*, 2016. **118**(4): p. 284-91.
155. Brill, M.J., et al., Semiphysiologically based pharmacokinetic model for midazolam and CYP3A mediated metabolite 1-OH-midazolam in morbidly obese and weight loss surgery patients. *CPT Pharmacometrics Syst Pharmacol*, 2016. **5**(1): p. 20-30.
156. Dong, D., et al., Morbid Obesity Alters Both Pharmacokinetics and Pharmacodynamics of Propofol: Dosing Recommendation for Anesthesia Induction. *Drug Metab Dispos*, 2016. **44**(10): p. 1579-83.
157. Vaughns, J.D., et al., Use of Fentanyl in Adolescents with Clinically Severe Obesity Undergoing Bariatric Surgery: A Pilot Study. *Paediatr Drugs*, 2017. **19**(3): p. 251-257.
158. Diepstraten, J., et al., An integrated population pharmacokinetic meta-analysis of propofol in morbidly obese and nonobese adults, adolescents, and children. *CPT Pharmacometrics Syst Pharmacol*, 2013. **2**(9): p. e73.
159. Goday Arno, A., et al., Pharmacokinetics in Morbid Obesity: Influence of Two Bariatric Surgery Techniques on Paracetamol and Caffeine Metabolism. *Obes Surg*, 2017. **27**(12): p. 3194-3201.
160. van Rongen, A., et al., Morbidly Obese Patients Exhibit Increased CYP2E1-Mediated Oxidation of Acetaminophen. *Clin Pharmacokinet*, 2016. **55**(7): p. 833-847.
161. Emery, M.G., et al., CYP2E1 activity before and after weight loss in morbidly obese subjects with nonalcoholic fatty liver disease. *Hepatology*, 2003. **38**(2): p. 428-35.
162. Cardoso-Júnior, A., et al., Gastric emptying of solids and semi-solids in morbidly obese and non-obese subjects: an assessment using the 13C-octanoic acid and 13C-acetic acid breath tests. *Obes Surg*, 2007. **17**(2): p. 236-41.
163. Benedek, I.H., et al., Serum alpha 1-acid glycoprotein and the binding of drugs in obesity. *Br J Clin Pharmacol*, 1983. **16**(6): p. 751-4.
164. Chalasani, N., et al., The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. *Hepatology*, 2018. **67**(1): p. 328-357.
165. Le, M.H., et al., 2019 Global NAFLD Prevalence: A Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol*, 2021.
166. Younossi, Z.M., et al., Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology*, 2016. **64**(1): p. 73-84.
167. Machado, M., P. Marques-Vidal, and H. Cortez-Pinto, Hepatic histology in obese patients undergoing bariatric surgery. *J Hepatol*, 2006. **45**(4): p. 600-6.
168. Targher, G., et al., The complex link between NAFLD and type 2 diabetes mellitus - mechanisms and treatments. *Nat Rev Gastroenterol Hepatol*, 2021. **18**(9): p. 599-612.
169. Jamwal, R. and B.J. Barlock, Nonalcoholic Fatty Liver Disease (NAFLD) and Hepatic Cytochrome P450 (CYP) Enzymes. *Pharmaceuticals (Basel)*, 2020. **13**(9).

170. Donato, M.T., et al., Potential impact of steatosis on cytochrome P450 enzymes of human hepatocytes isolated from fatty liver grafts. *Drug Metab Dispos*, 2006. **34**(9): p. 1556-62.
171. Fisher, C.D., et al., Hepatic cytochrome P450 enzyme alterations in humans with progressive stages of nonalcoholic fatty liver disease. *Drug Metab Dispos*, 2009. **37**(10): p. 2087-94.
172. Kolwankar, D., et al., Association between nonalcoholic hepatic steatosis and hepatic cytochrome P-450 3A activity. *Clin Gastroenterol Hepatol*, 2007. **5**(3): p. 388-93.
173. Donato, M.T., et al., Effects of steatosis on drug-metabolizing capability of primary human hepatocytes. *Toxicol In Vitro*, 2007. **21**(2): p. 271-6.
174. Weltman, M.D., et al., Hepatic cytochrome P450 2E1 is increased in patients with nonalcoholic steatohepatitis. *Hepatology*, 1998. **27**(1): p. 128-33.
175. Anavi, S., Z. Madar, and O. Tirosh, Non-alcoholic fatty liver disease, to struggle with the strangle: Oxygen availability in fatty livers. *Redox Biol*, 2017. **13**: p. 386-392.
176. Chatterjee, S., K. Khunti, and M.J. Davies, Type 2 diabetes. *Lancet*, 2017. **389**(10085): p. 2239-2251.
177. NCD Risk Factor Collaboration, Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4.4 million participants. *Lancet*, 2016. **387**(10027): p. 1513-1530.
178. Lascar, N., et al., Type 2 diabetes in adolescents and young adults. *Lancet Diabetes Endocrinol*, 2018. **6**(1): p. 69-80.
179. Lingvay, I., et al., Obesity management as a primary treatment goal for type 2 diabetes: time to reframe the conversation. *Lancet*, 2022. **399**(10322): p. 394-405.
180. Younossi, Z.M., et al., The global epidemiology of NAFLD and NASH in patients with type 2 diabetes: A systematic review and meta-analysis. *J Hepatol*, 2019. **71**(4): p. 793-801.
181. Tsalamandris, S., et al., The Role of Inflammation in Diabetes: Current Concepts and Future Perspectives. *Eur Cardiol*, 2019. **14**(1): p. 50-59.
182. Donath, M.Y. and S.E. Shoelson, Type 2 diabetes as an inflammatory disease. *Nat Rev Immunol*, 2011. **11**(2): p. 98-107.
183. Dostalek, M., et al., Significantly reduced cytochrome P450 3A4 expression and activity in liver from humans with diabetes mellitus. *Br J Pharmacol*, 2011. **163**(5): p. 937-47.
184. Marques, M.P., et al., Dynamic and kinetic disposition of nisoldipine enantiomers in hypertensive patients presenting with type-2 diabetes mellitus. *Eur J Clin Pharmacol*, 2002. **58**(9): p. 607-14.
185. Moisés, E.C., et al., Pharmacokinetics of lidocaine and its metabolite in peridural anesthesia administered to pregnant women with gestational diabetes mellitus. *Eur J Clin Pharmacol*, 2008. **64**(12): p. 1189-96.
186. Jacobson, P.A., et al., Novel polymorphisms associated with tacrolimus trough concentrations: results from a multicenter kidney transplant consortium. *Transplantation*, 2011. **91**(3): p. 300-8.
187. Angiolillo, D.J., et al., Platelet function profiles in patients with type 2 diabetes and coronary artery disease on combined aspirin and clopidogrel treatment. *Diabetes*, 2005. **54**(8): p. 2430-5.
188. Schuette, C., et al., The effect of clopidogrel on platelet activity in patients with and without type-2 diabetes mellitus: a comparative study. *Cardiovasc Diabetol*, 2015. **14**: p. 15.
189. Erlinge, D., et al., Patients with poor responsiveness to thienopyridine treatment or with diabetes have lower levels of circulating active metabolite, but their platelets

respond normally to active metabolite added ex vivo. *J Am Coll Cardiol*, 2008. **52**(24): p. 1968-77.
190. Urry, E., A. Jetter, and H.P. Landolt, Assessment of CYP1A2 enzyme activity in relation to type-2 diabetes and habitual caffeine intake. *Nutr Metab (Lond)*, 2016. **13**: p. 66.
191. Darakjian, L., et al., Chronic Inflammatory Status Observed in Patients with Type 2 Diabetes Induces Modulation of Cytochrome P450 Expression and Activity. *Int J Mol Sci*, 2021. **22**(9).
192. Jakobsen, G.S., et al., Association of Bariatric Surgery vs Medical Obesity Treatment With Long-term Medical Complications and Obesity-Related Comorbidities. *Jama*, 2018. **319**(3): p. 291-301.
193. Ikramuddin, S., et al., Lifestyle Intervention and Medical Management With vs Without Roux-en-Y Gastric Bypass and Control of Hemoglobin A1c, LDL Cholesterol, and Systolic Blood Pressure at 5 Years in the Diabetes Surgery Study. *Jama*, 2018. **319**(3): p. 266-278.
194. Aminian, A., et al., Association of Bariatric Surgery With Major Adverse Liver and Cardiovascular Outcomes in Patients With Biopsy-Proven Nonalcoholic Steatohepatitis. *Jama*, 2021. **326**(20): p. 2031-2042.
195. Syn, N.L., et al., Association of metabolic-bariatric surgery with long-term survival in adults with and without diabetes: a one-stage meta-analysis of matched cohort and prospective controlled studies with 174 772 participants. *Lancet*, 2021. **397**(10287): p. 1830-1841.
196. Mingrone, G., et al., Metabolic surgery versus conventional medical therapy in patients with type 2 diabetes: 10-year follow-up of an open-label, single-centre, randomised controlled trial. *Lancet*, 2021. **397**(10271): p. 293-304.
197. Rubino, F., et al., Metabolic Surgery in the Treatment Algorithm for Type 2 Diabetes: A Joint Statement by International Diabetes Organizations. *Diabetes Care*, 2016. **39**(6): p. 861-77.
198. Welbourn, R., et al., Bariatric Surgery Worldwide: Baseline Demographic Description and One-Year Outcomes from the Fourth IFSO Global Registry Report 2018. *Obes Surg*, 2019. **29**(3): p. 782-795.
199. Angrisani, L., et al., IFSO Worldwide Survey 2016: Primary, Endoluminal, and Revisional Procedures. *Obes Surg*, 2018. **28**(12): p. 3783-3794.
200. Pucci, A. and R.L. Batterham, Mechanisms underlying the weight loss effects of RYGB and SG: similar, yet different. *J Endocrinol Invest*, 2019. **42**(2): p. 117-128.
201. Seeras, K., R.J. Acho, and P.P. Lopez, Roux-en-Y Gastric Bypass Chronic Complications. StatPearls. 2022, *Treasure Island (FL)* StatPearls Publishing.
202. Angeles, P.C., et al., The influence of bariatric surgery on oral drug bioavailability in patients with obesity: A systematic review. *Obes Rev*, 2019. **20**(9): p. 1299-1311.
203. Hachon, L., et al., RYGB and Drug Disposition: How to Do Better? Analysis of Pharmacokinetic Studies and Recommendations for Clinical Practice. *Obes Surg*, 2017. **27**(4): p. 1076-1090.
204. Mitrov-Winkelmolen, L., et al., The Effect of Roux-en-Y Gastric Bypass Surgery in Morbidly Obese Patients on Pharmacokinetics of (Acetyl)Salicylic Acid and Omeprazole: the ERY-PAO Study. *Obes Surg*, 2016. **26**(9): p. 2051-2058.
205. Chan, L.N., et al., Proximal Roux-en-Y gastric bypass alters drug absorption pattern but not systemic exposure of CYP3A4 and P-glycoprotein substrates. *Pharmacotherapy*, 2015. **35**(4): p. 361-9.

206. Brill, M.J., et al., The Pharmacokinetics of the CYP3A Substrate Midazolam in Morbidly Obese Patients Before and One Year After Bariatric Surgery. *Pharm Res*, 2015. **32**(12): p. 3927-36.
207. Puris, E., et al., Laparoscopic Roux-en-Y gastric bypass surgery influenced pharmacokinetics of several drugs given as a cocktail with the highest impact observed for CYP1A2, CYP2C8 and CYP2E1 substrates. *Basic Clin Pharmacol Toxicol*, 2019. **125**(2): p. 123-132.
208. Lloret-Linares, C., et al., Effect of a Roux-en-Y gastric bypass on the pharmacokinetics of oral morphine using a population approach. *Clin Pharmacokinet*, 2014. **53**(10): p. 919-30.
209. Jakobsen, G.S., et al., Long-term effects of gastric bypass and duodenal switch on systemic exposure of atorvastatin. *Surg Endosc*, 2013. **27**(6): p. 2094-101.
210. Skottheim, I.B., et al., Significantly altered systemic exposure to atorvastatin acid following gastric bypass surgery in morbidly obese patients. *Clin Pharmacol Ther*, 2009. **86**(3): p. 311-8.
211. Schwenger, K.J.P., et al., In nonalcoholic fatty liver disease, Roux-en-Y gastric bypass improves liver histology while persistent disease is associated with lower improvements in waist circumference and glycemic control. *Surg Obes Relat Dis*, 2018. **14**(9): p. 1233-1239.
212. Pedersen, J.S., et al., Effects of Roux-en-Y Gastric Bypass and Sleeve Gastrectomy on Non-Alcoholic Fatty Liver Disease: A 12-Month Follow-Up Study with Paired Liver Biopsies. *J Clin Med*, 2021. **10**(17).
213. Lautenbach, A., et al., Long-Term Improvement of Chronic Low-Grade Inflammation After Bariatric Surgery. *Obes Surg*, 2021. **31**(7): p. 2913-2920.
214. Askarpour, M., et al., Effect of Bariatric Surgery on Serum Inflammatory Factors of Obese Patients: a Systematic Review and Meta-Analysis. *Obes Surg*, 2019. **29**(8): p. 2631-2647.
215. Portolés-Pérez, A., et al., Effect of obesity and Roux-en-Y gastric surgery on omeprazole pharmacokinetics. *Obesity Facts*, 2022.
216. Egeland, E.J., et al., Chronic Inhibition of CYP3A is Temporarily Reduced by Each Hemodialysis Session in Patients With End-Stage Renal Disease. *Clin Pharmacol Ther*, 2020. **108**(4): p. 866-873.
217. Størset, E., et al., The CYP3A biomarker 4β-hydroxycholesterol does not improve tacrolimus dose predictions early after kidney transplantation. *Br J Clin Pharmacol*, 2017. **83**(7): p. 1457-1465.
218. Wiśniewski, J.R. and D. Rakus, Multi-enzyme digestion FASP and the 'Total Protein Approach'-based absolute quantification of the Escherichia coli proteome. *J Proteomics*, 2014. **109**: p. 322-31.
219. Wiśniewski, J.R. and M. Mann, Consecutive proteolytic digestion in an enzyme reactor increases depth of proteomic and phosphoproteomic analysis. *Anal Chem*, 2012. **84**(6): p. 2631-7.
220. Krogstad, V., et al., A Comparative Analysis of Cytochrome P450 Activities in Paired Liver and Small Intestinal Samples from Patients with Obesity. *Drug Metab Dispos*, 2020. **48**(1): p. 8-17.
221. Kotronen, A., et al., Prediction of non-alcoholic fatty liver disease and liver fat using metabolic and genetic factors. *Gastroenterology*, 2009. **137**(3): p. 865-72.
222. Lloret-Linares, C., Pharmacokinetic considerations for patients with a history of bariatric surgery. *Expert Opin Drug Metab Toxicol*, 2017. **13**(5): p. 493-496.
223. Greenblatt, H.K. and D.J. Greenblatt, Altered drug disposition following bariatric surgery: a research challenge. *Clin Pharmacokinet*, 2015. **54**(6): p. 573-9.

224. Yoshinari, K., et al., Hepatic CYP3A expression is attenuated in obese mice fed a high-fat diet. *Pharm Res*, 2006. **23**(6): p. 1188-200.
225. Ghose, R., et al., Role of high-fat diet in regulation of gene expression of drug metabolizing enzymes and transporters. *Life Sci*, 2011. **89**(1-2): p. 57-64.
226. Salem, F., et al., Considering Age Variation When Coining Drugs as High versus Low Hepatic Extraction Ratio. *Drug Metab Dispos*, 2016. **44**(7): p. 1099-102.
227. van Rongen, A., et al., Higher Midazolam Clearance in Obese Adolescents Compared with Morbidly Obese Adults. *Clin Pharmacokinet*, 2018. **57**(5): p. 601-611.
228. Dundee, J.W., et al., Midazolam. A review of its pharmacological properties and therapeutic use. *Drugs*, 1984. **28**(6): p. 519-43.
229. Franken, L.G., et al., Hypoalbuminaemia and decreased midazolam clearance in terminally ill adult patients, an inflammatory effect? *Br J Clin Pharmacol*, 2017. **83**(8): p. 1701-1712.
230. Rostami-Hodjegan, A. and G.T. Tucker, The effects of portal shunts on intestinal cytochrome P450 3A activity. *Hepatology*, 2002. **35**(6): p. 1549-50; author reply 1550-1.
231. Gravel, S., et al., A Pilot Study towards the Impact of Type 2 Diabetes on the Expression and Activities of Drug Metabolizing Enzymes and Transporters in Human Duodenum. *Int J Mol Sci*, 2019. **20**(13).
232. Abernethy, D.R., et al., Prolongation of drug half-life due to obesity: studies of desmethyldiazepam (clorazepate). *J Pharm Sci*, 1982. **71**(8): p. 942-4.
233. Abernethy, D.R., et al., Alterations in drug distribution and clearance due to obesity. *J Pharmacol Exp Ther*, 1981. **217**(3): p. 681-5.
234. Sandvik, P., et al., Association between low body weight and cytochrome P-450 enzyme activity in patients with anorexia nervosa. *Pharmacol Res Perspect*, 2020. **8**(3): p. e00615.
235. Adithan, C., et al., Effect of type II diabetes mellitus on theophylline elimination. *Int J Clin Pharmacol Ther Toxicol*, 1989. **27**(5): p. 258-60.
236. Madsen, L.R., et al., Effect of Roux-en-Y gastric bypass surgery on diabetes remission and complications in individuals with type 2 diabetes: a Danish population-based matched cohort study. *Diabetologia*, 2019. **62**(4): p. 611-620.
237. Purnell, J.Q., et al., Diabetes Remission Status During Seven-year Follow-up of the Longitudinal Assessment of Bariatric Surgery Study. *J Clin Endocrinol Metab*, 2021. **106**(3): p. 774-788.
238. Tandra, S., et al., Pharmacokinetic and pharmacodynamic alterations in the Roux-en-Y gastric bypass recipients. *Ann Surg*, 2013. **258**(2): p. 262-9.
239. Abiru, S., et al., Serum cytokine and soluble cytokine receptor levels in patients with non-alcoholic steatohepatitis. *Liver Int*, 2006. **26**(1): p. 39-45.
240. Wandrer, F., et al., TNF-Receptor-1 inhibition reduces liver steatosis, hepatocellular injury and fibrosis in NAFLD mice. *Cell Death Dis*, 2020. **11**(3): p. 212.
241. Yang, J., et al., Cytochrome p450 turnover: regulation of synthesis and degradation, methods for determining rates, and implications for the prediction of drug interactions. *Curr Drug Metab*, 2008. **9**(5): p. 384-94.
242. Kaartinen, T.J.K., et al., Effect of High-Dose Esomeprazole on CYP1A2, CYP2C19, and CYP3A4 Activities in Humans: Evidence for Substantial and Long-lasting Inhibition of CYP2C19. *Clin Pharmacol Ther*, 2020. **108**(6): p. 1254-1264.
243. Dickmann, L.J., et al., Effects of interleukin-6 (IL-6) and an anti-IL-6 monoclonal antibody on drug-metabolizing enzymes in human hepatocyte culture. *Drug Metab Dispos*, 2011. **39**(8): p. 1415-22.

244. Madsen, J.L., S.B. Søndergaard, and S. Møller, Meal-induced changes in splanchnic blood flow and oxygen uptake in middle-aged healthy humans. *Scand J Gastroenterol*, 2006. **41**(1): p. 87-92.
245. McLean, A.J., et al., Food, splanchnic blood flow, and bioavailability of drugs subject to first-pass metabolism. *Clin Pharmacol Ther*, 1978. **24**(1): p. 5-10.
246. Rose, R.H., et al., Incorporation of the Time-Varying Postprandial Increase in Splanchnic Blood Flow into a PBPK Model to Predict the Effect of Food on the Pharmacokinetics of Orally Administered High-Extraction Drugs. *Aaps j*, 2017. **19**(4): p. 1205-1217.
247. Chalasani, N., et al., Hepatic and intestinal cytochrome P450 3A activity in cirrhosis: effects of transjugular intrahepatic portosystemic shunts. *Hepatology*, 2001. **34**(6): p. 1103-8.
248. Bornemann, L.D., et al., Influence of food on midazolam absorption. *J Clin Pharmacol*, 1986. **26**(1): p. 55-9.
249. Heizmann, P., M. Eckert, and W.H. Ziegler, Pharmacokinetics and bioavailability of midazolam in man. *Br J Clin Pharmacol*, 1983. **16 Suppl 1**(Suppl 1): p. 43s-49s.
250. Hohmann, N., et al., Midazolam microdose to determine systemic and pre-systemic metabolic CYP3A activity in humans. *Br J Clin Pharmacol*, 2015. **79**(2): p. 278-85.
251. Lee, J.I., et al., Application of semisimultaneous midazolam administration for hepatic and intestinal cytochrome P450 3A phenotyping. *Clin Pharmacol Ther*, 2002. **72**(6): p. 718-28.
252. van Rongen, A., et al., Population Pharmacokinetic Model Characterizing 24-Hour Variation in the Pharmacokinetics of Oral and Intravenous Midazolam in Healthy Volunteers. *CPT Pharmacometrics Syst Pharmacol*, 2015. **4**(8): p. 454-64.
253. Klein, K., et al., Pathway-Targeted Pharmacogenomics of CYP1A2 in Human Liver. *Front Pharmacol*, 2010. **1**: p. 129.
254. Gabbia, D., et al., Pregnane X receptor and constitutive androstane receptor modulate differently CYP3A-mediated metabolism in early- and late-stage cholestasis. *World J Gastroenterol*, 2017. **23**(42): p. 7519-7530.
255. Fukushima, S., et al., Effect of biliary obstruction and internal biliary drainage on hepatic cytochrome P450 isozymes in rats. *World J Gastroenterol*, 2008. **14**(16): p. 2556-60.
256. Gravel, S., et al., Use of 4β-Hydroxycholesterol Plasma Concentrations as an Endogenous Biomarker of CYP3A Activity: Clinical Validation in Individuals With Type 2 Diabetes. *Clin Pharmacol Ther*, 2019. **106**(4): p. 831-840.
257. Mannheimer, B., et al., No impact of vitamin D on the CYP3A biomarker 4β-hydroxycholesterol in patients with abnormal glucose regulation. *PLoS One*, 2015. **10**(4): p. e0121984.
258. Kharasch, E.D., et al., Menstrual cycle variability in midazolam pharmacokinetics. *J Clin Pharmacol*, 1999. **39**(3): p. 275-80.
259. Kharasch, E.D., et al., Intraindividual variability in male hepatic CYP3A4 activity assessed by alfentanil and midazolam clearance. *J Clin Pharmacol*, 1999. **39**(7): p. 664-9.
260. Matthaei, J., et al., Inherited and Acquired Determinants of Hepatic CYP3A Activity in Humans. *Front Genet*, 2020. **11**: p. 944.
261. Wegler, C., et al., Influence of Proteome Profiles and Intracellular Drug Exposure on Differences in CYP Activity in Donor-Matched Human Liver Microsomes and Hepatocytes. *Mol Pharm*, 2021. **18**(4): p. 1792-1805.

262. Tomalik-Scharte, D., et al., Population pharmacokinetic analysis of circadian rhythms in hepatic CYP3A activity using midazolam. *J Clin Pharmacol*, 2014. **54**(10): p. 1162-9.
263. Sheiner, L.B., B. Rosenberg, and V.V. Marathe, Estimation of population characteristics of pharmacokinetic parameters from routine clinical data. *J Pharmacokinet Biopharm*, 1977. **5**(5): p. 445-79.
264. Ette, E.I. and P.J. Williams, Population pharmacokinetics I: background, concepts, and models. *Ann Pharmacother*, 2004. **38**(10): p. 1702-6.
265. Yasar, U., et al., Pharmacokinetics of losartan and its metabolite E-3174 in relation to the CYP2C9 genotype. *Clin Pharmacol Ther*, 2002. **71**(1): p. 89-98.
266. McLachlan, L.A., B.B. Chaar, and I.S. Um, Pharmacokinetic changes post-bariatric surgery: A scoping review. *Obes Rev*, 2020. **21**(5): p. e12988.
267. Ochoa, D., et al., Effect of food on the pharmacokinetics of omeprazole, pantoprazole and rabeprazole. *BMC Pharmacol Toxicol*, 2020. **21**(1): p. 54.
268. Baldwin, R.M., et al., Increased omeprazole metabolism in carriers of the CYP2C19*17 allele; a pharmacokinetic study in healthy volunteers. *Br J Clin Pharmacol*, 2008. **65**(5): p. 767-74.
269. Dostalek, M., F. Akhlaghi, and M. Puzanovova, Effect of diabetes mellitus on pharmacokinetic and pharmacodynamic properties of drugs. *Clin Pharmacokinet*, 2012. **51**(8): p. 481-99.

I

Received: 2 April 2020 | Revised: 17 July 2020 | Accepted: 26 July 2020

DOI: 10.1111/cts.13142

ARTICLE

Short- and long-term effects of body weight loss following calorie restriction and gastric bypass on CYP3A-activity – a non-randomized three-armed controlled trial

Kine Eide Kvitne[1] | Ida Robertsen[1] | Eva Skovlund[2] | Hege Christensen[1] | Veronica Krogstad[1] | Christine Wegler[3,4] | Philip Carlo Angeles[5,6] | Birgit Malene Wollmann[7] | Kristine Hole[7] | Line Kristin Johnson[5] | Rune Sandbu[5,6] | Per Artursson[8] | Cecilia Karlsson[9,10] | Shalini Andersson[11] | Tommy B. Andersson[4] | Jøran Hjelmesæth[5,12] | Rasmus Jansson-Löfmark[4] | Anders Åsberg[1,13]

[1]Section for Pharmacology and Pharmaceutical Biosciences, Department of Pharmacy, University of Oslo, Oslo, Norway

[2]Department of Public Health and Nursing, Norwegian University of Science and Technology, NTNU, Trondheim, Norway

[3]Department of Pharmacy, Uppsala University, Uppsala, Sweden

[4]DMPK, Research and Early Development, Cardiovascular, Renal and Metabolism, BioPharmaceuticals R&D, AstraZeneca, Mölndal, Sweden

[5]Vestfold Hospital Trust, The Morbid Obesity Center, Tønsberg, Norway

[6]Department of Surgery, Vestfold Hospital Trust, Tønsberg, Norway

[7]Center for Psychopharmacology, Diakonhjemmet Hospital, Oslo, Norway

[8]Department of Pharmacy and Science for Life Laboratory, Uppsala University, Uppsala, Sweden

[9]Late-stage Development, Cardiovascular, Renal and Metabolism, BioPharmaceuticals R&D, AstraZeneca, Mölndal, Sweden

[10]Department of Molecular and Clinical Medicine, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

[11]Research and Early Development, Discovery Sciences, BioPharmaceuticals R&D, AstraZeneca, Mölndal, Sweden

[12]Department of Endocrinology, Morbid Obesity and Preventive Medicine, Institute of Clinical Medicine, University of Oslo, Oslo, Norway

[13]Department of Transplantation Medicine, Oslo University Hospital, Oslo, Norway

Correspondence
Anders Åsberg, Department of Transplantation Medicine, Oslo University Hospital, P.O. Box 4950 Nydalen, 0424 Oslo, Norway.
Email: anders.asberg@farmasi.uio.no

Funding information
This study was funded by the Vestfold Hospital Trust, Norway; Department of Pharmacy, University of Oslo, Norway; Swedish Research Council; and AstraZeneca, Sweden.

Abstract

It remains uncertain whether pharmacokinetic changes following Roux-en-Y gastric bypass (RYGB) can be attributed to surgery-induced gastrointestinal alterations per se and/or the subsequent weight loss. The aim was to compare short- and long-term effects of RYGB and calorie restriction on CYP3A-activity, and cross-sectionally compare CYP3A-activity with normal weight to overweight controls using midazolam as probe drug. This three-armed controlled trial included patients with severe obesity preparing for RYGB ($n = 41$) or diet-induced ($n = 41$) weight-loss, and controls ($n = 18$). Both weight-loss groups underwent a 3-week low-energy-diet (<1200 kcal/day) followed by a 6-week very-low-energy-diet or RYGB (both <800 kcal/day). Patients were followed for 2 years, with four

pharmacokinetic investigations using semisimultaneous oral and intravenous dosing to determine changes in midazolam absolute bioavailability and clearance, within and between groups. The RYGB and diet groups showed similar weight-loss at week 9 (13 ± 2.4% vs. 11 ± 3.6%), but differed substantially after 2 years (−30 ± 7.0% vs. −3.1 ± 6.3%). At baseline, mean absolute bioavailability and clearance of midazolam were similar in the RYGB and diet groups, but higher compared with controls. On average, absolute bioavailability was unaltered at week 9, but decreased by 40 ± 7.5% in the RYGB group and 32 ± 6.1% in the diet group at year 2 compared with baseline, with no between-group difference. No difference in clearance was observed over time, nor between groups. In conclusion, neither RYGB per se nor weight loss impacted absolute bioavailability or clearance of midazolam short term. Long term, absolute bioavailability was similarly decreased in both groups despite different weight loss, suggesting that the recovered CYP3A-activity is not only dependent on weight-loss through RYGB.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

The current literature indicates an inverse relationship between body mass index (BMI) and CYP3A-activity, and that systemic clearance of the CYP3A-probe midazolam increases after Roux-en-Y gastric bypass (RYGB). However, the knowledge regarding changes in drug disposition following weight loss in general, and bariatric surgery in particular, is sparse and inconclusive, making drug dosing challenging. In addition, it remains uncertain whether pharmacokinetic changes following bariatric surgery are attributed to the gastrointestinal alterations per se and/or the subsequent weight loss.

WHAT QUESTION DID THIS STUDY ADDRESS?

Do body weight, weight loss, and RYGB impact CYP3A-activity in vivo?

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

This study shows that neither short-term weight loss induced by RYGB or very-low-energy-diet, nor RYGB per se, impact absolute bioavailability or systemic clearance of midazolam.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

Our results suggests that dose adjustments of CYP3A substrate drugs, 30–50% of all drugs on the market, may not be necessary following nonsurgical and surgical weight loss neither in a short- nor long-term perspective.

INTRODUCTION

Obesity is a worldwide epidemic. Individuals with obesity often receive multiple medication for obesity-related comorbidities, such as diabetes, cardiovascular diseases, and non-alcoholic fatty liver disease (NAFLD).[1,2] Bariatric surgery is considered superior to nonsurgical intervention in terms of achieving long-lasting weight loss and improvement of comorbidities.[3,4] The anatomic and physiological alterations in the gastrointestinal tract following Roux-en-Y gastric bypass (RYGB)[5] may influence oral drug bioavailability and thus have consequences for postsurgery dosing. More specifically, the total surface area available for drug absorption is reduced and the proximal intestinal segments rich in cytochrome P450 (CYP) enzymes are bypassed.[6]

CYP3A is the most important drug metabolizing enzyme with regard to drug dosing, and accounts for the metabolism of 30–50% of clinically used drugs.[7,8] CYP3A is not only widely expressed in the liver, but also in the duodenum and proximal jejunum.[9] RYGB may therefore reduce first-pass metabolism, resulting in an increased oral bioavailability. Besides the gastrointestinal alterations, the subsequent weight loss may also influence the expression and activity of CYP3A enzymes in both the intestine

and liver. Obesity is associated with impaired CYP3A-activity,[6,10,11] and previous studies suggest an inverse relationship between body mass index (BMI) and CYP3A-activity,[12,13] possibly due to low-grade inflammation and NAFLD.[14–16] Because inflammation status and NAFLD improve after RYGB or nonsurgical weight loss,[17–20] both intestinal and hepatic CYP3A mediated drug metabolism may also recover.[21] An increase in intestinal CYP3A-activity is expected to decrease oral bioavailability of CYP3A substrates, unless other physiological parameters compensate for the higher activity. Clearance, however, is expected to increase with an increase in hepatic CYP3A activity, unless weight loss also results in other physiological changes that may influence clearance, such as lower hepatic blood flow. In studies investigating pharmacokinetic changes following bariatric surgery,[6,21–23] patients usually serve as their own controls and it is therefore not possible to disentangle the effect of bariatric surgery and the subsequent weight loss. To improve our understanding of the underlying mechanisms involved in restricting oral bioavailability, this is important to investigate.

RYGB may influence the pharmacokinetics of many drugs and thus have consequences for dosing recommendations. However, it remains uncertain whether such potential pharmacokinetic changes are attributed to the gastrointestinal alterations per se and/or the subsequent weight loss. To address this, we performed a three-armed controlled trial over 2 years with a dietary control group achieving a matched short-term weight loss as patients undergoing RYGB.[24] CYP3A activity was investigated using oral and intravenous dosing of midazolam, the clinical CYP3A probe of choice. The study objectives were to compare short- and long-term effects of surgical and nonsurgical calorie restriction on CYP3A activity, and to compare CYP3A activity in a control group of normal weight to overweight individuals with patients with severe obesity.

METHODS

Patients and study design

The COCKTAIL study is an open, nonrandomized, three-armed, single-center controlled study performed at Vestfold Hospital Trust in Norway, as previously described in detail.[24] In short, patients with severe obesity scheduled for weight loss treatment with RYGB or a restricted calorie diet based on clinical indications were included and followed prospectively for 2 years. Additionally, a cross-sectional control group of individuals with BMI 18.5–30 kg/m^2 scheduled for cholecystectomy was included. The study was approved by the Regional Committee for Medical and Health Research Ethics (2013/2379/REK) and performed in accordance with Good Clinical Practice and the Declaration of Helsinki (NCT02386917). Written informed consent was obtained prior to study participation.

Patients were eligible for inclusion based on the following inclusion criteria: greater than or equal to 18 years, BMI greater than or equal to 18.5 kg/m^2, and stable body weight (<5 kg weight change) over the last 3 months. Exclusion criteria included glomerular filtration rate less than 30 ml/min/1.73m^2, previous bariatric or upper gastrointestinal surgery, or treatment with substances that may influence midazolam pharmacokinetics in close approximation to the investigations.[24]

Study visits and procedures

The current analysis included data from the control group (baseline; week 0), and from the intervention groups at weeks 0, 3, and 9, and year 2. At baseline (week 0), all three groups were subjected to a 24-h pharmacokinetic investigation one day before intervention start. Both weight loss groups were prescribed a low-energy-diet (LED; <1200 kcal/day) for the first 3 weeks, followed by an additional 6 weeks of an isocaloric very-low-energy-diet (VLED; <800 kcal/day) or RYGB (<800 kcal/day). Thereafter, patients were treated according to local guidelines until the year 2 visit. A 24-h pharmacokinetic investigation was repeated at all three follow-up visits. For patients undergoing RYGB, small intestinal and hepatic biopsies were obtained on the day of surgery, as previously described.[13,25] Hepatic biopsies were also obtained from the control group during cholecystectomy.[13]

Patients abstained from food and drugs from 10:00 p.m. the evening before all pharmacokinetic investigations. On the study day, patients met at 07:30 a.m. for blood sampling, before 1.5 mg oral midazolam syrup was administered followed by 1.0 mg intravenous midazolam 4 h later. Blood samples were collected from a peripheral venous catheter at: 0.25, 0.5, 1, 1.5, 2, 3, 4, 4.25, 4.5, 5, 5.5, 6, 8, 10, 12, 23, and 24 h. Details regarding the pharmacokinetic investigations and clinical chemistry analyses are described in the Supplement.

Outcomes

The primary outcomes were short- and long-term changes in absolute bioavailability and clearance of midazolam as measures of both first-pass and systemic CYP3A-activity.[24] Secondary outcomes included CYP3A protein quantification in liver and intestine biopsies. Additional outcomes included full pharmacokinetic profiling; oral clearance (clearance/bioavailability), volume of distribution (V_d), terminal elimination half-life ($t_{1/2}$), maximum plasma

concentration (C_{max}), and time to reach maximum plasma concentration (T_{max}) after oral midazolam administration as well as concentrations of the endogenous CYP3A biomarker; 4β-hydroxycholesterol (4βOHC) formed from cholesterol via CYP3A metabolism.[26]

Analytical assays

Detailed descriptions of the analytical assays are presented in the Supplement. In brief, midazolam plasma concentrations were determined by validated ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS).[27] Within-series and between-series performance were assessed with resulting imprecision less than 12.3%, and the mean accuracy ranged from 99.3 to 104.3%. Plasma concentrations of 4βOHC was determined by an HPLC-MS method previously described,[28] with an added filtration step,[29] at the Center for Psychopharmacology, Diakonhjemmet Hospital, Oslo, Norway. Within- and between-series imprecision and inaccuracy were less than 15% at 10 ng/ml and less than 4% at 644 ng/ml.[28]

Protein quantification (intestine and liver)

Proteins were extracted from small intestinal and liver biopsies in an SDS-containing (2% w/v) lysis buffer and quantified, as previously described.[30,31] Details are presented in the Supplement, in short, samples were processed with the multi-enzyme digestion filter-aided sample preparation (MED-FASP) protocol, using LysC and trypsin.[32] Proteomics analysis was performed with Q Exactive HF or Q Exactive HF-X mass spectrometer in data dependent mode. MS data were processed with MaxQuant (version 1.6.10.43)[33] where proteins were identified by searching MS and MS/MS data of peptides against the human UniProtKB (UP000005640). Spectral raw intensities were normalized with variance stabilization (vsn),[34] and were subsequently used to calculate the protein concentrations using the Total Protein Approach.[35] Batch effects were removed by geometric mean centering of proteins from samples analyzed at different time points.

Data analysis

The data analysis comprised two subsequent steps. In step one, a population pharmacokinetic model was developed for characterization of individual pharmacokinetic profiles in order to retrieve individual pharmacokinetic parameters from each investigation. In step two, the individual pharmacokinetic parameters were used to assess changes over the study period using linear mixed effects modeling and visualizations.

Population pharmacokinetic modeling

Given the semisimultaneous oral and intravenous dosing of midazolam applied in this study, we developed a population pharmacokinetic model for description of individual pharmacokinetic profiles in order to retrieve individual pharmacokinetic parameters from all patients, including absolute bioavailability. A detailed description of the population pharmacokinetic model development is presented in the Supplement. In brief, a midazolam model parameterized to determine individual absolute bioavailability was developed with data from semisimultaneous administration of oral and intravenous midazolam. The modeling was performed using the nonparametric adaptive grid approach implemented in Pmetrics (version 1.5.2) for R (version 3.6.2).[36,37] A total of 5414 midazolam concentrations corresponding to 306 unique 24-h pharmacokinetic profiles were available from a total of 98 patients. A catenary three-compartment model[38,39] with absorption lag-time and first-order elimination from the central compartment (Figure S1) was developed and validated. Due to the sole interest in individual predictions of pharmacokinetic parameters and to avoid including the same potential covariate both in the pharmacokinetic population model and the statistical analysis (linear mixed effects model), no covariates were implemented in the population model. Results from the population pharmacokinetic modeling are described in the Supplement Figure S2–S4, and Table S1/S2.

Calculations

Posterior individual parameter values obtained from the final model were used to describe midazolam pharmacokinetics in each individual at each investigation. Absolute bioavailability, C_{max}, and T_{max} were obtained directly from the individual model predictions, whereas clearance, oral clearance, Vd, and $t_{1/2}$ were calculated from the individual model parameter estimates. Equations are described in the Supplement. NAFLD score was calculated as suggested by Kotronen et al.[40]

Statistical analyses and considerations

Student's *t*-tests were used for the cross-sectional analysis between patients with obesity and controls. Linear mixed effects models, with the pharmacokinetic parameters as dependent variable and visit and group as fixed effects

with individual intercepts (random effect), were used to compare delta change and 95% confidence interval (CI) from baseline at the different study visits within the RYGB and diet groups. All exploratory tests were two-sided at the 5% level without adjustment for multiplicity. To adjust for comparison between multiple study visits in the mixed effects model, CIs were adjusted with the Tukey method. To describe the relationship between variables, linear regression analyses were performed. All statistical analyses were performed using R (version 3.6.2).[37]

RESULTS

Patient characteristics

Between March 18, 2015, and May 22, 2017, 196 patients who were preparing for bariatric surgery, low calorie diet (<1200 kcal/day), or cholecystectomy, were screened for eligibility.[24] After exclusion of 88 ineligible patients, a total of 44, 44, and 20 patients were included in the RYGB, diet, and control group, respectively (Figure 1a). The first and last patient were included April 15, 2015, and June 29, 2017, respectively. Eight patients withdrew or were excluded before study start (week 0), leaving 41, 41, and 18 patients in the three groups starting treatment at week 0. Three patients did not undergo RYGB due to (1) liver cirrhosis (thus excluded from pharmacokinetic analyses), (2) intubation not possible, and (3) anaphylactic reaction. The latter two were hence excluded after the study visit at week 3. Finally, 13 patients (RYGB = 4 and diet = 9) withdrew or dropped out after week 9, leaving 34 (RYGB) and 30 (diet) patients who attended the 2-year follow-up. Due to technical difficulties, some patients were unable to supply evaluable pharmacokinetic profiles at all four study visits, whereas one patient in the diet group did not supply any pharmacokinetic profile. A total of 40 and 40 patients in the RYGB and diet groups, respectively, supplied at least one 24-h pharmacokinetic profile during the study period.

At baseline, sex, age, and ethnicity did not differ between the intervention groups and controls, but the control group had lower body weight (Figure 1b), alanine aminotransferase (ALT), and high-sensitivity C-reactive protein (hs-CRP) than the intervention groups (Table 1). Baseline characteristics did not differ between the intervention groups (Table 1).

Changes in body weight and selected laboratory measures

Body weight decreased similarly in the RYGB and diet groups from baseline to week 3 (4.8 ± 1.1% vs. 4.4 ± 2.0%), and from baseline to week 9 (13 ± 2.4% vs. 11 ± 3.6%). Between week 9 and year 2, mean body weight decreased in the RYGB group (20 ± 9.0%) but increased in the diet group (9.0 ± 8.0%) as several patients had almost returned to baseline body weight at year 2 (Figure 1c, Table S3).

Changes in selected laboratory measures are presented in Table S3. At week 9, hs-CRP levels were unaltered in both groups, but hs-CRP decreased in the RYGB group at year 2 ($p < 0.001$; Figure 1d). ALT increased between week 0 and week 9 in the RYGB group, whereas no change was observed in the diet group.

Baseline pharmacokinetics – severe obesity versus normal weight to overweight

In total, 96 pharmacokinetic profiles (RYGB = 38, diet = 40, and control = 18) were included in the cross-sectional analysis at baseline. Mean ±SD plots for pharmacokinetic parameters and variables in the RYGB, diet, and control groups at baseline are presented in Figure 2. Mean pharmacokinetic profiles are shown in Figure S5. Absolute bioavailability was 153% higher in patients with severe obesity ($n = 78$) compared with controls ($n = 18$, $p < 0.001$).

In patients with severe obesity, 4βOHC concentrations were 44% lower compared with normal weight to overweight controls ($p < 0.001$). In addition, patients with severe obesity exhibited a higher systemic clearance compared with controls (46%, $p = 0.003$). We observed positive associations between body weight and both absolute bioavailability ($\beta = 0.10$, $R^{2adj} = 0.04$) and clearance ($\beta = 0.10$, $R^{2adj} = 0.08$; Figure 3a, b, respectively).

In the patients undergoing RYGB and cholecystectomy ($n = 54$), regression analyses revealed no associations between individual hepatic CYP3A4 concentrations and midazolam clearance at the time of surgery (Figure 3e). However, significant associations were observed among body weight and hepatic CYP3A4 concentrations ($\beta = -0.09$, $R^{2adj} = 0.11$), hs-CRP, and hepatic CYP3A4 concentrations ($\beta = -0.48$, $R^{2adj} = 0.06$), NAFLD score and hepatic CYP3A4 concentrations ($\beta = -1.7$, $R^{2adj} = 0.16$), and 4βOHC and hepatic CYP3A4 concentrations ($\beta = 0.45$, $R^{2adj} = 0.11$; Figure 3f–i). The individual CYP3A4 concentrations in the jejunum ($n = 36$), information only available in the patients undergoing RYGB, showed a negative association with absolute bioavailability: $\beta = -1.1$, $R^{2adj} = 0.23$ (Figure 3d).

