Perinatal asphyxia:

investigating novel neuroprotective agents
and optimising current pharmacotherapy

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Perinatal asphyxia: investigating novel neuroprotective agents and optimising current pharmacotherapy

Perinatale asfyxie: onderzoek naar nieuwe neuroprotectieve stoffen en het optimaliseren van de huidige farmacotherapie
(met een samenvatting in het Nederlands)

Proefschrift

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“Even an end, has a start.”

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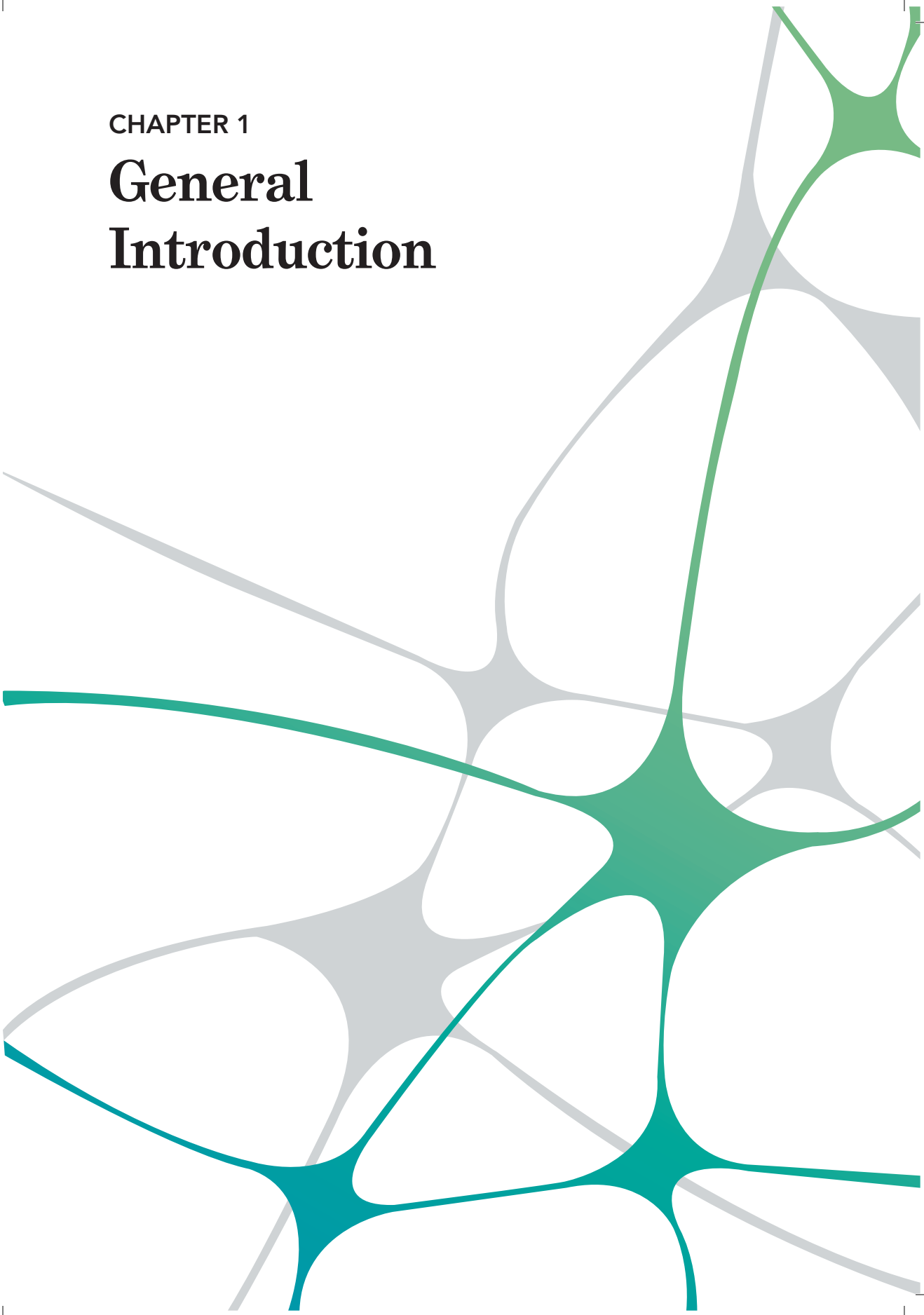
CHAPTER 1

General Introduction



Perinatal asphyxia

Perinatal asphyxia is a medical condition resulting from deprivation of oxygen around the time of birth. According to the World Health Organization (WHO), perinatal asphyxia is the fifth largest cause of death under the age of five. Worldwide, between 4 and 9 million neonates develop perinatal asphyxia each year. Of those, an estimated 1.2 million die and at least the same number develop severe complications such as epilepsy, cerebral palsy and developmental delay. The majority of these occur in developing countries. In countries with more advanced economies, the incidence of perinatal asphyxia is substantially lower due to high quality primary, obstetric and perinatal care. However, irrespective of a country's developmental status, perinatal asphyxia comes with a heavy long term health, social, and financial burden.(1–3)

The causes of perinatal asphyxia can be various. Perinatal infection, placental abruption, uterine rupture, umbilical cord entanglement, shoulder dystocia, maternal or fetal bleeding and breech presentation are amongst them, but often the reason remains unknown.(4) The diagnosis is based on a combination of clinical conditions of the neonate including a low Apgar score (<7), the need for resuscitation and/or the need for mechanical ventilation combined with laboratory values confirming the presence of a metabolic acidosis (cord blood pH, base deficit). Perinatal asphyxia can lead to widespread hypoxic-ischaemic damage to organ systems such as heart, lungs, liver and kidney, but central nervous system dysfunction is the biggest concern as the brain is least likely to have a quick or complete recovery.(5,6) In preterm neonates, brain damage predominantly manifests as diffuse white matter injury, whereas in term and near term neonates (gestational age (GA) of 36 weeks and higher) brain damage is characterised by hypoxic-ischaemic encephalopathy (HIE), also known as neonatal encephalopathy.(7,8) HIE can be defined by a (too) high Sarnat or Thompson score from one hour after birth or an abnormal aEEG.

(9–11) Upon diagnosis, neonates with moderate to severe HIE will be transferred to a neonatal intensive care unit for specialised treatment.(12–14)

Mechanisms of brain damage

Both clinical and experimental studies have shown that neuronal death occurs in two phases following the hypoxic-ischaemic event.(15) During the actual hypoxic-ischemic period, an excessive production of excitatory neurotransmitters promotes the activation of N-methyl D-aspartate and the opening of voltage-regulated ion channels, which results in an increase of intracellular calcium in neuronal cells. In addition to direct cell damage, activation of several pathways will lead to production of pro-radicals and accumulation of xanthine.(15) This first phase is called the primary energy failure.(16–18) The extent of this damage is directly related to the duration of the event and is often accompanied by clinical or subclinical seizures. After resuscitation and return of systemic circulation, a latent period follows during which cerebral energy metabolism initially returns to normal, but deteriorates between six and fifteen hours after the actual insult, initiating a second (delayed) phase of neuronal death.(19) This second phase is marked by cytotoxic oedema and mitochondrial failure.(14,20) During this phase, xanthine is metabolised to uric acid by xanthine oxidase, releasing large amounts of superoxide free radicals which play a key role in the formation of other harmful substances.(21–23) Reaction with non-protein bound iron and other pro-radicals results in the formation of hydroxyl free radical, the most aggressive free radical in nature known so far.(24) Furthermore, superoxide free radicals also react with nitric oxide to form peroxynitrate. Both hydroxyl free radicals and peroxynitrate are considered extremely toxic, setting a pre-apoptotic pathway in motion resulting in further (delayed) brain damage.(15,25)

This phase of secondary energy failure is associated with encephalopathy and increased seizure activity, and accounts for a significant proportion of the final

cell loss, which is further accentuated because neurotrophic growth factors are downregulated, inhibiting neuroregeneration.(18,26–28)

As the primary energy failure occurs around the time of birth, preventing cell damage during this first phase is extremely challenging. However, further damage caused by the secondary energy failure can be attenuated. The existence of the latent phase between the primary and secondary energy failures presents a therapeutic window of opportunity for interventions aimed at preventing brain damage and thereby reducing the incidence of death and long term disability.(15)

Management of perinatal asphyxia

Management of neonates with perinatal asphyxia starts with resuscitation in the delivery room. After stabilisation of the systemic circulation, the neonate is transferred to a neonatal intensive care unit (NICU) for optimal supportive care and treatment aimed to reduce the incidence of HIE-related death and disabilities such as cerebral palsy, hearing loss and neurodevelopmental delay in these neonates.

Therapeutic hypothermia

Currently, the only intervention with proven neuroprotective efficacy is therapeutic hypothermia (TH) in the early postnatal period. Since its introduction in the Netherlands and Belgium in 2008, the composite adverse outcome of death and disability as a result of HIE has been reduced from 60% to 45%. During whole body hypothermia, the neonates' core body temperature is reduced to 33.5 °C with a recommended range of 33-34 °C. This temperature is maintained for 72 hours, after which the neonate is gradually rewarmed to normothermia.(13) TH is indicated for neonates with a GA of at least 36.0 weeks suffering from HIE after perinatal asphyxia, and it is currently being explored in neonates with HIE and a GA of 35.0 weeks.(29) Currently in the Netherlands, between 100 and 150 neonates

yearly are treated with TH. In order to exhibit its neuroprotective properties, TH needs to be started as soon as possible, but preferably within six hours after birth.(14) The mechanism by which hypothermia provides neuroprotection has not been fully unraveled, but several possibilities have been described. Hypothermia may protect neurons by reducing the cerebral metabolic rate by attenuating the release of excitatory amino acids such as glutamate and dopamine, ameliorating the ischaemia-impaired uptake of glutamate and lowering production of toxic nitric oxide and free radicals.(14) Additionally, hypothermia reduces energy expenditure and reduces histological neuronal loss due to apoptosis. Although this intervention is not entirely without risks and complications such as sinus bradycardia, hypotension, thrombocytopenia and subcutaneous fat necrosis have been described, the benefits of cooling on the survival and neurodevelopment outweigh these short term adverse effects.(12–14)

Additional neuroprotection

Despite the success of TH, mortality in this population still resides around 30% while another 15% survives with serious disabilities, prompting clinicians and researchers worldwide with an incentive to explore novel neuroprotective interventions to further improve the outcomes of these neonates.(15,30) Although the majority of the interventions will be aimed at the secondary energy failure as postnatal administration is much more feasible than antenatal administration, some compounds may (also) be utilised to mitigate the primary energy failure. Allopurinol is a xanthine-oxidase inhibitor that is indicated for gout treatment in adults. Additionally, allopurinol can act as a free iron chelator and a scavenger of hydroxyl free radicals, especially when high dosages are used.(31) Its neuroprotective potential is highest when administered antenatally or, upon reoxygenation and reperfusion, in the (very) early postnatal period.(15) Melatonin, a hormone produced by the pineal gland to regulate the sleep-wake cycle, also exhibits anti-oxidative, anti-inflammatory and anti-apoptotic effects. Its neuroprotective properties can be

deployed in both the primary and secondary energy failure.(30,32) Erythropoietin and its recombinant derivative darbepoietin, and also mesenchymal stem cells exhibit anti-inflammatory properties in the early stage but most importantly have neurotrophic properties, promoting neurogenesis and repair in the later stage. (15,33–35)

One of the most promising targets for additional neuroprotection is the inhibition of nitric oxide synthase (NOS). NOS is an enzyme responsible for the production of nitric oxide and has three constitutions: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). eNOS is predominantly expressed in endothelial cells. Its foremost action is blood pressure control through vasodilatation, but it also has numerous other vasoprotective and anti-atherosclerotic effects. nNOS is constitutively expressed in central and peripheral neurons. Its function includes synaptic plasticity in the central nervous system and has been implicated in modulating physiological processes such as learning and memory formation. iNOS expression can be stimulated in virtually any cell or tissue in response to bacterial lipopolysaccharides, cytokines, and other agents. Once expressed, it generates large amounts of nitric oxide that have cytostatic effects on (parasitic) target cells. (36)

All three isoforms of nitric oxide synthase are upregulated in neonates suffering from HIE, although not simultaneously. Both eNOS and nNOS peak in the first few hours after birth while iNOS proliferates at a slower rate and is present from around twelve hours up until two days after birth.(37) While upregulation of eNOS following HIE is believed to be neuroprotective by maintaining pulmonary blood flow and ensuring adequate cerebral oxygenation, excessive activation of nNOS and iNOS is associated with deleterious effects on the brain. Compounds that selectively inhibit nNOS and iNOS whilst not affecting eNOS have a high potential for additional neonatal neuroprotection.(37–39)

A relatively new domain in this field is neuroprotection through inhalation of noble gases.(40) Xenon ventilation has been used in adults to induce anesthesia and sedation. Furthermore, it has been shown to antagonize the N-methyl D-aspartate receptor and to exhibit anti-apoptotic properties, marking its potential as a neuroprotective agent.(15,41) However, the first trial in neonates has shown disappointing results.(42) Major concerns with xenon ventilation in any setting are the high costs involved in the extraction of xenon from the earth's atmosphere. Moreover, in order to minimize the loss, application of xenon requires closed circuit ventilation with specialized and dedicated ventilators.(43,44) Argon, a much more abundant and therefore cheaper noble gas, also exhibits neuroprotective properties most likely via interaction with toll-like receptors although the exact mechanism has not yet been elucidated.(15,45) To date, argon ventilation has not been applied in humans.

Figure 1 summarises the destructive pathways caused by perinatal asphyxia as well as the potential neuroprotective strategies additional to TH.

Management of sedation and seizures

Adequate sedation is considered vital during treatment with TH. Like any other human being, a neonate will start to shiver when exposed to cold in order to maintain a body temperature of 37°C.(46) In an animal model for HIE, the neuroprotective effect of hypothermia was nullified in newborn piglets who were not sedated, due to the stress associated with shivering and the sensation of cold.(47) This effect has never been quantified in humans but for obvious ethical reasons, no clinical trials randomizing neonates between sedation and no sedation during TH have been performed. In the Netherlands, morphine is commonly prescribed conjoint with TH to provide analgesia and prevent stress, although other opioids

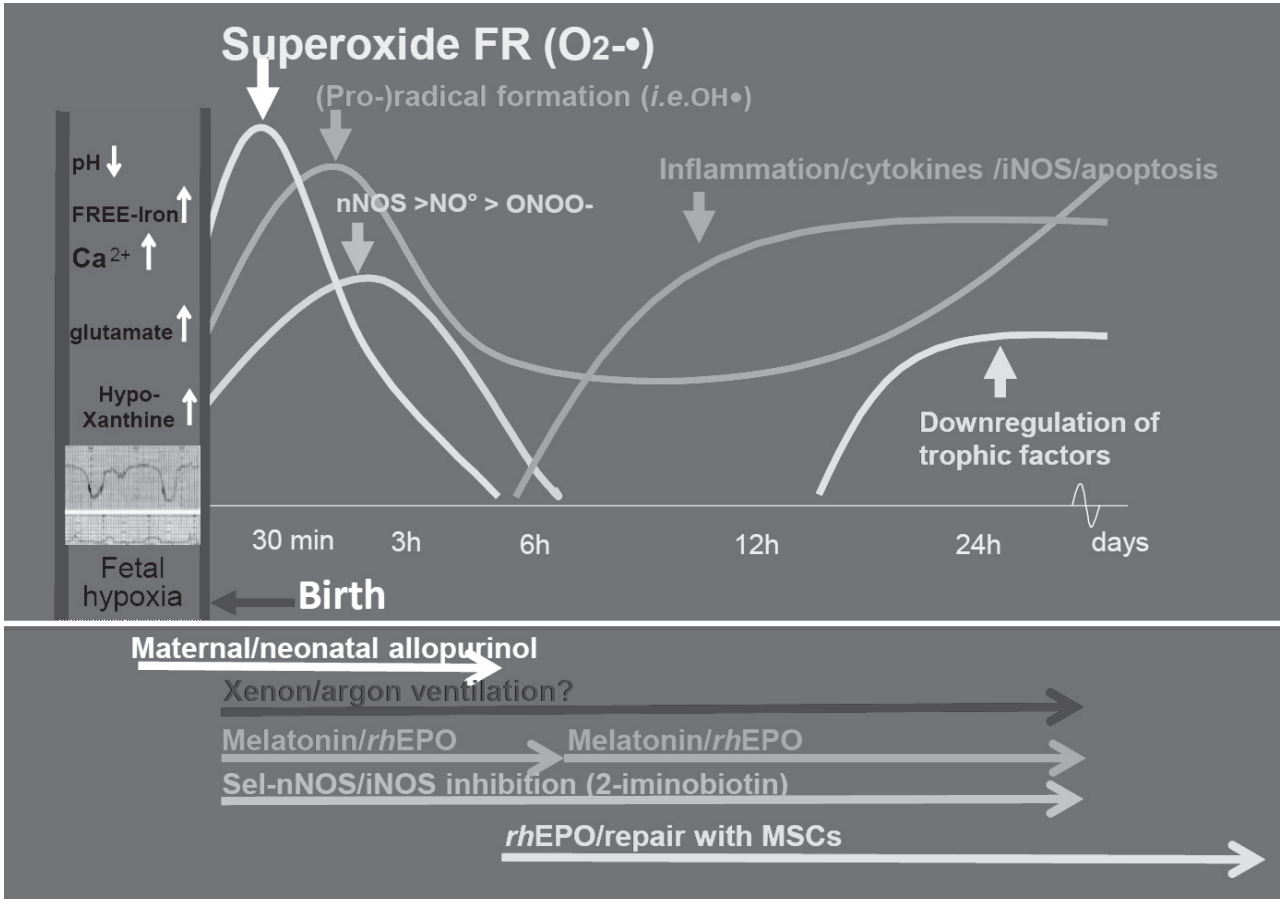


Figure 1: Time profile of the destructive pathways leading to neuronal damage following perinatal asphyxia (top panel) and the potential targets for neuroprotective therapy (bottom panel). Adapted from (15).

such as fentanyl can also be used.(48) When neonates require mechanical ventilation, morphine is often augmented with midazolam to ensure adequate sedation.(49) Neonatal opioid use has been associated with impaired cognitive and behavioural development in animals, and long term follow-up studies in humans suggest a possible negative effect in early childhood that does not persist later in life.(50–54) Even though successful management of both pain and discomfort is essential for neonatal recovery, it is wise to strive for the lowest possible effective opioid exposure to minimize any risk of short term and long term side effects.(55)

Seizure control is another key aspect in neonates with HIE, both during and after TH. Although part of the damage is primarily caused by the underlying pathology, it is generally accepted seizures themselves are also detrimental for the developing brain.(56) Therefore, treatment of both clinical and subclinical seizures is considered neuroprotective.(57,58)

Over the last few decades, an abundance of new anti-epileptic drugs (AEDs) with a wide variety of pharmacological targets have become available for the treatment of epilepsy in adults and older children.(59) In neonates, however, the positions of drugs of first, second and third choice are still reserved for older anticonvulsants with similar mechanisms of action.(60) Phenobarbital, midazolam, lidocaine and phenytoin have long-standing clinical experience but are also associated with both short term and long term adverse effects.(61) Short term side effects include respiratory depression and hypotension whilst prolonged use has been linked to long term cognitive and memory difficulties. Both phenobarbital and midazolam have been associated with direct neurotoxicity in animal models.(62,63)

Despite these concerns, the older anticonvulsants can rely on an extensive clinical experience. Another substantial advantage is the ability to administer these drugs intravenously, whereas the newer drugs are mostly limited to oral form. Until both

clinical efficacy and a lack of pro-apoptotic properties can firmly be established for a new drug such as levetiracetam, we must ensure optimal treatment of neonatal seizures with the older drugs.

Challenges in optimising pharmacotherapy

Several processes potentially affecting drug disposition are occurring simultaneously in neonates with HIE. Independent of HIE, functions responsible for drug metabolism and clearance mature at different rates over the first few days to weeks of life. For instance, glomerular filtration in neonates is impaired and reaches adults values in six to twelve months. Expression of phase I and phase II liver enzymes varies greatly in the neonatal period of life, with some already present within hours after birth whilst others proliferate much more slowly.(64,65) In addition, neonates with HIE have suffered a traumatic event around birth with varying degrees of multi-organ failure. After resuscitation and treatment at a neonatal intensive care unit, recovery of organ function can also play a part in changes in the pharmacokinetics (PK) and pharmacodynamics (PD) of drugs used in this population.(66,67)

Furthermore, alterations in (body) temperature can alter both PK and PD. A recent example of temperature dependent drug delivery in adults is hyperthermic intraperitoneal chemotherapy for the treatment of tumors in the abdominal cavity.(68,69) More often, however, hypothermia treatment is initiated on clinical indications with unknown effects on drugs used concomitantly. The effects of body temperature on drug disposition and drug effects are part of a domain known as thermopharmacology.(70) Over the past decade, neonatal thermopharmacology has focussed on the effects of TH on drugs frequently used in neonates with HIE such as antibiotics, sedatives and AEDs.(71–78)

TH has the potential to alter the PK of drugs in several ways. Absorption, distribution and protein and tissue binding capacity can be affected by alterations in body temperature. Most notably, and likely the most clinically relevant, is a potential effect on clearance.(70,79) TH decreases heart rate and cardiac output which will subsequently reduce perfusion of the liver and kidneys, organs responsible for drug clearance.(80,81) Hypothetically, compounds relying mostly on renal clearance will be affected in a similar matter. For hepatically cleared drugs, the largest impact can be expected on drugs with a high extraction ratio as clearance of these drugs predominantly depends on liver perfusion. The effect of TH on drugs with a low hepatic extraction ratio might be negligible. Additionally, hepatic clearance might also be affected by altered activity of liver enzymes responsible for phase I and phase II metabolism. Since most enzymatic processes exhibit temperature dependency, a lower body temperature might result in reduced enzyme activity and thus reduced clearance.(70) Upon rewarming, processes hampered by the hypothermic state can recover, possibly resulting in a swift surge in drug clearance.

All these factors will have to be taken into account when selecting the most appropriate dose of drugs necessary in neonates with HIE both during and after TH. To investigate the PK and PD of frequently used drugs in neonates with HIE during and after treatment with TH, the prospective multicentre cohort PharmaCool study (NTR2529) was designed and performed in all ten NICUs in the Netherlands and two in Belgium. Plasma concentrations of antibiotics, sedatives and AEDs were measured in blood samples collected on days 2-5 after birth.(48) PK of antibiotics has been investigated by a research group of the Amsterdam University Medical Center whereas the PK of sedatives and AEDs were investigated by a research group of the University Medical Center Utrecht and are part of this thesis.

Objectives of the thesis

The specific objectives of this thesis are:

- to explore promising novel neuroprotective agents in both an animal model for HIE (argon) as well as in neonates in combination with TH (2-iminobiotin);
- to optimise current pharmacotherapy during TH by assessing its influence on the PK of sedatives and AEDs (morphine, phenobarbital, midazolam and lidocaine) and to develop dosing guidelines for these drugs in neonates with HIE;
- to identify structural effects relating to the PK of all drugs in this population.

Outline of the thesis

In *chapter 2.1* the neuroprotective properties of several NOS inhibitors in animal models for hypoxic-ischaemic encephalopathy are being reviewed in order to select the most promising candidate to progress to human studies.

Chapter 2.2 describes the PK and short term safety of the selective iNOS and nNOS inhibitor 2-iminobiotin in addition to TH in asphyxiated neonates.

Chapter 2.3 reports on the safety of ventilation with the noble gas argon in asphyxiated newborn piglets with and without hypothermia treatment.

Chapter 3.1 describes the PK of morphine and its metabolites in neonates with HIE during and after TH and advises on a morphine starting dose in this population.

In *chapter 3.2* PK of phenobarbital and midazolam in neonates with HIE during and after TH are discussed including their drug-drug interaction and anti-epileptic effectiveness.

In *chapter 3.3* the use of lidocaine as an anticonvulsant in preterm neonates as well as normothermic and hypothermic term neonates with regards to PK, safety and evaluation of a previously developed dosing regimen is assessed.

In *chapter 4.1* clinical and pharmacological data from seven different drugs administered to neonates with HIE during and after TH are used to identify structural effects of body size, maturation, recovery of organ function and TH on PK in this population.

Chapter 5 discusses the findings of this thesis and the possible clinical implications. In addition, directions for future research are provided.

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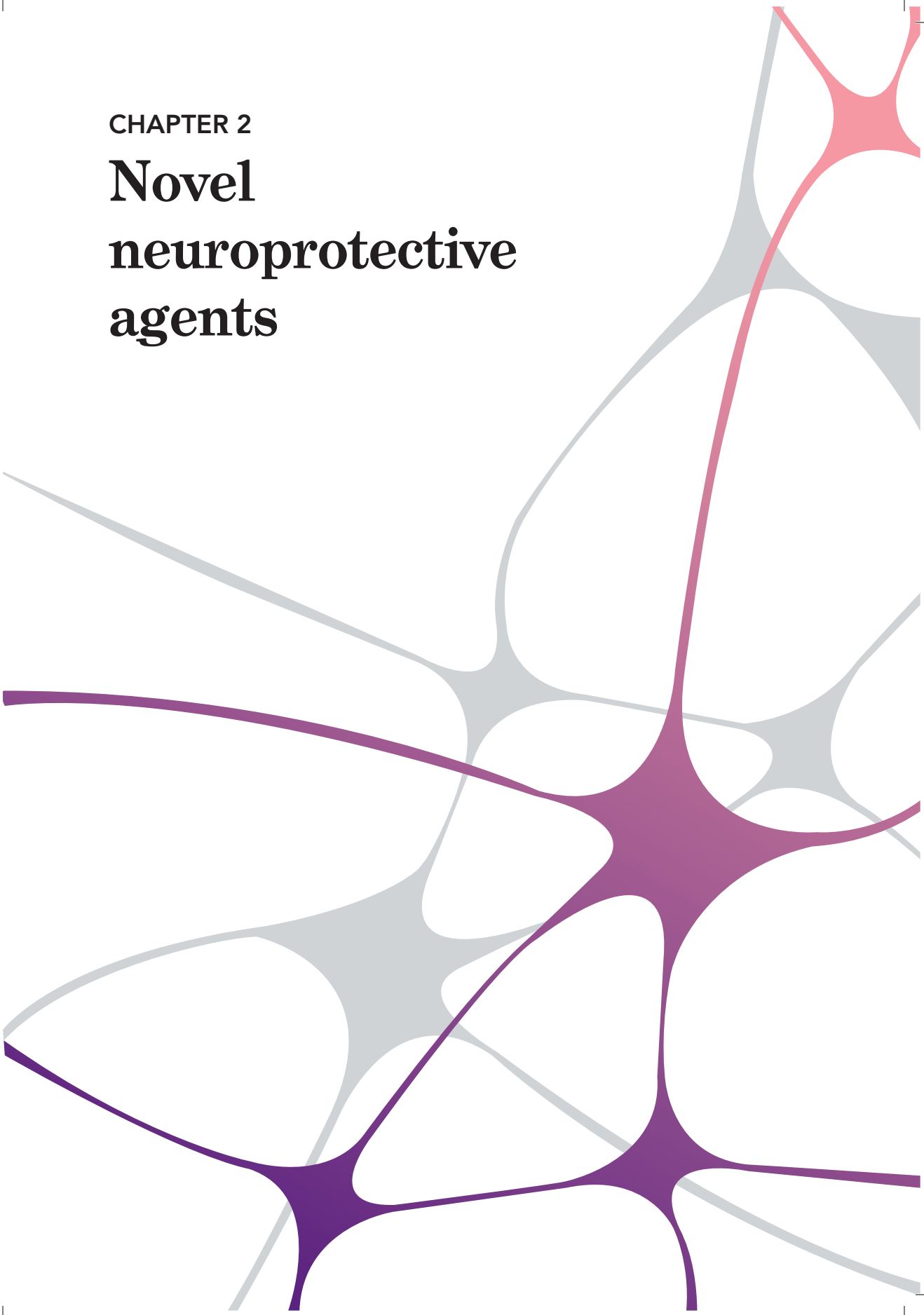
CHAPTER 2

Novel neuroprotective agents



Chapter 2.1

Nitric oxide synthase inhibition as a neuroprotective strategy following hypoxic-ischemic encephalopathy: evidence from animal studies

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Abstract

Background: Hypoxic-ischemic encephalopathy following perinatal asphyxia is a leading cause of neonatal death and disability worldwide. Treatment with therapeutic hypothermia reduced adverse outcome from 60% to 45%. Additional strategies are urgently needed to further improve the outcome of these neonates. Inhibition of nitric oxide synthase is a potential neuroprotective target. This article reviews the evidence of neuroprotection by nitric oxide synthesis inhibition in animal models.

Methods: Literature search using the EMBASE, Medline, Cochrane and PubMed databases. Studies comparing nitric oxide synthase inhibition to placebo with neuroprotective outcome measures in relevant animal models were included. Methodologic quality of the included studies was assessed.

Results: 26 studies were included using non-selective or selective nitric oxide synthase inhibition in rat, piglet, sheep or rabbit animal models. A large variety in outcome measures was reported. Outcome measures were grouped as either histological, biological or neurobehavioral. Both non-selective and selective inhibitors show neuroprotective properties in one or more outcome measures. Methodologic quality was either low or moderate for all studies.

Conclusion: Inhibition of nitric oxide synthesis is a promising strategy for additional neuroprotection. In humans, intervention can only take place after the onset of the hypoxic-ischemic event. Therefore, combined inhibition of neuronal and inducible nitric oxide synthase seems the most likely candidate for human clinical trials. Future studies should determine its safety and effectiveness in neonates as well as a potential sex specific neuroprotective effect. Researchers should strive to improve methodologic quality of animal intervention studies by using a systematic approach in conducting and reporting of these studies.

Introduction

Hypoxic-ischemic encephalopathy (HIE) following perinatal asphyxia (i.e. severe oxygen deprivation at birth) is one of the leading causes of neonatal death and adverse neuromotor outcome in term and near-term infants worldwide. In high-income countries, the incidence of HIE has been estimated between 0.5 and 1.0 for every thousand live births, although some sources have reported an incidence as high as 8 per 1000 live births.(1,2) In low and middle income countries, the incidence of HIE is higher, affecting more than 1.1 million babies annually.(3–5)

The overall burden of HIE is high in terms of quality-adjusted life years, years of life lost, and years lived with disability, not to mention a great financial cost for both society and the families involved.(6,7) With an estimated annual one million deaths worldwide, HIE is accountable for roughly 25% of all deaths in the neonatal period.(3,8)

Hypoxic-ischemic brain injury is not a single event evoked by the actual asphyxia but rather an ongoing process that leads to significant neuronal cell death over hours to days after the initial insult.(9,10) Several distinct phases have been identified in this process. The primary energy failure takes place during the hypoxic-ischemic event, resulting in failure of oxidative metabolism, cytotoxic edema and accumulation of excitotoxins.(11) After resuscitation and restoration of cerebral circulation, a latent phase, lasting approximately six hours, commences.(12,13) Subsequently, starting between 6-15 hours after asphyxia, the brain experiences a secondary energy failure that can last for days. This phase is marked by seizures, renewed cytotoxic edema, release of excitotoxins, impaired cerebral oxidative energy metabolism and finally, neuronal cell death.(14)

Currently, the only treatment that has proven to effectively reduce hypoxic-ischemic brain injury following perinatal asphyxia is the application of therapeutic hypothermia (TH). During TH the brain temperature is lowered to 33-34 °C which is maintained for 72 hours.(1) Since the introduction of TH, the combined adverse outcome of death and disability, such as hearing loss, cerebral palsy and other neuromotor disorders, has been reduced from approximately 60% to 45%.(15–17) TH has widely been implemented as standard of care treatment for moderate to severe HIE in high-income countries. However, TH needs to be started within six hours after birth, leaving clinicians with a narrow window for establishing the diagnosis and severity of HIE as well as transportation to a medical facility equipped for TH.(18) Additional neuroprotective strategies for HIE are urgently needed to augment TH but also as first line treatment option when hypothermia is not (yet) feasible.(3,4,19)

A potential target for (additional) neuroprotection in patients with HIE is inhibition of nitric oxide synthase (NOS, enzyme commission number 1.14.13.39). NOS is an enzyme catalyzing production of nitric oxide (NO) from L-arginine. After perinatal asphyxia, NO can react with the superoxide free radical to form toxic peroxynitrite, setting a pre-apoptotic pathway in motion resulting in neuronal loss.(10,20) Nitrotyrosine, an end product of this process, has been demonstrated post mortem in neonatal brain and spinal cord tissue after severe HIE.(21,22)

Three isoforms of NOS have been identified: endothelial (eNOS), neuronal (nNOS), and inducible NOS (iNOS).(23) All isoforms are upregulated after asphyxia; both nNOS and eNOS immediately after reperfusion and iNOS from several hours onwards.(24) While eNOS is regarded to be critical in maintaining pulmonary blood flow, preventing pulmonary hypertension and thereby maintaining adequate oxygenation of tissues throughout the body, excessive activation of nNOS and iNOS is associated with deleterious effects on the brain.(24,25) To illustrate, in mice genetically deficient of eNOS, infarct size after middle cerebral artery occlusion

(MCAO) is larger compared to wild type animals due to a reduction in regional cerebral blood flow.(26) In contrast, nNOS knockout mice are protected against hypoxic-ischemic brain injury while mice lacking iNOS showed a delayed reduction in brain injury.(27–32)

The aim of this study is to review the available evidence on NOS inhibition as a potential neuroprotective strategy in animal models translational for HIE and to identify one or more NOS inhibiting compounds that could evolve from preclinical to clinical studies in the near future.

Methods

Search strategy

Studies assessing the neuroprotective effects of NOS inhibitors in HIE-models were identified. A literature search using the EMBASE, Medline, Cochrane and PubMed databases was performed. The primary keywords were Animals (newborn), Hypoxia and Nitric Oxide Synthesis; the searches were limited to the English language. The complete search string is included in the Supplementary material. After exclusion of duplicates, titles and abstracts were screened independently by two researchers (LF and AC); final selection was made after full text evaluation. Any discrepancies were resolved by a third researcher (FG). Additionally, the reference lists of the retrieved articles were searched for additional studies.

Selection criteria

Studies were included based on the following inclusion criteria: animal models of a postnatal age in which brain development corresponds to near term or term brain development in humans, transient hypoxia or hypoxia-ischemia (HI), neuroprotection as outcome defined by histological, biochemical and/or neurobehavioral parameters

and inclusion of both a treatment group administering at least one NOS inhibitor and a control group that received sham treatment or consisted of untreated animals.

Data synthesis

The year of publication, name of first author, the class and type of NOS inhibitor, the animal model, the method used to achieve HI, the dose and number of animals in each treatment group, the type of control group and number of control animals, the timing of administration with regards to the HI insult and the results on the reported outcome parameters were recorded for each study. Each outcome parameter was categorized as either histological, biochemical or neurobehavioral.

