CYP3A activity in the pediatric population

A CyPed pilot study

Tine Marie Herlofsen

Thesis for the degree of Master of Pharmacy
45 credits

Section for Pharmacology and Pharmaceutical Biosciences
Department of Pharmacy
Faculty of Mathematics and Natural Sciences

UNIVERSITY OF OSLO

May 2021

CYP3A activity in the pediatric population

A CyPed pilot study

by

Tine Marie Herlofsen

Thesis for the degree of Master of Pharmacy

Section for Pharmacology and Pharmaceutical Biosciences

Department of Pharmacy

Faculty of Mathematics and Natural Sciences

UNIVERSITY OF OSLO

Supervisors

Associate professor Ida Robertsen

PhD student Kine Eide Kvitne

Professor Hege Christensen

2021

CYP3A activity in the pediatric population

Tine Marie Herlofsen

http://www.duo.uio.no/

Trykk: Reprosentralen, Universitetet i Oslo

Forord

Denne masteroppgaven er utført ved Avdeling for farmasøytisk biovitenskap, under veiledning av førsteamanuensis Ida Robertsen, doktorgradsstipendiat Kine Eide Kvitne og professor Hege Christensen. Arbeidet med masteroppgaven det siste året har vært svært lærerikt og jeg har hatt et fantastisk år sammen med PK-gruppa.

Jeg vil spesielt takke førsteamanuensis Ida Robertsen og doktorgradsstipendiat Kine Eide Kvitne for deres positive innstilling og støtte gjennom hele perioden. Jeg setter veldig stor pris på all hjelp og veiledning jeg har fått. Tusen takk Ida for at du alltid er tilgjengelig, tålmodig og ikke minst en humørspreder. Tusen takk Kine for at du tok meg så godt imot og for all hjelp jeg har fått. Ikke minst, takk for vennskapet som har utviklet seg. Tusen takk for at dere alltid har hatt så troa på meg.

Tusen takk til mine medstudenter Ole og Vilde for all hjelp, støtte og gode samtaler på masterkontoret. Dette året hadde ikke blitt det samme uten dere. Tusen takk Hege for ditt gode humør og oppløftende samtaler. Takk for gode tilbakemeldinger og hjelp med oppgaven. Tusen takk Markus for all digital hjelp og ikke minst god kaffe. Tusen takk til ingeniør Eline for all hjelp på lab og tillagning av diverse løsninger.

Til slutt vil jeg takke min kjære samboer, familie og venner for all støtte og motivasjon gjennom studiene.

Oslo, mai 2021

Tine Marie Herlofsen

List of abbreviations

ABC	ATP-binding cassette
ADME	Absorption, distribution, metabolism and excretion
ALAT	Alanine aminotransferase
ASAT	Aspartate aminotransferase
ATP	Adenosine triphosphate
AUC	Area under the concentration-time curve
CL	Clearance
C_{max}	Maximum concentration
C_{min}	Minimum concentration
CRF	Case report form
CRP	C-reactive protein
CV	Coefficient of variation
CYP	Cytochrome P450
d	Deuterium
E_H	Hepatic extraction ratio
EMA	European Medicines Agency
F	Bioavailability
GABA	γ-aminobutyric acid
GFR	Glomerular filtration rate

GI	Gastrointestinal
GST	Glutathione-S-transferase
LLOQ	Lower limit of quantification
NAT	*N*-acetyltransferase
NF-κB	Nuclear factor kappa B
OATP	Organic anion transporting polypeptide
P-gp	P-glycoprotein
PXR	Pregnane X receptor
QC	Quality control sample
RE	Relative deviation
RXRα	Retinoid X receptor alpha
SLC	Solute carrier
SNP	Single nucleotide polymorphism
T_{max}	Time to reach maximum concentration
UGT	Uridine diphosphate-glucuronosyltransferase
UHPLC-MS/MS	Ultra-high-performance liquid chromatography- tandem mass spectrometry
V_d	Volume of distribution

Abstract

Introduction: In the recent years, there has been an increased focus on the development and availability of effective and safe drugs for the pediatric population. As the cytochrome P450 (CYP) 3A subfamily is of significant relevance regarding the metabolism of the majority of drugs, it is of interest to understand how the activity of these enzymes develop in children. However, there is still important gaps in our knowledge regarding the ontogeny of essential CYP enzymes. The aim of this thesis was therefore to conduct a pilot study investigating the ontogeny of systemic (hepatic) CYP3A activity across different age groups by using midazolam as a probe drug.

Method: A pilot study including pediatric patients from the CyPed study was conducted. Patients were divided into four age-specific groups: 0-6 months, 6 months – 2 years, 2-5 years and 5-16 years, called A, B, C and D, respectively. Midazolam was used as a probe drug to determine CYP3A activity. Blood samples for determination of midazolam and metabolite plasma concentrations were obtained during midazolam infusion and after dose adjustment or drug withdrawal. A previously validated UHPLC-MS/MS method to quantify midazolam and the main metabolite concentrations was optimized and a partial validation was performed. Non-compartmental methods were used to determine pharmacokinetic parameters.

Results: Thirteen patients were included in this pilot study; 3 in age group A (median age 7 days), 6 in age group B (median age 10 months), 1 in age group C (2 years) and 3 in age group D (median age 8 years). The median estimated midazolam clearance was 0.63 L/h, 3.9 L/h and 20 L/h for group A, B and D respectively. For the patient in group C, clearance was 10.5 L/h. The optimized method showed that within-run and between-run coefficient of variation (CV) were <7.4% and <9.8% for midazolam and 1-hydroxymidazolam, respectively. Within-run and between-run mean accuracy was between 98% and 111% for midazolam and between 93% and 108% for 1-hydroxymidazolam.

Conclusion: The optimized method met the requirements in the guideline from European Medicines Agency (EMA) on bioanalytical method validation. The results imply that midazolam clearance, and thus CYP3A activity, increase with increasing age. However, the inter-individual variability was large with a 141-fold difference in midazolam clearance across the age groups. Only a small number of patients were included in this pilot study and inclusion of more patients will allow us to further explore the effect of age on CYP3A activity. In order

to fully explore the pharmacokinetic data from the CyPed-study it is vital to develop a population pharmacokinetic model of midazolam.

Sammendrag

Introduksjon: De siste årene har det blitt økt fokus på produksjon og tilgjengelighet av effektive og trygge legemidler for den pediatriske populasjonen. Ettersom Cytokrom P450 (CYP) 3A familien er av stor betydning når det kommer til metabolisme av legemidler, er det av stor interesse å forstå hvordan aktiviteten til disse enzymene utvikler seg hos barn. Selv om noe er kjent, er det fortsatt behov for mer kunnskap om hvordan aktiviteten av de essensielle CYP enzymene endrer seg over tid. Målet med denne oppgaven var derfor å utføre en pilotstudie for å undersøke hepatisk CYP3A aktivitet på tvers av aldersgrupper ved å bruke midazolam som et probelegemiddel.

Metode: En pilot studie ble utført ved å inkludere pasienter fra den pågående CyPed studien ved Oslo universitetssykehus. Pasienter ble delt inn i fire aldersspesifikke grupper; 0-6 måneder, 6 måneder – 2 år, 2-5 år og 5-16 år, kalt henholdsvis A, B, C og D. Midazolam ble brukt som et probelegemiddel for å bestemme CYP3A aktivitet. For å kunne bestemme plasmakonsentrasjoner av midazolam og hovedmetabolitt, 1-hydroxymidazolam, ble blodprøver tatt under kontinuerlig midazolam infusjon, og under nedtrapping av dose eller ved stopp av infusjon. En tidligere validert UHPLC-MS/MS metode for å kvantifisere plasmakonsentrasjoner av midazolam og metabolitten ble optimalisert og en delvalidering ble utført. Standard non-kompartment metoder ble brukt til å bestemme farmakokinetiske parametere.

Resultater: Tretten pasienter ble inkludert i CyPed pilot studien, 3 i aldersgruppe A (median alder 7 dager), 6 i aldersgruppe B (median alder 10 måneder), 1 i aldersgruppe C (2 år) og 3 i aldersgruppe D (median alder 8 år). Median estimert midazolam clearance var 0.63 L/h, 3.9 L/h and 20 L/h for henholdsvis gruppe A, B og D. Midazolam clearance for den ene pasienten i gruppe C var 10.5 L/h. Den optimaliserte metoden viste intra- og interdag variasjonskoeffisienter (CV) <7.4% og <9.8% for henholdsvis midazolam og 1-hydroxymidazolam. Gjennomsnittlig intra- og interdag nøyaktighet var mellom 98% og 111% for midazolam og mellom 93% og 108% for 1-hydroxymidazolam.

Konklusjon: Den optimaliserte metoden tilfredsstilte kravene til bioanalytisk metodevalidering fra det europeiske legemiddelbyrået (EMA). Resultatene indikerer at midazolam clearance, og dermed CYP3A aktivitet, øker med økende alder. Den inter-individuelle variabiliteten var

imidlertid stor, med en 141 ganger forskjell i midazolam clearance på tvers av aldersgruppene. Kun et lite antall pasienter ble inkludert i denne pilotstudien. Ved å inkludere flere pasienter kan effekten av alder på CYP3A-aktivitet undersøkes ytterligere. Erfaringer fra pilotstudien viste at det er nødvendig å utvikle en populasjonsfarmakokinetisk modell for midazolam for å få analysert de farmakokinetiske dataene på en tilfredsstillende måte.

Table of contents

1 Introduction

1.1 Inter-individual variability in drug response

The ultimate goal in pharmacotherapy is to find the most suitable drug for each patient in order to maximize benefits and minimize adverse events. However, even when applying individualized drug therapy, significant inter-individual variability in drug response still exists. Reasons for the high inter-individual variability in drug response are manifold, and it is a major challenge in clinical practice as it affects both efficacy and toxicity [1]. Drug response depends on both the pharmacokinetics and pharmacodynamics of a drug. Pharmacokinetics refers to the movement of a drug into, through and out of the body while pharmacodynamics describes the relationship between drug concentration at the site of action and the resulting effect. In the last decades, there has been an increased focus on how disease, genetics, environmental factors and age influences drug response. Despite this awareness, there is still insufficient knowledge regarding the impact of age on pharmacokinetics in pediatric patients. Thus, more knowledge regarding correct drug dosing in this population and the effect of age on pharmacokinetics are needed.

1.2 Pharmacokinetic variability

One of the major factors influencing a patient's response to any specific drug is pharmacokinetics. This term refers to the processes of drug absorption, distribution, metabolism and excretion (ADME) [1, 2]. Several physiological and environmental factors influence the fraction of administered dose reaching the systemic circulation, to what extent the drug is distributed to different tissues and how efficiently the drug is eliminated from the body. Pharmacokinetics is therefore an important source of inter-individual variability in drug response, and may to a large degree explain why the same dose of the same drug can lead to overexposure and adverse events in one patient and therapeutic failure in another patient.

A drug given orally must be absorbed through the membranes of the gastrointestinal tract, transported to the liver via the portal vein and thereafter enter the central compartment. Along this way, the drug may be metabolized by drug metabolizing enzymes located in both the small intestinal membrane and in the liver. The term bioavailability (F) describes the fraction or

percent of the administered dose that reaches the systemic circulation unchanged [1, 2]. Oral bioavailability can be described by Equation 1:

$$F = F_A * F_G * F_H \quad (1)$$

where F_A is the fraction of dose absorbed from the gastrointestinal lumen without being lost in feces or decomposed in the lumen. F_G is the fraction of dose that escapes presystemic metabolism in the gastrointestinal wall and/or excretion by efflux drug transporters. This fraction enters the portal vein and reaches the liver. F_H is the fraction of dose that reaches the systemic circulation without being a subject for presystemic metabolism in the liver or biliary excretion (**Figure 1**) [1]. The loss of drug through these tissues is known as first-pass metabolism or first-pass loss [1, 2]. Distribution describes the transfer of a drug from the site of measurement to peripheral tissues [1]. The apparent volume of distribution (V_d) is a useful parameter in estimating the dose required to achieve a given plasma concentration and can be described as the (apparent) volume into which a drug distributes in the body at equilibrium. Drugs are eliminated from the body by both metabolism and excretion. Total systemic clearance (CL) is the parameter relating the rate of elimination to the plasma concentration, and it is of major clinical relevance. The systemic exposure-time profile is a function of ADME and can be used to determine the total systemic exposure of a drug (area under the concentration-time curve, AUC).

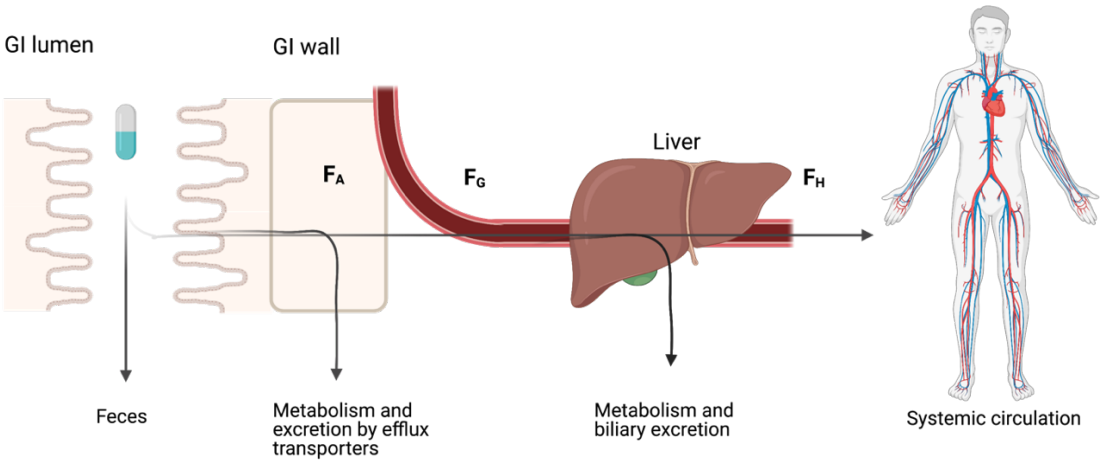


Figure 1. A drug given orally enters the gastrointestinal (GI) lumen where it can be absorbed through the GI wall or lost to feces. F_A is the fraction of dose absorbed from the GI lumen. Drug metabolizing enzymes and efflux drug transporters are located in the GI wall. F_G is the fraction of dose that enters the portal vein and escapes presystemic metabolism in the GI wall and/or excretion efflux transporters. F_H is the fraction of dose who reaches the systemic circulation without being a subject for presystemic metabolism by drug metabolizing enzymes in the liver or biliary excretion. The fraction entering the systemic circulation unchanged ($F_A \bullet F_G \bullet F_H$) is the oral bioavailability (F) of the drug.

1.3 Drug metabolism and transport

Drug metabolizing enzymes are highly abundant in the intestine and liver and are key determinants of drug disposition. Since a large proportion of therapeutic drugs are lipophilic, they do not pass readily into the urine and consequently may accumulate in the body [2]. The metabolism of drugs into more hydrophilic metabolites is therefore essential for their elimination from the body and for termination of the pharmacological activity (with some exceptions). In addition to the intestine and liver, drug metabolizing enzymes are also present to a lesser extent in the lungs and kidneys. Drug metabolism can be categorized as a phase 1 reaction or a phase 2 reaction [1-3]. A phase 1 reaction involves oxidation, reduction, or hydrolysis, which most importantly alternate the biological properties of the drug. Phase 2 reactions involve conjugation of the phase 1 product with a second molecule, usually glucuronic acid, which improves the water solubility remarkably. Important phase 2 enzymes include several superfamilies of conjugating enzymes, such as uridine diphosphate-glucuronosyltransferase (UGT), glutathione-S-transferase (GST) and *N*-acetyltransferase (NAT) [2]. The liver is the major organ responsible for drug metabolism, and the effectiveness

of the liver at removing the presented drug can be expressed as hepatic extraction ratio [1]. Drugs are often categorized to be drugs with low, intermediate, or high extraction ratio. Hepatic clearance of a drug with a low extraction ratio depends upon hepatic intrinsic clearance, while hepatic clearance of a drug with high extraction ratio depends upon hepatic blood flow.

Drug transporters and their interplay with drug metabolizing enzymes also play a substantial role in drug disposition [4, 5]. There are two major superfamilies of drug transporters, the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily and the solute carrier (SLC) superfamily [4, 6]. Most ABC transporters are active transporters, which means they rely on ATP hydrolysis to pump their substrates across membranes, and the majority are efflux transporters [4]. P-glycoprotein (P-gp) is one of the best recognized members of the ABC superfamily [4, 7]. P-gp is expressed in the enterocytes in the small intestine, the bile canalicular membrane of hepatocytes, renal proximal tubular cells and the capillary endothelial cells in the blood-brain barrier [8]. Expression of P-gp is associated with decreased absorption of orally administrated drugs, increased excretion of drugs into bile, increased excretion of drugs into the urine and decreased penetration of drugs into the brain [7, 9]. SLC transporters on the other hand are mainly involved in the uptake of small molecules into cells [4]. SLC transporters are primarily facilitative or secondary active [4, 6]. Facilitative transporters do not require energy since transport across membranes occurs down the electrochemical gradient. Secondary active transporters use energy from ion-gradients generated by ATP-dependent pumps to transport substrates against their concentration gradient. Organic anion transporting polypeptides (OATPs) are important SLC-transporters in humans and mediate transport of a wide range of substances, both endo- and exogenous [10]. OATP1B1 is expressed on the basolateral membrane of hepatocytes and plays an important role in the uptake of statins [10, 11].

1.3.1 Cytochrome P450 (CYP) enzymes

The cytochrome P450 (CYP) enzymes are the major enzymes responsible for the phase 1 metabolism of endogenous substances and xenobiotics, including the majority of drugs [3, 12, 13]. Of 57 functional human CYP enzymes, only a small fraction of these enzymes, belonging to the CYP1, CYP2 and CYP3 families, are responsible for the biotransformation of 70-80% of all clinically used drugs [13, 14]. The CYP enzymes are embedded in the phospholipid bilayer of the endoplasmic reticulum of the cells [2]. Major isoforms involved in drug metabolism are CYP1A2, CYP2C9, CYP2D6, CYP3A4, and CYP2C19 [13, 15, 16]. Genotyping has made it

possible to characterize many significant CYP genes and allowed us to uncover mutations and thus polymorphic forms of CYP enzymes [13, 17, 18]. Dependent on genotype, subpopulations are often classified as extensive metabolizers, intermediate metabolizers, poor metabolizers, or for some isoforms, ultrarapid metabolizers. Clinically, genotyping is most useful when it can predict phenotype [19]. However, both intrinsic and extrinsic factors influence the expression and activity of CYP enzymes resulting in altered drug disposition [13]. Phenoconversion of genotypic extensive metabolizers into phenotypic poor metabolizers typically results from drug-drug interactions [17]. This is well documented for the CYP enzymes.

1.3.2 CYP3A

The CYP3 family consists of the CYP3A subfamily with four CYP genes: CYP3A7, CYP3A4, CYP3A5 and CYP3A43, whereas CYP3A4 is the most abundant isoform [13, 20]. There is a high sequence similarity and a similar substrate specificity between the isoforms CYP3A4 and CYP3A5 [13]. CYP3A4/5 enzymes are of great importance since they metabolize 30-50% of all clinically used drugs [13, 21]. CYP3A is abundantly expressed in the human liver and represents 40% of the total hepatic CYP content, although there is a large inter-individual variability in the population (10-100 fold) [13, 20, 22]. CYP3A is also the major subfamily expressed in intestinal enterocytes, representing about 80% of the total CYP content in the small intestinal mucosa [22]. Similarly to hepatic CYP3A, there is considerable inter-individual variability in intestinal CYP3A. Although the total mass of enteric CYP3A only represents about 1% of that in the liver, intestinal CYP3A may contribute substantially, and sometimes equally with hepatic CYP3A to the overall first-pass metabolism [13, 22, 23].

1.3.3 Variability in CYP3A

Drugs metabolized by CYP3A show high inter-individual variability in drug response. The reason for this variability is multifactorial, including several extrinsic and intrinsic factors influencing the expression and activity of these enzymes. It is necessary to understand these factors in order to be able to optimize and individualize drug treatment.

Genetics

Several single nucleotide polymorphisms (SNPs) within the locus of CYP3A4 have been identified [12], but with a frequency of less than 1%. Only a small fraction of the large inter-individual variability in CYP3A4 expression can be explained by genetic polymorphism [20].

The decrease-of-function *CYP3A4*22* allele has a frequency above 1% and is therefore polymorphic, resulting in lower expression and activity of CYP3A4 [13, 24, 25]. As opposed to CYP3A4, CYP3A5 is highly polymorphic and most individuals do not express active enzyme [13]. Only about 10% of Caucasians express active CYP3A5 enzymes and are thus carriers of at least one *CYP3A5*1* allele. However, most Caucasians are carriers of the deficient allele *CYP3A5*3.* A higher frequency of *CYP3A5*1* is seen in the African and Asian populations. Individuals carrying active CYP3A5 have increased clearance of CYP3A4/5 substrates, and may therefore require higher doses of drugs metabolized by CYP3A enzymes.

Environmental factors

CYP3A activity is largely affected by environmental factors such as concomitant drug treatment and/or nutritional agents [13]. In a study by Christensen *et al*., with 10 healthy male volunteers, it was found that the systemic exposure of the CYP3A substrate diltiazem was increased after a single intake of the CYP3A inhibitor grapefruit juice (250 mL) compared to water, with considerable inter-individual variability [26]. There are several drugs on the market that inhibits CYP3A4/5 (e.g. erythromycin, ketoconazole, clarithromycin, fluconazole), resulting in decreased metabolism and clearance of CYP3A4/5 substrates [27, 28]. Inducers like rifampicin, phenytoin, St. John´s wort, valproic acid and glucocorticoids will increase clearance of CYP3A4/5 substrates, resulting in decreased plasma concentrations [3, 13, 29].

Disease

Other factors, as inflammation and organ failure, may also alter the expression and activity of CYP enzymes [17, 28, 30-36]. Previous studies have shown that critically ill patients may have a disease-induced change in drug clearance resulting in inter and intra-individual variability in drug exposure [27, 28, 30, 32, 33, 36, 37]. Disease may also alter hepatic blood flow because of alterations in cardiac output related to cardiac failure and/or mechanical ventilation [28, 38] In patients with liver failure, a heavy loss of hepatocytes may also affect hepatic drug clearance [28]. Accordingly, disease significantly influences the activity and expression of CYP3A.

Proinflammatory cytokines released during infection and inflammation depress the activity of certain drug metabolizing enzymes in an isoform-specific manner [17, 28, 30, 39]. Cytokines are produced by cells such as macrophages, B- and T-lymphocytes, endothelial cells and mast cells in response to infection or other inflammatory diseases [17]. The proinflammatory cytokines interleukin-6, interleukin-1 and tumor necrosis factor-alpha are considered to be the

key cytokines that induce change in liver protein expression and activity of drug metabolizing enzymes. The mechanism is not fully understood, but in brief, cytokines bind to receptors on the cell surface in target organs and activate intracellular signal systems that regulate gene transcription and biosynthesis in a range of enzymes and transporters [8, 17]. Cell components including the transcription factor nuclear factor-κB and the pregnane X receptor upon heterodimerization with the nuclear receptor, retinoid X receptor, are important for gene expression of CYP3A4 [8, 40, 41]. Cytokines have been shown to induce the production of nuclear factor-κB which directly disrupts the binding of the pregnane X receptor retinoid X receptor-alpha complex to its response element, resulting in suppression of CYP3A4 expression (**Figure 2**) [8, 41]. Altered drug disposition in pathophysiological conditions like diabetes, cancer, rheumatoid arthritis and infection due to changes in gene expression are well established. However, the clinical implication is difficult to predict since these effects depend upon the degree of inflammation and thus may change when the disease is treated [8, 40].

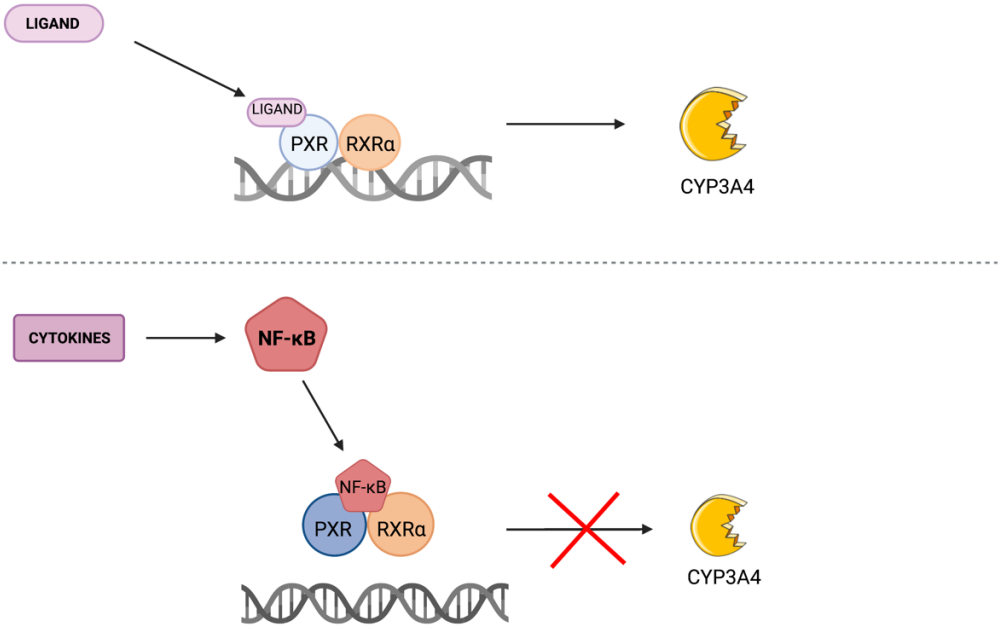


Figure 2. Under physiological conditions, ligands binds to the pregnane X receptor (PXR) which then heterodimerizes with the retinoid X receptor-alpha (RXRα) and binds to its response element leading to gene transcription and biosynthesis of CYP3A4. Proinflammatory cytokines released during inflammation or infection activates the production of the transcription factor nuclear factor- κB (NF- κB) which translocate into the nucleus and disrupts the binding of the PXR • RXRα complex to its response element leading to suppression of CYP3A4 expression.

1.3.4 Midazolam as a CYP3A probe drug

Midazolam is a short-acting benzodiazepine that exerts its effect by binding as a modulator to a subunit on the $GABA_A$ receptor, increasing the affinity and thus the inhibitory effect of the neurotransmitter γ-aminobutyric acid (GABA) [27, 42]. It has a rapid onset of action and a short time of duration [27, 43, 44]. Therefore, midazolam is well suited as a sedative, anticonvulsive, muscle relaxant and anxiolytic drug for pediatric patients in a variety of clinical settings, especially for mechanically ventilated patients. Furthermore, midazolam is a substrate for CYP3A4/5 and slightly CYP3A7 [27]. Since CYP3A7 is the main CYP enzyme in the fetal liver, it may contribute to clearance of midazolam in the first weeks of life [45, 46]. Midazolam is hydroxylated by the CYP3A isoenzymes to the major active metabolite 1-hydroxymidazolam and minimally to the inactive metabolites 4-hydroxymidazolam and 1,4-hydroxymidazolam (**Figure 3**) [42, 44, 47]. The metabolites are conjugated to the glucuronide forms and then excreted by the kidneys [27, 42]. Almost none of the intact midazolam are excreted in the urine [42, 44].