Changes in pharmacokinetics after VLED, RYGB, and weight loss

In total, 288 pharmacokinetic profiles from 80 patients (RYGB = 40 and diet = 40) were included in the

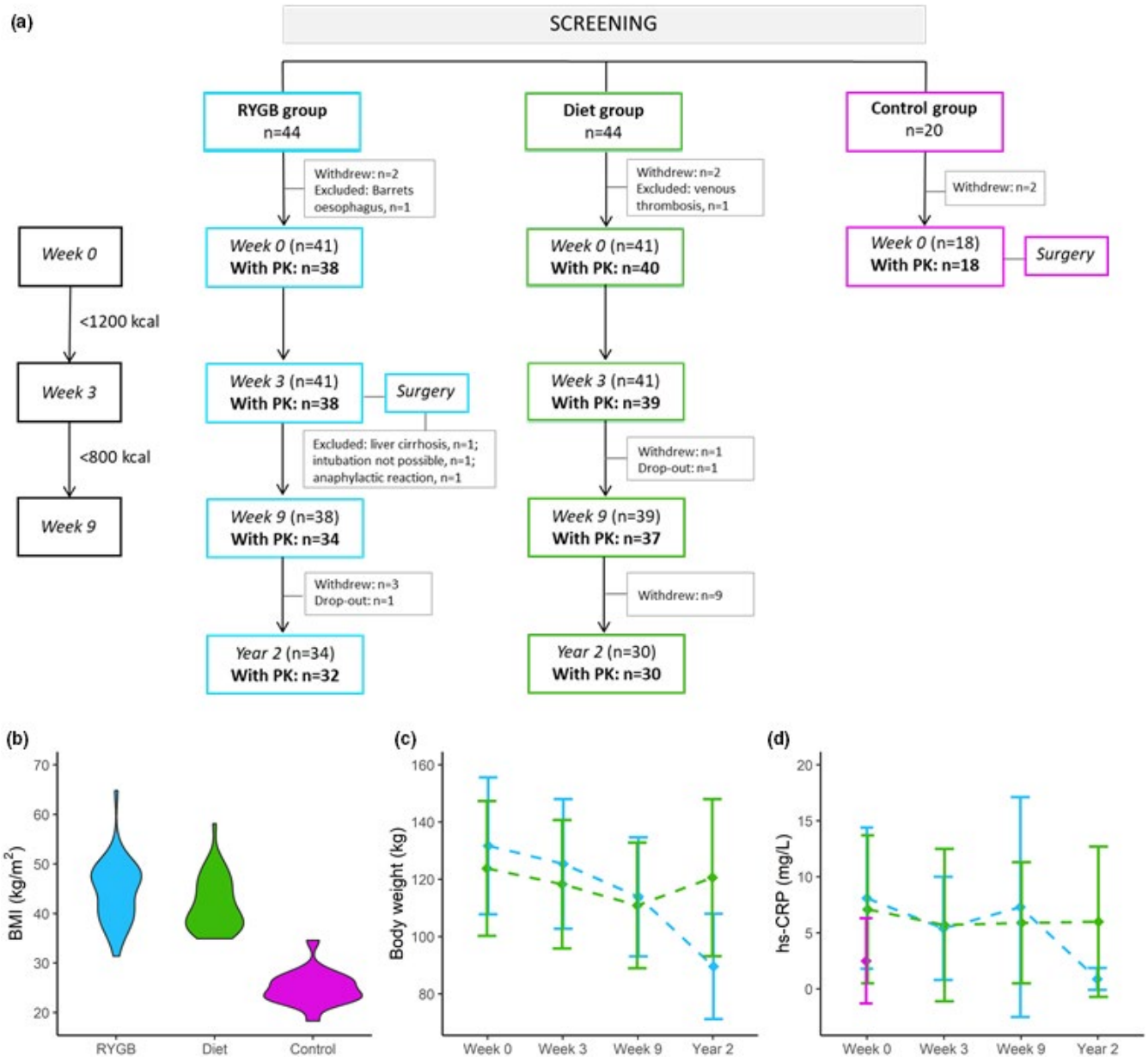

FIGURE 1 Patient flow-chart, study design, and clinical parameters. In panel (a), patient flow chart during the study period is shown. Number of patients with PK profiles are in bold. BMI in the RYGB group ($n = 41$), diet group ($n = 41$), and control group ($n = 18$) at baseline (week 0) is shown in panel b. Changes in total body weight and hs-CRP during the study period in patients with evaluable 24-h PK profiles are shown in panels c and d, respectively. Abbreviations: BMI, body mass index; hs-CRP, high-sensitivity C-reactive protein; PK, pharmacokinetic; RYGB, Roux-en-Y gastric bypass

longitudinal analysis, and of these, 56 patients supplied pharmacokinetic profiles at all four study visits. At baseline, absolute bioavailability and clearance did not differ between the intervention groups, but the RYGB group had a 32% lower oral clearance than the diet group ($p = 0.002$). Mean ±SD pharmacokinetic parameters for all groups at the different study visits are shown in Table 2. Short- and long-term pharmacokinetic outcomes are presented in Table 3.

Short-term effect of RYGB and diet on midazolam pharmacokinetics

Three weeks of LED did not change midazolam pharmacokinetics in the two intervention groups (Table 3). After an additional 6 weeks of VLED, absolute bioavailability and clearance were still unaltered in both groups and did not show difference between groups (Figure 2a,b). In the RYGB group, a 35 ± 2.6% shorter T_{max} and a 52 ± 15%

TABLE 1 Baseline (week 0) characteristics presented as mean ±SD or number of patients (%)

	RYGB $n = 41$	Diet $n = 41$	Control $n = 18$
Sex, n (male/female)	14/27	14/27	3/15
Age (years)	46 ± 9	49 ± 10	42 ± 15
Ethnicity, *n* (White/other)	41/0	40/1	17/1
Body weight (kg)	132 ± 24	124 ± 23	71 ± 11
BMI (kg/m^2)	44.5 ± 6.2	42.0 ± 5.4	25.0 ± 3.5
Albumin (g/L)	40 ± 2	40 ± 2	40 ± 2
Creatinine (μmol/L)	58 ± 11	59 ± 14	60 ± 12
AST (U/L)	29 ± 11	28 ± 15	25 ± 11
ALT (U/L)	34 ± 17	32 ± 18	22 ± 15
hs-CRP (mg/L)	8.1 ± 6.3	8.2 ± 9.6	2.5 ± 3.8

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; hs-CRP, high sensitivity C-reactive protein; RYGB, Roux-en-Y gastric bypass.

higher C_{max} was, however, observed 6 weeks after surgery (week 9; Figure 2f,g).

Long-term effect of RYGB and diet on midazolam pharmacokinetics

At year 2, absolute bioavailability was decreased in both the RYGB and diet groups by 40 ± 7.5% ($p < 0.001$) and 32 ± 6.1% ($p = 0.016$), respectively (Table 3, Figure 2a). In addition, both groups exhibited increases in 4βOHC concentrations: 60 ± 22% in the RYGB group ($p < 0.001$) versus 23 ± 6.8% in the diet group ($p = 0.018$; Table 3, Figure 2h). Mean difference between the groups at year 2 was 20% ($p = 0.009$). No difference in clearance was observed over time, nor between the groups (Table 3, Figure 2b). In the RYGB group, C_{max} after oral midazolam administration showed similar values as prior to surgery, but T_{max} was still shorter (−28%, $p < 0.001$; Table 3, Figure 2f,g).

Inter- and intra-individual variability

A large inter-individual variability was observed for all pharmacokinetic parameters and variables (Figure 2). Patients with severe obesity also exhibited a substantial intra-individual variability over time, as illustrated in Figure S6. The intra-individual variability was assessed as the coefficient of variation (CV) in patients with pharmacokinetic profiles at all of the three first study visits ($n = 69$). Of these patients with pharmacokinetic profiles at weeks 0, 3, and 9, 61% and 41% had a CV greater than 30% for absolute bioavailability and clearance, respectively.

DISCUSSION

The main finding of this three-armed, controlled study with 98 participants was that RYGB per se did not have any significant effect on absolute bioavailability or systemic clearance of midazolam short-term (week 9). Considering the rearrangement of the gastrointestinal tract leading to both loss of absorptive area and bypass of the most CYP3A rich part of the intestine, we expected to observe an increased oral bioavailability following RYGB, which has also been suggested in previous studies.[21,22,41] However, the unaltered absolute bioavailability and clearance of midazolam 6 weeks postsurgery suggests that neither the surgery induced gastrointestinal alterations, nor a moderate weight loss, influences midazolam pharmacokinetics in the short-term perspective. The unique dietary control group included in this study showing matched weight loss with the surgery group short-term (11% vs. 13%), further substantiates that such a significant weight loss seems to have limited clinical relevance considering dosing of CYP3A substrates, such as midazolam. Nevertheless, these findings may reduce the uncertainty for clinicians regarding the need for dose adjustments of CYP3A substrates in the early period after RYGB, but also following a moderate weight loss after nonsurgical calorie restriction.

The semisimultaneous administration of oral and intravenous midazolam revealed an almost three-fold higher absolute bioavailability in patients with severe obesity compared with normal weight to overweight individuals. Similar results for absolute bioavailability of midazolam have been shown in a smaller study by Brill et al. comparing patients with severe obesity and healthy normal weight controls.[10] We also observed that patients with severe obesity had an almost 50% higher clearance compared to normal weight with overweight controls. Considering previous literature on CYP3A-activity in patients with obesity,[10,11,21] the inverse association between hepatic CYP3A concentration and body weight (Figure 3f), and that patients with obesity had lower 4βOHC concentrations compared with controls, this was unexpected. However, patients with severe obesity have higher hepatic blood flow, possibly induced by a combination of increased blood volume, cardiac output, and liver volume.[42,43] Given that midazolam is a medium-to-high extraction ratio drug, hepatic blood flow will affect midazolam clearance and potentially, based on the present results, have a more significant role than CYP3A activity on clearance estimates in this patient population.[11,44] Regardless, this finding is in contrast to what Brill et al. observed in their study.[10] We can only speculate on the reasons for this discrepancy in midazolam clearance. In our study, the age of the patients in the different groups were comparable, whereas, in the study of Brill et al., the mean

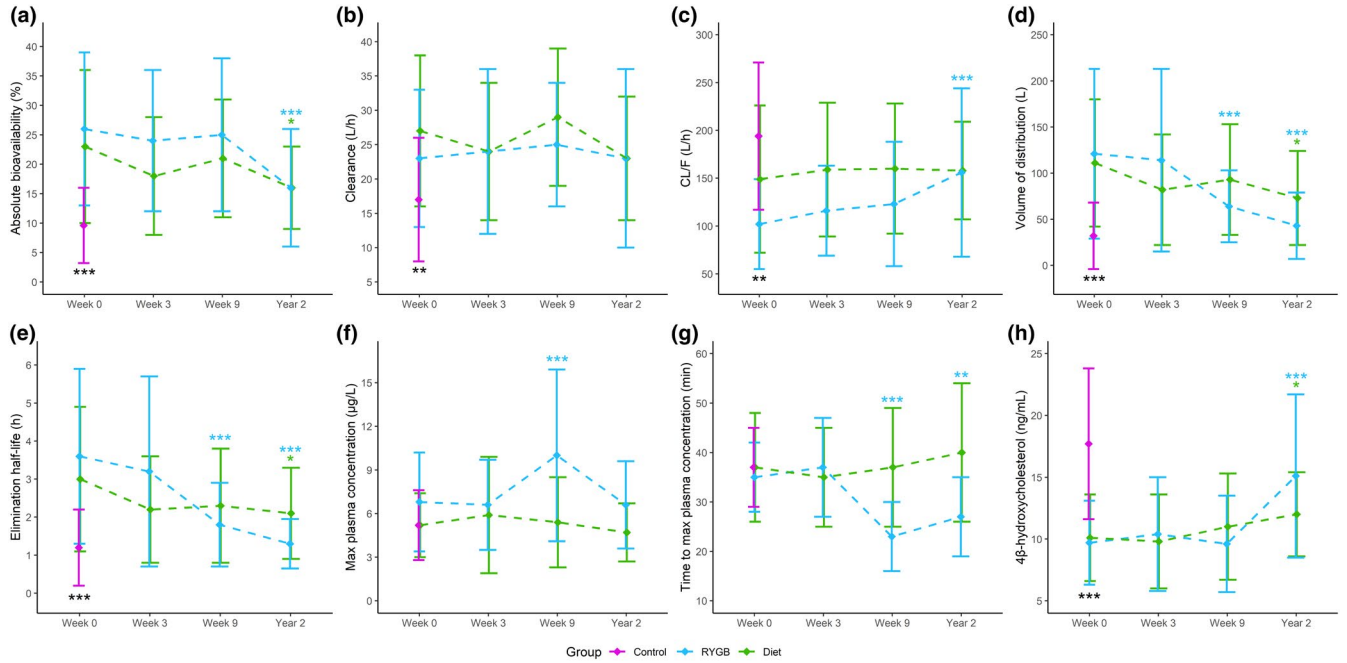

FIGURE 2 Pharmacokinetic changes during the study period. Mean ±standard deviation plots for midazolam (a) absolute bioavailability, (b) clearance, (c) oral clearance (CL/F), (d) volume of distribution, (e) elimination half-life, (f) maximum plasma concentration (C_{max}) after oral midazolam, (g) time to reach maximum plasma concentration (T_{max}) after oral midazolam, and (h) 4β-hydroxycholesterol at the different study visits. Statistically significant differences between patients with obesity versus control at baseline (from two-sided *t*-test) are given by black stars. Statistically significant differences were seen for all parameters, except from C_{max} and T_{max}. Statistically significant differences over time compared to baseline within the Roux-en-Y gastric bypass (RYGB) and diet groups (from linear mixed effects model) are given by blue and green stars, respectively. Difference within and between the two intervention groups at the different study visits are shown in Table 3. **p* value <0.05; ***p* value ≤0.01; ****p* value ≤0.001

age of the patients with obesity was similar to our patients (44 years), but their controls were considerable younger (mean age of 22 years). Younger individuals will typically have higher liver blood flow resulting in higher clearance of midazolam compared with older individuals.[11,44] Further, the present study supports previous findings of an inverse relationship between BMI and oral clearance of CYP3A substrates (Figure 3c).[10,12,13] Given that we assessed both absolute bioavailability and clearance, we can argue that the negative association between BMI and oral clearance is explained by both higher bioavailability and clearance with higher body weight, but that the effect on bioavailability was greater.

The protein quantification in the present study supports previous findings of a lower hepatic CYP3A expression and activity in patients with severe obesity[12,13] and in patients with NAFLD (Figure 3f,h).[15,16] The lack of short-term changes in absolute bioavailability and clearance of midazolam with body weight reduction in our study indicates that 9 weeks is not sufficient time for CYP3A activity to recover. Given the suggested mechanism of reduced CYP3A activity due to low-grade inflammation and NAFLD this appears plausible.[14–16] This also seems reasonable considering the hepatic CYP3A4 enzyme turnover half-life,[45] and that hs-CRP did not change in this period. The decreased absolute bioavailability in the RYGB group at year 2 (40%) supports the hypothesis that CYP3A activity is recovered following weight loss, given enough time, considering a sustained mean weight loss of 30%. However, other simultaneous physiological alterations, such as changed splanchnic and hepatic blood flow during substantial body weight loss, limit the true mechanistic understanding of the effects. The diet group also showed decreased bioavailability (32%) despite the fact that many of these patients had returned to their baseline body weight at year 2 (mean weight loss of 3% from baseline). Overall, this suggests that other mechanisms known to influence midazolam pharmacokinetics, such as gut microbiota,[46] may also be involved.

The lack of association between clearance and hepatic CYP3A4 expression observed in the present study (Figure 3e), further supports that hepatic blood flow may have a larger role in determining clearance of midazolam. Thus, CYP3A activity may be recovered, although clearance was unaltered at the 2-year study visit. The observed increase in 4βOHC concentrations in the present study supports a recovery of CYP3A activity. Additionally, this endogenous CYP3A biomarker was positively associated

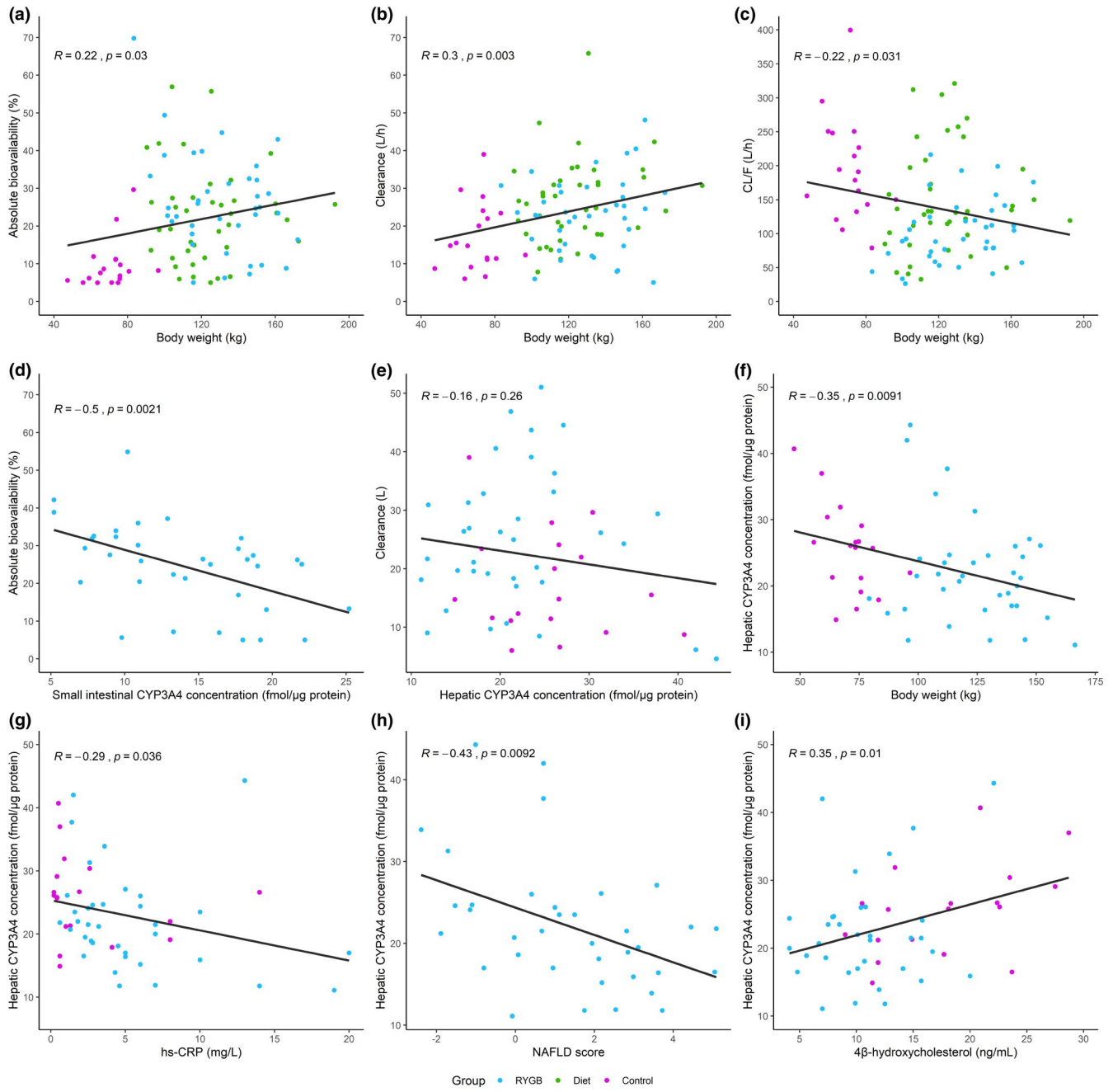

FIGURE 3 Association among body weight, CYP3A4 concentrations, and pharmacokinetics. Association between body weight and midazolam (a) absolute bioavailability, (b) clearance, and (c) oral clearance (CL/F) at baseline. Association between small intestinal CYP3A4 concentration and (d) midazolam absolute bioavailability at the time of surgery. Association between hepatic CYP3A4 concentration and (e) midazolam clearance at the time of surgery. Association among (f) body weight, (g) high sensitivity C-reactive protein (hs-CRP), (h) non-alcoholic fatty liver (NAFLD) score, and (i) 4β-hydroxycholesterol, and hepatic CYP3A concentrations at the time of surgery. R is the correlation coefficient. RYGB, Roux-en-Y gastric bypass

with hepatic CYP3A4 concentrations and negatively correlated with BMI, which has also been shown previously.[47]

The major strength of this study is the matched short-term weight loss between a group undergoing RYGB and a dietary control group, enabling us to separate the surgery effect per se on drug disposition from the subsequent weight loss effect. To our knowledge, this is the first study to achieve this. In addition, we were not limited to investigating the surrogate variable oral clearance as we assessed both absolute bioavailability and clearance using a semisimultaneous oral and intravenous administration of midazolam, the standard CYP3A in vivo probe. Additionally, our study has a relatively large sample size considering the study design, and it maintains a high quality compared to other

TABLE 2 PK variables and population pharmacokinetic model derived parameters at the different study visits in the RYGB, diet, and control group, respectively

	RYGB				Diet				Control
	Mean ±SD								
PPK parameter	Week 0 $n = 38$	Week 3 $n = 38$	Week 9 $n = 34$	Year 2 $n = 32$	Week 0 $n = 40$	Week 3 $n = 39$	Week 9 $n = 37$	Year 2 $n = 30$	Week 0 $n = 18$
Absolute bioavailability (%)[a]	26 ± 13	24 ± 12	25 ± 13	16 ± 10	23 ± 13	18 ± 10	21 ± 10	16 ± 7	9.6 ± 6.4
Clearance (L/h)[a]	23 ± 10	24 ± 12	25 ± 9	23 ± 13	27 ± 11	24 ± 10	29 ± 10	23 ± 9	17 ± 9
Oral clearance (L/h)[a]	102 ± 47	116 ± 47	123 ± 65	156 ± 88	149 ± 77	159 ± 70	160 ± 68	158 ± 51	194 ± 77
Volume of distribution (L)[a]	121 ± 92	114 ± 99	64 ± 39	43 ± 36	111 ± 69	82 ± 60	93 ± 60	73 ± 51	32 ± 36
Elimination half-life (h)[a]	3.6 ± 2.3	3.2 ± 2.5	1.8 ± 1.1	1.3 ± 0.65	3.0 ± 1.9	2.2 ± 1.4	2.3 ± 1.5	2.1 ± 1.2	1.2 ± 1.0
$C_{max,\ oral}$ (μg/L)[a]	6.8 ± 3.4	6.6 ± 3.1	10.0 ± 5.9	6.6 ± 3.0	5.2 ± 2.2	5.9 ± 4.0	5.4 ± 3.1	4.7 ± 2.0	5.2 ± 2.4
4β-hydroxycholesterol (ng/ml)	9.7 ± 3.4	10.4 ± 4.6	9.6 ± 3.9	15.1 ± 6.6	10.1 ± 3.5	9.8 ± 3.8	11.0 ± 4.3	12.0 ± 3.4	17.7 ± 6.1

Abbreviations: C_{max}, maximum plasma concentration after oral midazolam; PK, pharmacokinetic; RYGB, Roux-en-Y gastric bypass.

[a]Absolute bioavailability and C_{max} were obtained directly from the individual model predictions, whereas clearance, oral clearance, volume of distribution, and elimination half-life were calculated from model derived values.

studies with similar objectives.[10,21,23,48] In addition, the rich pharmacokinetic data (18 concentrations per 24-h profile) combined with population pharmacokinetic analyses, the high completion rate and the long-term follow-up, form a strong foundation for this thorough in-depth investigation of both short- (9 weeks) and long-term (2 years) effects.

The results in this study should, however, be interpreted in light of some important limitations. Although midazolam (a moderate to high extraction drug) is the preferred probe drug to study CYP3A activity in vivo, other mechanisms, such as alterations in blood flow and/or unbound fraction, will also impact midazolam pharmacokinetics. However, no other in vivo probes available will bypass this problem (e.g., a low extraction drug would bypass a hepatic blood flow effect but not provide detailed information on absolute oral bioavailability). In addition, the midazolam absolute bioavailability observed in the control group in our study (~10%) was lower than the large proportion of estimates reported in the literature, ranging from 20 to 70% in different populations and a range of doses.[10,27,49,50] We also observed a high intra-individual variability in midazolam pharmacokinetics, in addition to the expected high inter-individual variability.[21,23] The reason for the high intra-individual variability is unknown, although the pharmacokinetic population modeling analyses indicate that the initial volume of distribution may be a main contributor. It may be speculated that this might be due to the rapid changes in body composition in the early study period, resulting in very high midazolam concentrations immediately after intravenous dosing and thus low distribution volume estimates in some patients. We therefore applied model predicted area under the curve (AUC) to determine clearance, instead of deriving clearance from the individual model parameter estimates of elimination rate constant and distribution volume.

In conclusion, neither RYGB per se nor the subsequent weight loss impacted absolute bioavailability or systemic clearance of midazolam short term. Thus, our results suggest that, despite the extensive rearrangement of the gastrointestinal tract by RYGB, dose adjustments may not be necessary for the extensive group of drugs that is substrates for CYP3A metabolism in the early period after RYGB. In the long-term perspective, absolute bioavailability decreased in the RYGB group, suggesting that CYP3A activity is recovered following substantial weight loss. This is also supported by an increased concentration of 4βOHC at year 2. A decreased absolute bioavailability and an increased concentration of 4βOHC were also observed in the diet group, although the majority of these patients had returned to their baseline weight at year 2, suggesting that other unknown processes also have influenced the CYP3A activity. The large inter- and intra-individual pharmacokinetic variability should be

TABLE 3 Short- and long-term outcomes in PK parameters and variables from baseline to week 3, week 9, and year 2 in the RYGB group and diet group, respectively

	RYGB			Diet			Difference between groups[b]	
	Estimated mean difference (Δ) [95% CI][a]							
PK parameter	Week 0 – Week 3	Week 0 – Week 9	Week 0 – Year 2	Week 0 – Week 3	Week 0 – Week 9	Week 0 – Year 2	Week 9	Year 2
Absolute bioavailability (%)	−2.0 [−7.7, 3.7]	−1.0 [−6.9, 4.9]	**−10.0** *** [−16.1, −4.0]	−4.4 [−10.0, 1.1]	−2.1 [−7.8, 3.6]	**−7.0*** [−13.1, −0.97]	4.3 [−0.99, 9.6]	0.22 [−5.4, 5.8]
Clearance (L/h)	1.6 [−3.9, 7.1]	1.4 [−4.3, 7.1]	−0.66 [−6.5, 5.2]	−3.0 [−8.4, 2.4]	1.7 [−3.8, 7.2]	−3.6 [−9.4, 2.3]	−4.2 [−9.0, 0.69]	−0.93 [−6.1, 4.2]
Oral clearance, CL/F (L/h)	13 [−18, 45]	19 [−14, 51]	**54** *** [21, 87]	10 [−21, 40]	13 [−18, 44]	12 [−21, 45]	**−41**** [−71, −10]	−4.5 [−37, 28]
Volume of distribution (L)	5.1 [−41, 30]	**−58***** [−95, −21]	**−77***** [−114, −39]	−29 [−64, 5.9]	−20 [−55, 15]	**−41*** [−79, −3.3]	−29 [−60, 3.2]	−26 [−60, 7.3]
Elimination half-life, t½ (h)	−0.38 [−1.2, 0.49]	**−1.8***** [−2.7, −0.88]	**−2.2***** [−3.1, −1.3]	−0.75 [−1.6, 0.10]	−0.72 [−1.6, 0.14]	**−1.0*** [−1.9, −0.09]	−0.49 [−1.3, 0.30]	−0.65 [−1.5, 0.20]
$C_{max,\ oral}$ (μg/L)	−0.28 [−1.9, 1.3]	**3.2***** [1.6, 4.9]	−0.20 [−1.9, 1.5]	0.70 [−0.89, 2.3]	0.45 [−1.2, 2.1]	−0.33 [−2.1, 1.4]	**4.4***** [2.7, 6.0]	1.7 [0.0, 3.5]
4β-hydroxycholesterol (ng/ml)	0.69 [−1.1, 2.5]	−0.25 [−2.1, 1.6]	**5.3***** [3.4, 7.1]	−0.34 [−2.1, 1.4]	1.2 [−0.59, 2.9]	**2.2*** [0.26, 4.1]	−1.7 [−3.7, 0.28]	**2.8**** [0.70, 4.9]

Abbreviations: CI, confidence interval; CL/F, oral clearance; C_{max}, maximum plasma concentration after oral midazolam; PK, pharmacokinetic; RYGB, Roux-en-Y gastric bypass.

Bold values show significant differences.

The *p* values are calculated from Δ at week 3, week 9, and year 2 compared to baseline (week 0) within both groups and between groups at week 9 and year 2 (without adjustment for multiplicity). **p* value <0.05; ***p* value ≤0.01; ****p* value ≤0.001.

[a]Linear mixed model was used to estimate mean difference in change (Δ).

[b]Difference between groups is calculated with RYGB as reference group.

kept in mind when initiating CYP3A therapies within a broad range of BMI, and warrants further investigation in future clinical trials.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to the participants, the surgical staff, and the study personnel working on the COCKTAIL study at Vestfold Hospital Trust. We are also grateful for the laboratory assistance provided by Grete Hasvold and master students (Anders Ulvmoen, Peter Huy Vo Le, Martin Vu, Ingeborg Karbø, Maria Ilyas, and Louise Borgenhard) at the Department of Pharmacy. The authors thank Matthew McGee for proofreading the manuscript. The authors thank Sabry Razick and Frode Strømsvåg at the University Center Information Technology (USIT), University of Oslo for performing optimization of Pmetrics. The optimization of Pmetrics was funded and carried out as an advanced user support from UNINETT Sigma2, the National Infrastructure for High Performance Computing and Data Storage in Norway (project NN9736K). The authors also thank the Swedish Research Council, approval numbers 5715 and 01951 (C.W., T.B.A., and P.A.) for supporting the proteomics analyses.

CONFLICT OF INTEREST

S.A., C.K., and R.J.-L. are AstraZeneca employees and own shares in AstraZeneca. T.B.A. and C.W. are former AstraZeneca employees. All other authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

K.E.K., I.R., and A.Å. wrote the manuscript. J.H., A.Å., S.A., C.K., T.B.A., H.C., E.S., and R.S. designed the research. I.R., V.K., L.K.J., P.C., B.M.W., K.H., P.A., C.W., and R.J. performed the research. K.E.K., I.R., A.Å., E.S., C.W., and P.A. analyzed the data.

REFERENCES

1. Guh DP, Zhang W, Bansback N, Amarsi Z, Birmingham CL, Anis AH. The incidence of co-morbidities related to obesity and overweight: a systematic review and meta-analysis. *BMC Public Health*. 2009;9:88.
2. Machado M, Marques-Vidal P, Cortez-Pinto H. Hepatic histology in obese patients undergoing bariatric surgery. *J Hepatol*. 2006;45:600-606.
3. Colquitt JL, Pickett K, Loveman E, Frampton GK. Surgery for weight loss in adults. *Cochrane Database Sys Rev*. 2014;8:CD003641.
4. Jakobsen GS, Småstuen MC, Sandbu R, et al. Association of bariatric surgery vs medical obesity treatment with long-term medical complications and obesity-related comorbidities. *JAMA*. 2018;319:291-301.
5. Kral JG, Näslund E. Surgical treatment of obesity. *Nat Clin Pract Endocrinol Metab*. 2007;3:574-583.
6. Angeles PC, Robertsen I, Seeberg LT, et al. The influence of bariatric surgery on oral drug bioavailability in patients with obesity: A systematic review. *Obes Rev*. 2019;20:1299-1311.
7. Rendic S, Guengerich FP. Survey of human oxidoreductases and cytochrome P450 enzymes involved in the metabolism of xenobiotic and natural chemicals. *Chem Res Toxicol*. 2015;28:38-42.
8. Guengerich FP. Cytochrome P-450 3A4: regulation and role in drug metabolism. *Annu Rev Pharmacol Toxicol*. 1999;39:1-17.
9. Paine MF, Hart HL, Ludington SS, Haining RL, Rettie AE, Zeldin DC. The human intestinal cytochrome P450 "pie". *Drug Metab Dispos*. 2006;34:880-886.
10. Brill MJ, van Rongen A, Houwink API, et al. Midazolam pharmacokinetics in morbidly obese patients following semi-simultaneous oral and intravenous administration: a comparison with healthy volunteers. *Clin Pharmacokinet*. 2014;53:931-941.
11. van Rongen A, Brill MJE, Vaughns JD, et al. Higher midazolam clearance in obese adolescents compared with morbidly obese adults. *Clin Pharmacokinet*. 2018;57:601-611.
12. Ulvestad M, Skottheim IB, Jakobsen GS, et al. Impact of OATP1B1, MDR1, and CYP3A4 expression in liver and intestine on interpatient pharmacokinetic variability of atorvastatin in obese subjects. *Clin Pharma Ther*. 2013;93:275-282.
13. Krogstad V, Peric A, Robertsen I, et al. Correlation of body weight and composition with hepatic activities of cytochrome P450 enzymes. *J Pharm Sci*. 2021;110(1):432-437.
14. Jover R, Bort R, Gómez-Lechón MJ, Castell JV. Down-regulation of human CYP3A4 by the inflammatory signal interleukin-6: molecular mechanism and transcription factors involved. *FASEB J*. 2002;16:1799-1801.
15. Kolwankar D, Vuppalanchi R, Ethell B, et al. Association between nonalcoholic hepatic steatosis and hepatic cytochrome P-450 3A activity. *Clin Gastroenterol Hepatol*. 2007;5:388-393.
16. Woolsey SJ, Mansell SE, Kim RB, Tirona RG, Beaton MD. CYP3A activity and expression in nonalcoholic fatty liver disease. *Drug Metab Dispos*. 2015;43:1484-1490.
17. Rao SR. Inflammatory markers and bariatric surgery: a meta-analysis. *Inflamm Res*. 2012;61:789-807.
18. Schwenger KJP, Fischer SE, Jackson T, Okrainec A, Allard JP. In nonalcoholic fatty liver disease, Roux-en-Y gastric bypass improves liver histology while persistent disease is associated with lower improvements in waist circumference and glycemic control. *Surg Obes Relat Dis*. 2018;14:1233-1239.
19. Nicklas JM, Sacks FM, Smith SR, et al. Effect of dietary composition of weight loss diets on high-sensitivity c-reactive protein: the Randomized POUNDS LOST trial. *Obesity (Silver Spring)*. 2013;21:681-689.
20. Vilar-Gomez E, Martinez-Perez Y, Calzadilla-Bertot L, et al. Weight loss through lifestyle modification significantly reduces features of nonalcoholic steatohepatitis. *Gastroenterology*. 2015;149:367-78.e5:quiz e14–e15.
21. Brill MJ, van Rongen A, van Dongen EP, et al. The pharmacokinetics of the CYP3A substrate midazolam in morbidly obese patients before and one year after bariatric surgery. *Pharma Res*. 2015;32:3927-3936.
22. Skottheim IB, Stormark K, Christensen H, et al. Significantly altered systemic exposure to atorvastatin acid following gastric bypass surgery in morbidly obese patients. *Clin Pharmacol Ther*. 2009;86:311-318.

23. Chan LN, Lin YS, Tay-Sontheimer JC, et al. Proximal Roux-en-Y gastric bypass alters drug absorption pattern but not systemic exposure of CYP3A4 and P-glycoprotein substrates. *Pharmacotherapy*. 2015;35:361-369.
24. Hjelmesaeth J, Åsberg A, Andersson S, et al. Impact of body weight, low energy diet and gastric bypass on drug bioavailability, cardiovascular risk factors and metabolic biomarkers: protocol for an open, non-randomised, three-armed single centre study (COCKTAIL). *BMJ Open*. 2018;8:e021878.
25. Krogstad V, Peric A, Robertsen I, et al. A comparative analysis of cytochrome P450 activities in paired liver and small intestinal samples from patients with obesity. *Drug Metab Dispos*. 2020;48:8-17.
26. Bodin K, Andersson U, Rystedt E, et al. Metabolism of 4 beta -hydroxycholesterol in humans. *J Biol Chem*. 2002;277:31534-31540.
27. Egeland EJ, Witczak BJ, Zaré HK, Christensen H, Åsberg A, Robertsen I. Chronic inhibition of CYP3A is temporarily reduced by each hemodialysis session in patients with end-stage renal disease. *Clin Pharmacol Ther*. 2020;108(4):866-873.
28. Gjestad C, Huynh DK, Haslemo T, Molden E. 4β-hydroxycholesterol correlates with dose but not steady-state concentration of carbamazepine: indication of intestinal CYP3A in biomarker formation? *Br J Clin Pharmacol*. 2016;81:269-276.
29. Størset E, Hole K, Midtvedt K, Bergan S, Molden E, Åsberg A. The CYP3A biomarker 4β-hydroxycholesterol does not improve tacrolimus dose predictions early after kidney transplantation. *Br J Clin Pharmacol*. 2017;83:1457-1465.
30. Wegler C, Garcia LP, Klinting S, et al. Proteomics-informed prediction of rosuvastatin plasma profiles in patients with a wide range of body weight. *Clin Pharmacol Ther*. 2021;109(3):762-771.
31. Wegler C, Ölander M, Wiśniewski JR, et al. Global variability analysis of mRNA and protein concentrations across and within human tissues. *NAR Genom Bioinformatics*. 2019;2(1):lqz010.
32. Wiśniewski JR, Mann M. Consecutive proteolytic digestion in an enzyme reactor increases depth of proteomic and phosphoproteomic analysis. *Anal Chem*. 2012;84:2631-2637.
33. Tyanova S, Temu T, Cox J. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nat Protoc*. 2016;11:2301-2319.
34. Huber W, von Heydebreck A, Sültmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics*. 2002;18(Suppl 1):S96-S104.
35. Wiśniewski JR, Rakus D. Multi-enzyme digestion FASP and the 'Total Protein Approach'-based absolute quantification of the Escherichia coli proteome. *J Proteomics*. 2014;109:322-331.
36. Neely MN, van Guilder MG, Yamada WM, Schumitzky A, Jelliffe RW. Accurate detection of outliers and subpopulations with Pmetrics, a nonparametric and parametric pharmacometric modeling and simulation package for R. *Ther Drug Monit*. 2012;34:467-476.
37. R Foundation for Statistical Computing. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing; 2018.
38. Świętaszczyk C, Jødal L. Three-compartment pharmacokinetic models of radiotracers used in the GFR-determination - estimation of their parameters using the time-concentration curves. *Nucl Med Rev Cent East Eur*. 2019;22:60-68.
39. de Biasi J. Four open mammillary and catenary compartment models for pharmacokinetics studies. *J Biomed Eng*. 1989;11:467-470.
40. Kotronen A, Peltonen M, Hakkarainen A, et al. Prediction of non-alcoholic fatty liver disease and liver fat using metabolic and genetic factors. *Gastroenterology*. 2009;137:865-872.
41. Darwich AS, Pade D, Ammori BJ, Jamei M, Ashcroft DM, Rostami-Hodjegan A. A mechanistic pharmacokinetic model to assess modified oral drug bioavailability post bariatric surgery in morbidly obese patients: interplay between CYP3A gut wall metabolism, permeability and dissolution. *J Pharm Pharmacol*. 2012;64:1008-1024.
42. Sung-Joon CI-S, Dae-Duk K. Obesity-related physiological changes and their pharmacokinetic consequence. *J Pharma Investig*. 2013;43:161-169.
43. Smith A, Henriksen B, Cohen A. Pharmacokinetic considerations in Roux-en-Y gastric bypass patients. *Am J Health-system Pharmacy*. 2011;68:2241-2247.
44. Salem F, Abduljalil K, Kamiyama Y, Rostami-Hodjegan A. Considering age variation when coining drugs as high versus low hepatic extraction ratio. *Drug Metab Dispos*. 2016;44:1099-1102.
45. Yang J, Liao M, Shou M, et al. Cytochrome p450 turnover: regulation of synthesis and degradation, methods for determining rates, and implications for the prediction of drug interactions. *Curr Drug Metab*. 2008;9:384-394.
46. Togao M, Kawakami K, Otsuka J, Wagai G, Ohta-Takada Y, Kado S. Effects of gut microbiota on in vivo metabolism and tissue accumulation of cytochrome P450 3A metabolized drug: Midazolam. *Biopharm Drug Dispos*. 2020;41:275-282.
47. Hole K, Heiberg PL, Gjestad C, Mehus LL, Rø Ø, Molden E. Elevated 4β-hydroxycholesterol/cholesterol ratio in anorexia nervosa patients. *Pharmacol Res Perspect*. 2018;6:e00430.
48. Brill MJ, Välitalo P, Darwich A, et al. Semiphysiologically based pharmacokinetic model for midazolam and CYP3A mediated metabolite 1-OH-midazolam in morbidly obese and weight loss surgery patients. *CPT Pharmacometrics Syst Pharmacol*. 2016;5:20-30.
49. Heizmann P, Eckert M, Ziegler WH. Pharmacokinetics and bioavailability of midazolam in man. *Br J Clin Pharmacol*. 1983;16(Suppl 1):43s-s49.
50. Hohmann N, Kocheise F, Carls A, Burhenne J, Haefeli WE, Mikus G. Midazolam microdose to determine systemic and pre-systemic metabolic CYP3A activity in humans. *Br J Clin Pharmacol*. 2015;79:278-285.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Kvitne KE, Robertsen I, Skovlund E, et al. Short- and long-term effects of body weight loss following calorie restriction and gastric bypass on CYP3A-activity – a non-randomized three-armed controlled trial. *Clin Transl Sci*. 2022;15:221–233. https://doi.org/10.1111/cts.13142

Supplementary Material

Short- and long-term effects of body weight loss following calorie restriction and gastric bypass on CYP3A-activity – a non-randomized three-armed controlled trial

Authors:

Kine Eide Kvitne[1], Ida Robertsen[1], Eva Skovlund[2], Hege Christensen[1], Veronica Krogstad[1], Christine Wegler[3,4], Philip Carlo Angeles[5,6], Birgit Malene Wollmann[7], Kristine Hole[7], Line Kristin Johnson[5], Rune Sandbu[5,6], Per Artursson[8], Cecilia Karlsson[9,10], Shalini Andersson[11], Tommy B. Andersson[4], Jøran Hjelmesæth[5,12], Rasmus Jansson-Löfmark[4], Anders Åsberg[1,13]

Affiliations:

[1] *Section for Pharmacology and Pharmaceutical Biosciences, Department of Pharmacy, University of Oslo, PO 1068 Blindern, 0316 Oslo, Norway*

[2] *Department of Public Health and Nursing, Norwegian University of Science and Technology, NTNU, P.O.Box 8905, 7491 Trondheim, Norway*

[3] *Department of Pharmacy, Uppsala University, P.O.Box 580, SE-75123, Uppsala, Sweden*

[4] *DMPK, Research and Early Development, Cardiovascular, Renal and Metabolism, BioPharmaceuticals R&D, AstraZeneca, Pepparedsleden 1, 431 83 Mölndal, Sweden*

[5] *The Morbid Obesity Center, Vestfold Hospital Trust, P.O.Box 2168, 3103 Tønsberg, Norway*

[6] *Department of Surgery, Vestfold Hospital Trust, P.O.Box 2168, 3103 Tønsberg, Norway*

[7] *Center for Psychopharmacology, Diakonhjemmet Hospital, P.O.Box 23 Vinderen, 0319 Oslo, Norway*

[8] *Department of Pharmacy and Science for Life Laboratory, Uppsala University, P.O.Box 580, SE-75123, Uppsala, Sweden*

[9] *Late-stage Development, Cardiovascular, Renal and Metabolism, BioPharmaceuticals R&D, AstraZeneca, Pepparedsleden 1, 431 83 Mölndal, Sweden*

[10] *Department of Molecular and Clinical Medicine, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Vita Stråket 15, 413 45 Gothenburg, Sweden*

[11] *Research and Early Development, Discovery Sciences, BioPharmaceuticals R&D, AstraZeneca, Pepparedsleden 1, 431 83 Mölndal, Sweden*

[12] *Department of Endocrinology, Morbid Obesity and Preventive Medicine, Institute of Clinical Medicine, University of Oslo, P.O.Box 1171, 0318 Oslo, Norway*

[13] *Department of Transplantation Medicine, Oslo University Hospital, P.O.Box 4950 Nydalen, 0424 Oslo, Norway*

METHODS

Midazolam and clinical chemistry analyses

Blood samples were drawn in K2-EDTA vacutainer tubes on ice and centrifuged for 10 minutes at 4ºC (1,800 g). Plasma was separated into Cryovials and frozen within one hour at -70ºC until analysis. Plasma midazolam and 4β-hydroxycholesterol (4βOHC) concentrations were determined as detailed below and plasma concentrations of C-reactive protein (CRP) and high-sensitivity CRP (hs-CRP) were measured using immunoturbidimetry (Advia Chemistry XPT systems, Siemens) at Fürst Medical Laboratory, Oslo, Norway. Clinical chemistry analyses were performed in fresh blood samples at the Department of Laboratory Medicine, Vestfold Hospital Trust, Tønsberg, Norway.