Quality assessment

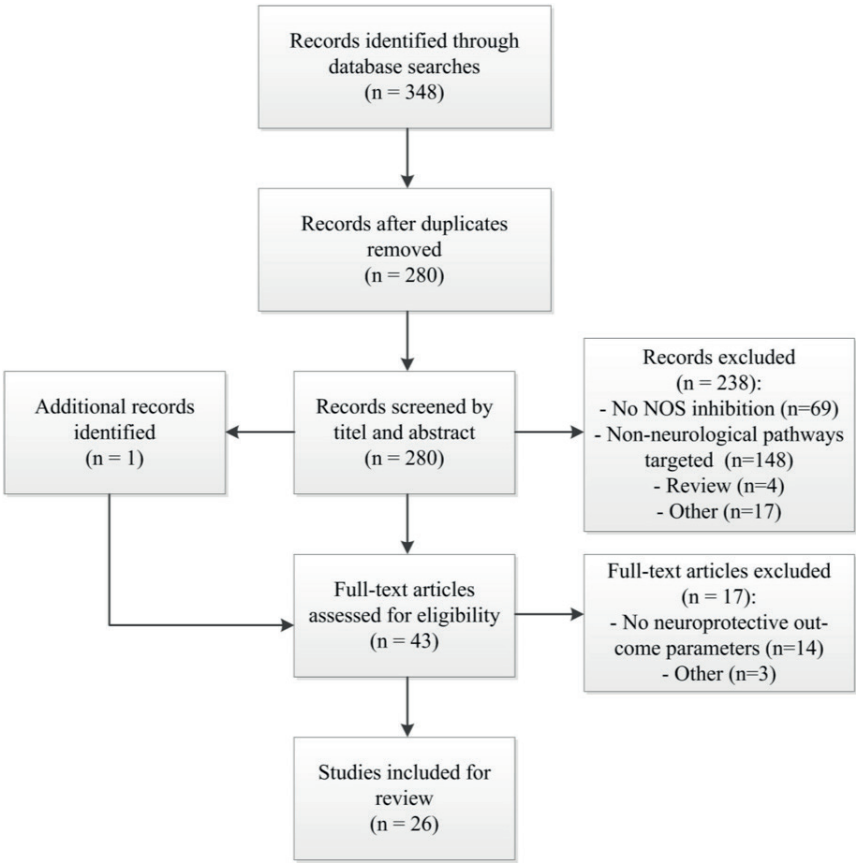
The methodological quality of the included articles was assessed using the SYRCLE's risk of bias (RoB) tool for animal intervention studies.(33) This tool is based on the Cochrane RoB tool and consists of ten items on which an article can be scored. Each item was scored zero, one or two points by two researchers (LF and AC) independently. This tool is based on the Cochrane RoB tool and consists of ten items on which an article can be assessed. Each item was scored zero, one or two points by two researchers (LF and AC) independently. If no evidence for adherence or evidence for non-adherence was found, a score of zero was awarded. When evidence for adherence was present but inconclusive, one point was scored. If the item was fully adhered to, two points were scored. Any discrepancies were resolved after consultation with a third researcher (AvdH). Because of the nature of the included studies and the timing of the interventions, 'allocation concealment' was deemed unfeasible and was not rated for any of the articles. Articles scoring 1-6 points were considered low quality, 7-12 points moderate quality and 13-18 points high quality. An example of the tool is included in the Supplementary material.

Results

Eligible studies

The search yielded a total of 348 studies; 280 studies after removal of duplicates. After screening of title and abstract, 238 articles were excluded. Screening of the reference lists identified one additional article. 43 articles were thus assessed in full detail. Of these, 26 were deemed eligible for inclusion (Figure 1); the data were extracted from these studies and these studies were assessed for methodological quality. Performing a meta-analysis was considered impossible because of the heterogeneity of the studies in outcome, administered NOS inhibitor and animal models.

Figure 1: study flow diagram; n = number of studies; NOS = nitric oxide synthase



Study characteristics

The included studies and their descriptive characteristics are summarized in Table 1. Eight studies (31%) tested a non-specific NOS inhibitor(34–41), another eight (31%) applied an nNOS specific inhibitor(42–49); three studies (12%) used an iNOS specific inhibitor(50–52) and six (23%) used an inhibitor of both nNOS and iNOS. (53–58) One study (3%) used separate groups for nNOS and iNOS inhibition.(59) Four different species of animals were used: rat (n=11, 42%), piglet (n=10, 38%), sheep (n=3, 12%) and rabbit (n=2, 8%).

Different models for HI were used, mostly dependent on the animal species. All rat studies applied the Vannucci-Rice model in P7-P14 pups. All newborn (P1-P5) piglet studies induced brain injury by hypoxia for 30-60 minutes, in 30% of studies combined with transient bilateral artery occlusion. In sheep aged 2-11 days (one study), hypoxia for 30 minutes was combined with hypotension for 5 minutes. Also two studies using sheep at 103-104 days gestation (term = 147 days) were included in which brain injury was induced by hypoxia due to occlusion of the umbilical cord for 25 minutes. In rabbits, fetuses (embryonic day 22, 70% gestation) were subjected to a HI event by uterine ischemia for 40 minutes.

The dosing regimen of the included studies is summarized in Table 2. Seventeen studies (65%, all non-specific or nNOS specific inhibitors) describe only a single administration and nine studies (35%, all iNOS of combined nNOS and iNOS inhibitors) described repeated dosing. With regards to timing of the intervention, twelve studies (46%) administered the (first) dose prior to the onset of the HI event; nine (35%) after the event and the remaining five (19%) incorporated groups with administration both before and after the event.

Table 1: study characteristics including risk of bias score. Studies are grouped by type of NOS inhibitor

Author, year	NOS-inhibitor class, type	Animal, age	HI method	Dose, no.	Control, no	Timing	Outcome		RoB L/M/H (score)
							Parameter H/B/N, NP yes/no	Result	
Ashraf (2002)[34]	Non-spec, NNLA	Piglet (unknown), 3-5 days	Hypoxia (FiO2 0.05-0.15) for 60 min	40 mg/kg, n=6	Untreated, n=9	Unknown time before insult	B, yes	Prevention of hypoxia induced upregulation of nitrated Bax protein vs untreated	L (5)
Blum-berg (1991)[35]	Non-spec, L-NAME	Rat (Wistar), 14 days	Right common artery ligation & hypoxia (FiO2 0.08) for 90 min	30 mg/kg, n=16	Vehicle, n=18	Directly after insult	H, no	No significant difference in size of infarction vs vehicle	M (8)
Groenen-daal (1999)[36]	Non-spec, NNLA	Piglet (Yorkshire), 1-3 days	Bilateral carotid artery occlusion & hypoxia (FiO2 0.07) for 60 min	40 mg/kg, n=5	Vehicle, n=5	60 min before insult	B, no	Worse cerebral energy status during and after HI vs vehicle (no change before HI)	L (6)
Nu-na-gami (1997)[37]	Non-spec, NNLA	Piglet (unknown), 2-4 days	Hypoxia (FiO2 0.07) for 60 min	40 mg/kg, n=5	Vehicle, n=5	60 min before insult	B, yes	Significant decrease in free radical formation of 65% vs vehicle; Preservation of Na-K-ATPase activity vs vehicle; Significant reduction in lipid peroxidation vs vehicle	M (7)
Zubrow (2002)[38]	Non-spec, NNLA	Piglet (Yorkshire), 2-4 days	Hypoxia (FiO2 0.07-0.09) for 60 min	40 mg/kg, n=7	Vehicle, n=6	60 min before insult	B, yes	significant decrease in amount of Bax protein and DNA fragmentation vs vehicle	L (6)
Hamada (1992)[39]	Non-spec, NNLA	Rat (Wistar), 7 days	Left carotid artery ligation & hypoxia (FiO2 0.08) for 150 min	2 mg/kg, n=12	Vehicle, n=12	90 min before insult	H, yes	reduction in cortical and striatal lesions vs vehicle	M (7)
				2 mg/kg, n=12	Vehicle, n=12	Directly after insult	H, no	no reduction in cortical and striatal lesions vs vehicle	
Trifiletti (1994)[40]	Non-spec, NNLA	Rat (Sprague–Dawley), 7 days	Left carotid artery ligation & hypoxia (FiO2 0.08) for unknown duration	50 mg/kg, n=6	Vehicle, n=6	15 h before insult	H, yes	89% reduction in ipsilateral/ contralateral weight ratio disparity vs vehicle	L (3)
				100 mg/kg, n=4			H, no	100% reduction in ipsilateral/ contralateral weight ratio disparity vs vehicle	

Author, year	NOS-inhibitor class, type	Animal, age	HI method	Dose, no.	Control, no	Timing	Outcome		RoB L/M/H (score)
							Parameter H/B/N, NP yes/no	Result	
Dorre-paal (1997)[41]	Non-spec, NNLA	Sheep (Rom-ney/ Suffolk), 2-11 days	Hypoxia (FiO2 0.06-0.08) for 30 min followed by MABP <35 mmHG for 5 min	10 mg/kg, n=6	Vehicle, n=6	Directly after insult	H, yes	Non-significant lower brain-body mass ratio vs vehicle; Non-significant decrease in necrotic purkinje cells vs vehicle	M (8)
							B, yes	Significant increase in cerebral metabolic oxygen rate vs vehicle; Significant recovery of electrocordial brain activity to baseline vs vehicle;	
				40 mg/kg, n=6			H, yes	Significant lower brain-body mass ratio vs vehicle; Non-significant decrease in necrotic purkinje cells vs vehicle	
							B, yes	Significant increase in cerebral metabolic oxygen rate vs vehicle	
							B, no	No change in recovery of electrocordial brain activity to baseline vs vehicle	
Ashraf (2004)[42]	nNOS, 7-NI	Piglet (York-shire), 2-4 days	Hypoxia (FiO2 0.05-0.15) for 60 min	Unknown, n=6	Untreated, n=5	Unknown time before insult	B, yes	Prevention of hypoxia induced decrease in protein tyrosine phosphatases activity vs untreated	L (5)
Drury (2013)[43]	nNOS, JI-10	Sheep (Rom-ney/ Suffolk), GA 103-104 (term = 147 days)	Complete umbil-ical cord occlu-sion for 25 min	0.044, n=8	Vehicle, n=8	15 min be-fore insult	H, yes	Partial neuronal and white matter protection after 7 days recovery vs vehicle	M (10)
							B, yes	Delay in the onset of seizures on EEG vs vehicle	
Drury (2014)[44]	nNOS, JI-10	Sheep (Rom-ney/ Suffolk), GA 103-104 (term = 147 days)	Complete umbil-ical cord occlu-sion for 25 min	0.022 mg/kg, n=8	Vehicle, n=8	30 min be-fore insult	H, yes	Significant reduction in loss of striatal phenotypic neurons vs vehicle	M (10)
Mishra (2006)[45]	nNOS, Ji-10	Piglet (York-shire), 3-5 days	Hypoxia (FiO2 0.06) for 60 min	1 mg/kg, n=5	Untreated, n=5	Directly after insult	B, yes	Decreased expression of Bax protein and DNA fragmentation vs untreated	L (6)

Author, year	NOS-inhibitor class, type	Animal, age	HI method	Dose, no.	Control, no	Timing	Outcome		RoB L/M/H (score)
							Parameter H/B/N, NP yes/no	Result	
Parikh (2003)[46]	nNOS, 7-NI	Piglet (unknown), 3-5 days	Hypoxia (FiO2 0.05-0.07) for 60 min	1 mg/kg, n=6	Untreated, n=5	Directly before insult	B, yes	Less caspase-3 activity and less DNA fragmentation vs untreated	L (6)
Ishida (2001)[47]	nNOS, 7-NI	Rat (CD), 7 days	Right common artery ligation & hypoxia (FiO2 0.08) for 120 min	50 mg/kg, n=?	Vehicle, n=?	30 min before insult	H, no	No neuroprotection vs vehicle	L (5)
				100 mg/kg, n=?		30 min before insult	H, yes	Significant reduction in the difference between the ipsilateral and contralateral cerebral hemisphere wet weights vs vehicle	
				50 mg/kg, n=?		15 min after insult	H, yes	No neuroprotection vs vehicle	
				100 mg/kg, n=?		15 min after insult	H, yes	No neuroprotection vs vehicle	
Ji (2009)[48]	nNOS, C5 or C6	Rabbit (New-Zealand White), embryonic day 22 (70% gestation)	Uterine ischeamia for 40 min	Unknown, n=?	Vehicle, n=?	30 min before insult	N, yes	Less fetal/neonatal deaths vs vehicle; Less neurobehavioural abnormalities vs vehicle; More normal kits at P1 vs vehicle	L (5)
	nNOS, C6			Unknown, n=?		30 min after insult	N, no	No difference in fetal/neonatal deaths vs vehicle; No difference in neurobehavioural abnormalities vs vehicle; No difference in normal kits at P1 vs vehicle	
Yu (2011)[49]	nNOS, 7-NI	Rabbit (New-Zealand White), embryonic day 22 (70% gestation)	Uterine ischeamia for 40 min	0.1575 umol/kg, n=?	Vehicle, n=?	30 min before insult	N, yes	Decrease in number of deaths vs vehicle; Significantly improved righting reflex vs vehicle	M (7)
	nNOS, JI-8			0.1575 umol/kg, n=?			N, yes	Significant increase in normal appearing kits vs vehicle; Significant decrease in severely affected and dead kits vs vehicle; Significantly improved smell, muscle tone and righting reflex vs vehicle; Overall better outcome vs 7-NI	

Author, year	NOS-inhibitor class, type	Animal, age	HI method	Dose, no.	Control, no	Timing	Outcome		RoB L/M/H (score)
							Parameter H/B/N, NP yes/no	Result	
Ikeno (2000)[50]	iNOS, S-MI	Rat (Wistar), 7 days	Right carotid artery ligation & hypoxia (FiO2 0.08) for 90 min	10 mg/kg, n=?	Vehicle, n=?	Directly before insult, repeated at 12, 24, 36 and 48 h	H, yes	Significantly decreased damage to the cerebral cortex vs vehicle	L (5)
Tsuji (2000)[51]	iNOS, AG	Rat (Wistar), 7 days	Left carotid artery ligation & hypoxia (Fio2 0.08) for 150 min	300 mg/kg, n=29	Vehicle, n=24	60 min before insult, repeated every 8 h, nine doses in total	H, yes	Significant reduction in cortical infarct volume of 89% vs vehicle; Significant reduction in striatal infarct volume of 90% vs vehicle	M (8)
Tutak (2005)[52]	iNOS, AG	Rat (Wistar), 7 days	Left carotid artery ligation & hypoxia for 150 min	300 mg/kg, n=18	Vehicle, n=18, 30 min after insult, repeated every 12h	30 min after insult, repeated every 12 h, six doses in total	H, no	No reduction in mean infarcted area vs vehicle	M (12)
	iNOS, IMC			0.2 mg/kg, n=20		30 min after insult, repeated every 8 h, nine doses in total	H, no	No reduction in mean infarcted area vs vehicle	
	iNOS, AG & IMC			300 mg/kg & 0.2 mg/kg, n=18		AG: 60m before insult; IMC: 30 m after insult, repeated every 8 h, nine doses in total	H, yes	Significant reduction in mean infarcted area vs vehicle	

Author, year	NOS-inhibitor class, type	Animal, age	HI method	Dose, no.	Control, no	Timing	Outcome		RoB L/M/H (score)
							Parameter H/B/N, NP yes/no	Result	
Peeters-Scholte (2002)[53]	nNOS & iNOS, 2-IB	Piglet (Dutch Store) 1-3 days	Bilateral carotid artery occlusion & hypoxia for 60 min	0.2 mg/kg, n=11	Vehicle, n=12	Directly after insult, repeated every 60 min, six doses in total	H, yes	90% reduction of vascular edema vs vehicle; 60-80% increase in normal neuronal cells vs vehicle	M (7)
							B, yes	90% improvement of cerebral energy state vs vehicle; Reduction of caspase-3 activity by 93% in cortex and 71% in striatum vs vehicle	
Peeters-Scholte (2002)[54]	nNOS & iNOS, 2-IB	Piglet (Dutch Store) 1-3 days	Bilateral carotid artery occlusion & hypoxia for 60 min	0.2 mg/kg, n=11	Vehicle, n=12	Directly after insult, repeated every 60 min, six doses in total	B, yes	Preservation of endogenous IGF-1 production vs vehicle; Reduction of caspase-3 activity vs vehicle;	L (4)
							B, no	No significant decrease in cytokine production vs vehicle	
Nijboer (2007)[55]	nNOS & iNOS, 2-IB	Rat (Wistar), 7 days	Right carotid artery ligation & hypoxia (FiO2 0.08) for 120 min	10 mg/kg, n=?	Vehicle, n=?	Directly after insult, repeated at 12 and 24 h	H, yes	Significantly higher ipsilateral/contralateral hippocampus area ratio vs vehicle in females only; Significant reduction in cortical and hippocampal lesions vs vehicle in females only;	M (11)
							B, yes	Significant reduction in cytochrome c release vs vehicle in females only; Decrease in caspase-3 activity vs vehicle in females only; No effect on nuclear translocation of apoptosis-inducing factor vs vehicle in both genders;	
							N, yes	Less deaths in female pups compared to male pups	

Author, year	NOS-inhibitor class, type	Animal, age	HI method	Dose, no.	Control, no	Timing	Outcome		RoB L/M/H (score)
							Parameter H/B/N, NP yes/no	Result	
Van der Tweel (2002)[56]	nNOS & iNOS, 7-NI & AG	Rat (Sprague–Dawley), 12 days	Right carotid artery ligation & hypoxia (FiO2 0.08) for 90 min	50 mg/kg & 100 mg/kg, n=24	Vehicle, n=24	Directly after insult, AG repeated every 12 h, four doses in total	H, yes	Significant reduction in brain damage to the ipsilateral hemisphere vs vehicle	M (12)
							B, no	No difference in HSP70 or cytokine mRNA expression vs vehicle	
Van der Tweel (2005)[57]	nNOS & iNOS, 2-IB	Rat (Wistar), 12 days	Right carotid artery ligation & hypoxia (FiO2 0.08) for 90 min	5.5 mg/kg, n=11	Vehicle, n=24	Directly after insult, repeated at 12 and 24 h	H, no	No difference in hippocampus and cortex neuropathology score vs vehicle; No difference in ipsilateral/contralateral hemisphere area ratio vs vehicle	M (8)
				10 mg/kg, n=10			H, yes	Significantly higher hippocampus and cortex neuropathology score vs vehicle;	
				30 mg/kg, n=20			H, no	No difference in ipsilateral/contralateral hemisphere area ratio vs vehicle	
							B, yes	Significantly higher hippocampus and cortex neuropathology score vs vehicle; Significantly higher ipsilateral/contralateral hemisphere area ratio vs vehicle;	
							B, yes	Significantly lower ipsilateral HSP70 level vs vehicle;	
				60 mg/kg, n=10			H, no	No difference in nitrotyrosine levels vs vehicle	

Author, year	NOS-inhibitor class, type	Animal, age	HI method	Dose, no.	Control, no	Timing	Outcome		RoB L/M/H (score)
							Parameter H/B/N, NP yes/no	Result	
Bjork-man (2013)[58]	nNOS & iNOS, 2-IB	Piglet (York-shire), new-born	Hypoxia (FiO2 0.02-0.04) for 30 min	0.1 mg/ kg, n=7	Vehicle, n=10	Directly after insult, repeated every 60 min, six doses in total	H, yes	Decreased nitration in thalamus, pa-rietal and temporal cortex vs vehicle	M (11)
							H, no	No difference in neuronal injury his-tology score	
							B, no	No difference in electrographical seizure activity at 48h vs vehicle; No difference in Caspase-3 activity vs vehicle	
							N, yes	Increased survival with normal EEG at 48h vs vehicle	
							N, no	No difference in neurobehavioural scores at 48h vs vehicle	
				0.2 mg/ kg, n=9			H, yes	Decreased nitration in thalamus, pa-rietal and temporal cortex vs vehicle	
							H, no	No difference in neuronal injury his-tology score	
							B, yes	Lower electrographical seizure activity at 48h vs vehicle	
							B, no	No difference in Caspase-3 activity vs vehicle	
							N, yes	Increased survival with normal EEG at 48h vs vehicle	
							N, no	No difference in neurobehavioural scores at 48h vs vehicle	
				1.0 mg/ kg, n=5			H, yes	Decreased nitration in thalamus, pa-rietal and temporal cortex vs vehicle	
							H, no	No difference in neuronal injury his-tology score	
							B, yes	Lower electrographical seizure activi-ty at 48h vs vehicle	
							B, no	No difference in caspase-3 activity vs vehicle	
							N, yes	Increased survival with normal EEG at 48h vs vehicle	
							N, no	No difference in neurobehavioural scores at 48h vs vehicle	

Author, year	NOS-inhibitor class, type	Animal, age	HI method	Dose, no.	Control, no	Timing	Outcome		RoB L/M/H (score)
							Parameter H/B/N, NP yes/no	Result	
Hsu (2014)[59]	nNOS, 7-NI	Rat (Sprague–Dawley), 7 days	Right carotid artery ligation & hypoxia (FiO2 0.08) for 120 min	75 mg/kg, n=?	Vehicle, n=?	30 min before insult	H, yes	Higher ipsilateral/contralateral cortical area ratio vs vehicle and AG; Significant increases in vascular density and decreases of BBB damage and microglia activation vs vehicle; Decrease in microvascular nitrosative stress vs vehicle and AG	M (7)
							B, yes	Increased cerebral perfusion vs vehicle and AG	
						3 h after insult	H, no	No difference in ipsilateral/ contralateral cortical area ratio vs vehicle	
	iNOS, AG			300 mg/kg, n=?		30 min before insult	H, yes	Higher ipsilateral/contralateral cortical area ratio vs vehicle; Significant increases in vascular density and decreases of BBB damage and microglia activation vs vehicle	
							H, no	No change in microvascular nitrosative stress vs vehicle	
							B, no	No change in cerebral perfusion vs vehicle	
						3 h after insult	H, yes	Higher ipsilateral/contralateral cortical area ratio vs vehicle and 7-NI	

non-spec = non-specific; NNLA = N-nitro-I-arginine; L-NAME = N-nitro-L-arginine methyl ester; nNOS = neuronal nitric oxide synthase; 7-NI = 7-nitro indazole; iNOS = inducible nitric oxide synthase; S-MI = S-methyl-isothiourea; AG = aminoguanidine; IMC = indomethacin; 2-IB = 2-iminobiotin; GA = gestational age; FiO2 = Fraction of inspired oxygen; MABP = mean arterial blood pressure; H = histological; B = biochemical; N = neurobehavioral; HI = hypoxia-ischemia; HSP70 = heat shock protein 70; BBB = blood brain barrier; RoB = risk of bias; L = low; M = moderate

Table 2: Dosing frequency and timing of intervention for the included studies

Timing of first dose to HI event	Dosing frequency	Type of inhibitor				Total no of studies	
		Non-specific	nNOS	iNOS	nNOS + iNOS		
Prior	Single	5	5	-	-	10	12
	Repeated	-	-	2	-	2	
Post	Single	2	1	-	-	3	9
	Repeated	-	-	-	6	6	
Both	Single	1	3*	1*	-	4*	5*
	Repeated	-	-	1	-	1	
Total		8	9	4	6	26	

*One study tested both an nNOS and iNOS inhibitior in separate groups

non-spec = non-specific; nNOS = neuronal nitric oxide synthase; iNOS = inducible nitric oxide synthase

Outcome

The results of the reported outcome parameters for each study are presented in Table 1. A wide variety of histological, biochemical and neurobehavioral outcome parameters were reported. Histological parameters included ipsilateral/contralateral weight ratio disparity and analysis of cortical and striatal lesions. Biochemical parameters included free radical formation and other biomarkers for neurological damage, but also cerebral energy status and electrocortical brain activity. Neurobehavioral parameters included overall survival, survival with normal EEG and results of neurobehavioral tests.

In the group of non-specific NOS inhibitors, administration prior to onset of the insult proved neuroprotective in 7/8 settings (88%), while administration directly after the insult was partially beneficial in 2/3 settings (67%).
For nNOS inhibitors, administration before the insult showed neuroprotective properties in 9/10 settings (90%) and when administered directly after the insult (1/1). When administration was delayed by 15 minutes of more, neuroprotective properties were lost (4/4).
When treatment with an iNOS inhibitor was started prior to the insult, neuroprotection was achieved (4/4). Administering the first dose after induction of HI showed neuroprotection in 33% of the settings (1/3). Hsu et al. administered the iNOS inhibitor aminoguanidine (AG) 30 min before and 3 h after the insult as a single dose. Both were neuroprotective compared to the control group, although less parameters were tested in the post insult treatment group.
All studies testing combined inhibition of nNOS and iNOS reported (partially) neuroprotective outcome. Van der Tweel et al. (2005) showed that 2-iminobiotin (2-IB) is neuroprotective in rats in a dose-dependent matter.

A direct comparison between two different inhibitors was made in two studies. Yu at al. reported superior neuroprotection of the novel nNOS inhibitor JI-8 compared to

7-nitro-indazole (7-NI) when administered prior to the insult in equimolar doses. Hsu et al. observed that both 7-NI and AG are neuroprotective when administered 30 min before HI and that7-NI is superior to AG in this setting. When the compounds were administered 3 h after HI, 7-NI lost its neuroprotective effect while AG remained neuroprotective compared to both vehicle and 7-NI.

Methodological quality

Eleven studies (42%) were ranked low quality, fifteen (58%) were considered moderate quality; none of the studies were ranked in the high quality group.
On average, RoB score was 7 (3-12). Overall, animal baseline characteristics, randomization for treatment allocation, blinding of investigators and/or outcome assessors and random selection for outcome assessment were often not mentioned and therefore scored zero.

Discussion

This systematic review shows that both selective and non-selective NOS inhibitors have neuroprotective qualities in various animal models of HI brain damage using histological, biochemical and neurobehavioral outcome parameters.
In animal studies, induction of the insult and administration of the potentially neuroprotective agent (before and/or after the insult) can be timed precisely. In contrast, this is not the case in clinical practice. The onset of perinatal asphyxia is often sudden and unpredictable. Therefore, administration of any drug before the onset of the insult is impossible and administration directly after the insult (i.e. directly after birth) highly improbable. All non-selective NOS inhibitors reviewed in this paper were administered prior to insult or directly after; there is no data on delayed administration. For selective nNOS inhibitors, neuroprotection was lost when administration was withheld by as little as 15 minutes. For selective iNOS inhibitors, administration before the insult shows greater neuroprotective

potential than treatment post-insult. The combination of nNOS and iNOS inhibition shows neuroprotective properties when administered after the insult in a repeated dosing regimen. Thus, combined nNOS/iNOS inhibition with a repeated dosing regimen seems the most potent strategy to advance into human clinical trials. In fact, several phase II studies with 2-IB are currently underway, in addition to TH (NTR5221) as well as without TH in low-income countries (NCT01626924, EudraCT2015-003063-12).

Important differences exist between the adult and the neonatal brain with regard to susceptibility to injury, plasticity and cell death pathways. Therefore, adult animal models are not suitable to examine neuroprotective interventions for HIE. Across species, key brain maturation events regarding susceptibility and regenerative capacities have been identified at different moments before and after birth and are related to the developmental stage of the human neonatal brain.(60–62) It is generally accepted that rats at postnatal days 7-14 (P7-14) are comparable to near term/term human neonates with regards to cerebral cortex development.(63,64) The Vannucci-Rice model of unilateral common carotid artery ligation followed by a period of systemic hypoxia results in apoptotic-necrotic cell degeneration in P7-14 rats, similar to HIE.(64–67) In term piglets aged 1-5 days, hypoxia leads to basal ganglia and somatosensory cortical injury largely comparable to damage seen in human neonates after perinatal asphyxia.(64,68,69) Introducing hypoxia-ischemia in utero to fetal rabbits provides animals with a motor phenotype similar to human cerebral palsy.(64,70) In term and preterm sheep models, hypoxia and asphyxia causes abnormalities in cerebral oxygen metabolism and hemodynamics as well as electrocortical brain activity comparable to human neonates after hypoxia-ischemia and basal ganglia injury representative for cerebral palsy.(71–73)

Of interest is the potential role of sex-specific cell death pathways involved in HIE and possible sex-specific neuroprotective therapies. In general, females seem to

be less susceptible to brain injury. This effect is seen across species, age groups and origin of injury.(74) In adult animal models, reduction in ischemic injury in females has been attributed to estradiol levels.(74) Although estradiol will not be as predominant in prepuberal animal models, there is evidence of sexual dimorphism regarding sex steroids in central nervous system development in mice and rats.(75,76) Other studies show sex-specific cell death pathways leading to brain injury after hypoxia-ischemia both in vitro and in vivo. For instance, there is evidence that brain injury after hypoxia-ischemia in males is evoked by caspase-independent pathways whereas in females, caspase-dependent pathways are responsible.(77–82) Therefore, neuroprotective agents such as NOS inhibitors that interact, either upstream or downstream, with the caspase-dependent pathway may only be effective in females.

The role of sex was only sparsely investigated in the studies included. For the majority of the studies (65%), the sex of the animals used was not reported. Six studies (23%) have used rats of both sexes but have not reported sex-specific outcome. Yu et al. reported no outcome differences between sex for 7-NI and JI-9 but this statement was not supported by statistical analysis, possibly due to the small sample size in each of the groups.(49) Nijboer et al. showed a statistically significant difference in histological and biochemical outcome parameters between sexes in rats, concluding that 2-IB was neuroprotective in female rats only. Other studies with different neuroprotective agents in both animals and humans also indicate a (potential) neuroprotective effect in females only.(81–84)

Methodological quality assessment using the SYRCLE's RoB tool resulted in only low and moderate scores. In each study, no information was available on one or more items, forcing a score of 0 in that area. It is uncertain whether these items were not adhered to during the experiment or simply not included in the final manuscript due to regulations imposed by the editorial guidelines of the publishing

journal. Unfortunately, it is not yet common practice to be as complete and precise in reporting data for animal studies as is for human studies.(33,85) However, since this problem was addressed in a commentary published in the Lancet in 2002, awareness has been steadily increasing.(86,87) Fourteen of the studies included in this review are published in or before 2002; seven (50%) scoring low and an equal number scoring moderate. For the twelve included studies published in 2003 or later, eight (67%) were awarded a moderate score. The SYRCLE's RoB tool proved to be an adequate tool to consistently score the methodological quality of the included studies. However, this tool was developed recently and experience is still sparse. We would like to encourage future researchers to adhere to the items listed in this tool when conducting and reporting animal intervention studies in order to improve the methodologic quality of these studies as well as to use this tool when attempting a systematic review of animal literature.

Despite the low to moderate methodological quality, the evidence presented in this systematic review still indicates NOS inhibition as a potential target for (additional) neuroprotection in human neonates after perinatal asphyxia. Combined inhibition of nNOS and iNOS started as soon as possible after birth and in a repeated dosing regimen seems to have the best evidence based on the combined outcome parameters, translation to clinical practice and methodological quality. Human studies with 2-IB, an inhibitor of both nNOS and iNOS, are currently taking place. Future clinical studies should make clear whether the sex-specific neuroprotective effect of drugs such as 2-IB observed in rats is present in humans as well. Furthermore, future studies are needed to determine the safety of 2-IB in neonates and its effectiveness both with and without TH.

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Conflict of interest statement

Frank van Bel, Floris Groenendaal and Cacha Peeters-Scholte are inventors of 2-iminobiotin as neuroprotective agent for neonates with HIE. Cacha Peeters-Scholte is consultant for and shareholder of Neurophyxia BV's-Hertogenbosch, The Netherlands. The other authors report no (potential) conflict of interest.

Supplementary materials

Search string used for the EMBASE database. Slight modifications were made for the other databases if required.

newborn OR fetus AND (piglet OR rat OR sheep OR mouse OR rabbit) AND ('brain hypoxia' OR asphyxia OR 'hypoxic ischemic encephalopathy' OR 'reperfusion injury':ti,ab OR 'fetus hypoxia' OR hypox:ti,ab) AND ('nitric oxide synthase' OR 'nitric oxide synthesis' OR 'nitric oxide synthase inhibitor') NOT ('heart muscle ischemia'/exp OR 'heart muscle ischemia') NOT 'pulmonary hypertension' NOT 'heart' NOT 'necrotizing enterocolitis' NOT 'alveolar' NOT retina NOT kidney NOT 'preeclampsia' AND [english]/lim*

Table S1: Example of the RoB tool. For each study, each item was awarded 0, 1 or 2 points based on the available information in the manuscript. Item 3 was dropped for all studies.

	Type of bias	Description	Score 0/1/2	Reason
1	Selection bias - Sequence generation	Was the allocation sequence adequately generated and applied?		
2	Selection bias - Baseline characteristics	Were the groups similar at baseline or were they adjusted for confounders in the analysis?		
3	*Selection bias - Allocation concealment*	*Was the allocation adequately concealed?*	*NA*	*Not scored*
4	Performance bias - Random housing	Were the animals randomly housed during the experiment?		
5	Performance bias - Blinding	Were the caregivers and/or investigators blinded from knowledge which intervention each animal received during the experiment?		
6	Detection bias - Random outcome assessment	Were animals selected at random for outcome assessment?		
7	Detection bias - Blinding	Was the outcome assessor blinded?		
8	Attrition bias - Incomplete outcome data	Were incomplete outcome data adequately addressed?		
9	Reporting bias - Selective outcome reporting	Are reports of the study free of selective outcome reporting?		
10	Other - Other sources of bias	Was the study apparently free of other problems that could result in high risk of bias?		
	Total		0-18	

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Chapter 2.2

Pharmacokinetics and short term safety of the selective NOS inhibitor 2-iminobiotin in asphyxiated neonates treated with therapeutic hypothermia

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Abstract

Background: Neonatal encephalopathy following perinatal asphyxia is a leading cause for neonatal death and disability, despite treatment with therapeutic hypothermia. 2-iminobiotin is a promising neuroprotective agent additional to therapeutic hypothermia to improve the outcome of these neonates.

Methods: In an open label study, pharmacokinetics and short term safety of 2-iminobiotin were investigated in neonates treated with therapeutic hypothermia. Group A (n=6) received four doses of 0.16 mg/kg intravenously q6h. Blood sampling for pharmacokinetic analysis and monitoring of vital signs for short term safety analysis were performed. Data from group A was used to determine the dose for group B, aiming at an $AUC_{0\text{-}48h}$ of 4800 ng*h/mL.

Results: Exposure in group A was higher than targeted (median $AUC_{0\text{-}48h}$ 9522 ng*h/mL); subsequently, group B (n=6) received eight doses of 0.08 mg/kg q6h (median $AUC_{0\text{-}48h}$ 4465 ng*h/mL). No changes in vital signs were observed and no adverse events related to 2-iminobiotin occurred.

Conclusion: This study indicates that 2-iminobiotin is well tolerated and not associated with any adverse events in neonates treated with therapeutic hypothermia after perinatal asphyxia. Target exposure was achieved with eight doses of 0.08 mg/kg q6h. Neuroprotective efficacy in combination with therapeutic hypothermia should be determined in further clinical trials.

Background

Neonatal encephalopathy (NE) after perinatal asphyxia remains one of the leading causes for neonatal death and other adverse outcomes in term and near term neonates, affecting between 0.5 and 2 of every 1000 live born children in high-income countries.(1–3) Currently, the only established strategy to reduce brain injury following perinatal asphyxia is therapeutic hypothermia (TH), i.e. lowering the core temperature of the infant to 33-34 °C for 72 hours by whole body cooling.(1) TH has reduced the composite adverse outcome of death and disability such as cerebral palsy, hearing loss and neurodevelopmental disorders from approximately 60% to 45%.(2,3) Combining TH with additional neuroprotective strategies is, however, urgently needed in order to further improve the outcome of these neonates.(4,5)

Molecular pathways contributing to NE have been partly unravelled during the last decades.(6) An excessive production of excitatory neurotransmitters promotes the activation of N-methyl D-aspartate and the opening of voltage-regulated ion channels. This results in an increase of intracellular calcium in neuronal cells causing mitochondrial failure, production of pro-radicals and accumulation of xanthine. Further steps lead to reaction of superoxide free radical with nitric oxide (NO) to form peroxynitrite.(4) End products of this process such as nitrotyrosine have been demonstrated post mortem in brain and spinal cord tissue of neonates who died after severe NE.(7,8)

Production of NO is catalysed by the enzyme nitric oxide synthase (NOS, enzyme commission number 1.14.13.39). Three isoforms of NOS have been identified: endothelial (eNOS), neuronal (nNOS), and inducible NOS (iNOS). All three isoforms are upregulated after NE; both nNOS and eNOS immediately after reperfusion, iNOS from approximately twelve hours onwards.(9) Excessive production of nNOS and iNOS has shown to be deleterious to the brain in several animal models for

NE.(10–13) Selective inhibition of nNOS and iNOS is therefore a potential target for additional neuroprotection.(4,14)

2-iminobiotin is a biotin analogue and has been identified as a selective inhibitor of nNOS and iNOS. 2-iminobiotin has shown favourable results in several preclinical studies regarding safety and efficacy and is available in a stable, inexpensive formulation suitable for human use. Therefore, it is a likely candidate to advance to clinical studies.(14–19) Based on data obtained in a piglet model for NE, target exposure to 2-iminobiotin was determined as an area under the concentration time curve from 0-48h ($AUC_{0\text{-}48h}$) of 4800 ng*h/mL as higher exposure in that study did not result in additional neuroprotection.(20)

A phase I dose escalating safety and tolerability study in healthy male volunteers showed mild adverse events (AEs) such as dizziness and headache at a dose of 12 mg/kg every four hours (six doses) that resolved quickly after discontinuation. No difference in AEs were observed compared to placebo at the lower 2-iminobiotin doses (0.6 to 6 mg/kg). No serious adverse events (SAEs) have been reported.(21)

No studies combining 2-iminobiotin with TH in neonates have been performed to date. The objective of this study was to investigate the pharmacokinetics (PK) and short term safety of 2-iminobiotin in asphyxiated neonates treated with TH. Results from this study will be used to determine the dose required in future clinical trials to achieve the target $AUC_{0\text{-}48h}$ associated with neuroprotection in animal models for NE.