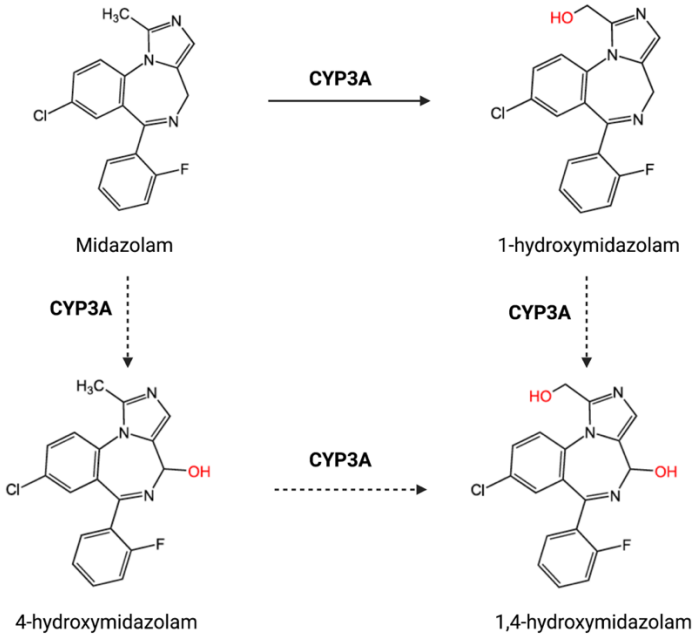


Figure 3. Structure and phase 1 metabolic pathways of midazolam. Midazolam is hydroxylated by the CYP3A isoenzymes to the major active metabolite 1-hydroxymidazolam and minimally to the inactive metabolites 4-hydroxymidazolam and 1,4-hydroxymidazolam.

Midazolam is considered to be the gold standard probe drug to determine CYP3A activity. The optimal method for examining real-time enzyme activity *in vivo* is phenotyping, where the metabolism of a specific probe drug is used to estimate the activity of the enzyme involved in the metabolism [19, 43, 48]. A probe drug is ideal when clearance directly correlates with intrinsic clearance by the specific enzyme investigated [43, 48, 49]. Furthermore, a probe drug should not be affected by variations in either protein binding nor hepatic blood flow. Thus, drugs with a low extraction ratio and minimally protein binding are most ideal. The absorption of probe drugs should not be affected by P-gp as it complicates the determination of the contribution of P-gp versus CYP3A on first-pass metabolism [50]. Finally, the probe drug must have an excellent safety profile for doses used in a clinical study situation, and a short duration of action is beneficial [43, 49]. As midazolam can be administered both intravenously and orally, it may be used to determine CYP3A activity in both the liver and intestine [19, 29, 43, 51]. When midazolam is administered intravenously, clearance will represent hepatic CYP3A activity. When midazolam is given both orally and intravenously, it will be possible to examine the contributions of both intestinal and hepatic CYP3A enzymes. Moreover, midazolam has an excellent safety profile at a dose providing detectable samples, and it has a short elimination half-life [37, 43]. This allows for easy estimation of pharmacokinetic parameters and variables [29].

The weaknesses of midazolam as a probe drug are its high protein binding (≈ 95%) and variable hepatic extraction ratio [43]. Rogers *et al*. calculated a mean extraction ratio of 0.55 from 24 healthy subjects, but with a range from 0.32-0.96 [48]. On average, midazolam is an intermediate extracted drug ($0.3 \leq E_H \leq 0.7$), where clearance may be dependent on clearance intrinsic, unbound fraction of drug and hepatic blood flow. In addition, midazolam as a probe drug requires multiple blood samples [51]. Accordingly, it may be difficult to study CYP3A activity in critically ill patients, due to great variability in their acute illness, comorbidities and co-administered drugs, which in turn can influence hepatic blood flow, protein binding and CYP3A enzyme activity [29, 43]. Despite these weaknesses, midazolam remains a well-studied, reliable and preferred probe drug for investigating CYP3A activity *in vivo* [19, 29, 43].

1.4 Drug therapy in the pediatric population

The vast majority of drugs are developed for use in the adult population, and a large amount of drugs/drug therapy prescribed to pediatric patients today are unlicensed or off-labeled [52]. That is prescribing a drug outside the terms of the license, for example in a different dose, as a different formulation, or for a different age group or disease. The off-label therapies increase the risks of adverse drug events, inadequate doses and the absence of a suitable pediatric formulation [53, 54]. Off-labeled therapy is often prescribed in a hospital setting [52]. In the last years, there has been an increased awareness on this topic, and the need for authorized medicinal products for children. The EU-regulation No 1901/2006 of the European parliament and the introduction of the pediatric investigation plan was established in 2006 [55]. The aim was to ensure that development of medicinal products potentially to be used for the pediatric population is actively investigated in this target population as well.

It may be challenging to find the optimal dose of a drug outside the terms of the license in a pediatric patient. The normal practice has been to linearly scale the adult dose to a pediatric dose solely based on the body weight (in milligrams per kilogram) or body surface area of the patient [56, 57]. However, this method has a tendency to overdose drugs in neonates and infants, because it does not take into account the overall organ function and that size itself does not reflect the impact of ontogeny on pharmacokinetics [56, 57].

1.4.1 Pharmacokinetics in the pediatric population

A child is not fully developed at birth, especially when it comes to physiology and biochemistry related to drug treatment [56]. Physiological changes occur rapidly, at least for the first decade of life, but these changes are not a linear process. There is no set classification of the pediatric population into age categories, but the following categorization has been suggested: neonates (0-27 days), infants and toddlers (>28 days – 23 months), children (2-11 years) and adolescent (12-16 years) (**Figure 4**) [58]. There is a difference in developmental growth between the different age categories, but it is also differences within the age categories and overlap between them [57]. **Table 1** summarizes the current knowledge regarding the effect of age on pharmacokinetics.

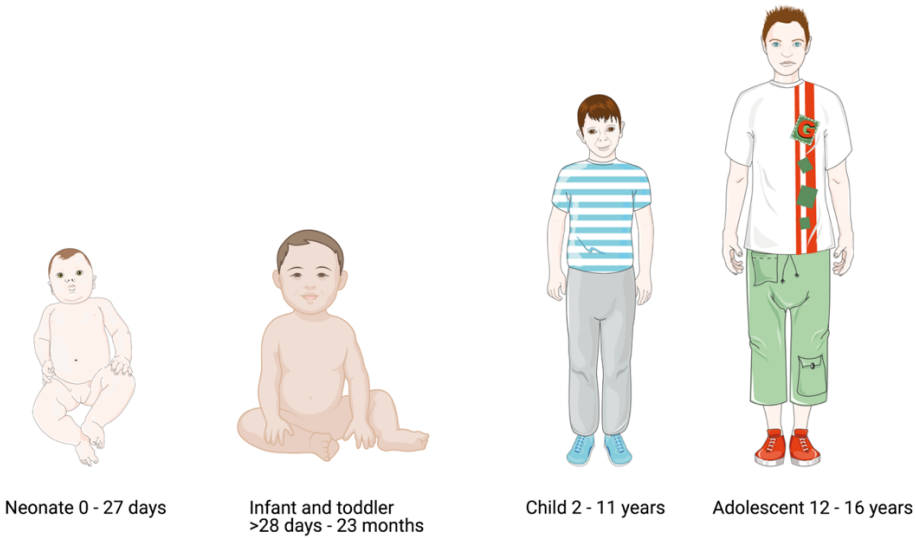


Figure 4. Classification of the pediatric population into age categories.

Absorption

Gastrointestinal absorption of drugs is influenced by physiological parameters including gastric pH, intestinal transit time and gastric emptying time, but also environmental factors like food and physicochemical properties of the drug [59]. The gastric pH is practically neutral at birth [59, 60]. Thereafter the pH slowly declines until it reaches adult values by 2 years of age, although there are some disagreements regarding the time required to reach adult values. Stomach acidity is decreased because of the frequent intake of milk in neonates and infants. These changes in gastric pH may result in increased absorption of weak bases and acid labile drugs in neonates compared with older children, even though most of the absorption of drugs takes place in the intestine [56, 61, 62]. There is a very limited understanding of the effect of age on gastric emptying time [59], but it is usually considered to be delayed in infants and toddlers [38, 61]. This may result in delayed time before reaching maximal plasma concentration of drugs, and thus altered plasma concentration profiles. Intestinal transit time appears to be shorter and more unpredictable in infants, resulting in incomplete absorption of sustained release products [38, 60]. However, other conflicting data indicate that mean intestinal transit time is similar in children and adults [59, 63].

Distribution

Neonates and infants have increased total body water- to- body fat ratio where total body water is about 78% of the newborn´s body weight compared to 60% of the adult´s body weight [56]. This contributes to an increase in the volume of distribution of hydrophilic drugs [56, 60, 64].

Consequently, water-soluble drugs need larger mg/kg loading doses to achieve therapeutic plasma- and tissue concentrations in the smallest children. Also, infants have a higher proportion of body fat compared to adolescents (22% at 12 months compared to 13% at 15 years), resulting in increased volume of distribution for lipophilic drugs in neonates and infants [56, 60]. Protein binding tends to be reduced in neonates and infants because of reduced albumin and α_1-acid glycoprotein concentrations, resulting in an increased unbound fraction of drugs, especially for highly protein-bound drugs, which in turn can lead to increased volume of distribution [64]. However, this is rarely clinically relevant.

Renal excretion

At birth, the kidneys are immature and thus the glomerular filtration rate (GFR) is low with a value close to 20 mL/min/1,73m^2 in term neonates [65], but it increases steadily to adult values are reached by 8-12 months of age [56, 62, 64]. Renal function is important to consider when dosing drugs that are eliminated through the kidneys. Tubular secretion is immature at birth but reaches adult values around 7 months of age [56].

Drug metabolism and transport

Maturation of hepatic drug metabolizing enzymes from birth plays a major role in non-renal drug clearance. Each individual isoenzyme has a unique maturation profile and may be absent at birth or present with low activity [56, 66, 67]. CYP3A7 is the predominant CYP enzyme detected in newborn liver, but it declines rapidly over time [31, 56, 59, 62, 66-69]. Conversely, hepatic CYP3A4 activity is low at birth, with increasing activity during the first weeks of life. CYP2C9 and CYP2C19 activity are rising slowly from birth to the first 6 months of life [59]. CYP2D6 activity is detectable hours after birth. CYP1A2 is the last hepatic CYP enzyme to appear with a slow developmental pattern after birth [59, 62]. CYP3A5 is expressed from birth, but with large inter-individual variability [66, 67]. It appears as the CYP enzymatic activity for CYP3A4, CYP1A2 and CYP2C9 exceeds adult values at approximately 1-2 years of life and decreases to adult levels at puberty [38, 57, 62, 64, 67, 68]. Higher weight-adjusted doses of drugs solely metabolized by these enzymes may thus be needed in children. P-gp is present in the intestine from birth, although the inter-individual variability is large [9]. Also, knowledge about the ontogeny of this efflux pump in the liver and intestine is sparse [45, 70]. In general, knowledge on the ontogeny of drug transporters during the early years of life is insufficient [5]. Infants, toddlers and young children have a larger size of liver expressed as a percentage of

body weight compared to adults, resulting in increased hepatic blood flow [59, 60, 64, 71, 72]. Accordingly, drugs with a high extraction ratio may have a higher drug clearance in this population.

Although most studies confirm the developmental patterns described above, there is still a need for increased knowledge on the maturation of drug metabolizing enzymes and the function of age on pharmacokinetics [31]. In addition, very few studies have been performed on the ontogeny of drug metabolizing enzymes in the intestine, and it appears that the ontogeny of CYP3A activity in the liver and intestine does not always change with age in parallel [49, 73]. More information is needed to be able to determine the effect on first-pass metabolism and thus bioavailability of drugs in the pediatric population.

Table 1. Summary of pharmacokinetic differences in the pediatric population compared to adults

	Developmental change	**Pharmacokinetic consequence**
Absorption	↑ Gastric pH	↑ Absorption of weak bases and acid labile drugs
	Delayed gastric emptying time	↑ T_{max}
	↑ ⇆ Intestinal transit time	Incomplete absorption of sustained release products
Distribution	↑ Body water- to body fat ratio in neonates and infants	↑ Volume of distribution of hydrophilic drugs
	↑ Body fat in infants compared to older children	↑ Volume of distribution for lipophilic drugs
	↓ Plasma proteins	↑ Free fraction of drug in plasma
Metabolism	Larger relative size of liver	↑ Hepatic clearance of drugs with a high extraction ratio
	Ontogeny of CYP enzymes	↑ ⇄ ↓ Hepatic clearance, different maturation profiles for individual isoenzymes
Elimination	↓ GFR in neonates	↓ Renal clearance
	Ontogeny of tubular transporters	↓ Tubular secretion in neonates

Abbreviations: CYP, cytochrome P450; GFR, glomerular filtration rate; T_{max}, time to reach maximum concentration

1.4.2 CYP3A activity in the pediatric population

Most of the pharmacokinetic studies examining midazolam clearance and thus CYP3A activity in the pediatric population are conducted in ill patients, and a large proportion of these studies have been conducted in critically ill patients at the pediatric intensive care unit. Admission to the pediatric intensive care unit for children 0-18 years is due to either acute critical illness, planned surgical procedures or illness that requires special monitoring and treatment [74]. Examples of diagnoses at admission are sepsis, respiratory failure, cardiac disorder and liver failure [28, 75]. During the hospital stay, patients receive several different drugs. Antibiotics, furosemide, ketamine, dexamethasone, benzodiazepines, bronchodilators and painkillers such as paracetamol, morphine and ibuprofen are widely used [52, 76, 77]. Patients may also need mechanical ventilation, which requires sedatives and analgesics [78, 79]. A normal combination is a benzodiazepine (often midazolam) and an opioid [78-81]. Both critical illness and comedication can affect CYP3A activity substantially.

Previous studies have reported a large inter-individual variability in midazolam clearance, and there is even greater variability in critically ill infants, children and adolescents [32, 33, 82-86]. In a review from 2015, including 25 articles, Altamimi *et al*. identified papers that described midazolam clearance in children [82]. The variability in clearance was expressed as the coefficient of variation (CV). The CV was greater in critically ill patients (18-170%) compared to non-critically ill patients (13-54%). The absolute range of midazolam clearance reported for critically ill patients was 0.1-67 mL/min/kg in the age group preterm neonates to 18-year-old adolescents. The absolute range of clearance reported for non-critically ill children from 2-11 years was 1.1-23 mL/min/kg. However, most of the articles presented data for critically ill children, and there were no corresponding amounts of data for non-critically ill children. Nevertheless, this review reflects the large inter-individual variability in midazolam clearance in patients through childhood.

In a study by Vet *et al*., the relationship between organ failure, inflammation and midazolam clearance in critically ill children admitted to the pediatric intensive care unit was investigated using a population pharmacokinetic model [28]. Midazolam was used as a probe drug to study CYP3A mediated drug metabolism. C-reactive protein (CRP) concentrations were used as an inflammatory marker. Vet *et al*. showed that a CRP concentration of 300 mg/L was associated with a 65% lower clearance of midazolam, compared to a CRP concentration of 10 mg/L. They also found that midazolam clearance decreased with an increasing number of failing organs.

Furthermore, Reed *et al.* studied the absolute bioavailability of midazolam in relatively healthy children 6 months – 12 years of age [87]. Average bioavailability was 36%, but there was considerable inter-individual variability, ranging from 9-71%. This may reflect both the ontogeny of hepatic and intestinal CYP3A enzymes and the inter-individual variability in CYP3A activity. Unfortunately, absolute bioavailability could only be determined in 6 patients. Brussee *et al.* also studied intestinal and hepatic CYP3A-mediated metabolism of midazolam in children 0-18 years old using a physiologically based pharmacokinetic model, in order to predict first-pass metabolism and bioavailability [73]. Median bioavailability of midazolam was found to be 20% (95% CI: 3.8-50%), and age-independent. They also concluded that the intrinsic CYP3A mediated intestinal clearance was substantially lower than the intrinsic hepatic clearance, and thus contributes less to the first-pass metabolism of midazolam in children compared to adults.

Differences in the development of CYP3A activity (both hepatic and intestinal) in pediatric patients may, in addition to disease, alter the pharmacokinetics of midazolam [32]. Although studies on midazolam clearance have previously been performed in children, there is still a need for more data to better understand the effect of age on pharmacokinetics and to be able to optimize drug dosing in the pediatric population.

1.5 Aim

In the recent years, there has been an increased focus on the development and availability of effective and safe drugs for the pediatric population. However, there is still important gaps in our knowledge regarding the ontogeny of essential CYP enzymes in both the intestine and liver in the pediatric population. As the CYP3A subfamily is of significant relevance regarding the metabolism of the majority of drugs, it is also of interest to understand how these enzymes develop in children.

The aim of this thesis was to conduct a pilot study investigating the ontogeny of systemic (hepatic) CYP3A activity across different age groups by using midazolam as a prode drug, and to optimize and partially validate an ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method to quantify midazolam and 1-hydroxymidazolam concentrations in plasma samples from the CyPed study.

2 Methods

2.1 Method optimization for determination of midazolam and metabolite plasma concentrations

Midazolam and 1-hydroxymidazolam concentrations were quantified in plasma samples from the CyPed-study by using a validated UHPLC-MS/MS method as previously described [88]. This method was developed to quantify midazolam and the metabolite plasma concentrations after administration of low doses of midazolam (1.5 mg oral and 1.0 mg intravenous) for CYP3A phenotyping in the adult population. Since the patients included in the CyPed-study received therapeutic doses of midazolam, the plasma concentrations of midazolam and the metabolite were expected to be outside the previously validated calibration range (0.1 to 100 ng/mL). Therefore, the sample preparation and calibration range needed modification in order to quantify the expected higher midazolam and 1-hydroxymidazolam plasma concentrations with satisfying accuracy and precision. Lists of materials, solutions and equipment used in the analysis are listed in the supplementary.

2.1.1 Original UHPLC-MS/MS method

The original and validated UHPLC-MS/MS method [88] was used to quantify midazolam and 1-hydroxymidazolam concentrations in plasma samples from 2-3 study participants in order to determine an expected concentration range for the plasma samples from the CyPed-study.

2.1.1.1 Sample preparation

Plasma samples and blank plasma were thawed at room temperature and then centrifuged for 5 minutes at 700 g together with the calibrators and quality control (QC) samples. Calibrators and QC solutions were prepared in methanol, and 30 µL of each calibrator and 80 µL of each QC sample were added to separate Eppendorf tubes (1.5 mL). They were then evaporated to dryness using N_2 gas and heat (60ºC) for 5-8 minutes. Thereafter, 150 µL of blank plasma were added to the Eppendorf tubes containing calibrators, while 400 µL of blank plasma was added to the Eppendorf tubes containing QC samples. One hundred µL of each calibrator and QC sample (3 parallels), as well as patient samples, were added to a 96-well tray with 0.5 mL wells. For protein precipitation, 200 µL of cold 95% acetonitrile and 5% methanol containing deuterated

internal standards of 2.5 ng/mL (midazolam-d6) and 0.5 ng/mL (1-hydroxymidazolam-d5) were added to each well. Self-adhesive aluminum foil was placed on the tray and samples were vortex-mixed for minimum 30 seconds, followed by 1 hour of storage at -20ºC. Samples were then centrifuged for 10 minutes at 4,000 rpm (4ºC) and 50 µL of supernatant was added to a Vanquish-tray. Next, 50 µL of mobile phase A was added to each well. Self-adhesive aluminum foil was placed on the Vanquish-tray to avoid evaporation before injection (5 µL) into the UHPLC-MS/MS system.

2.1.1.2 Calibrators and quality control samples

Calibrators and QC solutions were prepared as described in 2.1.1.1. For midazolam, the calibration curve consisted of ten concentration levels, ranging from 0 to100 ng/mL (0, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 75, 100 ng/mL). For 1-hydroxymidazolam, ten concentration levels were used for the calibration curve, ranging from 0 to 10 ng/mL (0, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 7.5, 10 ng/mL). QC samples were prepared independently from the calibrators at 4 concentrations levels: 0.25, 2.5, 25, 100 ng/mL for midazolam and 0.025, 0.25, 2.5, 10 ng/mL for 1-hydroxymidazolam. The calibration curve for midazolam and 1-hydroxymidazolam was best fitted with linear regression, weighting index 1/x and forced origin.

2.1.1.3 UHPLC-MS/MS analysis

The UHPLC-MS/MS system consisted of a Vanquish UPLC coupled to an Altis triple quadrupole mass spectrometer (Thermo-Fisher, Waltham, MA). Chromatographic separation was performed by UHPLC using an Accucore Vanquish C18, 2.1 x 50 mm reverse phase column (Thermo-Fisher). Gradient elution was performed with mobile phase A (5% acetonitrile and 10 mM ammonium formate) and mobile phase B (90% acetonitrile and 10% methanol) as presented in **Table 2**. The retention time for midazolam and 1-hydroxymidazolam was 0.66 minutes and 0.73 minutes respectively. The total run time was 4.5 minutes. Detection was performed by positive electrospray ionization tandem mass spectrometry. The MS settings with respect to gas and temperature are listed in **Table 3**.

Table 2. Gradient elution for UHPLC-method for separation of midazolam and 1-hydroxymidazolam

Time (min)	% Mobile phase A	% Mobile phase B	Flow rate (mL/min)
0.40	70%	30%	0.400
1.40	5%	95%	0.400
2.60	5%	95%	0.400
2.65	70%	30%	0.400
4.50	Stop run		

Table 3. Gas and temperature settings of the mass spectrometer

Sheath gas (Arb)	35
Aux gas (Arb	10
Sweep gas (Arb)	1
Ion transfer tube temp (ºC)	325
Vaporizer temp (ºC)	350

2.1.2 Optimized UHPLC-MS/MS method

As described, the sample preparation and the calibration curve range needed to be modified in order to quantify midazolam and the metabolite in samples from the CyPed-study. In the optimized sample preparation a lower volume of plasma was used, as higher concentrations of midazolam and 1-hydroxymidazolam were expected. In addition, calibrators and QC samples were prepared in plasma (as opposed to methanol) to simplify the sample preparation and make it more efficient. Besides these changes, no other modification to the previous method was performed.

2.1.2.1 Optimized sample preparation

The optimized sample preparation is illustrated in **Figure 5**. Plasma samples, calibrators and QC samples were thawed at room temperature and centrifuged for 5 minutes at 700 g. Twenty µL of each plasma sample, calibrator and QC samples (3 parallels) as well as 200 µL precipitation solution were added to a 96-well tray with 0.5 mL wells. Self-adhesive aluminum foil was placed on the tray and samples were vortex-mixed before storage at -20ºC for 1 hour. Samples were thereafter centrifuged for 10 minutes at 4,000 rpm (4ºC), and 20 µL of supernatant was added to a Vanquish-tray. Next, 100 µL of mobile phase A was added to each

well. Self-adhesive aluminum foil was placed on the Vanquish-tray to avoid evaporation before injection (5 µL) into the UHPLC-MS/MS system.

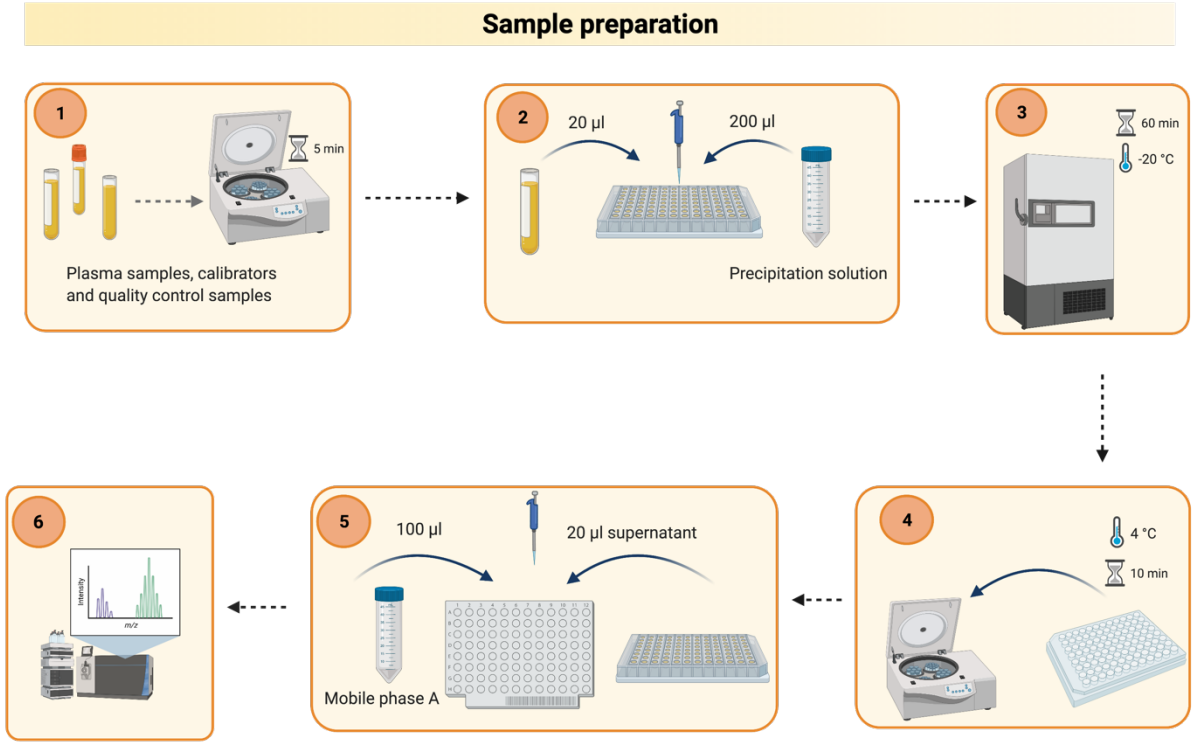


Figure 5. Overview of optimized sample preparation. Thawed plasma samples, calibrators and quality control (QC) samples were centrifuged for 5 minutes at 700 g. Twenty µL of each plasma sample, calibrator and QC samples (3 parallels) as well as 200 µL precipitation solution were added to a 96-well tray with 0.5 mL wells. Samples were kept at -20ºC for 1 hour. Samples were thereafter centrifuged for 10 minutes at 4,000 rpm (4ºC), and 20 µL of supernatant was added to a Vanquish-tray. Next, 100 µL of mobile phase A were added to each well. Self-adhesive aluminum foil was placed on the Vanquish-tray to avoid evaporation before injection (5 µL) into the UHPLC-MS/MS system.

2.1.2.2 Calibrators and quality control samples

A calibration curve was prepared to determine the unknown concentrations of the analytes in plasma samples from the patients in the CyPed-study. Calibrators and QC samples were prepared in plasma and thereafter prepared as described in 2.1.2.1. The calibration curve consisted of 8 different concentration levels of midazolam and 1-hydroxymidazolam ranging from 0 to 1000 ng/mL (0, 10, 25, 50, 100, 250, 500, 750, 1000 ng/mL) and 0 to 100 ng/mL (0, 1, 2.5, 5, 10, 25, 50, 75, 100 ng/mL) respectively. QC samples were prepared independently

from the calibrators at 4 concentrations levels: 25, 250, 500 and 1000 ng/mL for midazolam, and 2.5, 25, 50 and 100 ng/mL for 1-hydroxymidazolam.

2.1.2.3 UHPLC-MS/MS

UHPLC-MS/MS analysis was performed as described in 2.1.1.3.

2.1.3 Validation

A partial validation of the optimized method as described in 2.1.2 was performed in accordance with the guideline from European Medicines Agency (EMA) on bioanalytical method validation [89]. This included validation of calibration curve, the lower limit of quantification (LLOQ) and determination of accuracy and precision of the optimized method. Full validation of the original method has been performed previously [88].

2.1.3.1 Calibration curve

The choice of calibration model was determined by evaluating 5 different runs with calibrators and QC samples. Linear and quadratic curve fitting, with either weighting factor $1/x^2$, $1/x$ or without weighting, and with or without forced origin were investigated. Percentage relative deviation from nominal value (%RE) for the measured QC samples and the proportion of QC samples that did not satisfy the requirements (% QC fail) were emphasized. The calibration model with the lowest total of %RE and % QC fail was chosen.

2.1.3.2 Lower limit of quantification

The LLOQ, which is the lowest concentration of analyte in a sample that can be quantified with acceptable accuracy and precision, was determined for the optimized method [89]. The LLOQ was set as the lowest concentration in the calibration curve. The analyte signal of the LLOQ sample should be at least 5 times the signal of the blank sample.

2.1.3.3 Accuracy and imprecision

Accuracy and imprecision of the method were evaluated both within-run and between-run. Within-run accuracy and imprecision were determined by analyzing 5 parallels in a single run, and between-run accuracy and imprecision were determined by analyzing 3 parallels on 5

separate days. For accuracy, mean concentrations within ±15% of the nominal value were accepted, except for the LLOQ where mean concentrations within ±20% of the nominal value were accepted. For precision, CV less than 15% were accepted, except for the LLOQ where less than 20% were accepted.