Midazolam analytical assay

Midazolam plasma concentrations were determined by validated ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) methods (1). Sample preparation using liquid-liquid extraction was performed at the Department of Pharmacy, University of Oslo for all samples (n=5,414). In brief, 50 ng/mL deuterated midazolam (MDZ-d6) used as internal standard was added into 5 mL Eppendorf tubes and evaporated to dryness using nitrogen gas before 250 µL of plasma was added to each tube. A 0.5 M ammonia solution was then added in a 1:1 ratio (250 µL) and 1.0 mL of ethyl acetate was used as extraction solvent. The sample was mixed for 10 minutes (Invitrogen Hulamixer, Thermo Fischer, Scientific Inc) and centrifuged at 2,500 g for 10 minutes at room temperature (Heraeus Megafuge 16R-centrifuge, Thermo Fisher Scientific Inc). Extraction with ethyl acetate was performed twice. The resulting organic phase was then collected and evaporated to dryness using nitrogen gas and heat (60°C). Finally, the samples were reconstituted in 50 µL of 0.05 M ammonium acetate buffer with 5 % acetonitrile (mobile phase A, pH 4.4), vortexed and transferred to micro vials before UHPLC-MS/MS analysis. Due to limited analytical machine capacity, samples were analyzed with two different UHPLC-MS/MS; at the Department of Pharmacy, University of Oslo (n=2,597 samples) and at the Center for Psychopharmacology, Diakonhjemmet Hospital (n=2,817 samples). All samples from each patient (week 0, 3 and 9) were analyzed in batches at the same

laboratory. Inter-laboratory cross-validation was performed using clinical study samples (n=47), and the percentage bias was within ±12.5%. The UPLC-MS/MS system at the Department of Pharmacy consisted of a Vanquish UPLC coupled to an Altis triple quadrupole mass spectrometer (Thermo-Fisher, Waltham, MA). Positive electrospray ionization and selected reaction monitoring were performed with compound-tuned conditions. The mass to charge (m/z) transitions were 326.1 -> 249.1 and 326.1 -> 291.2 for midazolam, and 332.1 -> 252.1 and 332.1 -> 203.1 for midazolam-d6. The analytical column was an Accucore Vanquish C18, 2.1 x 50 mm reverse phase column (Thermo-Fisher). Mobile phase A consisting of 5% acetonitrile and 10 mM ammonium formate and mobile phase B consisting of 90% acetonitrile and 10% methanol were delivered in a gradient flow rate of 0.4 mL/min. The UPLC-MS/MS system at the Center for Psychopharmacology consisted of Acquity UPLC coupled to a Micromass Quattro micro tandem MS detector (Waters, Milford, MA). After positive electrospray ionization detection was obtained with multiple reaction monitoring at m/z 326.15 -> 391.15 (midazolam) and m/z 332.1 -> 252.1 (midazolam-d6). Chromatographic separation was achieved on a BEH C18 column RP-shield (1.7 μm, 1 × 100 mm) from Waters with mobile phases consisting of 10 mM ammonium acetate and acetonitrile. The flow rate was 0.2 mL/min.

Calibrators and quality control (QC) samples were prepared in blank plasma and analyzed in each analytical run. Eight calibrators in the range 0.1 to 20 ng/mL were applied, and back calculated values of calibrators within 80 to 120% were accepted. Lower limit of quantification (LLOQ) was 0.1 ng/mL and upper limit of quantification (ULOQ) was 20 ng/mL. Samples with midazolam concentrations above ULOQ were diluted in blank plasma and reanalyzed. Dilution integrity with dilution factors of 1/2, 1/5, 1/10, 1/20 and 1/50 was established; mean accuracy ranged from 88.9% to 103.8% and the imprecision was <4.5%. Within-series and between-series performance were assessed with resulting coefficient of variation <12.3% and the mean accuracy ranged from 99.3 to 104.3%.

4β-hydroxycholesterol

Plasma concentrations of 4βOHC was determined by an HPLC-MS method previously described (2), with an added filtration step (3), at the Center for Psychopharmacology, Diakonhjemmet Hospital, Oslo, Norway. 4βOHC was de-esterified from fatty acids by ethanolic sodium methoxide and separated from

plasma by liquid-liquid extraction with hexane. Extracts were evaporated to dryness using nitrogen and reconstituted in methanol before filtration. An Acquity UPLC coupled to a Micromass Quattro micro tandem MS detector (Waters, Milford, MA) was used for quantitative analysis. Chromatographic separation was achieved on a BEH C18 column RP-shield (1.7 μm, 1 × 100 mm) from Waters with a mobile phase of water and methanol. After atmospheric pressure chemical ionization, detection was obtained with multiple reaction monitoring at m/z 385.25 -> 367.45 (4βOHC) and m/z 392.30 -> 374.50 (4βOHC-d7; internal standard). The LLOQ was 10 ng/mL. Within- and between-series imprecision and inaccuracy were <15% at 10 ng/mL and <4% at 644 ng/mL (n = 6) (2).

Protein quantification (intestine and liver)

Small intestinal- and liver biopsies were homogenized in lysis buffer containing 2% (w/v) SDS, 50 mM DTT, and 100 mM Tris/HCl, pH 7.8 and proteins were denatured at 95 °C. Proteins were cleaned and digested with the multi-enzyme digestion filter-aided sample preparation (MED-FASP) protocol, using LysC and trypsin (4). Peptides were separated on a C18 column (50 cm, and 75 μm inner diameter) using a 2-hour acetonitrile gradient in 0.1% formic acid at a flow rate of 300 nL/min. The LC was coupled to a Q Exactive HF or Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific) operating in data dependent mode with survey scans at a resolution of 60 000, AGC target of 3 × 106, and maximum injection time of 20 ms. The top 15 most abundant isotope patterns were selected from the survey scan with an isolation window of 1.4 m/z and fragmented with nCE at 27. The MS/MS analysis was performed with a resolution of 15 000, AGC target of 1 × 105, and maximum injection time of 60 ms (5). The resulting MS data was analyzed with MaxQuant (version 1.6.10.43) (6), where proteins were identified by searching peptides against a decoy version of the human UniProtKB (UP000005640). Carbamidomethylation was set as a fixed modification, and protein false discovery rates were specified as 0.01, allowing a maximum of two missed cleavages. Spectral raw intensities were normalized with variance stabilization (vsn) (7), and were subsequently used to calculate the protein concentrations using the Total Protein Approach (8). Batch effects were removed by geometric mean centering of proteins from samples analyzed at different time points.

Population pharmacokinetic modeling

Midazolam model development and validation

A midazolam population pharmacokinetic model parameterized to determine individual absolute bioavailability was developed with data from semi-simultaneous administration of oral and intravenous midazolam (Midazolam hydrochloride syrup 2 mg/mL, Roxane Laboratories, Boehringer Ingelheim, Germany and Midazolam B. Braun 1 mg/mL injection/infusion, Braun, Germany). The modeling was performed using the nonparametric adaptive grid approach implemented in Pmetrics (version 1.5.2) for R (version 3.6.2) (9, 10). A total of 5,414 midazolam concentrations corresponding to 306 unique 24-hour pharmacokinetic profiles were available from in total 98 patients. The initial model development included investigation of different structural models to fit midazolam concentrations from normal- to overweight individuals (control group) and patients with severe obesity scheduled for weight loss treatment before and after intervention. To reduce computational time running models of this size and complexity, initial model development was performed by splitting the data and running sequential runs (MacBook Pro 2012, with 2.9 GHz Intel Core i7). Thus, the individual pharmacokinetic profiles were split into separate datasets based on study visit (week 0, 3, 9 and year 2) and group (intervention/control). To evaluate the validity of the structural model and the parameters, each separate dataset was split into a development dataset (75%) and a validation dataset (25%) by simple randomization. In the initial stages, modeling was performed on baseline data from the two intervention groups in the development dataset. Two- and three-compartmental mammillary (parallel) and catenary (in series) models (11, 12) with different absorption pattern (zero- and first order with and without lag-time) were evaluated, and the structural model that best fitted the data was taken further into development (34, 35). Both gamma (error = SD*γ) and lambda (error= $[SD^{2*}\lambda^{2}]^{0.5}$) error models were tested, where SD is the standard deviation of the analytical method; SD = $4.445597e^{-01} + 1.925945e^{-02}$*obs – $4.570195e^{-05}$*obs^2 where obs is the observed midazolam concentration. Midazolam plasma concentrations more than two standard deviations from the mean concentration at the specific time point were considered outliers. Outliers were assigned a larger error polynominal, and were hence less weighted during model regression. Optimization of parameter boundaries was performed separately for each dataset, to assure that a

potential change in midazolam pharmacokinetics between the different study visits were captured accurately. Covariates were not included in the model, due to rich data and solely interest in individual predictions and pharmacokinetic parameters. The Pmetrics function *PMcompare()* was applied to compare models during model development. Absolute- and relative predictive error and absolute- and relative root mean squared error (RMSE), in addition to Akaike information criteria (AIC), bias, imprecision and R^2 of the observed-predicted plots for the individual predicted values were used in combination to evaluate models. The relative predictive error (predicted concentration – observed concentration/observed concentration) was applied as the main validation metric in addition to individual imprecision and bias. In the final stages, all development- and validation datasets were merged into one development dataset and one validation dataset, respectively. The consolidated development model was set up using the most outer boundaries from the previous runs of the divided development datasets and run on a high-performance computing (HPC) cluster until convergence, utilizing a modified version of Pmetrics version 1.9.4. and 480126 initial support points. The Pmetrics optimization was performed as an Advanced User Support, provided by UNINETT Sigma2. The standard compute nodes are equipped with 40 cores and 192 GB memory each, with the full cluster boasting a theoretical performance (Rpeak) of 645 teraflops. The final consolidated development model was validated on the consolidated validation dataset using the final consolidated development model. The validated model was cycled until convergence with the complete dataset (development and validation combined) and used for pharmacokinetic calculations as presented below.

Pharmacokinetic calculations

Posterior individual parameter values obtained from the final model with the complete dataset were used to describe midazolam pharmacokinetics in each individual. Absolute bioavailability, C_{max} and T_{max} were obtained directly from the individual model predictions, while clearance, oral clearance (clearance/absolute bioavailability), volume of distribution and terminal elimination half-life ($T_{1/2}$) were calculated according to the equations below. The function *makeAUC()* in the Pmetrics package for R was used to calculate the model derived area under the concentration versus time curve from zero to infinity ($AUC_{0\text{-}inf}$).

$$\text{Clearance (L/h)} = \frac{((\text{Absolute bioavailability (F)} \times 1.5\text{mg}) + 1.0\text{mg})}{\text{AUC}_{0-\text{inf}}(\text{mg} \times \text{h/L})} \qquad \textit{Equation 1}$$

Clearance was estimated in this manner to minimize any potential correlation biases in the transfer rate constants.

$$\text{Oral clearance (L/h)} = \frac{\text{Clearance (L/h)}}{\text{Absolute bioavailability}} \qquad \textit{Equation 2}$$

$$\text{Volume of distribution (L)} = \text{V1} + \left(\frac{\text{K23}}{\text{K32}} \times \text{V1}\right) + \left(\frac{\text{K34}}{\text{K43}} \times \text{V1}\right) \qquad \textit{Equation 3}$$

Where V1 is volume of central compartment from the population pharmacokinetic model, K23 and K32 are transfer rate constants between compartment 2 and 3 and K34 and K43 are transfer rate constants between compartment 3 and 4.

$$\text{Elimination half} - \text{life (h)} = \text{LN}(2) \times \frac{\text{Volume of distribution (L)}}{\text{Clearance (L/h)}} \qquad \textit{Equation 4}$$

RESULTS

Population pharmacokinetic modeling

Midazolam model development and validation

A 2-compartment model did not fit the data well resulting in an AIC of 2,683 which was substantially higher than the other models evaluated and was thus discarded. The mammillary 3-compartment model with first order absorption with lag time and first order elimination from the central compartment showed an AIC of 2,189. The corresponding catenary 3-compartment model resulted in an AIC of 2,090 and was taken further in the model development (Figure S1). Absolute- and relative predictive error with resulting RMSE, number of support points as well as number of cycles for convergence are presented in Table S1. In the development dataset, mean relative predictive error using the final model was -6.6 ±30.4%. Based on the analyses of the validation dataset it was judged that the pharmacokinetic model adequately described data for its intended purpose. In the validation dataset, mean relative predictive error was -8.5 ±29.1%. The diagnostic plots of observed versus individual predicted concentration plots and residual plots in both the development dataset and validation dataset were considered as well, with no major bias (Figure S2 and S3). The weighted residuals were homogeneously distributed over the concentration range and time range, but the 23- and 24-hour midazolam concentrations were slightly under-predicted in the model (Figure S3). Visual predictive check (VPC) plots are not shown, due to solely interest in the individual predictions. The pharmacokinetic parameter values from the final model are presented in Table S2. Individual predicted profiles are shown in Figure S4.

TABLES

Table S1. An overview of the results from the development dataset, the validation dataset and the complete dataset using the final model.

	Development, n=75%	**Validation, n=25%**	**Complete dataset, n=100%**
PK-profiles, n	231	75	306
Absolute PE, µg/L	-0.08 ± 0.79	-0.10 ± 0.77	-0.08 ± 0.86
Absolute RMSE, µg/L	0.39 ± 0.69	0.39 ± 0.67	0.40 ± 0.77
Relative PE, %	-6.6 ± 30.4	-8.5 ± 29.1	-6.4 ± 30.3
Relative RMSE, %	17.1 ± 25.9	17.3 ± 25.0	17.1 ± 25.8
Converged after cycle, n	7,146	6,173	9,890
Support points, n	228	75	303

Values are presented as mean ± SD or number.
Abbreviations: PE, predictive error; PK, pharmacokinetic; RMSE, root mean squared error

Table S2. An overview of the pharmacokinetic parameter values from the final model based on the complete dataset (n=306 profiles).

	Parameter values (mean ± SD)	**Shrink** (%)
Ka, h^{-1}	3.21 ± 2.55	0.13
K23, h^{-1}	3.92 ± 2.60	0.32
K32, h^{-1}	17.4 ± 10.5	0.70
K34, h^{-1}	21.8 ± 12.8	0.61
K43, h^{-1}	1.92 ± 1.77	0.15
K20, h^{-1}	2.02 ± 1.69	0.03
V1, L	21.1 ± 16.3	0.06
Tlag, h	0.18 ± 0.11	0.48
FA	0.21 ± 0.12	0.06

Abbreviations: FA, absolute bioavailability; Ka, oral absorption rate constant; K20, elimination rate constant; K23 and K32, transfer rate constants between compartment 2 and 3; K34 and K43, transfer rate constants between compartment 3 and 4; Tlag, absorption lag time, V1, volume of central compartment.

Table S3. Changes in body weight and selected laboratory measures. Short- and long-term outcomes in clinical parameters for patients performing 24 hour pharmacokinetic investigations, from baseline to week 3, week 9 and year 2 in the RYGB group and diet group, respectively.

	RYGB			**Diet**			**Difference between groups**	
	Estimated mean difference (Δ) [95% CI]							
Clinical parameter	**Week 0 - Week 3**	**Week 0 - Week 9**	**Week 0 - Year 2**	**Week 0 - Week 3**	**Week 0 - Week 9**	**Week 0 - Year 2**	**Week 9**	**Year 2**
Body weight (kg)	**-6.3** [-9.5, -3.1]	**-17.5** [-20.7, -14.2]	**-37.8** [-41.1, -34.4]	**-5.6** [-8.6, -2.5]	**-13.4** [-16.5, -10.3]	**-3.5** [-6.9, -0.12]	2.8 [-7.4, 13.1]	**-27.4** [-37.7, -17.1]
BMI (kg/m^2)	**-2.1** [-3.2, -1.0]	**-6.0** [-7.1, -4.9]	**-12.9** [-14.1, -11.8]	**-1.9** [-2.9, -0.81]	**-4.5** [-5.5, -3.4]	**-1.2** [-2.3, -2.7]	1.1 [-1.5, 3.7]	**-9.2** [-11.8, -6.6]
Albumin (g/L)	-0.14 [-7.7, 7.4]	-1.5 [-9.3, 6.3]	-3.9 [-11.8, 4.0]	0.78 [-6.7, 8.2]	0.33 [-7.2, 7.9]	6.0 [-2.0, 14.0]	-1.5 [-7.6, 4.5]	**-9.6** [-16.1, -3.1]
Creatinine (µmol/L)	1.8 [-2.1, 5.7]	-0.60 [-4.6, 3.4]	1.9 [-2.2, 6.0]	2.3 [-1.5, 6.0]	0.78 [-3.1, 4.6]	**6.6** [2.4, 10.7]	-3.0 [-9.2, 3.2]	-6.3 [-12.6, 0.04]
AST (U/L)	4.0 [-0.95, 8.9]	3.2 [-1.9, 8.3]	-1.2 [-6.4, 4.0]	0.38 [-4.4, 5.2]	-3.1 [-7.9, 1.8]	-4.3 [-9.5, 0.95]	**6.2** [0.78, 11.6]	3.0 [-2.7, 8.6]
ALT (U/L)	7.8 [-0.23, 15.9]	**9.4** [1.0, 17.8]	-2.5 [-11.0, 6.1]	2.6 [-5.3, 10.5]	-2.0 [-10.0, 6.1]	-1.4 [-10.0, 7.3]	**13.6** [4.1, 23.0]	1.1 [-8.7, 10.9]
hs-CRP (mg/L)	**-2.9** [-5.4, -0.28]	-0.83 [-3.5, 1.8]	**-6.6** [-9.4, -3.9]	-1.2 [-3.7, 1.3]	-1.4 [-3.9, 1.2]	-0.79 [-3.5, 2.0]	1.7 [-1.3, 4.7]	**-4.7** [-7.8, -1.6]

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; hs-CRP, high sensitivity C-reactive protein; RYGB, Roux-en-Y gastric bypass
† Linear mixed model was used to estimate mean difference in change (Δ).

FIGURES

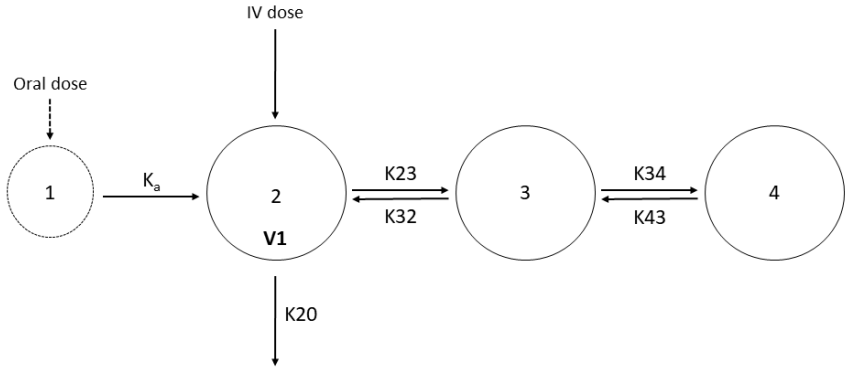

Figure S1. Schematic presentation of the structural population pharmacokinetic model. Abbreviations: K_a, oral absorption rate constant; K20, elimination rate constant; 1, transit compartment; 2, central compartment; 3, peripheral compartment 1; 4, peripheral compartment 2; K23 and K32, transfer rate constants between compartment 2 and 3; K34 and K43, transfer rate constants between compartment 3 and 4; V1, volume of central compartment.

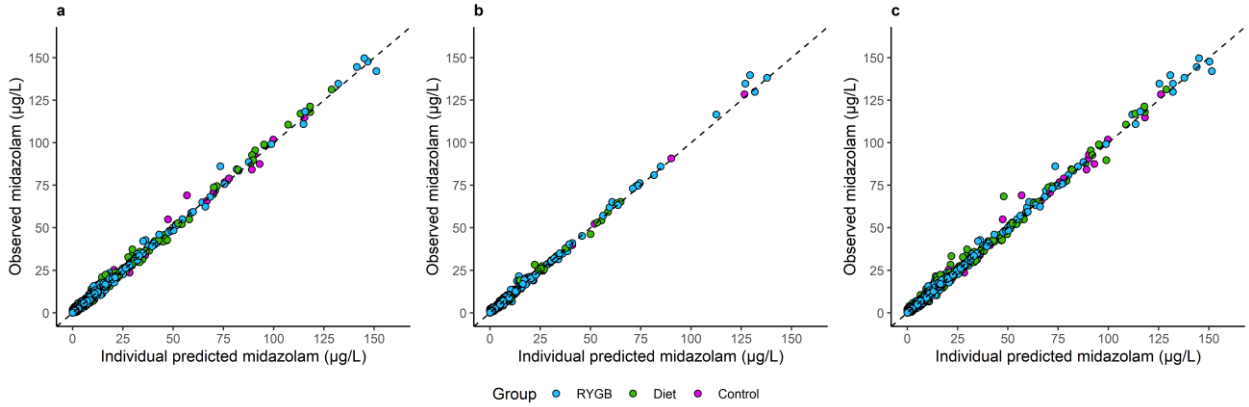

Figure S2: Observed-predicted plots. Individual predicted midazolam concentrations as a function of observed midazolam concentrations in the final model based on the a) development dataset, b) validation dataset and c) complete dataset.

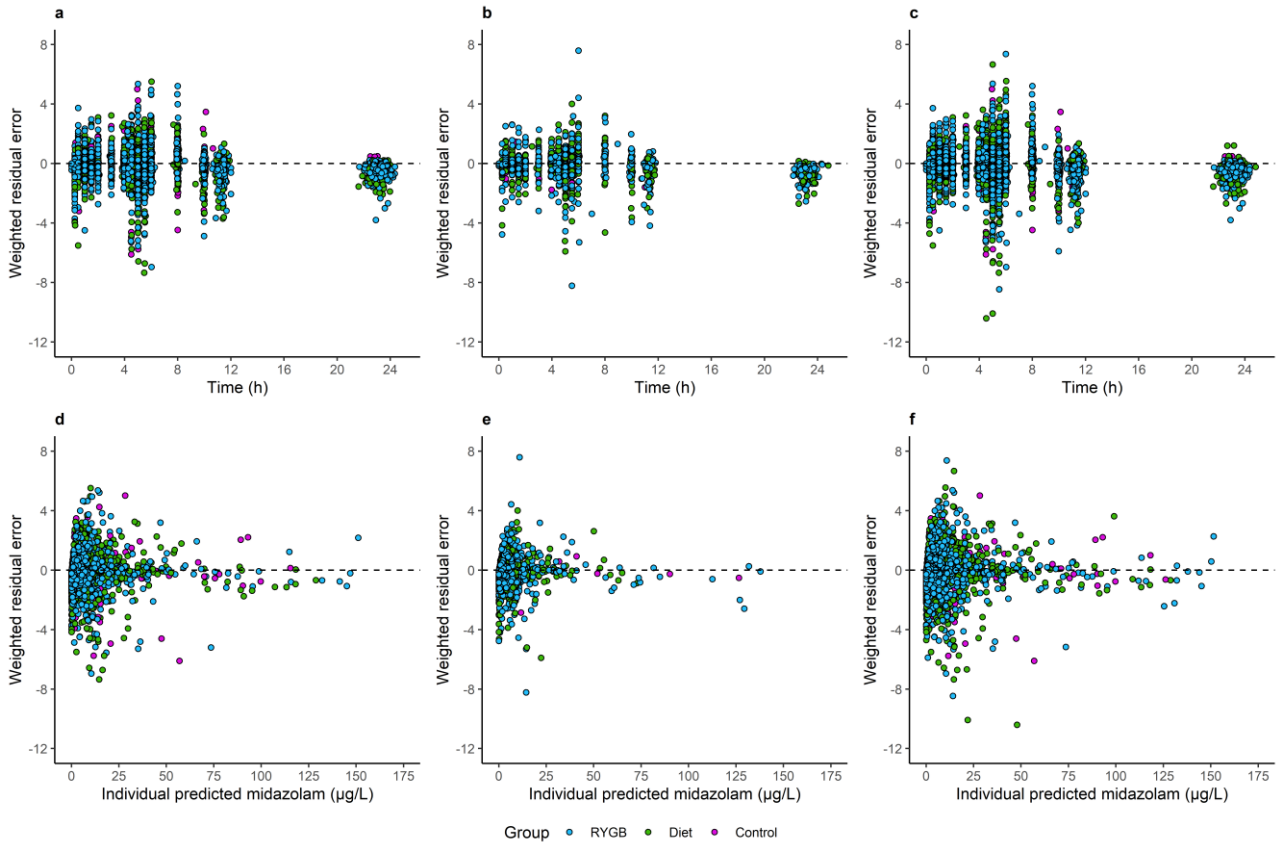

Figure S3: Residual plots. Weighted residuals as a function of time in the final model based on the development-, validation- and complete dataset are shown in a, b and c, respectively. Weighted residuals as a function of individual predicted midazolam concentrations in the final model based on the same datasets are shown in d, e and f, respectively.

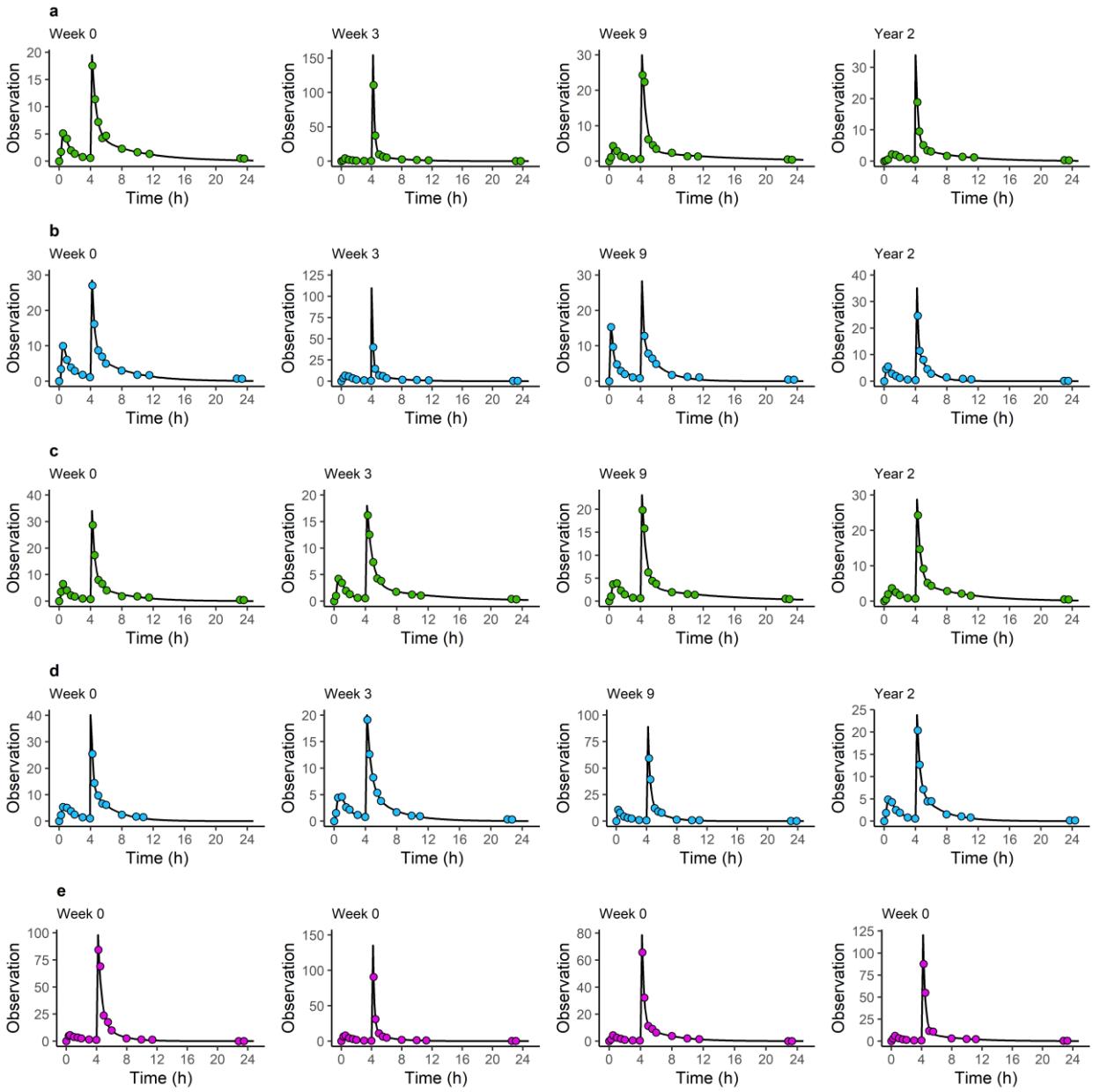

Figure S4: Individual predicted profiles for two diet patients (green) and two RYGB patients (blue) at the different study visits are shown in a, b, c and d. Individual predicted profiles for four different control patients scheduled for cholecystectomy (purple) are shown in e.

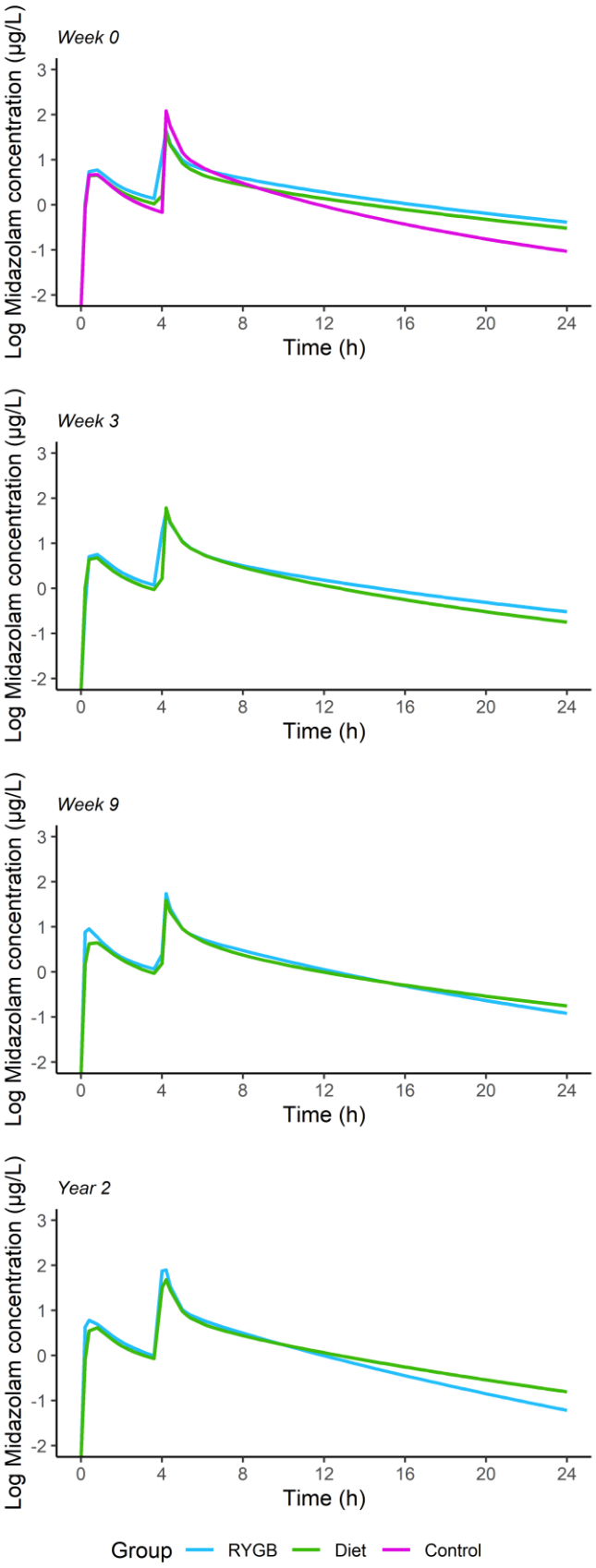

Figure S5. Mean plasma concentration time profiles of midazolam in the three groups at the different study visits (log-transformed y-axis).

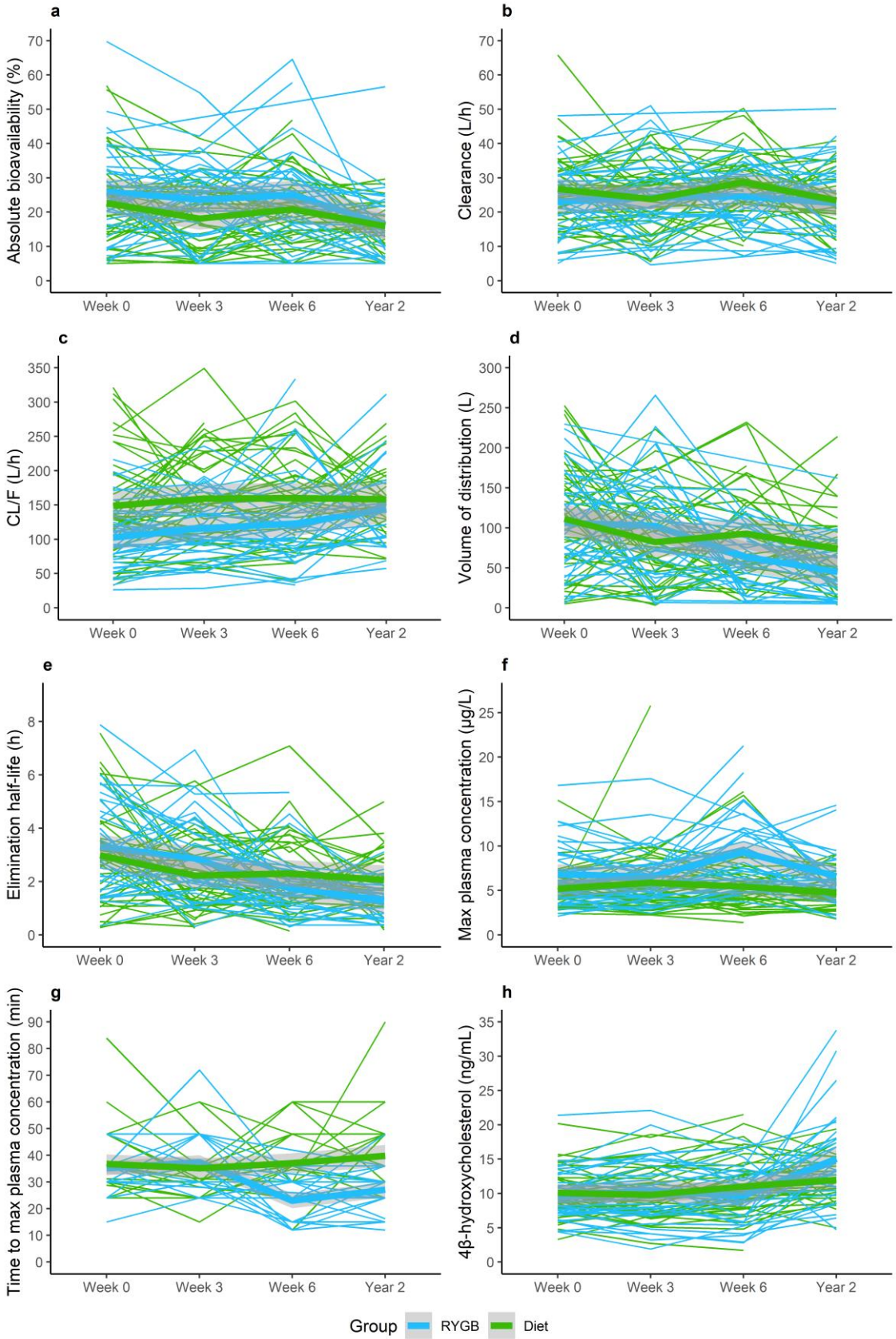

Figure S6. Individual plots for a) absolute bioavailability, b) clearance, c) oral clearance (CL/F), d) volume of distribution, e) elimination half-life, f) maximum plasma concentration (C_{max}) after oral midazolam, g) time to reach C_{max} (T_{max}) after oral midazolam and h) 4β-hydroxycholesterol at the different study visits. Extreme values were excluded from figure c (n=1), d (n=1), e (n=1), f (n=1) and i (n=1).

REFERENCES

(1) Egeland, E.J., Witczak, B.J., Zaré, H.K., Christensen, H., Åsberg, A. & Robertsen, I. Chronic Inhibition of CYP3A is Temporarily Reduced by Each Hemodialysis Session in Patients With End-Stage Renal Disease. *Clinical pharmacology and therapeutics*, (2020).

(2) Gjestad, C., Huynh, D.K., Haslemo, T. & Molden, E. 4β-hydroxycholesterol correlates with dose but not steady-state concentration of carbamazepine: indication of intestinal CYP3A in biomarker formation? *Br J Clin Pharmacol* **81**, 269-76 (2016).

(3) Størset, E., Hole, K., Midtvedt, K., Bergan, S., Molden, E. & Åsberg, A. The CYP3A biomarker 4β-hydroxycholesterol does not improve tacrolimus dose predictions early after kidney transplantation. *Br J Clin Pharmacol* **83**, 1457-65 (2017).

(4) Wiśniewski, J.R. & Mann, M. Consecutive proteolytic digestion in an enzyme reactor increases depth of proteomic and phosphoproteomic analysis. *Anal Chem* **84**, 2631-7 (2012).

(5) Wegler, C. *et al.* Global variability analysis of mRNA and protein concentrations across and within human tissues. *NAR Genomics and Bioinformatics* **2**, (2019).

(6) Tyanova, S., Temu, T. & Cox, J. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nat Protoc* **11**, 2301-19 (2016).

(7) Huber, W., von Heydebreck, A., Sültmann, H., Poustka, A. & Vingron, M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics* **18 Suppl 1**, S96-104 (2002).

(8) Wiśniewski, J.R. & Rakus, D. Multi-enzyme digestion FASP and the 'Total Protein Approach'-based absolute quantification of the Escherichia coli proteome. *J Proteomics* **109**, 322-31 (2014).

(9) R Foundation for Statistical Computing. R: A Language and Environment for Statistical Computing (Vienna, Austria, 2018).

(10) Neely, M.N., van Guilder, M.G., Yamada, W.M., Schumitzky, A. & Jelliffe, R.W. Accurate detection of outliers and subpopulations with Pmetrics, a nonparametric and parametric pharmacometric modeling and simulation package for R. *Ther Drug Monit* **34**, 467-76 (2012).

(11) Świętaszczyk, C. & Jødal, L. Three-compartment pharmacokinetic models of radiotracers used in the GFR-determination - estimation of their parameters using the time-concentration curves. *Nucl Med Rev Cent East Eur* **22**, 60-8 (2019).

(12) de Biasi, J. Four open mammillary and catenary compartment models for pharmacokinetics studies. *J Biomed Eng* **11**, 467-70 (1989).

Pharmacokinetic population model code

```
#Pri
Ka,0.1,10
K23,0.01,12
K32,0.01,36
K34,0.001,42
K43,0.001,7
K20,0.1,10
V20,1,70
Tlag0,0,0.5
FA0,0.05, 1

#Dif
XP(1) = -Ka*X(1)
XP(2) = Ka*X(1)+RATEIV(1)-K23*X(2)+K32*X(3)-K20*X(2)
XP(3) = K23*X(2)-K32*X(3)+K43*X(4)-K34*X(3)
XP(4) = K34*X(3)-K43*X(4)

#Sec
V2=V20

#Out
Y(1) = X(2)/V2

#F
FA(1)=FA0

#LAG
TLAG(1)=Tlag0

#Err
L=14.445597e-01, 1.925945e-02, 4.570195e-05, 0
```

II

Received: 23 January 2022 | Revised: 23 March 2022 | Accepted: 29 March 2022

DOI: 10.1111/bcp.15349

ORIGINAL ARTICLE

Short- and long-term effects of body weight, calorie restriction and gastric bypass on CYP1A2, CYP2C19 and CYP2C9 activity

Kine Eide Kvitne[1] | Veronica Krogstad[1] | Christine Wegler[2,3] | Line Kristin Johnson[4] | Marianne K. Kringen[5,6] | Markus Herberg Hovd[1] | Jens K. Hertel[4] | Maria Heijer[7] | Rune Sandbu[4,8] | Eva Skovlund[9] | Per Artursson[10] | Cecilia Karlsson[11,12] | Shalini Andersson[13] | Tommy B. Andersson[3] | Jøran Hjelmesæth[4,14] | Anders Åsberg[1,15] | Rasmus Jansson-Löfmark[3] | Hege Christensen[1] | Ida Robertsen[1]

[1]Section for Pharmacology and Pharmaceutical Biosciences, Department of Pharmacy, University of Oslo, Oslo, Norway

[2]Department of Pharmacy, Uppsala University, Uppsala, Sweden

[3]DMPK, Research and Early Development, Cardiovascular, Renal and Metabolism, BioPharmaceuticals R&D, AstraZeneca, Mölndal, Sweden

[4]The Morbid Obesity Center, Vestfold Hospital Trust, Tønsberg, Norway

[5]Center for Psychopharmacology, Diakonhjemmet Hospital, Oslo, Norway

[6]Department of Health Sciences, Oslo Metropolitan University, Oslo, Norway

[7]Clinical Pharmacology and Quantitative Pharmacology, Clinical Pharmacology & Safety Sciences, BioPharmaceuticals R&D, AstraZeneca, Mölndal, Sweden

[8]Department of Surgery, Vestfold Hospital Trust, Tønsberg, Norway

[9]Department of Public Health and Nursing, Norwegian University of Science and Technology, NTNU, Trondheim, Norway

[10]Department of Pharmacy and Science for Life Laboratory, Uppsala University, Uppsala, Sweden

[11]Late-stage Development, Cardiovascular, Renal and Metabolism, BioPharmaceuticals R&D, AstraZeneca, Mölndal, Sweden

[12]Department of Molecular and Clinical Medicine, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

[13]Research and Early Development, Discovery Sciences, BioPharmaceuticals R&D, AstraZeneca, Mölndal, Sweden

[14]Department of Endocrinology, Morbid Obesity and Preventive Medicine, Institute of Clinical Medicine, University of Oslo, Oslo, Norway

[15]Department of Transplantation Medicine, Oslo University Hospital, Oslo, Norway

Correspondence
Kine Eide Kvitne, Section for Pharmacology and Pharmaceutical Biosciences, Department of Pharmacy, University of Oslo, PO 1068 Blindern, 0316 Oslo, Norway.
Email: k.e.kvitne@farmasi.uio.no

Funding information
Swedish Research Council, Grant/Award Numbers: 01951, 5715; AstraZeneca, Sweden; Department of Pharmacy, University of Oslo, Norway; Vestfold Hospital Trust, Norway

Aim: Roux-en-Y gastric bypass (RYGB) may influence drug disposition due to surgery-induced gastrointestinal alterations and/or subsequent weight loss. The objective was to compare short- and long-term effects of RYGB and diet on the metabolic ratios of paraxanthine/caffeine (cytochrome P450 [CYP] 1A2 activity), 5-hydroxyomeprazole/omeprazole (CYP2C19 activity) and losartan/losartan carboxylic acid (CYP2C9 activity), and cross-sectionally compare these CYP-activities with normal-to-overweight controls.

Methods: This trial included patients with severe obesity preparing for RYGB (n = 40) or diet-induced (n = 41) weight loss, and controls (n = 18). Both weight loss

The authors confirm that the Principal Investigator for this paper is Jøran Hjelmesæth and that he had direct clinical responsibility for patients.

groups underwent a 3-week low-energy diet (<1200 kcal/day, weeks 0-3) followed by a 6-week very-low-energy diet or RYGB (both <800 kcal/day, weeks 3-9). Follow-up time was 2 years, with four pharmacokinetic investigations.