Methods

Setting and study design

A single centre open label phase II prospective study was conducted at the level III Neonatal Intensive Care Unit of the University Medical Center Utrecht, the Netherlands. (Near-)term neonates treated with TH after perinatal asphyxia and with the ability to receive the first dose of 2-iminobiotin within twelve hours after birth were eligible for inclusion. Exclusion criteria were major congenital malformations, inability to insert an indwelling catheter for administration of 2-iminobiotin or blood sampling. The study enrolled two groups (group A and B) of six evaluable patients each.

Dosing regimen

In group A, patients received four intravenous one minute bolus infusions of 0.16 mg/kg 2-iminobiotin at six hour intervals. This dose was expected to yield the target $AUC_{0\text{-}48h}$ of 4800 ng*h/mL when administered eight times at six hour intervals, based on simulations using data from preclinical and clinical studies.(20,21) A dose-escalation study in moderate and severely asphyxiated newborn piglets demonstrated efficacy using 2-iminobiotin with doses of 0.1, 0.2 and 1.0 mg/kg every four hours. Although the highest 2-iminobiotin dose was still significantly neuroprotective compared to placebo, a trend was seen towards less neuroprotection compared to the dose of 0.2 mg/kg, which was selected as the most promising for future clinical trials.(20) When combining PK data obtained from both animal models and adult humans, clearance appeared to increase linearly with body weight and a constant mg/kg dose achieved similar exposure across different species. Therefore, a dose of 0.2 mg/kg every four hours was predicted to be the optimal dose in normothermic neonates.(22) In a newborn piglet study investigating 2-iminobiotin during normothermia and hypothermia, clearance during hypothermia was estimated to be 2.5 fold lower compared to normothermic conditions (personal

communication H. Tjabbes, Neurophyxia BV). Using simulations, the dosing regimen of 0.2 mg/kg under normothermic conditions was compared to different dosing regimens under hypothermic conditions, aiming for an equal $AUC_{0\text{-}24h}$. The dosing regimen of 0.16 mg/kg/dose every six hours under hypothermia was predicted to achieve the desired target $AUC_{0\text{-}24h}$ of approximately 2400 ng*h/mL (Table 1).

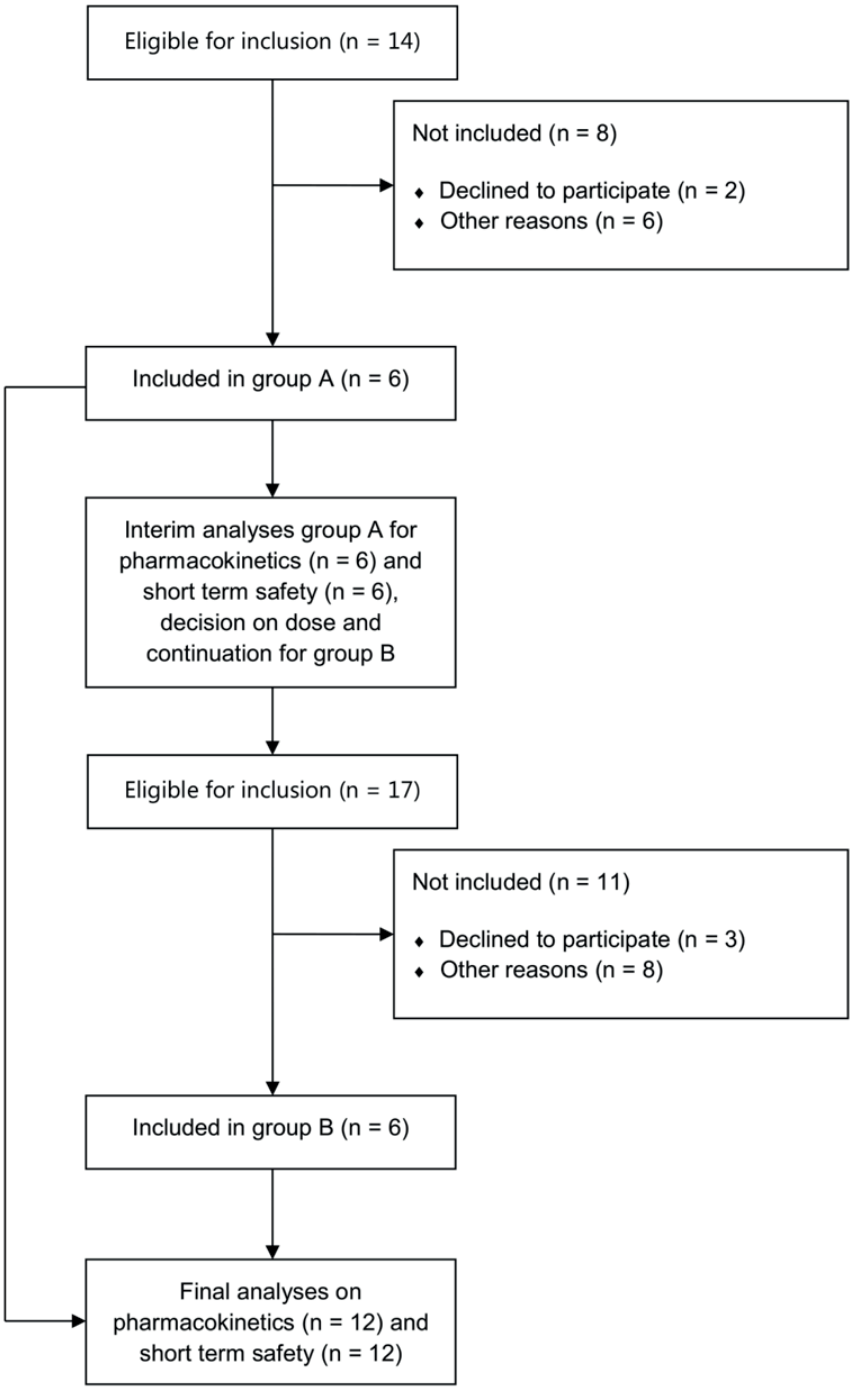
Table 1: Simulated 2-iminobiotin cumulative AUCs after each dose with different dosing regimens under normothermic and hypothermic conditions based on PK data obtained prior to this study.

	Cumulative 2-iminobiotin AUC (ng*h/ml)		
	0.2 mg/kg q4h	0.16 mg/kg q4h	0.16 mg/kg q6h
	normothermia	hypothermia	hypothermia
1	387	426	483
2	788	955	1085
3	1190	1556	1758
4	1592	2209	2475*
5	1994	2899	-
6	2396*	3616*	-

*$AUC_{0\text{-}24h}$
AUC = area under the concentration time curve

Upregulation of iNOS can be present for up to 48 hours after birth.(23) Therefore, the desired dosing interval was set to 48h with a corresponding $AUC_{0\text{-}48h}$ of 4800 ng*h/mL. However, due to uncertainties regarding PK and safety in this extremely vulnerable population, 2-iminobiotin administration was limited to four doses in group A, after which an interim analysis was conducted by an independent Data and Safety Monitoring Board (DSMB) assessing both PK and short term safety (for flowchart, see Figure 1). In group B, 2-iminobiotin was increased to eight doses at six-hour intervals.

Figure 1: Study flow diagram



This study was approved by the ethics committee of the University Medical Center Utrecht (16/471) and was registered in the European Clinical Trials Database (www.clinicaltrialsregister.eu: 2014-004265-25) and the Netherlands Trial Register (www.trialregister.nl: NTR5221). Written informed consent was obtained from both parents.

Study drug

2-iminobiotin was supplied as a 0.75 mg/mL solution for infusion in ready to administer vials containing NaCl 0.9% and a citrate buffer as vehicle to ensure solubility at pH 3.8-4.2 (Neurophyxia BV,
's-Hertogenbosch, the Netherlands).

Pharmacokinetic analysis

From each included neonate, five blood samples of 0.5 mL were drawn at the following scheduled times for PK evaluation: one peak sample 5 minutes after the first administration, two trough samples (one before the second administration and one before the last administration) and two samples at 1h and 3h after the last administration. 2-iminobiotin plasma concentrations were measured using an LC-MS/MS method validated in compliance with the Organisation for Economic Co-operation and Development Good Laboratory Practice guidelines.

The lower limit of quantification (LLQ) was 5 ng/ml and the calibration curves were linear from 5 to 5000 ng/ml. Between-run and within-run coefficients of variation were <20%. Samples were stored at -80°C until analyses.

A population pharmacokinetic model was developed from 2-iminobiotin using non-linear mixed effects modelling (NONMEM, version 7.3.; ICON, Ellicott City MD). In the interim analyses, PK data from group A was used to calculate the $AUC_{0\text{-}48h}$ if the current dose were to be extended to eight administrations at six-hour intervals. Subsequently, the dose required to achieve the target $AUC_{0\text{-}48h}$ with a dosing

regimen of eight administrations at six hour intervals was determined. Based on the PK data from the interim analyses, the DSMB advised on the dose for group B.

Short term safety analysis

Safety was assessed by close monitoring of vital signs such as heart rate, arterial blood pressure, transcutaneous oxygen saturation, rectal temperature, continuous neuromonitoring using 2-channel amplitude-integrated electroencephalography (aEEG) and near-infrared spectroscopy (NIRS), and recording of AEs and SAEs. Safety assessment included trend analysis of vital functions and an intrapatient comparison of vital functions in the fifteen minutes before compared to the thirty minutes after each administration of 2-iminobiotin. In group A (n = 6), four administration per patient led to a total of 24 evaluations of each vital sign. In group B (n = 6) eight administrations yielded a total of 48 evaluations. With this data, a clinically relevant change of more than 10% could be detected with a power of 80% for each variable. For each test, a p-value <0.05 was considered statistically significant. A paired t-test was performed to investigate any difference between the pre- and post-administration timeframes of 2-iminobiotin. When the parameter was not normally distributed (based on a Shapiro-Wilk test), a Wilcoxon rank test was performed. Statistical analyses was performed using SPSS (version 21.0.0, IBM Corp, Armonk NY). All SAEs and AEs were recorded and discussed by the study team and reported to the ethics committee according to national policy. Based on the short term safety data from the interim analyses, the DSMB advised on continuation of the study.

Results

Six patients were included in group A and six in group B. All patients concluded all study activities. All patients were treated with TH for 72h following perinatal asphyxia and ensuing NE according to the national protocol.(2) All administrations of 2-iminobiotin and all blood sampling took place during TH. Patient characteristics are presented in Table 2.

Table 2: Patient characteristics

Parameter	**Group A (n = 6)**	**Group B (n = 6)**	**Total (n = 12)**
BW; kg, mean (range)	3.49 (2.32 – 4.98)	3.18 (2.63 – 4.00)	3.34 (2.32 – 4.98)
GA (weeks, mean, range)	39.4 (37.1 – 41.3)	39.8 (36.9 – 41.4)	39.6 (36.9 – 41.4)
Male (n)	3	2	5
First pH (median, range)	6.92 (6.60 – 7.13)	6.82 (6.80 – 6.99)	6.85 (6.60 – 7.13)
Apgar score 5 min (median, range)	1.5 (0 – 5)	3 (2-5)	3 (0 – 5)
Thompson score 1h (median, range)	11.5 (7 – 18)	7 (2 – 11)	10 (2 – 18)
aEEG background pattern on admission			
Discontinuous normal voltage < 5 µV (n)	3	6	9
Burst suppression (n)	2	0	2
Flat trace (n)	1	0	1

BW = birth weight; GA = gestational age; aEEG = amplitude-integrated electroencephalogram

Pharmacokinetics

2-iminobiotin concentrations could be measured in all samples; no concentrations were below the LLQ. A preliminary population PK model was developed based on data obtained from patients in group A. A two-compartment structural PK model was superior to a one-compartment model (objective function value 559.8 vs 602.3, decrease of 42.5) and provided an adequate fit for the data. Estimation method was first order conditional estimation with interaction option in NONMEM. Interindividual

variability (IIV) could be detected on clearance; residual variability was proportional. Only body weight was considered as a potential covariate, which was not found to have a significant effect on clearance ($p>0.05$) and was therefore excluded from the model. The estimated clearance was used to compute the model predicted $AUC_{0\text{-}48h}$ for group A when treatment would be extended to eight administrations every six hours using the linear trapezoidal rule. Consecutively, the dose required to achieve the target

$AUC_{0\text{-}48h}$ with a dosing regimen of eight administrations at six-hour intervals was determined. The median $AUC_{0\text{-}48h}$ for group A was 9522 ng*h/mL (range 5176 – 22615 ng*h/mL), 198% of the target $AUC_{0\text{-}48h}$ of 4800 ng*h/mL. Simulations using the individual estimates based on this preliminary model predicted that eight administrations of 0.08 mg/kg every six hours would result in a median $AUC_{0\text{-}48h}$ of 4772 ng*h/mL (range 2588-11307). Therefore, this dosing regimen was applied in group B. The resulting median $AUC_{0\text{-}48h}$ for group B was 4465 ng*h/mL (range 2926 – 13816 ng*h/mL), close to the target $AUC_{0\text{-}48h}$.

Data from both groups was used to expand the preliminary model into the final population PK model. No structural changes to the preliminary model were required. The population estimate for clearance was 0.380 L/h; PK parameter estimates for the final model are shown in Table 3; Figure 2 illustrates the poor correlation between clearance and body weight. Exposure metrics were determined though high density simulation of the individual predictions; individual PK metrics are shown in Table 4. Figure 3 shows that the developed model provides an adequate individual and population fit for the PK data, albeit with considerable interpatient variability. Figure 4 shows the goodness of fit plots for the complete dataset and Figure 5 shows the visual predictive check for both treatment groups where the observations have been adequately captured by the model predictions.

Table 3: Population pharmacokinetic parameters for 2-iminobiotin in neonates based on data from both groups (n = 12)

Parameter	Estimate (% SE)	95% CI
Cl (L/h)	0.380 (25)	0.196 – 0.565
$V_{central}$ (L)	0.462 (28)	0.212 – 0.712
Q (L/h)	2.30 (44)	0.324 – 4.27
$V_{peripheral}$ (L)	1.23 (9.3)	1.01 – 1.46
Interindividual variability		
Cl, %	36.2 (55)	0 – 0.752
Residual variability		
Proportional	7.10 (26)	0.0350 – 0.107

Cl = clearance, V = volume of distribution, SE = standard error, CI = confidence interval

Figure 2: 2-iminobiotin clearance vs body weight. Black dots represent the individual predicted clearances; solid line indicates the spline

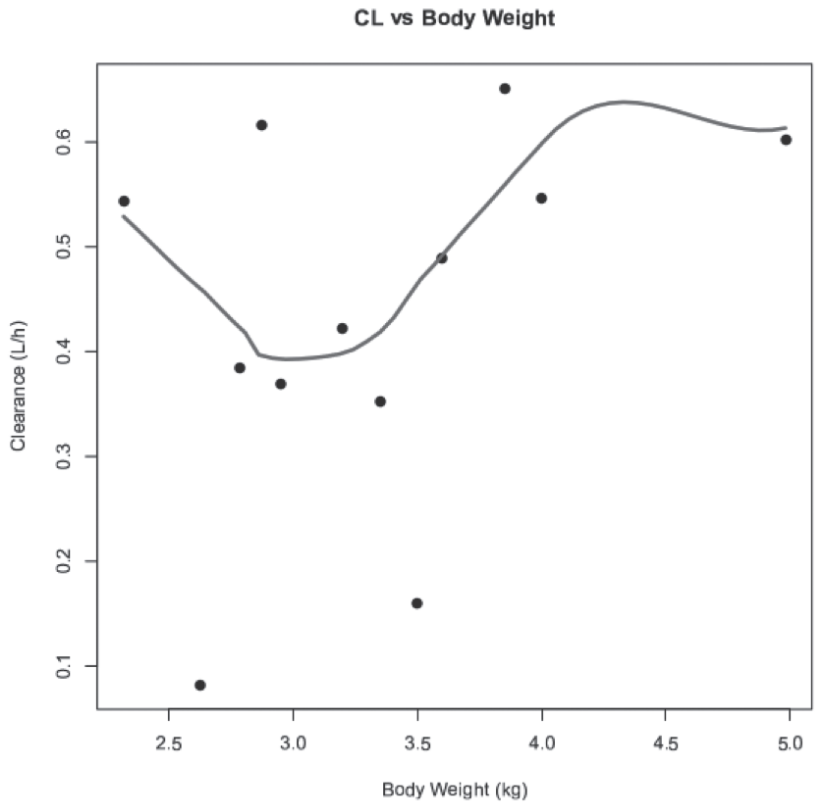


Table 4: Individual PK metrics

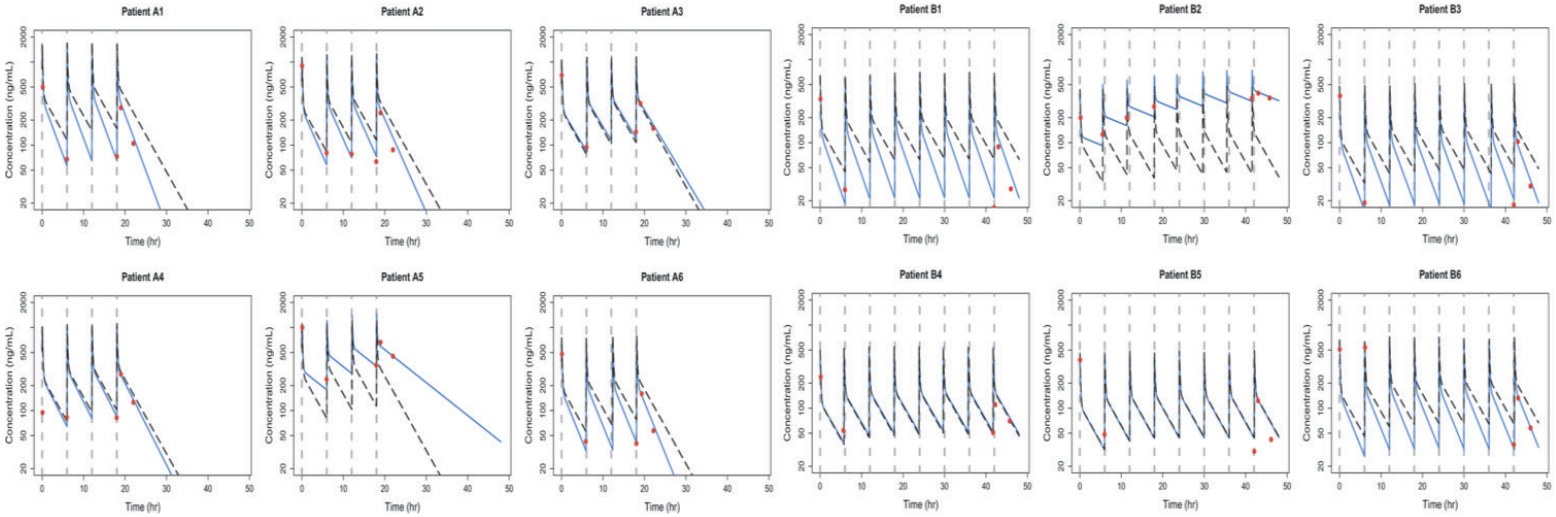
	C_{max} (ng/ml)	T_{max} (h)	$T_{1/2}$ (h)	AUC_{48h} (ng*h/ml)
Group A*				
Patient A1	498	0.17	2.3	10021
Patient A2	894	0.08	2.8	8839
Patient A3	690	0.08	3.8	11182
Patient A4	274	0.12	3.2	9068
Patient A5	1000	19.0	8.1	22615
Patient A6	479	0.10	2.6	5176
Mean	*693*	*3.26*	*3.8*	*11150*
Median	*594*	*0.11*	*3.0*	*9544*
Group B#				
Patient B1	336	0.08	2.1	3741
Patient B2	395	43.0	15	13816
Patient B3	366	0.13	2.2	2926
Patient B4	239	0.13	3.5	4959
Patient B5	382	0.08	3.3	4361
Patient B6	538	6.15	2.4	4570
Mean	*376*	*8.27*	*4.7*	*5729*
Median	*374*	*0.13*	*2.9*	*4465*

*Based on PK parameter estimates from group A only

#Based on PK parameter estimates from group A and B

Cmax = maximum plasma concentration; Tmax = time after first dose at which maximum plasma concentration is reached; T1/2 = elimination half-life; AUC_{48h} = area under the concentration time curve for 48 hours

Figure 3: Individual PK plots of patient in group A (left) and in group B (right). Grey dashed lines indicate the nominal dosing times; red dots are the observed 2-iminobiotin plasma concentrations; blue solid lines represent the individual model fit; black dashed lines represent the population model fit. PK = pharmacokinetica



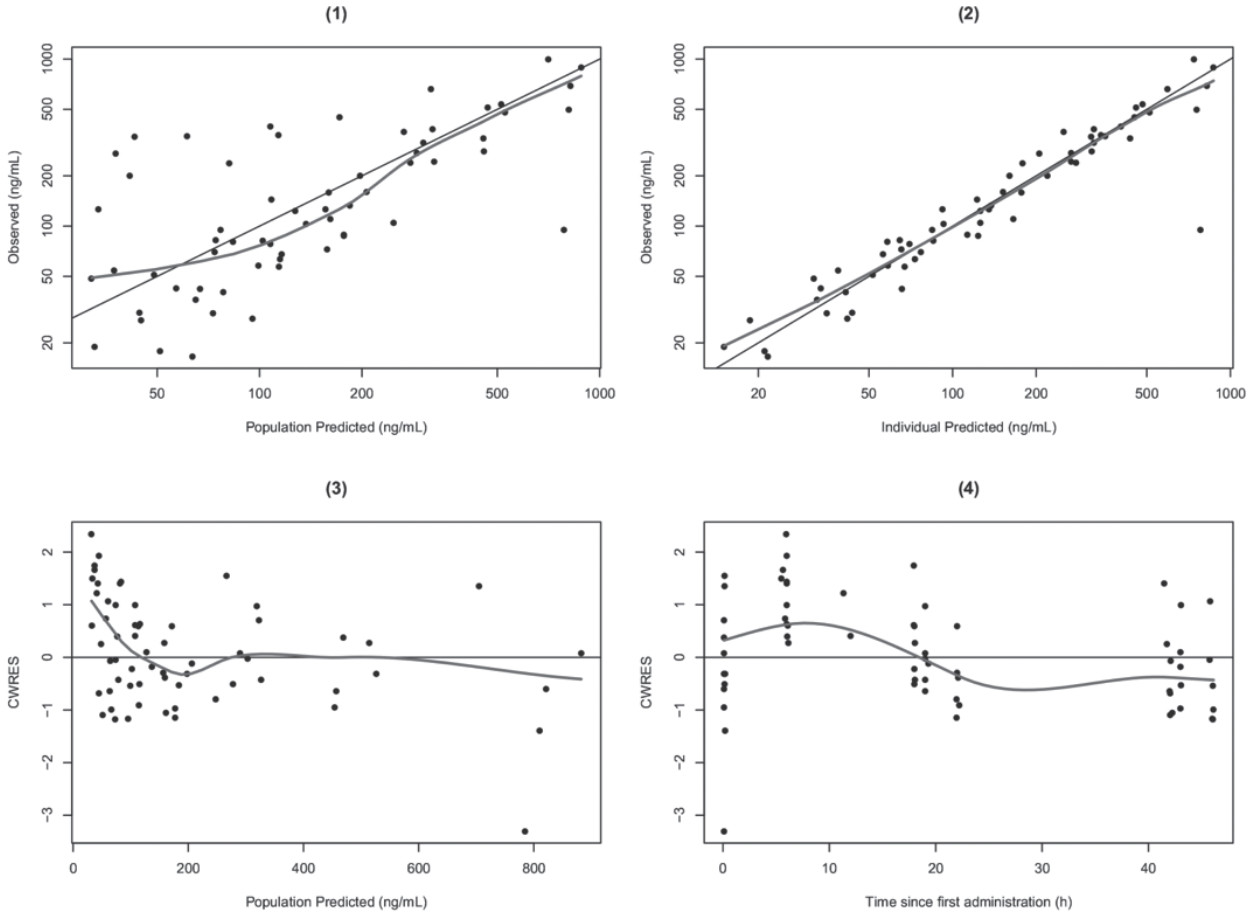
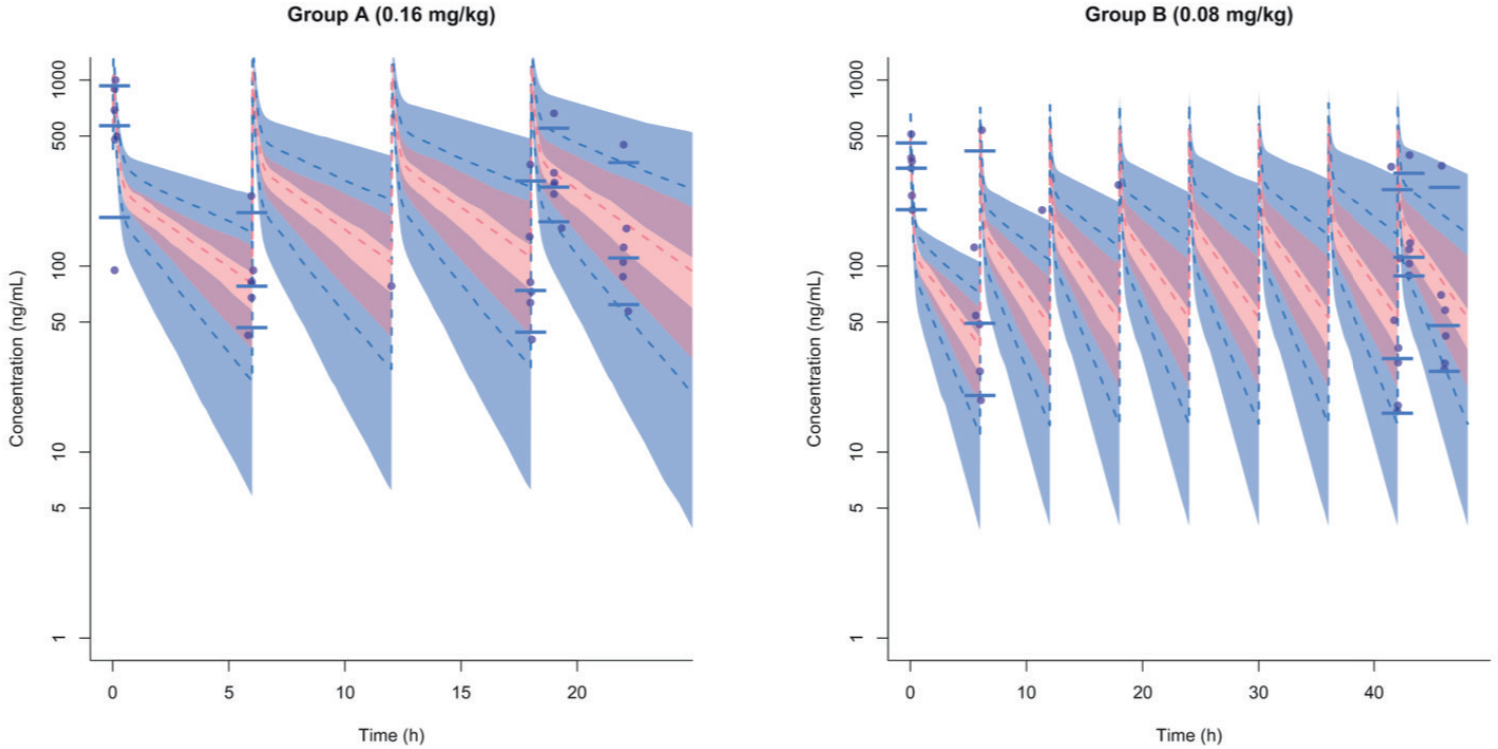


Figure 4: GoF plots for the final model. 1 = observed vs population predicted 2-iminobiotin plasma concentrations; 2 = observed vs individual predicted 2-iminobiotin plasma concentrations; 3 = conditional weighted residuals vs population predicted 2-iminobiotin plasma concentrations; 4 = conditional weighted residuals vs time after first administration. GoF = goodness-of-fit

Figure 5: VPC plots for group A (left) and group B (right). Purple dots represent the individual observations; dashed lines represent the 5th, 50th and 95th percentiles of the observations. Red shaded area represent the predicted 90% confidence interval around the 50th percentile, blue shaded areas represent the predicted 90% confidence intervals around the 5th and 95th percentiles. VPC = visual predictive check



Safety

The trends of heart rate, mean arterial blood pressure and rectal temperature during and after 2-iminobiotin administration are presented in Figure 6. No statistically significant or clinically relevant change in any vital sign (heart rate, blood pressure, ventilation rate, arterial oxygen saturation, cerebral oxygenation using NIRS, and mean, minimum and maximum aEEG background activity) was observed in the fifteen minutes before compared to the thirty minutes after 2-iminobiotin administration in either group.

Two SAEs were reported, both in group A. One patient (A4) died on day three after redirecting of care because of irreversible cerebral injury, and one patient (A5) had an abnormal MRI on day five demonstrating injury to the deep grey matter. Both were considered related to severe perinatal asphyxia, and not to the study medication. AEs reported were hyperkalemia (two patients), hypokalemia (three patients), elevated serum creatinine (SCr) and serum urea concentrations (one patient), elevated serum alanine transaminase and aspartate transaminase concentrations (eight patients), and elevated C-reactive protein concentrations (six patients). None were considered to be related to the study medication. As no concerns regarding short term safety arose in group A, no additional safety measures or protocol amendments were necessary for group B according the DSMB.

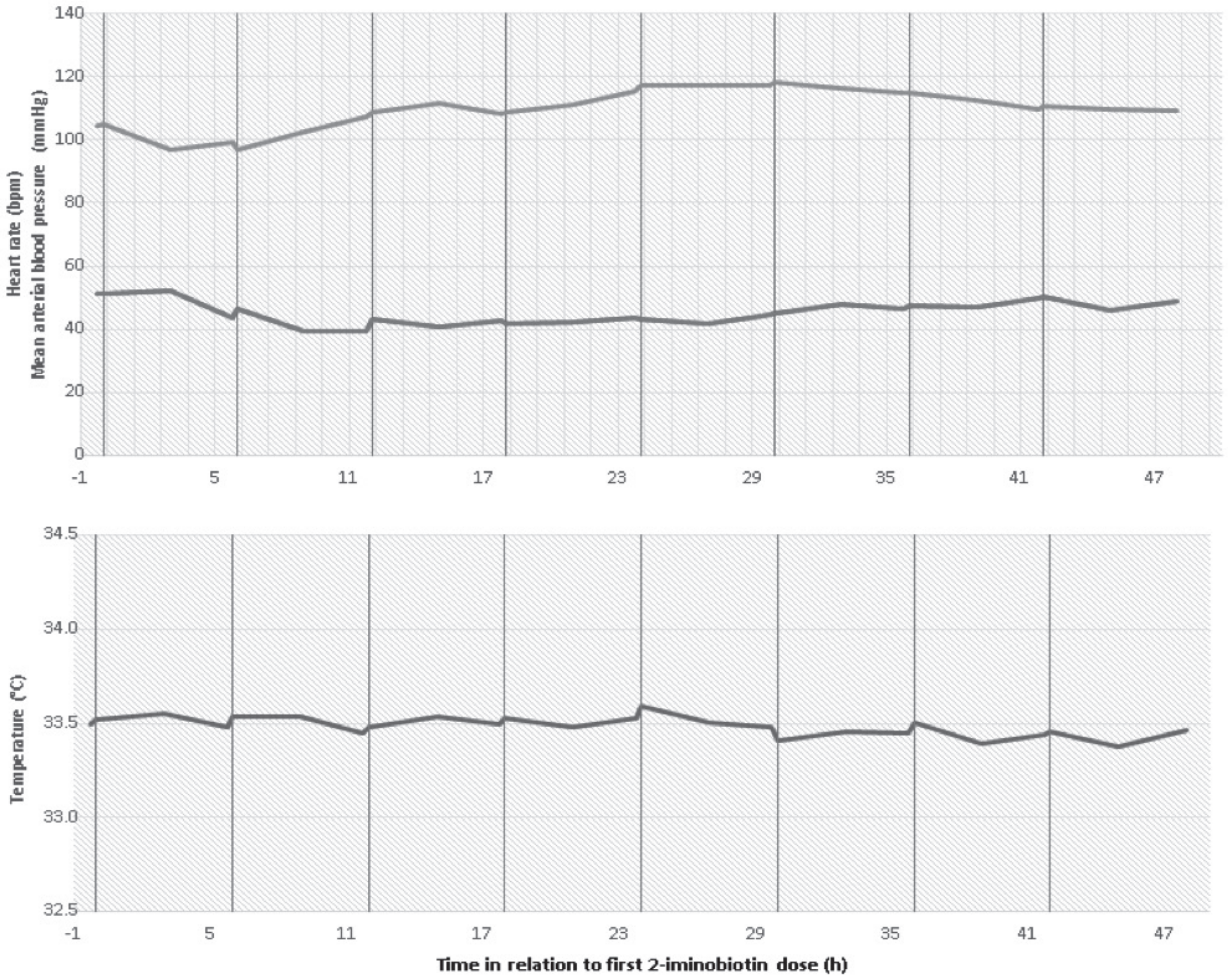


Figure 6: Trend of average heart rate (top), mean arterial blood pressure (middle) and temperature (bottom) during and after the 2-iminobiotin dosing period. Vertical lines represent 2-iminobiotin doses

Discussion

This is the first study to describe the short term safety and PK of 2-iminobiotin in (near-)term neonates treated with TH for moderate to severe NE. 2-iminobiotin exposure in terms of $AUC_{0\text{-}48h}$ in group A was much higher than anticipated. The dose for group A was determined with the best available evidence, using a predicted clearance in neonates with data derived from adult human volunteers as well as animal models translational for NE.(20,21) However, the animal models used to study 2-iminobiotin are considered translational for NE based on brain development and pattern of hypoxic-ischemic injury but may not accurately reflect maturation of other organs.(24,25) Therefore, immaturity of renal function in human neonates may not have been captured adequately in this prediction. The exponent describing the relationship between body weight and 2-iminobiotin clearance was estimated to be 1 based on interspecies scaling.(22) Using adult human data alone with a generally more accepted exponent of 0.75 and accounting for immaturity of renal function would have predicted a neonatal clearance much closer to the value estimated in this study.(26)

Treatment with TH has the potential to influence PK.(27) 2-iminobiotin does not undergo hepatic metabolism and is excreted renally in unchanged form. Clearance of predominantly renally excreted drugs were found to be impaired during TH compared to normothermia, most likely due to diminished kidney perfusion.(28) Although a potential effect of TH on 2-iminobiotin clearance was anticipated when selecting the dose for group A, underestimation of the effect of TH on 2-iminobiotin clearance may have contributed to the overexposure in group A. Additionally, renal clearance in asphyxiated neonates can be lower compared to non-asphyxiated neonates regardless of TH.(29)

Neonatal renal function is impaired compared to older children and adults but increases steadily over the first few weeks after birth.(30) However, as 2-iminobiotin

was administered in this study during the first two days after birth only, it is unlikely that maturation of kidney function will significantly impact 2-iminobiotin clearance in this timeframe. However, impaired kidney function as a result of perinatal asphyxia could also influence clearance and may contribute to the observed variation in clearance.(31) Figure 3 shows accumulation of 2-iminobiotin in patient A5 and B2. Patient B2 suffered from acute kidney injury (AKI) indicated by low urinary output, elevated SCr and urea concentrations until seven days after birth. Patient A5 had elevated SCr on day one after birth that normalized within 24 hours. The same situation was observed in patients A2, A3 and B5 who did not show 2-iminobiotin accumulation. As SCr concentrations during the first few days of life are confounded by maternal transfer, determination of individual renal function based on SCr is subject to a high degree of uncertainty in neonates. Although a 2-iminobiotin dose reduction might be necessary in patients with renal impairment to achieve the desired $AUC_{0\text{-}48h}$, the lack of a robust tool to accurately predict neonatal renal function on the first day after birth makes this clinically unfeasible. Additionally, data from this study suggests that other factors besides AKI may be responsible for 2-iminobiotin accumulation and that elevated SCr concentration on the first day of life will not always predict impaired 2-iminobiotin clearance.