2.2 The CyPed pilot study

The CyPed-study is an ongoing single-center, open, prospective, non-randomized study at the Pediatric Intensive Care Unit, Oslo University Hospital, Rikshospitalet. The primary study objective is to describe hepatic CYP3A phenotype in the pediatric population using midazolam as a probe drug for CYP3A activity. A total of 130 patients will be included in the study. The study is approved by the Regional Committee for Medical and Health Research Ethics (2019/31635/REK) and performed in accordance with the principles of the Declaration of Helsinki and the guidelines of Good Clinical Practice [90]. In this master thesis, a pilot study including 13 patients was performed.

Informed consent procedure

Information about the study was provided to the parents or legal representatives both orally and in writing when a patient was eligible for inclusion in the study. Written informed consent was obtained before any study-specific procedures were initiated (Supplementary). The parents or legal representatives were also informed that they are allowed to withdraw their children from the study at any time. Whenever possible, the children received information about their study participation afterwards using separate information sheets customized for pediatric patients; one for children aged 0-12 years and one for children aged 12-16 years (Supplementary).

2.2.1 Study design and population

The study design is presented in **Figure 6.** Patients aged 0-16 years scheduled to receive continuous treatment with intravenous midazolam as part of their standard treatment of care, for any medical reason or condition, were eligible for participation in the study. Patients were divided into four age-specific groups: 0-6 months, 6 months – 2 years, 2-5 years and 5-16 years, called A, B, C and D, respectively. The midazolam dose was individualized based on therapeutic effect and dosed according to the routine at the Pediatric Intensive Care Unit. Thus,

study participation did not interfere with any treatment procedures. Concomitant treatment with other drugs did not influence study participation. Exclusion criteria included conditions anticipated to interfere with hepatic and/or gastrointestinal drug disposition. Potential participants were identified by screening of patients hospitalized at the Pediatric Intensive Care Unit. The chief physician at the unit had the main responsibility for both screening and inclusion of patients.

The study consisted of two parts; a primary and a secondary investigation. For the primary investigation, blood samples were drawn during continuous midazolam infusion and after dose adjustment or withdrawal (**Figure 6**). The primary endpoint was to determine systemic clearance of the CYP3A probe drug midazolam. For the secondary investigation, a single dose of midazolam syrup was administered orally or through a nasogastric tube in close time to the continuous infusion (within ±5 days) (**Figure 6**). The objective of this was to investigate the relationship between intestinal and hepatic ontogeny of CYP3A activity, and was only performed in a subgroup of the included patients.

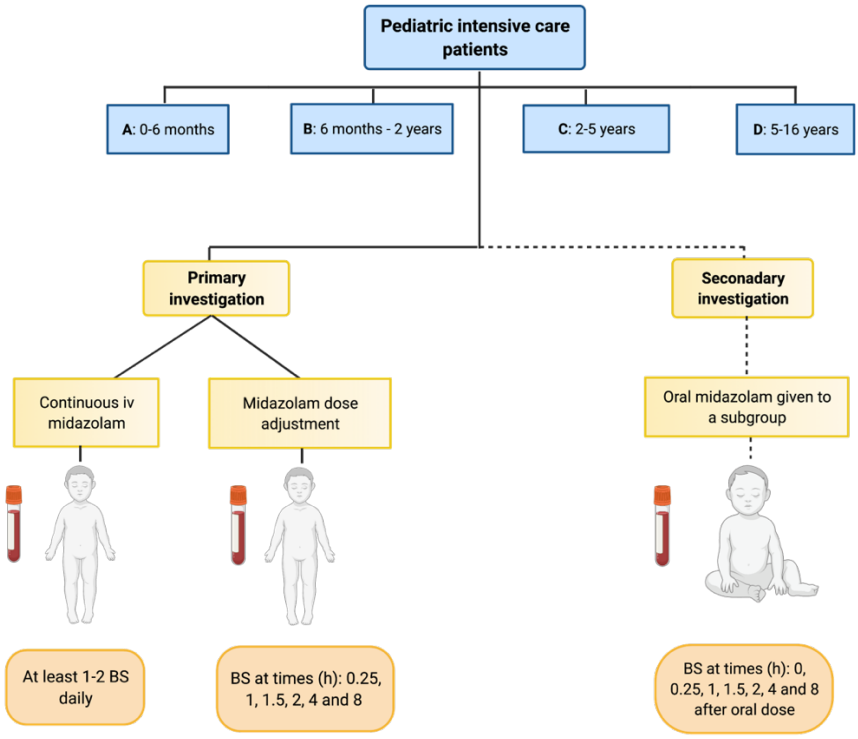


Figure 6. **Study design**. Patients hospitalized at the Pediatric Intensive Care Unit and scheduled to receive continuous infusion with midazolam as part of their treatment were eligible for inclusion in the study. Patients were categorized into four specific age groups; A: 0-6 months, B: 6 months – 2 years, C: 2-5 years and D: 5-16 years to ensure patients across the pediatric age range. Abbreviations: BS, blood samples; h, hours; iv, intravenous

2.2.2 Study procedures

Blood samples for determination of midazolam plasma concentration were obtained from an arterial tap during midazolam infusion and after dose adjustment or drug withdrawal. To limit the trial-related blood loss, only a small volume of blood (0.5 mL) was drawn for each sample. The remaining blood volume following a blood gas analysis, which was already a part of the patient monitoring were used as study specific blood samples. The blood volume drawn from each patient was thus kept to a minimum. At least 1-2 samples were obtained each day. One time during the study period, after midazolam dose adjustment or withdrawal, blood samples were collected at 0.25, 0.5, 1, 1.5, 2, 4 and 8 hours. The date and time for blood sampling and the midazolam infusion rate were documented in case report forms (CRFs) (Supplementary) by the hospital personal collecting the blood samples. In the subgroup of patients receiving an oral dose of midazolam (0.4 mg/kg or 1.5 mg/kg if ≥ 3.8 kg), blood samples were collected at 0, 0.25, 1, 1.5, 2, 4 and 8 hours. Blood samples for determination of midazolam plasma concentrations were drawn in 0.5 mL BD Microtainer vacutainer tubes (K2-EDTA) and centrifuged for 10 minutes at 4ºC (1,800 g) within maximum 72 hours after collection. Plasma was then transferred into cryovials and stored at -20ºC at the Oslo University Hospital, before transported to the Department of Pharmacy, University of Oslo where samples were stored at -80ºC until analysis.

In order to assess the potential impact of other drugs on CYP3A activity, all concomitant drug therapy during the study period for each patient was collected from MetaVision (software for electronic medical records). Relevant biochemistry and hematology blood samples were obtained by the hospital personal when considered necessary and analyzed at the Department of Clinical Chemistry, Oslo University Hospital, Rikshospitalet. For each patient, values of relevant biomarkers as well as age, weight and sex, were collected from DIPS (software for electronic patient record) and recorded. Changes in relevant biomarkers and a corresponding change in midazolam pharmacokinetics were examined for some patients. Medical records were used to determine the reason for admission to the Pediatric Intensive Care Unit, and the main reason for hospitalization was recorded. Based on the diagnosis, patients were categorized into main categories of disease.

2.2.3 Quantification of midazolam and metabolite concentrations

The concentration of midazolam and 1-hydroxymidazolam in plasma samples from the CyPed pilot study were quantified by using a calibration curve as described in 2.1.2.2. The plasma samples were prepared and analyzed as described in 2.1.2.1 and 2.1.1.3 respectively.

2.3 Pharmacokinetic calculations

Non-compartmental methods were used to determine the pharmacokinetics of midazolam and 1-hydroxymidazolam for the individual patient. Clearance for patients where steady-state was reached was calculated using the following equation:

$$\text{Clearance (L/h)} = \frac{\text{Infusion rate (mg/kg/h)}}{C_{ss}\ \text{(ng/mL)}} \quad (2)$$

where C_{ss} is the steady-state concentration achieved after approximately 5 times the elimination half-life. The elimination half-life from available literature was used to calculate the expected time to achieve steady-state [32, 83, 84]. At least 50 hours without changes in infusion rate was set as the cut-off value for time to reach steady-state. For patients who were not expected to have achieved C_{ss} due to few blood samples or frequent adjustment of the midazolam infusion rate, individual clearance estimates (CL_{ind}) were determined by using a clearance estimate for the population (CL_{pop}). CL_{pop} was determined in the following age groups A, B, C and D, using the calculated clearance from patients determined from actual steady-state concentrations (described above). Expected steady-state concentration (expected C_{ss}), with the given CL_{pop} was calculated using the following equation:

$$\text{Expected } C_{ss}\ \text{(ng/mL)} = \frac{\text{Infusion rate (mg/kg/h)}}{\text{CL}_{\text{pop}}\text{(L/h)}} \quad (3)$$

The difference between the expected steady-state concentration and all the actual measured concentrations, expressed as an individual factor, was determined for each actual concentration measurement:

$$\text{Factor} = \left(\frac{\text{Expected } C_{ss}}{\text{Actual measured concentration}}\right) \quad (4)$$

The mean factor between the expected steady-state concentration and the actual measured concentrations was calculated and further used to adjust the CL_{pop} to determine an individual clearance estimate (CL_{ind}):

$$CL_{ind} = CL_{pop} * \text{factor} \quad (5)$$

An individual mean metabolic ratio of 1-hydroxymidazolam/midazolam was calculated using all the available plasma concentrations for each patient. This average is reffered to as the metabolic ratio throughout this master thesis, unless otherwise is stated. If more than one sample/concentration measurement were available within a 12-h period, the mean concentration of these available concentrations were calculated and used to describe the plasma concentrations-time profiles.

RStudio (version 1.3.1093) was used to create plots and Biorender was used to create figures [91, 92]. Microsoft Excel (version 16.48) was used for all pharmacokinetic calculations. Results are expressed as median (absolute range) unless otherwise specified.

3 Results

3.1 Validation of the UHPLC-MS/MS method

3.1.1 Calibration curve

The calibration curves showed a quadratic relationship between concentration and peak height ratio. For midazolam, the calibration curve was best fitted by quadratic regression with a weighting factor of 1/x and without forced origin. The calibration curve for 1-hydroxymidazolam was best fitted by quadratic regression without weighting and with forced origin. R-squared (R^2) was >0.99 for both midazolam and 1-hydroxymidazolam (**Figure 7**). The chosen calibration model for midazolam (**Table 4**) and 1-hydroxymidazolam (**Table 5**) showed the lowest %RE and the lowest proportion of QC samples that did not satisfy the requirements (± 15% of nominal value or ± 20% for LLOQ).

Table 4. **Calibration curve models for midazolam**. The data are presented as absolute percentage relative deviation from nominal value (%RE) calculated for all QC samples, and the proportion of QC samples that did not satisfy the requirements (% QC fail) with the given calibration model.

Calibration model (type, origin, weighting)	Absolute %RE	% QC fail
Linear, ignore, equal	1199	32.4
Linear, ignore, 1/x	712	22.0
Linear, ignore, $1/x^2$	1379	50.0
Linear, force, equal	1012	35.3
Linear, force, 1/x	1015	41.2
Linear, force, $1/x^2$	1745	42.6
Quadratic, ignore, equal	838	25.0
Quadratic, ignore, 1/x	**498**	**7.4**
Quadratic, ignore, $1/x^2$	744	30.9
Quadratic, force, equal	709	17.7
Quadratic, force, 1/x	619	13.3
Quadratic, force, $1/x^2$	808	35.3

Table 5. Calibration curve models for 1-hydroxymidazolam. The data are presented as absolute percentage relative deviation from nominal value (%RE) calculated for all QC samples, and the proportion of QC samples that did not satisfy the requirements (% QC fail) with the given calibration model.

Calibration model (type, origin, weighting)	Absolute %RE	% QC fail
Linear, ignore, equal	921	25.0
Linear, ignore, 1/x	539	8.8
Linear, ignore, $1/x^2$	782	30.9
Linear, force, equal	626	16.2
Linear, force, 1/x	654	13.2
Linear, force, $1/x^2$	911	30.9
Quadratic, ignore, equal	618	11.8
Quadratic, ignore, 1/x	524	7.4
Quadratic, ignore, $1/x^2$	567	10.3
Quadratic, force, equal	**506**	**7.4**
Quadratic, force, 1/x	525	7.4
Quadratic, force, $1/x^2$	554	8.8

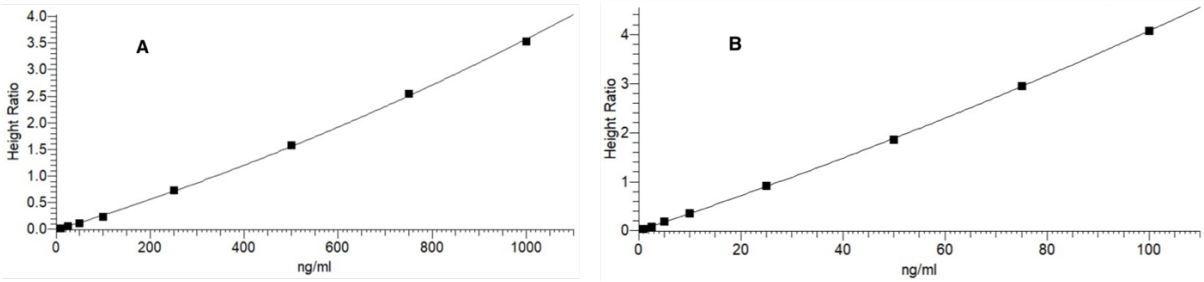


Figure 7. **Calibration curves for A) midazolam and B) 1-hydroxymidazolam**. The calibration curve for midazolam was best fitted by quadratic regression, weighting factor of 1/x and without forced origin, while the calibration curve for 1-hydroxymidazolam was best fitted by quadratic regression, without weighting factor and with forced origin.

3.1.2 Lower limit of quantification

A LLOQ of 10 ng/mL for midazolam and 1 ng/mL for 1-hydroxymidazolam resulted in a signal/noise ratio >313 and signal/noise ratio >121 respectively (**Figure 8**). The LLOQ showed an acceptable accuracy and precision for both midazolam and 1-hydroxymidazolam, as described in 3.1.3.

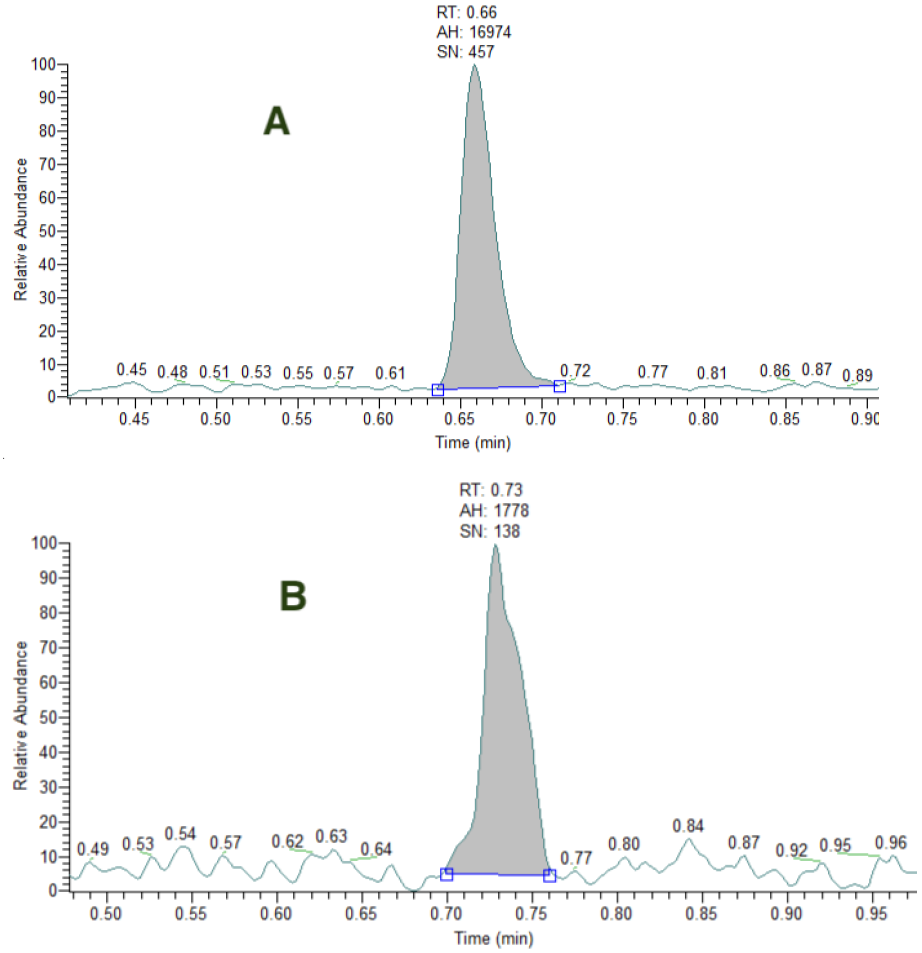


Figure 8. Chromatogram of lower limit of quantification (LLOQ) for A) midazolam with a signal/noise ratio of 457 and B) 1-hydroxymidazolam with a signal/noise ratio of 138.

3.1.3 Accuracy and imprecision

The partial validation of the optimized method showed acceptable accuracy and imprecision for both midazolam and 1-hydroxymidazolam, and met the requirements in the guideline from EMA on bioanalytical method validation (**Table 6**) [89]. The within-run and between-run CVs for midazolam were <4.7% and <7.4% for all QC concentrations, respectively. Within-run

mean accuracy for midazolam was between 97% and 109% and between-run mean accuracy was between 98% and 111% for all QC concentrations. For 1-hydroxymidazolam, the within-run and between-run CVs were <7.2% and <9.8% for all QC concentrations, respectively. Within-run mean accuracy for 1-hydroxymidazolam was between 93% and 101%, and between-run mean accuracy was between 97% and 108% for all QC concentrations.

Table 6. Within-run and between-run accuracy and imprecision for midazolam and 1-hydroxymidazolam.

		Within-run (n=5)[1]		**Between-run (n=15)[2]**	
	Nominal concentration (ng/mL)	Accuracy (mean, %)	Imprecision (CV,%)	Accuracy (mean, %)	Imprecision (CV,%)
Midazolam	25	97	4.7	98	7.4
	250	103	4.0	107	4.9
	500	109	4.2	111	4.7
	1000	103	1.4	104	4.8
1-hydroxymidazolam	2.5	93	5.4	97	9.8
	25	106	7.2	104	6.9
	50	110	6.5	108	5.2
	100	109	4.1	107	5.6

[1] Five parallels analyzed at the same day

[2] Five series of 3 parallels analyzed separate days

Abbreviations: CV, coefficient of variation

3.2 The CyPed pilot study

3.2.1 Patient characteristics

In this pilot study, 13 patients were included of whom 77% were boys and 23% were girls. Median age was 11 months (absolute range: 0 months – 15 years) and of the included patients, 3 were in age group A (0-6 months), 6 in age group B (6 months – 2 years), 1 in age group C (2-5 years) and 3 in age group D (5-16 years). Baseline characteristics of the patients and relevant biomarkers are summarized in **Table 7**. A large inter-individual variability in baseline CRP concentrations was observed, with an absolute range of 0-283 mg/L. The highest CRP

concentration observed was 297 mg/L. The highest concentration of aspartate aminotransferase (ALAT) and alanine aminotransferase (ASAT) observed was 539 U/L and 692 U/L, respectively. The median creatinine and albumin concentrations at baseline were similar in all age groups. Patients were admitted to the Pediatric Intensive Care Unit due to the following main categories of disease: cancer (n=2), gastrointestinal disorder (n=3), head injury (n=1), infection (n=4), respiratory disorder (n=2) and other disease (n=1), and the median length of stay at the intensive care unit was 10 days (absolute range: 5-51 days). The median length of study participation was 4 days (absolute range: 2-12 days).

Table 7. Demographic data of patients at baseline. Values are expressed as median (absolute range) or number of patients. Exact values are presented for group C since only one patient was included in this group.

	All n=13	**Group A n=3**	**Group B n=6**	**Group C n=1**	**Group D n=3**
Gender (male/female)	10/3	2/1	5/1	1/0	2/1
Age (days, months or years)	0 (0-15)	7 (5-9)[1]	10 (7-18)[2]	2	8 (6-15)
Body weight (kg)	10 (2.5-80.2)	3.5 (2.5-4)	9 (5.3-11.2)	11.5	28 (22-80.2)
ASAT (U/L)	46 (19-692)	34 (20-45)	91 (18-692)	19	48 (46-56)
ALAT (U/L)	26 (6-539)	13 (13-42)	61 (6-539)	27	26 (21-39)
Creatinine (µmol/L)	25 (13-46)	29 (25-40)	20 (13-27)	24	35 (13-46)
CRP (mg/L)	34 (0-283)	29 (0-49)	26 (3.4-162)	79	130 (2.1-283)
Albumin (g/L)	30 (21-38)	23 (21-31)	31 (24-38)	31	30 (28-32)

Abbreviations: ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; CRP, C-reactive protein

[1]Expressed as days

[2]Expressed as months

Concomitant drug therapy

The median number of drugs each patient received during study participation were 16 with an absolute range of 8 to 38 drugs (**Table 8**). All patients received treatment with at least one antibiotic, analgesic and diuretic drug. Both glucocorticoids and antihypertensive drugs were used by 77% of the study participants. Sixty-two percent of the patients used at least one hypnotics or sedative, one adrenergic drug and one muscle relaxant. Twenty-three percent of the patients received treatment with a CYP3A inhibitor and 85% with a CYP3A inducer (**Table 9**). The number of patients treated with a drug that inhibits or induces CYP3A activity were similar in age group A, C and D, but lower compared with age group B.

Table 8. Overview of concomitant drug treatment during the study period. Values for the ten most frequently used drug categories are shown, expressed as median (absolute range) or number of patients.

	All n=13	**Group A n=3**	**Group B n=6**	**Group C n=1**	**Group D n=3**
Number of total drugs	16 (8-38)	9 (8-22)	14 (12-27)	28	22 (18-38)
Antibiotics	13	3	6	1	3
Adrenergic drugs	8	1	3	1	3
Diuretics	13	3	6	1	3
Antithrombotic drugs	7	2	3	0	2
Antiemetics	7	1	3	1	2
Antihypertensive drugs	10	2	5	1	2
Glucocorticoids	10	2	5	1	2
Muscle relaxants	8	2	3	1	2
Anesthetics and analgesics	13	3	6	1	3
Hypnotics and sedatives	8	0	4	1	3

Table 9. Concomitant drug treatment with drugs that inhibit or induce the CYP3A activity. Values are expressed as number of patients who received an inhibitor or inducer.

	All n=13	**Group A n=3**	**Group B n=6**	**Group C n=1**	**Group D n=3**
Inhibitors					
Aprepitant*	1	0	0	0	1
Fluconazole	1	0	1	0	0
Posaconazole	1	0	1	0	0
Inducers					
Phenobarbital	1	1	0	0	0
Glucocorticoids	10	2	5	1	2

*Initially aprepitant inhibits the metabolism of midazolam via CYP3A4, but with prolonged treatment, the metabolism via CYP3A4 is induced.

3.2.2 Pharmacokinetics of midazolam and 1-hydroxymidazolam

Pharmacokinetic data of midazolam and 1-hydroxymidazolam are presented in **Table 10**. A total of 301 plasma samples were analyzed. Median samples per patient were 18 (absolute range: 4 to 61 samples). Median maximum midazolam plasma concentration (C_{max}) was 204 ng/mL in group A, 158 ng/mL in group B and 442 ng/mL in group D. Only one patient was included in group C, and the maximum midazolam plasma concentration for this patient was 291 ng/mL. Median minimum midazolam plasma concentration (C_{min}) was 38 ng/mL in group A, 5 ng/mL in group B and 90 ng/mL in group D. For the patient in group C, minimum midazolam plasma concentration was 2 ng/mL.

Clearance

The median estimated midazolam clearance was 0.63 L/h, 3.9 L/h and 20 L/h for group A, B and D, respectively. For the patient in group C, clearance was 10.5 L/h. There was a 141-fold difference in clearance between the patient with the lowest clearance (7 days old) compared with the patient with the highest clearance (15 years old). A positive association between age and clearance was seen in a simple linear regression analysis: $\beta = 3.8$, $R^2 = 0.8$, $P < 0.001$ (**Figure 9**) There was a 10-fold difference in clearance within the patients in group A and the clearance within group B differed by 2-fold. Further, the difference in clearance within group D was 23-fold. The estimated clearance for patients in each of the age groups are presented in **Figure 10**.

Table 10. Pharmacokinetic data of midazolam and 1-hydroxymidazolam. Data are presented as median (absolute range). Exact values are presented for group C since only 1 patient was included in this group.

	All n=13	**Group A n=3**	**Group B n=6**	**Group C n=1**	**Group D n=3**
Midazolam clearance (L/h)	3.41 (0.35-49)	0.63 (0.35-3.4)	3.9 (3.1-9.7)	10.5	20 (2.1-49)
Midazolam clearance (mL/min/kg)	9.8 (1.5-16)	4.1 (1.5-16)	8.0 (4.7-14)	15	10 (1.6-12)
Midazolam infusion rate (mg/h)[1]	0.66 (0.07-4.8)	0.10 (0.07-0.35)	0.54 (0.11-0.8)	1.6	2.2 (1.1-4.8)
Dose adjusted midazolam concentrations[2]	238 (28-2885)	1667 (221-2885)	241 (132-299)	89	182 (28-480)
C_{max} midazolam (ng/mL)	203 (47-585)	204 (111-411)	158 (47-207)	291	443 (148-585)
C_{min} midazolam (ng/mL)	27 (0.30-341)	38 (15-54)	5.4 (0.30-30)	2	90 (39-341)
C_{max} 1-hydroxymidazolam (ng/mL)	17 (2-280)	13 (8-17)	9 (2-60)	280	34 (23-142)
C_{min} 1-hydroxymidazolam (ng/mL)	0.93 (0.03-9.3)	2.1 (0.5-4.8)	0.63 (0.03-2.2)	0.71	9.1 (0.98-9.3)

Abbreviations: C_{max}, maximum concentration; C_{min}, minimum concentration

[1]Midazolam infusion rate at study start

[2]Mean midazolam concentration during that dosage/infusion rate (mg/h) at study start

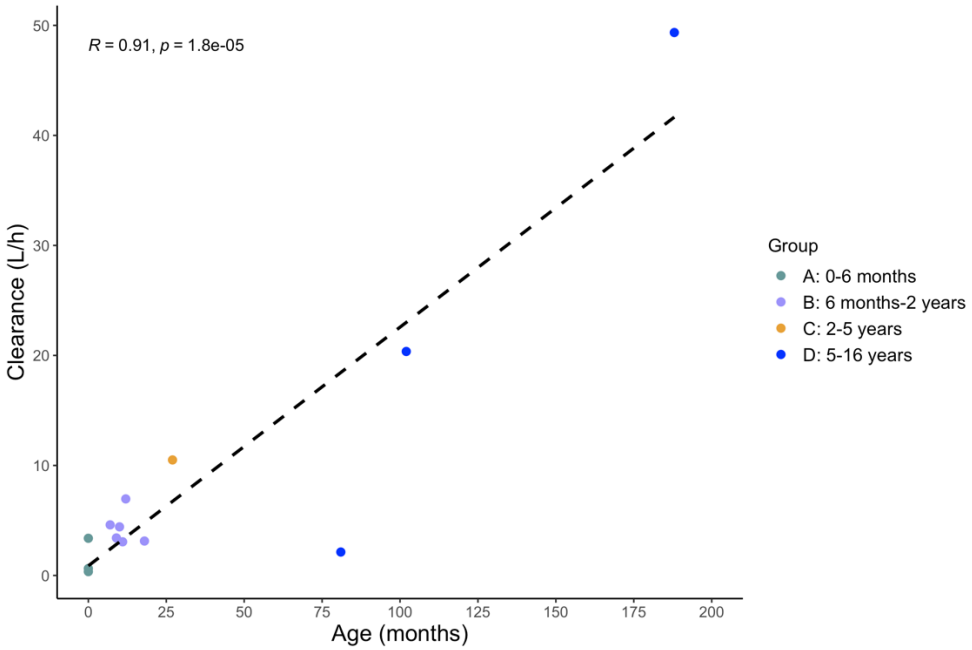


Figure 9. Simple linear regression between age and clearance.