Results: Mean ± SD weight loss from baseline was similar in the RYGB-group (13 ± 2.4%) and the diet group (10.5 ± 3.9%) at week 9, but differed at year 2 (RYGB −30 ± 6.9%, diet −3.1 ± 6.3%). From weeks 0 to 3, mean (95% confidence interval [CI]) CYP2C19 activity similarly increased in both groups (RYGB 43% [16, 55], diet 48% [22, 60]). Mean CYP2C19 activity increased by 30% (2.6, 43) after RYGB (weeks 3-9), but not in the diet-group (between-group difference −0.30 [−0.63, 0.03]). CYP2C19 activity remained elevated in the RYGB group at year 2. Baseline CYP2C19 activity was 2.7-fold higher in controls compared with patients with obesity, whereas no difference was observed in CYP1A2 and CYP2C9 activities.

Conclusion: Our findings suggest that CYP2C19 activity is lower in patients with obesity and increases following weight loss. This may be clinically relevant for drug dosing. No clinically significant effect on CYP1A2 and CYP2C9 activities was observed.

KEYWORDS
cytochrome P450, drug metabolism, gastric bypass, obesity, pharmacokinetics

1 | INTRODUCTION

The most important group of drug-metabolizing enzymes is the cytochrome P450 (CYP) superfamily, contributing to the metabolism and systemic exposure of approximately 75% of clinically used drugs.[1,2] The CYP isoforms considered to play a quantitatively important role in drug metabolism are CYP3A, **CYP1A2**, **CYP2C9**, CYP2C19 and CYP2D6.[1,2] These drug-metabolizing enzymes reside mainly in the liver, the primary drug-metabolizing organ.[3] All these isoforms, except for CYP1A2, are also expressed in the small intestine and thus are involved in restricting the oral bioavailability of substrate drugs.[4] There is a large interindividual variability in both the expression and activity of CYP enzymes[3,4] for various reasons, including genetic polymorphism, drug-drug interactions and disease state.[5,6]

Obesity is associated with altered pharmacokinetics of drugs.[7–9] These alterations may be related to obesity-associated conditions and disorders such as low-grade inflammation, nonalcoholic fatty liver disease (NAFLD), higher adipose tissue mass and higher hepatic blood flow due to increased blood volume and cardiac output.[10–12] Clearance mediated by CYP1A2, CYP2C19 and CYP2C9 appears to be similar or slightly increased in patients with obesity, as compared with normal weight subjects.[7,13,14] CYP2C19, CYP2C9 and CYP1A2 activities are also affected by genetic polymorphism and may differ substantially between individuals regardless of body weight.[5,15]

In obesity, a moderate weight loss is associated with improvement of several health-related outcomes.[16–18] However, the most effective treatment to achieve durable weight loss and improvement of comorbidities in patients with severe obesity is bariatric surgery.[19,20] Furthermore, after Roux-en-Y gastric bypass (RYGB), the surface area available for drug absorption is reduced and the proximal intestinal segments rich in CYP enzymes are bypassed.[21] The subsequent weight loss, followed by lower inflammation and reduced liver

What is already known about this subject

- There is growing evidence that body weight and gastric bypass may influence drug disposition, but the clinical relevance remains uncertain.
- Previous studies had small sample-sizes, and were uncontrolled and unable to disentangle the surgery effect itself from weight loss.

What this study adds

- Similar weight loss in the intervention groups enabled us to separate the surgery effect from the weightloss effect.
- CYP2C19 activity was lower in obesity and increased after weight loss.
- This should be taken into consideration for optimal dosing of CYP2C19 substrates.

fat content, may also influence the expression and activity of CYP enzymes.[22,23] We have previously shown that neither RYGB per se nor a moderate weight loss impacted CYP3A activity early after surgery, but that CYP3A activity increased following a substantial weight loss long term.[24]

Although there is growing evidence that body weight and bariatric surgery may affect drug dosing of various CYP substrates,[12,21] it remains to be shown whether pharmacokinetic changes after RYGB are due to the surgery itself or the subsequent weight loss. To disentangle these effects, we performed this nonrandomized controlled study including a dietary control group achieving a similar short-term weight loss as patients subjected to RYGB. The primary objective was to compare short- and long-term effects of surgical and nonsurgical calorie restriction on CYP1A2, CYP2C19 and CYP2C9 activities using caffeine, omeprazole, and losartan as probe drugs, respectively.[25] Additionally, we aimed to compare these CYP activities in normal to overweight controls and patients with obesity.

2 | METHODS

2.1 | Patients and study design

The present analyses are part of the extensive COCKTAIL study, where the metabolic and pharmacokinetic effects of RYGB and a very-low-energy diet (VLED) were investigated, which has been described in detail previously.[24,25] The COCKTAIL study was an open-label, nonrandomized, three-armed, single-center controlled study performed at Vestfold Hospital Trust in Norway and included investigations of four probe drugs of different CYP enzymes (CYP3A, CYP1A2, CYP2C19 and CYP2C9). The data on CYP3A activity, using midazolam as a probe drug, has been published previously.[24] Patients with severe obesity scheduled for weight loss treatment with RYGB or nonsurgical calorie restriction were included and followed prospectively for 2 years. A cross-sectional control group of mainly normal to overweight individuals scheduled for cholecystectomy was also included. The study was approved by the Regional Committee for Medical and Health Research Ethics (2013/2379/REK) and performed in accordance with Good Clinical Practice and the Declaration of Helsinki (NCT02386917). Written informed consent was obtained prior to study participation.

Patients aged 18 years or above with stable body weight for the last 3 months (<5 kg weight change) were eligible for inclusion in the study.[25] Key exclusion criteria included previous bariatric or upper gastrointestinal surgery and glomerular filtration rate <30 mL/min/1.73m^2. Full eligibility criteria are listed in the protocol.[25]

2.2 | Study visits and procedures

Data were included from the control group at week 0 (baseline) and from the weight loss groups at weeks 0, 3 and 9, and year 2. All three groups were subjected to a pharmacokinetic investigation of different probe drugs at baseline. In the weight loss groups, the pharmacokinetic investigation was repeated at all three follow-up visits. Both intervention groups started a 3-week low-energy diet (LED; <1200 kcal/day) immediately after the pharmacokinetic investigation at baseline, followed by a 6-week isocaloric (<800 kcal/day) VLED or RYGB. Thereafter, patients followed local treatment guidelines until the final study visit at year 2. For patients undergoing RYGB or cholecystectomy, liver biopsies were obtained at the time of surgery (RYGB, week 3; control, week 0) as previously described.[26]

On the pharmacokinetic investigational days, 100 mg of oral caffeine was administered first, followed by 25 mg of oral losartan and 20 mg of oral omeprazole 1 hour later. Blood samples were collected 3 and 4 hours after omeprazole and caffeine administration,[27] whereas urine was collected for 8 hours after losartan administration to determine the metabolic ratio of the respective probe drug. A detailed description can be found in the Supporting Information, Methods.

2.3 | Metabolic ratios and CYP activities

Metabolite drug ratio, with the metabolite as the numerator and the parent compound as the denominator, was used to estimate CYP1A2 and CYP2C19 activities in plasma. CYP1A2 activity was described by the plasma (4-hour) paraxanthine/caffeine ratio and CYP2C19 activity was described by the plasma (3-hour) 5-hydroxyomeprazole (5-OH-omeprazole)/omeprazole ratio. Drug metabolite ratio, with the parent compound as the numerator and the metabolite as the denominator, was used to estimate CYP2C9 activity in urine. CYP2C9 activity was described by the urinary (8-hour) losartan/losartan carboxylic acid (LCA) ratio. For the metabolic ratios calculated as the metabolite/drug ratio (CYP1A2 and CYP2C19), a higher ratio implies a higher CYP activity, while for the metabolic ratio calculated as the drug/metabolite ratio (CYP2C9), a higher ratio implies a lower CYP activity. Both the metabolite/drug ratios and the drug/metabolite ratio are referred to as metabolic ratios in the following.

2.4 | Bioanalytical assays

Plasma concentrations of caffeine, paraxanthine, omeprazole and 5-OH-omeprazole as well as the urinary concentration of losartan and LCA were determined by Covance Laboratories (Madison, Wisconsin, USA) using validated liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) methods. All methods demonstrated acceptable precision, accuracy and selectivity for the analytes in the appropriate matrices. The results from the in-study quality control samples and calibration standards were evaluated, and all three methods performed acceptably for this study. Details regarding the LC-MS/MS results and clinical chemistry analyses are described in the Supporting Information, Methods.

2.5 | Genotyping

Analysis of CYP1A2, CYP2C19 and CYP2C9 variant alleles was performed using Taqman-based real-time polymerase chain reaction assays implemented for routine pharmacogenetic analyses at the Center for Psychopharmacology, Diakonhjemmet Hospital. For this analysis, the following variant alleles were assessed: CYP1A2, the increased induction allele **1F* (rs762551); CYP2C19, the *null* alleles **2* (rs4244285), **3* (rs4986893) and **4* (rs28399504) and the gain-of-function allele **17* (rs12248560); CYP2C9, the reduced-function alleles **2* (rs1799853) and **3* (rs1057910). Patients were divided into the following subgroups based on genotype-predicted phenotype: normal, poor, intermediate, rapid and ultrarapid metabolizer. With the exception of *CYP2C9*3*, all alleles were in Hardy-Weinberg equilibrium.

2.6 | Protein quantification in hepatic biopsies

Proteins were quantified as previously described.[28] Briefly, proteins were extracted from liver biopsies in an SDS-containing (2% w/v) lysis buffer. Samples were processed with the multi-enzyme digestion filter-aided sample preparation protocol, using LysC and trypsin.[29] Proteomics analysis was performed with a Q Exactive HF/Q Exactive HF-X MS. MS data were processed with MaxQuant (version 1.6.10.43),[30] using the human UniProtKB. Spectral raw intensities were normalized with variance stabilization[31] and were subsequently used to calculate the protein concentrations using the total protein approach.[32] The proteomics analyses were supported by the Swedish Research Council, grant numbers 5715 and 01951.

2.7 | Data and statistical analysis

Due to the exploratory nature of the present analysis, no sample size calculation has been performed.[25] Normality of data was assessed using visual inspection of plots and the Shapiro-Wilk test. Student's *t*-test on log-transformed metabolic ratios was used in the cross-sectional analysis between controls and patients with obesity at baseline. Linear mixed effects models, with a log-transformed dependent variable, were used in the longitudinal analysis to estimate within-group changes and between-group (RYGB versus diet) differences in within-group changes. The metabolic ratios were treated as the dependent variable, while visit (time), group (RYGB and diet) and their interaction (visit × group) were treated as fixed effects. To account for individual variability, the unique patient id was used as a random effect (individual intercepts). The mixed effects models were adjusted for the log-transformed introduced bias and confidence intervals were adjusted using Tukey's method. Contrasts analyses were performed for parameters of interest. Patients characterized as poor metabolizers based on genotype, ie, *CYP2C19 *2/*2* or **2/*4* (RYGB, n = 3; diet, n = 1) had a metabolic ratio of almost zero at baseline. As the ratios in patients with *CYP2C19 *2/*2* or **2/*4* are not expected to change, these patients were excluded from the longitudinal analyses. The Spearman's rank order correlation test was used to describe the rank-based measure of association between variables. The NAFLD liver fat score and liver fat content were calculated according to Kotronen et al.[33] Values of NAFLD liver fat score greater than −0.640 were indicative of NAFLD. All statistical analyses were performed using R for windows (version 3.6.2) and a *P* value <.05 was considered statistically significant.[34]

2.8 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHARBPS Guide to PHARMACOLOGY, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22.[35]

3 | RESULTS

3.1 | Patient characteristics

A total of 108 patients were included in the study (RYGB = 44, diet = 44 and controls = 20).[24] Eight patients withdrew or were excluded before the study start, and one patient was excluded from all pharmacokinetic analyses due to severe liver cirrhosis. Thus, in the present analysis, 40, 41 and 18 patients were included in the RYGB, diet and control groups, respectively. Two of the patients in the RYGB group did not undergo surgery and were therefore excluded after week 3, and 13 patients (RYGB = 4, diet = 9) withdrew or dropped out after week 9. One losartan/LCA ratio was excluded from week 0 due to bioanalytical technicalities (diet group). Also, not all included patients were able to supply metabolic ratios at all four study visits (RYGB = 4, diet = 1) due to technical difficulties.

Patient characteristics and selected clinical measures of included patients, as well as CYP1A2, CYP2C19 and CYP2C9 genotype distribution, are given in Table 1. In short, all three groups were similar according to age and ethnicity. Mean body weight at baseline did not differ substantially between the RYGB and the diet group, while, in accordance with the inclusion criteria, body weight was lower in the control group (*P* < .05; Table 1). Also, patients with obesity had higher mean liver fat content and high-sensitivity C reactive protein (hs-CRP) compared with controls (all I < .05; Table 1). Seventy-three percent of the patients had NAFLD liver fat score >−0.640 (RYGB = 36, diet = 34, control = 2), indicative of NAFLD, at baseline.

3.2 | Changes in body weight and selected clinical measures

Mean total body weight loss from baseline was similar in the RYGB and diet groups at week 3 (4.8 ± 1.2% *vs* 4.4 ± 2.0%) and at week

TABLE 1 Patient characteristics at baseline

	RYGB (n = 40)	Diet (n = 41)	Control (n = 18)
Age (years)	46 ± 9.0	49 ± 10	42 ± 15
Sex (female/male)	27/13	27/14	15/3
Ethnicity (Caucasian/other)	40/0	40/1	17/1
Body weight (kg)	132 ± 24	124 ± 23	71 ± 11
BMI (kg/m^2)	45 ± 6	42 ± 5	25 ± 3
NAFLD liver fat score	2.7 ± 2.8	2.1 ± 2.8	−1.7 ± 1.0
Liver fat content (%)	11 ± 6.2	10 ± 7.2	2.5 ± 1.3
ALAT (U/L)	34 ± 17	32 ± 18	22 ± 15
Creatinine (μmol/L)	58 ± 11	59 ± 14	60 ± 12
Albumin (g/L)	40 ± 2.2	40 ± 2.1	40 ± 2.4
Hs-CRP (mg/L)	8.2 ± 6.2	8.2 ± 9.5	2.5 ± 3.8
CYP1A2 genotype (likely phenotype)			
*1/*1 or *1/*1F (NM)	19 (48%)	19 (46%)	11 (61%)
*1F/*1F (hyperinducer)	21 (53%)	22 (54%)	7 (39%)
CYP2C19 genotype (likely phenotype)			
*1/*1 (NM)	12 (30%)	20 (49%)	8 (44%)
*17/*17 or *1/*17 (UM/RM)	15 (38%)	15 (37%)	5 (28%)
*1/*2 or *2/*17 (IM)	10 (25%)	5 (12%)	5 (28%)
*2/*2 or *2/*4 (PM)	3 (7.5%)	1 (2.4%)	0 (0.0%)
CYP2C9 genotype (likely phenotype)			
*1/*1 or *1/*2 (NM)	36 (90%)	38 (93%)	17 (94%)
*1/*3 or *2/*2 (IM)	4 (10%)	3 (7.3%)	0 (0.0%)
*3/*3 (PM)	0 (0.0%)	0 (0.0%)	1 (5.6%)

Note: Data are presented as mean ± SD or number (%). Patient characteristics at baseline are given for patients supplying at least one metabolic ratio during the study period.
Abbreviations: ALAT, alanine aminotransferase; BMI, body mass index; CYP, cytochrome P450; hs-CRP, high-sensitivity C-reactive protein; IM, intermediate metabolizer; NAFLD, nonalcoholic fatty liver disease; NM, normal metabolizer; PM, poor metabolizer; RM, rapid metabolizer; RYGB, Roux-en-Y gastric bypass; UM, ultrarapid metabolizer.

9 following either RYGB (13 ± 2.4%) or VLED (10.5 ± 3.9%). Between the 9-week and 2-year follow-ups, mean body weight decreased 19 ± 8.9% in the RYGB group and increased 9.0 ± 8.0% in the diet group (Figure 1A). Mean NAFLD liver fat score and liver fat content decreased similarly in the two groups between baseline and week 9 (Figure 1B,C). By contrast, both liver fat measures decreased further in the RYGB group between week 9 and year 2, while in the diet group these measures increased to similar values as baseline.

3.3 | Changes in metabolic ratios after RYGB, strict diet and weight loss

The results of the mixed-effects model analysis of short- and long-term within-group changes in metabolic ratios are presented in Table 2. Observed metabolic ratios for CYP1A2, CYP2C19 and CYP2C9 as well as concentrations of the probe drugs and respective metabolite are presented in Supporting Information Table S1. Individual changes in metabolic ratios during the study period are shown in Figure 2. The paraxanthine/caffeine ratio (CYP1A2) was unaltered in both groups after 3 weeks of LED (Table 2 and Figure 1D). Following RYGB, no change was observed after 6 weeks (week 9), but a 19% (95% confidence interval [CI] 4.3, 30) mean increase in this ratio was observed at year 2 compared with week 9. In the diet group, mean paraxanthine/caffeine ratio was increased by 17% (95% CI 4.3, 28) at week 9 compared with baseline, with no further change at the 2-year follow-up. No between-group difference was observed in the within-group changes (Table 3) or at any of the study visits (Supporting Information Table S2).

During the initial 3-week LED, the mean 5-OH-omeprazole/omeprazole ratio (CYP2C19) increased similarly in the RYGB (43% [95% CI 16, 55]) and diet (48% [95% CI 22, 60]) groups (Table 2 and Figure 1E). Thereafter, the mean metabolic ratio increased by an additional 30% (95% CI 2.6, 43) for 6 weeks after RYGB, whereas no further change was observed after the 6-week VLED (Figure 1E), with no between-group difference (Table 3). Two years after treatment started, the mean 5-OH-omeprazole/omeprazole ratio was higher than baseline in RYGB patients, while it was unchanged in diet

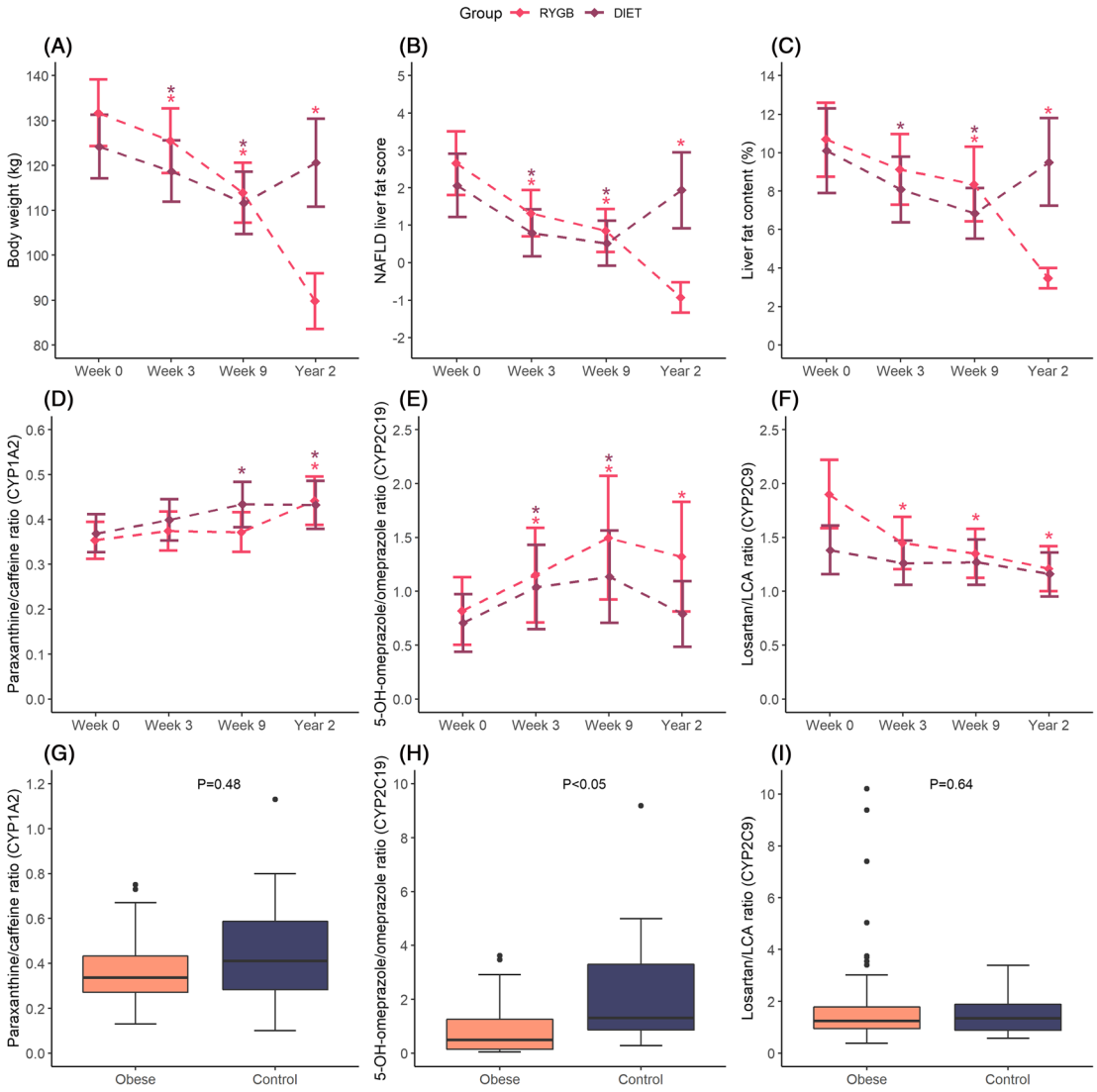

FIGURE 1 Body weight, liver measures and metabolic ratios. Changes in (A) total body weight[a], (B) NAFLD liver fat score[a], (C) liver fat content[a], (D) paraxanthine/caffeine ratio[a,b], (E) 5-OH-omeprazole/omeprazole ratio[a,b,c] and (F) losartan/LCA ratio[a,b] in the RYGB and diet groups. Linear mixed-effects models, with log-transformed dependent variables, were used to estimate mean change over time compared with baseline. Data are presented as mean (95% CI). Boxplots of the (G) paraxanthine/caffeine ratio, (H) 5-OH-omeprazole/omeprazole ratio and (I) losartan/LCA ratio[d] in patients with severe obesity (n = 81) and controls (n = 18) at baseline. Student's *t*-test on log-transformed data was used to compare patients with obesity and controls at week 0. [a]Statistically significant differences ($P < .05$) within group (RYGB or diet) compared with baseline are symbolized by *. [b]Predicted values from the mixed-effect model. [c]Patients with genotype *CYP2C19 *2/*2* or **2/*4* were excluded from the plot. [d]Due to visualization purposes, one losartan/LCA ratio in the control group (49.05) was excluded from plot i. CYP, cytochrome P450; LCA, losartan carboxylic acid; NAFLD, nonalcoholic fatty liver disease; RYGB, Roux-en-Y gastric bypass

patients (Table 2, Figures 1E and 2D), resulting in a statistically significant between-group difference (Table 3). At year 2, the mean metabolic ratio was almost 2-fold higher in the RYGB group compared with the diet group (Supporting Information Table S2).

At baseline, the mean losartan/LCA ratio (CYP2C9) was 1.4-fold higher in the RYGB group compared with the diet group (Supporting Information Table S2). The mean metabolic ratio decreased in the RYGB group at week 3 (24% [95% CI 4.2, 52] from baseline), but no further changes were observed after this study visit (Table 2). No change in the losartan/LCA ratio was observed in the diet group. Taken together, there was a statistically significant difference in within-group changes from baseline to year 2 (Table 3). However, no between-group difference in within-group changes was observed from week 3 to 9 or from week 3 to year 2.

3.4 | Effect of genotypes on changes in metabolic ratios over time

Changes in the metabolic ratios over time in different genotype subgroups are shown in Figure 3 and Supporting Information Table S3. In

TABLE 2 Short- and long-term outcomes in metabolic ratios within groups

	RYGB				
Metabolic ratio	**W0-W3**	**W0-W9**	**W0-Y2**	**W3-W9**	**W9-Y2**
Paraxanthine/caffeine (CYP1A2)	0.02 (−0.02, 0.07) *n = 39*	0.02 (−0.03, 0.06) *n = 36*	**0.09 (0.03, 0.14)** *n = 31*	−0.00 (−0.05, 0.04) *n = 36*	**0.07 (0.01, 0.13)** *n = 31*
5-OH-omeprazole/omeprazole (CYP2C19)[a]	**0.39 (0.09, 0.69)** *n = 36*	**0.79 (0.32, 1.3)** *n = 34*	**0.57 (0.18, 0.96)** *n = 28*	**0.39 (0.02, 0.77)** *n = 34*	−0.22 (−0.59, 0.16) *n = 29*
Losartan/LCA (CYP2C9)[b]	**−0.46 (−0.82, −0.09)** *n = 38*	**−0.55 (−0.91, −0.19)** *n = 36*	**−0.69 (−1.1, −0.33)** *n = 33*	−0.10 (−0.40, 0.20) *n = 36*	−0.14 (−0.43, 0.15) *n = 32*

Note: Data are presented as model estimated mean change (95% CI) between the different study visits in the RYGB group and diet group, respectively. Bold values show statistically significant differences ($P < .05$). Linear mixed-effects models, with a log-transformed dependent variable, were used to estimate mean difference in change. Not all included patients were able to supply metabolic ratios at all four study visits (RYGB = 4, diet = 1) due to technical difficulties.

Abbreviations: CYP, cytochrome P450; LCA, losartan carboxylic acid; RYGB, Roux-en-Y gastric bypass; W, week; Y, year.

[a]Patients with genotype *CYP2C19 *2/*2* or **2/*4* were excluded from the analysis.

[b]One losartan/LCA ratio was excluded from week 0 due to analytical technicalities (diet group).

TABLE 2 (Continued)

	Diet				
Metabolic ratio	**W0-W3**	**W0-W9**	**W0-Y2**	**W3-W9**	**W9-Y2**
Paraxanthine/caffeine (CYP1A2)	0.03 (−0.02, 0.08) *n = 41*	**0.06 (0.01, 0.11)** *n = 39*	**0.06 (0.01, 0.12)** *n = 30*	0.03 (−0.02, 0.09) *n = 39*	−0.00 (−0.06, 0.06) *n = 30*
5-OH-omeprazole/omeprazole (CYP2C19)[a]	**0.33 (0.10, 0.57)** *n = 39*	**0.43 (0.16, 0.70)** *n = 38*	0.08 (−0.09, 0.25) *n = 30*	0.09 (−0.13, 0.32) *n = 37*	**−0.35 (−0.61, −0.08)** *n = 30*
Losartan/LCA (CYP2C9)[b]	−0.12 (−0.39, 0.15) *n = 40*	−0.12 (−0.39, 0.16) *n = 38*	−0.23 (−0.52, −0.06) *n = 29*	0.00 (−0.26, 0.27) *n = 39*	−0.11 (−0.39, 0.17) *n = 30*

Note: Data are presented as model estimated mean change (95% CI) between the different study visits in the RYGB group and diet group, respectively. Bold values show statistically significant differences ($P < .05$). Linear mixed-effects models, with a log-transformed dependent variable, were used to estimate mean difference in change. Not all included patients were able to supply metabolic ratios at all four study visits (RYGB = 4, diet = 1) due to technical difficulties.

Abbreviations: CYP, cytochrome P450; LCA, losartan carboxylic acid; RYGB, Roux-en-Y gastric bypass; W, week; Y, year.

[a]Patients with genotype *CYP2C19 *2/*2* or **2/*4* were excluded from the analysis.

[b]One losartan/LCA ratio was excluded from week 0 due to analytical technicalities (diet group).

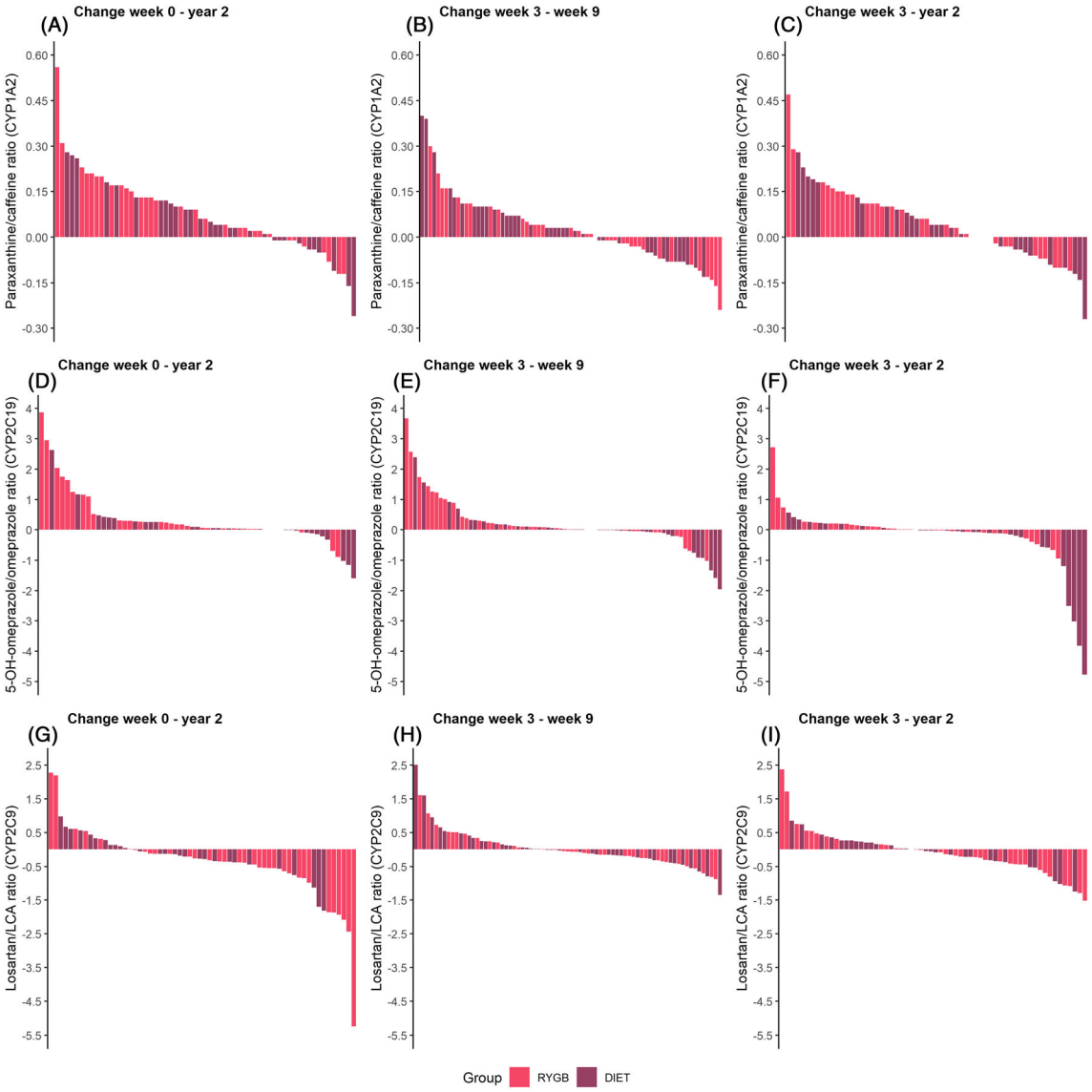

FIGURE 2 Individual variability in change in metabolic ratios between different study visits. Individual change in paraxanthine/caffeine ratio (CYP1A2) between (A) week 0 and year 2, (B) week 3 and week 9, and (C) week 3 and year 2 in 5-OH-omeprazole/omeprazole ratio (CYP2C19)[a] between (D) week 0 and year 2, (E) week 3 and week 9, and (F) week 3 and year 2, and losartan/LCA ratio (CYP2C9) between (G) week 0 and year 2, (H) week 3 and week 9, and (I) week 3 and year 2. Each bar represents the change within each patient. Note that the *y* axis range is different for each probe drug. [a]Patients with genotype *CYP2C19 *2/*2* or **2/*4* were excluded from the plot. CYP, cytochrome P450; LCA, losartan carboxylic acid; RYGB, Roux-en-Y gastric bypass

general, the metabolic ratios were in agreement with respective genotype subgroups. CYP2C19 ultrarapid (*CYP2C19*17/*17*) and rapid (*CYP2C19*1/*17*) metabolizers exhibited a numerically larger increase in mean 5-OH-omeprazole/omeprazole ratio during the study period compared with normal (*CYP2C19*1/*1*) and intermediate metabolizers (*CYP2C19*1/*2* or **2/*17*), with the largest increase observed in RYGB patients. Also, the decrease in this metabolic ratio from week 9 to year 2 in the diet group was only present in CYP2C19 ultrarapid or rapid metabolizers. In the RYGB group, intermediate metabolizers of CYP2C9 (*CYP2C9*1/*3* or **2/*2*) exhibited a numerically larger increase in CYP2C9 activity compared with normal metabolizers (*CYP2C9*1/*1* or **1/*2*).

3.5 | Comparison with normal to overweight controls

The mean 5-OH-omeprazole/omeprazole ratio (CYP2C19) was 2.7-fold higher in normal to overweight controls compared with patients with severe obesity at baseline ($I < .05$), whereas no

TABLE 3 Between-group differences in within-group changes in metabolic ratios

Metabolic ratio	RYGB versus diet		
	W0-Y2	**W3-W9**	**W3-Y2**
Paraxanthine/caffeine (CYP1A2)	−0.02 (−0.08, 0.03) *n* = *61*	0.04 (−0.02, 0.09) *n* = *75*	−0.03 (−0.09, 0.03) *n* = *61*
5-OH-omeprazole/omeprazole (CYP2C19)[a]	**−0.49 (−0.81, −0.17)** *n* = *58*	−0.30 (−0.63, 0.03) *n* = *71*	**−0.43 (−0.74, −0.12)** *n* = *57*
Losartan/LCA (CYP2C9)[b]	**0.47 (0.11, 0.82)** *n* = *62*	0.10 (−0.20, 0.41) *n* = *75*	0.13 (−0.18, 0.44) *n* = *62*

Note: Data are presented as model estimated mean difference in change (95% CI). Bold values show statistically significant differences ($P < .05$). Linear mixed-effects models, with a log-transformed dependent variable, were used to estimate between-group differences in within-group changes, with diet as reference group. Not all included patients were able to supply metabolic ratios at all four study visits (RYGB = 4, diet = 1) due to technical difficulties.
Abbreviations: CYP, cytochrome P450; LCA, losartan carboxylic acid; W, week; Y, year.
[a]Patients with genotype *CYP2C19 *2/*2* or **2/*4* were excluded from the analysis.
[b]One losartan/LCA ratio was excluded from week 0 due to analytical technicalities (diet group).

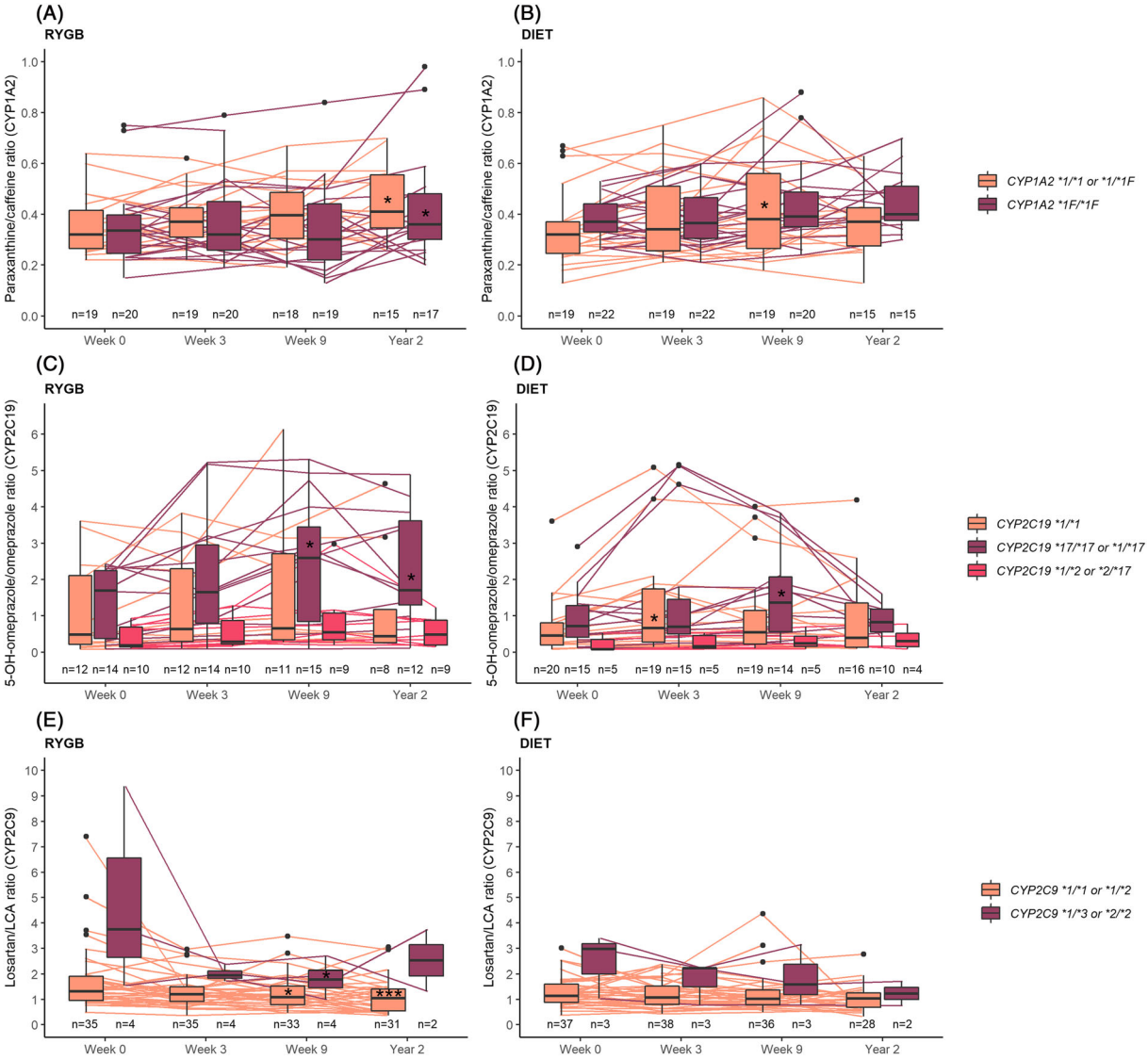

FIGURE 3 Changes in metabolic ratios during the study period based on genotype. Combined boxplots and individual plots of the ratio of (A, B) paraxanthine/caffeine, (C, D) 5-OH-omeprazole/omeprazole[a] and (E, F) losartan/LCA in the RYGB and diet groups, respectively, at the four study visits. [a]Patients with genotype *CYP2C19 *2/*2* or **2/*4* were excluded from the plot. CYP, cytochrome P450; LCA, losartan carboxylic acid; RYGB, Roux-en-Y gastric bypass

difference was observed in the ratio of paraxanthine/caffeine (CYP1A2) or losartan/LCA (CYP2C9) (Figure 1G-I).

3.6 | Association between hepatic CYP concentrations, metabolic ratios and clinical variables

There was a moderate association between hepatic CYP1A2, CYP2C19 and CYP2C9 concentrations and their respective metabolic ratios (Figure 4). Body weight was inversely associated with the ratio of 5-OH-omeprazole/omeprazole at baseline ($\rho = -0.24$, $P < .05$). Liver fat content was also negatively associated with the metabolic ratio for CYP2C19 activity ($\rho = -0.57$, $P < .05$). In the subset of patients undergoing RYGB or cholecystectomy, an inverse association was also observed between body weight and hepatic CYP1A2 concentration at the time of surgery ($\rho = -0.27$, $P = .05$) (RYGB, week 3; control, week 0). Detailed results are presented in Supporting Information Table S4. Finally, mean 5-OH-omeprazole/omeprazole ratio was 60% lower in patients with NAFLD liver fat score indicative of NAFLD ($P < .05$). There was no difference in the paraxanthine/caffeine or losartan/LCA ratios ($P = .81$ and $P = .48$, respectively).

4 | DISCUSSION

The main findings of this three-armed study are (i) CYP2C19 (omeprazole)-mediated metabolism is lower in patients with obesity compared with normal to overweight controls and (ii) CYP2C19-mediated metabolism increased following weight loss. By contrast, body weight, RYGB and weight loss did not impact CYP1A2 (caffeine)- and CYP2C9 (losartan)-mediated metabolism to any clinically relevant degree.

Our results provide evidence that CYP2C19-mediated metabolism is lower in patients with obesity and increases after weight loss. Previous literature on this topic is sparse, but studies have showed an unaltered or increased metabolism of CYP2C19 substrates in patients with obesity compared with nonobese individuals.[36,37] However, these drugs are not validated as probe drugs for CYP2C19 activity. We also show that CYP2C19 activity was lower in patients with NAFLD liver fat score indicative of NAFLD. In line with this, Fisher et al have also shown a decreased protein expression and enzymatic activity of CYP2C19 with progressive states of NAFLD.[38] We therefore suggest that the large reduction in liver fat content after RYGB or VLED may be an important mechanism for the increased CYP2C19-mediated metabolism following a moderate weight loss. Furthermore, we observed that the 5-OH-omeprazole/omeprazole ratio increased 6 weeks after RYGB (weeks 3-9), whereas no mean change was observed in diet patients in the same period despite a similar weight loss in the two groups. Considering the anatomical changes in the gastrointestinal tract following surgery, it is not unlikely that the single timepoint metabolic ratio to some degree has been influenced by, for example, alterations in the absorption, hence partly explaining the additional effect observed in the RYGB group. Nevertheless, an increased CYP2C19 activity after bariatric surgery is supported by two previous studies, which both observed a significantly lower systemic exposure of omeprazole 1 and 6 months, and approximately 2 months after RYGB, respectively.[39,40] Other studies have not provided evidence of an altered CYP2C19-mediated metabolism after bariatric surgery.[41,42] However, these studies had small sample sizes, lacked a proper control group and had less granular follow-up. Also, in an ex vivo activity assay of hepatic biopsies in a subset of the patients included in the present study, CYP2C19 activity was not associated with varying body mass index (BMI).[43] These inconsistent findings may be an effect of the variable enrichment of CYPs in the human liver microsomes used for the ex vivo activity assay.[44]

CYP1A2-mediated metabolism was not significantly different in patients with obesity and normal to overweight controls, which is in

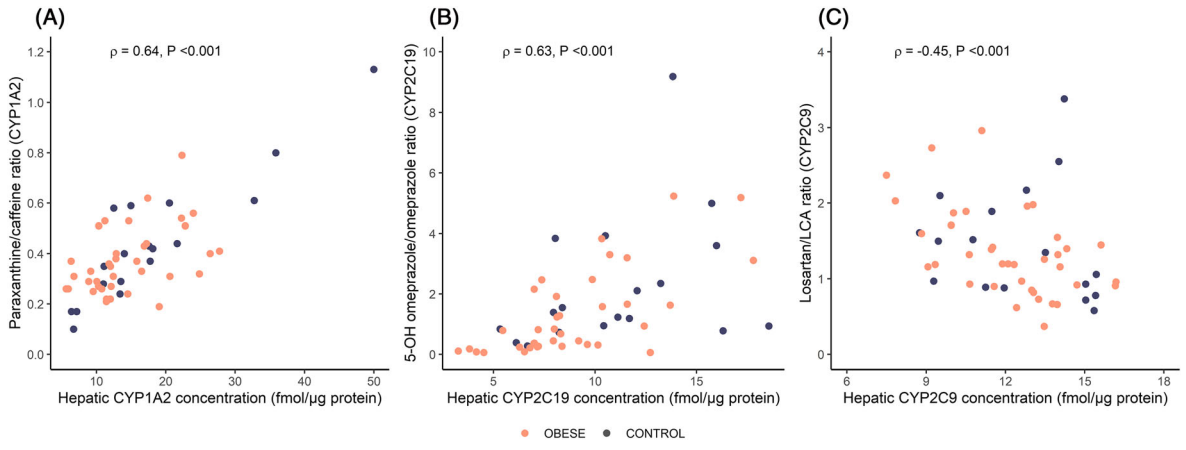

FIGURE 4 Association between hepatic (A) CYP1A2, (B) CYP2C19 and (C) CYP2C9 concentrations and respective metabolic ratios at the time of surgery for patients subjected to RYGB (week 3) or cholecystectomy (week 0). Spearman's rho (ρ) is the correlation coefficient. *P* values are from the Spearman rank correlation analysis. CYP, cytochrome P450; LCA, losartan carboxylic acid. Two patients were not able to supply metabolic ratios (RYGB = 2) due to technical difficulties. Due to visualization purposes, one losartan/LCA ratio in the control group (49.05) was excluded from plot (C)

line with previous studies.[13,14,45] Although clearance by CYP1A2 seems to be similar in individuals with different body weights, CYP1A2-mediated metabolism increased from baseline to week 9 in the diet group. This was not observed in the RYGB group, despite a similar weight loss, possibly due to unknown surgery-specific effects counteracting the true effect of weight loss on CYP1A2-mediated metabolism. This is supported by Rodriguez et al, who actually found a lower CYP1A2 activity 4 weeks after bariatric surgery, which then was recovered after 6 months.[13] In another study by Puris et al, an increased CYP1A2-mediated metabolism was observed 1 year after bariatric surgery,[42] which is in line with our findings in the RYGB group long term (year 2). Pro-inflammatory cytokines such as IL-6 have been reported to decrease the expression of CYP1A2 enzymes, and may explain the results long term as the inflammation status was lower in the RYGB group at year 2 compared with baseline.[46,47] However, we also observed a lack of association between body weight and CYP1A2 activity in this study. This has previously been described in the ex vivo activity assay of hepatic biopsies in a subset of the patients included in the present study.[43] Given the inverse association, although weak, between body weight and hepatic CYP1A2 expression, it is plausible that the increased activity following substantial weight loss long term is due to an increase in CYP1A2 expression. As the CYP1A2-mediated metabolism remained higher in the diet group at year 2 despite regained body weight, it may be hypothesized that the effect of weight loss is of more significance on CYP1A2 activity than body weight per se.