Even though the pharmacokinetic analysis was performed in a small group of neonates with sparse sampling, the population and consequently individual PK parameters could be determined with an adequate precision. An exception is the IIV on clearance, which could not be estimated reliably as the 95% confidence interval of the estimate includes zero (Table 2). However, addition of this random effect was necessary to obtain adequate individual fits. In this small dataset no relationship between body weight and clearance was detected. Current understanding of pharmacokinetics would imply that clearance is dependent on body weight and post-conception age.(32) All neonates in this study were term or near-term, therefore having a similar degree of maturation. The main aim of the population PK analysis

was to describe the pharmacokinetics of 2-iminobiotin in the individual neonates and to predict the dose required to achieve the target $AUC_{0\text{-}48h}$. As this study consists of only twelve neonates, little inference on the absence or presence of a body weight effect on the PK can be derived on the current data.

Based on the PK analyses of group A , the dose for group B was reduced to 0.08 mg/kg in order to achieve a median $AUC_{0\text{-}48h}$ of approximately 4800 ng*h/mL. Even though this target was achieved, three patients in group B had a lower $AUC_{0\text{-}48h}$ due to considerable interpatient variability in PK parameters. Choosing a higher dose for group B would have resulted in more patients above the predefined target $AUC_{0\text{-}48h}$. Because of the observation of less neuroprotection at a higher 2-iminobiotin dose in the piglet model, a higher median $AUC_{0\text{-}48h}$ in neonates was deemed undesirable. NOS promotes various physiological intracellular activities such as vasodilation, long term synaptic neurotransmission and smooth muscle relaxation.[36–38] eNOS is believed to be instrumental following perinatal asphyxia by maintaining pulmonary blood flow, preventing pulmonary hypertension and ensuring adequate cerebral oxygenation.(9,11) Inhibition of eNOS could therefore potentially be neurodegenerative.(33) In our study, no effect on respiratory and cardiovascular parameters was observed after administration of 2-iminobiotin. Both nNOS and iNOS are associated with brain damage and are present in excessive amounts following birth asphyxia. nNOS is upregulated immediately after reperfusion; interventions aimed at nNOS should therefore be administered as soon as possible. iNOS peaks around twelve hours after birth and can be present for up to 48 hours. (23) Animal data suggests that both nNOS and iNOS are upregulated during TH after hypoxia-ischemia, although TH might attenuate NOS expression.(34) Additionally, in vitro data indicates that 2-iminobiotin in combination with hypothermia attenuates hypoxia-induced neuronal cell damage.(35)

The aim of this study was to investigate the PK and short term safety of 2-iminobiotin

when combined with TH. We therefore allowed a twelve hour window between birth and first administration of 2-iminobiotin. In a future studies aiming at efficacy, start of 2-iminobiotin should preferentially be as soon as possible after birth and should be maintained for 48 hours to effectively inhibit both nNOS and iNOS production.

All events recorded as AEs or SAEs are common complications of the underlying disease and did not occur more often in our group when compared to the overall population of neonates treated with TH for NE in our hospital.(36) None of the complications were deemed related to 2-iminobiotin by the study team and the DSMB. Although this study was not designed for efficacy, it is noteworthy that the incidence of SAEs (death and deep gray matter injury, both one out of twelve (8.3%)) is lower compared to historical data in this population (around 30% mortality and 15% neurodevelopmental disorders).(1,2,36)

Since its introduction in 2008, TH has significantly improved the outcome of children with NE and has become the standard of care in western countries.(1–3) However, additional neuroprotective strategies to augment TH have not been established in the last decade. Several promising pharmacological interventions such as melatonin, erythropoietin and darbepoetin have recently made the transition from pre-clinical to explorative clinical studies (37–39) but their effect has yet to be confirmed in randomized, phase III clinical trials (NCT02621944, NCT03079167, NCT03071861). Phase II studies investigating topiramate and allopurinol in addition to TH are currently underway (NCT01765218, NCT03162653). Additionally, xenon ventilation in neonates is safe and feasible despite the need of closed circuit ventilation to minimize the loss of this expensive noble gas but a recent efficacy study showed disappointing results.(40) Argon, a much more abundant and less expensive noble gas has also shown neuroprotective properties in animal models but has not (yet) progressed to trials in humans.(41)

Over the past two decades studies have focused on safety and efficacy of 2-iminobiotin in cell cultures, animal models for NE, and humans. In the present study 2-iminobiotin exposure known to be effective in animals has been achieved in human neonates with NE during treatment with TH, although the PK parameters are derived from a small dataset. An open-label efficacy trial with 2-iminobiotin in combination with TH is at risk for introducing bias as the standard of care continues to evolve and patients might not be adequately matched to historical controls. Therefore, it is our opinion that a placebo-controlled (phase II) trial in addition to TH with eight 2-iminobiotin doses of 0.08 mg/kg/dose every six hours is the logical next step to examine the neuroprotective potential of 2-iminobiotin. This trial should include collection of PK data to further increase the knowledge of 2-iminobiotin PK in this vulnerable population. Allometric scaling and incorporation of maturation should be considered in future PK models.

Conclusion

Administration of 2-iminobiotin in (near-) term neonates treated with TH for NE is safe regarding short term clinical parameters and occurrence of (S)AEs. Estimated clearance of 2-iminobiotin in this population is 0.380 L/h, and a dose of 0.08 mg/kg every six hours is necessary to reach the desired median $AUC_{0\text{-}48h}$ of approximately 4800 ng*h/mL. Based on this data, a placebo-controlled trial investigating the efficacy of 2-iminobiotin during TH using a dose of 0.08 mg/kg every six hours with a total of eight doses is warranted.

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Disclosure

Cacha Peeters-Scholte, Frank van Bel and Floris Groenendaal are the inventors of 2-iminobiotin as a neuroprotective agent in neonates with hypoxic-ischemic encephalopathy.

Cacha Peeters-Scholte and Huibert Tjabbes are shareholders and consultants of Neurophyxia BV.

Peter Vis is employed at LAP&P Consultants BV.

All other authors declare no conflicts of interest

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Chapter 2.3

Neuroprotection by Argon Ventilation after Perinatal Asphyxia: A Safety Study in Newborn Piglets

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Abstract

Background: Hypothermia is ineffective in 45% of neonates with hypoxic-ischemic encephalopathy. Xenon has additive neuroprotective properties, but is expensive, and its application complicated. Argon gas is cheaper, easier to apply, and also has neuroprotective properties in experimental settings. The aim was to explore the safety of argon ventilation in newborn piglets.

Methods: Eight newborn piglets (weight 1.4-3.0 kg) were used. Heart rate, blood pressure, regional cerebral saturation, and electrocortical brain activity were measured continuously. All experiments had a 30 min. baseline period, followed by three 60 min. periods of argon ventilation alternated with 30 min argon washout periods. Two animals were ventilated with increasing concentrations of argon (1h 30%, 1h 50%, and 1h 80%), two were subjected to 60 min. hypoxia (FiO_2 0.08) before commencing 50% argon ventilation, and two animals received hypothermia following hypoxia as well as 50% argon ventilation. Two animals served as home cage controls and were terminated immediately.

Results: Argon ventilation did not result in a significant change of heart rate (mean ± s.d. -3.5 ± 3.6 bpm), blood pressure (-0.60 ± 1.11 mmHg), cerebral oxygen saturation (0.3 ± 0.9%), electrocortical brain activity (-0.4 ± 0.7 μV), or blood gas values. Argon ventilation resulted in elevated argon concentrations compared to the home cage controls (34.5, 25.4, and 22.4 vs. 7.3 μl/ml)

Conclusion: Ventilation with up to 80% argon during normoxia, and 50% argon after hypoxia did not affect heart rate, blood pressure, cerebral saturation and electrocortical brain activity. Clinical safety studies of argon ventilation in humans seem justified.

Introduction

Perinatal asphyxia in full term neonates is an important cause of mortality and morbidity. Moderate hypothermia is the standard neuroprotective treatment, but is still ineffective in 45% of cases.(1) This underlines the need for additional neuroprotective interventions.(2)

Brain injury following hypoxia-ischemia is partly mediated by activation of the N-methyl-D-aspartate (NMDA) subtype of the glutamate receptor.(3) The noble gas xenon exhibits non-competitive antagonism of this NDMA receptor and results of its use as an additive neuroprotective agent are promising.(4–6) However, xenon is an expensive gas and therefore requires complex closed-circuit ventilation systems. (7,8) This makes large scale clinical use of xenon less feasible.
Recently, argon gas has been shown to have neuroprotective properties in cell cultures of dissociated neurons, in organotypic hippocampal slice cultures, and in vivo models.(9–14) In contrast with xenon, argon is a more abundant and cheaper noble gas. It can be obtained as a pure gas of pharmaceutical quality at low costs and therefore does not require complex ventilator setups.

At present data on the safety of argon ventilation in larger animals is scarce. Therefore, the aim of the present study was to explore the safety of argon ventilation in newborn piglets.

Materials and methods

Animal preparation and instrumentation

The experimental protocol was approved by the Animal Care Committee of Utrecht University, The Netherlands. Eight newborn Dutch store piglets with a mean age of 4 days (range 2-7 days) and mean body weight of 2275 g (range 1400-3000 g)

were used. The animals were sedated using a intramuscular injection consisting of midazolam (0.7 mg/kg), ketamine (13 mg/kg), and atropine (0.02 mg/kg). Thiopental (4 mg/kg) was administered before endotracheal intubation.
An intravenous catheter was inserted in an ear vein for continuous infusion of glucose 10% and sedatives. General anesthesia was maintained throughout the experiment with midazolam (2 mg/kg/h) and morphine (40 µg/kg/h). Pancuronium bromide (1 mg/kg/h) was used for muscle paralysis.

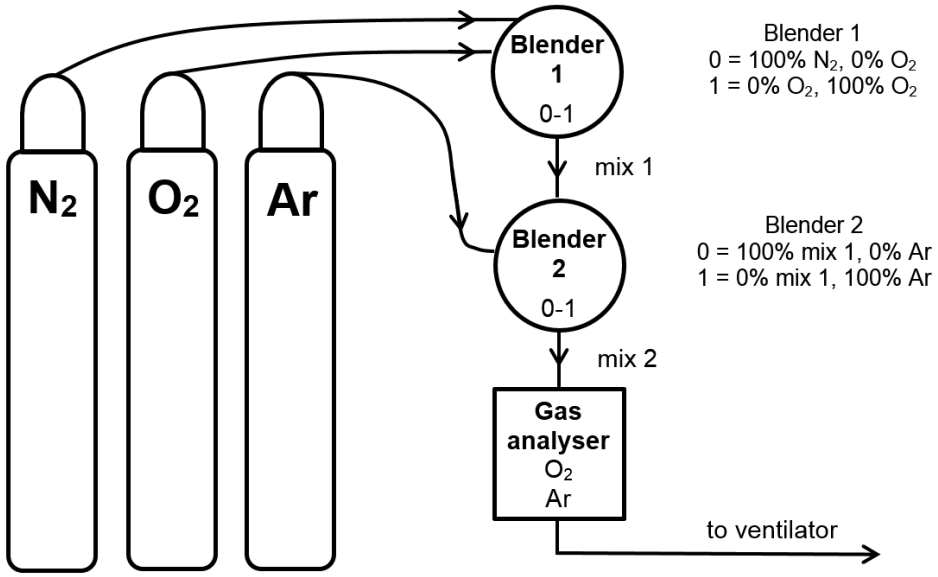
For monitoring mean arterial blood pressure (MABP) and sampling of arterial blood gasses, a catheter was surgically inserted in the right femoral artery. The blood was heparinized with 2.5 U/ml (2 ml/h). The total fluid administration rate was 5 ml/kg/h. Rectal temperature was maintained between 38.5 and 39.5 by using a heated table top matrass and a forced-air warming system (Bair Hugger®, Arizant Healthcare Inc., Eden Prairie, MN).

Argon ventilation
Medical grade argon, with a purity of > 99.995% was used during the animal experiments (Linde Gas Therapeutics Benelux BV, The Netherlands). The piglets were mechanically ventilated by using a continuous flow, pressure-controlled ventilator (Stephanie, Fritz Stephan GmbH, Gackenbach, Germany). The ventilator was technically adapted to enable ventilation with oxygen, argon, and nitrogen. The three gases were mixed by using two gas blenders. Mixture 1, containing oxygen and nitrogen, was mixed in the first blender. In the second blender, mixture 1 was blended with argon to obtain the final mixture (mixture 2). Figure 1 displays the setup for argon ventilation.

The final gas mixture was analyzed by a gas analyzer (KG6050 Dual gas analyzer, Hitech Instruments Ltd, Luton, Bedfordshire, United Kingdom) to quantify oxygen and argon quantity in the mixture. The gas analyzer has a resolution of 0.1%, and

an accuracy of ± 1.0% for oxygen and argon. These adjustments enabled gas concentrations of 0-100% for all three gasses (i.e. oxygen, nitrogen, and argon).

Figure 1: Schematic of ventilator setup.



Parameters for safety measurements

Heart rate (HR), MABP, arterial SO_2, end-tidal CO_2, and rectal temperature were monitored using a M1094B patient monitor (Philips, Best, The Netherlands). These physiological parameters were recorded and stored at 1Hz on a personal computer for off-line analysis (Poly 5, Inspector Research Systems, Amsterdam, The Netherlands).

An Olympic 6000 (Natus Medical Systems, Seattle, WA) cerebral function monitor was used to monitor electrocortical brain activity throughout the experiments. This monitor continuously records the amplitude-integrated electroencephalogram (aEEG), real time raw EEG, and electrode impedance at 100 Hz. The (a)EEG signal

was obtained from a pair of needle electrodes spaced 1.5 cm apart placed on the left side of the scalp with a central (frontal) reference electrode.(15,16)
The regional cerebral saturation ($rScO_2$) was monitored by using a two wavelength (730 and 810 nm) Near-InfraRed Spectrometer (INVOS 4100, Covidien, Mansfield, MA) with a transducer containing a light emitting diode and two distant sensors (i.e. at 30 and 40 mm).

The transducer was placed in a cross-cerebral configuration on shaved skin just between the eyes and ears. The sensor was fixated by using an elastic bandage and was carefully positioned to avoid contact with the aEEG electrodes.

Arterial pH, base excess, arterial pO_2 and pCO_2, glucose and lactate concentration were obtained by blood gas analysis. A blood sample was drawn during baseline and subsequently after each change in ventilation settings.

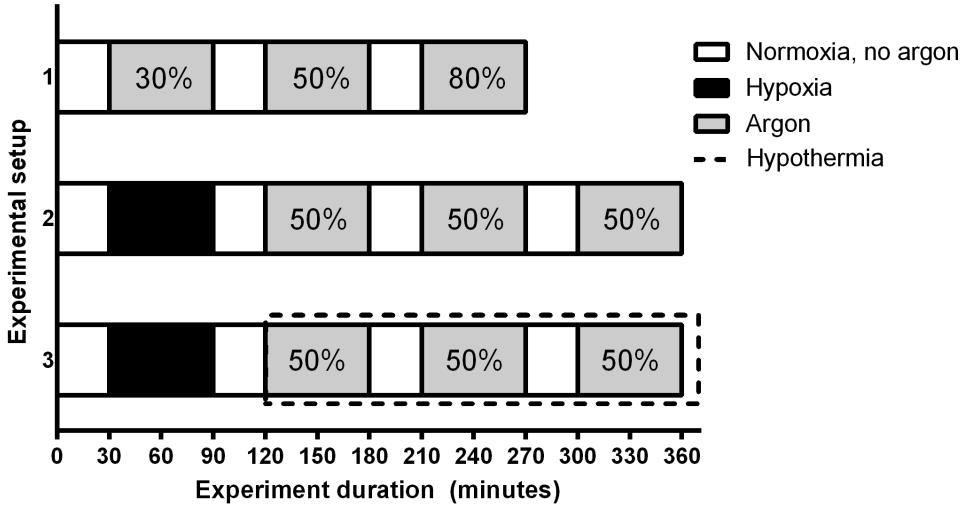
The Experimental Protocol
Two animals served as home cage controls for histopathologic examination (see below) and where therefore terminated using pentobarbital (100 mg/kg) upon arrival at the lab. In the remaining 6 animals the experiment contained a 30 min. baseline period after intubation as a washout of the drugs used for induction. Subsequently 3 periods of 60 min. argon ventilation were alternated by 30 min of normoxic ventilation without argon to allow for argon washout.

Figure 2 is a schematic representation of the three experimental setups that were used to explore the effect of argon ventilation in different clinical settings:

1. *Normoxia*. The first two animals were used to examine the effects of increasing concentrations of argon (i.e. 30%, 50%, and 80%) in normoxic, normothermic animals

2. *Hypoxia*. After the baseline period of 30 min, two additional animals were subjected to hypoxia by decreasing the FiO_2 to 0.08 for 60 min, after which 50% argon ventilation was started to study the effects of argon ventilation after hypoxia.
3. *Hypoxia + hypothermia*. The final two animals were subjected to 60 min of hypoxia, followed by therapeutic hypothermia in addition to 50% argon ventilation to mimic the most likely clinical setting in which argon might be used.

Figure 2: Schematic representation of the three steps of the experimental setup.



In the 4 hypoxic animals 50% argon (as opposed to 80% argon) was used since this is the maximum concentration likely to be used in a clinical setting where additional oxygen may be needed for neonates with perinatal asphyxia in addition to GBS infection or meconium aspiration. During therapeutic hypothermia, the rectal temperature of the two animals was maintained at 34-35° C. Hypothermia was achieved by using small packs of refrigerated saline. Animals were terminated after the third period of argon ventilation with an overdose pentobarbital (100 mg/kg i.v.).

Histopathology

Termination of the animals was directly followed by transcardial perfusion with 4% paraformaldehyde in phosphate buffered salt. Brains were post-fixed in 4% formaline for 5-7 days, dehydrated (30-100% ethanol), and subsequently embedded in paraffin. Coronal sections of 4 µm were cut at hippocampal level.

We studied neuronal damage in detail by assessing pyknotic nuclei in HE-stained sections as a marker of dying neurons and loss of MAP2 staining as a specific marker of neuronal integrity. [17,18] These markers might be signs of argon toxicity. Deparaffinised sections were stained with hematoxylin-eosin (HE; Klinipath, Duiven, the Netherlands) or were incubated with mouse-anti-MAP2 (microtubule associated protein 2) antibody (Exbio, Vestec, Czech Republic) followed by biotin-labeled horse-anti-mouse antibody (Vector Laboratories, Burliname, CA). Visualization was performed by using Vectastain ABC kit (Vector Laboratories) and diaminobenzidine (Sigma-Aldrich, Steinheim, Germany). Photographs were made using a Zeiss Axio Lab A1 microscope and Icc5 camera and analyzed using ZEN2012 software (Carl Zeiss, Oberkochen, Germany).

Argon analysis

To assess the concentration of argon that reached the blood, two 1 ml blood samples per animal were drawn during the final minutes of argon ventilation, just before termination. In addition, blood samples were also drawn from the control animals before termination. These samples were immediately transferred into headspace vials, sealed airtight, and frozen at -20°C until analysis. Two air samples were also taken to determine the argon concentration in lab air. Calibration samples were prepared in the University Medical Center Freiburg, Germany by adding 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µL of argon (Air Liquide Deutschland GmbH, Düsseldorf, Germany; purity > 99.999%,) to blank piglet blood using a gas tight syringe. Prior to analysis, animal samples and calibration samples were stored at

room temperature for 2.5 h to reach equilibrium. After equilibrium, argon (Ar, m/z 40) was measured using nitrogen (N¬¬2, m/z 28) as an internal standard. Ar/N2 ratios were plotted against added argon concentration (µl/ml).

Analysis was performed using an Agilent 6890N gas chromatography system combined with a Agilent 5973 Series mass selective detector (Agilent, Waldbronn, Germany) and a PAL headspace autosampler (CTC Analytics AG, Zwingen, Switzerland). The samples were incubated at 30 °C for 30 seconds and 250 µL gas from the headspace was injected into the gas chromatography – mass spectrometry (GC-MS) system (split injection mode 500:1).

Separation was performed on an PoraPLOT Q column (25 m x 0.25 mm) (Agilent, Waldbronn, Germany) using the following temperature gradient: 40°C for 3 min, increased to 210°C at 50°C/min and holding 210°C for 2.1 min. Helium 4.6 was used as carrier gas at a constant flow rate of 0.8 mL/min. The MS conditions were as follows: transfer line heater 280°C; ion source temperature 230°C; quadrupole temperature 150°C; electron impact ionization mode, and ionization energy 70 eV. Analysis was performed in full scan mode analyzing a mass range from 10 to 70 amu with a scan time of 0.2 seconds.

Data processing and analysis

The three periods of argon ventilation yielded 5 switches per animal (figure 2) and thereby 30 switches (i.e. 6 animals with argon, 5 switches per animal) in total. Data was used from representative segments of at least 15 min. that were selected at the beginning and at end of each argon or washout period. The change in physiological parameters was always calculated by subtracting the segment with argon ventilation from the segment without argon ventilation.

All physiological data were imported in SignalBase (v7.7.8, UMC Utrecht, The Netherlands) and analyzed together.

Statistics

The 5 argon-normoxia switches per animal and use of 6 animals (30 transitions) enabled us to detect a change of more than 10% in any parameter, which was considered clinically relevant, with a power of 80%. Statistical analysis was performed by using a mixed models approach in R (version 2.14.1; http://www.r-project.org) with the nlme package. The different physiological parameters were used as the dependent variable, the individual piglet was used as a random factor, and argon ventilation (y/n) during an epoch of recorded data, hypoxia in the experimental setup (y/n), and hypothermia in the experimental setup (y/n) were investigated as fixed factors. A p-value <0.05 was considered statistically significant.

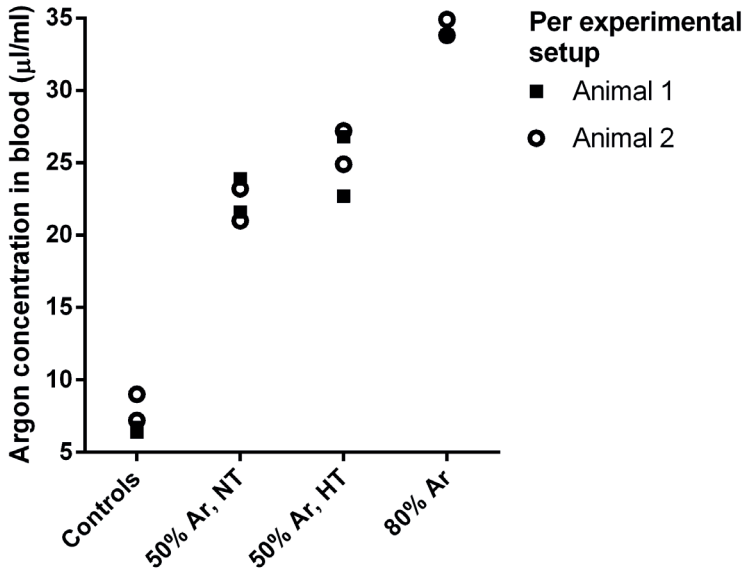
Results

Concentrations in blood

With the calibration samples a calibration line was made: Ar/N_2 ratio = 0.0003*blood concentration (µl/ml) + 0.0583, with a R^2 of 0.9949.

Results of the argon analysis in blood are demonstrated in figure 3. Argon concentrations were higher in piglets that were ventilated with 50% argon as compared to the control piglets (med. 23.6 µl/ml vs. 7.0 µl/ml) The value of the control piglets is extrapolated, and below the lowest value of the calibration line. In addition, there was a difference in argon concentrations between piglets that were ventilated with 80% argon as compared to piglets that were ventilated with 50% argon upon termination (med. 34.5 µl/ml vs. 23.6 µl/ml). Analysis of laboratory air did not show altered argon background concentrations at the time of the experiment (data not shown).

Figure 3: Concentrations of argon gas (µl/ml) in blood in home cage controls, animals ventilated under normoxic conditions, animals ventilated with argon after hypoxia, and animals ventilated with argon after hypoxia that subsequently underwent hypothermia. Separate animals within one experimental setup are displayed with different symbols (open circles and closed squares).



Physiology

Figure 4 displays representative recordings of physiological parameters of an animal that underwent hypoxia and subsequent hypothermia. During hypoxia HR and MABP increased, and $rScO_2$ decreased, whereas aEEG remained stable during the one hour hypoxia. In the hypoxic animals, hypoxia resulted in metabolic acidosis (pH 7.03 ± 0.09, base excess -19.9 ± 3.0 mmol/l, lactate 11.8 ± 1.5 mmol/l). These values normalized during re-oxygenation.

In the mixed model analysis, ventilation with argon did not result in a significant change of HR, mean arterial blood pressure, $rScO_2$ or aEEG (see table 1). Having received hypoxia, and undergoing hypothermia did not have significant effects on HR, aEEG, and $rScO_2$, but hypothermia did cause a decrease in MABP.

Figure 4: Recorded physiological data from representative animal receiving therapeutic hypothermia. Black horizontal bars represent periods of argon ventilation.

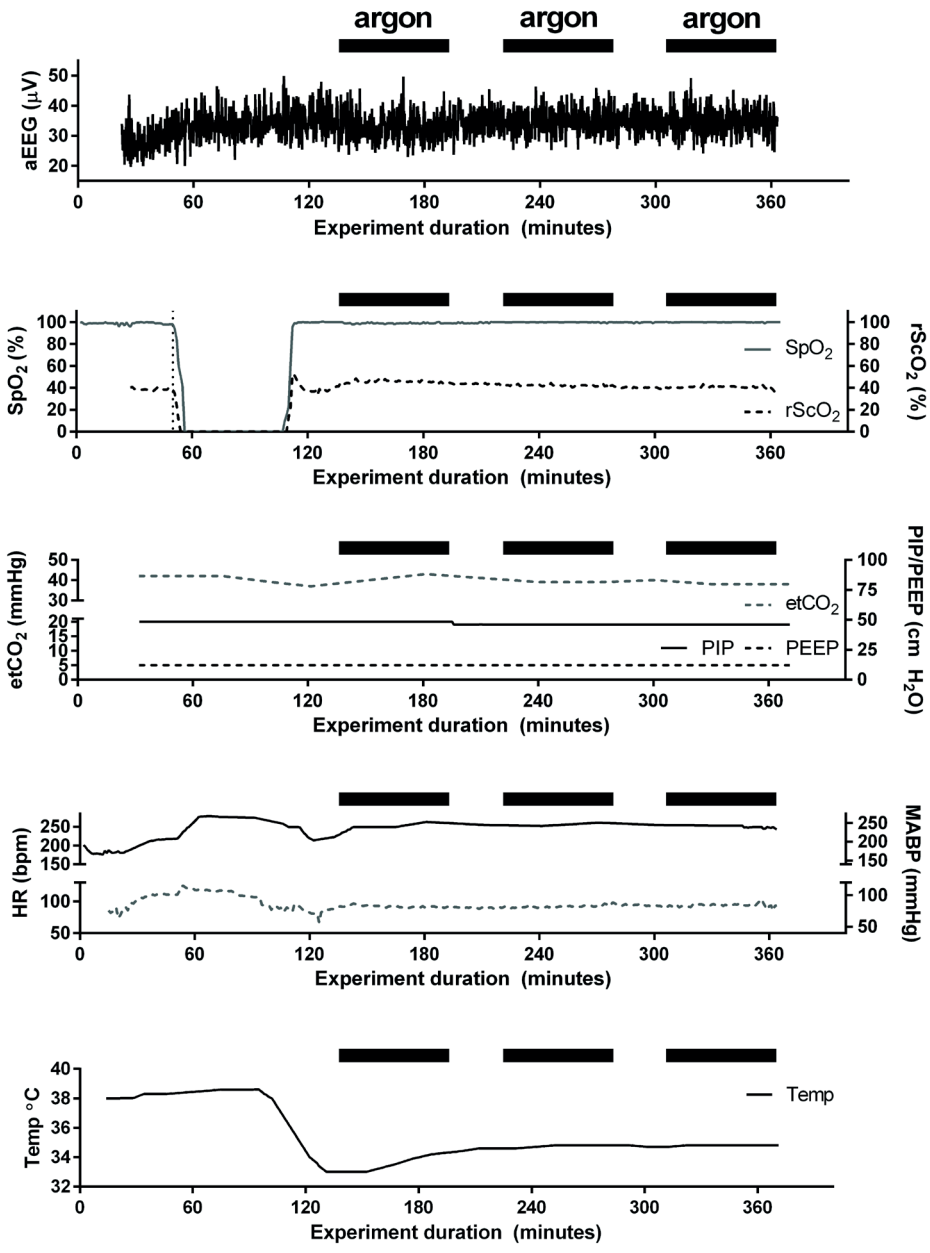


Figure 5 displays the absolute changes in physiological parameters induced by switching between argon ventilation and normoxic ventilation without argon, showing that the median change is always very close to zero.

Figure 5: Calculated differences in monitored parameters (normoventilation – argon ventilation) displayed as absolute difference. For reference, the median value of the 6 animals during baseline is added to the right of each panel. Each dot represents a single switch from argon to normoventilation or vice versa. Ag: argon, HI: hypoxia-ischemia, HT: hypothermia.

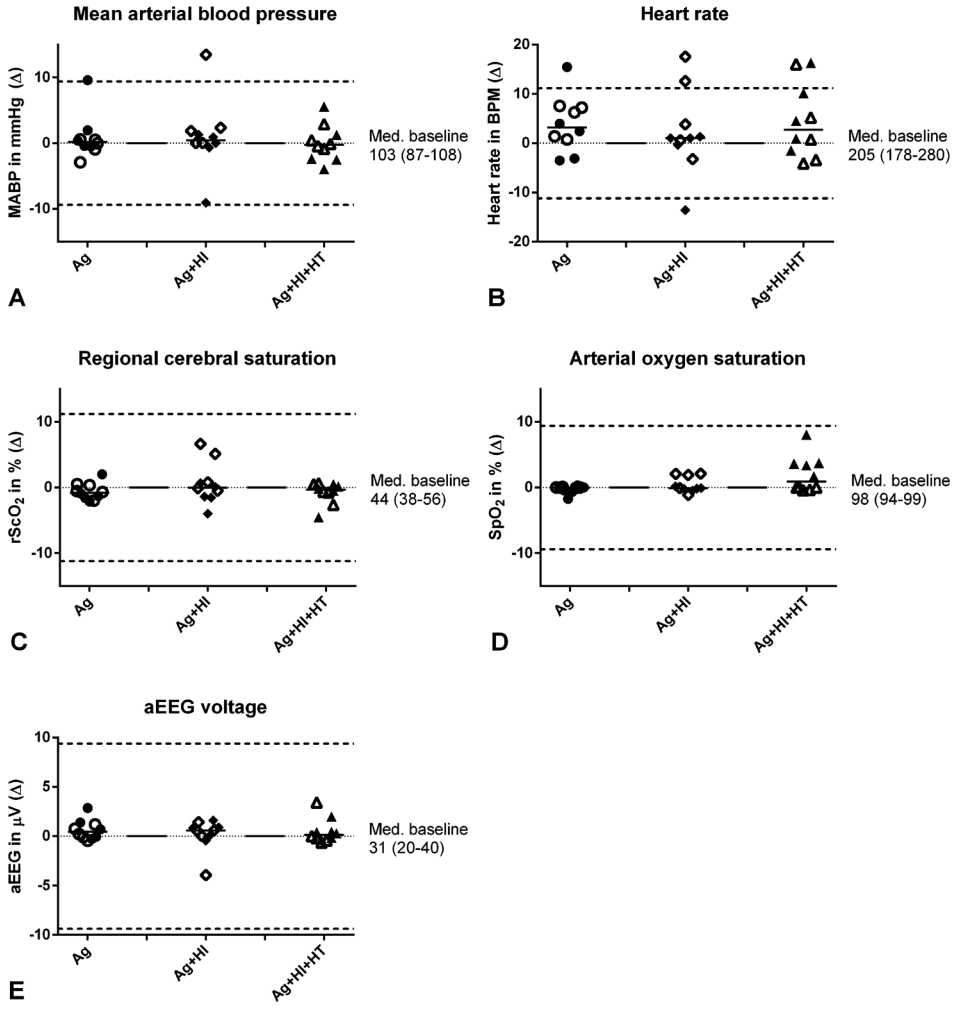
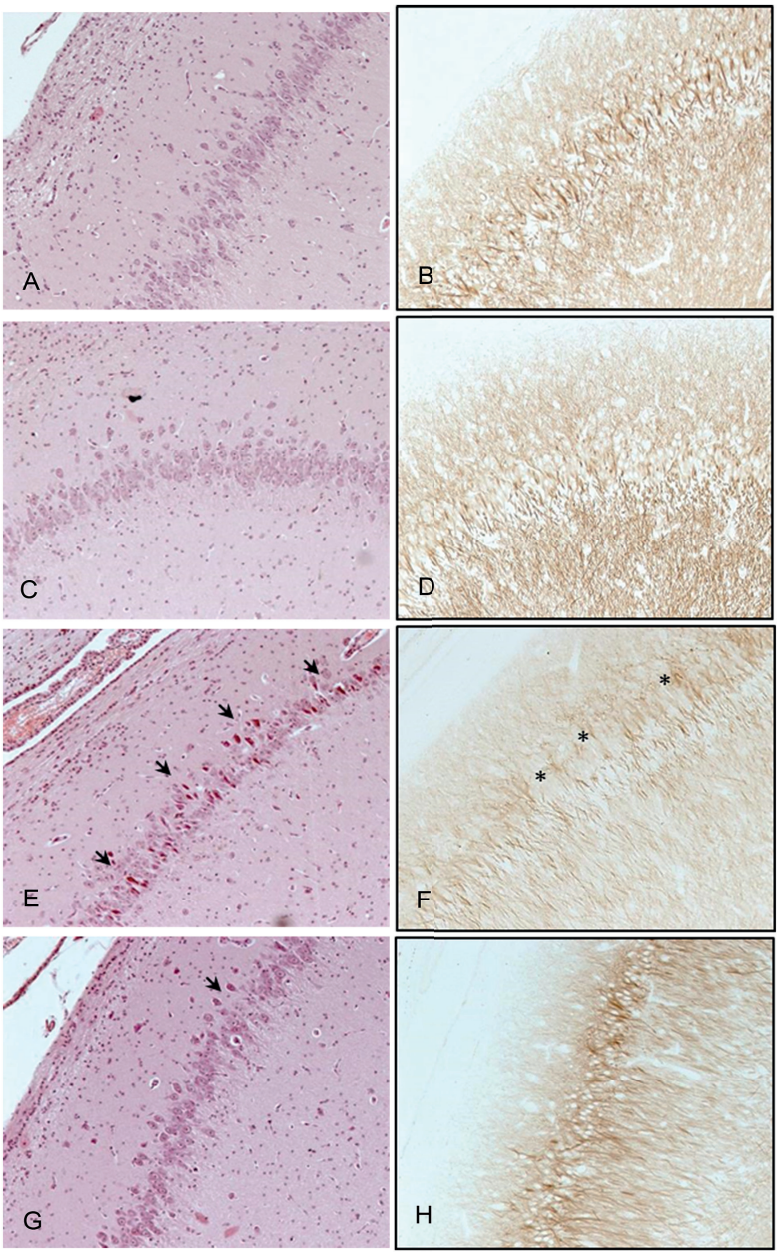


Figure 6: Hematoxylin-eosin staining (left) and microtubule associated protein 2 (right) stained slides of the hippocampus of (top-to bottom):

A,B: home cage controls, C,D: animals ventilated with argon under normoxic conditions, E,F: animals ventilated with argon after hypoxia, and G,H: animals ventilated with argon after hypoxia that subsequently underwent hypothermia. No differences between home cage controls and argon ventilated normoxic animals were demonstrated. Marked brain damage was seen in hypoxic animals demonstrated by pyknotic nuclei (arrowheads in panels E and G) and MAP2 loss (asterisks in panel F).