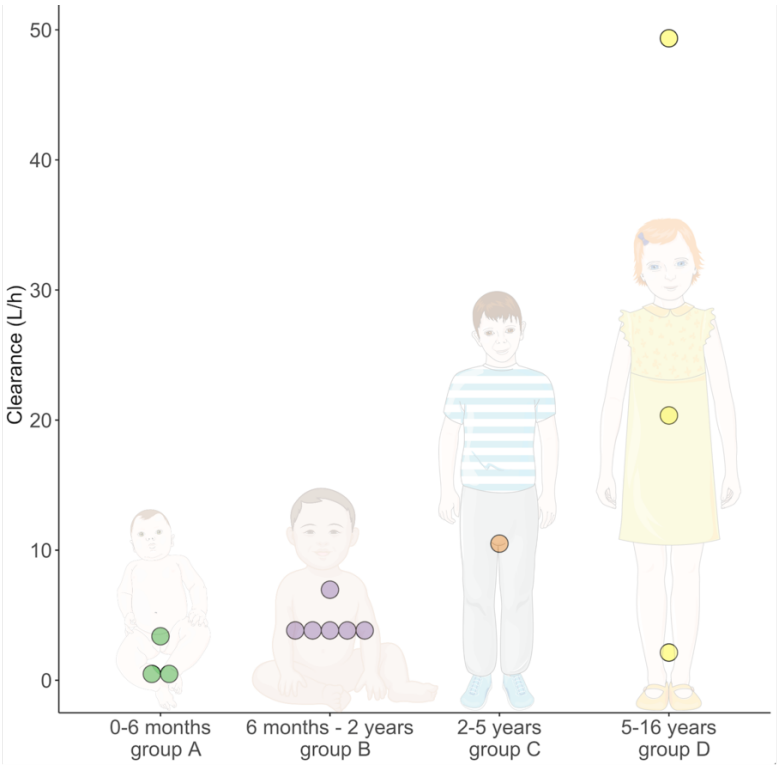


Figure 10. Estimated clearance for all patients presented by age group.

Metabolic ratio

There was a 8-fold difference in metabolic ratio between the patient with the lowest average metabolic ratio (9 months old, group B) compared with the patient with the highest metabolic ratio (2 years old, group C). The median metabolic ratio of 1-hydroxymidazolam/midazolam was 0.05, 0.08 and 0.08 for group A, B and D respectively. The metabolic ratio for the patient in group C was 0.3. Metabolic ratios for all patients are presented in **Figure 11**.

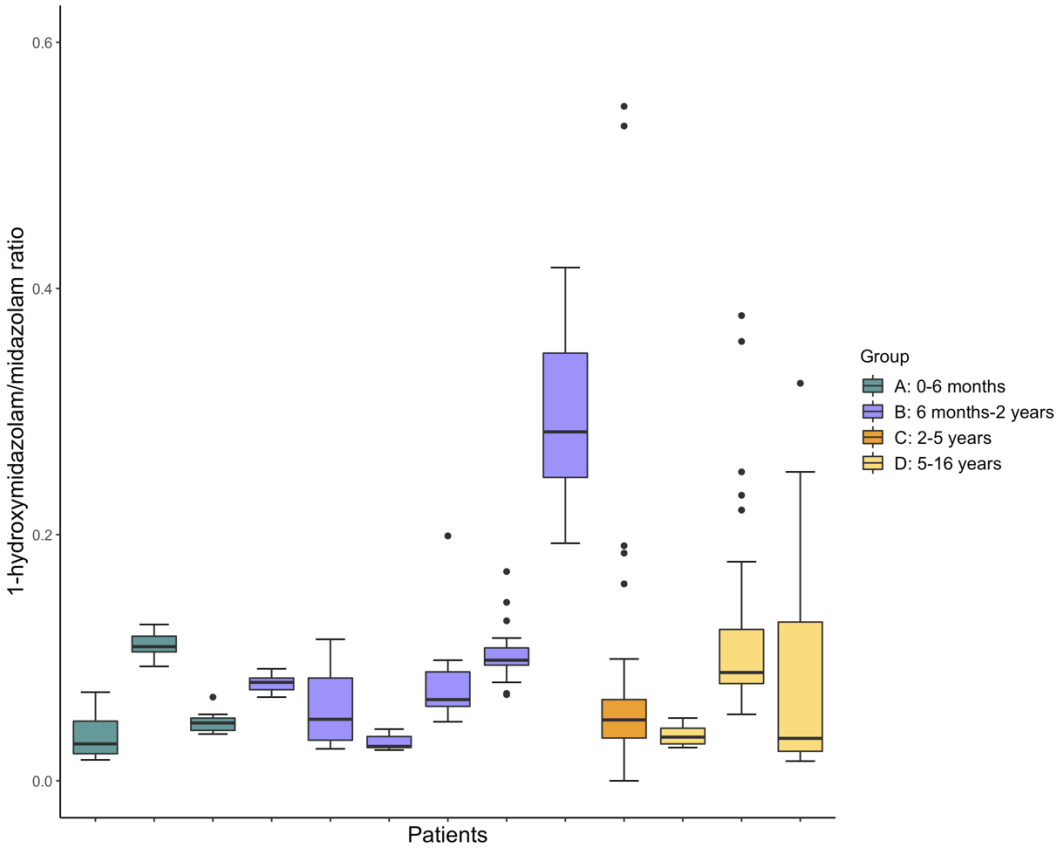


Figure 11. The ratio of 1-hydroxymidazolam/midazolam presented for each individual patient, using all respective plasma concentrations available. The number of available metabolic ratios ranged from 4 to 53.

Individual pharmacokinetic profiles

Individual plasma concentration-time profiles for midazolam and 1-hydroxymidazolam during continuous midazolam treatment are presented in **Figure 12** and **Figure 13**, respectively.

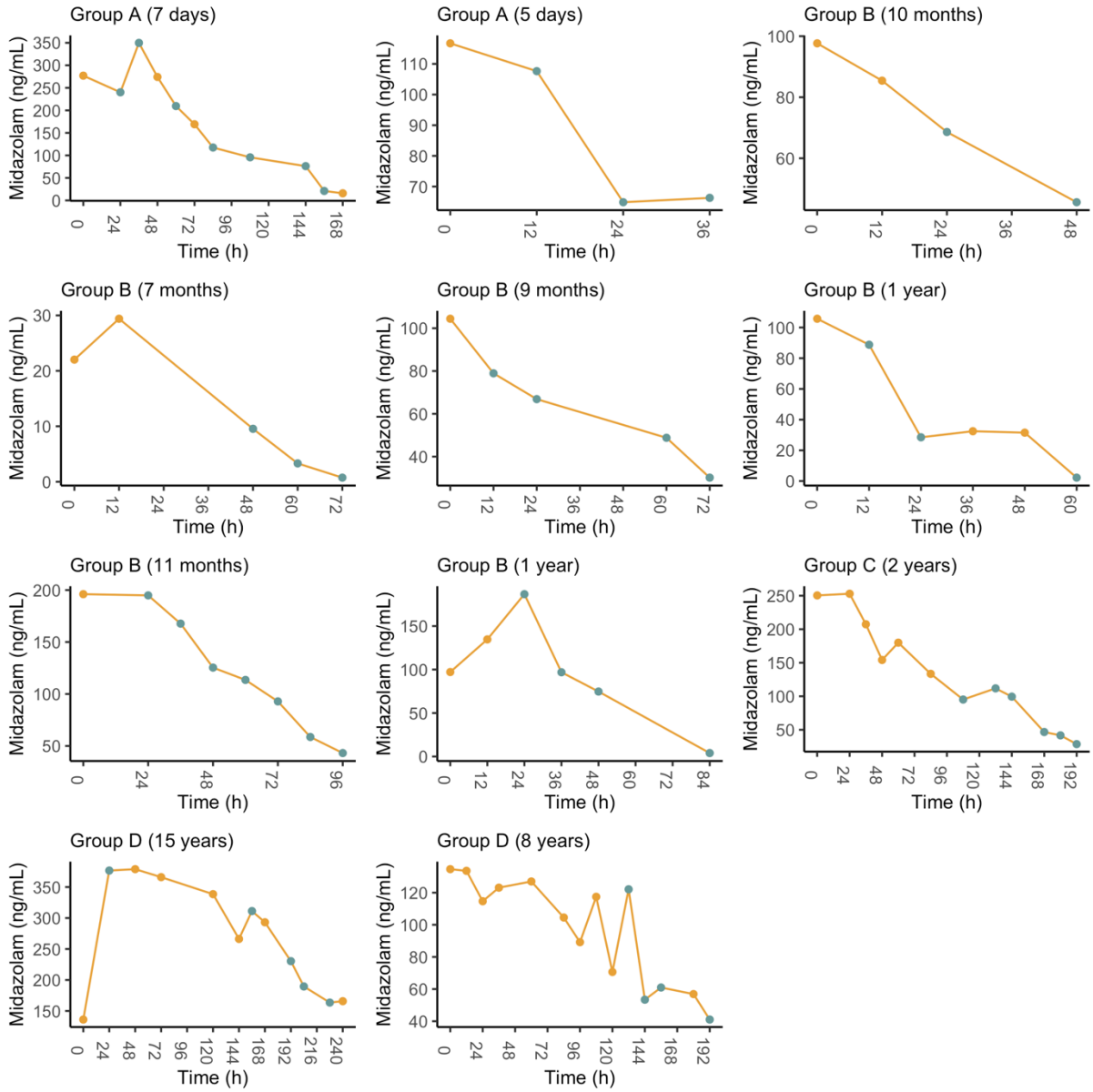


Figure 12. Individual plasma concentration-time profiles for midazolam during continuous. Two patients are excluded from the figure due to an insufficient number of blood samples (<2 samples) or ≤24h study period. Green dots represent adjustment in midazolam dosage.

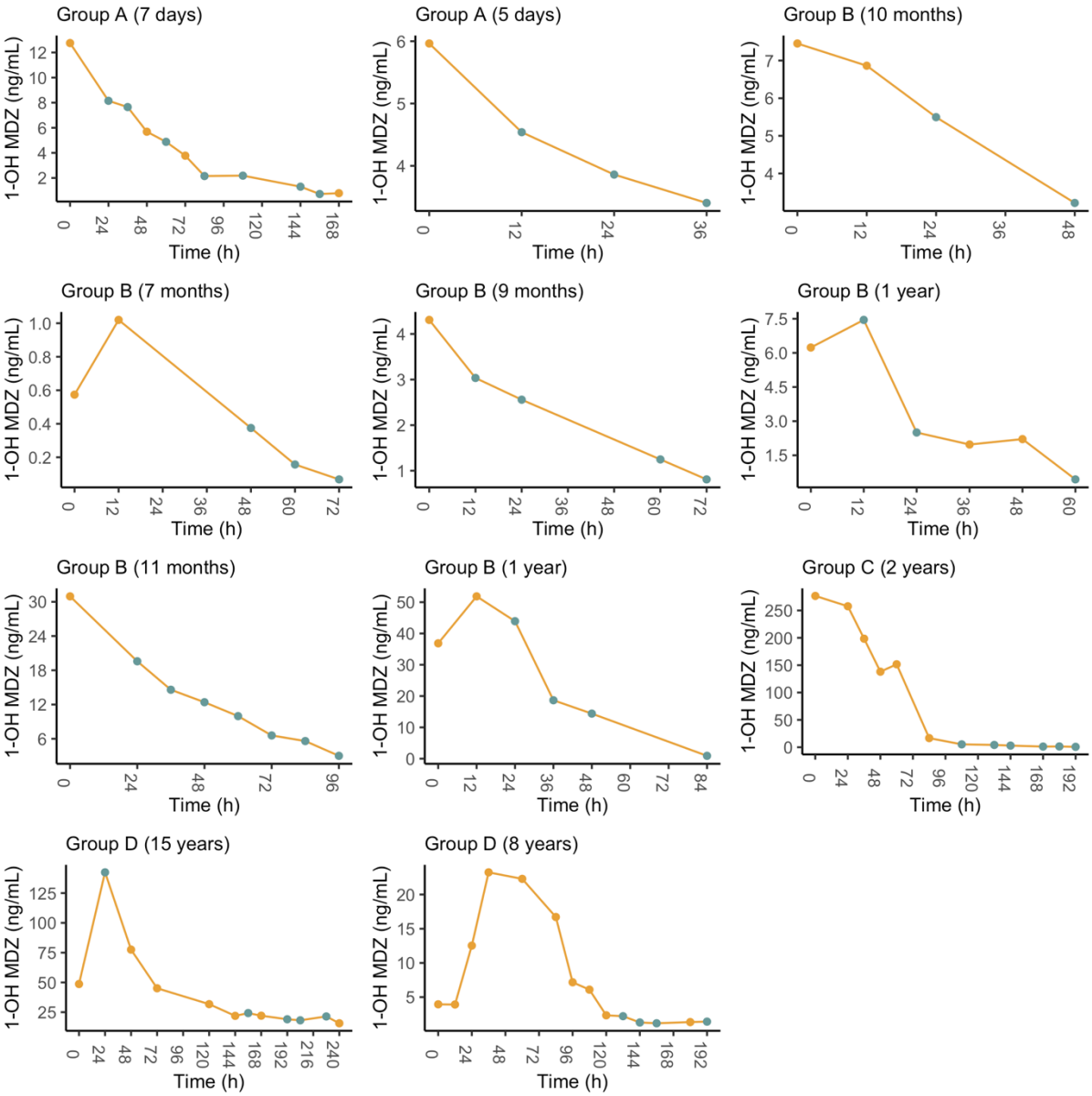


Figure 13. Individual plasma concentration-time profiles for 1-hydroxymidazolam during continuous. Two patients are excluded from the figure due to an insufficient number of blood samples (<2 samples) or ≤24h study period. Green dots represent adjustment in midazolam dosage. Abbreviations: 1-OH MDZ, 1-hydroxymidazolam

A change in midazolam concentration coincided with changes in relevant biomarkers (**Figure 14**). One of the patients showed decreasing CRP concentrations during the study period with a coincident change in midazolam concentrations the following days, despite the same dosage of midazolam. Another patient had high values of ALAT and ASAT, which decreased over time in a similar manner as the midazolam concentrations. The midazolam dosage was also adjusted daily, but the dose reduction was minor and could not alone explain the decreased midazolam concentrations.

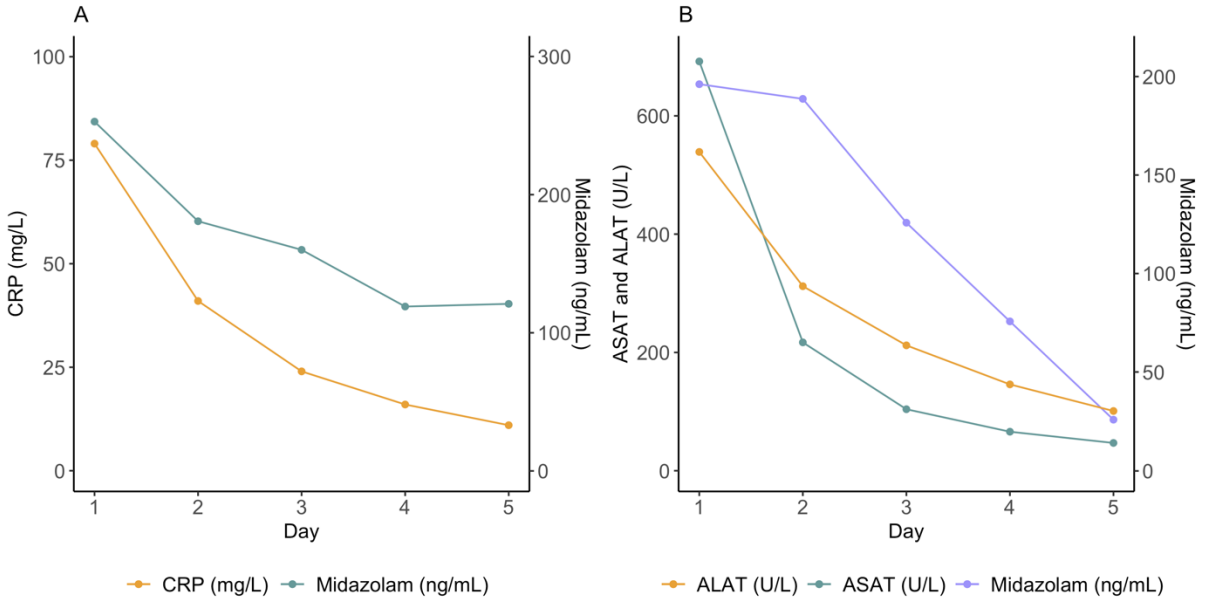


Figure 14. A change in midazolam concentration coincided with changes in relevant biomarkers. A) Reduction in CRP concentration and the corresponding reduction in midazolam concentration for one of the patients in the study, despite the same midazolam dosage. B) Reduction in alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) and the coincident reduction in midazolam concentration for one of the other patients. The midazolam dosage was adjusted daily, but the dose reduction was minor and could not alone explain the decreased midazolam concentrations.

Pharmacokinetic profiles following dose adjustment

Following dose reduction, the corresponding change in midazolam plasma concentration were negligible for all patients. Thus, it was not possible to calculate any elimination rate constant, nor any volume of distribution or elimination half-life. The plasma concentration-time profiles following dose adjustment are listed in the supplementary, Figure S1.

Oral midazolam dosing

One of the patients (group D) received an oral dose of midazolam syrup, but it was not possible to calculate absolute bioavailability of midazolam with non-compartmental methods. Thus, data from the secondary investigation are excluded from this master thesis.

4 Discussion

In this thesis, a previously validated UHPLC-MS/MS method was optimized to determine midazolam and 1-hydroxymidazolam concentrations in plasma samples from the CyPed-study. A partial validation of the optimized method was performed and requirements from EMA on validation of bioanalytical method was satisfied. There was a 141-fold difference in midazolam clearance across the different ages, with the lowest clearance observed in neonates (group A) and the highest clearance observed in adolescents (group D). This suggest that the CYP3A activity increases with increasing age.

4.1 Method optimization and validation

After validation, the optimized method was used to quantify midazolam and 1-hydroxymidazolam concentrations in plasma samples from the CyPed-study. As higher concentrations of midazolam and 1-hydroxymidazolam were expected in samples from the CyPed-study, lower volume of plasma were applied in the optimized sample preparation. The use of lower plasma volumes in the sample preparation gave satisfactory sensitivity. The main advantage of applying a method where small plasma volume is needed, is that it only requires a small blood sample volume, and thus limiting the trial-related blood loss. Smaller plasma volumes in sample preparation will also enable for reanalysis which is important in clinical trials. By preparing calibrators and QC samples in plasma instead of methanol, the method was both less time consuming and laborious. This made it possible to analyze several samples in a short time, and during one working day it was possible to analyze up to three 96-well trays (n=200). The calibration range covered the midazolam and metabolite concentration in all analyzed samples included in this thesis. Although only 13 patients were included in this pilot study, a wide age range and a large range of administered doses were covered. It is therefore likely that the calibration range will be sufficient.

4.2 The CyPed pilot study

4.2.1 Midazolam clearance and CYP3A activity

The midazolam clearance was highest in the oldest children (group D) and lowest in neonates (group A), indicating that the CYP3A activity increases with increasing age. However, the inter-individual variability in midazolam clearance was large within the different age groups, with the highest variability in the group 5-16 years, followed by the group 0-6 months. The large age difference in group D, from 5 to 16 years, may explain the 23-fold difference in midazolam clearance observed within this group. The 10-fold difference in midazolam clearance within the age group 0-6 months (group A) suggests that CYP3A activity changes rapidly during the first weeks of life. The lower inter-individual variability observed within the age group 6 months – 2 years (group B) may suggest that the CYP3A activity changes less at this age. The high inter-individual variability in midazolam clearance in the study population is not surprising, considering that the hepatic CYP3A expression is highly variable (>100-fold) also in the adult population [13].

The midazolam clearance reported in the present study is in agreement with previously published papers. Three neonates were included in group A, and the mean midazolam clearance in this group was in agreement with previous findings [32, 93, 94]. The midazolam clearance observed for toddlers and children 6 months – 2 years was also consisted with previous findings [32, 85]. The highest midazolam clearance reported in previous studies is observed in children between 2 and 11 years [85, 95]. Several previous studies have suggested that the CYP3A activity exceeds adult values at approximately 2 years of life and decreases to adult levels at puberty [38, 57, 62, 64, 67, 68]. In this master thesis, only one patient was included in the age group 2-5 years, and only 3 patients were included in the age group 5-16 years. Inclusion of more patients in these age groups are needed to validate these results in this pilot study. The mean midazolam clearance for adults is 300-500 mL/min [96]. The oldest participant in the CyPed pilot study was 15 years old and had a clearance of about 823 mL/min. Although more patients needs to be included in the present study before a proper comparison of the ontogeny of CYP3A activity during childhood can be made, it appears that our preliminary findings is in agreement with previously published studies.

4.2.2 CYP3A activity in neonates and infants (0-6 months)

In this pilot study, CYP3A activity, reflected by midazolam clearance, seems to be lower in the age group 0-6 months (group A). However, there was a 10-fold difference in clearance in this group of three neonates. One patient (9 days old) had a higher clearance compared with the two other patients in this group, and clearance was actually similar to the clearance observed in the age group 6 months – 2 years (group B). A possible explanation is that this specific patient received the CYP3A inducer phenobarbital during the entire study period, as well as a glucocorticoid one day. The induction of CYP3A activity by phenobarbital has a greater influence when midazolam is given orally, compared to intravenous administration [97]. The degree of induction is therefore difficult to assess, but it is still expected that the induction will have an effect on midazolam clearance even with intravenous dosing. CYP3A7 is the predominant CYP enzyme detected in the newborn liver, while hepatic CYP3A4 activity is low at birth with increasing activity during the first weeks of life [59, 62, 66-69] . CYP3A5 is also expressed from birth. All the included patients in group A were only a few days old (5-9 days), and midazolam clearance may therefore represent the activity of both CYP3A4, CYP3A5 and CYP3A7. No study participants have been genotyped in this master thesis, but genotyping of all the above-mentioned isoforms will be performed later.

Midazolam dosing is titrated to the desired level of sedation, assessed by the COMFORT scale [98]. The patients in the age group 0-6 months needed lower doses of midazolam to achieve a similar plasma concentration and sedation level as the older children (group B, C and D). This is not surprising and is emphasized by the fact that neonates had lower midazolam clearance as shown in the present study. Neonates have a decreased drug-protein binding because of decreased plasma concentrations of albumin and α_1-acid glycoprotein resulting in increased plasma concentration of unbound drug [64, 99]. Drug penetration into the central nervous system is more likely in neonates because of immature blood-brain barrier and a higher ratio of cerebral to systemic blood flow [99-101]. This, together with the lower CYP3A activity, and thus midazolam clearance, explains why neonates needs lower midazolam doses than older children. de Wildt *et al*. concluded that there is no pharmacokinetic-pharmacodynamic relationship for midazolam in pediatric intensive care patients [98]. Thus, it may not be possible to determine the optimal midazolam dose based on the plasma concentrations of midazolam. Pharmacokinetics and pharmacodynamics in the growing child are complicated. The

development of a pharmacokinetic-pharmacodynamic model or a physiological based pharmacokinetic model would be ideal to study this further.

4.2.3 CYP3A activity in toddlers and children (6 months–2 years and 2-5 years)

Group B had a 2-fold difference in midazolam clearance. As discussed previously, major changes in CYP3A activity occur during the first months of life. The results from this pilot study suggests that changes in CYP3A activity at the age of 6 months to 2 years are not that significant. The highest difference in midazolam clearance (2-fold) were observed between the two patients with similar age (>1 year). Both patients received treatment with glucocorticoids and a possible induction of CYP3A activity may therefore apply. Glucocorticoids dose-dependently induce the CYP3A activity, but the dose of glucocorticoids administered to patients in this pilot study has not been registered, and the induction is therefore not possible to assess [102, 103]. In addition, one of these patients had higher CRP concentrations compared with the other patient during the study period. Vet *et al*. studied the relationship between inflammation and midazolam clearance [28], and showed that a CRP concentration of 300 mg/L was associated with a 65% lower clearance of midazolam, compared with a CRP concentration of 10 mg/L. This may explain why the patient with higher CRP concentration had a 2-fold lower midazolam clearance compared to the other patient, despite the similar age.

Only one patient (2 years old) was included in group C (2-5 years), and this patient had a higher midazolam clearance compared with patients aged 6 months – 2 years (group B). It is possible that the increased clearance observed in this patient, and thus the higher CYP3A activity, is due to the higher age. However, more patients needs to be included in this age group before a proper comparison can be made. The patient received a glucocorticoid during the entire study period, which may also have influenced the clearance of midazolam. However, the degree of induction on CYP3A activity was not possible to assess.

4.2.4 CYP3A activity in children and adolescents (5-16 years)

The difference in midazolam clearance in the age group 5-16 years (group D) was 23-fold. The highest midazolam clearance was observed in the oldest study participate (15 years old). This patient did not receive any inhibitor or inducer of CYP3A, and a higher midazolam clearance may therefore reflect an actual higher CYP3A activity at this age. The lowest clearance in this group was observed in a 6-year-old patient, admitted to the Pediatric Intensive Care Unit due to an infection, which developed into sepsis, with corresponding CRP concentrations up to almost 300 mg/L. The low CYP3A activity in the 6-year-old is probably mainly due to critical illness and infection, not only because of an age-dependent activity. Several authors have shown that CYP3A activity is inversely correlated with CRP levels [104-107]. Similar explanation may apply to the third patient included in this group as this 8-year-old child had cancer. Inflammation due to cancer has been shown to exert a modest change in CYP3A activity [30, 107]. The lower midazolam clearance in this patient compared to the 15-year-old may be due to the disease itself or an age-dependent impact on CYP3A activity, or possibly a combination of both. In addition, the patient received treatment with the antiemetic aprepitant on and off throughout the study period. Aprepitant is initially a CYP3A inhibitor, and then becomes an inducer with long-term treatment [108, 109]. This patient received aprepitant one day at a time, with a few days off before the next dosage, and it is therefore expected that aprepitant behaves like an inhibitor in this situation. The effect is more significant when midazolam administered as an oral dose, and as the effect is rarely clinically relevant when midazolam is given intravenously, this may not be a plausible explanation for a lower midazolam clearance in this patient.

4.2.5 The effect of changes in biomarkers on midazolam concentrations

Two patients had changes in midazolam plasma concentrations that coincided with changes in relevant biomarkers. One patient had high values of ALAT and ASAT, which decreased over time in a similar manner as the midazolam concentrations. ASAT and ALAT are biomarkers used to examine liver function, and increased concentrations are found in the bloodstream in case of liver cell damage [110, 111]. A loss of hepatocytes because of cell damage may affect hepatic drug clearance, and may explain why this patient had a lower midazolam clearance compared with the other patients in this age group (6 months – 2 years). The values of ASAT and ALAT were above the reference range, and decreased during the study period. The

coincident fall in midazolam concentration can be explained by an improvement in liver function and thus an increase in CYP3A activity, reflected by an increased midazolam clearance. The midazolam dosage was also adjusted daily, but the dose reduction was minor and could not alone explain the decreased midazolam concentrations.

Another patient showed decreasing CRP concentrations during the study period with a coincident change in midazolam concentrations the following days, despite the same dosage of midazolam. This patient was hospitalized due to an infection with respiratory syncytial (RS) virus, and during the stay, CRP concentrations decreased. Midazolam concentration decreased correspondingly despite no change in infusion rate. As previously mentioned, several authors have shown that CYP3A activity is inversely correlated with CRP levels. A possible explanation for the observed reduction in midazolam concentration may therefore be an increased clearance as the patient recovers from the infection, due to a recovery of CYP3A activity. Other inflammatory markers, as interleukin-6, may correlate better than CRP with the downregulated CYP3A activity [33], but interleukin-6 concentrations were not measured in this pilot study.