The lack of association between body weight and both CYP2C9 activity and hepatic CYP2C9 concentrations suggests that CYP2C9-mediated metabolism is not influenced by body weight or RYGB. In the present study, RYGB patients exhibited a higher CYP2C9 activity at baseline compared with diet patients, which was not expected. This difference diminished over time as CYP2C9-mediated metabolism decreased significantly in the RYGB group after 3 weeks of LED, before stabilizing, whereas no changes were seen in the diet group. Other studies have not provided evidence of an altered CYP2C9-mediated metabolism in different body weight ranges or following bariatric surgery.[13,42,45]

It is well known that genetic polymorphism is of significant importance for the interindividual variability in CYP2C19 and CYP2C9 activity.[5] We observed that changes in CYP-mediated metabolism after surgical or nonsurgical calorie restriction to a certain degree were dependent on genotype. Statistically significant changes in CYP2C19-mediated metabolism over time were mainly present in ultrarapid and rapid metabolizers. This may suggest that individuals carrying the *CYP2C19*17* genetic variant are more susceptible to changes in CYP2C19 activity with fluctuations in body weight. As expected, no significant changes in metabolic ratios were observed in poor metabolizers of CYP2C19 and CYP2C9, but considering the small number of patients in this category we should be careful to conclude.

Major strengths of this study include large sample size, both short-term and long-term study investigations, and genotyping of all patients. The inclusion of proteomics data also strengthens the findings. By inclusion of a dietary control group (with a short-term weight loss comparable to that in patients subjected to RYGB), we were able to separate the surgery effect from the weight loss effect. The results of this study should, however, be interpreted in light of its important limitations. The single timepoint metabolic ratios used in the present study do not provide information about all the pharmacokinetic changes of the probe drugs following RYGB or weight loss. The difference in both absorption rate and extent is common following RYGB[14,40,41,48–51] and could also differ between normal-weight individuals and patients with obesity.[8,12] The single timepoint metabolic ratio used in the present study has not been validated in patients with obesity nor following RYGB. Hence, we cannot rule out that changes following weight loss or RYGB have influenced the metabolic ratios. This is especially relevant for omeprazole, which displays a complex absorption profile, and previous studies have shown both an increased and a decreased absorption of omeprazole following RYGB.[39,40] Presuming that the expression of CYP2C19 also reflects CYP2C19 activity, the findings in this study support that the 3-hour 5-OH-omeprazole/omeprazole ratio actually reflects CYP2C19-mediated metabolism. Second, in addition to the CYP2C19-mediated metabolism, omeprazole is also, to a lesser extent, metabolized by CYP3A4.[52] A metabolic shift in the metabolism of omeprazole, however, was not suspected in the present study as we have previously shown that CYP3A activity is not significantly altered after RYGB or weight loss in the early period after surgery.[24] Finally, we have used surrogate measures of liver fat content, which may underestimate the effect of RYGB and weight loss on NAFLD early after surgery.

In conclusion, this study showed that CYP2C19-mediated metabolism was lower in patients with severe obesity compared with normal to overweight controls, and increased after weight loss. By contrast, body weight, RYGB and weight loss did not impact CYP1A2- and CYP2C9-mediated metabolism to any clinically relevant degree. The effect on CYP2C19-mediated metabolism may be of clinical importance for dosing of drugs with clearance primarily dependent on CYP2C19.

ACKNOWLEDGMENTS

The authors would like to thank the participants, the surgical staff and the study personnel working on the COCKTAIL study at Vestfold Hospital Trust. The authors also thanks the Swedish Research Council, approval numbers 5715 and 01951 (C.W., T.B.A. and P.A.) for supporting the proteomics analyses. Vestfold Hospital Trust, Norway; Department of Pharmacy, University of Oslo, Norway; and AstraZeneca, Sweden. The Swedish Research Council, grant numbers 5715 and 01951 supported the proteomics analyses.

COMPETING INTERESTS

S.A., M.H., C.K. and R.J.-L. are AstraZeneca employees and own shares in AstraZeneca, while C.W. and T.B.A. are former AstraZeneca employees. K.E.K., I.R., E.S., H.C., V.K., M.K.K., M.H.H., J.K.H., L.K.J., R.S., P.A., J.H. and A.Å. have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

J.H., A.Å., S.A., C.K., T.B.A., H.C., E.S., and R.S. designed the study. I.R., V.K., L.K.J., M.K.K., J.K.H., P.A., R.J.L, and C.W. performed the research. K.E.K., I.R., E.S., and M.H.H. analyzed the data. K.E.K. and I. R. wrote the manuscript. All authors contributed to critically reviewing the manuscript and gave their final approval for submission.

DATA AVAILABILITY STATEMENT

Access to data collected from this study, including anonymized individual participant data, may potentially be made available following publication on e-mail request to the corresponding author. After approval of a proposal, data will be shared with investigators whose proposed use of the data has been approved by the COCKTAIL steering committee, according to the consent given by the participants and Norwegian laws and legislations.

ORCID

Kine Eide Kvitne https://orcid.org/0000-0001-8118-7660
Christine Wegler https://orcid.org/0000-0002-2810-7518
Markus Herberg Hovd https://orcid.org/0000-0002-6077-0934
Anders Åsberg https://orcid.org/0000-0002-0628-1769
Ida Robertsen https://orcid.org/0000-0001-9401-7716

REFERENCES

1. Williams JA, Hyland R, Jones BC, et al. Drug-drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUCi/AUC) ratios. *Drug Metab Dispos*. 2004;32(11):1201-1208. doi:10.1124/dmd.104.000794
2. Rendic S, Guengerich FP. Survey of human oxidoreductases and cytochrome P450 enzymes involved in the metabolism of xenobiotic and natural chemicals. *Chem Res Toxicol*. 2015;28(1):38-42. doi:10.1021/tx500444e
3. Achour B, Barber J, Rostami-Hodjegan A. Expression of hepatic drug-metabolizing cytochrome p450 enzymes and their intercorrelations: a meta-analysis. *Drug Metab Dispos*. 2014;42(8):1349-1356. doi:10.1124/dmd.114.058834
4. Paine MF, Hart HL, Ludington SS, Haining RL, Rettie AE, Zeldin DC. The human intestinal cytochrome P450 "pie". *Drug Metab Dispos*. 2006;34(5):880-886. doi:10.1124/dmd.105.008672
5. Zanger UM, Schwab M. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther*. 2013;138(1):103-141. doi:10.1016/j.pharmthera.2012.12.007
6. Morgan ET, Goralski KB, Piquette-Miller M, et al. Regulation of drug-metabolizing enzymes and transporters in infection, inflammation, and cancer. *Drug Metab Dispos*. 2008;36(2):205-216. doi:10.1124/dmd.107.018747
7. Brill MJ, Diepstraten J, van Rongen A, van Kralingen S, van den Anker JN, Knibbe CAJ. Impact of obesity on drug metabolism and elimination in adults and children. *Clin Pharmacokinet*. 2012;51(5):277-304. doi:10.2165/11599410-000000000-00000
8. Brill MJ, van Rongen A, Houwink API, et al. Midazolam pharmacokinetics in morbidly obese patients following semi-simultaneous oral and intravenous administration: a comparison with healthy volunteers. *Clin Pharmacokinet*. 2014;53(10):931-941. doi:10.1007/s40262-014-0166-x
9. Abernethy DR, Greenblatt DJ, Divoll M, Smith RB, Shader RI. The influence of obesity on the pharmacokinetics of oral alprazolam and triazolam. *Clin Pharmacokinet*. 1984;9(2):177-183. doi:10.2165/00003088-198409020-00005
10. Wellen KE, Hotamisligil GS. Obesity-induced inflammatory changes in adipose tissue. *J Clin Invest*. 2003;112(12):1785-1788. doi:10.1172/JCI20514
11. Machado M, Marques-Vidal P, Cortez-Pinto H. Hepatic histology in obese patients undergoing bariatric surgery. *J Hepatol*. 2006;45(4):600-606. doi:10.1016/j.jhep.2006.06.013
12. Smit C, de Hoogd S, Brüggemann RJM, Knibbe CAJ. Obesity and drug pharmacology: a review of the influence of obesity on pharmacokinetic and pharmacodynamic parameters. *Expert Opin Drug Metab Toxicol*. 2018;14(3):275-285. doi:10.1080/17425255.2018.1440287
13. Rodríguez-Morató J, Goday A, Langohr K, et al. Short- and medium-term impact of bariatric surgery on the activities of CYP2D6, CYP3A4, CYP2C9, and CYP1A2 in morbid obesity. *Sci Rep*. 2019;9(1):20405. doi:10.1038/s41598-019-57002-9
14. Goday Arno A, Farré M, Rodríguez-Morató J, et al. Pharmacokinetics in morbid obesity: influence of two bariatric surgery techniques on paracetamol and caffeine metabolism. *Obes Surg*. 2017;27(12):3194-3201. doi:10.1007/s11695-017-2745-z
15. Koonrungsesomboon N, Khatsri R, Wongchompoo P, Teekachunhatean S. The impact of genetic polymorphisms on CYP1A2 activity in humans: a systematic review and meta-analysis. *Pharmacogenomics J*. 2018;18(6):760-768. doi:10.1038/s41397-017-0011-3
16. Apovian CM, Aronne LJ, Bessesen DH, et al. Pharmacological management of obesity: an endocrine Society clinical practice guideline. *J Clin Endocrinol Metab*. 2015;100(2):342-362. doi:10.1210/jc.2014-3415
17. American College of Cardiology/American Heart Association Task Force on Practice Guidelines. O.E.P. 2013, Expert Panel Report: Guidelines (2013) for the management of overweight and obesity in adults. *Obesity (Silver Spring)*. 2014;22(Suppl 2):S41-S410.
18. Garvey WT, Mechanick JI, Brett EM, et al. American Accociation of Clinical Endocrinologists and American College of Endocrinology comprehensive clinical practice guidelines for medical care of patients with obesity. *Endocr Pract*. 2016;22(Suppl 3):1-203. doi:10.4158/EP161365.GL
19. Colquitt JL, Pickett K, Loveman E, Frampton GK, Cochrane Metabolic and Endocrine Disorders Group. Surgery for weight loss in adults. *Cochrane Database Syst Rev*. 2014;(8):Cd003641. doi:10.1002/14651858.CD003641.pub4
20. Jakobsen GS, Småstuen MC, Sandbu R, et al. Association of bariatric surgery vs medical obesity treatment with long-term medical complications and obesity-related comorbidities. *JAMA*. 2018;319(3):291-301. doi:10.1001/jama.2017.21055
21. Angeles PC, Robertsen I, Seeberg LT, et al. The influence of bariatric surgery on oral drug bioavailability in patients with obesity: A systematic review. *Obes Rev*. 2019;20(9):1299-1311. doi:10.1111/obr.12869
22. Rao SR. Inflammatory markers and bariatric surgery: a meta-analysis. *Inflamm Res*. 2012;61(8):789-807. doi:10.1007/s00011-012-0473-3
23. Schwenger KJP, Fischer SE, Jackson T, Okrainec A, Allard JP. In nonalcoholic fatty liver disease, Roux-en-Y gastric bypass improves liver histology while persistent disease is associated with lower improvements in waist circumference and glycemic control. *Surg Obes Relat Dis*. 2018;14(9):1233-1239. doi:10.1016/j.soard.2018.06.007
24. Kvitne KE, Robertsen I, Skovlund E, et al. Short- and long-term effects of body weight loss following calorie restriction and gastric bypass on CYP3A-activity – a non-randomized three-armed controlled trial. *Clin Transl Sci*. 2021;15(1):221-233. doi:10.1111/cts.13142
25. Hjelmesæth J, Åsberg A, Andersson S, et al. Impact of body weight, low energy diet and gastric bypass on drug bioavailability, cardiovascular risk factors and metabolic biomarkers: protocol for an open,

non-randomised, three-armed single centre study (COCKTAIL). *BMJ Open*. 2018;8(5):e021878. doi:10.1136/bmjopen-2018-021878

26. Krogstad V, Peric A, Robertsen I, et al. Correlation of body weight and composition with hepatic activities of cytochrome P450 enzymes. *J Pharm Sci*. 2021;110(1):432-437. doi:10.1016/j.xphs.2020.10.027
27. Christensen M, Andersson K, Dalén P, et al. The Karolinska cocktail for phenotyping of five human cytochrome P450 enzymes. *Clin Pharmacol Ther*. 2003;73(6):517-528. doi:10.1016/S0009-9236(03)00050-X
28. Wegler C, Ölander M, Wiśniewski JR, et al. Global variability analysis of mRNA and protein concentrations across and within human tissues. *NAR Genom Bioinf*. 2019;2(1):lqz010. doi:10.1093/nargab/lqz010
29. Wiśniewski JR, Mann M. Consecutive proteolytic digestion in an enzyme reactor increases depth of proteomic and phosphoproteomic analysis. *Anal Chem*. 2012;84(6):2631-2637. doi:10.1021/ac300006b
30. Tyanova S, Temu T, Cox J. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nat Protoc*. 2016;11(12):2301-2319. doi:10.1038/nprot.2016.136
31. Huber W, von Heydebreck A, Sultmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics*. 2002;18(Suppl 1):S96-S104. doi:10.1093/bioinformatics/18.suppl_1.S96
32. Wiśniewski JR, Rakus D. Multi-enzyme digestion FASP and the 'Total Protein Approach'-based absolute quantification of the *Escherichia coli* proteome. *J Proteomics*. 2014;109:322-331. doi:10.1016/j.jprot.2014.07.012
33. Kotronen A, Peltonen M, Hakkarainen A, et al. Prediction of non-alcoholic fatty liver disease and liver fat using metabolic and genetic factors. *Gastroenterology*. 2009;137(3):865-872. doi:10.1053/j.gastro.2009.06.005
34. R Foundation for Statistical Computing, *R: A Language and Environment for Statistical Computing* 2018, Vienna, Austria.
35. Alexander SP, Fabbro D, Kelly E, et al. The Concise Guide to PHARMACOLOGY 2021/22: Enzymes. *Br J Pharmacol*. 2021;178(Suppl 1):S313-s411. doi:10.1111/bph.15542
36. Abernethy DR, Greenblatt DJ, Divoll M, Shader RI. Prolongation of drug half-life due to obesity: studies of desmethyldiazepam (clorazepate). *J Pharm Sci*. 1982;71(8):942-944. doi:10.1002/jps.2600710827
37. Abernethy DR, Greenblatt DJ, Divoll M, Harmatz JS, Shader RI. Alterations in drug distribution and clearance due to obesity. *J Pharmacol Exp Ther*. 1981;217(3):681-685.
38. Fisher CD, Lickteig AJ, Augustine LM, et al. Hepatic cytochrome P450 enzyme alterations in humans with progressive stages of non-alcoholic fatty liver disease. *Drug Metab Dispos*. 2009;37(10):2087-2094. doi:10.1124/dmd.109.027466
39. Portolés-Pérez A, Paterna ABR, Sánchez Pernaute A, et al. Effect of obesity and Roux-en-Y gastric surgery on omeprazole pharmacokinetics. *Obes Facts*. 2022;15(2):271-280. doi:10.1159/000521570
40. Mitrov-Winkelmolen L, van Buul-Gast MCW, Swank DJ, et al.. The effect of Roux-en-Y gastric bypass surgery in morbidly obese patients on pharmacokinetics of (acetyl)salicylic acid and omeprazole: the ERY-PAO study. *Obes Surg*. 2016;26(9):2051-2058. doi:10.1007/s11695-016-2065-8
41. Tandra S, Chalasani N, Jones DR, Mattar S, Hall SD, Vuppalanchi R. Pharmacokinetic and pharmacodynamic alterations in the Roux-en-Y gastric bypass recipients. *Ann Surg*. 2013;258(2):262-269. doi:10.1097/SLA.0b013e31827a0e82
42. Puris E, Pasanen M, Ranta VP, et al. Laparoscopic Roux-en-Y gastric bypass surgery influenced pharmacokinetics of several drugs given as a cocktail with the highest impact observed for CYP1A2, CYP2C8 and CYP2E1 substrates. *Basic Clin Pharmacol Toxicol*. 2019;125(2):123-132. doi:10.1111/bcpt.13234
43. Krogstad V, Peric A, Robertsen I, et al. Correlation of body weight and composition with hepatic activities of cytochrome P450 enzymes. *J Pharm Sci*. 2020;110(1):432-437. doi:10.1016/j.xphs.2020.10.027
44. Wegler C, Matsson P, Krogstad V, et al. Influence of proteome profiles and intracellular drug exposure on differences in CYP activity in donor-matched human liver microsomes and hepatocytes. *Mol Pharm*. 2021;18(4):1792-1805. doi:10.1021/acs.molpharmaceut.1c00053
45. Sandvik P, Lydersen S, Hegstad S, Spigset O. Association between low body weight and cytochrome P-450 enzyme activity in patients with anorexia nervosa. *Pharmacol Res Perspect*. 2020;8(3):e00615. doi:10.1002/prp2.615
46. Dickmann LJ, Patel SK, Rock DA, Wienkers LC, Slatter JG. Effects of interleukin-6 (IL-6) and an anti-IL-6 monoclonal antibody on drug-metabolizing enzymes in human hepatocyte culture. *Drug Metab Dispos*. 2011;39(8):1415-1422. doi:10.1124/dmd.111.038679
47. Klein M, Thomas M, Hofmann U, Seehofer D, Damm G, Zanger UM. A systematic comparison of the impact of inflammatory signaling on absorption, distribution, metabolism, and excretion gene expression and activity in primary human hepatocytes and HepaRG cells. *Drug Metab Dispos*. 2015;43(2):273-283. doi:10.1124/dmd.114.060962
48. McLachlan LA, Chaar BB, Um IS. Pharmacokinetic changes post-bariatric surgery: A scoping review. *Obes Rev*. 2020;21(5):e12988. doi:10.1111/obr.12988
49. Brill MJ, van Rongen A, van Dongen EP, et al. The pharmacokinetics of the CYP3A substrate midazolam in morbidly obese patients before and one year after bariatric surgery. *Pharm Res*. 2015;32(12):3927-3936. doi:10.1007/s11095-015-1752-9
50. Chan LN, Lin YS, Tay-Sontheimer JC, et al. Proximal Roux-en-Y gastric bypass alters drug absorption pattern but not systemic exposure of CYP3A4 and P-glycoprotein substrates. *Pharmacotherapy*. 2015;35(4):361-369. doi:10.1002/phar.1560
51. Lloret-Linares C, Hirt D, Bardin C, et al. Effect of a Roux-en-Y gastric bypass on the pharmacokinetics of oral morphine using a population approach. *Clin Pharmacokinet*. 2014;53(10):919-930. doi:10.1007/s40262-014-0163-0
52. Ishizaki T, Horai Y. Review article: cytochrome P450 and the metabolism of proton pump inhibitors--emphasis on rabeprazole. *Aliment Pharmacol Ther*. 1999;13(Suppl 3):27-36. doi:10.1046/j.1365-2036.1999.00022.x

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Kvitne KE, Krogstad V, Wegler C, et al. Short- and long-term effects of body weight, calorie restriction and gastric bypass on CYP1A2, CYP2C19 and CYP2C9 activity. *Br J Clin Pharmacol*. 2022;1-13. doi:10.1111/bcp.15349

Supplementary Information

Short- and long-term effects of body weight, calorie restriction, and gastric bypass on CYP1A2-, CYP2C19-, and CYP2C9 activity

British Journal of Clinical Pharmacology

Kine Eide Kvitne[1], Veronica Krogstad[1], Christine Wegler[2,3], Line Kristin Johnson[4], Marianne K Kringen[5,6], Markus Herberg Hovd[1], Jens K. Hertel[4], Maria Heijer[7], Rune Sandbu[4,8], Eva Skovlund[9], Per Artursson[10], Cecilia Karlsson[11,12], Shalini Andersson[13], Tommy B. Andersson[3], Jøran Hjelmesæth[4,14], Anders Åsberg[1,15], Rasmus Jansson-Löfmark[3], Hege Christensen[1], Ida Robertsen[1]

CORRESPONDING AUTHOR

Kine Eide Kvitne

Section for Pharmacology and Pharmaceutical Biosciences, Department of Pharmacy, University of Oslo, Norway

E-mail: k.e.kvitne@farmasi.uio.no

METHODS

Study visits and procedures

On the pharmacokinetic investigational days, patients met at the clinic at 07.30 a.m. for insertion of a peripheral venous catheter and blood sampling. Thereafter, 100 mg oral caffeine was administered at 08.00 a.m., followed by 25 mg oral losartan and 20 mg oral omeprazole one hour later. Concomitant drug therapy was considered for each participant, and drugs considered to influence the pharmacokinetics of the probe drugs were discontinued seven half-lives before all pharmacokinetic investigations. Food items with potential drug-interfering effects (e.g. grapefruit, pomelo, and St. John's wort) were not allowed three weeks prior to the pharmacokinetic investigations. The patients abstained from food and all other drugs from 22.00 pm the evening before. Caffeine was not allowed from 36 hours before and through four hours after caffeine administration. Blood samples were collected three and four hours after omeprazole and caffeine administration to determine the 3-hour and 4-hour metabolic ratio of the respective probe drug. The blood samples were drawn in K2-EDTA vacutainer tubes on ice and centrifuged for 10 minutes at 4ºC (1,800 g). Plasma was separated into Cryovials and frozen within one hour at -70ºC until analysis. Urine was collected in a container for eight hours to determine the 8-hour urinary metabolic ratio of losartan. The total volume of urine collected was recorded and an aliquot of 10 mL urine was frozen immediately at -70 ºC.

Bioanalytical assays

Plasma concentrations of caffeine, paraxanthine, omeprazole, and 5-OH-omeprazole as well as urinary concentration of losartan and losartan carboxylic acid (LCA) were determined by Covance Laboratories (Madison, Wisconsin, USA) using validated liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) methods. All methods demonstrated acceptable precision, accuracy and selectivity for the analytes in the appropriate matrices. The results from the in-study quality control (QC) samples and calibration standards were evaluated, and it was concluded that all three methods performed acceptably for this study. For both caffeine and paraxanthine, the calibration curve ranged from 25 to 20 000 ng/mL. The inter-run precisions, assessed as coefficient of variations (CV), were <4.6% and <4.5% for caffeine and paraxanthine, respectively. The inter-run accuracies for the study QC samples

ranged between 94.7% and 98.7% for caffeine and between 96.7% and 100.0% for paraxanthine. The calibration curve of losartan and LCA ranged from 20 to 20 000 nmol/L and 10 to 10 000 nmol/L, respectively. For losartan, the inter-run precision was <9.8% and inter-run accuracies varied between 95.6% and 103.0%, and for LCA, the inter-run precision was <10.6% and inter-run accuracies were between 96.9 and 100.2%. In the omeprazole and 5-OH-omeprazole assay, the calibration curve ranged from 0.500 to 1000 ng/mL for both analytes. Inter-run precisions were <9.8% and <14.9% and inter-run accuracies were between 96.9% and 101.2% and 98.0% and 102.4% for omeprazole and 5-OH-omeprazole, respectively.

Clinical chemistry analyses

Plasma concentrations of high sensitivity C-reactive protein (hs-CRP) were measured using immunoturbidimetry (Advia Chemistry XPT systems, Siemens) at Fürst Medical Laboratory, Oslo, Norway. Clinical chemistry analyses were performed in fresh blood samples at the Department of Laboratory Medicine, Vestfold Hospital Trust, Tønsberg, Norway.

RESULTS

Table S1. Observed metabolic ratios, and concentrations of the probe drugs and respective metabolite at the study visits in the RYGB-, diet- and control group, respectively. Data are presented as mean ± SD.

	RYGB				DIET				CONTROL
Metabolic ratio	**Week 0**	**Week 3**	**Week 9**	**Year 2**	**Week 0**	**Week 3**	**Week 9**	**Year 2**	**Week 0**
	n=39	*n=39*	*n=37*	*n=32*	*n=41*	*n=41*	*n=39*	*n=30*	*n=18*
Paraxanthine/caffeine (CYP1A2)	0.36 ± 0.14	0.38 ± 0.14	0.37 ± 0.16	0.44 ± 0.18	0.37 ± 0.12	0.40 ± 0.14	0.44 ± 0.18	0.41 ± 0.13	0.44 ± 0.25
Paraxanthine (ng/mL)	492 ± 183	502 ± 161	621 ± 363	618 ± 254	468 ± 123	518 ± 189	622 ± 388	540 ± 177	774 ± 452
Caffeine (ng/mL)	1467 ± 542	1399 ± 377	1742 ± 737	1516 ± 625	1394 ± 545	1398 ± 479	1501 ± 708	1386 ± 481	2103 ± 1566
	n=39	*n=39*	*n=37*	*n=32*	*n=41*	*n=40*	*n=39*	*n=30*	*n=18*
5-OH-omeprazole/ omeprazole (CYP2C19) [a]	0.95 ± 1.0	1.3 ± 1.4	1.7 ± 1.7	1.4 ± 1.5	0.72 ± 0.78	1.2 ± 1.5	1.1 ± 1.2	0.84 ± 0.91	2.2 ± 2.2
5-OH-omeprazole (ng/mL) [a]	82 ± 48	102 ± 49	89 ± 35	58 ± 18	106 ± 57	99 ± 52	86 ± 33	80 ± 39	106 ± 57
Omeprazole (ng/mL) [a]	249 ± 231	232 ± 206	174 ± 168	162 ± 167	309 ± 211	225 ± 195	203 ± 174	216 ± 193	105 ± 108
	n=39	*n=39*	*n=37*	*n=33*	*n=40*	*n=41*	*n=39*	*n=30*	*n=18*
Losartan/LCA (CYP2C9) [b]	2.2 ± 2.2	1.4 ± 0.62	1.3 ± 0.70	1.2 ± 0.80	1.4 ± 0.70	1.2 ± 0.57	1.3 ± 0.80	1.1 ± 0.51	4.1 ± 11
Losartan (nmol/L) [b]	1239 ± 958	1039 ± 698	2585 ± 2377	831 ± 797	1049 ± 667	748 ± 443	839 ± 557	879 ± 715	1163 ± 758
LCA (nmol/L) [b]	865 ± 783	853 ± 616	2057 ± 1610	844 ± 906	912 ± 730	655 ± 359	752 ± 424	866 ± 580	883 ± 623

Abbreviations: CYP, cytochrome P450; LCA, losartan carboxylic acid, RYGB, Roux-en-Y gastric bypass
Not all included patients were able to supply metabolic ratios at all four study visits (RYGB=4, diet=1) due to technical difficulties.
[a] CYP2C19 poor metabolizers are included.
[b] One losartan/LCA ratio (and hence the respective drug- and metabolite concentrations) was excluded from week 0 due to analytical technicalities (diet group).

Table S2. Between-group differences in metabolic ratios. Data are presented as model estimated mean difference [95% CI] between the RYGB group and diet group at week 0 (baseline), 3 and 9 and year 2.

	RYGB versus DIET			
Metabolic ratio	**Week 0**	**Week 3**	**Week 9**	**Year 2**
Paraxanthine/caffeine (CYP1A2)	0.02 [-0.04, 0.07] *n=80*	0.02 [-0.04, 0.09] *n=80*	0.06 [-0.01, 0.13] *n=76*	-0.01 [-0.09, 0.07] *n=62*
5-OH omeprazole/omeprazole (CYP2C19) [a]	-0.21 [-0.62, 0.21] *n=80*	-0.27 [-0.87, 0.34] *n=79*	-0.57 [-1.3, 0.18] *n=76*	**-0.70** **[-1.3, -0.08]** *n=62*
Losartan/LCA [b] (CYP2C9)	**-0.52** **[-0.91, -0.13]** *n=79*	-0.18 [-0.50, 0.13] *n=80*	-0.08 [-0.39, 0.23] *n=76*	-0.05 [-0.35, 0.24] *n=63*

Abbreviations: CYP, cytochrome P450; LCA, losartan carboxylic acid; RYGB, Roux-en-Y gastric bypass

Bold values show statistically significant differences ($p<0.05$). Linear mixed effects models, with log-transformed dependent variable, were used to estimate differences between the RYGB group and diet group at the different study visits, with diet as reference group. Not all included patients were able to supply metabolic ratios at all four study visits (RYGB=4, diet=1) due to technical difficulties.

[a] Patients with genotype *CYP2C19 *2/*2* or **2/*4* were excluded from the analysis.

[b] One losartan/LCA ratio was excluded from week 0 due to analytical technicalities (diet group).

Table S3. Short- and long-term changes in metabolic ratios based on genotype. Data are presented as model estimated mean difference in change [95% CI] from baseline (W0) to week 3 (W3), week 9 (W9) and year 2 (Y2) in the RYGB group and diet group, respectively.

	RYGB					DIET				
Metabolic ratio	**W0 – W3**	**W0 – W9**	**W0 – Y2**	**W3 – W9**	**W9 – Y2**	**W0 – W3**	**W0 – W9**	**W0 – Y2**	**W3 – W9**	**W9 – Y2**
Paraxanthine/caffeine										
*CYP1A2 *1/*1 or*1/*1F (normal metabolizer)*	0.03 [-0.04, 0.10]	0.04 [-0.03, 0.12]	**0.09 [0.00, 0.18]**	0.01 [-0.07, 0.09]	0.05 [-0.04, 0.14]	0.06 [-0.01, 0.12]	**0.07 [0.01, 0.14]**	0.05 [-0.01, 0.12]	0.02 [-0.05, 0.08]	-0.02 [-0.09, 0.05]
*CYP1A2 *1F/*1F* (hyperinducer)	0.01 [-0.05, 0.08]	-0.00 [-0.07, 0.06]	**0.08 [0.00, 0.16]**	-0.01 [-0.08, 0.05]	**0.09 [0.01, 0.16]**	0.00 [-0.06, 0.07]	0.05 [-0.02, 0.12]	0.08 [-0.00, 0.16]	0.05 [-0.02, 0.12]	0.02 [-0.06, 0.10]
5-OH omeprazole/ omeprazole [a]										
*CYP2C19 *1/*1 (normal metabolizer)*	0.31 [-0.12, 0.74]	0.72 [-0.02, 1.5]	0.70 [-0.08, 1.5]	0.41 [-0.19, 1.0]	-0.03 [-0.66, 0.61]	**0.37 [0.03, 0.71]**	0.29 [-0.01, 0.59]	0.09 [-0.14, 0.0.32]	-0.08 [-0.39, 0.23]	-0.21 [-0.50, 0.09]
*CYP2C19 *17/*17 or *1/*17 (ultrarapid- or rapid metabolizer)*	0.67 [-0.05, 1.4]	**1.1 [0.12, 2.2]**	**0.93 [0.00, 1.9]**	0.47 [-0.31, 1.3]	-0.21 [-1.0, 0.58]	0.36 [-0.12, 0.85]	**0.77 [0.04, 1.5]**	-0.02 [-0.42, 0.38]	0.40 [-0.23, 1.0]	**-0.79 [-1.5, -0.03]**
*CYP2C19 *1/*2 or *2/*17 (intermediate metabolizer)*	0.19 [-0.07, 0.46]	0.42 [-0.04, 0.87]	0.19 [-0.08, 0.46]	0.22 [-0.13, 0.57]	-0.22 [-0.58, 0.13]	0.08 [-0.11, 0.27]	0.15 [-0.11, 0.41]	0.10 [-0.12, 0.33]	0.07 [-0.16, 0.30]	-0.05 [-0.29, 0.19]
Losartan/LCA [b]										
*CYP2C9 *1/*1 or *1/*2 (normal metabolizer)*	-0.31 [-0.68, 0.06]	**-0.39 [-0.76, -0.02]**	**-0.57 [-0.92, -0.21]**	-0.08 [-0.40, 0.24]	-0.18 [-0.48, 0.12]	-0.09 [-0.33, 0.16]	-0.08 [-0.33, 0.16]	-0.18 [-0.44, 0.07]	0.00 [-0.23, 0.24]	-0.10 [-0.35, 0.15]
*CYP2C9 *1/*3 or *2/*2 (intermediate metabolizer)*	-3.3 [-6.6, 0.08]	**-3.6 [-6.9, -0.20]**	-2.7 [-6.4, 0.94]	-0.31 [-1.8, 1.2]	0.84 [-1.5, 3.1]	-0.69 [-2.2, 0.80]	-0.67 [-2.2, 0.82]	-0.92 [-2.5, 0.64]	0.02 [-1.2, 1.2]	-0.26 [-1.5, 1.0]

Abbreviations: CYP, cytochrome P450; LCA, losartan carboxylic acid; RYGB, Roux-en-Y gastric bypass

Bold values show statistically significant differences ($p<0.05$). Linear mixed effects models, with log-transformed dependent variable, were used to estimate mean difference (Δ) in change. Not all included patients were able to supply metabolic ratios at all four study visits (RYGB=4, diet=1) due to technical difficulties.

[a] Patients with genotype *CYP2C19 *2/*2* or *2/*4* were excluded from the analysis.

[b] One losartan/LCA ratio was excluded from week 0 due to analytical technicalities (diet group).

Table S4. Associations between body weight, NAFLD liver fat score, liver fat content, and (1) the different metabolic ratios at baseline (week 0), and (2) hepatic CYP1A2-, CYP2C19-, and CYP2C9 concentrations in individual liver biopsies at the time of surgery (RYGB; week 3, control; week 0). Spearman's rho (ρ) is the correlation coefficient.

	Week 0						**Time of surgery** [a]					
	Paraxanthine/ caffeine ratio (CYP1A2 activity) n=97		5-OH omeprazole/ omeprazole ratio (CYP2C19 activity) n=97		Losartan/LCA ratio (CYP2C9 activity) n=96 [b]		Hepatic CYP1A2 (fmol/µg protein) n=54 [a]		Hepatic CYP2C19 (fmol/µg protein) n=54 [a]		Hepatic CYP2C9 (fmol/µg protein) n=54 [a]	
Independent variable	**ρ**	**p-value**	**ρ**	**p-value**	**ρ**	**p-value**	**ρ**	**p-value**	**ρ**	**p-value**	**ρ**	**p-value**
Body weight (kg)	0.05	0.60	**-0.24**	**0.02**	0.10	0.35	**-0.27**	**0.05**	-0.17	0.20	0.00	0.98
Liver fat content (%)	-0.07	0.49	**-0.57**	**<0.001**	-0.11	0.31	-0.18	0.20	-0.26	0.06	-0.18	0.18

Abbreviations: CYP, cytochrome P450; LCA, losartan carboxylic acid; NAFLD, non-alcoholic fatty liver disease

Bold values show statistically significant differences (p<0.05). P-values are from the Spearman rank correlation analysis. Three patients were not able to supply metabolic ratios or liver fat indexes (RYGB=2, control=1) due to technical difficulties.

[a] Liver biopsies were only available in patients subjected to RYGB or cholecystectomy (control).

[b] One losartan/LCA ratio was excluded from week 0 due to analytical technicalities (diet group).

III

Received: 30 May 2022 | Revised: 18 August 2022 | Accepted: 18 August 2022

DOI: 10.1111/cts.13394

ARTICLE

Impact of type 2 diabetes on in vivo activities and protein expressions of cytochrome P450 in patients with obesity

Kine Eide Kvitne[1] | **Anders Åsberg**[1,2] | **Line K. Johnson**[3] | **Christine Wegler**[4,5] | **Jens K. Hertel**[3] | **Per Artursson**[6] | **Cecilia Karlsson**[7,8] | **Shalini Andersson**[9] | **Rune Sandbu**[3,10] | **Eva Skovlund**[11] | **Hege Christensen**[1] | **Rasmus Jansson-Löfmark**[5] | **Jøran Hjelmesæth**[3,12] | **Ida Robertsen**[1]

[1]Section for Pharmacology and Pharmaceutical Biosciences, Department of Pharmacy, University of Oslo, Oslo, Norway

[2]Department of Transplantation Medicine, Oslo University Hospital, Oslo, Norway

[3]The Morbid Obesity Center, Vestfold Hospital Trust, Tønsberg, Norway

[4]Department of Pharmacy, Uppsala University, Uppsala, Sweden

[5]DMPK, Research and Early Development, Cardiovascular, Renal and Metabolism (CVRM), BioPharmaceuticals R&D, AstraZeneca, Gothenburg, Sweden

[6]Department of Pharmacy and Science for Life Laboratory, Uppsala University, Uppsala, Sweden

[7]Late-stage Development, Cardiovascular, Renal and Metabolism (CVRM), BioPharmaceuticals R&D, AstraZeneca, Gothenburg, Sweden

[8]Department of Molecular and Clinical Medicine, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

[9]Oligonucleotide Discovery, Discovery Sciences, R&D, AstraZeneca, Gothenburg, Sweden

[10]Department of Surgery, Vestfold Hospital Trust, Tønsberg, Norway

[11]Department of Public Health and Nursing, Norwegian University of Science and Technology, NTNU, Trondheim, Norway

Abstract

Previous studies have not accounted for the close link between type 2 diabetes mellitus (T2DM) and obesity when investigating the impact of T2DM on cytochrome P450 (CYP) activities. The aim was to investigate the effect of T2DM on in vivo activities and protein expressions of CYP2C19, CYP3A, CYP1A2, and CYP2C9 in patients with obesity. A total of 99 patients from the COCKTAIL study (NCT02386917) were included in this cross-sectional analysis; 29 with T2DM and obesity (*T2DM-obesity*), 53 with obesity without T2DM (*obesity*), and 17 controls without T2DM and obesity (*controls*). CYP activities were assessed after the administration of a cocktail of probe drugs including omeprazole (CYP2C19), midazolam (CYP3A), caffeine (CYP1A2), and losartan (CYP2C9). Jejunal and liver biopsies were also obtained to determine protein concentrations of the respective CYPs. CYP2C19 activity and jejunal CYP2C19 concentration were 63% (−0.39 [95% CI: −0.82, −0.09]) and 40% (−0.09 fmol/μg protein [95% CI: −0.18, −0.003]) lower in *T2DM-obesity* compared with the *obesity* group, respectively. By contrast, there were no differences in the in vivo activities and protein concentrations of CYP3A, CYP1A2, and CYP2C9. Multivariable regression analyses also indicated that T2DM was associated with interindividual variability in CYP2C19 activity, but not CYP3A, CYP1A2, and CYP2C9 activities. The findings indicate that T2DM has a significant downregulating impact on CYP2C19 activity, but not on CYP3A, CYP1A2, and CYP2C9 activities and protein concentrations in patients with obesity. Hence, the effect of T2DM seems to be isoform-specific.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

The current literature suggests that type 2 diabetes mellitus (T2DM) alters cytochrome P450 (CYP) activities in an iso-specific manner. However, an important limitation of previous studies investigating the impact of T2DM on CYP activities is that the close link between T2DM and obesity has not have been accounted for.

[12]Department of Endocrinology, Morbid Obesity and Preventive Medicine, Institute of Clinical Medicine, University of Oslo, Oslo, Norway

Correspondence
Kine Eide Kvitne, Department of Pharmacy, University of Oslo, Oslo, Norway.
Email: k.e.kvitne@farmasi.uio.no

WHAT QUESTION DID THIS STUDY ADDRESS?
Does T2DM impact in vivo activities and protein expressions of CYP2C19, CYP3A, CYP1A2, and CYP2C9 in patients with obesity?

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
This study suggests that in vivo CYP2C19 activity is lower in patients with T2DM and obesity compared with patients with obesity only. T2DM does not seem to impact CYP3A, CYP1A2, and CYP2C9 activities and protein concentrations in patients with obesity.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
The risk of treatment failure (prodrugs) and adverse effects (active drugs) should be borne in mind when prescribing drugs dependent on CYP2C19-mediated metabolism in patients with T2DM and obesity.

INTRODUCTION

The cytochrome P450 (CYP) superfamily is involved in the metabolism of more than half of all clinically used drugs on the market, making it the most important group of drug-metabolizing enzymes.[1,2] Among all CYP isoforms identified, only a small number is considered to have a central role in drug metabolism.[3] These include CYP3A, CYP2C19, CYP2C9, CYP1A2, CYP2B6, CYP2D6, and CYP2E1. CYP isoforms are particularly important for the hepatic clearance of substrate drugs, but may also contribute significantly to the first-pass metabolism of orally administered drugs.[4] There is substantial interindividual variability in CYP-mediated metabolism due to a combination of factors, including genetics, environment, and diseases.[3,5]

Type 2 diabetes mellitus (T2DM) is a growing health issue worldwide, mainly caused by obesity leading to insulin resistance and impaired β-cell function.[6,7] The prevalence of nonalcoholic fatty liver disease (NAFLD) is particularly high in people with obesity and/or T2DM,[8,9] who typically also have elevated levels of inflammatory cytokines.[8,10,11] The drug response in patients with T2DM is often variable, and different, compared with other patient populations,[12–14] possibly due to altered activity and expression of drug-metabolizing enzymes.

A recent study investigating the impact of T2DM on different CYP activities showed that alterations in CYP activity were isoform-specific.[15] For instance, CYP3A and CYP2C19 activities were lower in patients with T2DM compared with individuals without T2DM, and elevated levels of inflammatory cytokines were suggested as an important mechanism.[15] However, chronic low-grade inflammation is also central in the pathophysiology of obesity, the main preventable cause of T2DM.[6,7] Hence, an important limitation of the current literature is that the obesity component has not been accounted for when the effect of T2DM on different CYP activities has been investigated. Previous studies have in fact provided evidence of lower CYP3A activity in patients with obesity compared with individuals without obesity.[16–19] Additionally, we have recently shown that patients with obesity (with or without T2DM) had lower CYP2C19 activity than mainly normal weight controls.[20] There is also evidence of lower CYP3A and CYP2C19 activity in patients with NAFLD,[20,21] and since obesity, T2DM, and NAFLD often coexist,[8] this further complicates the interpretation of previous findings.

In view of this, the primary objective was to investigate the impact of T2DM on in vivo activities and protein expressions of CYP2C19, CYP3A, CYP1A2, and CYP2C9, by comparing patients with obesity and T2DM with their respective counterparts without T2DM. Secondary, we aimed to investigate the effect of covariates such as NAFLD, body mass index (BMI), and inflammatory markers on these CYP activities within a wide body weight range.