Histology

In histopathologic examination, HE staining of the hippocampus did not demonstrate an increase in pyknotic cell nuclei in piglets that were ventilated with argon under normoxic conditions as compared to the control piglets (Figure 6, panels A vs. C). The amount of MAP2 staining in the hippocampus was also comparable between the control piglets and piglets ventilated under normoxic conditions (Figure 6, panels B vs. D).

Figure 6 also presents HE and MAP2 stained hippocampal slides of piglets that underwent hypoxia before argon ventilation. Brain injury could be demonstrated in HE slides by pyknotic cell nuclei in multiple areas of the hippocampus (Figure 6, panels E and G). In addition, there was a loss in MAP2 most markedly seen in piglets undergoing hypoxia without subsequently receiving hypothermia. (Figure 6, panel F). Although less severe, HE and MAP2 stained slides of the cerebral cortex had an aspect similar to the hippocampus, with pyknotic cell nuclei and MAP2 loss (data not shown).

Discussion

In the present study, the potential effect of argon ventilation on important physiological parameters was explored. A subgroup of animals was subjected to hypoxia and subsequent hypothermia to mimic the conditions during which argon will be used in clinical settings. Being exposed to hypoxia, and undergoing hypothermia tended to show some effects on HR and blood pressure. However, argon ventilation did not have any effect on these parameters. In addition, argon did not have negative effects on important cerebral functions such as aEEG or cerebral saturation, and did not seem to induce additional brain injury as assessed by histopathology in normoxic animals.

Our results show a dose-dependent systemic exposure for argon and that this exposure can be quantified in blood samples. Furthermore, increased blood argon concentrations following argon ventilation up to 80% in normoxic animals, and of 50% in hypoxic animals did not have any effect on important physiological parameters as mentioned above. In hypoxic animals the dose of 50% argon was used since this is the concentration most likely to be used in a clinical setting where additional oxygen may be needed for neonates with pulmonary problems in addition to perinatal asphyxia.

Previous studies have focused on the noble gas xenon for additive neuroprotection. Xenon demonstrated stable hemodynamics, independent of induced hypothermia. (17) Moreover, xenon ventilation up to 18 hours has been shown to be feasible in the human neonatal population in addition to hypothermia.(18) It is worth noting that xenon ventilation requires complex ventilators that recirculate the xenon gas because of the price of xenon. On top of this disadvantage, xenon has anesthetic properties at normobaric pressures that could potentially influence the neurological state of the patient, thereby complicating the assessment of the (neurological) condition of the patient by clinical examination or neuromonitoring (i.e. near-infrared spectroscopy (NIRS) or aEEG).(19)

Argon has the advantage over xenon that it is more abundant in the earth's atmosphere and therefore cheaper to produce and can be used for ventilation by only slightly modifying existing (neonatal) ventilators. Another advantage is that argon only has anesthetic properties at hyperbaric pressures and thus would not influence the neurological status of the patient in the intended clinical setting (i.e. 50% ventilation at normobaric pressures). In the past inhalation of 79% argon has been used in humans to measure coronary and myocardial blood flow [22,23], and recently it has been used in athletes to enhance performance (see World Anti-Doping Agency: www.wada-ama.org).

The present study was not designed to assess the neuroprotective effect of argon ventilation, as we did not include a group that underwent hypoxia without subsequent argon ventilation. However, the neuroprotective effect of argon ventilation has already been shown in pure neuronal cell cultures and organotypic hippocampal slice cultures following glucose deprivation and traumatic brain injury.(9,10) More recently, it was shown that argon significantly decreased infarction volumes and improved composite adverse outcome after unilateral internal carotid artery ligation and subsequent hypoxia in a rodent model.(12) In addition, the neuroprotective effect of argon has been demonstrated both in rodent and porcine models of cardiopulmonary resuscitation following cardiac arrest.(13,14) In contrast to xenon, the underlying mechanisms of neuroprotection by argon are not yet elucidated, but direct actions on specific cell death pathways in myocardial ischemia have been suggested.(20) Potential mechanisms include an increase of Bcl-2, which promotes cell survival.(12) In addition, argon modifies extracellular signal-regulated kinase 1/2 signaling in neurons and glial cells.(21) Another possible mechanism might be an indirect effect mediated by the GABAA receptor.(22) Based on our observations and the previous, safe use of argon in adults in the past, as well as the efficacy studies described above, further safety studies in human neonates with perinatal asphyxia seem warranted.

Although the current study has a limited sample size, it was specifically designed to detect a 10% difference in any parameter with a power of 80%.

In conclusion, we have demonstrated that inhalation of up to 80% argon in normoxic animals and of 50% in hypoxic animals did not affect blood pressure, heart rate, cerebral saturation and electrocortical brain activity in normoxic newborn piglets, and following hypoxia with subsequent therapeutic hypothermia. We therefore suggest that clinical safety studies in human neonates seem justified.

Acknowledgements

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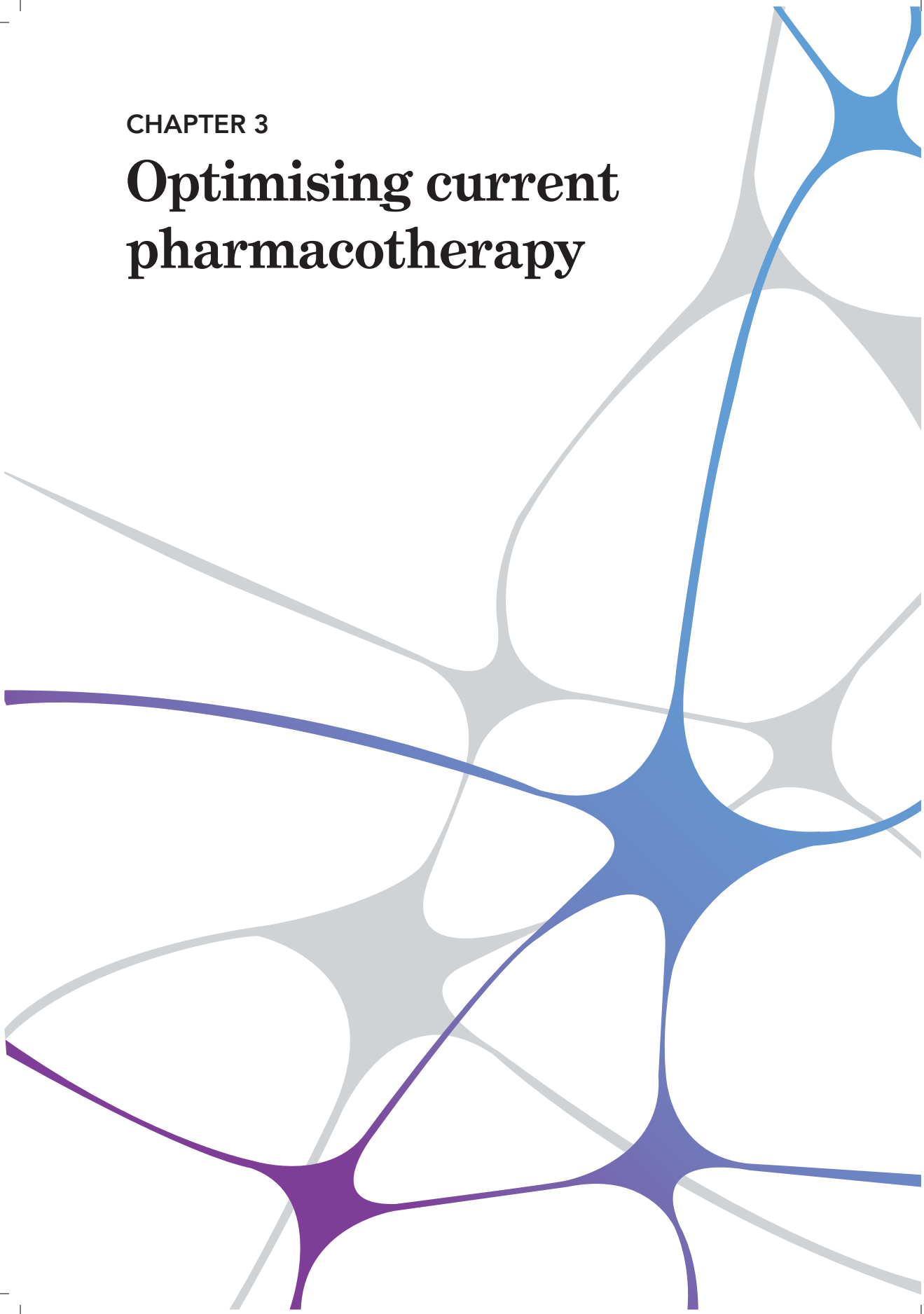
CHAPTER 3

Optimising current pharmacotherapy



Chapter 3.1

Pharmacokinetics of morphine in encephalopathic neonates treated with therapeutic hypothermia

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Abstract

Objective: Morphine is a commonly used drug in encephalopathic neonates treated with therapeutic hypothermia after perinatal asphyxia. Pharmacokinetics and optimal dosing of morphine in this population are largely unknown. The objective of this study was to describe pharmacokinetics of morphine and its metabolites morphine-3-glucuronide and morphine-6-glucuronide in encephalopathic neonates treated with therapeutic hypothermia and to develop pharmacokinetics based dosing guidelines for this population.

Study design: Term and near-term encephalopathic neonates treated with therapeutic hypothermia and receiving morphine were included in two multicentre cohort studies between 2008-2010 (SHIVER) and 2010-2014 (PharmaCool). Data were collected during hypothermia and rewarming, including blood samples for quantification of morphine and its metabolites. Parental informed consent was obtained for all participants.

Results: 244 patients (GA mean (sd) 39.8 (1.6) weeks, BW mean (sd) 3,428 (613) g, male 61.5%) were included. Morphine clearance was reduced under hypothermia (33.5°C) by 6.89%/°C (95% CI 5.37%/°C – 8.41%/°C, $p<0.001$) and metabolite clearance by 4.91%/°C (95% CI 3.53%/°C – 6.22%/°C, $p<0.001$) compared to normothermia (36.5°C). Simulations showed that a loading dose of 50 µg/kg followed by continuous infusion of 5 µg/kg/h resulted in morphine plasma concentrations in the desired range (between 10 and 40 µg/L) during hypothermia.

Conclusions: Clearance of morphine and its metabolites in neonates is affected by therapeutic hypothermia. The regimen suggested by the simulations will be sufficient in the majority of patients. However, due to the large interpatient variability a higher dose might be necessary in individual patients to achieve the desired effect.

Introduction

Hypoxic-ischemic encephalopathy (HIE) following perinatal asphyxia is one of the leading causes of death and disability in term and near term neonates. Therapeutic hypothermia (TH, lowering the core temperature to 33-34° C for 72h) is an established neuroprotective strategy and has become standard of care for these patients in developed countries.(1,2) In the Netherlands, approximately 150-200 neonates receive this treatment annually using whole-body cooling.(3)

Morphine is a commonly used drug in hypothermic neonates to provide analgesia and sedation, and is considered an important drug since stress may reduce the neuroprotective effects of TH.(4) Morphine undergoes extensive hepatic metabolism and its predominant metabolite is morphine-3-glucuronide (M3G) which is non-sedative. The less abundant metabolite morphine-6-glucuronide (M6G) is pharmacologically active with similar or greater sedative and analgesic effects compared to the parent compound.(5,6) Both glucuronide metabolites are formed by UDP glucuronosyltransferase 2B7 (UGT2B7).(5) The UGT2B7 enzyme activity in neonates is less than 10% of that in adults, but increases rapidly during the first days after birth.(7,8) Both metabolites are eliminated through the kidneys.(5) At birth, renal function is underdeveloped compared to older children and adults. In the first few weeks of life, a steady increase in renal function can be seen.(8) Thus, maturation of kidney function might influence metabolite elimination.(8,9)

Hypothermia might influence numerous physiological processes involved in drug metabolism. Hypothermia reduces cardiac output and increases vascular resistance, which leads to decreased liver perfusion. Decreased liver perfusion might result in decreased drug clearance, especially in drugs with a high hepatic extraction ratio. Furthermore, the activity of liver enzymes such as cytochrome P450 and UGT27B can be declined during TH resulting in impaired clearance. Likewise, TH

can decrease renal drug clearance by reducing kidney perfusion and subsequent glomerular filtration but also through changes in tubular secretion and reabsorption.(10–12) Additionally, pharmacokinetics (PK) of drugs administered to these neonates may be altered due to hypoxia-ischemia related multi-organ failure.(13,14) In recent years, studies have been conducted that investigated the PK of frequently used drugs in neonates undergoing TH. The findings of these studies have led to dose recommendations for several antibiotics and anticonvulsive drugs.(15–21)

Morphine PK in normothermic neonates has been investigated in several studies, mostly involving preterm and term neonates following major thoracic and abdominal surgery.(22) Neonates with a postnatal age (PNA) below 10 days had a markedly reduced morphine clearance compared to older children which has been attributed to impaired glucuronidation. This effect was independent of birth weight (BW) or gestational age (GA). Maintenance dose in this group was reduced by 50% compared to older children to achieve morphine plasma concentrations between 10 and 40 µg/L.(9) This dosing algorithm has been prospectively validated and body weight has been shown to accurately predict morphine clearance across the entire paediatric population.(23–25)

Morphine PK in neonates with HIE undergoing TH has only sparsely been investigated. Róka et al. (2008) found elevated morphine plasma concentrations in neonates treated with TH (N=10) compared to non-asphyxiated normothermic controls (N=6) with similar infusion rates and cumulative doses.(26) Frymoyer et al. (2016) developed a population PK model for morphine, M3G and M6G during TH using data from 20 neonates. They concluded that morphine clearance during TH was lower compared to previous studies in normothermic asphyxiated neonates and advised a loading dose of 50 µg/kg followed by 5 µg/kg continuous infusion.(27) Both studies did not include data during and after rewarming. Additional characterisation of morphine PK using a larger dataset is imperative to guide

clinicians in the application of this widely used and important drug in this critically ill population.

The objective of the present study was to describe the PK of morphine and its metabolites in neonates with HIE both during and after TH using nonlinear mixed effect modelling and to develop pharmacokinetics based dosing guidelines based on a large dataset obtained from two multicentre studies conducted in the Netherlands and Belgium.

Patients and methods

Setting, study design and study population

The open label prospective SHIVER study was performed in the tertiary neonatal intensive care units (NICU) of the University Medical Center Utrecht, Utrecht and Isala Clinics, Zwolle. The open label prospective PharmaCool study was conducted in twelve tertiary NICUs in the Netherlands and Belgium.(28) In both studies, term neonates undergoing TH for HIE were eligible for inclusion. According to national treatment protocol, neonates with a GA between 36.0 and 42.0 weeks were cooled within 6 hours after birth to a core temperature of 33.5°C (accepted range 33.0-34.0 °C) for 72 hours. Thereafter, patients were slowly (0.4°C/hour) rewarmed to normothermia (36.5°C). After rewarming, body temperature was stabilized at 36.5°C for 24 hours.(3) Exclusion criteria were severe congenital malformations, encephalopathy due to other causes than perinatal asphyxia and the absence of central venous or arterial access for non-invasive blood sampling. From each included patient, written parental informed consent was obtained. Inclusion took place between 2008-2010 (SHIVER) and 2010-2014 (PharmaCool). In total, 339 patients were screened and 277 included. For the present study, neonates participating in either study and receiving intravenous morphine were selected. Data analysis was completed in 2018. The SHIVER study was approved by the

Institutional Review Board (IRB) of the University Medical Center Utrecht (no. 08/404) and subsequently approved by the IRB of the Isala Clinics, Zwolle. The PharmaCool study was approved by the IRB of the Academic Medical Center Amsterdam (no. 10/255) and subsequently approved by the IRBs of the VU Medical Center Amsterdam, University Medical Center Utrecht, Leiden University Medical Center, Erasmus Medical Center Rotterdam, Maxima Medical Center Veldhoven, Maastricht University Medical Center, Radboud University Medical Center Nijmegen, Isala Clincs Zwolle, University Medical Center Groningen, University Hospital Gent and University Hospital Brussels.

Morphine dosing and administration

In both studies, morphine was administrated as morphine hydrochloride according to local protocols and/or the attending physician's discretion as an intravenous continuous infusion, often preceded by a loading dose. Morphine was generally started at the onset of TH or shortly before. Dose adjustments, including administration of any additional loading dose, were based on each patient's clinical condition and were not influenced by the study protocol.

Pharmacokinetic sampling and bioanalysis

From all patients, 1 ml blood samples were obtained from an indwelling catheter on four consecutive days, both during hypothermia and rewarming/normothermia. Sampling was scheduled at designated time points at 24 hours intervals. This limited sampling strategy was designed to minimize patient risk while still obtaining sufficient information to achieve the study objective. In the SHIVER study, residual material from blood samples taken for clinical care were available for some patients. Plasma concentrations of morphine, M3G and M6G were determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The lower limit of quantification (LLQ) was 10 µg/L for morphine and M3G and 5 µg/L for M6G. The calibration curves were linear from 10 to 1000 µg/L for morphine, 10 to 600 µg/L

for M3G and 5 to 200 µg/L for M6G. Between-run and within-run coefficients of variation were <5% for morphine and M3G and <8% for M6G. Samples were stored at -80°C until analyses at the Clinical Pharmaceutical and Toxicological Laboratory of the Department of Clinical Pharmacy of the University Medical Center Utrecht, the Netherlands.

Population pharmacokinetic analysis

A population pharmacokinetic model was developed from morphine, M3G, and M6G concentration–time data using the nonlinear mixed effect modelling program NONMEM (version 7.3, Icon Development Solutions) with R (version 3.4.1), Xpose (version 4) for data visualization and Piraña for run management. (29) Morphine hydrochloride (molecular weight (MW) 321.8 g/mol) doses were converted to morphine base (MW 285.3 g/mol) and consecutively, all units of dose and concentration for morphine, M3G and M6G (MW 461.5 g/mol) were converted to µmol and µmol/L, respectively for the purpose of the pharmacokinetic analysis. BW was used as a descriptor for body size in our population and was related to pharmacokinetic parameters using allometric relationships. The exponent defining the relationship of BW and clearance (Cl) was fixed to 0.75 and the exponent defining the relationship of BW and volume of distribution (V) was fixed to 1. The fractions of morphine converted to the metabolite M3G and M6G in neonates under hypothermia were unknown (FM3G and FM6G, respectively); therefore, parameters relative to F were estimated (e.g. ClM3G/FM3G and VM3G/FM3G). Based on previously published pharmacokinetic models of morphine in neonates(27,30), one- and two-compartment models for morphine and subsequent one-compartment models for both metabolites were tested as a structural model. Morphine and metabolite data were fitted simultaneously.

To study the effect of hypothermia on pharmacokinetics, a dynamic model of temperature over time was included, which allowed prediction of the actual body

temperature at each moment of sampling. For all patients, the reported start and end times of TH were used to determine the period of TH treatment. Body temperature during hypothermia was set at 33.5°C, with consecutive rewarming at 0.4°C/hour (i.e. rewarming time 7.5h) until 36.5°C after which body temperature was set to 36.5°C for the remainder of the study time. Calculated body temperature for each plasma sample was subsequently included in the PK model.
As renal function may be an important determinant for metabolite clearance and given that renal function cannot be estimated from a single serum creatinine (SCr) measurement in neonates, a model for SCr was developed using daily SCr values taken for clinical care from all patients. In this model, the elimination rate of SCr was used as a surrogate marker for renal function. PNA and GA were tested as covariates on both morphine and metabolite clearance. Inclusion of covariates was guided by effect size, biological plausibility and statistical significance (using the likelihood ratio test which assesses the difference in the NONMEM objective function value (OFV), which is equal to minus twice the log likelihood, with a p-value of <0.05 as cut-off for significance).

Interindividual variability (IIV) was modelled using a proportional model and tested on all parameters. Covariance between IIV components was included based on physiological plausibility and graphical exploration. A proportional error model was used to model residual unexplained variability. For each compound, separate error models were used. Parameter precision was assessed with sampling importance resampling (SIR).(31) Internal validation of the final model was evaluated by computing the normalized prediction distribution errors (NPDE, 1000 simulations). (32) Both graphical (e.g. goodness-of-fit plots, visual predictive check) and statistical model evaluation procedures were used to assess model adequacy.

Dosing regimen development

Simulations were conducted to test four different dosing regimens using the

parameter estimates from the final pharmacokinetic model. To create the simulation dataset, the patient characteristics of each neonate included in this study were replicated five times. Morphine loading dose was simulated at PNA 4 hours, immediately followed by continuous infusion. The following dosing regimens were evaluated, based on the current clinical practice: 1. loading dose of 50 µg/kg followed by continuous infusion of 5 µg/kg/h; 2. loading dose of 50 µg/kg, continuous infusion of 10 µg/kg/h; 3. loading dose of 100 µg/kg, continuous infusion of 5 µg/kg/h; 4. loading dose of 100 µg/kg, continuous infusion of 10 µg/kg/h. The dynamic temperature model was used to introduce TH. For each neonate in the simulation dataset, TH (body temperature of 33.5°C for 72 hours) was simulated to start at PNA 5 hours, after which rewarming commenced at 0.4°C/hour. After rewarming, body temperature was fixed to 36.5°C for the remainder of the simulations. Hourly plasma concentrations were predicted until PNA 120 hours. Morphine plasma concentrations between 10 and 40 µg/L were considered effective and safe.

Results

Patient characteristics

For 244 neonates morphine dosing information and at least one morphine plasma concentration was available for analysis (Table 1). In general, loading doses between 50 and 100 µg/kg were given, followed by continuous infusion with doses varying between 5 and 25 µg/kg/h.

A total of 853 blood samples were analyzed (median 4 samples per patient, range 1-11). Samples with measurements below LLQ for all compounds (n = 23) were excluded from further analyses, leaving 830 viable samples. Of these, 550 (66.3%) were drawn during the hypothermic phase. For 18 patients (7.4%), only one sample was available. Plasma concentrations for morphine varied between 10.0 and 371.2 µg/L (Fig 1); for M3G between 11.0 and 930.6 µg/L and for M6G between 5.1

and 211.2 µg/L (S1 Fig). In one patient (0.41%), morphine plasma concentrations exceeding 300 µg/L were reached.

Table 1: Patient characteristics

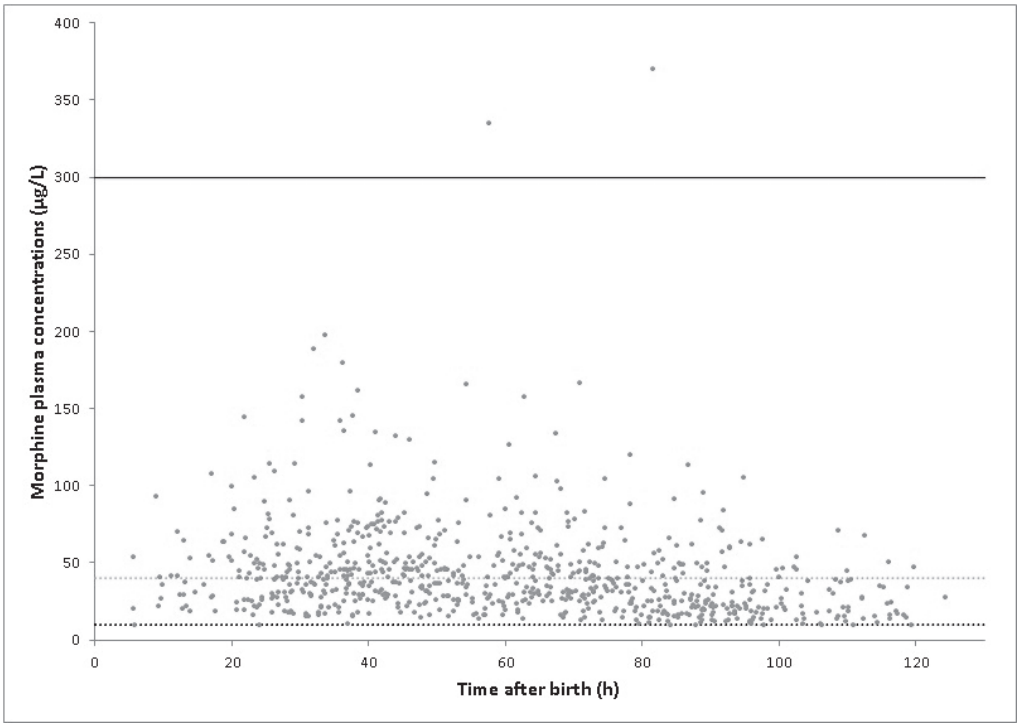
Parameter	**Patients (N = 244)**
Gestational age; wk, mean ± sd	39.8 ± 1.6
Birth weight; g, mean ± sd	3,428 ± 613
Birth weight ≤ 2500 g; n (%)	16 (6.6%)
Male; n (%)	150 (61.5%)
pH*; median (IQR)	6.96 (6.80 – 7.09)
Base Excess*; mmol/L, median (IQR)	-17 (-12.0 – -21.9)
Lactate*; mmol/L, median (IQR)	13.6 (9.0 – 18.2)
Thompson score#; median (IQR)	9.5 (8.0 – 12.0)
aEEG on admission#	
Continuous normal voltage; n (%)	30 (12.3%)
Discontinuous normal voltage; n (%) *of whom < 5 µV; n (%)*	102 (41.8%) 35 (14.3%)
Burst suppression; n (%)	58 (23.8%)
Continuous low voltage; n (%)	10 (4.1%)
Flat trace; n (%)	27 (11.1%)
Unknown; n (%)	17 (7.0%)
Mortality; n (%)	58 (23.8%)

sd = standard deviation, IQR = interquartile range

*Value measured in umbilical cord blood or, if unavailable, from arterial or venous blood within 1h after birth

#Encephalopathy was characterized by a Thompson score of >7 1h after birth or an abnormal aEEG on admission to a level III NICU

Figure 1: Observed morphine plasma concentrations (µg/L). Dotted lines indicate the proposed therapeutic window of 10-40 µg/L; solid line indicates the potentially toxic limit of 300 µg/L.



Population pharmacokinetic analysis

A one-compartment model for morphine and subsequent one-compartment models for both metabolites provided the best fit for the data. Pharmacokinetic parameter estimates of the final model are shown in Table 2.

Introduction of a peripheral compartment for morphine resulted in an unstable model with unrealistic intercompartmental clearance. GA and PNA were identified as covariates on morphine clearance (GA: $p<0.001$, PNA: $p<0.001$), but not on metabolite clearance. Morphine clearance was increased by 50.4% at PNA 5 days, compared to birth (increase of 0.42%/h, 95%CI 0.297%/h – 0.582%/h); at birth, morphine clearance in a neonate with GA 36 weeks was 46% lower compared to GA

Table 2: Final model pharmacokinetic parameter estimates and SIR results

	Morphine		M3G§		M6G§	
Parameter	Estimate	SIR* 95% CI	Estimate	SIR* 95% CI	Estimate	SIR* 95% CI
Cl, l/h[#]	0.899	0.797 – 0.985	0.456	0.424 – 0.492	1.73	1.61 – 1.87
V, l[#]	8.88	7.87 – 9.92	0.264	0.089 – 0.384	4.53	3.64 – 5.39
PNA on Cl; %/h	0.420	0.297 – 0.582	NA	NA	NA	NA
GA on Cl; %/d	1.66	1.30 – 1.94	NA	NA	NA	NA
TEMP on Cl; %/°C	6.89	5.37 – 8.41	4.91[$]	3.53 – 6.22[$]	4.91[$]	3.53 – 6.22[$]
Interindividual variability						
Cl, variance (rsd)	0.224 (47.3%)	0.185 - 0.276	0.291[$] (53.9%)	0.240-0.356[$]	0.291[$] (53.9%)	0.240-0.356[$]
V, variance (rsd)	0.464 (68.1%)	0.364 – 0.602	NA	NA	NA	NA
Covariance interindividual variability $Cl_{morphine}/Cl_{metabolites}$						
Covariance (correlation coefficient)	0.117 (46.0%)	0.0799 – 0.161				
Residual variability						
Proportional, variance (rsd)	0.0498 (22.3%)	0.0437 – 0.0574	0.0914 (30.2%)	0.0798 – 0.105	0.101 (31.8%)	0.0888 – 0.115

Final model:

$Cl_{MORPHINE}$ = 0.899 x (BW/3.5)$^{0.75}$ x (1 + 0.0042 x PNA) x (1 + 0.0166 x (GA-280)) x (1 + 0.0689 x (TEMP-36.5))

$V_{MORPHINE}$ = 8.88 x (BW/3.5)^{1}

Cl_{M3G}/F_{M3G} = 0.456 x (BW/3.5)$^{0.75}$ x (1 + 0.0491 x (TEMP-36.5)

V_{M3G}/F_{M3G} = 0.264 x (BW/3.5)^{1}

Cl_{M6G}/F_{M6G} = 1.73 x (BW/3.5)$^{0.75}$ x (1 + 0.0491 x (TEMP-36.5)

V_{M6G}/F^{M6G} = 4.53 x (BW/3.5)^{1}

§All metabolite estimates are relative to formation fraction FM3G and FM6G, resp.

*Ten iterations; no. of samples 1000,1000,1000,1000,1000,1000,2000,2000,2000,2000; no. of resamples 200,200,400,400,500, 500,1000,1000,1000,1000

#Estimates for neonate with BW 3.5 kg, GA 280 days, PNA 0h and TEMP 36.5°C

$Single estimate for both metabolites

V = volume of distribution, Cl = clearance, PNA = postnatal age, GA = gestational age, TEMP = body temperature, M3G = morphine-3-glucuronide, M6G = morphine-6-glucuronide, SIR = sampling importance resampling, BW = birth weight, NA = not applicable, rsd = relative standard deviation

40 weeks, while clearance in a neonate with GA 42 weeks is 23% higher (difference of 1.66%/d, 95%CI 1.30% – 1.94%). The elimination rate of SCr was introduced as a covariate on the clearance of the metabolites as a measure of renal function. The influence of this covariate was non-significant and therefore excluded from the final model.

Subsequently, the dynamic model of temperature over time was included as covariate on Cl. The influence of body temperature on clearance was separated into an effect on $Cl_{MORPHINE}$ (a combination of hepatic and renal clearance) and $CL_{METABOLITES}$ (renal clearance). As the effect of body temperature on M3G and M6G clearance were similar and separate effects for both metabolites did not improve model performance, this was estimated as a single effect in the final model. Morphine clearance during hypothermia was decreased by 20.7% ($p<0.001$, 6.89%/°C, 95% CI 5.37%/°C – 8.41%/°C) compared to normothermia. Metabolite clearance during hypothermia was decreased by 14.7% ($p<0.001$, 4.91%/°C, 95% CI 3.53%/°C – 6.22%/°C).

The influence of BW, GA, PNA and temperature on the average morphine clearance is predicted by the final model are depicted in Figure 2.

Model evaluation demonstrated that the final model was adequate in describing the data. Goodness-of-fit plots of observed versus population and individual predicted concentrations showed no systematic deviation and the weighted residuals were homogeneously scattered for both parent and metabolites (S2-S4 Figs). NPDE plots for morphine, M3G and M6G indicate that the NPDE follows the normal distribution and that the model does not contain major bias (S5-S7 Figs).

Dosing regimen

Morphine plasma concentrations after various dosing regimens were predicted

Figure 2: Average predicted morphine clearance over time before, during and after TH for neonates with BW 3.5 and GA 36, 38, 40 and 42 weeks, respectively (left) and for neonates with GA 40 weeks and BW 2.5, 3.0, 3.5 and 4.0 kg, respectively (right). Solid vertical lines represent the start and end of TH (33.5°C) simulated between 5h and 77h after birth; dashed vertical line indicates the return to normothermia (36.5 °C) with rewarming simulated at. 0.4 °C/h; TH = therapeutic hypothermia, BW = birth weight GA = gestational age.