4.2.6 Metabolic ratio

The inter-individual variation in the 1-hydroxymidazolam/midazolam ratio was 8-fold across all age groups in the present pilot study. However, the average metabolic ratio in each age group did not increase with an increasing age, as shown for midazolam clearance. The ratio between the parent drug and the metabolite should also reflect the CYP3A activity to a certain degree. However, several challenges exist by using this ratio to investigate CYP3A activity. First, midazolam is hydroxylated by the CYP3A isoenzymes to other metabolites than 1-hydroxymidazolam. In addition, 1-hydroxymidazolam is more unstable than midazolam in plasma samples, and there is a possibility of degradation during sample preparation and analysis. Thus, the metabolic ratio of 1-hydroxymidazolam/midazolam appears to be less relevant compared with midazolam clearance in investigating changes in CYP3A activity.

4.2.7 Midazolam as a probe drug in the pediatric population

The patients at the Pediatric Intensive Care Unit are a heterogeneous group with a range of different diseases and concomitant drug therapy, including CYP3A inducers and inhibitors. They also received blood transfusions, albumin transfusions and bolus doses of midazolam during the study period. In addition, the inter-individual variability in concentrations of relevant biomarkers was considerable, and all of these factors contributes to complicating the interpretation of pharmacokinetic parameters. Theoretically, a blood transfusion may lead to a dilution of the blood resulting in lower plasma concentrations of midazolam. By using steady-state concentration to calculate clearance, decreased plasma concentration of midazolam due to blood dilution may affect the calculated clearance. However, for the patients receiving a blood transfusion, plasma samples used to calculate the steady-state concentration were either collected a few days before or after the transfusion. Thus, the blood transfusion should not have affected the results. Midazolam is to a large extent bound to albumin. Since most people have an excess of albumin, the protein binding of midazolam should therefore not be affected by the albumin transfusion.

As mentioned earlier, midazolam is an intermediate extracted drug on average. Rogers *et al*. have presented a mean midazolam extraction ratio of 0.55 from 24 healthy adults, ranging from 0.32-0.96 [48]. The extraction ratio of a drug should not be considered an inherent drug property without considering the effect of age [112]. The extraction ratio of a drug is expected to change with age, and Salem *et al*. found that the hepatic extraction ratio of midazolam increased with age and that midazolam was a drug with low extraction ratio in neonates. Inter-individual variation in the hepatic extraction ratio of midazolam is expected in the present study. Therefore, midazolam clearance can be affected by altered blood flow in some of the patients. Mechanical ventilation can reduce cardiac out, resulting in reduced blood flow to the liver [82]. Shock following acute trauma and admission to an intensive care unit may also result in reduced hepatic blood flow. However, it is not possible to assess if midazolam clearance has been affected by blood flow alterations, as changes in blood flow have not been measured in the present study.

4.2.8 Lessons learned from the pilot study and further perspectives

To minimize the collection of unnecessary blood volumes, samples were taken in connection with other necessary laboratory analyzes, and the time for sampling could thus not be standardized. In addition, the dosage of midazolam was frequently changed in the majority of patients during the study period. This challenged the clearance estimation by non-compartmental analysis. Furthermore, the change in midazolam plasma concentration following the dose reduction were negligible for all patients. Thus, it was not possible to calculate any elimination rate constant, nor any volume of distribution or elimination half-life using non-compartmental analysis. In order to fully explore the pharmacokinetic data from the CyPed study it is vital to develop a population pharmacokinetic model of midazolam. In these models both clearance and the other pharmacokinetic parameters could be more accurately predicted compared to non-compartmental analysis as these models do not rely on steady state conditions. In addition, the effect of covariates as age, weight and inflammation status (CRP) on midazolam clearance can be investigated. A pharmacokinetic population model also provide a possibility to simulate different dosing strategies in different age groups. To investigate the effect of intestinal CYP3A activity, more patients receiving an oral dose of midazolam should be included.

In addition, the use of capillary sampling by volumetric absorptive microsampling (VAMS) could be explored as an alternative blood sampling method in studies requiring frequent blood sampling as the CyPed study [113]. Blood is applied on to VAMS tip, with limited invasiveness and discomfort, directly from a finger-prick, heel-prick, or indirectly from a blood syringe. A small accurate volume of blood can thus be collected, limiting the trial-related blood loss without inflicting unnecessary discomfort on the patient. This technology would therefore be an advantage for blood sampling in pediatric patients.

Clinical studies regarding use and dosing of drugs in the pediatric population are of great importance as the pharmacokinetics and pharmacodynamics in children and adults are not always similar. The CyPed-study will continue to include patients and investigate the CYP3A activity in pediatric patients, hopefully provide more insight into the effect of age on pharmacokinetics and thus dosing of CYP3A substrates.

5 Conclusion

The results from the CyPed pilot study imply that midazolam clearance, and thus CYP3A activity, increase with increasing age. However, the inter-individual variability was large with a 141-fold difference in midazolam clearance across the age groups. Only a small number of patients were included in this pilot study and inclusion of more patients will allow us to further explore the effect of age on CYP3A activity. In order to fully explore the pharmacokinetic data from the CyPed-study it is vital to develop a population pharmacokinetic model of midazolam. The partial validation of the optimized method to determine midazolam and 1-hydroxymidazolam concentrations in plasma samples met the requirements in the guideline from EMA on bioanalytical method validation. The optimized method covered the full concentration range of midazolam and the metabolite in plasma samples from the CyPed pilot study, and the smaller plasma volumes needed in the sample preparation enables a reduction of trial-related blood loss.

References

1. Rowland, M., et al., *Clinical pharmacokinetics and pharmacodynamics : concepts and applications*. 4th ed. ed. 2011, Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins.
2. Buxton, I.L.O., *Pharmacokinetics: The Dynamics of Drug Absorption, Distribution, Metabolism, and Elimination*, in *Goodman & Gilman's: The Pharmacological Basis of Therapeutics, 13e*, L.L. Brunton, R. Hilal-Dandan, and B.C. Knollmann, Editors. 2017, McGraw-Hill Education: New York, NY.
3. Gonzalez, F.J., M. Coughtrie, and R.H. Tukey, *Drug Metabolism*, in *Goodman & Gilman's: The Pharmacological Basis of Therapeutics, 13e*, L.L. Brunton, R. Hilal-Dandan, and B.C. Knollmann, Editors. 2017, McGraw-Hill Education: New York, NY.
4. Giacomini, K.M. and Y. Sugiyama, *Membrane Transporters and Drug Response*, in *Goodman & Gilman's: The Pharmacological Basis of Therapeutics, 13e*, L.L. Brunton, R. Hilal-Dandan, and B.C. Knollmann, Editors. 2017, McGraw-Hill Education: New York, NY.
5. Elmorsi, Y., J. Barber, and A. Rostami-Hodjegan, *Ontogeny of Hepatic Drug Transporters and Relevance to Drugs Used in Pediatrics.* Drug Metabolism and Disposition, 2016. **44**(7): p. 992.
6. Lin, L., et al., *SLC transporters as therapeutic targets: emerging opportunities.* Nat Rev Drug Discov, 2015. **14**(8): p. 543-60.
7. Strolin Benedetti, M., R. Whomsley, and E.L. Baltes, *Differences in absorption, distribution, metabolism and excretion of xenobiotics between the paediatric and adult populations.* Expert Opinion on Drug Metabolism & Toxicology, 2005. **1**(3): p. 447-471.
8. Christensen, H. and M. Hermann, *Immunological Response as a Source to Variability in Drug Metabolism and Transport.* Frontiers in Pharmacology, 2012. **3**(8).
9. Hawwa, A.F. and J.C. McElnay, *Impact of ATP-binding cassette, subfamily B, member 1 pharmacogenetics on tacrolimus-associated nephrotoxicity and dosage requirements in paediatric patients with liver transplant.* Expert Opin Drug Saf, 2011. **10**(1): p. 9-22.
10. Nie, Y., et al., *Genetic polymorphisms of human hepatic OATPs: functional consequences and effect on drug pharmacokinetics.* Xenobiotica, 2020. **50**(3): p. 297-317.
11. Hua, W.J., W.X. Hua, and H.J. Fang, *The Role of OATP1B1 and BCRP in Pharmacokinetics and DDI of Novel Statins.* Cardiovascular Therapeutics, 2012. **30**(5): p. e234-e241.
12. Klein, K. and U.M. Zanger, *Pharmacogenomics of Cytochrome P450 3A4: Recent Progress Toward the "Missing Heritability" Problem.* Front Genet, 2013. **4**: p. 12.
13. 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.
14. Guengerich, F.P., *Cytochrome p450 and chemical toxicology.* Chem Res Toxicol, 2008. **21**(1): p. 70-83.
15. Kumar, G.N. and S. Surapaneni, *Role of drug metabolism in drug discovery and development.* Med Res Rev, 2001. **21**(5): p. 397-411.

16. 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.
17. Shah, R.R. and R.L. Smith, *Inflammation-induced phenoconversion of polymorphic drug metabolizing enzymes: hypothesis with implications for personalized medicine.* Drug Metab Dispos, 2015. **43**(3): p. 400-10.
18. Brockmöller, J., et al., *Pharmacogenetic diagnostics of cytochrome P450 polymorphisms in clinical drug development and in drug treatment.* Pharmacogenomics, 2000. **1**(2): p. 125-151.
19. 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.
20. Wang, D., et al., *Intronic polymorphism in CYP3A4 affects hepatic expression and response to statin drugs.* The pharmacogenomics journal, 2011. **11**(4): p. 274-286.
21. Guengerich, F.P., *CYTOCHROME P-450 3A4: Regulation and Role in Drug Metabolism.* Annual Review of Pharmacology and Toxicology, 1999. **39**(1): p. 1-17.
22. Paine, M.F., et al., *The human intestinal cytochrome P450 "pie".* Drug Metab Dispos, 2006. **34**(5): p. 880-6.
23. Krogstad, V., et al., *A Comparative Analysis of Cytochrome P450 Activities in Paired Liver and Small Intestinal Samples from Patients with Obesity.* Drug Metabolism and Disposition, 2020. **48**(1): p. 8.
24. Kitzmiller, J.P., et al., *CYP3A4*22 and CYP3A5*3 are associated with increased levels of plasma simvastatin concentrations in the cholesterol and pharmacogenetics study cohort.* Pharmacogenet Genomics, 2014. **24**(10): p. 486-91.
25. Abdel-Kahaar, E., et al., *The Impact of CYP3A4*22 on Tacrolimus Pharmacokinetics and Outcome in Clinical Practice at a Single Kidney Transplant Center.* Front Genet, 2019. **10**: p. 871.
26. Christensen, H., et al., *Coadministration of grapefruit juice increases systemic exposure of diltiazem in healthy volunteers.* European Journal of Clinical Pharmacology, 2002. **58**(8): p. 515-520.
27. Blumer, J.L., *Clinical pharmacology of midazolam in infants and children.* Clin Pharmacokinet, 1998. **35**(1): p. 37-47.
28. Vet, N.J., et al., *Inflammation and Organ Failure Severely Affect Midazolam Clearance in Critically Ill Children.* Am J Respir Crit Care Med, 2016. **194**(1): p. 58-66.
29. Chung, E., et al., *Comparison of midazolam and simvastatin as cytochrome P450 3A probes.* Clin Pharmacol Ther, 2006. **79**(4): p. 350-61.
30. Coutant, D., et al., *Understanding Disease–Drug Interactions in Cancer Patients: Implications for Dosing Within the Therapeutic Window.* Clinical Pharmacology & Therapeutics, 2015. **98**(1): p. 76-86.
31. Ince, I., et al., *Developmental Changes in the Expression and Function of Cytochrome P450 3A Isoforms: Evidence from In Vitro and In Vivo Investigations.* Clinical Pharmacokinetics, 2013. **52**(5): p. 333-345.
32. de Wildt, S.N., et al., *Population pharmacokinetics and metabolism of midazolam in pediatric intensive care patients.* Crit Care Med, 2003. **31**(7): p. 1952-8.
33. Vet, N.J., et al., *The effect of critical illness and inflammation on midazolam therapy in children.* Pediatr Crit Care Med, 2012. **13**(1): p. e48-50.

34. Carcillo, J.A., et al., *Cytochrome P450 mediated-drug metabolism is reduced in children with sepsis-induced multiple organ failure.* Intensive Care Medicine, 2003. **29**(6): p. 980-984.
35. Aitken, A.E., T.A. Richardson, and E.T. Morgan, *Regulation of drug-metabolizing enzymes and transporters in inflammation.* Annu Rev Pharmacol Toxicol, 2006. **46**: p. 123-49.
36. Brussee, J.M., et al., *Predicting CYP3A-mediated midazolam metabolism in critically ill neonates, infants, children and adults with inflammation and organ failure.* Br J Clin Pharmacol, 2018. **84**(2): p. 358-368.
37. Kirwan, C.J., et al., *Acute kidney injury reduces the hepatic metabolism of midazolam in critically ill patients.* Intensive Care Medicine, 2012. **38**(1): p. 76-84.
38. Bartelink, I.H., et al., *Guidelines on paediatric dosing on the basis of developmental physiology and pharmacokinetic considerations.* Clin Pharmacokinet, 2006. **45**(11): p. 1077-97.
39. Gravel, S., et al., *Modulation of CYP450 Activities in Patients With Type 2 Diabetes.* Clinical Pharmacology & Therapeutics, 2019. **106**(6): p. 1280-1289.
40. Gandhi, A., B. Moorthy, and R. Ghose, *Drug disposition in pathophysiological conditions.* Curr Drug Metab, 2012. **13**(9): p. 1327-44.
41. Gu, X., et al., *Role of NF-κB in Regulation of PXR-mediated Gene Expression: A MECHANISM FOR THE SUPPRESSION OF CYTOCHROME P-450 3A4 BY PROINFLAMMATORY AGENTS** Journal of Biological Chemistry, 2006. **281**(26): p. 17882-17889.
42. Reves, J.G., et al., *Midazolam: Pharmacology and Uses.* Anesthesiology, 1985. **62**(3): p. 310-324.
43. 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 Opinion on Drug Metabolism & Toxicology, 2010. **6**(6): p. 761-771.
44. 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.
45. Björkman, S., *Prediction of Cytochrome P450-Mediated Hepatic Drug Clearance in Neonates, Infants and Children.* Clinical Pharmacokinetics, 2006. **45**(1): p. 1-11.
46. Christopher Gorski, J., et al., *Regioselective biotransformation of midazolam by members of the human cytochrome P450 3A (CYP3A) subfamily.* Biochemical Pharmacology, 1994. **47**(9): p. 1643-1653.
47. Nordt, S.P. and R.F. Clark, *Midazolam: A review of therapeutic uses and toxicity.* The Journal of Emergency Medicine, 1997. **15**(3): p. 357-365.
48. Rogers, J.F., et al., *An evaluation of the suitability of intravenous midazolam as an in vivo marker for hepatic cytochrome P4503A activity.* Clinical Pharmacology & Therapeutics, 2003. **73**(3): p. 153-158.
49. Watkins, P.B., *Noninvasive tests of CYP3A enzymes.* Pharmacogenetics, 1994. **4**(4): p. 171-84.
50. Lund, M., T.S. Petersen, and K.P. Dalhoff, *Clinical Implications of P-Glycoprotein Modulation in Drug-Drug Interactions.* Drugs, 2017. **77**(8): p. 859-883.
51. Thummel, K.E., et al., *Use of midazolam as a human cytochrome P450 3A probe: I. In vitro-in vivo correlations in liver transplant patients.* J Pharmacol Exp Ther, 1994. **271**(1): p. 549-56.

52. Nir-Neuman, H., et al., *Unlicensed and Off-Label Medication Use in Pediatric and Neonatal Intensive Care Units: No Change Over a Decade.* Advances in Therapy, 2018. **35**(7): p. 1122-1132.
53. Stephenson, T., *Medicines for children—the last century and the next.* Archives of Disease in Childhood, 2001. **85**(3): p. 177-179.
54. Teigen, A., et al., *Off-label and unlicensed medicines to hospitalised children in Norway.* J Pharm Pharmacol, 2017. **69**(4): p. 432-438.
55. European Medicines Agency. *REGULATION (EC) No 1901/2006 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 12 December 2006 on medicinal products for paediatric use and amending Regulation (EEC) No 1768/92, Directive 2001/20/EC, Directive 2001/83/EC and Regulation (EC) No 726/2004* 2006 [cited 2020 19 November]; Available from: https://ec.europa.eu/health/sites/health/files/files/eudralex/vol-1/reg_2006_1901/reg_2006_1901_en.pdf.
56. Mahmood, I., *Dosing in children: a critical review of the pharmacokinetic allometric scaling and modelling approaches in paediatric drug development and clinical settings.* Clin Pharmacokinet, 2014. **53**(4): p. 327-46.
57. Cella, M., et al., *What is the right dose for children?* Br J Clin Pharmacol, 2010. **70**(4): p. 597-603.
58. European Medicines Agency. *ICH Topic E 11 Clinical Investigation of Medicinal Products in the Paediatric Population Step 5 NOTE FOR GUIDANCE ON CLINICAL INVESTIGATION OF MEDICINAL PRODUCTS IN THE PAEDIATRIC POPULATION (CPMP/ICH/2711/99)*. 2001 [cited 2020 19 November]; Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/international-conference-harmonisation-technical-requirements-registration-pharmaceuticals-human-use_en-1.pdf.
59. van den Anker, J., et al., *Developmental Changes in Pharmacokinetics and Pharmacodynamics.* The Journal of Clinical Pharmacology, 2018. **58**(S10): p. S10-S25.
60. Batchelor, H.K. and J.F. Marriott, *Paediatric pharmacokinetics: key considerations.* Br J Clin Pharmacol, 2015. **79**(3): p. 395-404.
61. Debotton, N. and A. Dahan, *A mechanistic approach to understanding oral drug absorption in pediatrics: an overview of fundamentals.* Drug Discov Today, 2014. **19**(9): p. 1322-36.
62. Kearns, G.L., et al., *Developmental Pharmacology — Drug Disposition, Action, and Therapy in Infants and Children.* New England Journal of Medicine, 2003. **349**(12): p. 1157-1167.
63. Maharaj, A.R. and A.N. Edginton, *Examining Small Intestinal Transit Time as a Function of Age: Is There Evidence to Support Age-Dependent Differences among Children?* Drug Metab Dispos, 2016. **44**(7): p. 1080-9.
64. Anderson, G.D., *Developmental pharmacokinetics.* Semin Pediatr Neurol, 2010. **17**(4): p. 208-13.
65. Basalely, A., D. Liu, and F.J. Kaskel, *Big equation for small kidneys: a newly proposed model to estimate neonatal GFR.* Pediatric Nephrology, 2020. **35**(4): p. 543-546.
66. de Wildt, S.N., *Profound changes in drug metabolism enzymes and possible effects on drug therapy in neonates and children.* Expert Opin Drug Metab Toxicol, 2011. **7**(8): p. 935-48.

67. de Wildt, S.N., et al., *Cytochrome P450 3A: ontogeny and drug disposition.* Clin Pharmacokinet, 1999. **37**(6): p. 485-505.
68. Leeder, J.S. and G.L. Kearns, *Pharmacogenetics in pediatrics. Implications for practice.* Pediatr Clin North Am, 1997. **44**(1): p. 55-77.
69. Lacroix, D., et al., *Expression of CYP3A in the human liver--evidence that the shift between CYP3A7 and CYP3A4 occurs immediately after birth.* Eur J Biochem, 1997. **247**(2): p. 625-34.
70. Fakhoury, M., et al., *LOCALIZATION AND mRNA EXPRESSION OF CYP3A AND P-GLYCOPROTEIN IN HUMAN DUODENUM AS A FUNCTION OF AGE.* Drug Metabolism and Disposition, 2005. **33**(11): p. 1603.
71. Krekels, E.H.J., et al., *Chapter 8 - Hepatic Drug Metabolism in Pediatric Patients*, in *Drug Metabolism in Diseases*, W. Xie, Editor. 2017, Academic Press: Boston. p. 181-206.
72. Murry, D.J., et al., *Liver volume as a determinant of drug clearance in children and adolescents.* Drug Metabolism and Disposition, 1995. **23**(10): p. 1110.
73. Brussee, J.M., et al., *Characterization of Intestinal and Hepatic CYP3A-Mediated Metabolism of Midazolam in Children Using a Physiological Population Pharmacokinetic Modelling Approach.* Pharmaceutical Research, 2018. **35**(9): p. 182.
74. Oslo Universitetssykehus. *Seksjon for intensivbehandling av barn, Rikshospitalet*. [cited 2021 18 January]; Available from: https://oslo-universitetssykehus.no/avdelinger/akuttklinikken/postoperativ-og-intensivavdelingen/barneintensiv-rikshospitalet/seksjon-for-intensivbehandling-av-barn#utstyr-vi-bruker.
75. Frivold, G., B. Dale, and Å. Slettebø, *Family members' experiences of being cared for by nurses and physicians in Norwegian intensive care units: A phenomenological hermeneutical study.* Intensive and Critical Care Nursing, 2015. **31**(4): p. 232-240.
76. Conroy, S., et al., *Survey of unlicensed and off label drug use in paediatric wards in European countries.* BMJ, 2000. **320**(7227): p. 79.
77. Yang, C.P., et al., *Food and Drug Administration approval for medications used in the pediatric intensive care unit: A continuing conundrum.* Pediatric Critical Care Medicine, 2011. **12**(5): p. e195-e199.
78. Wolf, A., et al., *Prospective multicentre randomised, double-blind, equivalence study comparing clonidine and midazolam as intravenous sedative agents in critically ill children: the SLEEPS (Safety profiLe, Efficacy and Equivalence in Paediatric intensive care Sedation) study.* Health Technol Assess, 2014. **18**(71): p. 1-212.
79. Kudchadkar, S.R., O.A. Aljohani, and N.M. Punjabi, *Sleep of critically ill children in the pediatric intensive care unit: A systematic review.* Sleep Medicine Reviews, 2014. **18**(2): p. 103-110.
80. Valkenburg, A.J., et al., *Sedation With Midazolam After Cardiac Surgery in Children With and Without Down Syndrome: A Pharmacokinetic-Pharmacodynamic Study.* Pediatric Critical Care Medicine, 9000. **Online First**.
81. Playfor, S., et al., *Consensus guidelines on sedation and analgesia in critically ill children.* Intensive Care Med, 2006. **32**(8): p. 1125-36.
82. Altamimi, M.I., H. Sammons, and I. Choonara, *Inter-individual variation in midazolam clearance in children.* Arch Dis Child, 2015. **100**(1): p. 95-100.
83. Nahara, M.C., et al., *Pharmacokinetics of midazolam in critically ill pediatric patients.* Eur J Drug Metab Pharmacokinet, 2000. **25**(3-4): p. 219-21.

84. de Wildt, S.N., et al., *Pharmacokinetics and metabolism of oral midazolam in preterm infants.* Br J Clin Pharmacol, 2002. **53**(4): p. 390-2.
85. Hughes, J., et al., *Steady-State Plasma Concentrations of Midazolam in Critically Ill Infants and Children.* Annals of Pharmacotherapy, 1996. **30**(1): p. 27-30.
86. Tolia, V., et al., *Pharmacokinetic and pharmacodynamic study of midazolam in children during esophagogastroduodenoscopy.* The Journal of Pediatrics, 1991. **119**(3): p. 467-471.
87. Reed, M.D., et al., *The single-dose pharmacokinetics of midazolam and its primary metabolite in pediatric patients after oral and intravenous administration.* J Clin Pharmacol, 2001. **41**(12): p. 1359-69.
88. Egeland, E.J., et al., *Chronic Inhibition of CYP3A is Temporarily Reduced by Each Hemodialysis Session in Patients With End-Stage Renal Disease.* Clinical Pharmacology & Therapeutics, 2020. **108**(4): p. 866-873.
89. European Medicines Agency. *Guideline on bioanalytical method validation*. 2011 [cited 2021 25 February]; Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf.
90. European Medicines Agency. *Guideline for good clinical practice E6(R2)*. 2016 [cited 2021 6 April]; Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/ich-e-6-r2-guideline-good-clinical-practice-step-5_en.pdf.
91. Rstudio Team, *RStudio: Integrated Development Environment for R*.
92. *BioRender*. Available from: https://app.biorender.com/.
93. Ahsman, M.J., et al., *Population pharmacokinetics of midazolam and its metabolites during venoarterial extracorporeal membrane oxygenation in neonates.* Clin Pharmacokinet, 2010. **49**(6): p. 407-19.
94. Burtin, P., et al., *Population pharmacokinetics of midazolam in neonates.* Clin Pharmacol Ther, 1994. **56**(6 Pt 1): p. 615-25.
95. Muchohi, S.N., et al., *Pharmacokinetics and clinical efficacy of midazolam in children with severe malaria and convulsions.* Br J Clin Pharmacol, 2008. **66**(4): p. 529-38.
96. Felleskatalogen.no. *Midazolam Accord*. 2020 [cited 2021 5 May]; Available from: https://www.felleskatalogen.no/medisin/midazolam-accord-accord-591458.
97. Backman, J.T., et al., *Concentrations and effects of oral midazolam are greatly reduced in patients treated with carbamazepine or phenytoin.* Epilepsia, 1996. **37**(3): p. 253-7.
98. de Wildt, S.N., et al., *Pharmacodynamics of midazolam in pediatric intensive care patients.* Ther Drug Monit, 2005. **27**(1): p. 98-102.
99. Ku, L.C. and P.B. Smith, *Dosing in neonates: special considerations in physiology and trial design.* Pediatr Res, 2015. **77**(1-1): p. 2-9.
100. Lam, J., et al., *The ontogeny of P-glycoprotein in the developing human blood–brain barrier: implication for opioid toxicity in neonates.* Pediatric Research, 2015. **78**(4): p. 417-421.
101. Anagnostakis, D., et al., *Blood-brain barrier permeability in "healthy" infected and stressed neonates.* The Journal of Pediatrics, 1992. **121**(2): p. 291-294.
102. Villikka, K., K.T. Kivistö, and P.J. Neuvonen, *The effect of dexamethasone on the pharmacokinetics of triazolam.* Pharmacol Toxicol, 1998. **83**(3): p. 135-8.
103. McCune, J.S., et al., *In vivo and in vitro induction of human cytochrome P4503A4 by dexamethasone.* Clinical Pharmacology & Therapeutics, 2000. **68**(4): p. 356-366.

104. Molanaei, H., et al., *Metabolism of alprazolam (a marker of CYP3A4) in hemodialysis patients with persistent inflammation.* European Journal of Clinical Pharmacology, 2012. **68**(5): p. 571-577.
105. Lenoir, C., et al., *Impact of Acute Inflammation on Cytochromes P450 Activity Assessed by the Geneva Cocktail.* Clin Pharmacol Ther, 2020.
106. Molanaei, H., et al., *Inflammation down-regulates CYP3A4-catalysed drug metabolism in hemodialysis patients.* BMC Pharmacol Toxicol, 2018. **19**(1): p. 33.
107. 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.* British Journal of Cancer, 2002. **87**(3): p. 277-280.
108. Shadle, C.R., et al., *Evaluation of Potential Inductive Effects of Aprepitant on Cytochrome P450 3A4 and 2C9 Activity.* The Journal of Clinical Pharmacology, 2004. **44**(3): p. 215-223.
109. Majumdar, A.K., et al., *Effects of aprepitant on cytochrome P450 3A4 activity using midazolam as a probe.* Clin Pharmacol Ther, 2003. **74**(2): p. 150-6.
110. Hagve, T.-A. and J.P. Berg, *Klinisk biokjemi og fysiologi*. 5. utg. [i.e. 16. utg.]. ed. 2015, Oslo: Gyldendal Akademisk.
111. Brukerhandboken.no. *Nasjonal brukerhåndbok i Medisinsk Biokjemi*. [cited 2020 11 december]; Available from: http://brukerhandboken.no/index.php.
112. Salem, F., et al., *Considering Age Variation When Coining Drugs as High versus Low Hepatic Extraction Ratio.* Drug Metabolism and Disposition, 2016. **44**(7): p. 1099.
113. Abu-Rabie, P., et al., *Validation of methods for determining pediatric midazolam using wet whole blood and volumetric absorptive microsampling.* Bioanalysis, 2019. **11**(19): p. 1737-1754.