METHODS

Participants

A total of 108 patients were included in the COCKTAIL study, an open-label, three-armed, single-center, controlled study (NCT02386917).[16] Of these, six patients withdrew and two patients were excluded before study start.[16] Also, one patient was excluded from the pharmacokinetic analysis due to severe liver cirrhosis, leaving 99 patients that were included in the present cross-sectional exploratory post hoc analysis. Inclusion and exclusion criteria of the COCKTAIL study have been published previously in a protocol article.[22] The study population included patients with obesity scheduled for weight loss treatment with Roux-en-Y-gastric bypass (RYGB) or non-surgical calorie restriction based on clinical indications, and a control group of mainly normal

weight individuals scheduled for cholecystectomy. The study design included a stratification for diabetic status of patients with obesity.[22] The impact of RYGB and strict diet on the activities and protein concentrations of central CYP enzymes in the same study population have been published previously.[16,20] The COCKTAIL study was approved by the Regional Committee for Medical and Health Research Ethics (2013/2379/REK), performed according to the Declaration of Helsinki, and all patients gave written informed consent as part of the COCKTAIL study.

In the present analysis, patients were divided into three groups based on diabetes status and BMI: (1) patients with T2DM and obesity (BMI ≥ 30 kg/m^2) labeled *T2DM-obesity*, (2) patients with obesity, but not T2DM, labeled *obesity*, and (3) controls without obesity (BMI < 30 kg/m^2) and without T2DM labeled *controls*. T2DM was diagnosed according to the following criteria: glycated haemoglobin (HbA1c) ≥48 mmol/mol (6.5%), or antidiabetic drug treatment, or previously diagnosed with T2DM treated with lifestyle interventions. The *T2DM-obesity* group and the *obesity* group underwent a 3-week low-energy diet (<1200 kcal/day) before the in-depth study investigation, whereas the *controls* did not undergo any defined diet. None of the patients received treatment with medications and/or other substances known to influence the pharmacokinetics of the probe drugs used in this study.

Study investigation and procedures

On the study day, patients first met for blood sampling at 7:30 a.m. before they received 100 mg oral caffeine (8:00 a.m.), followed by 1.5 mg oral midazolam syrup, 25 mg oral losartan, and 20 mg oral omeprazole 1 h later (9:00 a.m.). Intravenous midazolam (1.0 mg) was administered 4 h after the oral midazolam syrup (1:00 p.m.). Blood samples for analysis of omeprazole, caffeine, and their metabolites were collected 3 and 4 h after administration of the respective drug (12:00 a.m.). For midazolam the blood samples were collected before and 0.25, 0.5, 1, 1.5, 2, 3, 4, 4.25, 4.5, 5, 5.5, 6, 8, 10, 12, 23, and 24-h after the administration of oral midazolam syrup. The blood samples were drawn in K2-EDTA vacutainer tubes and centrifuged for 10 min at 4°C (1800 ***g***). Plasma was separated into Cryovials and frozen within 1 h at −70°C until analysis. Urine was collected in a container for 8 h after the administration of losartan, and the total volume was recorded. An aliquot of 10 ml urine was frozen immediately after sampling at −70°C. The pharmacokinetic investigation has been described in more detail previously.[16,20] Also, in the patients subjected to RYGB, paired jejunal and liver biopsies were obtained during surgery. This has been described in detail previously.[23,24] The biopsies were transferred into cryotubes, snap-frozen in liquid nitrogen directly upon sampling, and stored at −70°C until analysis.

Standard clinical chemistry analyses were performed in fresh blood samples at the Department of Laboratory Medicine, Vestfold Hospital Trust, Tønsberg, Norway. Plasma concentrations of high-sensitivity C-reactive protein (hs-CRP) were measured using immunoturbidimetry (Advia Chemistry XPT systems, Siemens) at Fürst Medical Laboratory, Oslo, Norway. Plasma concentration of inflammation markers representing various types of immune responses,[15] namely interferon (IFN)-γ, interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α), were analyzed using multiplex bead-based immunoassays (Bio-Techne, UK) based on xMAP technology (Luminex, Austin, TX) at the Department of Medical Biochemistry, Diakonhjemmet Hospital, Oslo, Norway. Body weight (kg) and body composition were measured with the Inbody 720 Body Composition Analyzer (Biospace, Korea). Waist and hip circumference were measured with a stretch-resistant tape parallel to the floor and midway between the 12th rib and the iliac crest, and around the widest portion of the buttocks, respectively.

Estimation of CYP activities

Absolute bioavailability and systemic clearance of midazolam and plasma concentrations of the endogenous CYP3A biomarker 4β-hydroxycholesterol (4βOHC) were used to estimate CYP3A activity. Midazolam absolute bioavailability and systemic clearance were determined using a population pharmacokinetic model, which has been described in detail previously.[16] In short, the modeling was performed using the nonparametric adaptive grid approach implemented in Pmetrics (version 1.5.2) for R (version 3.6.2).[25,26] A catenary three-compartment model with absorption lag-time and first-order elimination from the central compartment described the data adequately for the purpose of the present analysis. CYP1A2 activity was described by the 4-h plasma paraxanthine/caffeine ratio, CYP2C19 activity by the 3-h plasma 5-hydroxyomeprazole (5-OH-omeprazole)/omeprazole ratio, and CYP2C9 activity by the 8-h urine losartan/losartan carboxylic acid (LCA) ratio. For the metabolic ratios calculated as metabolite/drug ratio (CYP1A2 and CYP2C19), a higher ratio implies higher CYP activity, while for the metabolic ratio calculated as drug/metabolite ratio (CYP2C9), a higher ratio implies lower CYP activity.

Bioanalytical assays

Midazolam

A previously validated ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS) method was used to determine plasma concentrations of midazolam and has been described in detail previously.[27] Briefly, liquid–liquid sample extraction was used as sample preparation. Eight calibrators in the range of 0.1–20 ng/ml were applied, and back-calculated values of calibrators within 80–120% were accepted. The lower limit of quantification (LLOQ) was 0.1 ng/ml, and the upper limit of quantification (ULOQ) was 20 ng/ml. Samples with midazolam concentrations above ULOQ were diluted in blank plasma and reanalyzed. Dilution integrity with dilution factors of 1/2, 1/5, 1/10, 1/20, and 1/50 was established; mean accuracy ranged from 88.9% to 103.8%, and the imprecision was <4.5%. Within-series and between-series performance were assessed with the resulting coefficient of variation (CV) <12.3%, and the mean accuracy ranged from 99.3% to 104.3%.

Caffeine, omeprazole, and losartan

As previously described, plasma concentrations of caffeine, paraxanthine, omeprazole, and 5-OH-omeprazole, and urinary concentration of losartan and LCA were determined by Covance Laboratories (Madison, WI) using validated LC–MS/MS methods.[20] The inter-run precisions, assessed as CVs, were <4.6% and <4.5% for caffeine and paraxanthine, respectively. The inter-run accuracies ranged between 94.7% and 98.7% for caffeine and between 96.7% and 100.0% for paraxanthine. For losartan the inter-run precision was <9.8% and inter-run accuracies varied between 95.6% and 103.0%, and for LCA the inter-run precision was <10.6% and inter-run accuracies were between 96.9% and 100.2%. In the omeprazole and 5-OH-omeprazole assay, inter-run CVs were <9.8% and <14.9% and inter-run accuracies were between 96.9% and 101.2% and 98.0% and 102.4% for omeprazole and 5-OH-omeprazole, respectively.

4β-Hydroxycholesterol (4βOHC)

A previously described assay,[28] with an added filtration step,[29] was used to determine plasma concentrations of 4βOHC. In brief, 4βOHC was de-esterified from fatty acids by ethanolic sodium methoxide and isolated from plasma by liquid–liquid extraction with hexane. Extracts were evaporated by nitrogen and reconstituted in methanol before filtration. For the quantitative analysis, a UPLC followed by an MS detector (Waters, Milford, MA) was used. Chromatographic separation was achieved on a BEH C18 column RP-shield (1.7 µm, 1×100 mm) from Waters with a mobile phase of water and methanol. After atmospheric pressure chemical ionization, detection was obtained with multiple reaction monitoring at *m/z* 385.25–>367.45 (4βOHC) and *m/z* 392.30–>374.50 (4βOHC-D7; internal standard). The LLOQ was 10 ng/ml. Intra- and interday imprecision and inaccuracy were <15% at 10 ng/ml and <4% at 644 ng/ml ($n = 6$).[28]

Protein quantification of CYP enzymes in liver and jejunal biopsies

The protein quantification has been described previously.[30] In brief, proteins were extracted from liver and jejunal biopsies in a sodium dodecyl sulfate (SDS)-containing (2% w/v) lysis buffer, and processed with the multi-enzyme digestion filter-aided sample preparation protocol, using LysC and trypsin. Proteomics analysis was performed with Q Exactive HF or Q Exactive HF-X. MS data were processed with MaxQuant, using the human UniProtKB. Spectral raw intensities were normalized with variance stabilization. The protein concentrations were calculated using the total protein approach.

Genotype analyses

Taqman-based real-time polymerase chain reaction assays implemented for routine pharmacogenetic analyses at the Center for Psychopharmacology, Diakonhjemmet Hospital, Norway were used to analyze the following variant alleles: CYP1A2; the increased induction allele **1F* (rs762551), CYP2C9; the reduced function alleles **2* (rs1799853) and **3* (rs1057910), CYP2C19; the null alleles **2* (rs4244285), **3* (rs4986893), and **4* (rs28399504) and the gain-of-function allele **17* (rs12248560), CYP3A; the reduced function allele **22* (rs35599367) and CYP3A5; and the null allele **3* (rs776746). The following subgroups were used to describe genotype-predicted-phenotype: normal, ultrarapid/rapid, intermediate, and poor metabolizer. Except for *CYP2C9*3*, all alleles were in Hardy–Weinberg equilibrium.

Data and statistical analysis

Visual inspection of plots and Shapiro Wilk's test were used to evaluate the normality of the data. Wilcoxon rank-sum test was used to compare differences between (1) the *T2DM-obesity* group and *obesity* group and (2) the *obesity* group and the *controls*. Fisher's exact test was used to compare genotype-predicted-phenotype distribution between the

groups. Multivariable regression analyses were performed to explore the effects of BMI, T2DM (yes/no), and NAFLD (yes/no) on the various CYP activities. The multivariable regression analyses were adjusted for age, sex (male/female), and genotype-predicted-phenotype (i.e., genotypic normal, rapid/ultrarapid, intermediate, and poor metabolizer) effects. Inflammatory markers with a p value <0.25 in the univariate regression analyses were candidates for inclusion in the final multivariable model. Potential impact of the 3-week low-energy diet was investigated in a subanalysis. Linear mixed-effects models with a log-transformed dependent variable (PK parameter) were used to estimate the between-group difference (*T2DM-obesity* vs. *obesity*) in change over time (weeks 0–3). Visit (time), diabetes status (yes/no), and their interaction (visit × diabetes status) were treated as fixed effects. The unique patient ID was used as a random effect, and the confidence interval (CI) was adjusted using Tukey's method. Insulin resistance was estimated using the homeostasis model assessment (HOMA) and calculated by the following equation: fasting insulin (pmol/L) × fasting glucose (mmol/L)/135.[31] The NAFLD liver fat score (NAFLD-LFS) was calculated according to Kotronen et al.[32] Values of NAFLD-LFS greater than −0.640 were indicative of NAFLD. A p value <0.05 was considered statistically significant, and all statistical analyses were performed using R for Windows (version 4.1.2).[26]

RESULTS

Patients

A total of 99 patients (30% males) were included in the present analysis; 29 in the *T2DM-obesity* group, 53 in the *obesity* group, and 17 in the *controls*. Patient characteristics are presented in Table 1. Mean age was higher in the *T2DM-obesity* group compared with the *obesity* group (8 years [95% CI: 4, 12]), whereas mean

TABLE 1 Patient characteristics

Characteristic	T2DM-obesity ($n = 29$)	Obesity ($n = 53$)	Controls ($n = 17$)
Age (years)	52 ± 8	45 ± 9	42 ± 15
Sex (male/female)	8/21	19/34	3/14
Body weight (kg)	109 ± 17	128 ± 23	70 ± 11
BMI (kg/m^2)	38 ± 4.9	43 ± 5.6	24 ± 2.6
Waist circumference (cm)	119 ± 10	124 ± 13	81 ± 16
Hip circumference (cm)	120 ± 13	131 ± 12	96 ± 16
Waist−hip ratio	0.99 ± 0.09	0.95 ± 0.10	0.85 ± 0.06
HbA1c (mmol/mol)	50 ± 10	36 ± 3.4	35 ± 2.7
Glucose (mmol/L)	6.7 ± 1.4	4.9 ± 0.45	4.7 ± 0.40
Insulin (pmol/L)	122 ± 43	100 ± 51	50 ± 22
HOMA-IR	5.9 ± 3.0	3.4 ± 1.9	1.6 ± 0.81
Total cholesterol (mmol/L)	4.0 ± 1.1	3.9 ± 0.8	4.4 ± 0.9
hs-CRP (mg/L)	4.5 ± 4.2	6.3 ± 6.6	2.4 ± 3.9
ALT (U/L)	39 ± 23	37 ± 21	23 ± 15
NAFLD	29 (100)	33 (62)	1 (6)
Comorbidities			
Hypertension	15 (52)	17 (32)	0 (0)
Heart disease[a]	2 (7)	1 (2)	0 (0)
Depression/anxiety	9 (31)	14 (26)	0 (0)
Asthma/COPD	8 (28)	9 (17)	0 (0)
Autoimmune diseases[b]	1 (3)	2 (4)	0 (0)

Note: Data are described as mean ± standard deviation or absolute numbers (%).

Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; COPD, chronic obstructive pulmonary disease; HbA1c, glycated hemoglobin; hs-CRP, high-sensitivity C-reactive protein; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance; NAFLD, nonalcoholic fatty liver disease; T2DM, type 2 diabetes mellitus.

[a]Includes coronary artery bypass surgery, myocardial infarction, and percutaneous coronary intervention.

[b]Includes rheumatoid arthritis, Bechterew's disease (ankylosing spondylitis), inflammatory bowel disease, coeliac disease, and polymyalgia rheumatica.

BMI was slightly lower (−4.6 kg/m^2 [95% CI: −7.0, −2.1]; Table 1). In the *T2DM-obesity* group, all patients (100%) had NAFLD-LFS indicative of NAFLD, whereas in the *obesity* group and the *controls* the proportions were 62% and 6%, respectively. Among the patients with *T2DM-obesity*, 69% received treatment with one or more glucose-lowering drugs; metformin (n = 13), sodium-glucose co-transporter 2 (SGLT2) inhibitors (n = 5), glucagon-like peptide-1 (GLP-1) analogues (n = 4), and/or a combination of metformin and dipeptidyl peptidase 4 (DPP-4) inhibitors (n = 8). The CYP genotype distribution is given in Table 2. There were no significant between-group differences in genotype-predicted-phenotype distribution between the three groups.

CYP2C19

Table 3 reports the activities and protein concentrations of the various CYP enzymes in the *T2DM-obesity*, *obesity*, and *control* groups. The median 5-OH-omeprazole/omeprazole ratio was 63% lower in the *T2DM-obesity* group compared with the *obesity* group (Figure 1a, Table 4). Jejunal CYP2C19 concentrations, available in a subset of the patients (n = 34), were 40% lower in the *T2DM-obesity* group compared with the *obesity* group, while there was no statistically significant difference in hepatic CYP2C19 concentrations (n = 39; Figure 1b,c, Table 4). Results from the regression model including all three study groups (n = 99) suggested that interindividual variability in CYP2C19 activity (5-OH-omeprazole/omeprazole ratio) was associated with both T2DM and NAFLD, but not BMI (Table 5). None of the inflammatory markers were included in the final model, due to the lack of significance and/or influence of the β-estimates. The final model explained 43% of the variability in CYP2C19 activity. Results from the univariate regression analysis of covariates are presented in Table S1.

CYP3A

There were no differences in any of the in vivo CYP3A metrics or in jejunal or hepatic CYP3A concentration between the *T2DM-obesity* group and the *obesity* group (Table 4). The multivariable regression analyses showed that interindividual variability in midazolam absolute bioavailability, systemic clearance, as well as 4βOHC concentrations, were associated with BMI (Table 5). For absolute bioavailability of midazolam and 4βOHC

TABLE 2 Genotype distribution in the three study groups

Genotype (likely phenotype)	T2DM-obesity (n = 29)	Obesity (n = 53)	Controls (n = 17)
CYP2C19 genotype			
**1/*1* (NM)	12 (41)	21 (40)	7 (41)
**17/*17* or **1/*17* (UM/RM)	8 (28)	22 (41)	5 (29)
**1/*2* or **2/*17* (IM)	7 (24)	8 (15)	5 (29)
**2/*2* or **2/*4* (PM)	2 (7)	2 (4)	0 (0)
CYP3A4 genotype			
**1/*1* (NM)	27 (93)	50 (94)	15 (88)
**1/*22* (IM)	2 (7)	3 (6)	2 (12)
CYP3A5 genotype			
**1/*3* (IM)	2 (7)	6 (11)	2 (12)
**3/*3* (PM)	27 (93)	47 (89)	15 (88)
CYP1A2 genotype			
**1/*1* or **1/*1F* (NM)	14 (48)	25 (47)	10 (59)
**1F/*1F* (hyperinducer)	15 (52)	28 (53)	7 (41)
CYP2C9 genotype			
**1/*1* or **1/*2* (NM)	26 (90)	49 (92)	16 (94)
**1/*3* or **2/*2* (IM)	3 (10)	4 (8)	0 (0)
**3/*3* (PM)	0 (0)	0 (0)	1 (6)

Note: Data are presented as absolute numbers (%).

Abbreviations: CYP, cytochrome P450; IM, intermediate metabolizer; NM, normal metabolizer; PM, poor metabolizer; RM, rapid metabolizer; UM, ultrarapid metabolizer.

TABLE 3 Activities and expressions of CYP2C19, CYP3A, CYP1A2, and CYP2C9 in the three study groups

Parameter	T2DM-obesity ($n = 29$)	Obesity ($n = 53$)	Controls ($n = 17$)
CYP2C19			
5-OH-omeprazole/omeprazole	0.76 ± 0.92	1.5 ± 1.6	2.3 ± 2.3
Jejunal CYP2C19 (fmol/μg protein)[a,b]	0.14 ± 0.10	0.24 ± 0.15	NA
Hepatic CYP2C19 (fmol/μg protein)[c]	7.9 ± 2.5	9.3 ± 3.5	11 ± 3.9
CYP3A			
MDZ absolute bioavailability (%)	25 ± 12	20 ± 10	8.4 ± 4.2
MDZ systemic clearance (L/h)	25 ± 10	24 ± 11	17 ± 9.1
4βOHC (ng/mL)	10 ± 4.6	9.8 ± 3.8	18 ± 6.1
Jejunal CYP3A4 (fmol/μg protein)[a]	13 ± 4.9	14 ± 5.3	NA
Hepatic CYP3A4 (fmol/μg protein)[c]	19 ± 6.6	23 ± 8.1	26 ± 6.7
CYP1A2			
Paraxanthine/caffeine	0.41 ± 0.15	0.38 ± 0.14	0.45 ± 0.26
Hepatic CYP1A2 (fmol/μg protein)[c]	14 ± 5.6	14 ± 6.2	18 ± 11
CYP2C9			
LCA/losartan	1.2 ± 0.53	1.4 ± 0.62	4.3 ± 12
Jejunal CYP2C9 (fmol/μg protein)[a]	3.2 ± 6.1	1.8 ± 0.75	NA
Hepatic CYP2C9 (fmol/μg protein)[c]	12 ± 2.5	12 ± 2.2	12 ± 3.3

Note: Data are presented as mean ± standard deviation.

Abbreviations: 4βOHC, 4β-hydroxycholesterol; CYP, cytochrome P450; MDZ, midazolam; NA, not available; T2DM, type 2 diabetes mellitus.

[a]Jejunal biopsies were only available in 37 patients subjected to Roux-en-Y-gastric bypass (RYGB).

[b]Jejunal CYP2C19 concentration was missing in 3 patients due to technical error ($n = 34$).

[c]Hepatic biopsies were only available in 56 patients subjected to RYGB or cholecystectomy.

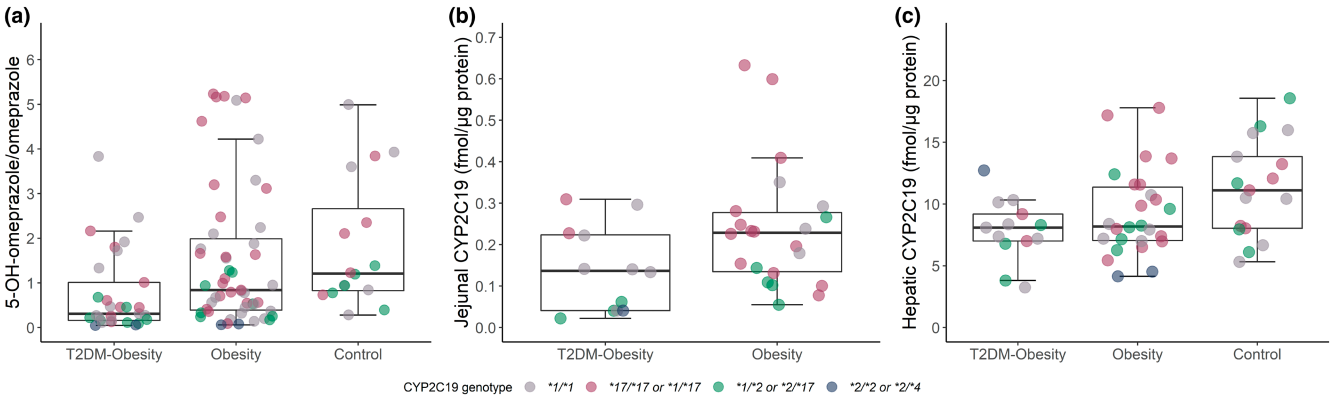

FIGURE 1 In vivo CYP2C19 activity and jejunal and hepatic protein expression in paired samples. Boxplot with individual points of (a) the 5-OH-omeprazole/omeprazole ratio (in vivo CYP2C19 activity), (b) jejunal CYP2C19 concentration, and (c) hepatic CYP2C19 concentration. The dots are colored according to genotype. CYP, cytochrome P450; T2DM, type 2 diabetes mellitus. Jejunal CYP2C19 concentration was only available in 34 patients subjected to Roux-en-Y-gastric bypass (RYGB). Hepatic CYP2C19 concentration was only available in 56 patients subjected to RYGB or cholecystectomy.

concentrations, the interindividual variability was also associated with T2DM and NAFLD, respectively. The covariates included in the final models could only explain 19%, 6%, and 25% of the variability in midazolam absolute bioavailability, systemic clearance, and 4βOHC concentrations, respectively.

CYP1A2 and CYP2C9

No differences in the paraxanthine/caffeine ratio or losartan/LCA ratio, nor intestinal (only CYP2C9) and hepatic CYP1A2 and CYP2C9 concentrations between the *T2DM-obesity* group and the *obesity* group were observed

TABLE 4 Between-group differences in protein expression and in vivo activities of CYP2C19, CYP3A, CYP1A2, and CYP2C9

Parameter	T2DM-obesity vs. Obesity
CYP2C19[a]	
5-OH-omeprazole/omeprazole	**−0.39 [−0.82, −0.09]**
Jejunal CYP2C19 (fmol/μg protein)[b,c]	**−0.09 [−0.18, −0.003]**
Hepatic CYP2C19 (fmol/μg protein)[d]	−0.83 [−3.4, 0.97]
CYP3A[e]	
MDZ absolute bioavailability (%)	4.4 [−1.2, 11]
MDZ systemic clearance (L/h)	1.9 [−3.7, 7.0]
4βOHC (ng/ml)	0.70 [−1.2, 2.7]
Jejunal CYP3A4 (fmol/μg protein)[b]	−1.6 [−5.6, 2.0]
Hepatic CYP3A4 (fmol/μg protein)[d]	−3.3 [−7.3, 0.81]
CYP1A2[f]	
Paraxanthine/caffeine	0.02 [−0.03, 0.08]
Hepatic CYP1A2 (fmol/μg protein)[d]	−0.11 [−3.9, 3.8]
CYP2C9[g]	
LCA/losartan	−0.16 [−0.42, 0.09]
Jejunal CYP2C9 (fmol/μg protein)[b]	1.5 [1.2, 2.0]
Hepatic CYP2C9 (fmol/μg protein)[d]	0.04 [−1.7, 1.7]

Note: Data are presented as median [95% confidence interval]. Wilcoxon rank-sum test was used to compare differences between the groups. A p value <0.05 was considered statistically significant (shown in bold).

Abbreviations: 4βOHC, 4β-hydroxycholesterol CYP; cytochrome P450; LCA, losartan carboxylic acid; MDZ, midazolam; NA, not available; T2DM, type 2 diabetes mellitus.

[a]CYP2C19 activity was described by the 3-h plasma 5-OH-omeprazole/omeprazole ratio.

[b]Jejunal biopsies were only available in 37 patients subjected to Roux-en-Y-gastric bypass (RYGB).

[c]Jejunal CYP2C19 concentration was missing in 3 patients due to technical error ($n = 34$).

[d]Hepatic biopsies were only available in 56 patients subjected to RYGB or cholecystectomy.

[e]CYP3A activity was described by absolute bioavailability and systemic clearance of midazolam and plasma concentrations of the endogenous CYP3A4 biomarker 4βOHC.

[f]CYP1A2 activity was described by the 4-h plasma paraxanthine/caffeine ratio.

[g]CYP2C9 activity was described by the 8-h urine losartan/LCA ratio.

(Table 4). The regression analyses supported that T2DM has no relevant effect on CYP1A2 (paraxanthine/caffeine ratio) or CYP2C9 activities (losartan/LCA ratio) (Table 5). The inflammatory marker TNF-α was associated with some of the interindividual variability in both CYP1A2 and CYP2C9 activities and was thus included in the final model. Genotype-predicted-phenotype also explained a significant part of the variability in CYP2C9 activity. The covariates included in the final model for CYP1A2 and CYP2C9 explained 10% and 48% of the interindividual variability, respectively.

Impact of the low-energy diet

Before the diet intervention, the median 5-OH-omeprazole/omeprazole ratio was 55% lower in the *T2DM-obesity* group compared with the *obesity* group: −0.27 [95% CI: −0.50, −0.03]. None of the other markers investigated showed any significant difference between these groups before the diet intervention ($p > 0.10$). During the 3-week diet intervention, the *obesity* group had a significantly larger increase in the 5-OH-omeprazole/omeprazole ratio compared with the *T2DM-obesity* group: 0.28 [95% CI: 0.05, 0.51]. For the paraxanthine/caffeine ratio, mean difference in change between the *obesity* group and the *T2DM-obesity* group during the diet intervention was −0.05 [95% CI: −0.09, −0.01]. There was no between-group difference in within-group change in midazolam absolute bioavailability, systemic clearance, 4βOHC concentration, or losartan/LCA ratio between weeks 0 and 3 (all $p > 0.23$).

DISCUSSION

In this comprehensive analysis including data from 99 patients we were able to study the impact of T2DM on CYP2C19, CYP3A, CYP1A2, and CYP2C9 activities and

TABLE 5 Multivariable analysis of CYP1A2, CYP2C9, CYP2C19, and CYP3A activities

CYP450 isoform	Covariates	Parameter estimate (β) ± SE	*p* value	Adjusted R^2
CYP2C19 (log 5-OH-omeprazole/omeprazole)	BMI (kg/m^2)	−0.000 ± 0.013	0.99	0.43
	T2DM (yes)	−0.64 ± 0.26	0.016	
	NAFLD (yes)	−0.63 ± 0.25	0.015	
CYP3A (MDZ absolute bioavailability, %)	BMI (kg/m^2)	0.030 ± 0.009	0.0011	0.19
	T2DM (yes)	0.40 ± 0.18	0.033	
	NAFLD (yes)	−0.027 ± 0.18	0.88	
CYP3A (MDZ systemic clearance, L/h)	BMI (kg/m^2)	0.015 ± 0.007	0.041	0.06
	T2DM (yes)	0.11 ± 0.15	0.46	
	NAFLD (yes)	−0.027 ± 0.15	0.86	
CYP3A (log 4βOHC, ng/ml)	BMI (kg/m^2)	−0.020 ± 0.006	0.0017	0.25
	T2DM (yes)	−0.012 ± 0.12	0.92	
	NAFLD (yes)	−0.27 ± 0.12	0.029	
CYP1A2 (log paraxanthine/caffeine)	BMI (kg/m^2)	−0.007 ± 0.005	0.21	0.10
	T2DM (yes)	0.14 ± 0.11	0.20	
	NAFLD (yes)	−0.14 ± 0.11	0.19	
	Log TNF-α (pg/ml)	0.074 ± 0.034	0.032	
CYP2C9 (log losartan/LCA)	BMI (kg/m^2)	−0.001 ± 0.006	0.89	0.48
	T2DM (yes)	0.013 ± 0.12	0.92	
	NAFLD (yes)	−0.24 ± 0.12	0.050	
	Log TNF-α (pg/ml)	0.081 ± 0.038	0.037	

Note: The models were adjusted for age, sex (male/female), and genotype-predicted-phenotype (categorized according to Table 2: CYP2C19: *1/*1 (normal), *17/*17 or *1/*17 (ultrarapid/rapid), *1/*2 or *2/*17 (intermediate), and *2/*2 or *2/*4 (poor); CYP3A4: *1/*1 (normal) and *1/*22 (intermediate); CYP3A5: *1/*3 (intermediate) and *3/*3 (poor); CYP1A2: *1/*1 or *1/*1F (normal) and *1F/*1F (hyperinducer); CYP2C9: *1/*1 or *1/*2 (normal), *1/*3 or *2/*2 (intermediate), and *3/*3 (poor)).

Abbreviations: 4βOHC, 4β-hydroxycholesterol; 5-OH-omeprazole, 5-hydroxyomeprazole; BMI, body mass index; CYP, cytochrome P450; LCA, losartan carboxylic acid; MDZ, midazolam; NAFLD, nonalcoholic fatty liver disease; TNF-α, tumor necrosis factor-α; T2DM, type 2 diabetes mellitus.

protein expression in primarily an obese population with and without T2DM. The main finding was that CYP2C19 activity was lower in patients with T2DM and obesity than in patients with obesity only. By contrast, T2DM had no impact on in vivo activities and/or protein expressions of CYP3A, CYP1A2, and CYP2C9.

Our results showed that CYP2C19 activity was ~60% lower in the patients with T2DM and obesity compared with the patients with obesity only. In agreement with our finding, Gravel et al. also found approximately 50% lower CYP2C19 activity in patients with T2DM compared with non-T2DM individuals with a wide body weight range.[15] A lower CYP2C19 activity in patients with T2DM is also supported by other studies showing an inadequate effect of clopidogrel at studied doses, a prodrug which to a large extent is activated by CYP2C19, in this patient population.[13,33,34] We have previously shown that patients with NAFLD have decreased CYP2C19 activity.[20] The regression analyses in this present analysis supported that NAFLD is involved in the downregulation of CYP2C19 activity, while BMI does not appear to be of significance. Even though higher levels of inflammatory markers have been suggested as an important mechanism for the downregulation of CYP2C19 activity in patients with T2DM,[15] none of the inflammation markers investigated in the present study could explain the additional effect of T2DM on CYP2C19 activity. The intestinal CYP2C19 protein expression was lower in the patients with T2DM and obesity than the patients with obesity only. Interestingly, there was no statistically significant difference in hepatic CYP2C19 expression. However, it may be that the study was not powered to show an effect, given that the proteomics data were only available in a subset of the patients.

We did not observe any difference in CYP3A activity or hepatic or intestinal CYP3A expression between the patients with T2DM and obesity and the patients with obesity only. The regression analyses supported that T2DM has no effect on hepatic CYP3A, but it may explain some of the interindividual variability in absolute bioavailability of midazolam. Taken together, the data in this study indicate that BMI, but not T2DM, is of importance for the downregulation of CYP3A activity in patients with

obesity. By contrast, Gravel et al. showed lower CYP3A activity in patients with T2DM compared with non-T2DM individuals.[15] Yet, in their study, the comparison was done between two groups with significantly different BMI (mean of 29.1 kg/m^2 in patients with T2DM vs. 25.7 kg/m^2 in non-T2DM). Hence, the lower CYP3A activity may actually be explained by the higher BMI rather than T2DM. The regression analyses in the present analysis supported this hypothesis. That obesity may play a role in such patients has been proposed previously,[35] and several studies have shown a decreased CYP3A activity and protein expression with increasing BMI.[24,36,37] Gravel et al. have also reported that T2DM did not have any effect on CYP3A expression and ex vivo activity in intestinal biopsies compared to patients without T2DM,[38] which is in line with the results in the present analysis. Higher levels of inflammatory markers such as IL-6, IL-1β, and TNF-α in obesity and T2DM have been shown previously.[6,11] It also appears that CYP3A is more susceptible to downregulation by inflammatory markers such as IL-6 compared to other CYP enzymes.[39] Yet, none of the inflammatory markers in the present study could explain the variability in CYP3A activity. Nevertheless, inflammation may play an important role in the downregulation of CYP3A with increasing BMI.

Our results also indicate that T2DM does not impact the expression and in vivo activity of CYP1A2 and CYP2C9 significantly. The literature to date has been inconclusive with respect to the effect of T2DM on CYP1A2 and CYP2C9 activity, although there have been some indications of an increased activity.[15,40,41] For CYP1A2, the inconsistent findings may be due to different gender distribution, as there are some indications of increased CYP1A2 activity in men.[3]

The major strength of this study is the stratified inclusion of patients with obesity with and without T2DM and a control group of primarily normal weight individuals without T2DM. Hence, we were in the unique position to investigate the effect of T2DM in an obese population, thus limiting the confounding effect of obesity on CYP activities. Another important strength is that we have matched in vivo activity data and proteomics data from liver and intestinal samples. Limitations of this study are, first, that a single-time point metabolic ratio for CYP2C19, CYP2C9, and CYP1A2 does not provide detailed information about pharmacokinetic processes. The absorption rate and extent may differ in the BMI range investigated, and the absorption may be delayed in patients with diabetes.[42,43] Thus, we cannot rule out that this has influenced the metabolic ratios. However, our data on oral midazolam did not indicate any difference in absorption rate or lag-time in the present study. Second, as the protein concentrations from the biopsies may not be entirely representative of the CYP content in the whole organ (liver and intestine) this complicates in vitro–in vivo extrapolation.[44] Third, an important limitation of this study includes not adjusting for multiple testing, which may increase the likelihood of type 1 errors. Also, no sample size calculation was performed due to the exploratory nature of the study. Hence, the study may not have been powered to detect a statistically significant difference (alpha = 0.05), thus increasing the likelihood of type 2 errors. Fourth, the patients with obesity (with and without T2DM) were subjected to a 3-week low-energy diet which may have influenced the results. However, the results from the subanalysis showed that the diet intervention had no effect on CYP3A and CYP2C9 activities, whereas the effect on CYP1A2 activity was minor, but statistically significant. The diet intervention had a larger impact on CYP2C19 activity in the patients with obesity without T2DM than the patients with both obesity and T2DM. Thus, the difference in the median 5-OH-omeprazole/omeprazole ratio was numerically larger after the diet than prior to the diet (63% vs. 55%). Accordingly, we do not believe that the diet intervention has influenced the results to any significant degree. Finally, we used surrogate measures of NAFLD, and hence it was not possible to determine any degree of steatosis, and we only measured systemic inflammation, not local inflammation in the liver.

In conclusion, in vivo CYP2C19 activity was lower in patients with T2DM and obesity compared with patients with obesity only, while there were no differences in CYP3A, CYP1A2, and CYP2C9 in vivo activities and protein expressions. Thus, the effect of T2DM on CYP activities is isoform-specific. It also appears that CYP2C19 activity is downregulated in patients with NAFLD. These findings should be borne in mind when prescribing drugs dependent on CYP2C19-mediated metabolism in patients with T2DM and obesity.

AUTHOR CONTRIBUTIONS

K.E.K. and I.R wrote the manuscript. K.E.K., I.R., J.H., A.Å., S.A., C.K., H.C., E.S., R.S., and R.J.-L. designed the research. I.R., L.K.J., J.K.H, P.A., and C.W. performed the research. K.E.K. and I.R. analyzed the data.

ACKNOWLEDGMENTS

The authors would like to thank the participants, the surgical staff, and the study personnel working on the COCKTAIL study at Vestfold Hospital Trust. The authors also thank the Swedish Research Council (Approval Numbers 5715 and 01951) (C.W. and P.A.) for supporting the proteomics analyses.

FUNDING INFORMATION

Study funding was received from Vestfold Hospital Trust, Norway; Department of Pharmacy, University of Oslo, Norway; and AstraZeneca, Sweden.

CONFLICT OF INTEREST

C.K., S.A., and R.J.-L. are AstraZeneca employees and own shares in AstraZeneca, while C.W. is a former AstraZeneca employee. All other authors declared no competing interests for this work.

ORCID

Kine Eide Kvitne https://orcid.org/0000-0001-8118-7660
Anders Åsberg https://orcid.org/0000-0002-0628-1769
Cecilia Karlsson https://orcid.org/0000-0002-4299-8775

REFERENCES

1. Williams JA, Hyland R, Jones BC, et al. Drug–drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUCi/AUC) ratios. *Drug Metab Dispos*. 2004;32(11):1201-1208. doi:10.1124/dmd.104.000794
2. Rendic S, Guengerich FP. Survey of human oxidoreductases and cytochrome P450 enzymes involved in the metabolism of xenobiotic and natural chemicals. *Chem Res Toxicol*. 2015;28(1):38-42. doi:10.1021/tx500444e
3. Zanger UM, Schwab M. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther*. 2013;138(1):103-141. doi:10.1016/j.pharmthera.2012.12.007
4. Paine MF, Hart HL, Ludington SS, Haining RL, Rettie AE, Zeldin DC. The human intestinal cytochrome P450 "pie". *Drug Metab Dispos*. 2006;34(5):880-886. doi:10.1124/dmd.105.008672
5. Morgan ET, Goralski KB, Piquette-Miller M, et al. Regulation of drug-metabolizing enzymes and transporters in infection, inflammation, and cancer. *Drug Metab Dispos*. 2008;36(2):205-216. doi:10.1124/dmd.107.018747
6. Zatterale F, Longo M, Naderi J, et al. Chronic adipose tissue inflammation linking obesity to insulin resistance and type 2 diabetes. *Front Physiol*. 2019;10:1607. doi:10.3389/fphys.2019.01607
7. Lingvay I, Sumithran P, Cohen RV, le Roux CW. Obesity management as a primary treatment goal for type 2 diabetes: time to reframe the conversation. *Lancet*. 2022;399(10322):394-405. doi:10.1016/s0140-6736(21)01919-x
8. Stefan N, Cusi K. A global view of the interplay between non-alcoholic fatty liver disease and diabetes. *Lancet Diabetes Endocrinol*. 2022;10(4):284-296. doi:10.1016/s2213-8587(22)00003-1
9. Seeberg KA, Borgeraas H, Hofsø D, et al. Gastric bypass versus sleeve gastrectomy in type 2 diabetes: effects on hepatic steatosis and fibrosis: a randomized controlled trial. *Ann Intern Med*. 2022;175(1):74-83. doi:10.7326/m21-1962
10. Donath MY, Shoelson SE. Type 2 diabetes as an inflammatory disease. *Nat Rev Immunol*. 2011;11(2):98-107. doi:10.1038/nri2925
11. Rohm TV, Meier DT, Olefsky JM, Donath MY. Inflammation in obesity, diabetes, and related disorders. *Immunity*. 2022;55(1):31-55. doi:10.1016/j.immuni.2021.12.013
12. Dostalek M, Sam WJ, Paryani KR, Macwan JS, Gohh RY, Akhlaghi F. Diabetes mellitus reduces the clearance of atorvastatin lactone: results of a population pharmacokinetic analysis in renal transplant recipients and in vitro studies using human liver microsomes. *Clin Pharmacokinet*. 2012;51(9):591-606. doi:10.2165/11632690-000000000-00000
13. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, et al. Platelet function profiles in patients with type 2 diabetes and coronary artery disease on combined aspirin and clopidogrel treatment. *Diabetes*. 2005;54(8):2430-2435. doi:10.2337/diabetes.54.8.2430
14. Akhlaghi F, Dostalek M, Falck P, et al. The concentration of cyclosporine metabolites is significantly lower in kidney transplant recipients with diabetes mellitus. *Ther Drug Monit*. 2012;34(1):38-45. doi:10.1097/FTD.0b013e318241ac71
15. Gravel S, Chiasson JL, Turgeon J, Grangeon A, Michaud V. Modulation of CYP450 activities in patients with type 2 diabetes. *Clin Pharmacol Ther*. 2019;106(6):1280-1289. doi:10.1002/cpt.1496
16. Kvitne KE, Robertsen I, Skovlund E, et al. Short- and long-term effects of body weight loss following calorie restriction and gastric bypass on CYP3A-activity – a non-randomized three-armed controlled trial. *Clin Transl Sci*. 2022;15(1):221-233. doi:10.1111/cts.13142
17. Brill MJ, van Rongen A, Houwink AP, et al. Midazolam pharmacokinetics in morbidly obese patients following semi-simultaneous oral and intravenous administration: a comparison with healthy volunteers. *Clin Pharmacokinet*. 2014;53(10):931-941. doi:10.1007/s40262-014-0166-x
18. Brill MJ, Välitalo PA, Darwich AS, et al. Semiphysiologically based pharmacokinetic model for midazolam and CYP3A mediated metabolite 1-OH-midazolam in morbidly obese and weight loss surgery patients. *CPT Pharmacometrics Syst Pharmacol*. 2016;5(1):20-30. doi:10.1002/psp4.12048
19. Rodríguez-Morató J, Goday A, Langohr K, et al. Short- and medium-term impact of bariatric surgery on the activities of CYP2D6, CYP3A4, CYP2C9, and CYP1A2 in morbid obesity. *Sci Rep*. 2019;9(1):20405. doi:10.1038/s41598-019-57002-9
20. Kvitne KE, Krogstad V, Wegler C, et al. Short- and long-term effects of body weight, calorie restriction and gastric bypass on CYP1A2, CYP2C19 and CYP2C9 activity. *Br J Clin Pharmacol*. 2022;88:4121-4133.
21. Woolsey SJ, Mansell SE, Kim RB, Tirona RG, Beaton MD. CYP3A activity and expression in nonalcoholic fatty liver disease. *Drug Metab Dispos*. 2015;43(10):1484-1490. doi:10.1124/dmd.115.065979
22. Hjelmesaeth J, Asberg A, Andersson S, et al. Impact of body weight, low energy diet and gastric bypass on drug bioavailability, cardiovascular risk factors and metabolic biomarkers: protocol for an open, non-randomised, three-armed single centre study (COCKTAIL). *BMJ Open*. 2018;8(5):e021878. doi:10.1136/bmjopen-2018-021878
23. Krogstad V, Peric A, Robertsen I, et al. A comparative analysis of cytochrome P450 activities in paired liver and small intestinal samples from patients with obesity. *Drug Metab Dispos*. 2020;48(1):8-17. doi:10.1124/dmd.119.087940
24. Krogstad V, Peric A, Robertsen I, et al. Correlation of body weight and composition with hepatic activities of cytochrome P450 enzymes. *J Pharm Sci*. 2021;110(1):432-437. doi:10.1016/j.xphs.2020.10.027
25. Neely MN, van Guilder MG, Yamada WM, Schumitzky A, Jelliffe RW. Accurate detection of outliers and subpopulations with Pmetrics, a nonparametric and parametric pharmacometric modeling and simulation package for R. *Ther Drug Monit*. 2012;34(4):467-476. doi:10.1097/FTD.0b013e31825c4ba6

26. R Core Team. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing; 2021.
27. Egeland EJ, Witczak BJ, Zaré HK, Christensen H, Åsberg A, Robertsen I. Chronic inhibition of CYP3A is temporarily reduced by each hemodialysis session in patients with end-stage renal disease. *Clin Pharmacol Ther*. 2020;108(4):866-873. doi:10.1002/cpt.1875
28. Gjestad C, Huynh DK, Haslemo T, Molden E. 4β-Hydroxycholesterol correlates with dose but not steady-state concentration of carbamazepine: indication of intestinal CYP3A in biomarker formation? *Br J Clin Pharmacol*. 2016;81(2):269-276. doi:10.1111/bcp.12833
29. Størset E, Hole K, Midtvedt K, Bergan S, Molden E, Åsberg A. The CYP3A biomarker 4β-hydroxycholesterol does not improve tacrolimus dose predictions early after kidney transplantation. *Br J Clin Pharmacol*. 2017;83(7):1457-1465. doi:10.1111/bcp.13248
30. Wegler C, Wiśniewski JR, Robertsen I, et al. Drug disposition protein quantification in matched human jejunum and liver from donors with obesity. *Clin Pharmacol Ther*. 2022;111:1142-1154.
31. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985;28(7):412-419. doi:10.1007/bf00280883
32. Kotronen A, Peltonen M, Hakkarainen A, et al. Prediction of non-alcoholic fatty liver disease and liver fat using metabolic and genetic factors. *Gastroenterology*. 2009;137(3):865-872. doi:10.1053/j.gastro.2009.06.005
33. Erlinge D, Varenhorst C, Braun OO, et al. Patients with poor responsiveness to thienopyridine treatment or with diabetes have lower levels of circulating active metabolite, but their platelets respond normally to active metabolite added ex vivo. *J Am Coll Cardiol*. 2008;52(24):1968-1977. doi:10.1016/j.jacc.2008.07.068
34. Schuette C, Steffens D, Witkowski M, et al. The effect of clopidogrel on platelet activity in patients with and without type-2 diabetes mellitus: a comparative study. *Cardiovasc Diabetol*. 2015;14:15. doi:10.1186/s12933-015-0182-7
35. Dostalek M, Court MH, Yan B, Akhlaghi F. Significantly reduced cytochrome P450 3A4 expression and activity in liver from humans with diabetes mellitus. *Br J Pharmacol*. 2011;163(5):937-947. doi:10.1111/j.1476-5381.2011.01270.x
36. Ulvestad M, Skottheim IB, Jakobsen GS, et al. Impact of OATP1B1, MDR1, and CYP3A4 expression in liver and intestine on interpatient pharmacokinetic variability of atorvastatin in obese subjects. *Clin Pharmacol Ther*. 2013;93(3):275-282. doi:10.1038/clpt.2012.261
37. Woolsey SJ, Beaton MD, Choi YH, et al. Relationships between endogenous plasma biomarkers of constitutive cytochrome P450 3A activity and single-time-point oral midazolam microdose phenotype in healthy subjects. *Basic Clin Pharmacol Toxicol*. 2016;118(4):284-291. doi:10.1111/bcpt.12492
38. Gravel S, Panzini B, Belanger F, Turgeon J, Michaud V. A pilot study towards the impact of type 2 diabetes on the expression and activities of drug metabolizing enzymes and transporters in human duodenum. *Int J Mol Sci*. 2019;20(13):3257. doi:10.3390/ijms20133257
39. de Jong LM, Jiskoot W, Swen JJ, Manson ML. Distinct effects of inflammation on cytochrome P450 regulation and drug metabolism: lessons from experimental models and a potential role for pharmacogenetics. *Genes (Basel)*. 2020;11(12):1509. doi:10.3390/genes11121509
40. Urry E, Jetter A, Landolt HP. Assessment of CYP1A2 enzyme activity in relation to type-2 diabetes and habitual caffeine intake. *Nutr Metab (Lond)*. 2016;13:66. doi:10.1186/s12986-016-0126-6
41. Adithan C, Sriram G, Swaminathan RP, Krishnan M, Bapna JS, Chandrasekar S. Effect of type II diabetes mellitus on theophylline elimination. *Int J Clin Pharmacol Ther Toxicol*. 1989;27(5):258-260.
42. Dostalek M, Akhlaghi F, Puzanovova M. Effect of diabetes mellitus on pharmacokinetic and pharmacodynamic properties of drugs. *Clin Pharmacokinet*. 2012;51(8):481-499. doi:10.2165/11631900-000000000-00000
43. Smit C, De Hoogd S, Brüggemann RJM, Knibbe CAJ. Obesity and drug pharmacology: a review of the influence of obesity on pharmacokinetic and pharmacodynamic parameters. *Expert Opin Drug Metab Toxicol*. 2018;14(3):275-285. doi:10.1080/17425255.2018.1440287
44. Wegler C, Matsson P, Krogstad V, et al. Influence of proteome profiles and intracellular drug exposure on differences in CYP activity in donor-matched human liver microsomes and hepatocytes. *Mol Pharm*. 2021;18(4):1792-1805. doi:10.1021/acs.molpharmaceut.1c00053

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Kvitne KE, Åsberg A, Johnson LK, et al. Impact of type 2 diabetes on in vivo activities and protein expressions of cytochrome P450 in patients with obesity. *Clin Transl Sci*. 2022;00:1-12. doi:10.1111/cts.13394

Supplementary Information

Impact of type 2 diabetes on *in vivo* activities and protein expressions of cytochrome P450 in patients with obesity

Clinical and Translational Science

Kine Eide Kvitne[1], Anders Åsberg[1,2], Line K. Johnson[3], Christine Wegler[4,5], Jens K. Hertel[3], Per Artursson[6], Cecilia Karlsson[7,8], Shalini Andersson[9], Rune Sandbu[3,10], Eva Skovlund[11], Hege Christensen[1], Rasmus Jansson-Löfmark[5], Jøran Hjelmesæth[3,12], Ida Robertsen[1]

CORRESPONDING AUTHOR

Kine Eide Kvitne

Department of Pharmacy, University of Oslo, P.O. Box 1068 Blindern, 0316 Oslo, Norway

E-mail: k.e.kvitne@farmasi.uio.no

Table S1. Univariate regression analysis of covariates. Values are presented as β ± SE (*P* value).