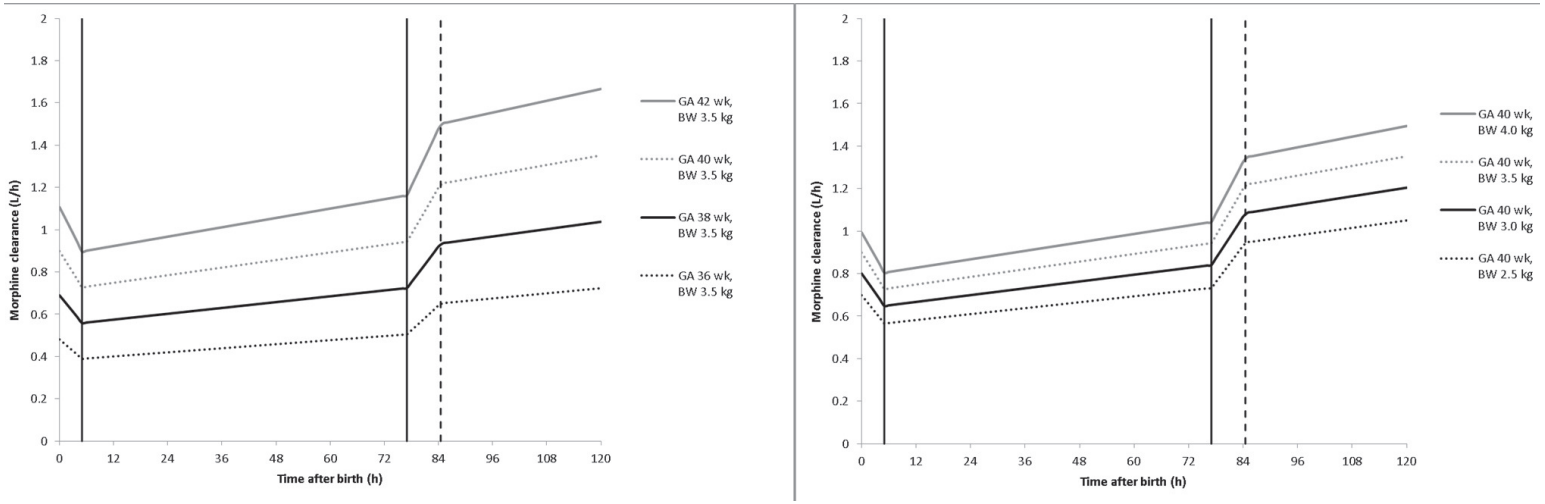
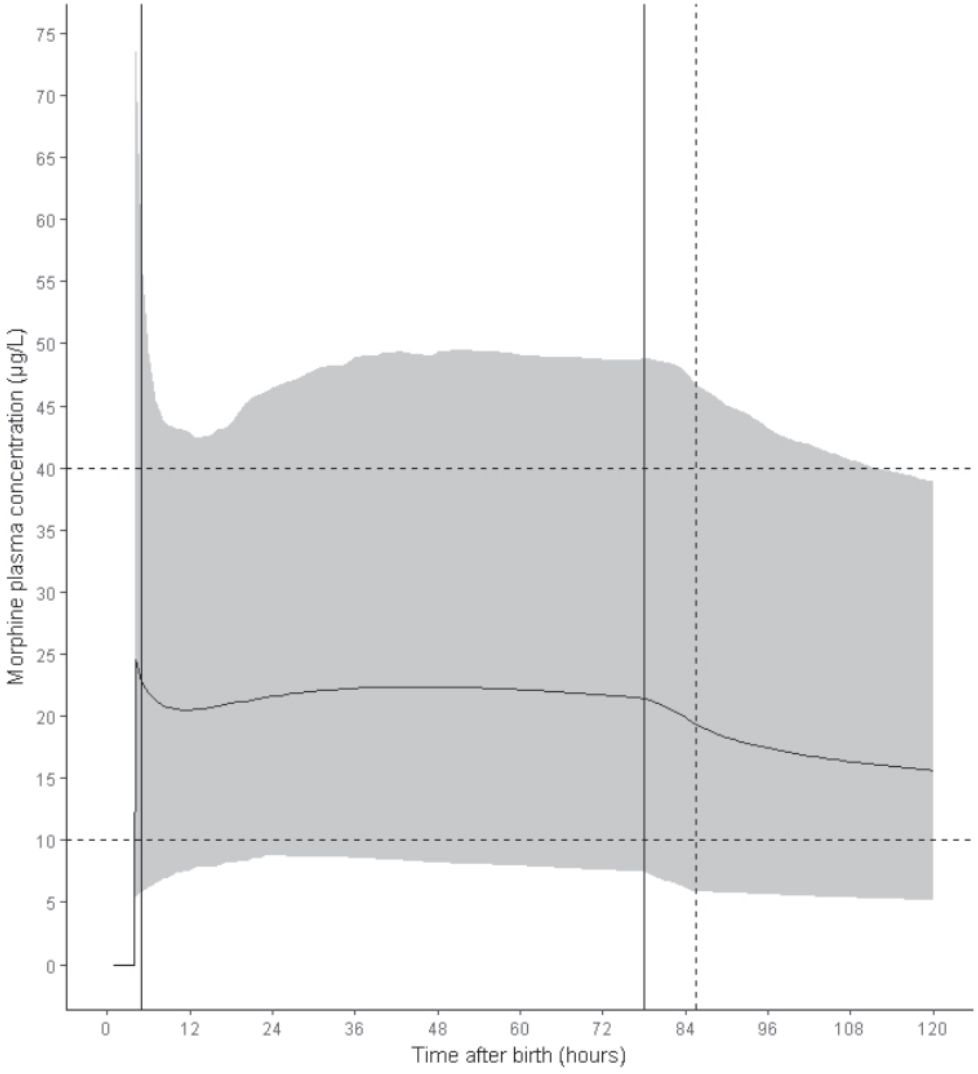


Figure 3: Simulated morphine plasma concentrations of the proposed dosing regimen of 5 µg/kg/h after loading dose of 50 µg/kg. Solid line indicates the mean morphine plasma concentration; gray area represents the 95% prediction interval. Dotted horizontal lines indicate the proposed therapeutic window of 10-40 µg/L. Solid vertical lines indicate the start and end of TH (33.5°C) simulated between 5h and 77h after birth; dashed vertical line indicates the return to normothermia (36.5 °C) with rewarming simulated at 0.4 °C/h; TH = therapeutic hypothermia



using a simulation dataset of 1220 patients and the final PK parameter estimates. In all simulations, morphine clearance was markedly influenced by PNA and TH. Immediately after rewarming, average morphine clearance was increased by 63.4% compared to clearance at the start of TH. Of this increase, 29.6% could be attributed to an effect of PNA. A maintenance dose of 5 μg/kg/h preceded by a loading dose of 50 μg/kg resulted in plasma concentrations between 10 and 40 μg/L at PNA 12h in 88.2% of patients, while 7.8% of patients were below 10 μg/L and 4.0% above 40 μg/L. At PNA 48h, morphine plasma concentration exceeded 40 μg/L in 6.8% of patients. As clearance is not constant but increased over time, no steady state in morphine plasma concentration was reached in the first five days of life. At PNA 77 hours, TH was stopped resulting in an additional increase in clearance and drop in plasma concentration (Fig 3). Plasma concentrations for both metabolites accumulated during TH but reached steady state once clearance increased under normothermic conditions (S8 Fig). Morphine plasma concentrations for the other simulated dosing regimens are included as a supplement (S9 Fig).

Discussion

This study combining data from two large multicenter studies shows that clearance of both morphine and its metabolites is reduced during hypothermia in neonates with HIE compared to normothermia. Furthermore, the impact of BW, GA and PNA on the PK of morphine and its metabolites has been quantified. Reduction in clearance during TH is most likely caused by a decrease in perfusion of the liver and kidneys. Additionally, an effect of TH on activity of UGT2B7, the enzyme responsible for metabolizing morphine into M3G and M6G, might explain why morphine clearance is more strongly affected than metabolite clearance.

Although a therapeutic window for morphine plasma concentrations has not been firmly established, especially in neonates undergoing TH, the best available

evidence suggests a preferred range between 10 and 40 µg/L, while levels above 300 µg/L have been associated with respiratory depression and prolonged mechanical ventilation.(25,33–38) Based on the simulations performed in this study, a starting dose of 50 µg/kg followed by 5 µg/kg/h is recommended to achieve morphine plasma concentrations between 10 and 40 µg/L, although the large interpatient variability (47.3% for Cl and 68.1% for V, Table 2) might lead to higher (>40 µg/l) plasma concentrations in individual patients. Contrarily, a higher morphine dose may be needed in some patients to ensure effective treatment. Clinicians should not be reluctant to increase the maintenance dose if the starting dose proves inadequate for a patient's clinical condition both during and, if morphine is not stopped simultaneously with TH, after hypothermia.

The current practice rarely leads to plasma concentrations exceeding the potentially toxic upper limit of 300 µg/L, but may lead to unnecessary high morphine exposure. Neonatal opioid use has been associated with impaired cognitive and behavioral development in animal studies.(39) Long term follow-up studies in humans suggest a possible negative effect in early childhood that does not persist later in life.(40–43) Conversely, adequate management of pain and discomfort is needed to improve recovery, to ensure the effectiveness of TH and to prevent adverse physiological responses such as changes in intrathoracic or arterial pressure and vasoconstriction of vital organs.(4,38,39)

Morphine PK was best described using a one-compartment model for morphine and subsequent one-compartment models for each of the glucuronide metabolites. Previously, PK of the parent compound has been adequately described using both one-compartment(44,45) and two-compartment models.(9,23,27) A recently published meta-model combining data obtained in neonates and older children from five separate studies proposed a one-compartment model for morphine PK.(30) Metabolite PK was adequately described using one-compartment models

for each metabolite in all studies.(9,23,27,44) Parameter estimates from this model extrapolated to a neonate with GA 40 weeks and BW 3.5 kg result in a higher morphine clearance (1.54 l/h) and a lower volume of distribution (5.25 l) compared to our findings.(30) This might be explained by the fact that our patient population consisted of critically ill term neonates admitted to a NICU. The meta-model incorporated data form both term and preterm neonates and from older children and adults as well. In all included studies, morphine was administered for post-operative pain. Morphine parameter estimates reported in a small dataset by Frymoyer et al. in the same population are in accordance with our findings, despite the differences in the underlying PK model (Cl 0.765 l/h, V 8.02 l).(27) Also, the impact of TH on morphine clearance in our study is similar to the effect found by Róka et al., who compared hypothermic neonates to non-asphyxiated normothermic controls using a non-parametric approach (Cl 0.69 l/h vs 0.89 l/h, decrease of 22.5%).(26)

GA was identified as a significant covariate on morphine clearance despite the relatively narrow range of GA (36-42 weeks) in this population. Previous reports investigating the PK of other drugs in the PharmaCool study population have reported similar effects.(18–20) In the present study, this might be explained by a lower baseline UGT2B7 activity in neonates with a lower GA. However, due to the large interpatient variability, this finding did not translate into a dosing advise differentiated by GA. For each of the situations presented in Figure 2, the simulated dosing regimen yields average morphine plasma concentrations between 10 and 40 µg/L. The majority of patients included in this study had a GA between 38 and 41 weeks (202/244, 82.8%); a lower mg/kg dose for neonates with a GA of 36 and 37 weeks only marginally improved the fraction of patients within the therapeutic window at PNA 12 hours. Furthermore, a differentiated dosing regimen based on GA within this relatively small subpopulation of NICU patients is deemed undesirable as this will be error-prone. Therefore, the proposed dosing regimen is advised for all neonates treated with TH after HIE. Currently, TH is explored in preterm neonates

(GA 34-35 weeks) as well.(46) Extrapolation of our results to these neonates should be done with caution due to the absence of this patient population in the current dataset.

Our data show an increase in morphine clearance during the first five days after birth. This effect could be identified independently of the effect of body temperature. The increase of clearance over time can be attributed to maturation of UGT2B7. Maturation of this enzyme in normothermic neonates has been described for morphine but also for other drugs predominantly glucuronidated by UGT2B7.(9,47–50) Recovery of organ function after perinatal asphyxia might also play a role in this observed increase in clearance during the first days of life. During asphyxia, the liver is deprived of oxygen, resulting in hepatocyte damage. Alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) levels are commonly used to indicate hepatocyte damage and both are frequently elevated in neonates with HIE. Peak levels are reached within 72 hours and normalize within the first two weeks of life, indicating recovery of liver function in this timeframe.(51–53) Unfortunately, ALAT and ASAT are poor predictors for hepatic drug metabolism and cannot be reliably used to predict hepatic clearance. Therefore, it was not possible to distinguish between enzyme maturation and recovery of liver damage.

Introducing temperature as a continuous covariate allowed for a more precise estimate of the effect of body temperature on the pharmacokinetics of morphine and its metabolites since also samples during rewarming were available. Additionally, including temperature as a dichotomous covariate proved to be a less adequate fit for the data. Some assumptions had to be made for this dynamic model. Firstly, we assumed that the average body temperature of each neonate during TH was 33.5°C and that possible fluctuations between 33.0 and 34.0°C would have a negligible effect. Body temperature during TH was therefore fixed to 33.5°C. Secondly, we assumed that rewarming for each neonate occurred according to national protocol at

0.4°C/hour. In clinical practice, rewarming is sometimes slowed or halted if seizures occur during rewarming. As this information was not available in our dataset, rewarming for each neonate was set at 0.4°C/hour. Thirdly, as body temperature after TH is stabilized at 36.5°C for 24 hours, we opted for a fixed body temperature of 36.5°C for each neonate after rewarming. As morphine is primarily administrated to prevent stress during hypothermia and is often stopped with or shortly after TH, we believe that this assumption is an accurate representation of clinical practice. Lastly, we assume a linear effect between body temperature and clearance and therefore report and effect per °C. Although we have no evidence for non-linearity, the limited sampling strategy was insufficient to exclude this. However, in event of non-linearity, the reported effect is an average effect per °C between 33.5 and 36.5°C and does not alter the overall effect of TH on clearance.

SCr is a specific marker for renal function and is widely used in adults to predict reduced clearance of renally excreted drugs. In neonates, SCr levels in the first few days of life are confounded by maternal SCr levels due to maternal transfer. We considered a dynamic model of SCr over time a better predictor of changes in renal function. No relationship between serum creatinine and renal clearance of the metabolites could be identified. Additionally, increased metabolite clearance over time in the first five days of life could not be observed. This effect was found in the same population for amoxicillin and benzylpenicillin, drugs that are predominantly excreted renally in unmetabolized form.(18,20) Data collection up to five days after birth might have been too short to detect maturation of renal function since steady state M3G and M6G plasma concentrations are not reached during hypothermia (S8 Fig). Also, as maturation of renal function in the first few days after birth occurs simultaneously with TH, the effect of maturation on metabolite clearance might not be distinguishable from the effect of hypothermia.

Pharmacodynamics (PD) end points of morphine were not incorporated in the final model. Although the COMFORT-B score, as indicator for pain and stress, was

routinely recorded in this population, the timing of this score in relation to morphine dosing was often unclear. Pain expression in hypothermic neonates differs from normothermic neonates, making it uncertain whether the COMFORT-B score is suitable for treatment evaluation in this population.(38,54,55) Additionally, this scale has not been developed to distinguish between adequate treatment effect (eg. adequate sedation) and supratherapeutic effects (eg. oversedation).
Strengths of this study are the large number of included patients recruited from twelve tertiary NICUs in two countries, making this study population representative for all neonates treated with TH after HIE. The dosing regimen advised in this study corresponds with the dosing advise postulated by Frymoyer et al.(27) Reconstructing the full profile of hypothermia and rewarming enabled us to accurately assess the influence of body temperature on clearance. In all participating centers, TH is applied using a uniform cooling device and with a joint treatment protocol, thereby decreasing the chance of treatment variation.(3)

Limitations are the lack of PD end points associated with morphine. Clinicians are insufficiently supported by robust tools to facilitate morphine dose adjustments. Development and validation of such an instrument should be the focus of future research. Subsequently, this tool could be used to prospectively validate our dosing regimen. Ideally, future PD studies will also incorporate M6G plasma concentrations for determining exposure-response as the contribution of this active metabolite to the effects attributed to morphine in neonates are largely unknown.

For ethical reasons, it was not possible to answer this research question using a prospective randomized controlled trial comparing hypothermic with non-hypothermic patients. Also, comparison to an adequate historical control group is not feasible as morphine nor metabolite plasma concentrations are available from before 2008.

Conclusion

Clearance of morphine and its metabolites is reduced in neonates treated with TH for HIE. Even though the current clinical practice only very rarely leads to morphine plasma concentrations exceeding 300 µg/L, a relatively low starting dose of 50 µg/kg followed by continuous infusion of 5 µg/kg/h is recommended in critically ill neonates treated with TH for HIE. However, due to the large interpatient variability, the uncertainty regarding the supposed therapeutic window and the undesirable effect of discomfort in this population, a higher maintenance dose may be required if the starting dose proves inadequate for the clinical condition of the individual patient.

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Figure S1: Observed plasma concentrations for M3G (left) and M3G (right). M3G = morphine-3-glucuronde, M6G = morphine-6-glucuronide

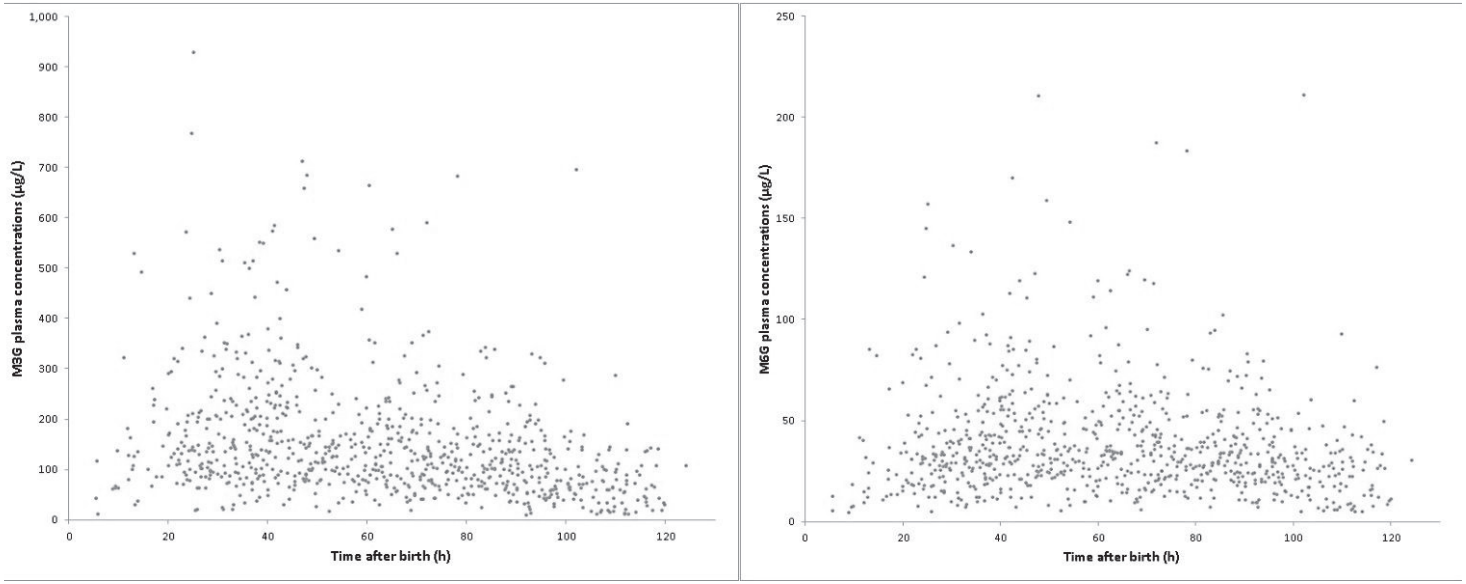


Figure S2: Morphine goodness-of-fit plots. A = observed vs population predicted plasma concentrations; B = observed vs individual predicted plasma concentrations; C = population conditional weighted residuals vs population predicted plasma concentrations; D = population conditional weighted residuals vs time after birth; solid line indicates the linear regression line

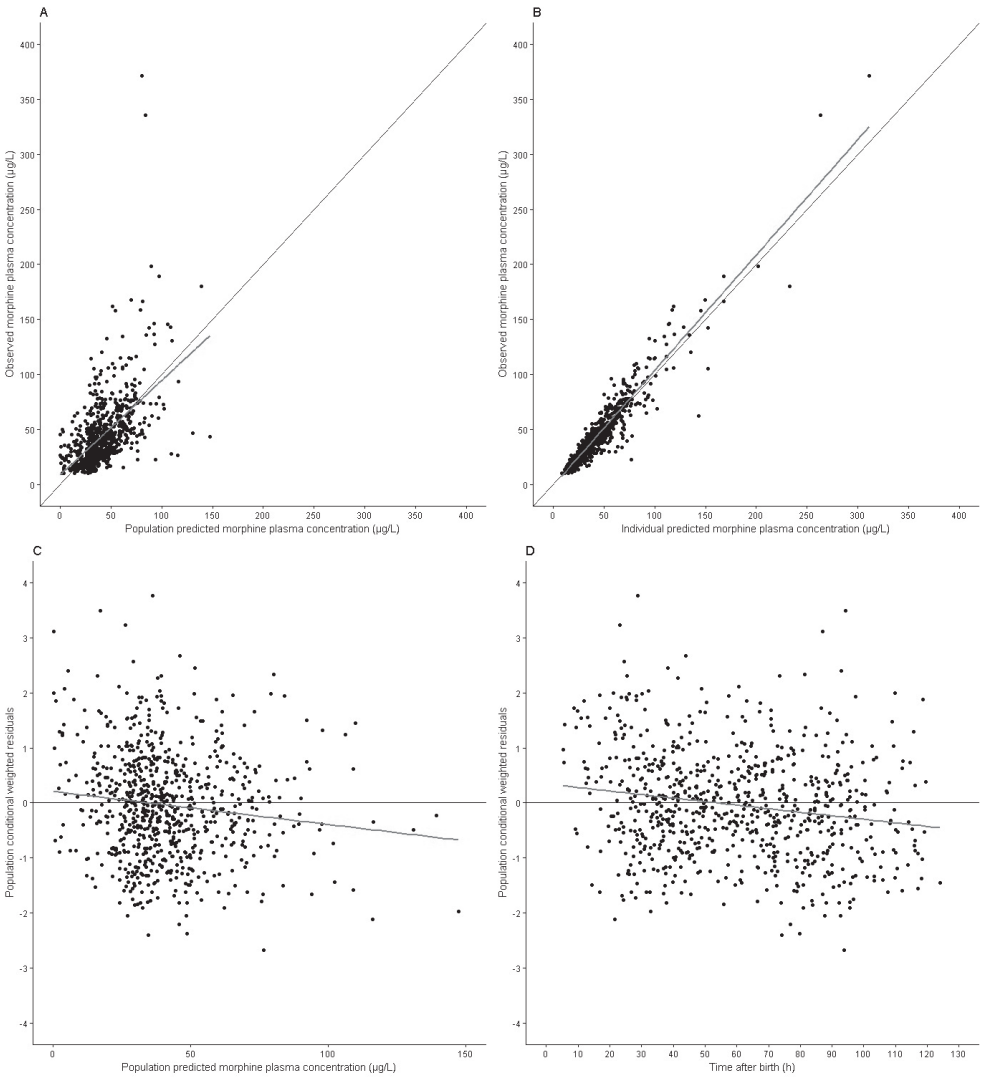


Figure S3: M3G goodness-of-fit plots. A = observed vs population predicted plasma concentrations; B = observed vs individual predicted plasma concentrations; C = population conditional weighted residuals vs population predicted plasma concentrations; D = population conditional weighted residuals vs time after birth; M3G = morphine-3-glucuronide; solid line indicates the linear regression line

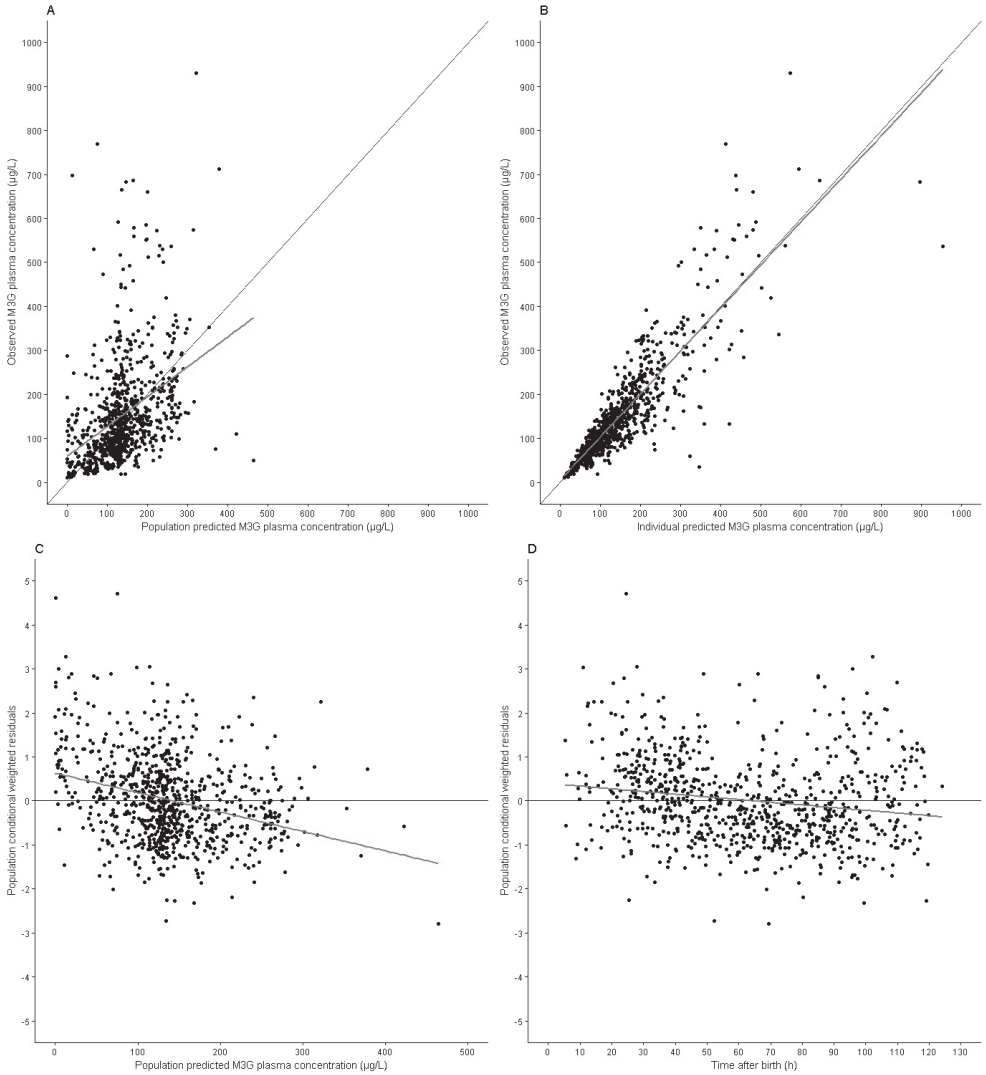


Figure S4: M6G goodness-of-fit plots. A = observed vs population predicted plasma concentrations; B = observed vs individual predicted plasma concentrations; C = population conditional weighted residuals vs population predicted plasma concentrations; D = population conditional weighted residuals vs time after birth; M6G = morphine-6-glucuronide; solid line indicates the linear regression line

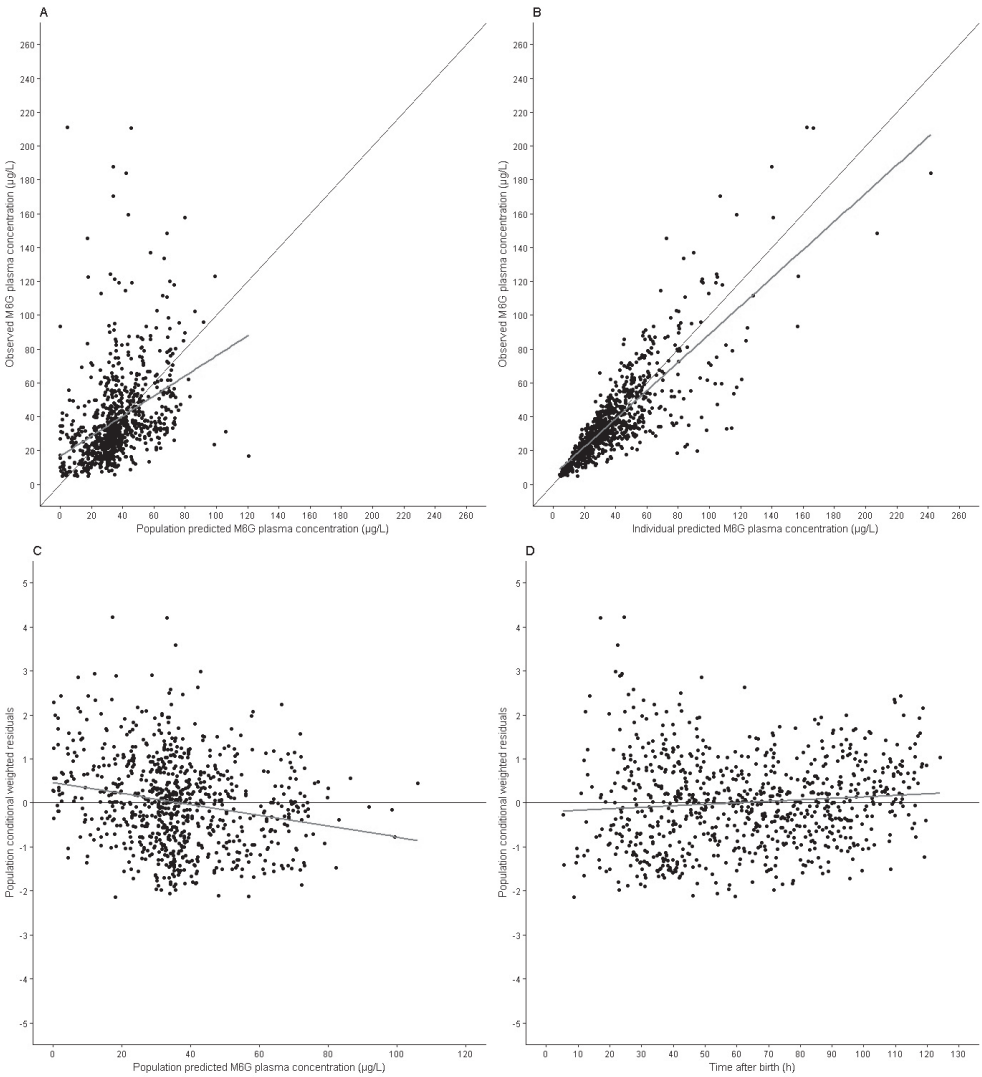


Figure S5: Normalised prediction distribution errors (NPDEs) of the final pharmacokinetic model for morphine. A = kernel density plot of NPDE with a normal, Gaussian distribution overlaid for comparative purposes; B = Q-Q plot of theoretical quantiles vs sample quantiles; C = NPDE vs Time; D = NPDE vs predicted plasma concentrations; solid lines in figures C and D represent the observed median, 5th and 95th percentiles, red box represent the predicted 90% confidence interval around the median, blue boxes represent the predicted 90% confidence intervals around the 5th and 95th percentiles

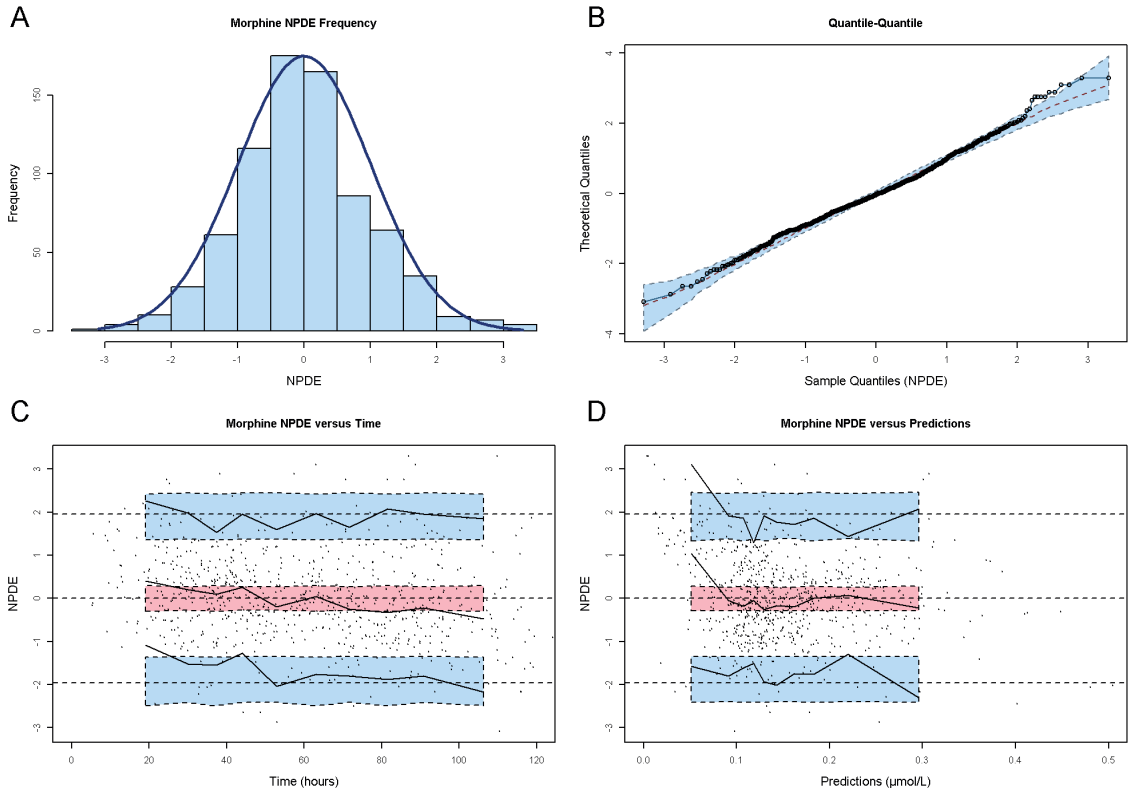


Figure S6: Normalised prediction distribution errors (NPDEs) of the final pharmacokinetic model for M3G. A = kernel density plot of NPDE with a normal, Gaussian distribution overlaid for comparative purposes; B = Q-Q plot of theoretical quantiles vs sample quantiles; C = NPDE vs Time; D = NPDE vs predicted plasma concentrations; M3G = morphine-3-glucuronide; solid lines in figures C and D represent the observed median, 5th and 95th percentiles, red box represent the predicted 90% confidence interval around the median, blue boxes represent the predicted 90% confidence intervals around the 5th and 95th percentiles

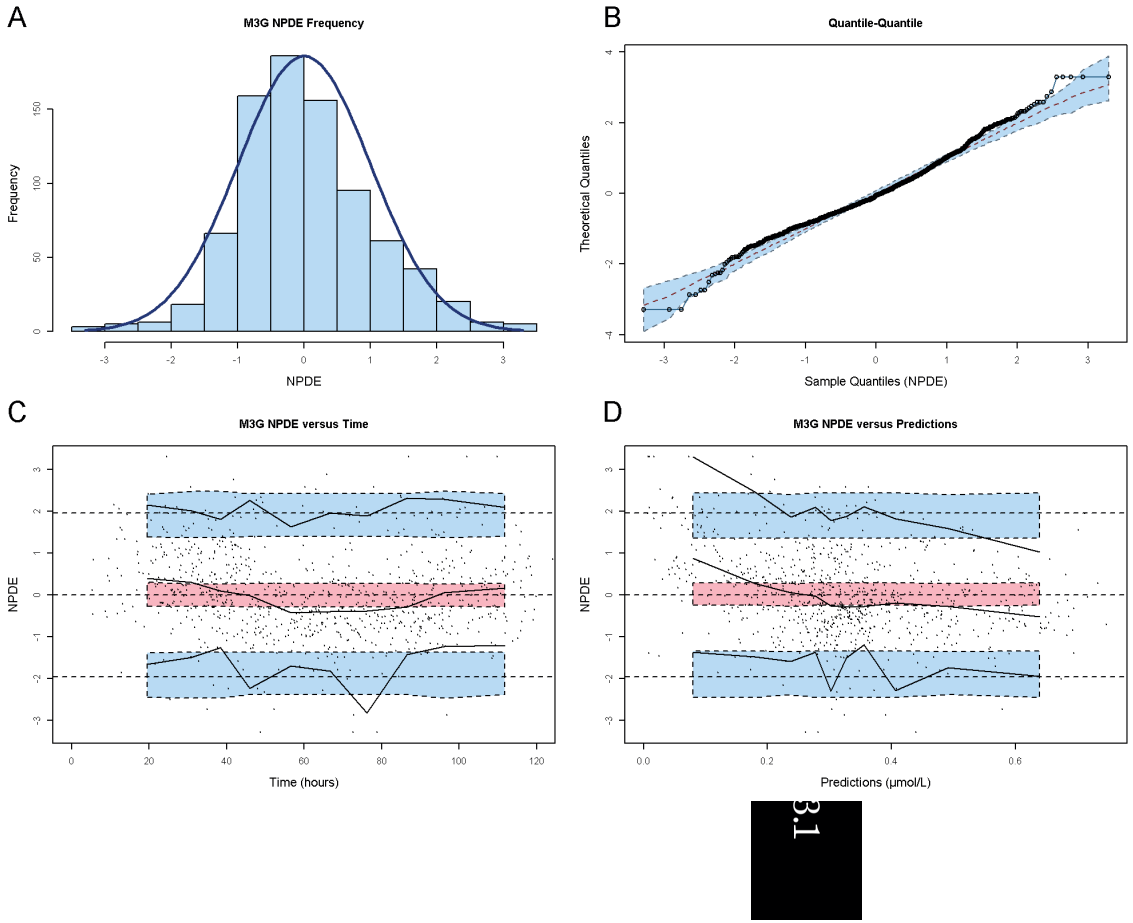


Figure S7: Normalised prediction distribution errors (NPDEs) of the final pharmacokinetic model for M6G. A = kernel density plot of NPDE with a normal, Gaussian distribution overlaid for comparative purposes; B = Q-Q plot of theoretical quantiles vs sample quantiles; C = NPDE vs Time; D = NPDE vs predicted plasma concentrations; M6G = morphine-6-glucuronide; solid lines in figures C and D represent the observed median, 5th and 95th percentiles, red box represent the predicted 90% confidence interval around the median, blue boxes represent the predicted 90% confidence intervals around the 5th and 95th percentiles