Supplementary

Relevant biomarkers

Alanine aminotransferase and aspartate aminotransferase

Alanine aminotransferase (ALAT) is found in various tissues, but especially with high activity in the liver, and is thus a relatively specific hepatic biomarker. It is measured clinically to examine liver function, and in case of liver cell damage, increased amounts of ALAT are found in the bloodstream [110, 111]. Aspartate aminotransferase (ASAT) is not as specific biomarker for the liver, since it is also found with relatively high activity elsewhere. However, it is often a marker of more severe liver damage, and it is very common to measure the ASAT/ALAT ratio in plasma when examining liver function [110, 111].

Creatinine

Creatinine is a breakdown product from muscle, and the amount of creatinine produced is closely correlated with the individual´s muscle mass. With constant muscle mass and constant creatinine intake, we can assume that plasma creatinine would vary inversely with GFR, and can thus be used to assess renal function [110, 111].

C-reactive protein

C-reactive protein (CRP) is an acute-phase protein produced in the liver, and an increase in plasma CRP can be seen shortly after acute onset of disease. CRP is today the most widely used measure of inflammation, and is very often used to assess the degree of inflammation in infectious and non-infectious conditions [111].

Materials

Chemicals

Chemicals	Manufacturer
Midazolam	*Toronto Research Chemicals, Ontario, Canada*
Midazolam-d6	*Toronto Research Chemicals, Ontario, Canada*
1-hydroxymidazolam	*Toronto Research Chemicals, Ontario, Canada*
1-hydroxymidazolam-d5	*Toronto Research Chemicals, Ontario, Canada*
Acetonitrile (Hypergrade for LC-MS)	*Merck, Germany*
Methanol (CH_3OH) (Hypergrade for LC-MS)	*Merck, Germany*
Ammonia solution (NH_3) 25%	*Merck, Germany*
Formic acid (HCOOH) 98%	*Merck, Germany*
Nitrogen gas (N_2)	*AGA Progas AS, Oslo, Norway*

Equipment

Equipment	Manufacturer
MegaBlock 96-well tray, 500 µl volume	*Sarstedt, Nümbrecht, Germany*
Aluminum foil for 96-well tray	*VWR, Pennsylvania, USA*
Vanquish tray, 96-well	*VWR, Pennsylvania, USA*
Eppendorf tubes	*Eppendorf, Hamburg, Germany*
Whirlimixer (Vortex Genie 2)	*Scientific Industries (ELMIS), NY, USA*
Pipette tips	*Thermo-Fisher Scientific, Waltham, MA, USA*
Combitips for dispenser pipette	*Eppendorf, Hamburg, Germany*
Pipettes (Finnpipette)	*Thermo-Fisher Scientific, Waltham, MA, USA*
Eppendorf dispenser pipette	*Eppendorf, Hamburg, Germany*
Centrifuge (Heraeus Megafuge 16R-centrifuge)	*Thermo-Fisher Scientific, Waltham, MA, USA*
Heat block/sample concentrator (Dri-Block ® DB-3D)	*Techne, Cole-Parmer, Vernon Hills, IL, USA*
Vanquish flex UHPLC	*Thermo-Fisher Scientific, Waltham, MA, USA*
TSQ Altis mass spectrometer	*Thermo-Fisher Scientific, Waltham, MA, USA*
Column: Accucore Vanquish C18, 2.1 × 50 mm reverse phase column	*Thermo-Fisher Scientific, Waltham, MA, USA*

Solutions

Mobile phase A: 10 mM ammonium acetate buffer (pH=3, 5% acetonitrile)

Chemicals	Volume
MQ water	950 mL
Acetonitrile (Hypergrade for LC-MS)	50 mL
Ammonia (NH_3) 25%	150 µL
Formic acid (HCOOH) 98%	384 µL

Mobile phase B: 90% acetonitrile and 10% methanol

Chemicals	Volume
Methanol (CH_3OH) (Hypergrade for LC-MS)	100 mL
Acetonitril (Hypergrade for LC-MS)	900 mL

Precipitation solution containing internal standard

Chemicals	Volume
Midazolam-d6 25ng/mL	0.125 mL
1-hydroxymidazolam-d5 5ng/mL	0.250 mL
Acetonitrile 95% (Hypergrade for LC-MS)	47.5 mL
Methanol 5% (Hypergrade for LC-MS)	2.125 mL

Software

Software analysis instrument	Xcalibur (version 3.1, Thermo Scientific
Software statistics	Microsoft Excel (version 16.48) RStudio (version 1.3.1093)

Individual data

Table S1. Pharmacokinetic data of midazolam and 1-hydroxymidazolam. Data are presented as median (range)

	Clearance (L/h)	Average mdz concentration (ng/mL)	Average 1-OH mdz concentration (ng/mL)	Average ratio of 1-OH mdz/mdz
Group A				
Patient 5	0.35	190 (16-411)	7 (0.5-17)	0.036
Patient 6	3.38	76 (38-111)	8 (5-13)	0.11
Patient 12	0.63	116 (54-204)	5 (2-8)	0.048
Group B				
Patient 4	4.42	58 (27-129)	5 (2-10)	0.08
Patient 7	4.6	15 (0.3-36)	0.7 (0.03-1)	0.056
Patient 8	3.41	113 (30-203)	4 (0.8-6)	0.032
Patient 9	6.96	73 (2-112)	5 (0.4-9)	0.08
Patient 10	3.06	149 (9-207)	16 (0.9-35)	0.103
Patient 13	3.13	100 (0.7-187)	30 (0.2-60)	0.293
Group C				
Patient 1	10.5	122 (2-291)	53 (0.7-280)	0.268
Group D				
Patient 2	2.13	481 (341-584)	18 (9-23)	0.037
Patient 3	49.35	283 (90-443)	34 (9-143)	0.117
Patient 11	20.36	90 (39-148)	8 (1-38)	0.078

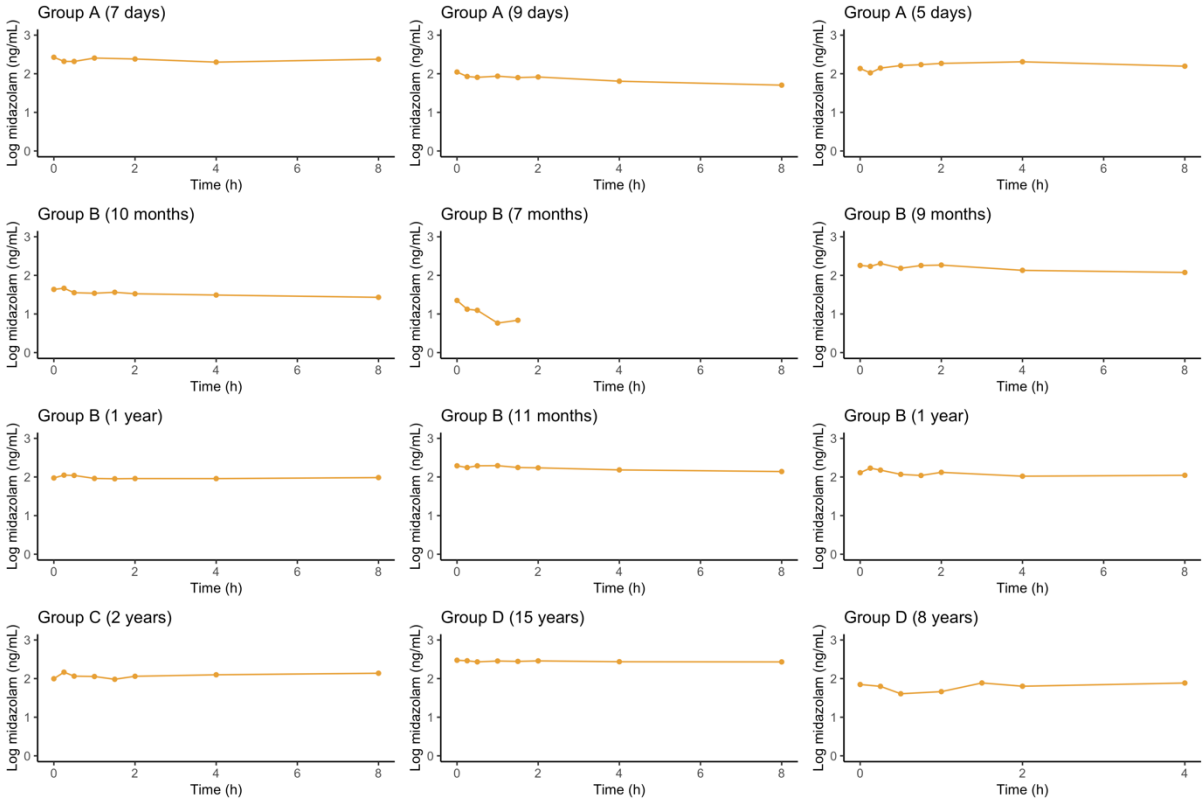


Figure S1. Individual concentration-time profiles for midazolam after dose reduction

Forespørsel om deltakelse i forskningsprosjektet:

«CyPed-studien»
En åpen studie for å undersøke CYP3A-aktivitet i barn

Dette er en forespørsel til deg som foresatt om du ønsker å la barnet ditt delta i et forskningsprosjekt vi holder på med her på barneintensiven på Oslo universitetssykehus (OUS), Rikshospitalet. I forskningsprosjektet skal vi undersøke naturlige fysiologiske endringer i evnen til å bryte ned legemidler som skjer i barneårene. Økt kunnskap på dette område er viktig for at vi skal kunne optimalisere legemiddelbehandlingen og sikre riktig dosering av legemidler også til barn. For mange legemidler finnes ikke slik kunnskap og man må bruke data fra voksne, noe som ofte ikke medfører korrekte doser til barn. Du får denne forespørselen fordi barnet ditt er innlagt på barneintensiven på OUS Rikshospitalet og får et legemiddel som heter midazolam som del av den kliniske behandlingen. Studien er en nasjonal studie som gjennomføres på OUS Rikshospitalet, og er tatt initiativ til av leger ved sykehuset og forskere ved Farmasøytisk institutt, Universitetet i Oslo.

FORMÅLET MED STUDIEN

Dosering av legemidler til barn kan være utfordrende. Dette skyldes blant annet at det i barneårene skjer store fysiologiske endringer i kroppen, som for eksempel modning av enzymer som bryter ned legemidler. Slike enzymer kalles legemiddelmetaboliserende enzymer, og er av stor betydning for hva som skjer med et legemiddel fra det gis til det er ute av kroppen. Et av de viktigste legemiddelmetaboliserende enzymene er CYP3A. Variasjon i CYP3A-aktivitet kan medføre opp til 40 ganger forskjellig legemiddeleksponering ved samme dose legemiddel til ulike individer. Dette kan gi utslag i bivirkninger hos noen pasienter og manglende effekt hos andre pasienter. Mange barn i dag bruker legemidler som metaboliseres av CYP3A, og økt kunnskap om funksjonen til dette enzymet i barn av ulike aldre er viktig for å kunne dosere legemidlene riktig. I denne studien ønsker vi derfor å undersøke CYP3A-aktiviteten i barn i ulike aldersgrupper.

HVA INNEBÆRER STUDIEN

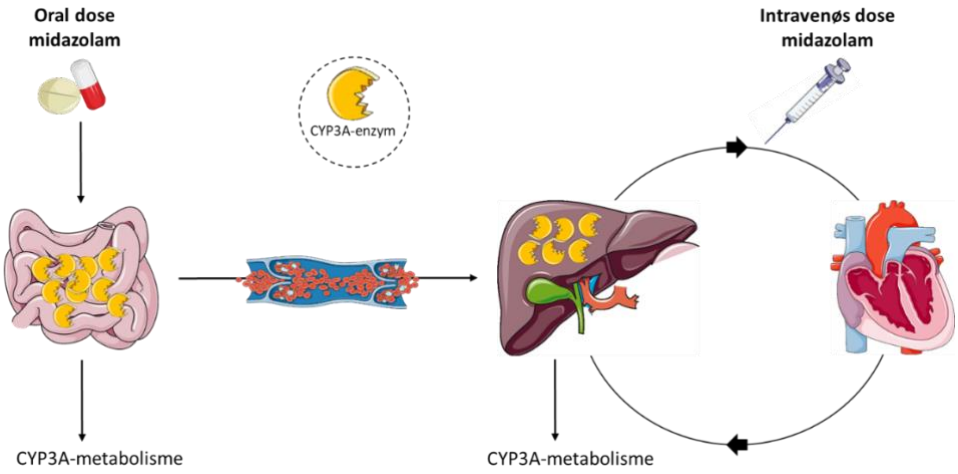
Legemiddelet vi bruker for å undersøke CYP3A-aktiviteten heter midazolam. Dette legemiddelet er allerede en del av behandlingen som barnet ditt får, så en eventuell deltakelse vil ikke påvirke den ordinære behandlingen. Deltakelse i studien innebærer at det vil tas noen ekstra blodprøver fra barnet. Alle prøver som tas i forbindelse med studien vil bli tatt mens barnet ditt er inneliggende på OUS Rikshospitalet. Barnet ditt vil ikke bli utsatt for noe ekstra stikking i forbindelse med blodprøvetakingen, da hun/han allerede har inneliggende arteriekran/ sentralt venekateter som blod kan tappes direkte fra. Prøvetakingen er derfor ikke forbundet med smerte eller ubehag.

Midazolam er et legemiddel som vi som regel gir som kontinuerlig infusjon (dvs. rett i blodåren) på avdelingen over flere dager, før vi etter hvert trapper det gradvis ned. Vi kommer til å ta 1-2 blodprøver a 0,5 mL om dagen for å måle konsentrasjonen av midazolam i blodet. I tillegg kommer vi til å ta noen flere blodprøver den dagen barnet ditt skal endre dosen eller slutte med midazolam, maks 8 stk á 0,5 mL. Den totale mengden blod som tas i forbindelse med studien utgjør mindre enn 3% av det totale blodvolumet. Dette er mye mindre enn det som tas rutinemessig, og vil ikke ha noen negative konsekvenser for barnet ditt. I en av blodprøvene skal vi også analysere hvilke gener barnet ditt har som er involvert i nedbrytingen av midazolam.

FRIVILLIG TILLEGG TIL DELTAKELSE

CYP3A er viktig for metabolisme av legemidler både i tarm og lever. For å kunne si noe om CYP3A-aktiviteten i tarm, hvilket er viktig med tanke på dosering av tabletter, trenger vi også blodprøver etter en dose oral midazolam. Etter den akutte fasen og når den intravenøse midazolaminfusjonen er avsluttet vil det også bli

vurdert, dersom barnet ditt har normal tarmfunksjon, å gi en enkeltdose med midazolam mikstur. Effektene/bivirkningene er de samme som for intravenøs midazolam. En ekstra dose oral midazolam med påfølgende blodprøver vil derfor ikke ha noen negative konsekvenser for barnet ditt utover det man eventuelt vil forvente av den ordinære behandlingen. Dersom du samtykker til at vi kan gi en dose oral midazolam i tillegg, kan du krysse av for dette på samtykkeskjemaet på siste side.



MULIGE FORDELER OG ULEMPER

Deltakelse i denne studien vil bidra til å øke kunnskapen om hvordan legemiddelmetabolismen endrer seg i barneårene. En bedre forståelse på disse områdene vil være viktig for å kunne optimalisere legemiddelbehandlingen og sikre riktig dosering av mange legemidler til barn. Den individuelle nytten for barnet ditt ved å delta i denne studien er begrenset.

Ulempene ved å delta er at studien innebærer en del ekstra blodprøvetakinger, men dette er ikke forbundet med noe ekstra ubehag eller andre negative konsekvenser for pasienten.

FRIVILLIG DELTAKELSE OG MULIGHET FOR Å TREKKE SITT SAMTYKKE

Det er frivillig å delta i studien. Dersom du ønsker at barnet ditt skal delta, undertegner du samtykkeerklæringen på siste side. Du kan når som helst og uten å oppgi noen grunn trekke ditt samtykke. Dette vil ikke få konsekvenser for barnets videre behandling. Dersom du trekker barnet ditt fra prosjektet, kan du kreve å få slettet innsamlede prøver og opplysninger, med mindre opplysningene allerede er inngått i analyser eller brukt i vitenskapelige publikasjoner. Dersom du senere ønsker å trekke barnet ditt eller har spørsmål til prosjektet, kan du kontakte overlege Hasse K. Zare (tel: 90 54 38 78) eller 1. amanuensis Ida Robertsen (tel: 99 22 75 69)/ stipendiat Kine Eide Kvitne (tel: 45422704).

HVA SKJER MED OPPLYSNINGENE OM BARNET DITT?

All informasjon som innhentes om barnet ditt i forbindelse med denne studien vil bli behandlet konfidensielt. Kontrollmyndigheter vil imidlertid kunne ha behov for å sjekke at opplysninger gitt i studien stemmer overens med opplysninger i pasientjournalen din. Dette gjøres for å sikre studiens kvalitet. Du har rett til innsyn i hvilke opplysninger som er registrert om barnet ditt, og rett til å få korrigert eventuelle feil i de opplysningene som er registrert. Opplysningene som registreres om barnet ditt skal kun brukes slik som beskrevet i hensikten med prosjektet.

All informasjon om barnet ditt vil bli behandlet uten navn og fødselsnummer eller andre direkte gjenkjennende opplysninger. En kode knytter barnet ditt til sine opplysninger gjennom en navneliste. Det er kun personell med

ansvar for studien som har tilgang til denne listen. All personale som håndterer opplysninger om barnet ditt har taushetsplikt. Opplysningene om deg vil lagres i 5 år etter at studien er ferdig, og deretter bli anonymisert i henhold til gjeldende regelverk.

Resultatene fra studien vil bli publisert i vitenskapelige tidsskrifter etter at studien er ferdig. Det vil ikke være mulig å identifisere barnet ditt i resultatene av studien når disse publiseres.

HVA SKJER MED PRØVENE AV BARNET DITT?

Blodprøvene som tas av barnet ditt skal oppbevares i en forskningsbiobank tilknyttet studien («CyPed»), og kan kun brukes til de analysene som er nevnt i denne informasjonen. Det vil i praksis si at de blir oppbevart i en fryser hos oss (på Rikshospitalet). Hvis du velger å trekke barnet ditt fra studien vil vi ikke ta flere prøver av hun/han, men de prøvene som allerede er samlet inn vil bli brukt i studien og vil ikke bli destruert. Ansvarshavende for biobanken er overlege Hasse K. Zare (e-mail: haskhi@ous-hf.no, tel: 90 54 38 78). Materiale skal destrueres ved prosjektslutt.

FORSIKRING

Deltakere i denne studien vil være dekket av en forsikring i henhold til Pasientskadeloven.

Denne skriftlige informasjonen skal ledsages av muntlig informasjon. Har du spørsmål kan du kontakte ansvarlig studielege. Du vil kopi av denne informasjonen og denne bør du spare på.

Ansvarlig studielege og hovedansvarlig for denne studien og biobanken er overlege dr. med. Hasse K. Zare (e-mail: haskhi@ous-hf.no, tel: 90 54 38 78).

GODKJENNING

Studien er forhåndsgodkjent av Regional komité for medisinsk og helsefaglig forskningsetikk (saksnr. 31635), og Personvernombudet ved Oslo universitetssykehus, Rikshospitalet.

Etter ny personopplysningslov har behandlingsansvarlig institusjon (Oslo universitetssykehus) og prosjektleder Hasse K. Zare et selvstendig ansvar for å sikre at behandlingen av dine opplysninger har et lovlig grunnlag. Dette prosjektet har rettslig grunnlag i EUs personvernforordning artikkel 6 nr. 1a og artikkel 9 nr. 2a og ditt samtykke. Du har rett til å klage på behandlingen av barnet ditt sine opplysninger til Datatilsynet.

Du kan ta kontakt med institusjonens personvernombud (Tor Åsmund Martinsen, tlf.: 23 01 50 22, e-post: toamar@ous-hf.no) dersom du har spørsmål om behandlingen av barnet ditt sine personopplysninger i prosjektet.

KONTAKTOPPLYSNINGER

Dersom du har spørsmål til prosjektet kan du ta kontakt med overlege Hasse K. Zare (e-mail: haskhi@ous-hf.no, tel: 90 54 38 78)/1. amanuensis Ida Robertsen (e-mail: ida.robertsen@farmasi.uio.no, tel: 99 22 75 69)/stipendiat Kine Eide Kvitne (e-mail: k.e.kvitne@farmasi.uio.no, tel: 45 42 27 04).

JEG SAMTYKKER TIL AT BARNET MITT KAN DELTA I STUDIEN OG TIL AT BARNET MITT SINE PERSONOPPLYSNINGER OG BIOLOGISKE MATERIALE BRUKES SLIK DET ER BESKREVET

Som foresatte til_ (Fullt navn) samtykker vi til at hun/han kan delta i prosjektet

Sted og dato	Foresattes signatur
	Foresattes navn med trykte bokstaver
Sted og dato	Foresattes signatur
	Foresattes navn med trykte bokstaver

FRIVILLIG TILLEGG TIL DELTAKELSE:

Jeg samtykker også til at det kan gis en oral dose midazolam til barnet mitt slik som beskrevet på side 1 i denne pasientinformasjonen, dersom studieansvarlig lege mener pasienten oppfyller inklusjonskriteriene for dette.

JA ☐ NEI ☐

JEG BEKREFTER Å HA GITT INFORMASJON OM STUDIEN

Sted og dato	Signatur
	Rolle i prosjektet

Forespørsel om deltakelse i forskningsprosjektet:

«CyPed-studien»

En studie for å undersøke hvordan medisinen midazolam brytes ned i kroppen til barn i ulike aldre

HVORFOR BLIR DU SPURT OM Å VÆRE MED?

Du har blitt inkludert i denne studien fordi du er innlagt på barneintensiven på OUS Rikshospitalet og har fått en medisin (midazolam) som vi ønsker å se nærmere på. Ved å lære mer om hvordan din kropp kvitter seg med denne medisinen vil det bli lettere for oss å vite hvilken dose av medisiner som vi skal gi til deg og andre barn.

HVA VIL SKJE DERSOM DU DELTAR?

Siden du er med i denne studien har vi tatt noen ekstra blodprøver fra deg mens du har vært på sykehuset. Disse har blitt tatt fra en kran som du allerede har inne i en av blodårene dine, slik at vi ikke har trengt å stikke deg. Det gjør ikke vondt å ta blod fra denne kranen. Vi har tatt blodprøver av deg 1-2 ganger om dagen. Den dagen du sluttet å bruke midazolam tok vi litt flere blodprøver av deg, maks 8 stk.

Du har mest sannsynlig sovet når vi tok blodprøver av deg. Derfor har du sannsynligvis ikke merket at vi tok prøvene. Foreldrene dine har på forhånd sagt at det var greit at vi tok disse prøvene av deg.

Andre barn i lignende situasjon som deg kommer også til å være med i denne studien.

HVA VIL SKJE DERSOM DU IKKE DELTAR

Det er helt frivillig å delta i studien. Du kan når som helst og uten å oppgi noen grunn si ifra om at du ikke lengre ønsker å være med i studien. Dette vil ikke ha noen betydning for din videre behandling og oppfølging.

Forespørsel om deltakelse i forskningsprosjektet:

«CyPed-studien»

En studie for å undersøke hvordan medisinen midazolam brytes ned i kroppen til barn i ulike aldre

HVORFOR BLIR DU SPURT OM Å VÆRE MED?

Du har blitt inkludert i denne studien fordi du er innlagt på barneintensiven på OUS Rikshospitalet og har fått en medisin (midazolam) som vi ønsker å se nærmere på. Vi vil i denne studien lære mer om hva som skjer med denne medisinen fra den gis til den er ute av kroppen. Det kan da bli lettere for oss å vite hvordan vi best gir denne og andre medisiner som brytes ned i kroppen på samme måte til deg og andre barn. At en medisin brytes ned betyr at medisinen forsvinner ut fra kroppen din.

BAKGRUNN OG HENSIKT MED STUDIEN

I kroppen har vi noe som heter enzymer, og noen av disse enzymene bryter ned medisiner. Enzymene har derfor betydning for hvor lenge en medisin som du har fått kan bli i kroppen din. I barneårene skjer det ganske store endringer i disse enzymene, noe vi ønsker å se nærmere på. I denne studien skal vi derfor undersøke dette i barn i ulike aldersgrupper. Mer kunnskap på dette område er viktig for at vi skal kunne gi riktig dose medisin til barn i alle aldre.

HVA INNEBÆRER STUDIEN

Siden du er med i denne studien har vi tatt noen ekstra blodprøver fra deg. Disse har blitt tatt fra en kran som du allerede har inne i en av blodårene dine, slik at vi ikke har trengt å stikke deg. Det gjør ikke vondt å ta blod fra denne kranen. Du har mest sannsynlig sovet når vi tok blodprøver fra deg. Derfor har du sannsynligvis ikke merket at vi tok prøver fra deg.

Alle blodprøver i forbindelse med studien har blitt tatt mens du allikevel var på sykehuset, og du har derfor ikke måttet bruke noe ekstra tid på å delta. Vi har tatt 1-2 blodprøver av deg om dagen. I tillegg har vi tatt litt flere blodprøver den dagen du sluttet med midazolam, maks 8 stk. Mengden blod som har blitt tatt er veldig liten (mindre enn 3% av ditt totale blodvolum), og vil ikke ha noen negative konsekvenser for deg.

Medisinen som vi bruker for å se på hvilke endringer som skjer i barneårene (midazolam) er allerede en del av behandlingen som du får eller har fått. En eventuell deltakelse har derfor ikke påvirket den ordinære behandlingen din.

MULIGE FORDELER OG ULEMPER

Ved å delta i denne studien kan du hjelpe oss å lære mer om hvordan medisiner brytes ned i kroppen i barn i ulike aldre. Dette er viktig informasjon for å vite hvordan vi best gir medisiner til deg og andre barn.

Ulempen med å delta er at vi må ta noen ekstra blodprøver fra deg. Dette er ikke forbundet med smerte/ubehag, og bør ikke ha noen negative konsekvenser for deg.

HVA SKJER MED PRØVENE OG INFORMASJONEN OM DEG?

All informasjon som registreres, inkludert prøver som er tas av deg, skal kun brukes slik som beskrevet i denne pasientinformasjonen. Alle opplysningene og prøvene vil bli behandlet uten navn og fødselsnummer eller andre direkte gjenkjennende opplysninger. Det vil derfor ikke være mulig å identifisere deg i resultatene av studien når disse publiseres i vitenskapelige tidsskrifter. Alt personale som håndterer opplysninger om deg har taushetsplikt.

FRIVILLIG DELTAKELSE OG MULIGHET TIL Å TREKKE SEG FRA STUDIEN

Det er frivillig å delta i studien. Du kan når som helst og uten å oppgi noen grunn trekke ditt samtykke til å delta i studien. Dette vil ikke få konsekvenser for din videre behandling og oppfølging.

Dersom du ønsker å trekke deg fra studien eller har andre spørsmål knyttet til forskningsprosjektet kan du eller dine foresatte kontakte: overlege Hasse K. Zare (tel: 90 54 38 78) eller 1.amanuensis Ida Robertsen (tel: 99 22 75 69)/stipendiat Kine Eide Kvitne (tel: 45422704).

A

CyPed-studien

EN STUDIE FOR Å UNDERSØKE CYP3A-AKTIVITET I BARN

Kontaktpersoner:

Kine Eide Kvitne **Tlf. 45422704**

Ida Robertsen Tlf. 99227569

Ikke nøl med å ta kontakt, når som helst på døgnet, dersom det skulle være noe.