Covariates	**CYP2C19** (log 5-OH-omeprazole/ omeprazole)	**CYP3A** (log MDZ absolute bioavailability, %)	**CYP3A** (log MDZ systemic clearance, L/h)	**CYP3A** (log 4βOHC, ng/mL)	**CYP1A2** (log paraxanthine/ caffeine)	**CYP2C9** (log losartan/LCA)
BMI (kg/m^2)	-0.013 ± 0.015 (0.40)	0.029 ± 0.008 (<0.001)	0.016 ± 0.007 (0.017)	-0.026 ± 0.005 (<0.001)	-0.006 ± 0.005 (0.19)	-0.010 ± 0.007 (0.16)
T2DM (yes)	-0.93 ± 0.26 (<0.001)	0.44 ± 0.16 (0.006)	0.16 ± 0.13 (0.19)	-0.14 ± 0.11 (0.21)	0.058 ± 0.089 (0.52)	-0.19 ± 0.13 (0.15)
NAFLD (yes)	-1.04 ± 0.24 (<0.001)	0.40 ± 0.15 (0.009)	0.17 ± 0.12 (0.16)	-0.38 ± 0.10 (<0.001)	-0.054 ± 0.086 (0.53)	-0.32 ± 0.12 (0.012)
Log IFN-γ (pg/mL)	0.20 ± 0.10 (0.056)	0.019 ± 0.060 (0.76)	0.044 ± 0.046 (0.34)	-0.007 ± 0.041 (0.87)	0.028 ± 0.033 (0.41)	0.046 ± 0.050 (0.36)
Log IL-1β (pg/mL)	0.062 ± 0.067 (0.36)	0.013 ± 0.039 (0.73)	0.051 ± 0.029 (0.087)	0.029 ± 0.027 (0.28)	0.011 ± 0.022 (0.61)	0.039 ± 0.032 (0.23)
Log IL-6 (pg/mL)	-0.05 ± 0.096 (0.58)	0.055 ± 0.055 (0.32)	0.034 ± 0.042 (0.42)	-0.033 ± 0.039 (0.40)	0.034 ± 0.031 (0.28)	0.017 ± 0.046 (0.72)
Log TNF-α (pg/mL)	0.077 ± 0.11 (0.48)	0.053 ± 0.062 (0.40)	0.090 ± 0.047 (0.056)	0.019 ± 0.04 (0.66)	0.069 ± 0.034 (0.046)	0.074 ± 0.052 (0.15)

Abbreviations: BMI, body mass index; CYP, cytochrome P450; IFN-γ, interferon-γ; IL, interleukin; LCA, losartan carboxylic acid; MDZ, midazolam; NAFLD, nonalcoholic fatty liver disease; TNF-α, tumor necrosis factor-α; T2DM, type 2 diabetes mellitus; 4βOHC, 4β-hydroxycholesterol; 5-OH-omeprazole, 5-hydroxyomeprazol

IV

European Journal of Clinical Pharmacology
https://doi.org/10.1007/s00228-022-03336-9

PHARMACOKINETICS AND DISPOSITION

Correlations between 4β-hydroxycholesterol and hepatic and intestinal CYP3A4: protein expression, microsomal ex vivo activity, and in vivo activity in patients with a wide body weight range

Kine Eide Kvitne[1] · Kristine Hole[2,3] · Veronica Krogstad[1] · Birgit Malene Wollmann[2] · Christine Wegler[4,5] · Line K. Johnson[6] · Jens K. Hertel[6] · Per Artursson[7] · Cecilia Karlsson[8,9] · Shalini Andersson[10] · Tommy B. Andersson[5] · Rune Sandbu[6,11] · Jøran Hjelmesæth[6,12] · Eva Skovlund[13] · Hege Christensen[1] · Rasmus Jansson-Löfmark[5] · Anders Åsberg[1,14] · Espen Molden[1,2] · Ida Robertsen[1]

Received: 6 April 2022 / Accepted: 14 May 2022

Abstract

Purpose Variability in cytochrome P450 3A4 (CYP3A4) metabolism is mainly caused by non-genetic factors, hence providing a need for accurate phenotype biomarkers. Although 4β-hydroxycholesterol (4βOHC) is a promising endogenous CYP3A4 biomarker, additional investigations are required to evaluate its ability to predict CYP3A4 activity. This study investigated the correlations between 4βOHC concentrations and hepatic and intestinal CYP3A4 protein expression and ex vivo microsomal activity in paired liver and jejunum samples, as well as in vivo CYP3A4 phenotyping (midazolam) in patients with a wide body weight range.

Methods The patients ($n=96$; 78 with obesity and 18 normal or overweight individuals) were included from the COCKTAIL-study (NCT02386917). Plasma samples for analysis of 4βOHC and midazolam concentrations, and liver ($n=56$) and jejunal ($n=38$) biopsies were obtained. The biopsies for determination of CYP3A4 protein concentration and microsomal activity were obtained during gastric bypass or cholecystectomy. In vivo CYP3A4 phenotyping was performed using semi-simultaneous oral (1.5 mg) and intravenous (1.0 mg) midazolam.

Results 4βOHC concentrations were positively correlated with hepatic microsomal CYP3A4 activity ($\rho=0.53$, $p<0.001$), and hepatic CYP3A4 concentrations ($\rho=0.30$, $p=0.027$), but not with intestinal CYP3A4 concentrations ($\rho=0.18$, $p=0.28$) or intestinal microsomal CYP3A4 activity ($\rho=0.15$, $p=0.53$). 4βOHC concentrations correlated weakly with midazolam absolute bioavailability ($\rho=-0.23$, $p=0.027$) and apparent oral clearance ($\rho=0.28$, $p=0.008$), but not with systemic clearance ($\rho=-0.03$, $p=0.81$).

Conclusion These findings suggest that 4βOHC concentrations reflect hepatic, but not intestinal, CYP3A4 activity. Further studies should investigate the potential value of 4βOHC as an endogenous biomarker for individual dose requirements of intravenously administered CYP3A4 substrate drugs.

Trial registration Clinical.Trials.gov identifier: NCT02386917.

Keywords 4β-Hydroxycholesterol · CYP3A4 · Midazolam pharmacokinetics · Drug metabolism · Proteomics

Introduction

The cytochrome P450 (CYP) 3A subfamily, consisting mainly of CYP3A4 and the polymorphic CYP3A5 [1], plays a significant role in the metabolism of 30–50% of clinically used drugs [2, 3]. Due to its abundant expression in both the liver and small intestine [4], CYP3A4 contributes significantly to the first-pass and systemic metabolism of substrate drugs. Hence, CYP3A4 is an important determinant for oral bioavailability and systemic clearance and thereby systemic drug exposure. There is substantial inter-individual variability in CYP3A4 activity, mainly due to environmental factors, disease state, and drug-drug interactions, leading to differences in dose requirements between patients. Conditions such as obesity and non-alcoholic fatty liver disease

✉ Kine Eide Kvitne
k.e.kvitne@farmasi.uio.no

Extended author information available on the last page of the article

Published online: 01 June 2022

(NAFLD) are associated with a lower expression and activity of CYP3A4, and several studies suggest that body weight is inversely associated with CYP3A4 activity [5–10]. So far, genetic factors seem to be of limited importance for the substantial variability in CYP3A4 phenotype [11, 12]. Thus, non-genetic biomarkers are warranted to study individual variability in CYP3A4 activity in humans.

The current gold standard method to assess in vivo CYP3A4 activity is midazolam, a selective CYP3A4 phenotypic probe drug in vivo with a short half-life [13–16]. However, since midazolam is a medium-to-high extraction ratio drug, systemic clearance may be affected by changes in protein binding, hepatic blood flow, and intrinsic clearance (CL_{int}), the latter representing metabolic capacity [17–19]. Previous studies have suggested that midazolam pharmacokinetics may be influenced by an increased hepatic blood flow in patients with obesity [20, 21]. Thus, in selected patient populations, the use of midazolam for CYP3A4 phenotyping may be challenging as it does not necessarily reflect CYP3A4 activity. 4β-hydroxycholesterol (4βOHC), a cholesterol metabolite mainly produced by CYP3A4, is proposed as a promising endogenous biomarker for CYP3A4 [22, 23]. There are practical advantages with an endogenous biomarker such as 4βOHC; it is non-invasive, and only a single blood sample is required, making it more convenient for measuring CYP3A4 activity than traditional pharmacokinetic studies with CYP3A4 probe drugs. To assess its suitability as a marker for CYP3A4 phenotype, several studies have investigated the correlation between plasma concentrations of 4βOHC and apparent oral clearance (CL/F) or systemic clearance of midazolam, but the results are conflicting [24–27]. The current literature suggests that 4βOHC concentrations reflect inter-individual variability in CYP3A4 activity and have mainly been suggested as a marker for CYP3A4 induction [28–30]. Still, the long elimination half-life may limit the ability to observe acute changes in CYP3A4 activity [31]. Also, the contribution of intestinal CYP3A4 in the formation of 4βOHC remains unclear [32]. The potential use of 4βOHC as a biomarker for individualized oral dosing of CYP3A4 substrates has thus been debated [33–35].

Additional investigations are required before 4βOHC can be accepted as a validated biomarker. As part of the primary endpoint in the COCKTAIL study [32], we evaluated 4βOHC as an endogenous biomarker for CYP3A4 activity by investigating the correlations between 4βOHC and different CYP3A4 metrics: (1) CYP3A4 expression and (2) ex vivo microsomal CYP3A4 activity in paired liver and jejunum biopsies and (3) absolute bioavailability, apparent oral clearance, and systemic clearance of midazolam in vivo.

Methods

Patients

In total, 96 patients from the open-label, three-armed COCKTAIL-study (NCT02386917) were included in the present analysis [36, 37]. The study population included 78 patients with severe obesity (BMI -> 40 or 35–40 kg/m^2 combined with at least one obesity-related comorbidity) scheduled for weight loss treatment with Roux-en-Y-gastric bypass (RYGB) ($n = 38$) or non-surgical calorie restriction ($n = 40$), and 18 mainly normal to overweight individuals scheduled for cholecystectomy (BMI 18.5–30 kg/m^2). The patients with severe obesity were subjected to a 3-week low-energy diet (< 1200 kcal/day) before the study investigation, whereas the normal to overweight individuals were not subjected to any defined diet beforehand. Medications, including statins, and/or other substances that are known to alter CYP3A4 activity/midazolam pharmacokinetics were discontinued at least seven half-lives before the investigational day, and none of the patients used any CYP3A4 inducers or time-dependent inhibitors. The study was approved by the Regional Committee for Medical and Health Research Ethics (2013/2379/REK) and complied with the Declaration of Helsinki. All patients signed a written informed consent before study participation.

Study investigation

Blood samples for determination of plasma 4βOHC concentrations and a 24-h pharmacokinetic investigation using semi-simultaneous oral and intravenous dosing of midazolam were collected from all patients. From 10:00 p.m. before the investigation, patients abstained from food, drink (except water), and drugs. On the investigational day, patients first met for blood sampling before 1.5 mg oral midazolam syrup was administered, followed by 1.0 mg intravenous midazolam 4 h later. Blood samples for determination of midazolam plasma concentrations were collected from a peripheral venous catheter before and at 0.25, 0.5, 1, 1.5, 2, 3, 4, 4.25, 2.5, 5, 5.5, 6, 8, 10, 12, 23, and 24 h following oral dosing. The pharmacokinetic investigation has been described in more detail previously [37]. In vivo midazolam pharmacokinetic data were available in 92 patients. The day after the study investigation, paired liver and jejunum biopsies were obtained during RYGB ($n = 38$), and liver biopsies were obtained during cholecystectomy ($n = 18$), as previously described [9, 38].

Body composition was measured with Inbody 720, Body Composition Analyzer (Biospace, Korea). The NAFLD liver fat score was calculated as suggested by Kotronen et al. [39]. Standard clinical chemistry analyses were performed in fresh blood samples at the Department of Laboratory Medicine, Vestfold Hospital Trust, Tønsberg, Norway. Plasma concentrations of high-sensitivity C-reactive protein (hs-CRP) were measured using immunoturbidimetry (Advia Chemistry XPT systems, Siemens) at Fürst Medical Laboratory, Oslo, Norway. Blood samples for determination of 4βOHC and midazolam concentrations were drawn in K2-EDTA vacutainer tubes on ice and centrifuged for 10 min at 4 °C (1,800 g). Plasma was separated into Cryovials and frozen within 1 h at − 70 °C until analysis.

Bioanalytical assays

4βOHC

4βOHC plasma concentrations were determined by a previously described assay [40] with an added filtration step [41]. In short, 4βOHC was de-esterified from fatty acids by ethanolic sodium methoxide and isolated from plasma by liquid–liquid extraction with hexane. Extracts were evaporated by nitrogen and reconstituted in methanol before filtration. After methanol reconstitution, the samples were frozen at − 20 °C for 15 min, and filtered in specialized Eppendorf filter tubes (Costar Spin-x HPLC Micro Centrifuge Filter, 0,2 µM Nylon Filter) through centrifugation (2,000 g for 6 min at 2 °C). The filtered extract was used for UPLC-MS/MS analysis. An Acquity ultra-performance liquid chromatograph (UPLC) followed by a Micromass Quattro micro tandem mass spectrometry (MS) detector (Waters, Milford, MA) was used for quantitative analysis. Chromatographic separation was achieved on a BEH C18 column RP-shield (1.7 µm, 1 × 100 mm) from Waters with a mobile phase of water and methanol. After atmospheric pressure chemical ionization, detection was obtained with multiple reaction monitoring at m/z 385.25 -> 367.45 (4βOHC) and m/z 392.30 -> 374.50 (4βOHC-D7; internal standard). The lower limit of quantification (LLOQ) was 10 ng/mL. Intra- and interday imprecision and inaccuracy were < 15% at 10 ng/mL and < 4% at 644 ng/mL ($n = 6$) [40]. All samples were stored at − 70 °C between sampling and analysis.

Midazolam

The plasma concentrations of midazolam were determined by liquid–liquid sample extraction followed by a previously validated UHPLC-MS/MS method [42]. The sample preparation in short, 50 ng/mL internal standard (deuterated midazolam (MDZ-d6)), was added to a 5-mL Eppendorf tube, evaporated to dryness by nitrogen gas followed by subsequent addition of 250 µL of sample plasma. Aqueous ammonia (0.5 M) was added to the tubes in a 1:1 ratio (250 µL) and agitated prior to the addition of the extracting solvent (1.0 mL of ethyl acetate). The sample was mixed for 10 min (Invitrogen Hulamixer, Thermo Fischer, Scientific Inc) prior to being centrifuged at 2,500 g for 10 min at room temperature (Heraeus Megafuge 16R-centrifuge, Thermo Fisher Scientific Inc). The extraction was carried out twice before the organic phase was transferred to a new Eppendorf tube, evaporated to dryness under nitrogen (60 °C), and subsequently reconstituted in 50 µL 5% ACN in 0.05 M ammonium acetate buffer (pH 4.4). The sample was mixed and transferred to micro vials before UHPLC-MS/MS analysis.

The UHPLC-MS/MS method was carried out in two labs due to limited capacity. The instrument in lab 1 was a Thermo Fisher Scientific Vanquish HPLC coupled to a Thermo Fisher Accucore (C18 2.1 × 50 mm) analytical column and a Thermo Fisher Scientific Altis QqQ MS. In lab 2, a Waters (Waters, Milford, MA) Acquity UPLC with a BEH C18 RP-shield (1.7 µm, 1 × 100 mm) analytical column coupled to a Micromass Quattro micro tandem MS was used. The chromatography was carried out with a flow rate of 0.4 mL/min and 0.2 mL/min and gradient elution for lab 1 and lab 2, respectively. Mobile phase A was 5% acetonitrile in 10 mM ammonium formate, and mobile phase B was 90% acetonitrile and 10% methanol. Positive electrospray ionization and selected reaction monitoring were performed with compound-tuned conditions. For both labs, acquisition was carried out by the MRM transitions m/z 326.1 -> m/z 249.1 and m/z 326.1 -> m/z 291.2 for midazolam, and m/z 332.1 -> m/z 252.1 and m/z 332.1 -> m/z 203.1 for midazolam-d6 in positive mode.

Inter-laboratory cross-validation was performed using clinical study samples, and the bias was within ± 12.5%. Calibrators and quality control (QC) samples were prepared in blank plasma and analyzed in each analytical run. Eight calibrators in the range of 0.1 to 20 ng/mL were applied, and back-calculated values of calibrators within 80 to 120% were accepted. LLOQ was 0.1 ng/mL, and the upper limit of quantification (ULOQ) was 20 ng/mL. Samples with midazolam concentrations above ULOQ were diluted in blank plasma and reanalyzed. Dilution integrity with dilution factors of 1/2, 1/5, 1/10, 1/20, and 1/50 was established; mean accuracy ranged from 88.9 to 103.8%, and the imprecision was < 4.5%. Within-series and between-series performance were assessed with the resulting coefficient of variation < 12.3%, and the mean accuracy ranged from 99.3 to 104.3%.

Liver and jejunum biopsies

The biopsies were transferred into individual cryotubes, snap-frozen in liquid nitrogen directly upon sampling, and stored at − 80 °C until analysis.

CYP3A4 protein quantification

Proteins were extracted from liver and jejunum biopsies in an SDS-containing (2% w/v) lysis buffer and quantified as previously described [43]. In short, samples were processed with the multi-enzyme digestion filter-aided sample preparation protocol, using LysC and trypsin [44]. Proteomics analysis was performed with Q Exactive HF or Q Exactive HF-X. MS data were processed with MaxQuant (version 1.6.10.43) [45], using the human UniProtKB. Spectral raw intensities were normalized with variance stabilization [46] and were subsequently used to calculate the protein concentrations using the total protein approach [47].

Preparation of microsomes and ex vivo CYP3A4 activity assay

Methods for quantifying CYP3A4 activity in liver and jejunum biopsies have been described previously [38]. In brief, liver and jejunum biopsies were prepared with a Potter–Elvehjem homogenizer, and differential centrifugation was used to isolate microsomal fractions. Midazolam was used as a probe drug and the formation of the metabolite 1′-hydroxymidazolam as a probe reaction for CYP3A4 activity. Activity incubation was carried out at 37 °C for 20 min, and midazolam was added in eight different concentrations. The incubation was terminated using ice-cold acetonitrile containing internal standard. Quantification of 1′-hydroxymidazolam was performed using a triple quadrupole mass spectrometer coupled to an ACQUITY UPLC I-class system (Waters XevoTM TQ-S; Waters Corporation, Milford, MA) equipped with an electrospray ionization source as previously described [38].

Genotype analyses

CYP3A4 and *CYP3A5* variant alleles were analyzed using Taqman-based real-time polymerase chain reaction assays implemented for routine pharmacogenetic analyses at the Center for Psychopharmacology, Diakonhjemmet Hospital, Norway. *CYP3A4*, the reduced function allele **22* (rs35599367), and *CYP3A5*, the *null* allele **3* (rs776746), were included in the analysis. All alleles were in Hardy–Weinberg equilibrium.

Pharmacokinetic calculations

In vivo absolute bioavailability, apparent oral clearance, and systemic clearance of midazolam from the pharmacokinetic investigation were determined using a previously developed population pharmacokinetic model [37]. Briefly, the modeling was performed using the non-parametric adaptive grid approach implemented in Pmetrics (version 1.5.2) for R (version 3.6.2) [48, 49]. A catenary three-compartment model with absorption lag-time and first-order elimination from the central compartment described the data adequately. The enzyme kinetic parameters from the ex vivo CYP3A4 activity investigation were determined using untransformed data and GraphPad prism 7 by fitting the reaction velocity versus the substrate concentration data to the Michaelis–Menten model or the substrate inhibition model, as previously described [38]. The Michaelis constant (K_m) values were adjusted for the fraction of unbound drug in microsomes, which was predicted from the physicochemical properties of the substrates and microsomal protein concentration using the Simcyp prediction tool (https://members.simcyp.com/account/tools/fumic). The unbound CL_{int} ($CL_{int,u}$) was calculated from the ratio of maximum velocity (V_{max}) to the unbound K_m. Due to limited capacity, the hepatic and jejunum microsomal $CL_{int,u}$ were determined in 20 RYGB patients.

Data and statistical analysis

Neither 4βOHC nor most of the other variables passed the normality tests (including the Shapiro–Wilk test and visual inspection of plots), and non-parametric statistical tests were therefore used in the statistical analyses. Spearman's rank-order correlation test was used to describe the rank-based measure of association. Wilcoxon rank-sum test was used to compare patients with obesity with normal to overweight individuals. A p value < 0.05 was considered statically significant. Data are presented as median difference (95% confidence interval (CI)) if not otherwise stated. Demographic data are presented as mean ± standard deviation (SD). All statistical analyses were performed using R for Windows (version 4.1.2) [49].

Results

Patient characteristics

Patient characteristics at the investigational day are given in Table 1. The patients had a mean age of 46 ± 11 years, 70% were women, and 98% were Caucasian. The majority (94%) were genotypic normal CYP3A4 metabolizers

Table 1 Patient characteristics at the study investigation. Data are described as mean ± SD or absolute numbers (%)

	Patients with obesity ***n*** **= 78**	**Normal to overweight individuals** ***n*** **= 18**
Age (years)	47 ± 10	42 ± 15
Sex (male/female)	26/52	3/15
Body weight (kg)	121 ± 22	71 ± 11
BMI (kg/m^2)	41 ± 5.8	25 ± 3.5
Total cholesterol (mmol/L)	3.9 ± 0.95	4.4 ± 0.87
Triglycerides (mmol/L)	1.3 ± 0.59	0.93 ± 0.47
LDL (mmol/L)	2.4 ± 0.82	2.5 ± 0.77
HDL (mmol(L)	0.96 ± 0.20	1.5 ± 0.36
hs-CRP (mg/L)	5.7 ± 6.0	2.5 ± 3.8
ALT (U/L)	39 ± 21	22 ± 15
NAFLD liver fat score	1.1 ± 2.0	− 1.7 ± 1.1
CYP3A4 genotype (likely phenotype)		
**1/*1* (normal metabolizer)	74 (95%)	16 (89%)
**1/*22* (intermediate metabolizer)	4 (5%)	2 (11%)
CYP3A5 genotype (likely phenotype)		
**1/*3* (intermediate metabolizer)	7 (9%)	3 (17%)
**3/*3* (poor metabolizer)	71 (91%)	15 (83%)

ALT alanine aminotransferase, *BMI* body mass index, *CYP* cytochrome P450, *HDL* high-density lipoproteins, *hs-CRP* high-sensitivity C-reactive protein, *LDL* low-density lipoproteins, *NAFLD* non-alcoholic fatty liver disease

(*CYP3A4 *1/*1*), and 10% also expressed functional CYP3A5 (*CYP3A5 *1/*3*). In line with the inclusion criteria, mean BMI was higher in patients with obesity compared with normal to overweight individuals. Patients with obesity had lower serum total cholesterol compared with the normal to overweight individuals (− 0.60 mmol/L [95% CI: − 1.0, − 0.00]) but higher NAFLD liver fat score (2.8 [95% CI: 1.9, 3.6]) and hs-CRP (2.1 mg/L [95% CI: 1.1, 3.9]). Twenty percent of the patients received treatment with cholesterol-lowering drugs (mainly statins) (patients with obesity = 19, normal to overweight = 0).

Correlations between 4βOHC and CYP3A4 metrics

4βOHC concentrations were positively correlated with both hepatic CYP3A4 concentrations ($\rho = 0.30$, $p = 0.027$) and hepatic microsomal $CL_{int,u}$ ($\rho = 0.53$, $p < 0.001$), but not with systemic midazolam clearance ($\rho = -0.03$, p = 0.81) (Fig. 1). The lack of correlation between 4βOHC concentrations and systemic midazolam clearance was also shown when the patients with obesity and the normal to overweight individuals were analyzed separately (Supplementary Fig. S1). Hepatic microsomal activity was significantly correlated with hepatic CYP3A4 concentrations ($\rho = 0.51$, $p = 0.001$), but no correlation was observed between systemic midazolam clearance and hepatic microsomal $CL_{int,u}$ (Supplementary Fig. S2). 4βOHC concentrations were not significantly correlated with intestinal CYP3A4 concentrations or intestinal microsomal $CL_{int,u}$ (Fig. 2c and d). However, 4βOHC plasma concentrations showed an inverse correlation with absolute bioavailability of midazolam ($\rho = -0.23$, $p = 0.027$), and a positive correlation with apparent oral clearance ($\rho = 0.28$, $p = 0.008$) (Fig. 2a and b). Absolute values of the different CYP3A metrics are reported in Supplementary Table S1.

In the patients with obesity, neither 4βOHC concentrations, systemic midazolam clearance, nor absolute bioavailability changed during the 3-week low-energy diet period (Supplementary Fig. S3). However, due to the low-fat low-energy diet and weight loss, the total cholesterol was lowered in this period. All correlations were thus repeated using the 4βOHC/cholesterol ratio (4βOHC/C). The results from these analyses showed a similar pattern (Supplementary Figs. S4 and S5).

Impact of genotype and patient characteristics on 4βOHC

An analysis of 4βOHC concentrations based on genotype did not indicate elevated 4βOHC in the individuals expressing *CYP3A5 *1/*3* compared with *CYP3A5 *3/*3* expressors, neither in normal to overweight individuals ($p = 0.95$) nor in patients with obesity ($p = 0.33$) (Supplementary Fig. 6a and b). However, 4βOHC concentrations were lower in patients with obesity expressing *CYP3A4 *1/*22* compared

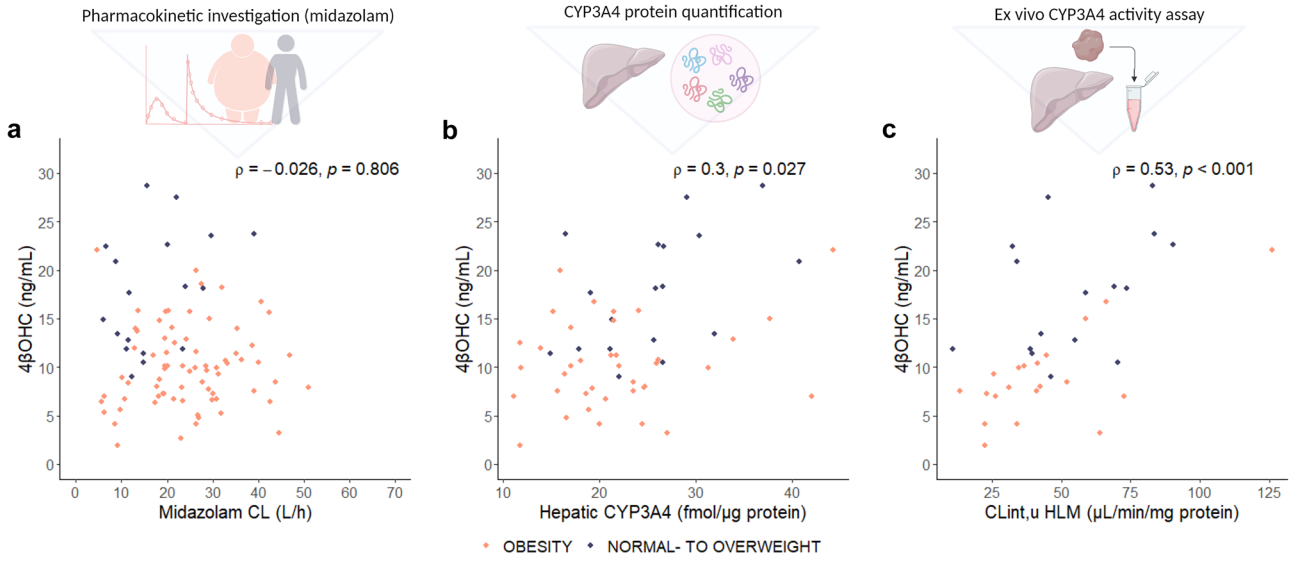

Fig. 1 Hepatic CYP3A4 metrics. Correlation between (**a**) systemic midazolam clearance (CL) and 4βOHC concentrations[a], (**b**) hepatic CYP3A4 concentration[b] and 4βOHC concentrations, and (**c**) clearance intrinsic for midazolam 1'-hydroxylation in human liver microsomes[c] and 4βOHC concentrations. Spearman's rho (ρ) is the correlation coefficient, and the p value is from the Spearman rank correlation analysis. [a]Available in 92 patients (patients with obesity = 74, normal to overweight individiuals = 18). [b]Available in 56 patients (patients with obesity = 38, normal to overweight individuals = 18). [c]Available in 36 patients (RYGB = 20, normal to overweight individuals = 16). *Abbreviations: $CL_{int,u,}$ clearance intrinsic unbound; CYP, cytochrome P450; HLM, human liver microsomes; 4βOHC, 4-beta hydroxycholesterol*

with *CYP3A4 *1/*1* ($p = 0.024$), whereas in the normal to overweight individuals, the difference did not reach statistical significance ($p = 0.12$) (Supplementary Fig. S6c and d). BMI was inversely correlated with 4βOHC concentrations: $\rho = -0.39$ ($p < 0.001$) (Fig. 3a), and median 4βOHC was 1.9-fold higher in normal to overweight individuals compared with patients with obesity (median difference 7.4 ng/mL [95% CI: 4.4, 11]) (Supplementary Fig. S7a). Both NAFLD liver fat score and hs-CRP were inversely correlated with 4βOHC: $\rho = -0.35$ ($p < 0.001$) and $\rho = -0.23$ ($p = 0.027$), respectively (Fig. 3b and c). Absolute bioavailability and systemic clearance of midazolam were 2.8-fold and 1.6-fold higher in the patients with obesity compared with the normal to overweight individuals (13% [95% CI:

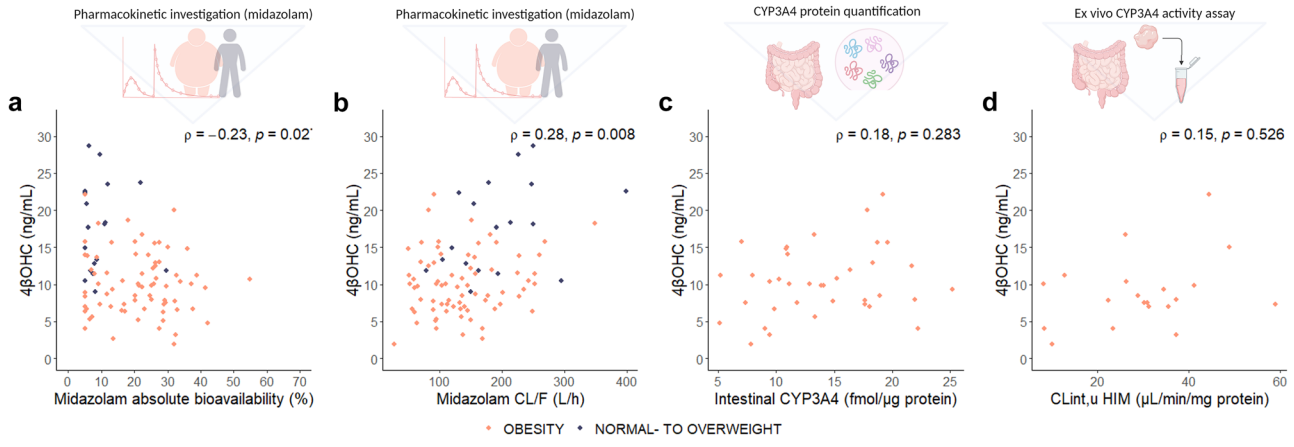

Fig. 2 Intestinal CYP3A4 metrics. Correlation between (**a**) midazolam absolute bioavailability[a] and 4βOHC concentrations, (**b**) apparent oral midazolam clearance (CL/F)[a] and 4βOHC concentrations, (**c**) jejunum CYP3A4 concentration[b] and 4βOHC concentrations, and (**d**) clearance intrinsic for midazolam 1′-hydroxylation in human intestinal microsomes[c] and 4βOHC concentrations. Spearman's rho (ρ) is the correlation coefficient, and the p value is from the Spearman rank correlation analysis. [a]Available in 92 patients (patients with obesity = 74, normal to overweight individuals = 18). [b]Available in 37 RYGB-patients. [c]Available in 20 RYGB-patients. *Abbreviations: $CL_{int,u,}$ clearance intrinsic unbound; CYP, cytochrome P450; HIM, human intestinal microsomes; 4βOHC, 4-beta hydroxycholesterol*

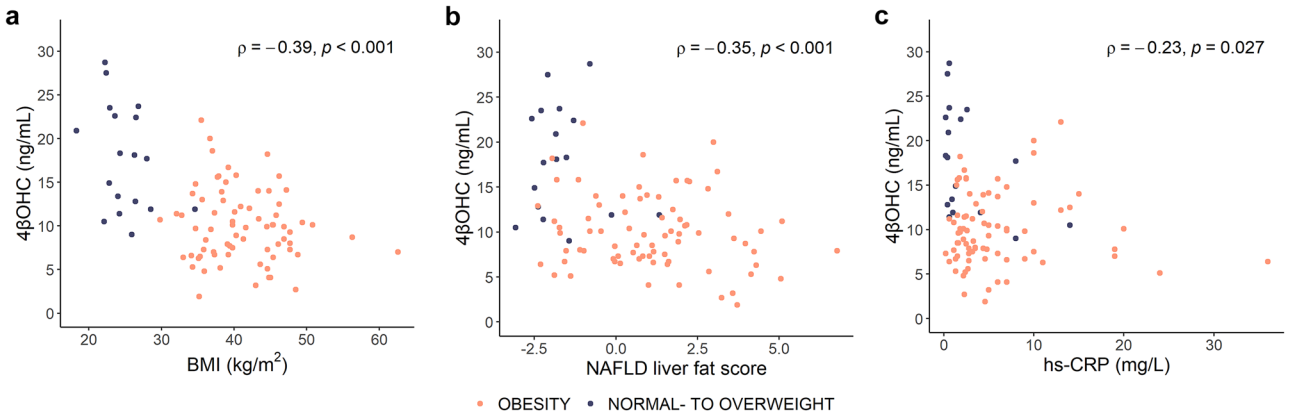

Fig. 3 Clinical variables and 4βOHC. Correlation between (**a**) BMI and 4βOHC concentrations ($n=96$), (**b**) NAFLD liver fat score and 4βOHC concentrations ($n=95$), and (**c**) hs-CRP and 4βOHC concentrations ($n=96$). Spearman's rho (ρ) is the correlation coefficient, and the p value is from the Spearman rank correlation analysis. *Abbreviations: BMI, body mass index; hs-CRP, high-sensitivity C-reactive protein; NAFLD, non-alcoholic fatty liver disease; 4βOHC, 4-beta hydroxycholesterol*

5.6, 18] and 7.7 L/h [95% CI: 2.3, 13], respectively) (Supplementary Fig. S7b and c). There was no sex difference in 4βOHC concentrations in the study population (median difference between females and males: 1.8 ng/mL [95% CI: −0.30, 3.9]).

Discussion

In this study, a comprehensive investigation of 4βOHC as an endogenous biomarker for CYP3A4 activity was performed. To our knowledge, this is the first study where 4βOHC has been compared with three other CYP3A4 metrics: intestinal and hepatic microsomal CYP3A4 activity and protein expression in paired liver and jejunum samples, as well as absolute bioavailability, apparent oral clearance, and systemic clearance of midazolam in vivo. The main findings were that 4βOHC concentrations were positively correlated with hepatic microsomal CYP3A4 activity and hepatic CYP3A4 expression. Equally important, this study also provides evidence of a negligible contribution from intestinal CYP3A4 to the formation of 4βOHC.

The moderate correlation between 4βOHC concentrations and hepatic microsomal activity ($CL_{int,u}$) supports that 4βOHC concentrations reflect individual CYP3A4 metabolic capacity and provides evidence that 4βOHC is a suitable marker for hepatic CYP3A4 phenotype. However, there was no correlation between 4βOHC concentrations and systemic clearance of midazolam, the gold standard method to assess CYP3A4 activity. The correlation between these two metrics has been investigated in multiple studies previously, with conflicting findings varying from no correlation as in this study to a weak or moderate correlation [24–27, 50, 51]. Even though systemic clearance of midazolam currently is the preferred method for CYP3A4 phenotyping, it may have some important limitations in special populations, e.g., patients with obesity, as shown in the present study. Surprisingly, we observed no correlation between hepatic microsomal activity ($CL_{int,u}$) and in vivo systemic midazolam clearance, suggesting that systemic midazolam clearance does not solely reflect CYP3A4 activity in the present population. In contrast to hepatic microsomal $CL_{int,u}$, in vivo systemic midazolam clearance may also be influenced by differences in hepatic blood flow given its medium to high extraction ratio [17–19] which may be especially relevant in patients with chronic diseases or in case of simultaneous physiological alterations. We have previously hypothesized that hepatic blood flow may have a more pronounced impact on systemic midazolam clearance than CYP3A4 activity in patients with obesity due to higher liver blood flow in this patient population compared with normal-weight individuals [20]. Brill et al. have also suggested that blood flow alterations seem to influence midazolam pharmacokinetics in patients with obesity [21]. This may at least partly explain why patients with obesity had lower 4βOHC concentrations in combination with higher systemic midazolam clearance compared with normal to overweight individuals, and also the lack of correlation between 4βOHC and systemic midazolam clearance in the present study. 4βOHC may therefore be an especially relevant biomarker to include in midazolam-based CYP3A4 phenotyping studies in specific patient populations where physiological factors such as altered blood flow may challenge the interpretation of the results. Additionally, there are some methodological considerations with the ex vivo CYP3A activity assay that should be taken into consideration. Microsomal activity in small liver tissue samples may not be representative of the CYP3A4 activity across the whole liver [52], the unbound fraction was

estimated, and it was not accounted for variability in microsomal protein per gram liver and microsomal recovery.

Information regarding the role of intestinal CYP3A4 in 4βOHC formation has been lacking [32, 53], and uncertainty about the intestinal contribution seems to be an important limitation with 4βOHC phenotyping. We showed that neither intestinal CYP3A4 expression nor intestinal microsomal CYP3A4 activity ($CL_{int,u}$) was correlated with 4βOHC concentrations. Even though there was a weak, but significant correlation between 4βOHC concentrations and (1) midazolam absolute bioavailability, and (2) apparent oral clearance, but no correlation with systemic clearance, these results indicate that intestinal CYP3A4 has a negligible role in the formation of 4βOHC. There have been some indications of a possible contribution from intestinal CYP3A4 in the formation of 4βOHC [40, 54]. However, a more recent study indicates that intestinal CYP3A4 is unlikely to be of significant importance for the 4βOHC formation [55], which is in line with our findings. Hence, it seems that 4βOHC is mainly appropriate for hepatic CYP3A4 phenotyping, but the relatively small sample size in our study should be kept in mind when interpreting these findings.