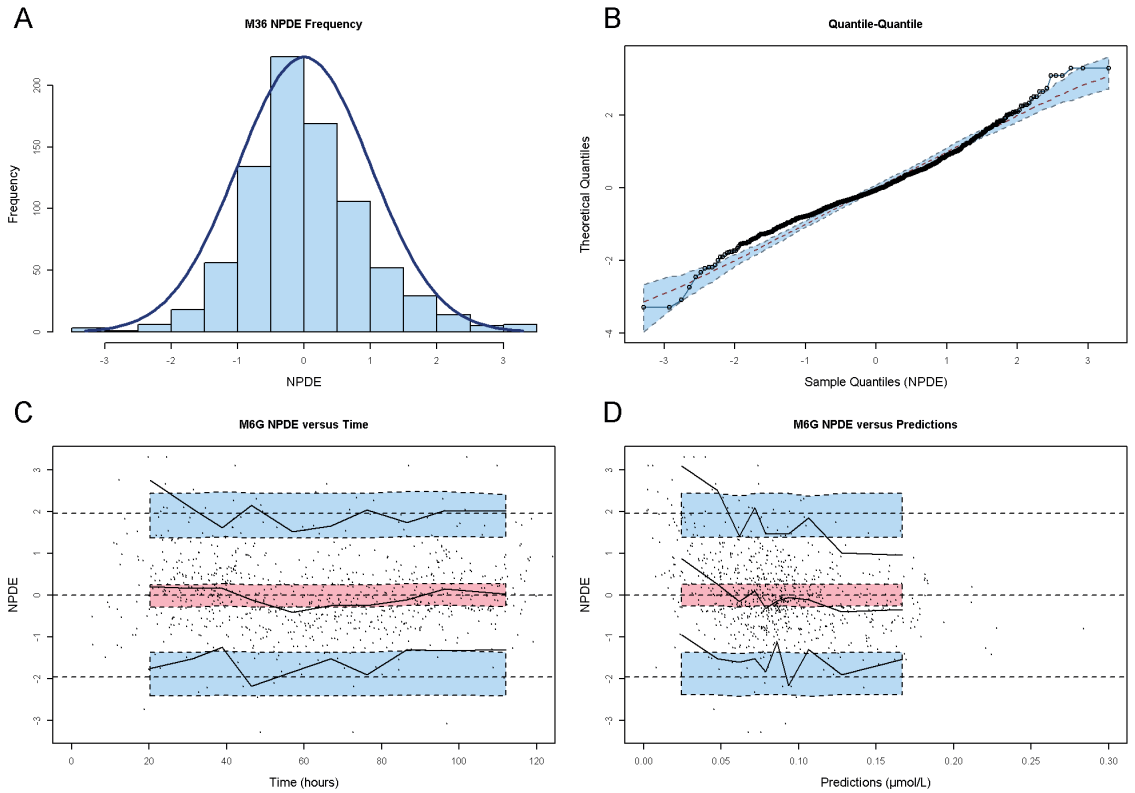


Figure S8: Simulated plasma concentration time profiles for M3G (left) and M6G (right) of the proposed morphine dosing regimen of 5 µg/kg/h after loading dose of 50 µg/kg. Solid line indicates the mean plasma concentration; gray area represents the 95% prediction interval. M3G = morphine-3-glucuronde, M6G = morphine-6-glucuronide

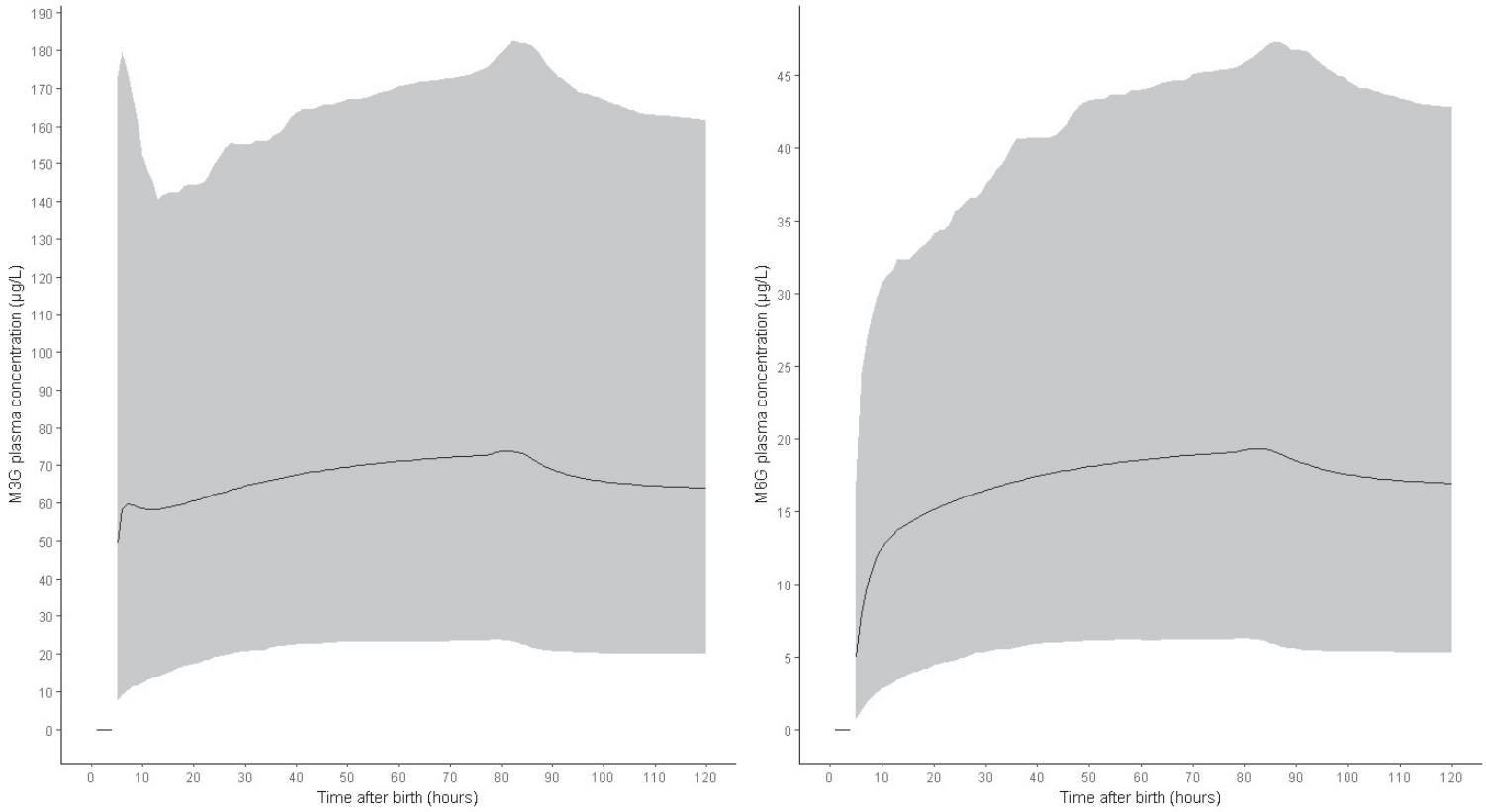
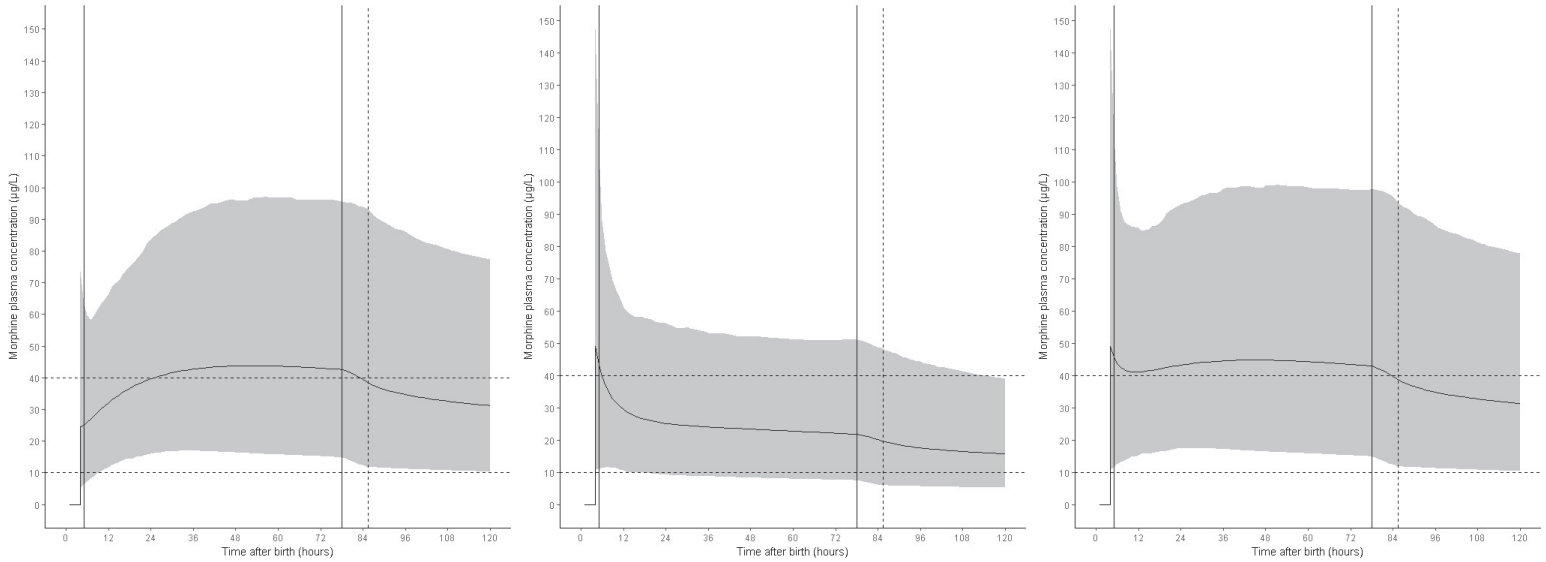


Figure S9: Simulated morphine plasma concentrations of the dosing regimens of 10 µg/kg/h after loading dose of 50 µg/kg (left), 5 µg/kg/h after loading dose of 100 µg/kg (center) and 10 µg/kg/h after loading dose of 100 µg/kg (right). Solid line indicates the mean morphine plasma concentration; gray area represents the 95% prediction interval. Dotted horizontal lines indicate the proposed therapeutic window of 10-40 µg/L. Solid vertical lines indicate the start and end of TH (33.5°C) simulated between 5h and 77h after birth; dashed vertical line indicates the return to normothermia (36.5 °C) with rewarming simulated at 0.4 °C/h; TH = therapeutic hypothermia



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Chapter 3.2

Phenobarbital, midazolam pharmacokinetics, effectiveness and drug-drug interaction in asphyxiated neonates undergoing therapeutic hypothermia

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Abstract

Background: Phenobarbital and midazolam are commonly used drugs in (near-) term neonates treated with therapeutic hypothermia for hypoxic-ischaemic encephalopathy, for sedation and/or as anti-epileptic drug. Phenobarbital is an inducer of CYP3A while midazolam is a CYP3A substrate. Therefore, co-treatment with phenobarbital might impact midazolam clearance.

Objectives: To assess pharmacokinetics and clinical anti-epileptic effectiveness of phenobarbital and midazolam in asphyxiated neonates and to develop dosing guidelines.

Methods: Data were collected in the prospective multicentre PharmaCool study. In the present study, neonates treated with therapeutic hypothermia and receiving midazolam and/or phenobarbital were included. Plasma concentrations of phenobarbital and midazolam including its metabolites were determined in blood samples drawn on days 2-5 after birth. Pharmacokinetic analyses were performed using non-linear mixed effects modelling; clinical effectiveness was defined as no use of additional anti-epileptic drugs.

Results: Data were available from 113 (phenobarbital) and 118 (midazolam) neonates; 68 were treated with both medications. Only clearance of 1-hydroxy midazolam was influenced by hypothermia. Phenobarbital co-administration increased midazolam clearance by a factor 2.3 (95% CI 1.9 – 2.9, $p < 0.05$). Anticonvulsant effectiveness was 65.5% for phenobarbital and 37.1% for add-on midazolam.

Conclusions: Therapeutic hypothermia does not influence clearance of phenobarbital or midazolam in (near-)term neonates with hypoxic-ischaemic encephalopathy. A phenobarbital dose of 30 mg/kg is advised to reach therapeutic concentrations.

Phenobarbital co-administration significantly increased midazolam clearance. Should phenobarbital be substituted by non-CYP3A inducers as first line anticonvulsant, a 50% lower midazolam maintenance dose might be appropriate to avoid excessive exposure during the first days after birth.

Introduction

Hypoxic-ischemic encephalopathy (HIE) caused by perinatal asphyxia is a serious clinical condition with significant morbidity and mortality in (near-)term neonates. Globally, the incidence varies between 0.5 and 20 of every 1000 live born neonates.(1) Therapeutic hypothermia (TH) is an established neuroprotective treatment which has markedly reduced the composite adverse outcome of death and neurodevelopmental disorders. In the Netherlands, 150-200 neonates are eligible for this treatment annually.(2,3)

Phenobarbital and midazolam are commonly prescribed drugs in this vulnerable population. Phenobarbital is a first line anti-epileptic drug (AED). It acts through stimulation of the γ-aminobutyric acid (GABA) receptors in the central nervous system which leads to a postsynaptic increase of chloride ions and thereby reducing neuronal excitability.(4) Phenobarbital has a half-life of approximately a week in neonates. Therefore, it can be administered as single or rapidly consecutive bolus administrations up to 40 mg/kg. Plasma concentrations between 20 and 40 mg/L are considered effective and safe.(5) Midazolam is a benzodiazepine which also interacts with the GABA receptor. It is used as a second line AED when phenobarbital is ineffective.(6) Additionally, it is used for sedation for instance in neonates who require mechanical ventilation.(7) Midazolam has a relatively short half-life of several hours in neonates and is usually administrated via continuous infusion. For sedation, doses around 0.1 mg/kg/h are often sufficient while as an AED, doses up to or even exceeding 0.3 mg/kg/h have been used. The therapeutic window for midazolam is not well defined but plasma concentrations of at least 0.1 mg/L are required for both indications. Higher plasma concentrations are associated with increased AED efficacy.(8) Levels above 2.4 mg/L are considered toxic.(9)

Midazolam undergoes hepatic metabolism by cytochrome P450 (CYP) 3A into 1-hydroxymidazolam (OHM). OHM is further metabolized into to hydroxymidazolam glucuronide (HMG) which is excreted renally. Both metabolites are pharmacologically active and accumulation has been associated with prolonged sedation.(10,11) Phenobarbital is known as a potent inducer of several CYP enzymes in adults, including CYP3A.(12)

Pharmacokinetics (PK) of drugs in neonates differs from older children and adults due to immaturity of the involved organs. CYP expression is impaired at birth but is subject to (rapid) maturation in the first few days of life.(13,14) Midazolam clearance could potentially be increased if phenobarbital is also administered, but it is uncertain whether this drug-drug interaction is present in neonates. Induction of midazolam clearance might have important consequences for the use of this drug in this population since adequate control of neonatal seizures is important to reduce the risk of neurological disabilities.(15–17)

Hypothermia could influence various physiological processes relevant for PK such as organ perfusion, protein binding and (metabolic) enzymatic activity.(18–20) Previous studies from our group have assessed the effect of TH on PK of both phenobarbital and midazolam in this population using data from two tertiary neonatal intensive care units (NICU) as well as clinical efficacy as AED.(5,8) Clearance of neither drug was found to be affected by TH. Sufficient seizure control was achieved in 66% of all neonates with phenobarbital monotherapy. When midazolam was started as a second line AED, efficacy was 23%.

The objective of the present study was to expand the current PK knowledge of phenobarbital and midazolam in neonates undergoing TH as treatment for HIE, to evaluate the previously developed models with an external dataset, to assess the effectiveness of each AED and to develop PK based dosing guidelines. In post-hoc

analyses, the influence of phenobarbital co-administration on midazolam clearance was investigated.

Methods

Setting and study population

The multi-centre prospective study PharmaCool was designed to investigate the pharmacokinetics of frequently used drugs during TH and rewarming in neonates suffering from HIE. In- and exclusion criteria have been described previously.(21) Parental informed consent was obtained in all cases. Choice of therapy and drug dosing was not influenced by the study protocol. The PharmaCool study was approved by the Ethics Committees of all twelve participating NICUs in the Netherlands and Belgium.

Dosing and administration

Phenobarbital was dosed as a single or repeated bolus short infusion of 10 of 20 mg/kg, up to a cumulative dose of 40 mg/kg. Midazolam was administrated as continuous infusion, both for sedation and as AED, with a starting dose of 0.05 mg/kg/h for sedation and 0.1 mg/kg/h for seizure control and titrated to effect. Both regimens can be preceded by a loading dose of 0.05-0.1 mg/kg.

Sampling and bioanalysis

Blood samples were drawn once daily on days 2-5 after birth both during hypothermia and rewarming/normothermia.(21) Plasma concentrations of phenobarbital, midazolam, OHM and HMG were determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Details are available in the Appendix.

Population pharmacokinetic analyses

PK analyses were performed using non-linear mixed effects modelling NONMEM (version 7.3, Icon Development Solutions).(22) Birth weight was used as a descriptor for body size and was related to pharmacokinetic parameters using allometric relationships. Based on previous publications from our group, a one-compartment model for phenobarbital and a one-compartment model for midazolam with consecutive one-compartment models for both metabolites were used as structural models.(5,8) Gestational age (GA), postnatal age (PNA) and body temperature (TEMP) were tested as covariates on clearance in both models. Phenobarbital co-medication was tested as covariate on clearance in the midazolam model. Parameter precision was assessed with sampling importance resampling.(23)

Anticonvulsant effectiveness

Treatment with phenobarbital was considered effective if no additional AED was started. In patients receiving midazolam for seizure control, effectiveness as second line AED was defined as no requirement for a third line AED.

Dosing guideline development

Several dosing regimens based on the current clinical practice were simulated using the parameter estimates of the final pharmacokinetic models. Phenobarbital single doses of 20, 30 and 40 mg/kg were evaluated. Midazolam was tested with a loading dose of 0.05 or 0.1 mg/kg followed by continuous infusions varying between 0.05 and 0.3 mg/kg/h.

Results

Patient characteristics

Phenobarbital data were available for 113 patients. Cumulative doses varied between 4.9 and 62.6 mg/kg. Midazolam data were available from 118 patients

(sedative n = 83, AED n = 35). Of these, 68 (57.6%) were also treated with phenobarbital. Midazolam maintenance dose for sedation rarely exceeded 0.15 mg/kg/h. Highest midazolam maintenance dose as AED was 0.45 mg/kg/h. Patient characteristics are presented in Table 1.

Table 1: Patient characteristics

	Phenobarbital (n=113)	**Midazolam (n=118)**	
Parameter		**PB YES (n = 68)**	**PB NO (n = 50)**
Gestational age; wk, mean ± sd	39.8 ± 1.7	40.2 ± 1.4	39.8 ± 1.6
Birth weight; g, mean ± sd	3,382 ± 582	3,450 ± 540	3,495 ± 674
Male; n (%)	63 (55.8%)	35 (51.5 %)	36 (72 %)
pH*; median (IQR)	7.02 (6.85-7.15)	6.98 (6.80-7.10)	6.90 (6.80 – 7.05)
Lactate*; mmol/L, median (IQR)	13.0 (9.0 – 18.2)	14.1 (11.4 – 16.6)	12.0 (7.8 – 14.6)
Base Excess*; mmol/L, median (IQR)	-18.0 (-12.0 – -22.0)	-18.0 (-22.0 – -12.0)	-18.5 (-21 – -14.3)
Thompson score#; median (IQR)	9 (8 – 13)	10 (8 – 14)	9 (7 – 10)
Midazolam sedation only; n (%)	-	33 (48.3%)	50 (100%)
Midazolam AED; n (%)	-	35 (51.5%)	0 (0%)
aEEG on admission#			
Continuous normal voltage; n (%)	16 (14.2%)	8 (11.8%)	10 (20.0%)
Discontinuous normal voltage; n (%) *of whom < 5 µV; n (%)*	45 (39.8%) 16 (14.2%)	20 (29.4%) 9 (13.2%)	29 (58.0 %) 14 (28.0%)
Burst suppression; n (%)	26 (23.0%)	21 (30.9%)	6 (12.0%)
Continuous low voltage; n (%)	5 (4.4%)	4 (5.9 %)	1 (2.0 %)
Flat trace; n (%)	17 (15.0%)	13 (19.1%)	1 (2.0 %)
Unknown; n (%)	4 (3.5%)	2 (2.9%)	3 (6.0 %)
Mortality; n (%)	32 (28.3%)	27 (39.7%)	1 (2.0 %)

*Value measured in umbilical cord blood or, if unavailable, form arterial or venous blood within 1h after birth

#Encephalopathy was characterized by a Thompson score of >7 1h after birth or an abnormal aEEG (i.e. background pattern DNV <5 µV or worse or seizures) on admission to a level III NICU

PB = phenobarbital co-medication; sd = standard deviation, IQR = interquartile range, AED = anti-epileptic drug, aEEG = amplitude-integrated electroencephalogram

Phenobarbital plasma concentrations were measured in 378 samples of which 219 (57.9%) were taken during TH. Plasma concentrations varied between 9.1 and 52.6 mg/L (Figure 1). Plasma concentrations of midazolam, OHM and HMG were measured in 376 samples of which 214 (56.9%) were taken during TH. Plasma concentrations for midazolam varied between 0.02 and 3.25 mg/L (Figure 2), for OHM between 0.02 and 1.05 mg/L and for HMG between 0.02 and 8.34 mg/L (Appendix).

Population pharmacokinetic analyses

Phenobarbital PK was best described by a one compartment model. No influence of GA, PNA or TEMP could be detected on clearance. Midazolam PK was described by a one compartment model with subsequent one compartment models for OHM and HMG. GA and PNA did not affect clearance of any compound; TEMP significantly influenced only OHM clearance; clearance during TH was reduced by 25.7% ($p< 0.05$, 8.6%/°C, 95% CI 5.6%/°C – 11.5%/°C). Phenobarbital co-medication significantly influenced midazolam clearance. In absence of phenobarbital, midazolam clearance for a neonate of 3.5 kg was 0.35 L/h (95% CI 0.29 – 0.41 L/h). In patients with phenobarbital co-medication, midazolam clearance was 2.3 fold higher ($p<0.05$, 95% CI 1.9 – 2.9). This effect was consistent over the entire study period and independent of phenobarbital dose, TH or indication for midazolam use. Pharmacokinetic parameter estimates of the final models are shown in Table 2. Further details of the population PK analyses are available in the Appendix.

Anticonvulsant effectiveness

Seizure control with phenobarbital monotherapy was achieved in 74 patients (65.5%). 35 patients received midazolam as second line AED. Of these, 22 (62.9%) also received lidocaine, levetiracetam and/or clonazepam as additional AED. Midazolam was considered effective in the remaining 13 (37.1%) neonates. AED effectiveness is summarised in Table 3.

Figure 1: Observed phenobarbital plasma concentrations versus time after birth. Dotted lines indicate the proposed therapeutic window of 20-40 mg/L.

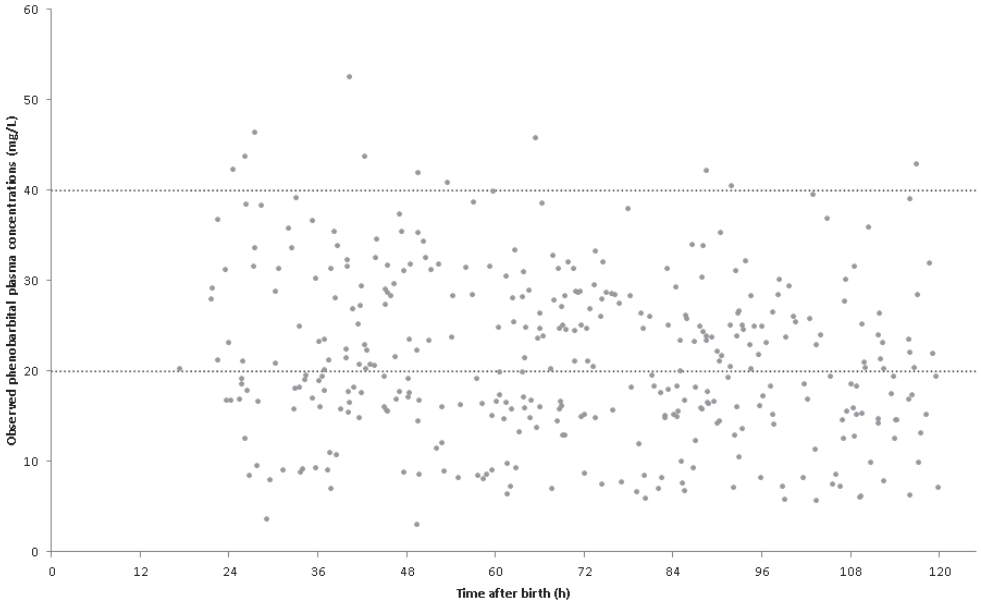


Figure 2: Observed midazolam plasma concentrations versus time after birth. Dotted line indicates the minimal effective plasma concentration of 0.1 mg/L. Solid line represent toxic upper limit of 2.4 mg/L.

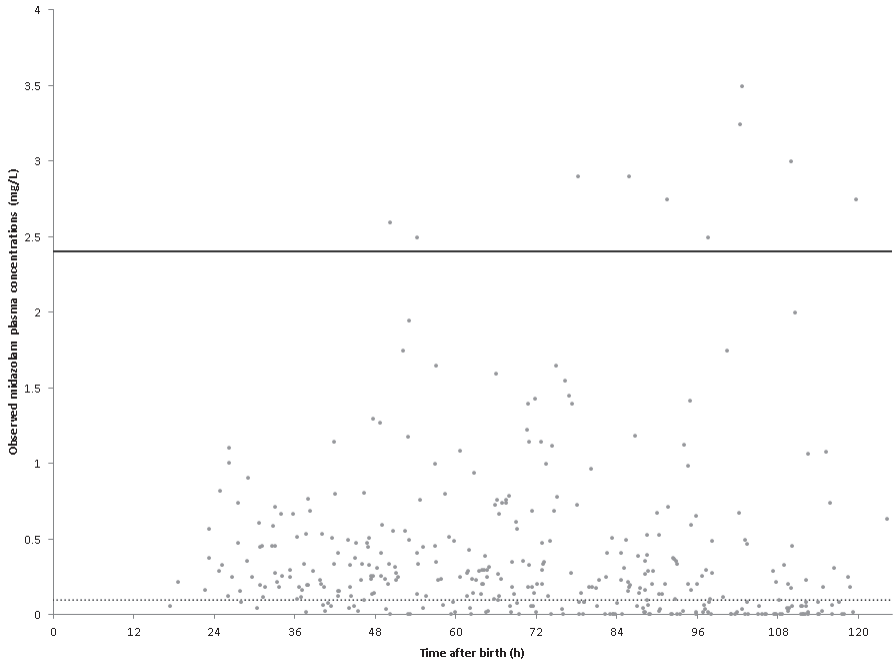


Table 2: Final model pharmacokinetic parameter estimates and SIR results

	Phenobarbital		**Midazolam**		**OHM**[§]		**HMG**[§]	
Parameter	*Estimate*	*SIR* 95% CI*	*Estimate*	*SIR# 95% CI*	*Estimate*	*SIR# 95% CI*	*Estimate*	*SIR# 95% CI*
Cl, l/h[$]	10.3	8.38 – 12.1	0.353	0.286 – 0.441	3.39	2.75 – 4.01	0.191	0.170 – 0.214
V, l[$]	3.60	3.43 – 3.79	5.42	4.49 – 6.81	4.18 (fixed)	NA (fixed)	1.06	0.834 – 1.27
TEMP on Cl, %/°C	NA	NA	NA	NA	8.58	5.63 – 11.5	NA	NA
PB on Cl, fold	NA	NA	2.33	1.88 – 2.92	NA	NA	NA	NA
Interindividual variability								
Cl, variance (rsd)	0.287 (54%)	0.148 – 0.498	0.628 (79.2%)	0.479 – 0.847	0.633 (79.6%)	0.472 – 0.818	0.241 (49.1%)	0.174 – 0.338
V, variance (rsd)	0.0442 (36%)	0.0305 – 0.0659	0.934 (96.6%)	0.583 – 1.27	NA (fixed)	NA (fixed)	0.837 (91.5%)	0.353 – 1.33
Residual variability								
Additional, mg/L (rse)	2.52 (31%)	2.32 – 2.79	0.01 (fixed)	NA (fixed)	0.01 (fixed)	NA (fixed)	0.01 (fixed)	NA (fixed)
Proportional, variance (rsd)	NA	NA	0.126 (35.5%)	0.107 – 0.164	0.0857 (29.3%)	0.0683 – 0.119	0.0871 (29.5%)	0.0729 – 0.112
Covariance interindividual variability on Cl			*Midazolam/OHM*		*Midazolam/HMG*		*OHM/HMG*	
			Estimate	SIR# 95% CI	Estimate	SIR# 95% CI	Estimate	SIR# 95% CI
Covariance (correlation coefficient)			0.500 (79.0%)	0.378 – 0.652	0.214 (55.0%)	0.133 – 0.310	0.233 (60.0%)	0.147 – 0.327

§All metabolite estimates are relative to formation fraction FOHM and FHMG, resp.
*Five iterations; no. of samples 4000, 4000, 4000, 4000, 4000; no. of resamples 1000, 1000,1000,1000,2000
#Six iterations, no. of samples 4000, 4000, 4000, 4000, 4000, 4000; no. of resamples 1000, 1000,1000,1000,1000,1000
§Estimates for neonate with a birth weight of 3.5 kg
Cl = clearance, V = volume of distribution, TEMP = body temperature, PB = phenobarbital co-medication, OHM = 1-hydroxymidazolam, HMG = hydoxymidazolam glucuronide, SIR = sampling importance resampling, NA = not applicable, rsd = relative standard deviation, rse = relative standard error

Table 3: effectiveness of phenobarbital and midazolam as anti-epileptic drugs

	Effective, n (%)	**Ineffective, n (%)**
Phenobarbital (n = 113)	74 (65.5%)	39* (34.5%)
Midazolam (n = 35)	13 (37.1%)	22 (62.9 %)

*4 patients unresponsive to phenobarbital received lidocaine instead of midazolam as second line anti-epileptic drug

Dosing guideline development

Simulation datasets were created by replicating the patient characteristics of each neonate in the original dataset nine times, yielding simulation datasets of 1017 patients for phenobarbital and 1062 patients for midazolam. These datasets were used with the final PK parameter estimates to predict plasma concentrations after various dosing regimens.

Figure 3 shows predicted phenobarbital concentration-time curves after doses of 20, 30 and 40 mg/kg at PNA 4h. With 20 mg/kg, 46.7% of patients is within the proposed therapeutic range (20-40 mg/kg) directly after bolus infusion, dropping to 18.7% at PNA 48h. 30 mg/kg results in 90.2% within the therapeutic range directly after infusion and 85.4% at PNA 48h. A dose of 40 mg/kg leads to 53.2% within the therapeutic range directly after infusion and 80.7% at PNA 48h.

Figure 4 shows predicted concentration-time curves of midazolam with and without phenobarbital co-administration after a loading dose of 0.1 mg/kg followed by continuous infusion of 0.15 mg/kg/h.

Discussion

Data from this study confirms the previous findings that TH does not influence phenobarbital or midazolam clearance in neonates suffering from HIE. Clearance of OHM is reduced during TH compared to normothermia; clinicians should be aware that prolonged sedation could occur after cessation of midazolam during TH. Phenobarbital co-medication was found to significantly increase midazolam clearance. Although this effect has not been described previously, it has most likely been present in clinical practice for decades as both midazolam and phenobarbital are commonly used in neonates, often concomitantly for the treatment of seizures. Midazolam is usually titrated to the desired effect and/or tolerability of

Figure 3: Simulated phenobarbital plasma concentrations after a dose of 20 mg/kg (left), 30 mg/kg (centre) and 40 mg/kg (right). Solid lines indicate the mean phenobarbital plasma concentrations. Dotted lines indicate the proposed therapeutic window; grey area represents the 95% prediction interval.