Pasientnummer: | | | |

Pasientinitialer: | | | |

BLODPRØVETAKING UNDER KONTINUERLIG MIDAZOLAM INFUSJON

- Noter ned dato og nøyaktig tidspunkt for når blodgassprøven ble tatt i tabellen under
- Kryss av for om prøven er tatt venøst eller arterielt
- Husk å sjekke at prøvenummeret på etiketten på EDTA-røret stemmer med prøvenummeret i tabellen
- Det resterende blodet (min 0,25 mL, maks 0,5 mL) fra blodgassprøyten overføres til ferdigmerkede EDTA-rør
- Vend røret 8-10 ganger
- Sett røret i merket stativ (CyPed)
- Sett stativet med blodprøven(e) på benken i basen

Blodprøve-nummer	Dato	Midazolam infusjonshastighet (mg/kg/time)	Tid blodprøvetaking (kl.) (tidspunktet når prøven faktisk ble tatt)	Arterielt (A) eller venøst (V)
A-01			:	A ☐ V ☐
A-02			:	A ☐ V ☐
A-03			:	A ☐ V ☐
A-04			:	A ☐ V ☐
A-05			:	A ☐ V ☐
A-06			:	A ☐ V ☐
A-07			:	A ☐ V ☐
A-08			:	A ☐ V ☐
A-09			:	A ☐ V ☐

Blodprøve-nummer	Dato	Midazolam infusjonshastighet (mg/kg/time)	Tid blodprøvetaking (kl.) (tidspunktet når prøven faktisk ble tatt)	Arterielt (A) eller venøst (V)
A-10			:	A ☐ V ☐
A-11			:	A ☐ V ☐
A-12			:	A ☐ V ☐
A-13			:	A ☐ V ☐
A-14			:	A ☐ V ☐
A-15			:	A ☐ V ☐
A-16			:	A ☐ V ☐
A-17			:	A ☐ V ☐
A-18			:	A ☐ V ☐
A-19			:	A ☐ V ☐
A-20			:	A ☐ V ☐
A-21			:	A ☐ V ☐
A-22			:	A ☐ V ☐
A-23			:	A ☐ V ☐
A-24			:	A ☐ V ☐
A-25			:	A ☐ V ☐
A-26			:	A ☐ V ☐
A-27			:	A ☐ V ☐
A-28			:	A ☐ V ☐

Blodprøve-nummer	Dato	Midazolam infusjonshastighet (mg/kg/time)	Tid blodprøvetaking (kl.) (tidspunktet når prøven faktisk ble tatt)	Arterielt (A) eller venøst (V)
A-29			:	A ☐ V ☐
A-30			:	A ☐ V ☐
A-31			:	A ☐ V ☐
A-32			:	A ☐ V ☐
A-33			:	A ☐ V ☐
A-34			:	A ☐ V ☐
A-35			:	A ☐ V ☐
A-36			:	A ☐ V ☐
A-37			:	A ☐ V ☐
A-38			:	A ☐ V ☐
A-39			:	A ☐ V ☐
A-40			:	A ☐ V ☐
A-41			:	A ☐ V ☐
A-42			:	A ☐ V ☐
A-43			:	A ☐ V ☐
A-44			:	A ☐ V ☐
A-45			:	A ☐ V ☐
A-46			:	A ☐ V ☐
A-47			:	A ☐ V ☐

Blodprøve-nummer	Dato	Midazolam infusjonshastighet (mg/kg/time)	Tid blodprøvetaking (kl.) (tidspunktet når prøven faktisk ble tatt)	Arterielt (A) eller venøst (V)
A-48			:	A ☐ V ☐
A-49			:	A ☐ V ☐
A-50			:	A ☐ V ☐
A-51			:	A ☐ V ☐
A-52			:	A ☐ V ☐
A-53			:	A ☐ V ☐
A-54			:	A ☐ V ☐
A-55			:	A ☐ V ☐
A-56			:	A ☐ V ☐
A-57			:	A ☐ V ☐
A-58			:	A ☐ V ☐
A-59			:	A ☐ V ☐
A-60			:	A ☐ V ☐
A-61			:	A ☐ V ☐
A-62			:	A ☐ V ☐
A-63			:	A ☐ V ☐
A-64			:	A ☐ V ☐
A-65			:	A ☐ V ☐
A-66			:	A ☐ V ☐

B

CyPed-studien

EN STUDIE FOR Å UNDERSØKE CYP3A-AKTIVITET I BARN

Kontaktpersoner:

Kine Eide Kvitne **Tlf. 45422704**

Ida Robertsen Tlf. 99227569

Ikke nøl med å ta kontakt, når som helst på døgnet, dersom det skulle være noe.

Pasientnummer: | | | | Dato: | | | |

Pasientinitialer: | | | |

BLODPRØVETAKING ETTER DOSEREDUKSJON/SEPONERING AV MIDAZOLAM INFUSJON

- Noter ned nøyaktig tidspunkt for når blodgassprøven ble tatt i tabellen
- Kryss av for om prøven er tatt venøst eller arterielt
- Husk å sjekke at prøvenummeret på etiketten på EDTA-røret stemmer med prøvenummeret i tabellen
- Det resterende blodet (min 0,25 mL, maks 0,5 mL) fra blodgassprøyten overføres til ferdigmerkede EDTA-rør
- Vend blodprøverøret 8-10 ganger
- Sett blodprøverørene i merket stativ (CyPed)
- Sett stativet med blodprøven(e) på benken i basen

Blodprøve-nummer	Tid blodprøve (time)	Faktisk tid blodprøven ble tatt (kl.)	Arterielt (A) eller venøst (V)
B-01	**0.0**	**:**	**A** ☐ **V** ☐
	Tid midazolam dosereduksjon/ seponering **Kl. _____:_____**	**Opprinnelig dose (før dosereduksjon):** **_____ mg/kg/time** **Ny dose (etter dosereduksjon):** **_____ mg/kg/time**	
B-02	**0.25** (:)	**:**	**A** ☐ **V** ☐
B-03	**0.5** (:)	**:**	**A** ☐ **V** ☐
B-04	**1.0** (:)	**:**	**A** ☐ **V** ☐
B-05	**1.5** (:)	**:**	**A** ☐ **V** ☐
B-06	**2** (:)	**:**	**A** ☐ **V** ☐
B-07	**4** (:)	**:**	**A** ☐ **V** ☐
B-08	**8** (:)	**:**	**A** ☐ **V** ☐

Info: det skal tas blodprøver 0.25, 0.5, 1, 1.5, 2, 4 og 8 timer etter dosereduksjon eller seponering av midazolam infusjon. Dette gjøres en gang i løpet av nedtrappingsforløpet.

C

CyPed-studien

EN STUDIE FOR Å UNDERSØKE CYP3A-AKTIVITET I BARN

Kontaktpersoner:

Kine Eide Kvitne **Tlf. 45422704**

Ida Robertsen Tlf. 99227569

Ikke nøl med å ta kontakt, når som helst på døgnet, dersom det skulle være noe.

Pasientnummer: | | | | Dato: | | | |

Pasientinitialer: | | | |

BLODPRØVETAKING ETTER ORAL MIDAZOLAM

- Noter ned nøyaktig tidspunkt for når blodgassprøven ble tatt i tabellen
- Kryss av for om prøven er tatt venøst eller arterielt
- Husk å sjekke at prøvenummeret på etiketten på EDTA-røret stemmer med prøvenummeret i tabellen
- Det resterende blodet (min 0,25 mL, maks 0,5 mL) fra blodgassprøyten overføres til ferdigmerkede EDTA-rør
- Vend blodprøverøret 8-10 ganger
- Sett blodprøverørene i merket stativ (CyPed)
- Sett stativet med blodprøven(e) på benken i basen

Dose oral midazolam: _____ mg

Blodprøve-nummer	Tid blodprøve (time)	Faktisk tid blodprøven ble tatt (kl.)	Arterielt (A) eller venøst (V)
C-01	0.0	:	A ☐ V ☐
	Tid midazolam administrering Kl. _____:_____		
C-02	0.25 (:)	:	A ☐ V ☐
C-03	0.5 (:)	:	A ☐ V ☐
C-04	1.0 (:)	:	A ☐ V ☐
C-05	1.5 (:)	:	A ☐ V ☐
C-06	2 (:)	:	A ☐ V ☐
C-07	4 (:)	:	A ☐ V ☐
C-08	8 (:)	:	A ☐ V ☐

Info: det skal tas blodprøver 0.25, 0.5, 1, 1.5, 2, 4 og 8 timer etter administrering av oral midazolam.

CYP3A ACTIVITY IN PEDIATRIC PATIENTS

Protocol Identification Number: CyPed-study

Principal Investigator:

Hasse Khiabani Zare
Department of Pharmacology,
Oslo University Hospital, Rikshospitalet
Sognsvannveien 20, 0372 Oslo, Norway
E-mail: haskhi@ous-hf.no

PROTOCOL VERSION NO. 2
13.08.2019

CONTACT DETAILS

Principal investigator:

Hasse Khiabani Zare, MD, PhD
Department of Pharmacology,
Oslo University Hospital, Rikshospitalet
Sognsvannveien 20, 0372 Oslo, Norway
E-mail: haskhi@ous-hf.no

Co-investigator:

Gunnar Kristoffer Bentsen, MD, PhD
Department of Anesthesiology,
Oslo University Hospital, Rikshospitalet
Sognsvannveien 20, 0372 Oslo
E-mail: gbentsen@ous-hf.no

Co-investigator

Kine Eide Kvitne, MSc Pharm, PhD student
Department of Pharmacy, Section of Pharmacology and
Pharmaceutical Biosciences, University of Oslo
Sem Sælandsvei 3, 0316 Oslo, Norway
E-mail: k.e.kvitne@farmasi.uio.no

Co-investigator

Ida Robertsen, Associate Professor, PhD
Department of Pharmacy, Section of Pharmacology and
Pharmaceutical Biosciences, University of Oslo.
Sem Sælandsvei 3, 0316 Oslo, Norway
E-mail: ida.robertsen@farmasi.uio.no

Co-investigator:

Anders Åsberg, Professor, PhD
Department of Transplantation Medicine**,** Oslo University Hospital, Rikshospitalet
Sognsvannveien 20, 0372 Oslo, Norway
E-mail: anders.asberg@ous-hf.no

Co-investigator

Hege Christensen, Professor, PhD
Department of Pharmacy, Section of Pharmacology and
Pharmaceutical Biosciences, University of Oslo.
Sem Sælandsvei 3, 0316 Oslo, Norway
E-mail: hege.christensen@farmasi.uio.no

Monitor:

Aase Jorun Klaveness
Department of Pharmacology, Oslo University Hospital
Sognsvannveien 20, 0424 Oslo
E-mail: klaveness@ddl.no

SIGNATURE PAGE

Title CYP3A activity in pediatric patients

Protocol ID no: CyPed-study

I hereby declare that I will conduct the study in compliance with the Protocol, ICH GCP and the applicable regulatory requirements:

To be signed, the coordinating investigator and the principal investigators.

Name	Title	Role	Signature	Date
Hasse Khiabani Zare	MD, PhD	Principal Investigator (OUS)		
Ida Robertsen	Associate Professor, PhD	Coordinating Investigator (UiO)		

PROTOCOL SYNOPSIS

Protocol title: CYP3A activity in pediatric patients

Principal investigator	Hasse Khiabani Zare Department of Pharmacology, Oslo University Hospital, Rikshospitalet Sognsvannveien 20, 0372 Oslo, Norway E-mail: haskhi@ous-hf.no
Phase and study type	Single-center, prospective, open, non-randomized
Center:	Oslo University Hospital, Rikshospitalet
Study Period:	Estimated date of first patient enrolled: Q4 2019 Anticipated recruitment period: 18 months Estimated date of last patient completed: Q1 2021
Investigational Duration:	During continuous midazolam infusion and up to 5 days after discontinuing the infusion in a subgroup of patients
Follow-up:	Patients will be followed the entire hospital stay.
Objectives	Main study objective: This study aims to determine ontogeny of systemic (i.e. hepatic) CYP3A activity in the pediatric population. Secondary objectives: To investigate the relationship between intestinal and hepatic ontogeny of CYP3A activity in the pediatric population. To investigate the impact of different polymorphism in CYP3A isforms on CYP3A activity
Endpoints:	Primary endpoint: Systemic clearance of the CYP3A probe drug midazolam in pediatric patients from intravenous (iv) data. Secondary endpoint: Presystemic clearance of the CYP3A probe drug midazolam in pediatric patients from peroral (po) and iv data. Association between different polymorphism in CYP3A isoforms and systemic midazolam clearance

Study Design:	Single-center, prospective, open, non-randomized.
Main Inclusion Criteria:	– Pediatric patients at the Pediatric Intensive Care Unit (Oslo University Hospital Rikshospitalet) who are scheduled to receive midazolam treatment for any medical reason or condition. This study will not interfere with the indication for therapy. – Age 0-16 years.
Main Exclusion Criteria	– Conditions anticipated to interfere with gastrointestinal and/or hepatic drug disposition up to the discretion of the investigator.
Sample Size:	Based on the number of patients hospitalized and receiving iv midazolam at the Pediatric Intensive Care Unit in 2018, we expect to include 130 patients.
Efficacy Assessments:	Plasma concentrations of midazolam
Safety Assessments:	Hematology, biochemistry and vital signs

TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND DEFINITIONS OF TERMS

Abbreviation or special term	Explanation
AE	Adverse Event
AUC	Area Under the Time-Concentration Curve
CI	Coordinating investigator
CL	Total Clearance
CL/F	Apparent Oral Clearance
CRF	Case Report Form (electronic/paper)
CSA	Clinical Study Agreement
CYP	Cytochrome P450
DAE	Discontinuation due to Adverse Event
EC	Ethics Committee, synonymous to Institutional Review Board (IRB) and Independent Ethics Committee (IEC)
GCP	Good Clinical Practice
iv	Intravenous
IB	Investigator's Brochure
ICF	Informed Consent Form
ICH	International Conference on Harmonization
po	Peroral
SAE	Serious Adverse Event
SOP	Standard Operating Procedure

1 INTRODUCTION

1.1 Background

Correct dosing of drugs in pediatric patients is challenging and less evidence-based than for adults [1]. This is partly because pediatric patients to a less extent have been included in clinical drug trials, most probably because it previously was perceived as ethically problematic to include pediatric patients in research. EU-regulation No 1901/2006 of the European parliament and the introduction of the paediatric investigation plan was a very important step toward ensuring that the development of medicinal products that are potentially to be used for the pediatric population is actively investigated in this target population as well [2]. But there is still insufficient knowledge regarding pharmacokinetics in pediatric patients and in the absence of suitably adapted medicinal products for the pediatric population, medicinal product authorized for adult population are being used daily in a comprehensive way. This could lead to increased risks of adverse reactions, ineffective treatment through under-dosage as well as non-availability to the pediatric population of therapeutic advances. Therefore, more knowledge regarding correct drug dosing in the pediatric population and the effect of age on pharmacokinetics are needed.

Drug metabolizing enzymes influences the exposure of different drugs. Cytochrome P450 (CYP) enzymes are involved in the metabolism of 75% of therapeutic drugs [3]. Approximately 30-50% of clinically used drugs today are metabolized by the CYP subfamily CYP3A, including the isoforms CYP3A4, CYP3A5 and CYP3A7 [4]. CYP3A enzymes accounts for 80% and 40% of the total amount of CYP enzymes in the small intestine and in the liver in adults, respectively [5]. Presystemic metabolism by CYP3A enzymes in the gastro intestinal tract, together with first pass metabolism in the liver, restricts the amount of drug reaching the systemic circulation. Interindividual variability in drug metabolizing enzyme activity, such as age-related differences in expression between pediatric patients and adults, can thus affect the oral bioavailability and elimination of drugs. For instance, CYP3A-enzymes in the small intestine and the liver mature at different rates and thus the contribution to first-pass metabolism may differ by age [6]. CYP3A7 is the major CYP isoform in the liver during the fetal period and early at birth [7], but is later replaced by CYP3A4, which increases rapidly the first months of life [8]. There are many physiologically changes occuring after birth, but these changes are not proportional to body weight. Therefore, traditional methods such as dosing of drugs based solely on body weight scaling are not an appropriate approach. Further understanding of CYP3A phenotype in children and how this phenotype may change over time (i.e. age) is important for drug dosing in this population.

Different probe drugs can be used to phenotype metabolizing enzymes in vivo. One of the best validated probe drugs to assess CYP3A activity is midazolam [9-12], a short acting benzodiazepine with amnesic, anxiolytic and sedative properties [13]. Midazolam is almost exclusively metabolized by CYP3A enzymes, and is not a substrat for the drug transporter P-glycoprotein [14, 15]. When administered intravenously, its pharmacokinetic pattern is dependent on hepatic CYP3A activity while when given orally its pharmacokinetics depends on both hepatic and intestinal CYP3A activity [16, 17]. Thus, midazolam can be used to predict both intestinal and hepatic CYP3A phenotype, which is of interest regarding their role in both first pass metabolism and systemic elimination [18]. A recent study found a considerably lower intestinal clearance than hepatic clearance of midazolam in relatively healthy children aged 1-18 years using a physiological based population pharmacokinetic modelling approach [6]. Thus, CYP3A enzymes in the gut wall seem to contribute less to the first pass metabolism in children than CYP3A enzymes in the liver. Several studies have assessed midazolam clearence in different pediatric patient populations, and the inter-individual variability is high [6, 19-21]. This variability seems to be even greater in critically ill children [21], probably because factors such as inflammation and organ failure affects the pharmacokinetics of midazolam [22]. Regarding oral bioavailability in the pediatric population, Brussee *et al.* found that hepatic bioavailability increases with age, while intestinal bioavailability increases the first 5 years of life, but then decreases until adulthood [6]. The inter-individual variability in oral midazolam bioavailability was high, which also has been described in another study with midazolam in children [23]. To our knowledge, no studies have determined the absolute bioavailability of midazolam in the pediatric population.

1.2 Rationale for the Study

Drugs that are metabolized by or inhibit CYP3A enzymes are widely used in the pediatric population, but dosing guidelines are often missing. Since dosing based on body weight scaling is unsuitable, a better understanding of CYP3A phenotype across the pediatric age range is important for future dosing guidelines.

1.3 Benefit-risk evaluation

The personal advantage for the patients participating in this study is limited. On a group level, the participants will contribute to a broader knowledge regarding the CYP3A activity in different age groups. This could potentially lead to improved dosing of substrate drugs for the pediatric population in the future.

All patients will receive midazolam as part of their standard treatment of care. A few additional blood samples will be drawn in the study period. Blood sampling will be obtained from peripheral venous catheter/arterial tap/central venous catheter and the patients will be sedated, thus limiting the discomfort of blood sampling. Additionally, only a small volume of blood (0.5 mL) will be drawn for each sample and the total trial-related blood loss will never exceed the maximal blood loss recommended for investigations in the pediatric population according to the European Commission and the European Medicines Agency (EMA) [24, 25].

A subgroup of the included patients will also receive a single dose of midazolam syrup orally or through a nasogastric tube after discontinuation of the iv midazolam to determine the intestinal CYP3A activity. This will be in addition to standard treatment of care. The midazolam syrup dose will be 0,4 mg/kg for patients <3.8 kg (which is the standard dose for patients below 25 kg scheduled for treatment with po midazolam at the Pediatric intensive care unit at OUS Rikshospitalet), and 1.5 mg for included patients ≥3.8 kg. The po dose is therefore considered a low dose, and the risk of adverse events is considered to be limited.

2 STUDY OBJECTIVES AND RELATED ENDPOINTS

The overall study objective is to describe intestinal and hepatic CYP3A phenotype in the pediatric population using midazolam as a probe drug for CYP3A activity.

2.1 Primary Endpoint

To investigate systemic clearance of the CYP3A probe drug midazolam (iv) in pediatric patients.

2.2 Secondary Endpoints

- To investigate presystemic clearance of the CYP3A probe drug midazolam in pediatric patients from po- and iv data.
- Association between different polymorphism in CYP3A isoforms and systemic midazolam clearance

3 OVERALL STUDY DESIGN

The study is a single-center, prospective, open, non-randomized study including pediatric patients aged 0-16 years at the Pediatric Intensive Care Unit at the Oslo University Hospital, Rikshospitalet.

Midazolam will be used as a probe drug for determination of CYP3A phenotype. Patients receiving continuous midazolam infusion in individualized, therapeutic doses as part of their intensive care treatment will be included regardless of concomitant treatment with other drugs. Blood samples for determination of midazolam concentrations will be obtained during the continuous infusion and after change in the infusion rate/ withdrawal. In order to assess the potential impact of concomitant drugs on CYP3A activity, all concomitant therapy will be recorded in CRFs.

In addition to standard treatment of care, in a subgroup of patients, a single dose of midazolam syrup (defined as po midazolam) will be administered orally or through a nasogastric tube relatively close in time to the continuous infusion (within ±5 days) to estimate presystemic clearance.

Patients will be divided in age specific groups; 0-6 months, 6 months-2 years, 2-5 years and 5-16 years called A, B, C and D respectively.

Study Period	Estimated date of first patient enrolled: Q4 2019
	Anticipated recruitment period: 18 months
	Estimated date of last patient completed: Q1 2019
Follow-up:	Patients will be followed the entire hospital stay.

4 STUDY POPULATION

4.1 Selection of Study Population

Pediatric patients hospitalized at the Pediatric Intensiv Care Unit and scheduled to receive continuous infusion with midazolam treatment for any medical reason or condition are eligible for inclusion in the study. Patients contributing with data following po midazolam dosing should have normal bowel function up to the discretion of the investigator.

This study will not interfere with the indication for therapy. The investigational days will be conducted at Oslo University Hospital, Rikshospitalet. Written informed consent will be obtained from the patient and/or the parents/legal representative on behalf of the patient according to the Declaration of Helsinki and "Good Clinical Practice" (GCP). The children will be involved in the conversations and the concent process together with both parents, whenever possible and appropriate. Also, own customized patient information and consent sheets will be given to the children when appropriate. Both parents/legal representative and/or the patient and the investigator will sign the patient information, which will be kept on file. Patient data will be recorded in Case Report Forms (CRF), data will be analyzed using de-identified codes and all information will be handled confidentially.

4.2 Number of Patients

Up to 130 patients will be included in this trial. Eventual dropouts will be replaced.

The aim is that at least 20% of the included patients also will be included in the following subgroup: continuous midazolam infusion and one additional single dose of po midazolam syrup (0.4 mg/kg or 1.5 mg if ≥3.8 kg) within ±5 days after discontinuing the iv midazolam.

4.3 Inclusion Criteria

Patients will be recruited from the Pediatric Intensive Care Unit at the Oslo University Hospital, Rikshospitalet.

All of the following conditions must apply to the prospective patient at screening prior to study start (e.g.):

- Patients scheduled to receive continuous treatment with iv midazolam for any medical condition
- Age 0-16 years
- Signed informed consent must be obtained and documented according to ICH GCP and national/local regulations

4.4 Exclusion Criteria

Patients will be excluded from the study if they meet any of the following criteria:

– Conditions anticipated to interfere with hepatic (all) and/or gastrointestinal (po data contribution) drug disposition up to the discretion of the investigator

5 STUDY DESIGN AND PROCEDURES

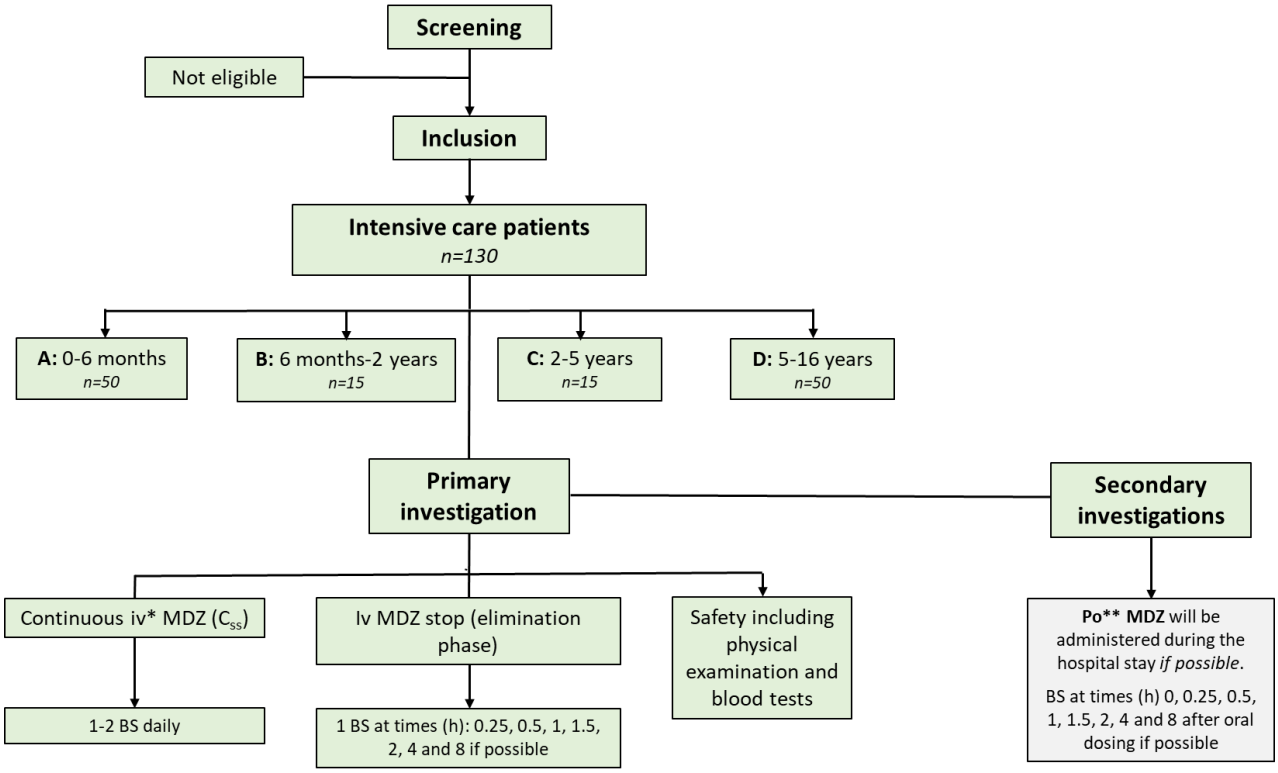


Figure 1: Trial flow chart for group 1 (A)
Abbreviations: BS, blood samples; h, hour; iv, intravenous; MDZ, midazolam; po, peroral
*Therapeutic doses of intravenous midazolam
**Midazolam syrup (0.4 mg/kg for patients <3.8 kg and 1.5 mg for patients ≥3.8 kg) administered orally or through a nasogastric tube to a subgroup of patients

Flow chart

	Screening	Hospital stay
Informed consent	X	
Inclusion/exclusion Evaluation	X	
Medical history	X	
Prior treatment		X
Record of concomitant medication		X
Physical examination[1]	X	X
Blood samples[2]	X	X
PK blood samples[3,4]		X
Adverse events		X
Follow-up		X

1. General appearance, skin, height, weight, blood pressure, pulse
2. Standard clinical blood samples when considered required.
3. 1-2 blood samples daily during continuous midazolam infusion (C_{ss}) and at times (h): 0.25, 0.5, 1, 1.5, 2, 4 and 8 (if possible) after discontinuing the midazolam infusion.
4. For patients receiving an additional po dose of midazolam syrup, blood samples will be collected at times 0, 0.25, 0.5, 1, 1.5, 2, 4 and 8 (if possible) hours after the administration.

5.1 In study period

All investigations will be performed at Oslo University Hospital, Rikshospitalet.

Screening

The screening of patients and inclusion will be performed at the Pediatric Intensive Care Unit at Oslo University Hospital, Rikshospitalet. Patient records at the Unit will be screened by study personnel for identifying potential participants.

Informed consent

Written informed consent must have been given voluntarily by each patient or his/her parents/legal representatives on behalf of the patient before any study specific procedures are initiated. The following tests will be done at screening:

Clinical status

Medical history (including disease history and corresponding treatment details), physical examination e.g. vital signs (weight, height, blood pressure, temperature and pulse) will be obtained when appropriate.

Concomitant medication

All concomitant medication (incl. vitamins, herbal preparation and other "over-the-counter" drugs) used by the patient will be recorded in the CRF during the hospital stay.

Laboratory analysis

Necessary biochemistry and hematology blood samples will be taken when considered required and analyzed at the respective hospital clinical chemistry departments.

All eligibility criteria should be assessed together with relevant baseline parameters prior to study inclusion (inclusion/exclusion criteria).

5.1.1 During the study period

Midazolam will be administered intravenously, and if possible orally. The iv dose will be dosed according to the individualized routine of the Pediatric Intensive Care Unit, i.e. the study team will not interfere or be involved in the treatment procedures. However, the po dose will be in addition to standard treatment. Blood samples will be obtained from a peripheral venous catheter/arterial tap/central venous catheter that has not been used for administering midazolam. The patients must be fasting for at least 2 hours before the po dose of midazolam is administered.