In this study, we also report a negative correlation between BMI and 4βOHC, and that patients with obesity have significantly lower 4βOHC concentrations compared with normal to overweight individuals. Previous studies have also found an inverse correlation between BMI and 4βOHC/C, and body weight and 4βOHC/C [10, 25]. Intestinal and hepatic CYP3A4 expression as well as $CL_{int,u}$ using midazolam as a probe drug are also reported to be negatively correlated with BMI [8, 9]. This supports that patients with obesity have a lower CYP3A4 activity compared with normal weight individuals [7]. The normal to overweight individuals in this study had similar values of 4βOHC compared to reported values for corresponding Caucasians in other studies [10, 23, 51, 56]. Lower levels of 4βOHC were also observed by Gravel et al. in patients with a mean BMI of ~ 29 kg/m^2, although the authors suggested that this was due to type 2 diabetes, not obesity [51], and by Woolsey et al. in patients with a mean BMI of ~ 33 kg/m^2 and NAFLD [5]. CYP3A4 activity seems to be suppressed during inflammation [57, 58], and in agreement with this, we also found a weak inverse correlation between 4βOHC concentrations and the inflammation marker hs-CRP. Björkhem-Bergman et al. also reported a similar correlation in a study with patients with increased susceptibility to respiratory infections [59]. Furthermore, and in agreement with a previous study in patients with NAFLD and obesity [5], we observed a weak inverse correlation between NAFLD liver fat score and 4βOHC concentrations, suggesting decreased CYP3A4 activity in patients with NAFLD.

Most of the cholesterol formation to 4βOHC is accounted for by CYP3A4 metabolism, whereas the contribution from CYP3A5 is more ambiguous [12, 22, 23, 60, 61]. Hole et al. found no significant effect of CYP3A5 genotype on 4βOHC concentrations in a similar study population as ours and suggested that CYP3A5 has a limited role in the formation of 4βOHC in vivo [12]. In agreement with this, we did not observe any significant difference in 4βOHC concentrations between individuals expressing functional (*CYP3A5*1/*3*) and non-functional CYP3A5 (*CYP3A5*3/*3*). Nevertheless, we do not believe that the heterozygote *CYP3A5*1* carriers in our study population (~ 10%) have impacted the interpretation of the results in this study to any significant degree. This is supported by the fact that there were no statistically significant differences between the heterozygote *CYP3A5*1* carriers and the homozygote *CYP3A5*3* carriers in any of the other metrics investigated. Also, there was a tendency of lower 4βOHC concentrations in *CYP3A4*22* carriers (reduced function), but the interpretation is challenged by the low number of individuals carrying this allele. Overall, these results are in line with previous studies [12, 25, 50] and support that 4βOHC primarily seems to be a biomarker for CYP3A4 phenotype.

The major strength of this study includes the comprehensive investigation of three different CYP3A4 metrics in relation to 4βOHC, all obtained from the same individuals at the same time point. The patients with obesity were subjected to a 3-week low-energy diet before the study investigation, which may have influenced the results. However, as neither 4βOHC concentrations, absolute bioavailability, nor systemic clearance of midazolam changed from before to after the diet, a change in the CYP3A4 phenotype during this short period seems unlikely. Nevertheless, in addition to the fact that 20% of the patients used cholesterol-lowering drugs, cholesterol levels also decreased following the diet, which led to a significant increase in 4βOHC/C in the patients with obesity. Several studies report the 4βOHC/C ratio to account for abnormal or changing cholesterol levels. However, the concentration of 4βOHC is less than 0.002% of total cholesterol [53]. We decided to report 4βOHC concentrations, as most individuals had normal cholesterol levels, but analyses using 4βOHC/C yielded similar results. Also, Diczfalusy et al. have previously shown that variability in cholesterol levels only explains 9% of the variability in 4βOHC concentrations [23]. Another limitation includes not adjusting for multiple testing, which may increase the likelihood of type 1 errors. Additionally, the results in this study may not be applicable in other populations, as the majority of the study population were patients with obesity.

In summary, this comprehensive study showed that 4βOHC plasma concentrations reflect hepatic, but not intestinal CYP3A4 activity well. Hence, 4βOHC may be a valuable biomarker for individualized dosing of intravenously administered CYP3A4 substrate drugs. Additionally,

4βOHC is a valuable supplement to traditional phenotyping with probe drugs such as midazolam given its easy implementation and complementary information when other factors, such as disease state or simultaneous physiological alterations, are expected to influence the pharmacokinetics of CYP3A4 probe drugs.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s00228-022-03336-9.

Acknowledgements The authors would like to thank the participants, the surgical staff, and the study personnel working on the COCKTAIL study at Vestfold Hospital Trust. The authors also thank the Swedish Research Council, approval numbers 5715 and 01951 (C.W., T.B.A., and P.A.), for supporting the proteomics analyses.

Author contribution J.H., A.Å., S.A., C.K., T.B.A., H.C., E.S., R.S., and R.J. conceived and designed the COCKTAIL-study. A.Å., E.M., K.H., B.M.W., K.E.K., and I.R. designed the present research. I.R., V.K., L.K.J., K.H., B.M.W., J.K.H, P.A., and C.W. performed the research. K.E.K. and I.R. analyzed the data. K.E.K. and I.R. wrote the manuscript. All authors contributed to critically reviewing the manuscript, and gave their final approval for submission.

Funding Open access funding provided by University of Oslo (incl Oslo University Hospital). The authors would like to thank the following: Vestfold Hospital Trust, Norway; Department of Pharmacy, University of Oslo, Norway; and AstraZeneca, Sweden.

Data availability Access to data collected from this study, including anonymized individual-participant data, may potentially be made available following publication upon e-mail request to the corresponding author. After approval of a proposal, data will be shared with investigators whose proposed use of the data has been approved by the COCKTAIL steering committee, according to the consent given by the participants and Norwegian laws and legislations.

Declarations

Ethics approval The study was approved by the Regional Committee for Medical and Health Research Ethics (2013/2379/REK) and complied with the Declaration of Helsinki.

Conflict of interest C. Karlsson, S. Andersson, and R. Jansson-Löfmark are AstraZeneca employees and own shares in AstraZeneca, while C. Wegler and T.B. Andersson are former AstraZeneca employees. K.E. Kvitne, I. Robertsen, K. Hole, B.M. Wollmann, L.K. Johnson, J.K. Hertel, R. Sandbu, P. Artursson, E. Molden, E. Skovlund, H. Christensen, V. Krogstad, J. Hjelmesæth, and A. Åsberg have no conflict of interest to declare.

References

1. Zanger UM, Schwab M (2013) Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacol Ther 138(1):103–141
2. Rendic S, Guengerich FP (2015) Survey of human oxidoreductases and cytochrome P450 enzymes involved in the metabolism of xenobiotic and natural chemicals. Chem Res Toxicol 28(1):38–42
3. Guengerich FP (1999) Cytochrome P-450 3A4: regulation and role in drug metabolism. Annu Rev Pharmacol Toxicol 39:1–17
4. Paine MF et al (2006) The human intestinal cytochrome P450 "pie." Drug Metab Dispos 34(5):880–886
5. Woolsey SJ et al (2015) CYP3A activity and expression in nonalcoholic fatty liver disease. Drug Metab Dispos 43(10):1484–1490
6. Kolwankar D et al (2007) Association between nonalcoholic hepatic steatosis and hepatic cytochrome P-450 3A activity. Clin Gastroenterol Hepatol 5(3):388–393
7. Brill MJ et al (2014) Midazolam pharmacokinetics in morbidly obese patients following semi-simultaneous oral and intravenous administration: a comparison with healthy volunteers. Clin Pharmacokinet 53(10):931–941
8. Ulvestad M et al (2013) Impact of OATP1B1, MDR1, and CYP3A4 expression in liver and intestine on interpatient pharmacokinetic variability of atorvastatin in obese subjects. Clin Pharmacol Ther 93(3):275–282
9. Krogstad V et al (2021) Correlation of body weight and composition with hepatic activities of cytochrome P450 enzymes. J Pharm Sci 110(1):432–437
10. Hole K et al (2018) Elevated 4β-hydroxycholesterol/cholesterol ratio in anorexia nervosa patients. Pharmacol Res Perspect 6(5):e00430
11. Klein K, Zanger UM (2013) Pharmacogenomics of cytochrome P450 3A4: recent progress toward the "Missing Heritability" problem. Front Genet 4:12
12. Hole K et al (2017) Impact of genetic and nongenetic factors on interindividual variability in 4β-hydroxycholesterol concentration. Eur J Clin Pharmacol 73(3):317–324
13. Heizmann P, Eckert M, Ziegler WH (1983) Pharmacokinetics and bioavailability of midazolam in man. Br J Clin Pharmacol 16 Suppl 1(Suppl 1):43s-49s
14. de Jonge H et al (2013) Impact of CYP3A5 genotype on tacrolimus versus midazolam clearance in renal transplant recipients: new insights in CYP3A5-mediated drug metabolism. Pharmacogenomics 14(12):1467–1480
15. Yu KS et al (2004) Effect of the CYP3A5 genotype on the pharmacokinetics of intravenous midazolam during inhibited and induced metabolic states. Clin Pharmacol Ther 76(2):104–112
16. Kharasch ED et al (2007) Influence of CYP3A5 genotype on the pharmacokinetics and pharmacodynamics of the cytochrome P4503A probes alfentanil and midazolam. Clin Pharmacol Ther 82(4):410–426
17. Thummel KE et al (1996) Oral first-pass elimination of midazolam involves both gastrointestinal and hepatic CYP3A-mediated metabolism. Clin Pharmacol Ther 59(5):491–502
18. Klotz U, Ziegler G (1982) Physiologic and temporal variation in hepatic elimination of midazolam. Clin Pharmacol Ther 32(1):107–112
19. Rogers JF et al (2003) An evaluation of the suitability of intravenous midazolam as an in vivo marker for hepatic cytochrome P4503A activity. Clin Pharmacol Ther 73(3):153–158

20. Kvitne KE et al (2022) Short- and long-term effects of body weight loss following calorie restriction and gastric bypass on CYP3A-activity - a non-randomized three-armed controlled trial. Clin Transl Sci 15(1):221–233
21. Brill MJ et al (2016) Semiphysiologically based pharmacokinetic model for midazolam and CYP3A mediated metabolite 1-OH-midazolam in morbidly obese and weight loss surgery patients. CPT Pharmacometrics Syst Pharmacol 5(1):20–30
22. Bodin K et al (2002) Metabolism of 4 beta -hydroxycholesterol in humans. J Biol Chem 277(35):31534–31540
23. Diczfalusy U et al (2008) 4Beta-hydroxycholesterol is a new endogenous CYP3A marker: relationship to CYP3A5 genotype, quinine 3-hydroxylation and sex in Koreans. Swedes and Tanzanians Pharmacogenet Genomics 18(3):201–208
24. Shin KH et al (2013) Evaluation of endogenous metabolic markers of hepatic CYP3A activity using metabolic profiling and midazolam clearance. Clin Pharmacol Ther 94(5):601–609
25. Woolsey SJ et al (2016) Relationships between endogenous plasma biomarkers of constitutive cytochrome P450 3A activity and single-time-point oral midazolam microdose phenotype in healthy subjects. Basic Clin Pharmacol Toxicol 118(4):284–291
26. Tomalik-Scharte D et al (2009) Plasma 4beta-hydroxycholesterol: an endogenous CYP3A metric? Clin Pharmacol Ther 86(2):147–153
27. Björkhem-Bergman L et al (2013) Comparison of endogenous 4β-hydroxycholesterol with midazolam as markers for CYP3A4 induction by rifampicin. Drug Metab Dispos 41(8):1488–1493
28. Bodin K et al (2001) Antiepileptic drugs increase plasma levels of 4beta-hydroxycholesterol in humans: evidence for involvement of cytochrome p450 3A4. J Biol Chem 276(42):38685–38689
29. Josephson F et al (2008) CYP3A induction and inhibition by different antiretroviral regimens reflected by changes in plasma 4beta-hydroxycholesterol levels. Eur J Clin Pharmacol 64(8):775–781
30. Kanebratt KP et al (2008) Cytochrome P450 induction by rifampicin in healthy subjects: determination using the Karolinska cocktail and the endogenous CYP3A4 marker 4beta-hydroxycholesterol. Clin Pharmacol Ther 84(5):589–594
31. Diczfalusy U et al (2009) 4beta-hydroxycholesterol as an endogenous marker for CYP3A4/5 activity. Stability and half-life of elimination after induction with rifampicin. Br J Clin Pharmacol 67(1):38–43
32. Penzak SR, Rojas-Fernandez C (2019) 4β-Hydroxycholesterol as an endogenous biomarker for CYP3A Activity: literature review and critical evaluation. J Clin Pharmacol 59(5):611–624
33. Neuhoff S, Tucker GT (2018) Was 4β-hydroxycholesterol ever going to be a useful marker of CYP3A4 activity? Br J Clin Pharmacol 84(7):1620–1621
34. Gjestad C et al (2018) Gjestad et al. reply to 'Was 4β-hydroxycholesterol ever going to be a useful marker of CYP3A4 activity?' by Neuhoff and Tucker. Br J Clin Pharmacol 84(7):1624–1625
35. Kuypers DRJ, Vanhove T (2018) Kuypers and Vanhove reply to 'Was 4β-hydroxycholesterol ever going to be a useful marker of CYP3A4 activity?' by Neuhoff and Tucker. Br J Clin Pharmacol 84(7):1622–1623
36. Hjelmesaeth J et al (2018) Impact of body weight, low energy diet and gastric bypass on drug bioavailability, cardiovascular risk factors and metabolic biomarkers: protocol for an open, nossn-randomised, three-armed single centre study (COCKTAIL). BMJ Open 8(5):e021878
37. Kvitne KE et al (2021) Short- and long-term effects of body weight loss following calorie restriction and gastric bypass on CYP3A-activity - a non-randomized three-armed controlled trial. Clin Transl Sci
38. Krogstad V et al (2020) A comparative analysis of cytochrome P450 activities in paired liver and small intestinal samples from patients with obesity. Drug Metab Dispos 48(1):8–17
39. Kotronen A et al (2009) Prediction of non-alcoholic fatty liver disease and liver fat using metabolic and genetic factors. Gastroenterology 137(3):865–872
40. Gjestad C et al (2016) 4β-hydroxycholesterol correlates with dose but not steady-state concentration of carbamazepine: indication of intestinal CYP3A in biomarker formation? Br J Clin Pharmacol 81(2):269–276
41. Størset E et al (2017) The CYP3A biomarker 4β-hydroxycholesterol does not improve tacrolimus dose predictions early after kidney transplantation. Br J Clin Pharmacol 83(7): 1457–1465
42. Egeland EJ et al (2020) Chronic inhibition of CYP3A is temporarily reduced by each hemodialysis session in patients with end-stage renal disease. Clin Pharmacol Ther
43. Wegler C et al (2022) Drug disposition protein quantification in matched human jejunum and liver from donors with obesity. Clin Pharmacol Ther
44. Wiśniewski JR, Mann M (2012) Consecutive proteolytic digestion in an enzyme reactor increases depth of proteomic and phosphoproteomic analysis. Anal Chem 84(6):2631–2637
45. Tyanova S, Temu T, Cox J (2016) The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. Nat Protoc 11(12):2301–2319
46. Huber W et al (2002) Variance stabilization applied to microarray data calibration and to the quantification of differential expression. Bioinformatics 18(Suppl 1):S96-104
47. Wiśniewski JR, Rakus D (2014) Multi-enzyme digestion FASP and the 'Total Protein Approach'-based absolute quantification of the Escherichia coli proteome. J Proteomics 109:322–331
48. Neely MN et al (2012) Accurate detection of outliers and subpopulations with Pmetrics, a nonparametric and parametric pharmacometric modeling and simulation package for R. Ther Drug Monit 34(4):467–476
49. R Foundation for Statistical Computing (2018) R: a language and environment for statistical computing. Vienna, Austria
50. Vanhove T et al (2016) Comparative performance of oral midazolam clearance and plasma 4β-hydroxycholesterol to explain interindividual variability in tacrolimus clearance. Br J Clin Pharmacol 82(6):1539–1549
51. Gravel S et al (2019) use of 4β-hydroxycholesterol plasma concentrations as an endogenous biomarker of CYP3A activity: clinical validation in individuals with type 2 diabetes. Clin Pharmacol Ther 106(4):831–840
52. Wegler C et al (2021) Influence of proteome profiles and intracellular drug exposure on differences in CYP activity in donor-matched human liver microsomes and hepatocytes. Mol Pharm 18(4):1792–1805
53. Mao J et al (2017) Perspective: 4β-hydroxycholesterol as an emerging endogenous biomarker of hepatic CYP3A. Drug Metab Rev 49(1):18–34
54. Gjestad C et al (2017) 4β-Hydroxycholesterol level significantly correlates with steady-state serum concentration of the CYP3A4 substrate quetiapine in psychiatric patients. Br J Clin Pharmacol 83(11):2398–2405
55. Gjestad C et al (2019) Effect of grapefruit juice intake on serum level of the endogenous CYP3A4 metabolite 4β-hydroxycholesterol-an interaction study in healthy volunteers. Aaps j 21(4):58
56. Mannheimer B et al (2015) No impact of vitamin D on the CYP3A biomarker 4β-hydroxycholesterol in patients with abnormal glucose regulation. PLoS ONE 10(4):e0121984
57. Jover R et al (2002) Down-regulation of human CYP3A4 by the inflammatory signal interleukin-6: molecular mechanism and transcription factors involved. Faseb j 16(13):1799–1801

58. Morgan ET et al (2008) Regulation of drug-metabolizing enzymes and transporters in infection, inflammation, and cancer. Drug Metab Dispos 36(2):205–216
59. Björkhem-Bergman L et al (2013) Serum levels of 25-hydroxy-vitamin D and the CYP3A biomarker 4β-hydroxycholesterol in a high-dose vitamin D supplementation study. Drug Metab Dispos 41(4):704–708
60. Nitta SI et al (2018) Evaluation of 4β-hydroxycholesterol and 25-hydroxycholesterol as endogenous biomarkers of CYP3A4: study with CYP3A-humanized mice. Aaps j 20(3):61
61. Gebeyehu E et al (2011) Sex and CYP3A5 genotype influence total CYP3A activity: high CYP3A activity and a unique distribution of CYP3A5 variant alleles in Ethiopians. Pharmacogenomics J 11(2):130–137

Authors and Affiliations

Kine Eide Kvitne[1] · Kristine Hole[2,3] · Veronica Krogstad[1] · Birgit Malene Wollmann[2] · Christine Wegler[4,5] · Line K. Johnson[6] · Jens K. Hertel[6] · Per Artursson[7] · Cecilia Karlsson[8,9] · Shalini Andersson[10] · Tommy B. Andersson[5] · Rune Sandbu[6,11] · Jøran Hjelmesæth[6,12] · Eva Skovlund[13] · Hege Christensen[1] · Rasmus Jansson-Löfmark[5] · Anders Åsberg[1,14] · Espen Molden[1,2] · Ida Robertsen[1]

1 Section for Pharmacology and Pharmaceutical Biosciences, Department of Pharmacy, University of Oslo, Blindern, P.O. Box 1068, 0316 Oslo, Norway

2 Center for Psychopharmacology, Diakonhjemmet Hospital, Oslo, Norway

3 Department of Life Sciences and Health, Oslo Metropolitan University, Oslo, Norway

4 Department of Pharmacy, Uppsala University, Uppsala, Sweden

5 DMPK, Research and Early Development, Cardiovascular, Renal and Metabolism (CVRM), AstraZeneca, BioPharmaceuticals R&D, Gothenburg, Sweden

6 The Morbid Obesity Center, Vestfold Hospital Trust, Tønsberg, Norway

7 Department of Pharmacy and Science for Life Laboratory, Uppsala University, Uppsala, Sweden

8 Clinical Metabolism, Cardiovascular, Renal and Metabolism (CVRM), Late-Stage Development, AstraZeneca, BioPharmaceuticals R&D, Gothenburg, Sweden

9 Department of Molecular and Clinical Medicine, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

10 Oligonucleotide Discovery, Discovery Sciences, R&D, AstraZeneca, Gothenburg, Sweden

11 Deparment of Surgery, Vestfold Hospital Trust, Tønsberg, Norway

12 Department of Endocrinology, Morbid Obesity and Preventive Medicine, Institute of Clinical Medicine, University of Oslo, Oslo, Norway

13 Department of Public Health and Nursing, Norwegian University of Science and Technology, NTNU, Trondheim, Norway

14 Department of Transplant Medicine, Oslo University Hospital, Oslo, Norway

Supplementary Information

Correlations between 4β-hydroxycholesterol and hepatic and intestinal CYP3A4; protein expression, microsomal *ex vivo* activity, and *in vivo* activity in patients with a wide body weight range

European Journal of Clinical Pharmacology

Kine Eide Kvitne[1], Kristine Hole[2,3], Veronica Krogstad[1], Birgit Malene Wollmann[2], Christine Wegler[4,5], Line K. Johnson[6], Jens K. Hertel[6], Per Artursson[7], Cecilia Karlsson[8,9], Shalini Andersson[10], Tommy B. Andersson[5], Rune Sandbu[6,11], Jøran Hjelmesæth[6,12] Eva Skovlund[13], Hege Christensen[1], Rasmus Jansson-Löfmark[5], Anders Åsberg[1,14], Espen Molden[1,2], Ida Robertsen[1]

Affiliations:

[1]*Section for Pharmacology and Pharmaceutical Biosciences, Department of Pharmacy, University of Oslo, Norway*

[2]*Center for Psychopharmacology, Diakonhjemmet Hospital, Oslo, Norway.*

[3]*Department of Life Sciences and Health, Oslo Metropolitan University, Oslo, Norway*

[4]*Department of Pharmacy, Uppsala University, Sweden*

[5]*DMPK, Research and Early Development, Cardiovascular, Renal and Metabolism (CVRM), BioPharmaceuticals R&D, AstraZeneca, Gothenburg, Sweden*

[6]*The Morbid Obesity Center, Vestfold Hospital Trust, Tønsberg, Norway*

[7]*Department of Pharmacy and Science for Life Laboratory, Uppsala University, Sweden*

[8]*Clinical Metabolism, Late-stage Development, Cardiovascular, Renal and Metabolism (CVRM), BioPharmaceuticals R&D, AstraZeneca, Gothenburg, Sweden*

[9]*Department of Molecular and Clinical Medicine, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden*

[10]*Oligonucleotide Discovery, Discovery Sciences, R&D, AstraZeneca, Gothenburg, Sweden*

[11]*Deparment of Surgery, Vestfold Hospital Trust, Tønsberg, Norway*

[12]*Department of Endocrinology, Morbid Obesity and Preventive Medicine, Institute of Clinical Medicine, University of Oslo, Norway*

[12]*Department of Public Health and Nursing, Norwegian University of Science and Technology, NTNU, Trondheim, Norway*

[13]*Department of Transplant Medicine, Oslo University Hospital, Oslo, Norway*

Corresponding author:

Kine Eide Kvitne

Department of Pharmacy, University of Oslo, P.O. Box 1068 Blindern, 0316 Oslo, Norway

E-mail: k.e.kvitne@farmasi.uio.no

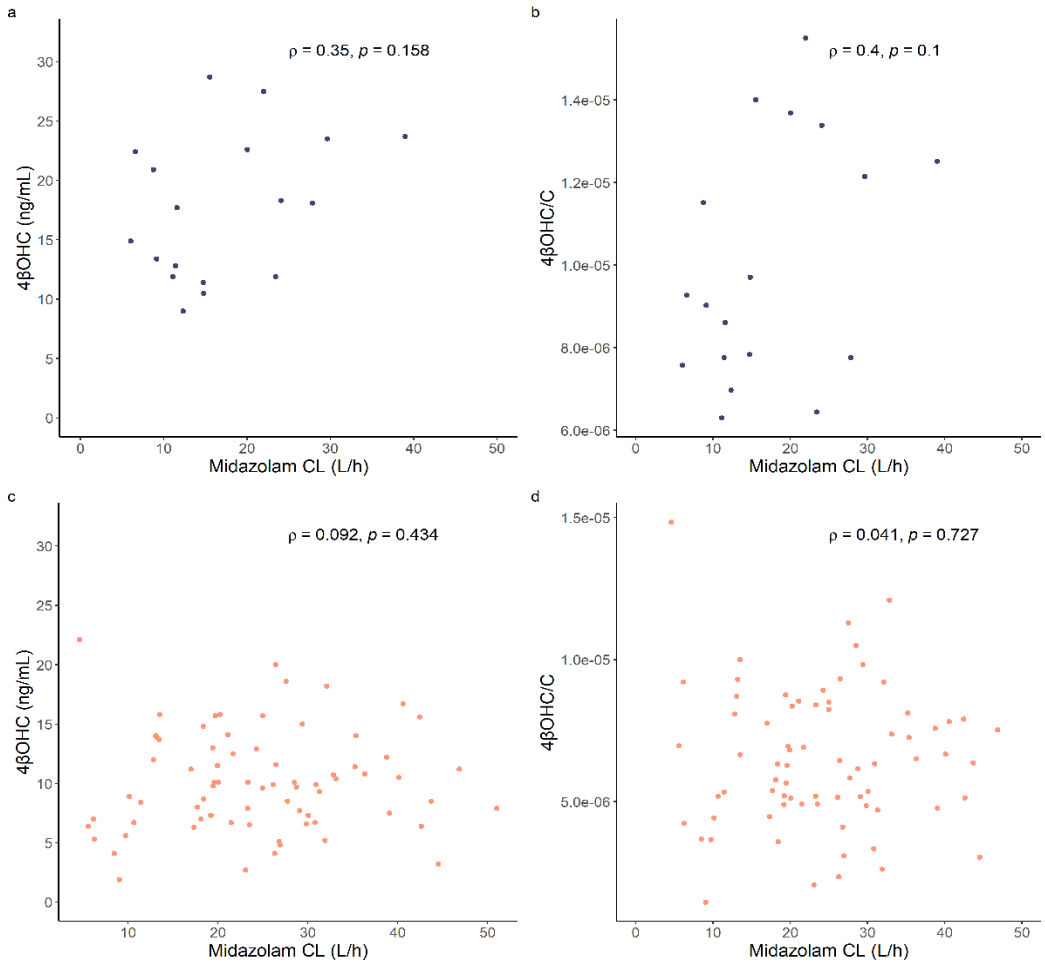

Fig S1. Association between systemic midazolam clearance and (a) 4βOHC concentrations, and (b) 4βOHC/C in normal- to overweight individuals (n=18). Association between systemic midazolam clearance and (c) 4βOHC concentrations, and (d) 4βOHC/C in patients with obesity (n=78). Spearman's rho (ρ) is the correlation coefficient, and the p value is from the Spearman rank correlation analysis

Abbreviations: 4βOHC, 4-beta hydroxycholesterol; 4βOHC/C, 4-beta hydroxycholesterol/cholesterol ratio

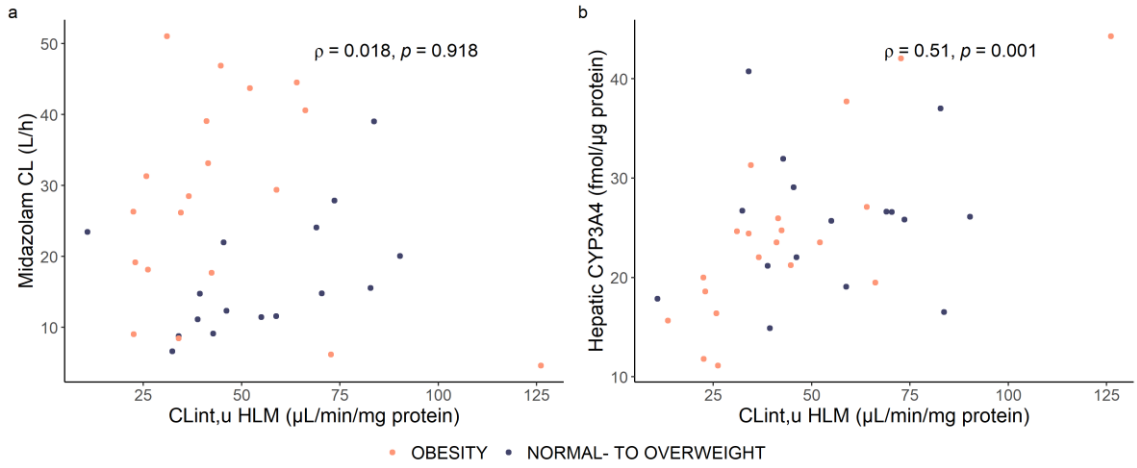

Fig. S2. Association between clearance intrinsic for midazolam 1'-hydroxylation in human liver microsomes and (a) systemic midazolam clearance (n=36), and (b) hepatic CYP3A4 expression (n=36). Spearman's rho (ρ) is the correlation coefficient, and the p value is from the Spearman rank correlation analysis

Abbreviations: $CL_{int,u}$, clearance intrinsic, unbound; CYP, cytochrome P450; HLM, human liver microsomes

Table S1. CYP3A metrics in the two study groups. Data are presented as median [IQR].

	Patients with obesity n=78	**Normal- to overweight individuals** n=18
Absolute bioavailability (%) [a]	22 [12, 29]	7.8 [5.7, 11]
Systemic clearance (L/h) [a]	24 [18, 31]	15 [11, 23]
Apparent oral clearance (L/h) [a]	134 [93, 173]	185 [145, 243]
4βOHC (ng/mL)	9.7 [7.1, 12]	18 [12, 23]
Hepatic CYP3A4 (fmol/µg protein) [b]	21 [17, 25]	26 [21, 28]
$CL_{int,u}$ HLM (µL/min/mg protein) [c]	39 [26, 54]	51 [39, 71]
Intestinal CYP3A4 (fmol/µg protein) [d]	13 [9.8, 18]	*NA*
$CL_{int,u}$ HIM (µL/min/mg protein) [e]	31 [23, 37]	*NA*

Abbreviations: $CL_{int,u}$, clearance intrinsic unbound; CYP, cytochrome P450; HIM, human intestinal microsomes; HLM, human liver microsomes; 4βOHC, 4β-hydroxycholesterol

[a] Midazolam population pharmacokinetic model derived parameters

[b] Only available in 56 individuals (obesity).

[c] Only available in 36 individuals (obesity).

[d] Only available in 37 individuals (obesity).

[e] Only available in 20 individuals (obesity).

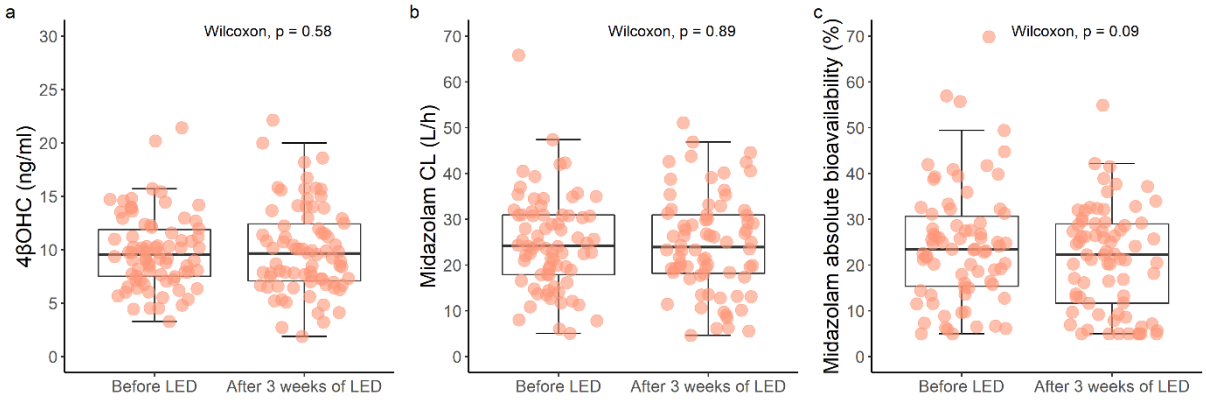

Fig. S3. CYP3A4 metrics before and after three weeks of LED. Boxplot with individual points of (a) 4βOHC concentrations (n=96), (b) systemic midazolam clearance (n=92), and (c) midazolam absolute bioavailability (n=92) in patients with severe obesity. Wilcoxon signed-rank test was used to compare change from week 0 to week 3

Abbreviations: 4βOHC, 4-beta hydroxycholesterol

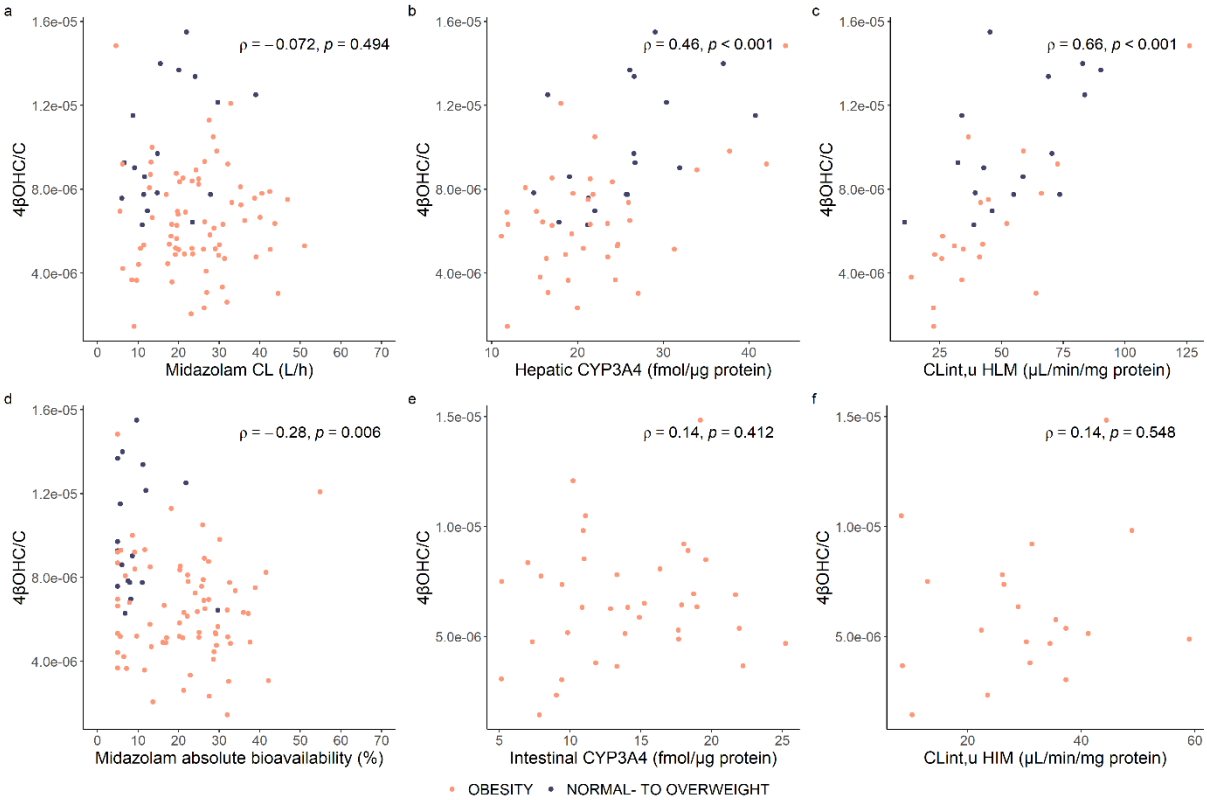

Fig. S4. CYP3A4 metrics and 4βOHC/C. Association between (a) systemic midazolam clearance and 4βOHC/C (n=92), (b) hepatic CYP3A4 expression and 4βOHC/C (n=56), (c) clearance intrinsic for midazolam 1'-hydroxylation in human liver microsomes and 4βOHC/C (n=36), (d) midazolam absolute bioavailability and 4βOHC/C (n=92), (e) jejunum CYP3A4 expression and 4βOHC/C (n=37), and (f) clearance intrinsic for midazolam 1'-hydroxylation in human intestinal microsomes and 4βOHC/C (20). Spearman's rho (ρ) is the correlation coefficient, and the p value is from the Spearman rank correlation analysis.

Abbreviations: $CL_{int,u}$, clearance intrinsic, unbound; CYP, cytochrome P450; HIM, human intestinal microsomes; HLM, human liver microsomes; 4βOHC/C, 4-beta hydroxycholesterol/cholesterol ratio

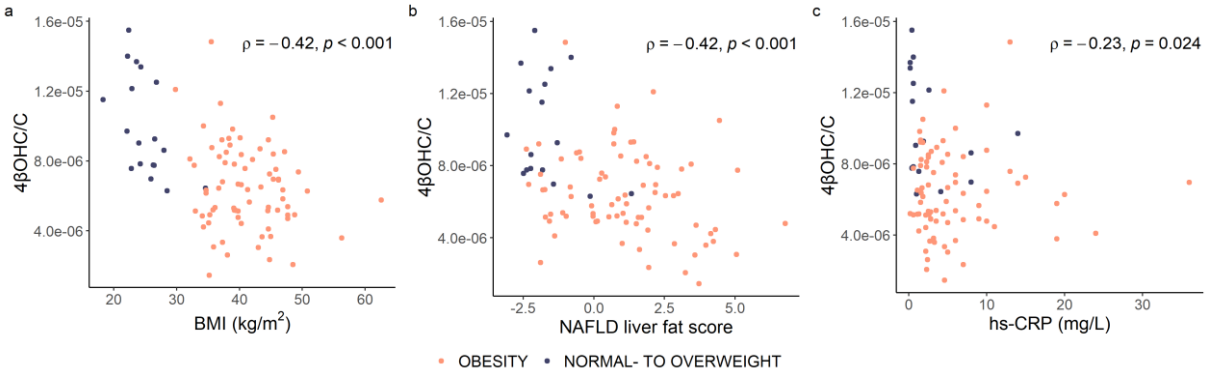

Fig. S5. Clinical variables and 4βOHC/C. Association between (a) BMI and 4βOHC/C (n=96), (b) NAFLD liver fat score and 4βOHC/C (n=95), and (c) high-sensitivity C-reactive protein and 4βOHC/C (n=96). Spearman's rho (ρ) is the correlation coefficient, and the p value is from the Spearman rank correlation analysis

Abbreviations: BMI, body mass index; hs-CRP, high-sensitivity C-reactive protein; NAFLD, non-alcoholic fatty liver disease; 4βOHC/C, 4-beta hydroxycholesterol/cholesterol ratio

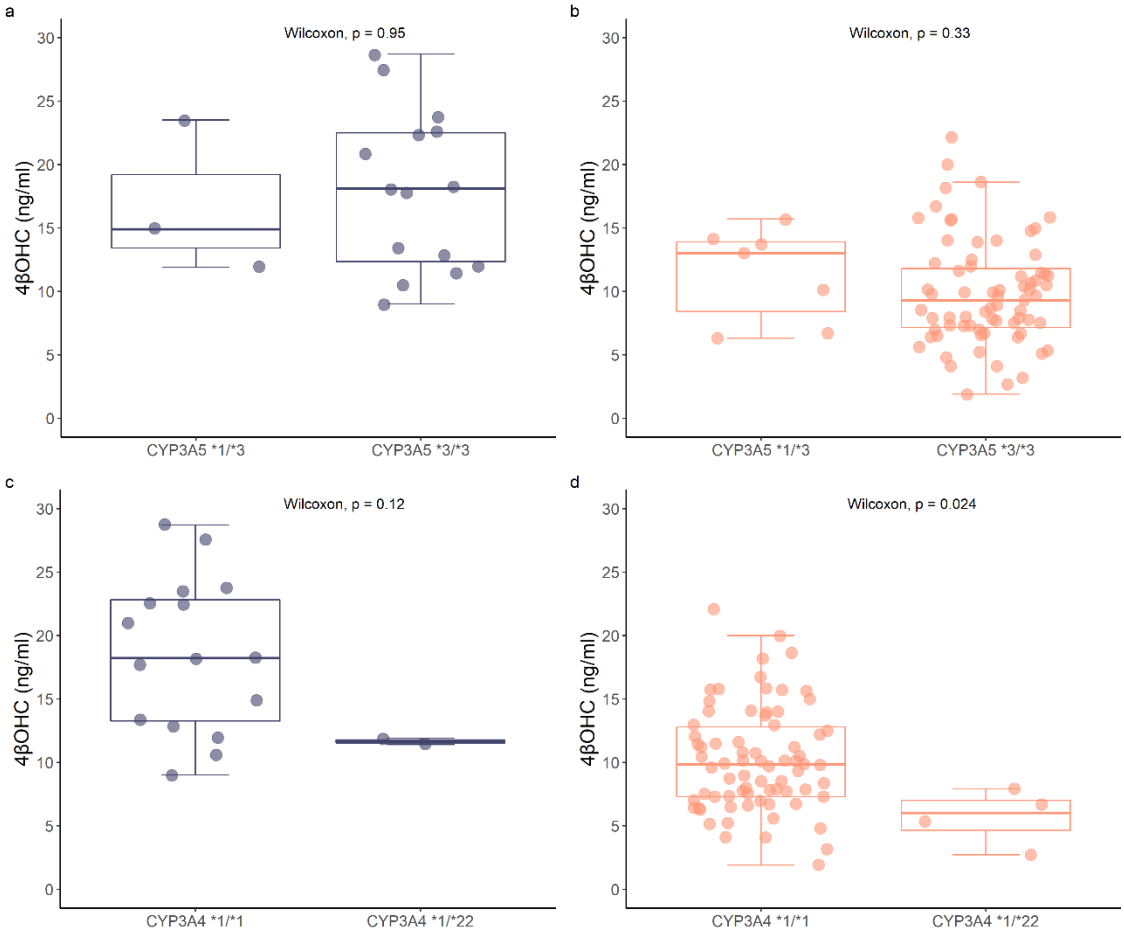

Fig. S6. 4βOHC and genotype. 4βOHC concentrations in (a) normal- to overweight individuals with *CYP3A5 *1/*3* (n=3) or *CYP3A5 *3/*3* (n=15) genotype, (b) patients with obesity with *CYP3A5 *1/*3* (n=7) or *CYP3A5 *3/*3* (n=71) genotype, (c) normal- to overweight individuals with *CYP3A4 *1/*1* (n=16) and *CYP3A4 *1/*22* (n=2), and (d) patients with obesity *CYP3A4 *1/*1* (n=74) and *CYP3A4 *1/*22* (n=4). Wilcoxon rank-sum test was used to compare the two groups

Abbreviations: CYP, cytochrome P450; 4βOHC, 4-beta hydroxycholesterol

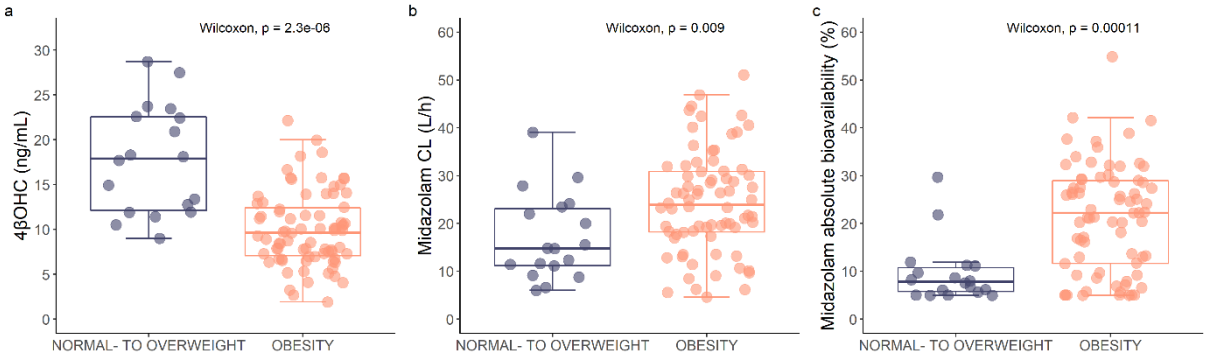

Fig. S7. CYP3A4 metrics in patients with obesity and normal- to overweight individuals. Boxplot with individual points of (a) 4βOHC concentrations (n=96), (b) systemic midazolam clearance (n=92), and (c) midazolam absolute bioavailability (n=92). Wilcoxon rank-sum test was used to compare the two groups

Abbreviations: 4βOHC, 4-beta hydroxycholesterol