In neonates, according to the European Commission and the EMA, trial-related blood loss should not exceed 1% of the total blood volume at any single time and 3% during a 4-week period [24, 25]. The total blood volume is estimated to 80-90 mL blood/kg. Therefore, blood loss from trial related blood samples will not exceed 7.0 mL (neonates >2.9 kg) during the entire investigation period in group A (0-6 months). The volume of each trial related blood sample will be ~0.5 mL, which is within the recommandations according to EMA.

Assessment of CYP3A activity and data collection

Blood sampling will be performed 1-2 times daily during the continuous midazolam infusion and 0.25, 0.5, 1, 1.5, 2, 4 and 8 hours after withdrawal (midazolam infusion stop) or change in infusion rate, if possible.

In cases of a po dose of midazolam relatively close in time to iv midazolam (within ±5 days), blood samples will be collected at times 0, 0.25, 0.5, 1, 1.5, 2, 4 and 8 hours after administration of the po dose.

5.1.2 After End of investigation (Follow-up)

Patients will continue their standard clinical follow-up at Oslo University Hospital, Rikshospitalet after completion of the investigations.

5.2 Safety of the probe

Midazolam has a short duration of action and low toxicity, and is used as a sedative in the intensive care unit or before surgical and non-surgical procedures across the pediatric age range. Midazolam is frequently used at the Department of Pediatric medicine at Oslo University Hospital, Rikshospitalet, and only patients scheduled for midazolam treatment for any medical reason will be included in this study. Midazolam has also been used as a probe drug in previous pharmacokinetic studies in pediatric patients [6, 20, 26, 27].

The pediatric population is often more susceptible for adverse events, and midazolam administration will therefore be monitored carefully and doses titrated slowly, according to standard procedures at the department. Coadministration of opioids or other CNS-depressants increases the risk for oversedation and lower doses of midazolam may be required. Otherwise, no special precautions need to be considered in the pediatric population.

5.3 Concomitant Medication

All concomitant medication (incl. vitamins, herbal preparation and other "over-the-counter" drugs) used by the patient will be recorded in the patient's file and CRF.

5.4 Subject Numbering

Each subject is identified in the study by a unique subject number that is assigned when subject and/or parents/legal representatives signs the Informed Consent Form. Once assigned the subject number cannot be reused for any other subject.

5.5 Criteria for Patient Discontinuation

Patients may be discontinued from study treatment and assessments at any time. Specific reasons for discontinuing a patient for this study are:

- Voluntary discontinuation by the patient and/or the parents/legal representatives on behalf of the patient who is at any time free to discontinue his/her participation in the study, without prejudice to further treatment.
- Safety reason as judged by the Investigators
- Major protocol deviation
- Incorrect enrollment ie, the patient does not meet the required inclusion/exclusion criteria for the study
- Deterioration in the patients' condition which in the opinion of the Principal Investigators warrants study medication discontinuation (to be records as an AE or under Investigator Discretion)

5.6 Procedures for Discontinuation

5.6.1 Patient Discontinuation

Patients who withdraw or are withdrawn from the study will continue their standard clinical follow-up at Oslo University Hospital, Rikshospitalet. The reason for discontinuation will be recorded in the CRF.

Patients withdrawn from the study due to AEs will be monitored until all symptoms are gone.

Patients who withdraw from the study, regardless of reason, will be replaced.

5.6.2 Trial Discontinuation

The whole trial may be discontinued at the discretion of the CI or the sponsor in the event of any of the following:

- Occurrence of AEs unknown to date in respect of their nature, severity and duration
- Medical or ethical reasons affecting the continued performance of the trial
- Difficulties in the recruitment of patients

The sponsor and coordinating investigator will inform all investigators and the Ethics Committees of the termination of the trial along with the reasons for such action. If the study is terminated early on grounds of safety the Ethics Committees will be informed within 15 days.

5.7 Laboratory Tests

The blood samples for determination of midazolam concentrations will be drawn in 0.5 mL BD Microtainer blood collection tubes (K2-EDTA), centrifuged immediately for 10 minutes at 4ºC (1800 g). Plasma is then transferred into cryovials and stored at -70°C within 1 hour from sampling until analyses.

Plasma concentrations of midazolam and the main metabolite (1-OH-midazolam) will be analyzed at the Department of Pharmacy, University of Oslo, with a validated UPLC-MS/MS method.

Clinical chemistry analyses will be performed at the Department of Medical Biochemistry, Oslo University Hospital, Rikshospitalet.

Whole blood (0.5 ml EDTA) will be drawn once during the study for determination of genotypes of relevant proteins invoved in drug metabolism and transport. These analyses will be performed at the Department of Medical Biochemistry, Oslo University Hospital, Rikshospitalet.

6 ASSESSMENTS

6.1 Assessment of CYP3A activity

Blood samples for determination of midazolam plasma concentrations will be collected 1-2 times daily during midazolam infusion and 0.25, 0.5, 1, 1.5, 2, 4 and 8 hours after ending the midazolam infusion and/or after change of the infusion rate. For patients receiving an additional po dose of midazolam, blood samples will be collected at times 0, 0.25, 0.5, 1, 1.5, 2, 4 and 8 hours after the administration, if possible. The total number of trial-related blood samples will not exceed 8 samples á 0.5 mL within 24 hours. The total amount of blood obtained during the investigational period will not exceed 3% of the total blood volume according to the recommandations from EMA.

6.2 Safety and Tolerability Assessments

Safety will be monitored by the assessments described below as well as the collection of AEs during the study period. Significant findings that are present prior to the signing of informed consent must be included in the relevant medical history/current medical condition page of the CRF. For details on AE collection and reporting, refer to Section 8.3 and 8.4.

Hematology and biochemistry assessments will be taken when considered clinically required.

Laboratory evaluation:

Local laboratory (and their respective reference ranges) will be used for the analysis of hematology and biochemistry specimens collected.

7 SAFETY MONITORING AND REPORTING

The investigator is responsible for the detection and documentation of events meeting the criteria and definition of an adverse event (AE) or serious adverse event (SAE). Each patient will be instructed to contact the investigator immediately should they manifest any signs or symptoms they perceive as serious.

The methods for collection of safety data are described below.

7.1 Definitions

7.1.1 Adverse Event (AE)

An AE is any untoward medical occurrence in a patient administered a pharmaceutical product and which does not necessarily have a causal relationship with this treatment.

An adverse event (AE) can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal (investigational) product, whether or not related to the medicinal (investigational) product.

The term AE is used to include both serious and non-serious AEs.

If an abnormal laboratory value/vital sign are associated with clinical signs and symptoms, the sign/symptom should be reported as an AE and the associated laboratory result/vital sign should be considered additional information that must be collected on the relevant CRF.

7.1.2 Serious Adverse Event (SAE)

Any untoward medical occurrence that at any dose:

- Results in death
- Is immediately life-threatening
- Requires in-patient hospitalization or prolongation of existing hospitalization
- Results in persistent or significant disability or incapacity
- Is a congenital abnormality or birth defect
- Is an important medical event that may jeopardize the subject or may require medical intervention to prevent one of the outcomes listed above.

Medical and scientific judgment is to be exercised in deciding on the seriousness of a case. Important medical events may not be immediately life-threatening or result in death or hospitalization, but may jeopardize the subject or may require intervention to prevent one of the listed outcomes in the definitions above. In such situations, or in doubtful cases, the case should be considered as serious. Hospitalization for administrative reason (for observation or social reasons) is allowed at the investigator's discretion and will not qualify as serious unless there is an associated adverse event warranting hospitalization.

A pre-planned hospitalization admission (ie, elective or scheduled surgery arranged prior to the start of treatment) for pre-existing condition is not considered to be a serious adverse event.

7.1.3 Suspected Unexpected Serious Adverse Reaction (SUSAR)

Adverse Reaction: all untoward and unintended responses to an investigational medicinal product related to any dose administered;

Unexpected Adverse Reaction: an adverse reaction, the nature or severity of which is not consistent with the applicable product information.

Suspected Unexpected Serious Adverse Reaction: SAE (see section 7.1.2) that is unexpected as defined in section 7.2 and possibly related to the investigational medicinal product(s).

7.2 Expected Adverse Events

The administered doses of midazolam will be therapeutic doses, or less. Based on clinical experience the risk of adverse events is low. Expected adverse events may occur according to the adverse events listed in the SmPC. Midazolam doses will be titrated gradually up and down to avoid oversedation, withdrawal reactions or other potential adverse events, and the participants will be carefully monitored at all time. Otherwise, no special precations needs to be considered in pediatric patients.

7.3 Time Period for Reporting AE and SAE

For each patient the standard time period for collecting and recording AE and SAEs will begin at the first investigational day and will continue for at least 7 days following the last dose of study treatment for each patient.

During the course of the study all AEs and SAEs will be proactively followed up for each patient; events should be followed up to resolution, unless the event is considered by the investigator to be unlikely to resolve due to the underlying disease. Every effort should be made to obtain a resolution for all events, even if the events continue after discontinuation/study completion.

7.4 Recording of Adverse Events

If the patient has experienced adverse event(s), the investigator will record the following information in the CRF:

- The nature of the event(s) will be described by the investigator in precise standard medical terminology (i.e. not necessarily the exact words used by the patient).

- The duration of the event will be described in terms of event onset date and event ended data.

- The intensity of the adverse event:

    - Mild: The AE is transient and easily tolerated by the subject
    - Moderate: The AE causes the subject discomfort and interrupts the subjects usual activities
    - Severe: The AE causes considerable interference with subjects usual activities and may be incapacitating of life-threatening

- The Causal relationship of the event to the study medication will be assessed as one of the following:

  Unrelated:

  There is not a temporal relationship to investigational product administration (too early, or late, or investigational product not taken), or there is a reasonable causal relationship between non-investigational product, concurrent disease, or circumstance and the AE.

  Unlikely:

  There is a temporal relationship to investigational product administration, but there is not a reasonable causal relationship between the investigational product and the AE.

  Possible:

  There is reasonable causal relationship between the investigational product and the AE. Dechallenge information is lacking or unclear.

  Probable:

There is a reasonable causal relationship between the investigational product and the AE. The event responds to dechallenge. Rechallenge is not required.

Definite:

There is a reasonable causal relationship between the investigational product and the AE.

- Action taken
- The outcome of the adverse event – whether the event is resolved or still ongoing.

It is important to distinguish between serious and severe AEs. Severity is a measure of intensity whereas seriousness is defined by the criteria in Section 7.1. An AE of severe intensity need not necessarily be considered serious. For example, nausea that persists for several hours may be considered severe nausea, but is not an SAE. On the other hand, a stroke that results in only a limited degree of disability may be considered a mild stroke, but would be an SAE.

7.5 Reporting Procedure

7.5.1 AEs and SAEs

All adverse events and serious adverse events that should be reported as defined in section 7.1.1 will be recorded in the patient's CRF. All SAE will be followed up until resolution.

SAEs will be reported by the Principal Investigator, Hasse Khiabani Zare at tel +47 90543878 or e-mail haskhi@ous-hf.no, within 24 hours after the site has gained knowledge of the SAE. Every SAE must be documented by the investigator on the SAE pages (to be found in in Trial Master File). The Serious Adverse Event Report Form must be completed, signed and sent to the sponsor. The initial report shall promptly be followed by detailed, written reports if necessary. The initial and follow-up reports shall identify the trial subjects by unique code numbers assigned to the latter.

The CI keep detailed records of all SAEs reported by the investigators and will perform an evaluation with respect to causality and expectedness. Based on, among other, SAE reports the sponsor will evaluate whether the risk/benefit ratio associated with study is changed.

7.5.2 SUSARs

SUSARs will be reported to the Ethics Committee according to national regulation. The following timelines should be followed:

The sponsor will ensure that all relevant information about suspected serious unexpected adverse reactions that are fatal or life-threatening is recorded and reported as soon as possible to the Ethics Committee in any case no later than seven (7) days after knowledge by the sponsor of such a case, and that relevant follow-up information is subsequently communicated within an additional eight (8) days.

All other suspected serious unexpected adverse reactions will be reported to the Ethics Committee concerned as soon as possible but within a maximum of fifteen (15) days of first knowledge by the sponsor.

SUSARs will be reported using the CIOMS form since “CyPed” is not connected to EudraVigilance.

7.5.3 Annual Safety Report

Once a year throughout the clinical trial, the sponsor will provide the Ethics committee with an annual safety update. The format will comply with national requirements.

7.5.4 Clinical Study Report

The adverse events and serious adverse events occurring during the study will be discussed in the safety evaluation part of the Clinical Study Report.

7.6 Procedures in Case of Emergency

The investigator is responsible for assuring that there are procedures and expertise available to cope with emergencies during the study. The study is not blinded and code breaking procedures are therefore unnecessary. The patients are closely monitored the entire investigational days at the Department of Pediatric medicine at Oslo University Hospital, Rikshospitalet.

8 DATA MANAGEMENT AND MONITORING

8.1 Case Report Forms (CRFs)

The designated investigator staff will enter the data required by the protocol into the Case report forms (CRF). The Coordinating Investigator is responsible for assuring that data entered into the CRF is complete, accurate, and that entry is performed in a timely manner. The signature of the investigator will attest the accuracy of the data on each CRF. If any assessments are omitted, the reason for such omissions will be noted on the CRFs. Corrections, with the reason for the corrections will also be recorded.

After database lock, the investigator will receive a CD-ROM or paper copies of the subject data for archiving at the investigational site.

8.2 Source Data

Source data are all information in original records and certified copies of original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source data are contained in source documents (original records or certified copies).

The medical records for each patient should contain information which is important for the patient's safety and continued care, and to fulfill the requirement that critical study data should be verifiable.

To achieve this, the medical records of each patient should clearly describe at least:

- That the patient is participating in the study, e.g. by including the enrollment number and the study code or other study identification;
- Date when Informed Consent was obtained from the patient and statement that patient received a copy of the signed and dated Informed Consent;
- Results of all assessments confirming a patient's eligibility for the study;
- Diseases (past and current; both the disease studied and others, as relevant);
- Surgical history, as relevant;
- Treatments withdrawn/withheld due to participation in the study;
- Results of assessments performed during the study;
- Treatments given, changes in treatments during the study and the time points for the changes;

- Non-Serious Adverse Events and Serious Adverse Events (if any) including causality assessments;
- Date of, and reason for, discontinuation from study treatment;
- Date of, and reason for, withdrawal from study;
- Date of death and cause of death, if available;
- Additional information according to local regulations and practice.

8.3 Study Monitoring

The investigator will be visited on a regular basis by the Clinical Study Monitor, who will check the following:

- Informed consent process
- Reporting of adverse events and all other safety data
- Adherence to protocol
- Maintenance of required regulatory documents
- Study Supply accountability
- Facilities and equipments (example: laboratory, pharmacy)
- Data completion on the CRFs including source data verification (SDV).

The monitor will review all signed patient informed concent and 20% of the CRFs for accuracy and completeness and will ask the site staff to adjust any discrepancies as required.

Sponsor's representatives (e.g. monitors, auditors) and/or competent authorities will be allowed access to source data for source data verification in which case a review of those parts of the hospital records relevant to the study will be required.

8.4 Confidentiality

The investigator shall arrange for the secure retention of the patient identification and the code list. Patient files shall be kept for the maximum period of time permitted by each hospital. The study documentation (CRFs, Site File etc) shall be retained and stored during the study and for 15 years after study closure. All information concerning the study will be stored in a safe place inaccessible to unauthorized personnel.

8.5 Database management

Initial data recording will be performed using a paper based CRF, except for laboratory values, which will be retrieved directly from digital medical records. Data will be entered locally at Oslo University Hospital, Rikshospitalet into a computer database at the Oslo University Hospital's scientific server (K:). To promote data quality, all the digital data will be compared to the source after initial database entering, either by another person and/or at separate day.

We will code our specimens, and individual charts with the patient's unique study identification number. The institution solution to handle the key linking a patient's identity to his or her study ID will be used (Medinsight database solution).

All study documentation will be stored electronically on the K://Sensitiv-server of the institution. This server has access restriction based on predefined users which cannot be changed without application to Personvernombudet. Upon study

completion the paper-based materials will be moved to a locked cabinet at the Department of Pharmacology, Oslo University Hospital, Rikshospitalet.

9 STATISTICAL METHODS AND DATA ANALYSIS

9.1 Determination of Sample Size

Since the present study primarily is a descriptive study, no strict sample size calculation can be done. Based on the number of patients hospitalized at the Pediatric Intensive Care Unit and receiving continuous midazolam infusion in 2018, we expect to include 130 patients. Based on these numbers, most patients hospitalized at the Pediatric Intensive Care Unit are <6 months or >5 years, and more patients will therefore be included in groups A and D.

Group A (0-6 months): 50
Group B (6 months-2 years): 15
Group C (2-5 years): 15
Group D (5-16 years): 50

We aim that at least 20% of the included patients in each age group also will be included in the subgroup receiving po midazolam to assure a good descriptive power of variability in intestinal (and hepatic) CYP3A activity.

9.2 Population for Analysis

The following populations will be considered for the analyses:

- Per-protocol population (PP): Includes all subjects who have completed the investigational day.
- Safety population: Includes all subjects who have received at least one dose of study probes. Subjects who withdraw from the study will be included in the safety analysis. A list of withdrawn subjects, preferably with the reasons for withdrawal, will be made.

9.3 Planned analyses

The main statistical analysis is planned when all patients have completed the investigational days, but subanalyses of group C and D will be allowed when these age groups are completed.

- The planned number of patients have been included
- All included patients have either finalized their last assessment or has/is withdrawn according to protocol procedures
- All data have been entered, verified and validated according to the data management plan

Deviation from the original statistical plan will be described and justified in the Clinical Study Report. Amendments to plan can be done until day of DB lock.

9.4 Determination of CYP3A activity

Hepatic CYP3A activity will be assessed by determining systemic clearance of midazolam. In the subgroup of patients receiving a po dose of midazolam, in addition to iv therapy, also the presystemic clearance will be determined to derive the intestinal and hepatic CYP3A activity separately. Non-parametric pharmacokinetic population modelling (Pmetrics, Laboratory of Applied Pharmacokinetics and Bioinformatics) will be utilized to determine systemic and presystemic clearance of midazolam.

To investigate any associations between change in systemic- and presystemic clearance and age, regression analyses will be performed. Individually predicted systemic and presystemic clearance from the pharmacokinetic population modelling will be used.

The potential impact of concomitant drugs on the CYP3A activity will be assessed by performing sensitivity analyses and these data will be separated from the main analysis. The criteria to indicate a relevant effect on CYP3A activity is if the addition of the concomitant drug as a covariate in the midazolam model result in a lower AIC (akaike information cirteria)- and RMSE (root mean squared error) values.

Measured values more than 2 standard deviations from the average value at the specific time point will be excluded from the analyses. All measured concentrations may be subjected to reanalysis based on suspicion of false concentrations by any reason. This is however only allowed before any pharmacokinetic analyses have been started.

Midazolam concentrations wil be included in the population pharmacokinetic analysis even if below the lower limit of determination as it is wheigthed by the analytical variance in the modelling procedure.

9.5 Statistical Analysis

All statistical tests will be performed applying a 5% significance level unless otherwise stated.

9.5.1 Descriptive statistics

Descriptive statistics will be presented as mean ± standard deviation or median (IQR/absolute range) for continuous values (e.g. age, weight) and as frequency counts and percentages for categorical data (e.g. gender).

10 STUDY MANAGEMENT

10.1 Investigator Delegation Procedure

The coordinating investigator is responsible for making and updating a “delegation of tasks” listing all the involved co-workers and their role in the project. She will ensure that appropriate training relevant to the study is given to all of these staff, and that any new information of relevance to the performance of this study is forwarded to the staff involved.

10.2 Protocol Adherence

Investigators ascertain they will apply due diligence to avoid protocol deviations.
All significant protocol deviations will be recorded and reported in the Clinical Study Report (CSR).

10.3 Study Amendments

If it is necessary for the study protocol to be amended, the amendment and/or a new version of the study protocol (Amended Protocol) must be notified to and approved by the Competent Authority and the Ethics Committee according to EU and national regulations.

10.4 Audit and Inspections

Authorized representatives of a Competent Authority and Ethics Committee may visit the centre to perform inspections, including source data verification. Likewise the representatives from sponsor may visit the center to perform an audit. The purpose of an audit or inspection is to systematically and independently examine all study-related activities and documents to determine whether these activities were conducted, and data were recorded, analyzed, and accurately reported according to the protocol, Good Clinical Practice (ICH GCP), and any applicable regulatory requirements. The

coordinating investigator will ensure that the inspectors and auditors will be provided with access to source data/documents.

11 ETHICAL AND REGULATORY REQUIREMENTS

The study will be conducted in accordance with ethical principles that have their origin in the Declaration of Helsinki and are consistent with ICH/Good Clinical Practice and applicable regulatory requirements. Registration of patient data will be carried out in accordance with national personal data laws.

11.1 Ethics Committee Approval

The study protocol, including the patient information and informed consent form to be used, must be approved by the regional ethics committee before enrollment of any patients into the study.

The investigator is responsible for informing the ethics committee of any serious and unexpected adverse events and/or major amendments to the protocol as per national requirements.

11.2 Other Regulatory Approvals

The protocol is not subjected to approval from other competent authorities in Norway before commencement of the study.

The protocol will be registered in www.clinicaltrials.gov before inclusion of the first patient.

11.3 Informed Consent Procedure

The investigator is responsible for giving the parents/legal representatives on behalf of the patient and, if appropriate, the patient, full and adequate verbal and written information about the nature, purpose, possible risk and benefit of the study. They will be informed as to the strict confidentiality of their patient data, but that their medical records may be reviewed for trial purposes by authorized individuals other than their treating physician.

It will be emphasized that the participation is voluntary and that the patient is allowed to refuse further participation in the protocol whenever she/he wants. This will not prejudice the patient's subsequent care. Documented informed consent must be obtained for all patients included in the study before they are registered in the study. This will be done in accordance with the national and local regulatory requirements. The investigator is responsible for obtaining signed informed consent.

A copy of the patient information and consent will be given to the patients. The signed and dated patient consent forms will be filed in the Investigator Site File binder and also scanned to be part of the patient's electronic medical record at the hospital.

11.4 Subject Identification

The investigator is responsible for keeping a list of all patients (who have received study treatment or undergone any study specific procedure) including patient's date of birth and personal number, full names and last known addresses.

The patients will be identified in the CRFs by patient number, initials and date of birth.

12 TRIAL SPONSORSHIP AND FINANCING

The study is financed by a good collaboration between the involved departments at the Oslo University Hospital and University of Oslo.

13 TRIAL INSURANCE

The patients are insured according to the Act of Product Responsibility ("Produktansvarsloven").

14 PUBLICATION POLICY

Upon study completion and finalization of the study report the results of this study will either be submitted for publication and/or posted in a publicly assessable database of clinical study results.

The results of this study will also be submitted to the Ethics Committee according to EU and national regulations.

All personnel who have contributed significantly with the planning and performance of the study (Vancouver convention 1988) may be included in the list of authors. Author position according to workload.

15 REFERENCES

1. Baber, N. and D. Pritchard, *Dose estimation for children.* Br J Clin Pharmacol, 2003. **56**(5): p. 489-93.
2. European Medicines Agency. *REGULATION (EC) No 1901/2006 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 12 December 2006 on medicinal products for paediatric use and amending Regulation (EEC) No 1768/92, Directive 2001/20/EC, Directive 2001/83/EC and Regulation (EC) No 726/2004* 2006 [cited 2019 16 May]; Available from: https://ec.europa.eu/health//sites/health/files/files/eudralex/vol-1/reg_2006_1901/reg_2006_1901_en.pdf.
3. Guengerich, F.P., *Cytochrome p450 and chemical toxicology.* Chem Res Toxicol, 2008. **21**(1): p. 70-83.
4. Guengerich, F.P., *Cytochrome P-450 3A4: regulation and role in drug metabolism.* Annu Rev Pharmacol Toxicol, 1999. **39**: p. 1-17.
5. Paine, M.F., et al., *The human intestinal cytochrome P450 "pie".* Drug Metab Dispos, 2006. **34**(5): p. 880-6.
6. Brussee, J.M., et al., *Characterization of Intestinal and Hepatic CYP3A-Mediated Metabolism of Midazolam in Children Using a Physiological Population Pharmacokinetic Modelling Approach.* Pharm Res, 2018. **35**(9): p. 182.
7. de Wildt, S.N., et al., *Cytochrome P450 3A: ontogeny and drug disposition.* Clin Pharmacokinet, 1999. **37**(6): p. 485-505.
8. Bjorkman, S., *Prediction of cytochrome p450-mediated hepatic drug clearance in neonates, infants and children : how accurate are available scaling methods?* Clin Pharmacokinet, 2006. **45**(1): p. 1-11.
9. de Wildt, S.N., S. Ito, and G. Koren, *Challenges for drug studies in children: CYP3A phenotyping as example.* Drug Discov Today, 2009. **14**(1-2): p. 6-15.
10. Chung, E., et al., *Comparison of midazolam and simvastatin as cytochrome P450 3A probes.* Clin Pharmacol Ther, 2006. **79**(4): p. 350-61.
11. 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.
12. 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.
13. Blumer, J.L., *Clinical pharmacology of midazolam in infants and children.* Clin Pharmacokinet, 1998. **35**(1): p. 37-47.
14. Gorski, J.C., et al., *Regioselective biotransformation of midazolam by members of the human cytochrome P450 3A (CYP3A) subfamily.* Biochem Pharmacol, 1994. **47**(9): p. 1643-53.
15. Takano, M., et al., *Interaction with P-glycoprotein and transport of erythromycin, midazolam and ketoconazole in Caco-2 cells.* Eur J Pharmacol, 1998. **358**(3): p. 289-94.
16. 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.
17. Paine, M.F., et al., *First-pass metabolism of midazolam by the human intestine.* Clin Pharmacol Ther, 1996. **60**(1): p. 14-24.
18. 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.
19. Altamimi, M.I., H. Sammons, and I. Choonara, *Inter-individual variation in midazolam clearance in children.* Arch Dis Child, 2015. **100**(1): p. 95-100.
20. Peeters, M.Y., et al., *Pharmacokinetics and pharmacodynamics of midazolam and metabolites in nonventilated infants after craniofacial surgery.* Anesthesiology, 2006. **105**(6): p. 1135-46.
21. Ince, I., et al., *Critical illness is a major determinant of midazolam clearance in children aged 1 month to 17 years.* Ther Drug Monit, 2012. **34**(4): p. 381-9.
22. Vet, N.J., et al., *Inflammation and Organ Failure Severely Affect Midazolam Clearance in Critically Ill Children.* Am J Respir Crit Care Med, 2016. **194**(1): p. 58-66.
23. Reed, M.D., et al., *The single-dose pharmacokinetics of midazolam and its primary metabolite in pediatric patients after oral and intravenous administration.* J Clin Pharmacol, 2001. **41**(12): p. 1359-69.
24. European Commission. *ETHICAL CONSIDERATIONS FOR CLINICAL TRIALS ON MEDICINAL PRODUCTS CONDUCTED WITH THE PAEDIATRIC POPULATION* 2008 [cited 2019 24 April]; Available from: https://ec.europa.eu/health/sites/health/files/files/eudralex/vol-10/ethical_considerations_en.pdf.
25. European Medicines Agency. *GUIDELINE ON THE INVESTIGATION OF MEDICINAL PRODUCTS IN THE TERM AND PRETERM NEONATE* 2007 [cited 2019 24 April]; Available from:

https://www.ema.europa.eu/en/documents/scientific-guideline/draft-guideline-investigation-medicinal-products-term-preterm-neonate_en.pdf.
26. Brussee, J.M., et al., *First-Pass CYP3A-Mediated Metabolism of Midazolam in the Gut Wall and Liver in Preterm Neonates.* CPT Pharmacometrics Syst Pharmacol, 2018. **7**(6): p. 374-383.
27. de Wildt, S.N., et al., *Population pharmacokinetics and metabolism of midazolam in pediatric intensive care patients.* Crit Care Med, 2003. **31**(7): p. 1952-8.