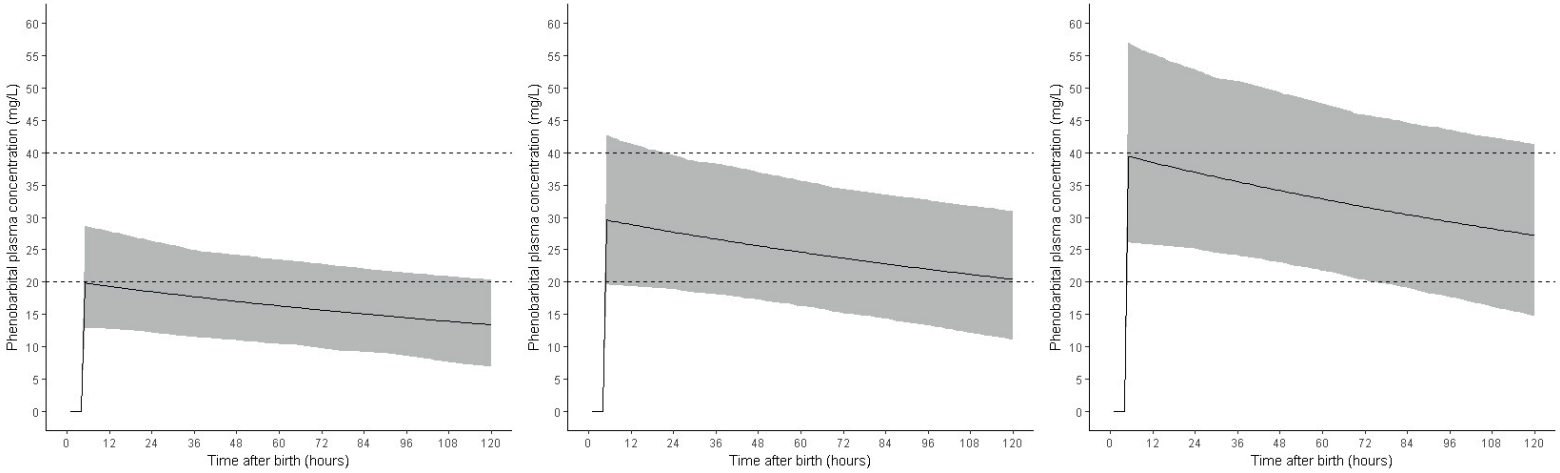
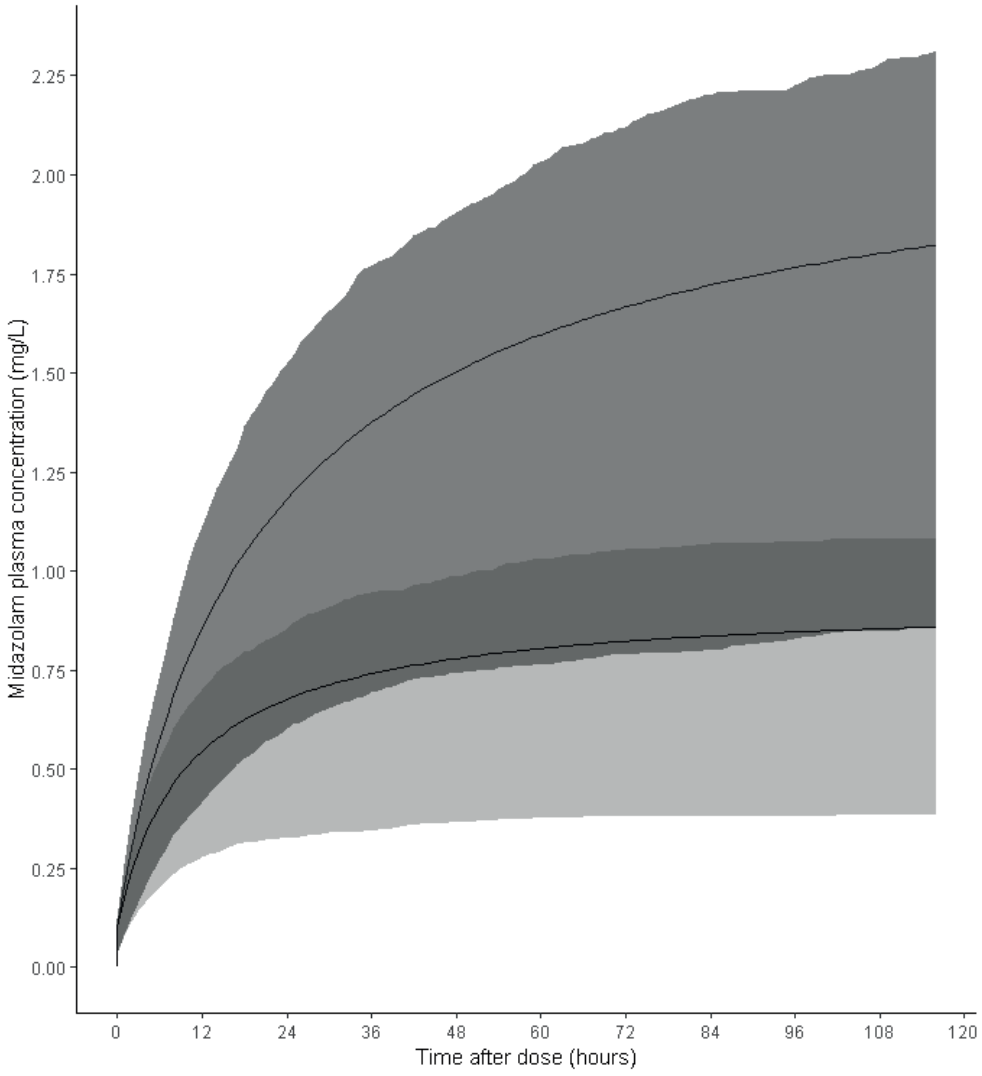


Figure 4: Simulated midazolam plasma concentrations after a loading dose of 0.1 mg/kg followed by continuous infusion of 0.15 mg/kg/h. Upper solid line indicates the mean midazolam plasma concentration without phenobarbital co-medication. Bottom solid line indicates the mean midazolam plasma concentration with phenobarbital co-medication; grey areas represent the interquartile ranges.



side effects independent of concomitant treatment with phenobarbital. Although adequate sedation and seizure control are crucial in neonates undergoing TH for HIE, concerns have been raised about both phenobarbital and midazolam use. Phenobarbital has been associated with neuronal toxicity in neonatal animal models and long term cognitive and motor impairment in humans. Therefore, alternative first line AEDs such as levetiracetam are currently being investigated.(24) As levetiracetam does not induce CYP3A, no influence on midazolam clearance is expected. In this situation, a reduction in midazolam maintenance dose by 50% is necessary to achieve similar plasma concentrations. Overexposure of midazolam should be avoided to minimise the risk of side effects such as hypotension and subsequent cerebral hypoperfusion when cerebral autoregulation is lost, and prolonged NICU admission.(6,8,25,26)

CYP3A is the most abundant subfamily of cytochrome P450 isozymes in the human liver and consists of at least three isoforms: CYP3A4, CYP3A5, and CYP3A7.(27) CYP3A4 activity is relatively low at birth but increases over the first few weeks of life and reaches adult capacity between 6-12 months after birth. In adult livers, it accounts for 30-40% of all CYP content. (27)(28) CYP3A5 is present at a much lower level compared to CYP3A4 and shows large interindividual variability. No maturational pattern of CYP3A5 has been identified.(28) CYP3A7 is the major CYP isoform detected in embryonic, foetal and newborn liver but decreases thereafter. Compounds metabolized by CYP3A4 in adults are most likely primarily metabolized by CYP3A7 in neonates and infants up to 3 months of age.(28)(29)

Induction of CYP enzymes is caused by an increase in gene transcription followed by upregulation of enzyme production. Unlike CYP inhibition, which is an almost immediate response, it is believed that CYP induction is a slower regulatory process which accumulates over time.(30) In adults, clinically relevant CYP3A induction has been described within 24 hours after administration.(31) In asphyxiated neonates,

both phenobarbital and midazolam are often administrated within the first few hours after birth. In this study, blood sampling commenced on the second day after birth.

As a time dependent effect of phenobarbital on midazolam clearance was not identified, we hypothesize that the increasing effect of phenobarbital on CYP3A production in this population is clinically relevant as early as 24 hours after birth. In the past, phenobarbital has been used to treat hyperbilirubinaemia in predominantly preterm infants by inducing glucuronidation of unconjugated bilirubin.(32) Although OHM is glucuronated into HMG, no effect of phenobarbital co-medication on OHM clearance was identified. It is possible that glucuronidation is more developed in term neonates and that induction of glucuronidation is only relevant in preterm neonates as phenobarbital was unsuccessful in preventing hyperbilirubinaemia in term neonates.(33) Also, to our knowledge no drug-drug interactions between phenobarbital and drugs undergoing glucuronidation have been described in humans.

Table 1 clearly shows a difference in characteristics between midazolam patients with and without phenobarbital co-administration. Neonates with phenobarbital co-medication had a more suppressed aEEG on admission and higher mortality, suggesting a more severe disease state. However, increased midazolam clearance in this group by induction of CYP3A indicates that hepatic metabolic capacity is unaffected even in the most severe HIE cases.

Based on simulations performed in this study, a phenobarbital loading dose of 30 mg/kg is recommended to achieve plasma concentrations within the therapeutic window. An additional dose of 10 mg/kg can be given if seizures persist. This advice is in line with a recent study investigating phenobarbital PK in non-asphyxiated term and preterm neonates.(34) Phenobarbital efficacy is comparable to previous reports.(4,5)

No changes to the midazolam dosing regimens are required in the current clinical practice. However, should phenobarbital be replaced as first line AED, midazolam for additional seizure control should be titrated more carefully. Although responsiveness to midazolam in this study was higher than previously reported, efficacy as second line AED remains limited.(4,8)

The data for this study were collected in neonates with a gestational age of ≥ 36 weeks treated with TH for HIE. However, we believe that the interaction between phenobarbital and midazolam can be extrapolated to (near-)term neonates in general. As liver function might be hampered by TH and/or HIE, the magnitude of the effect in non-asphyxiated normothermic neonates could be even be greater. Extrapolation to preterm neonates should be done with caution due to possible maturational differences in CYP enzymes.(13)

Conclusion

PK of phenobarbital and midazolam is unaffected by TH in (near-)term neonates treated with TH for HIE and clinical efficacy is comparable to previous reports. Phenobarbital significantly increased midazolam clearance. Should phenobarbital be substituted by non-CYP inducing drugs as first line anticonvulsant, a lower midazolam dose is necessary to avoid excessive exposure across the entire neonatal population.

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Conflicts of interest

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Supplementary materials

Bioanalyses

Plasma concentrations of phenobarbital were determined using a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. The lower limit of quantification (LLQ) was 2.73 mg/L. The calibration curves were linear from 2.73 to 58.4 mg/L. Between-run and within-run coefficients of variation were <9%.
Plasma concentrations of midazolam, OHM and HMG were determined using a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. The LLQ was 0.02 mg/L for midazolam, OHM and HMG. The calibration curves were linear from 0.02 to 1.5 mg/L for midazolam and OHM and between 0.02 and 10.0 mg/L for HMG. Between-run and within-run coefficients of variation were <2% for midazolam and <5% for OHM and HMG. Samples were stored at -80°C until analyses at the Clinical Pharmaceutical and Toxicological Laboratory of the Department of Clinical Pharmacy of the University Medical Center Utrecht, the Netherlands. Samples with initial results above the range of linearity were diluted and reanalysed. Figure 1 shows the observed OHM and HMG plasma concentrations.

Population pharmacokinetic analyses

PK analyses were performed using non-linear mixed effects modelling NONMEM (version 7.3, Icon Development Solutions) with R (version 3.4.1), Xpose (version 4) for data visualization and Piraña for run management. In both models, the exponent defining the relationship of BW and clearance (Cl) was fixed to 0.75 and the exponent defining the relationship of BW and volume of distribution (V) was fixed to 1. In the phenobarbital model, an additive error model was used to model residual unexplained variability.
In order to simultaneously fit the midazolam, OHM and HMG data, all units of dose and concentration for were converted to µmol and µmol/L, respectively. The

fractions (F) of midazolam converted to OHM and HMG were unknown; metabolite parameters are therefore estimated relative to F. For midazolam, 48 measurements (12.8%) were below the LLQ; for OHM, 100 measurements (36.1%) and for HMG, 2 measurements (0.53%). To account for below LLQ data, the M3 method was used.[35] For each compound, the additive error was fixed on LLQ/2. Separate proportional error models were used to model residual unexplained variability. Because the formation rate for OHM is slower than its elimination rate, OHM volume of distribution could not be estimated and was fixed to 4.18 L based on literature.[8] Model evaluation demonstrated that the final models adequately described the data, although with considerable variability on both clearance and volume of distribution of all compounds. Goodness-of-fit plots of observed versus population and individual predicted concentrations showed no systematic deviation and the weighted residuals were homogeneously scattered for all compounds (Figures S2-S5).

Figure S1: Observed OHM (left) and HMG (right) plasma concentrations versus time after birth.

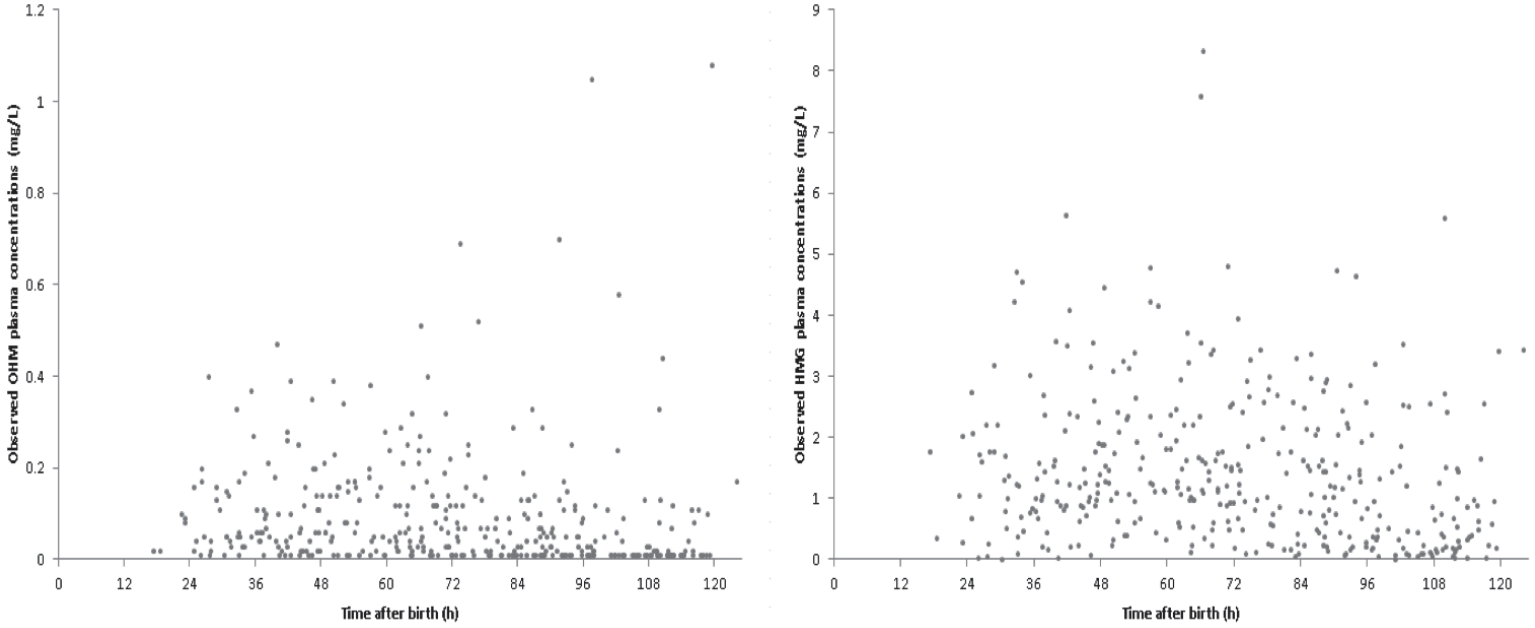


Figure S2: Phenobarbital goodness-of-fit plots. A = observed vs population predicted plasma concentrations; B = observed vs individual predicted plasma concentrations; C = population conditional weighted residuals vs population predicted plasma concentrations; D = population conditional weighted residuals vs time after birth;

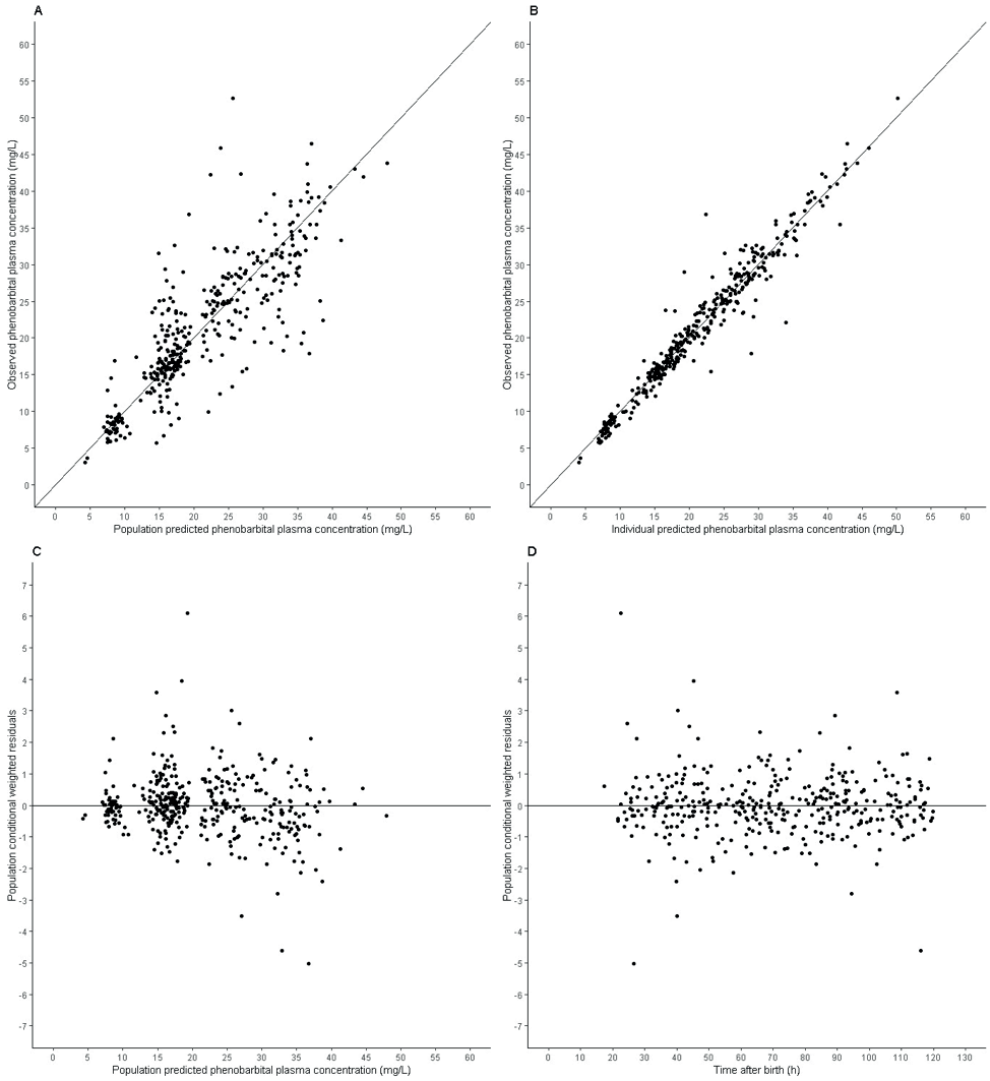


Figure S3: Midazolam goodness-of-fit plots. A = observed vs population predicted plasma concentrations; B = observed vs individual predicted plasma concentrations; C = population conditional weighted residuals vs population predicted plasma concentrations; D = population conditional weighted residuals vs time after birth;

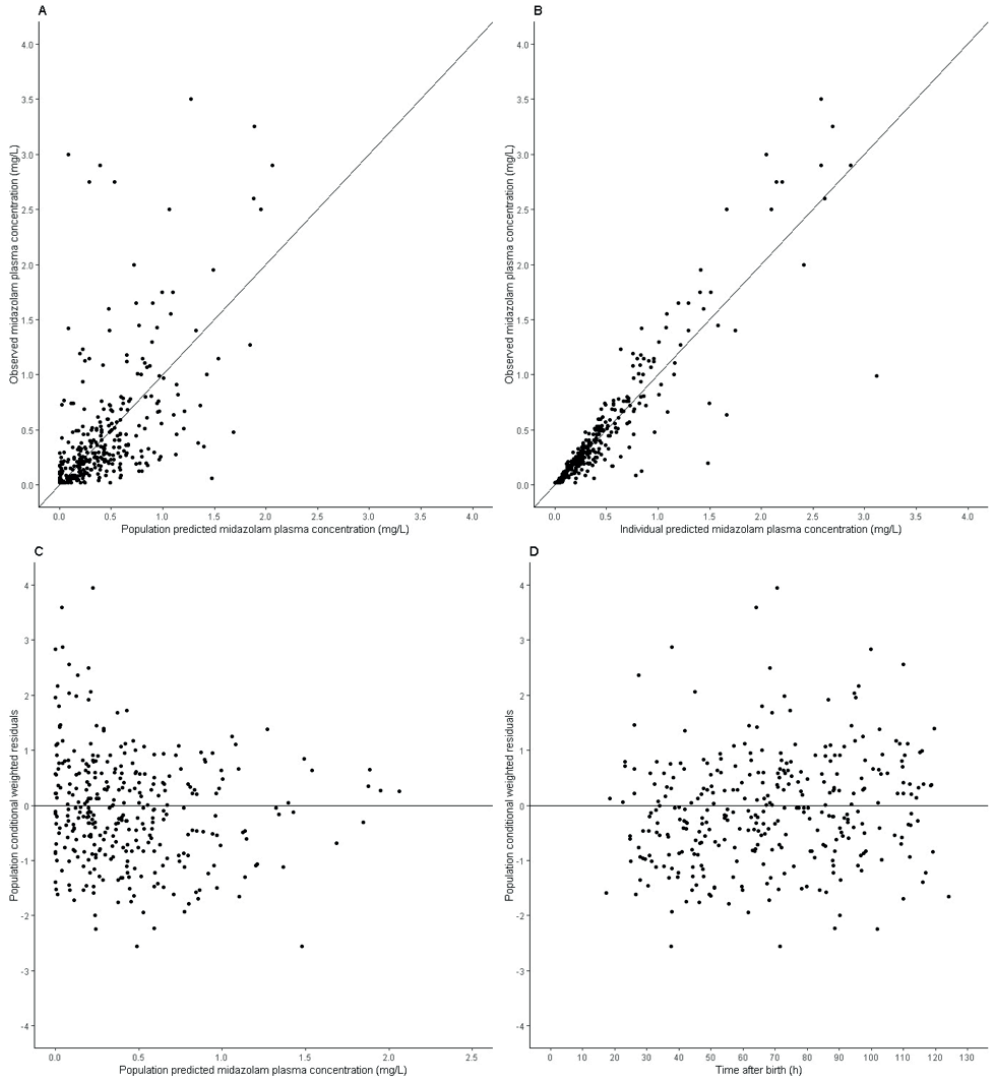


Figure S4: OHM goodness-of-fit plots. A = observed vs population predicted plasma concentrations; B = observed vs individual predicted plasma concentrations; C = population conditional weighted residuals vs population predicted plasma concentrations; D = population conditional weighted residuals vs time after birth; OHM = 1-hydroxymidazolam

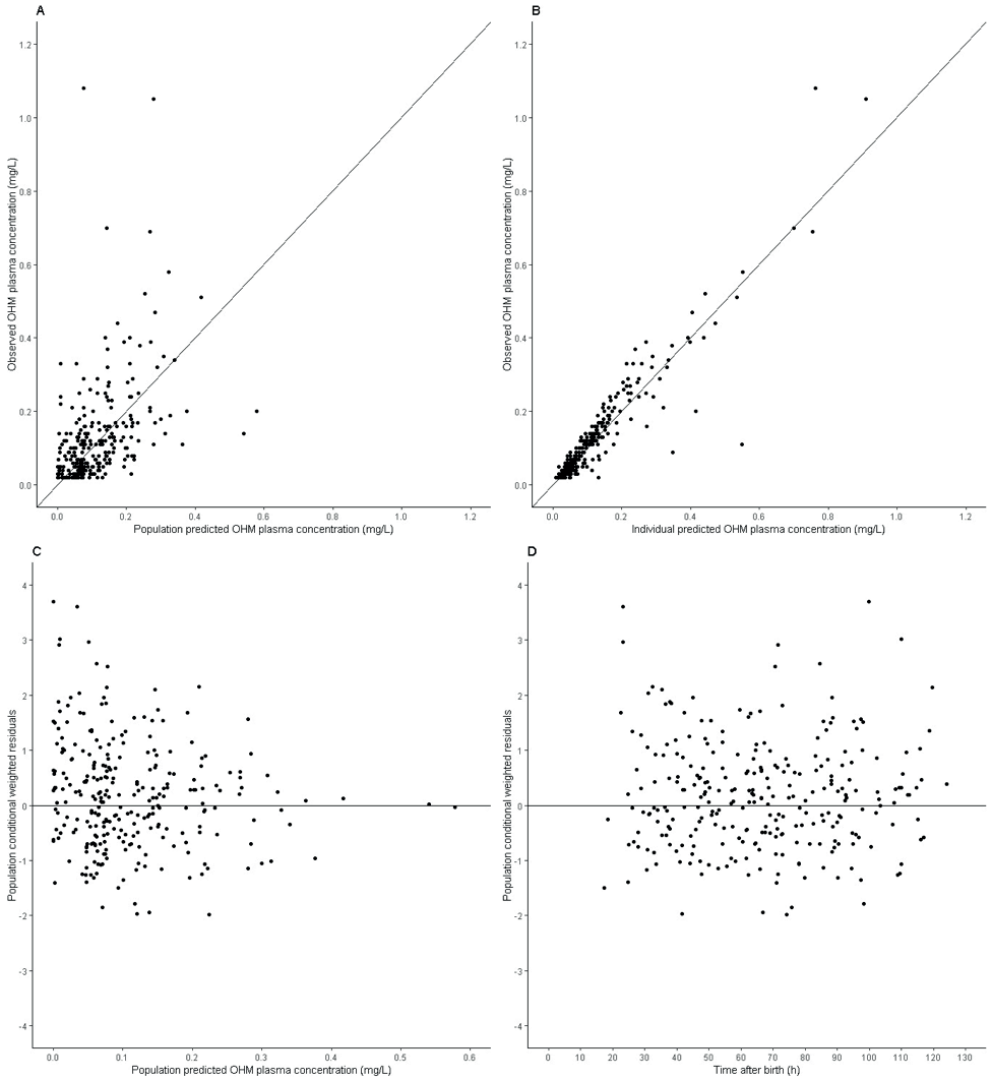
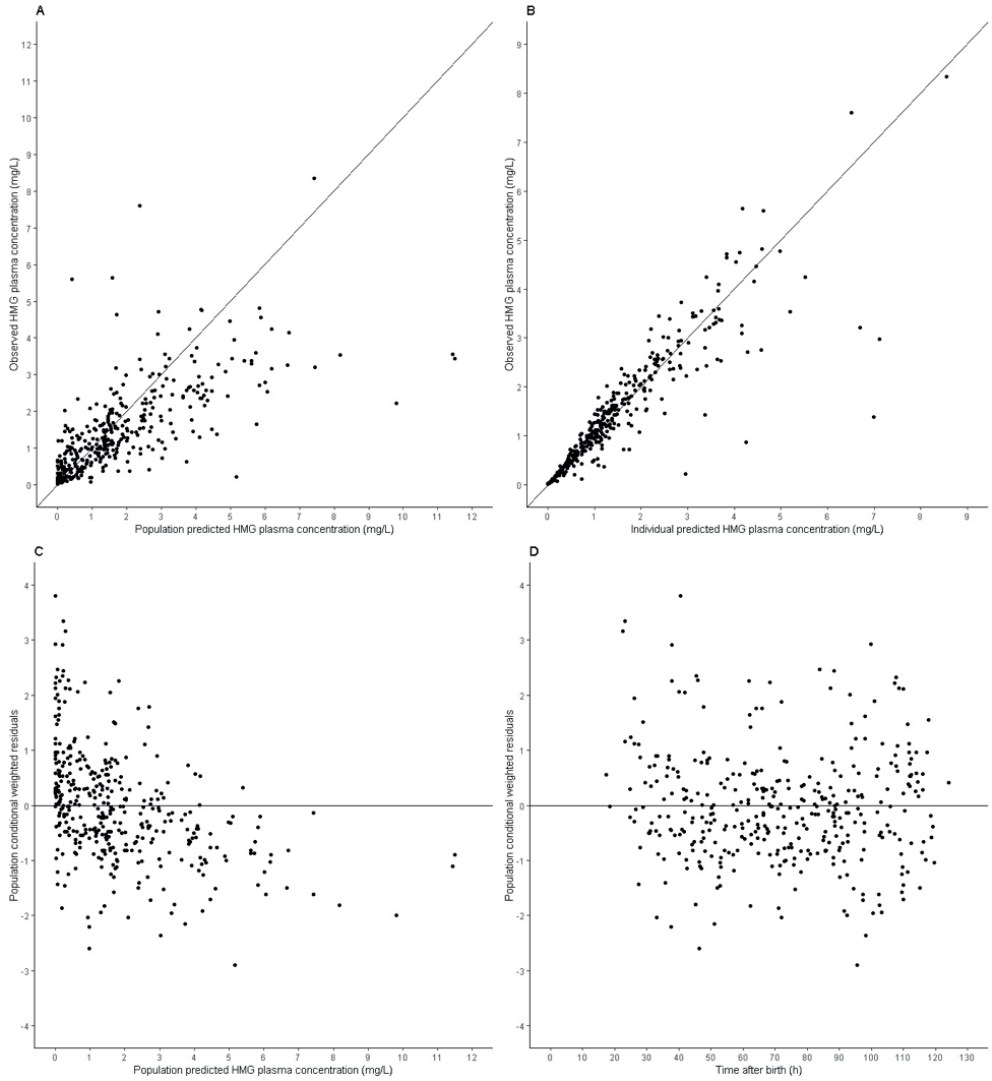


Figure S5: HMG goodness-of-fit plots. A = observed vs population predicted plasma concentrations; B = observed vs individual predicted plasma concentrations; C = population conditional weighted residuals vs population predicted plasma concentrations; D = population conditional weighted residuals vs time after birth; HMG = hydroxymidazolam glucuronide



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Chapter 3.3

Lidocaine as treatment for neonatal seizures: evaluation of a previously developed population pharmacokinetic models and dosing regimen

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Manuscript submitted

Abstract

Background and objective: lidocaine is used for the treatment of neonatal seizures in both preterm and term neonates refractory to other anticonvulsants. It is an effective anticonvulsant in this population but it has also been associated with cardiac toxicity. Previous studies have reported the pharmacokinetics of lidocaine in neonates and have a dosing regimen for effective and safe lidocaine use in this population. The objective of this study was to evaluate the previously developed pharmacokinetics models and dosing regimen. As a secondary objective, lidocaine effectiveness and safety were assessed.

Methods: clinical and pharmacological data from preterm neonates as well as (near-)term neonates with and without therapeutic hypothermia receiving lidocaine were included for the present study. Pharmacokinetic analyses were performed using non-linear mixed effects modelling . Simulations were done to evaluate the developed dosing regimen. Lidocaine was considered effective if no additional anticonvulsant was required and safe if no cardiac adverse events occurred.

Results: data was available for 159 neonates; 50 (31.4%) preterms and 109 term neonates of whom 49 (30.8%) were treated with therapeutic hypothermia for hypoxic-ischaemic encephalopathy. Lidocaine clearance increased with increasing postmenstrual age (0.69%/day, 95% 0.54% – 0.84%). During therapeutic hypothermia (33.5 °C), lidocaine clearance was reduced by 21.8% (7.26%/°C, 95% CI 1.63% – 11.2%) compared to normothermia (36.5 °C). Simulations showed that the novel dosing regimen leads to adequate average lidocaine plasma concentrations. Effectiveness and safety were assessed in 92 neonates. Overall effectiveness was 53.3% (49/92) and 56.5% (13/23) for neonates receiving the novel dosing regimen. No cardiac toxicity was observed.

Conclusion: lidocaine pharmacokinetics was adequately described across the entire neonatal age range. With the novel dosing regimen, lidocaine can provide effective and safe treatment for neonatal seizures.

Introduction

Epileptiform activity is the most common neurological disorder in the neonatal period of life and is often caused by underlying brain pathology such as hypoxic-ischemic encephalopathy (HIE), infections or intracranial hemorrhage.(1–3) Neonatal seizures are associated with an increased risk of long-term morbidity, including epilepsy and neurodevelopmental disorders.(4–8) Therefore, adequate treatment of seizures in neonates is important to prevent and reduce brain damage.(9–12)

Lidocaine, an amine derivative of cocaine, is used as a local anaesthetic and as a class 1b antiarrhythmic drug in both paediatric and adult patients.(13–16) However, it is also used as an anticonvulsant in neonates refractory to one or more other anti-epileptic drugs (AED) such as phenobarbital and midazolam.(17) Lidocaine has proven to be effective in several observational studies in both term and preterm neonates and is favoured as second or third line anticonvulsant therapy in several European countries. The most important theoretical safety risk of lidocaine is cardiac toxicity (bradycardia, tachycardia, arrhythmias or asystole), which could more likely occur at plasma concentrations exceeding 9 mg/L. (18–22)

Lidocaine is metabolized in the liver by the cytochrome p450 (CYP) enzyme system, predominantly by CYP1A2 and to a lesser extent by CYP3A. Monoethylglycinexylidide (MEGX) is the most prominent metabolite and is eliminated renally. Accumulation of MEGX has been associated with recurrent seizure activity. (23) An effect of gestational age (GA), postnatal age (PNA) and/or postmenstrual age (PMA) on the pharmacokinetics (PK) for lidocaine and MEGX can be expected given the physiological changes responsible for maturation of CYP1A2 and CYP3A activity as well as renal function in neonates.(24) In addition, therapeutic hypothermia (TH), lowering the core temperature to 33-34 °C for 72 hours which is the standard of care for term and near term neonates (GA ≥ 36 weeks) with HIE

to reduce the incidence of death and neurological disabilities,(25,26) can have an additional effect on lidocaine clearance due to reduction in organ perfusion and/or enzymatic activity secondary to TH.(27–31)

Previous studies from our group have investigated lidocaine PK in both preterm and term neonates with and without TH treatment. Lidocaine clearance was lower in preterm neonates compared to term neonates.(32) As GA in this population is closely related to body weight (BW), a novel dosing regimen differentiated by BW was developed for safe (lidocaine plasma concentrations <9 mg/L) and effective lidocaine use across the entire neonatal population (Table 1). Compared to the previously used dosing regimens, the loading phase was reduced from six to four hours.(18,32) In (near-)term neonates, TH was found to reduce lidocaine clearance by 24% compared to normothermia. Therefore, a further reduction of the loading phase to 3.5 hours during TH is incorporated in this dosing regimen.(33) However, we have only been able to investigate lidocaine PK in relatively small numbers of neonates and never simultaneously across the entire neonatal treatment spectrum. Furthermore, the developed dosing regimen has not been evaluated.

The aim of the present study was, therefore, to evaluate the previously developed population pharmacokinetic models and dosing regimen in a larger dataset encompassing preterm neonates and (near-)term neonates with and without TH treatment. As a secondary objective, anti-epileptic effectiveness and the incidence of cardiac toxicity was assessed.

Patients and methods

Setting and study population

For the current study, data collected in the multi-centre prospective cohort SHIVER study(33) and a historical dataset collected as part of routine clinical care(32) (both

Table 1: Novel lidocaine dosing regimen

	Bolus		**Loading phase**		**Maintenance phase I**		**Maintenance phase II**	
Weight	Dose	Duration	Dose	Duration	Dose	Duration	Dose	Duration
Normothermia								
< 1.6 kg	2 mg/kg	10 min	5 mg/kg	4 hours	2.5 mg/kg	12 hours	1.25 mg/kg	12 hours
1.6 – 2.6 kg	2 mg/kg	10 min	6 mg/kg	4 hours	3 mg/kg	12 hours	1.5 mg/kg	12 hours
> 2.6 kg	2 mg/kg	10 min	7 mg/kg	4 hours	3.5 mg/kg	12 hours	1.75 mg/kg	12 hours
Hypothermia								
< 2.5 kg	2 mg/kg	10 min	6 mg/kg	3.5 hours	3 mg/kg	12 hours	1.5 mg/kg	12 hours
≥ 2.5 kg	2 mg/kg	10 min	7 mg/kg	3.5 hours	3.5 mg/kg	12 hours	1.75 mg/kg	12 hours

previously published) were combined with data collected from patients receiving lidocaine in the multi-centre prospective cohort PharmaCool study and a novel dataset collected for routine clinical care. The SHIVER study and the PharmaCool study both included (near-)term neonates treated with TH for HIE; neonates with congenital disorders were excluded. Full in- and exclusion criteria have been published previously.(34,35) In both studies, written parental informed consent was obtained for each included neonate. The datasets collected during routine clinical care consisted of preterm and term neonates receiving lidocaine for treatment of neonatal seizures at the Neonatal Intensive Care Unit (NICU) of the University Medical Center Utrecht/Wilhelmina Children's Hospital between 2004 and 2018. An overview of the included neonates in the two studies and the two clinical care cohorts is shown in Table 2.

Table 2: overview of the included neonates per study and cohort

	Time period	No. of patients	Age groups, TH yes/no	Dosing regimen(s)	Sampling
Clinical care cohort 1	2004-2008	46	preterm and term neonates, no TH	See [32]	Between 4-6h and between 10-12h after start of lidocaine
SHIVER study	2008-2010	21*	(near-)term neonates, all treated with TH	See [33]	Once daily on days 2-5 after birth
PharmaCool study#	2010-2014	22	(near-)term neonates, all treated with TH	Various, none receiving the novel dosing regimen	Once daily on days 2-5 after birth
Clinical care cohort 2#	2010-2018	70	preterm and term neonates, 6 treated with TH	Various, 23 neonates receiving the novel dosing regimen	Between 4-6h and between 10-12h after start of lidocaine

*one patient from in the SHIVER study was excluded because the exact timing of TH could not be retrieved

#included for assessment of effectiveness and safety

TH = therapeutic hypothermia

Lidocaine dosing

In all neonates, lidocaine was prescribed as second- or third line AED for seizures refractory to midazolam and/or phenobarbital. Lidocaine was administrated as continuous intravenous infusion according to local clinical protocols. In the SHIVER and PharmaCool studies, choice of therapy or dosing regimen was not influenced by the study protocol. Neonates in the second clinical care cohort received the novel dosing regimen from 2015 onwards.

Sampling and bioanalysis

In the SHIVER and PharmaCool studies, once daily blood samples were drawn on days 2-5 after birth. In the two clinical care cohorts, blood samples were drawn between four and six hours and between ten and twelve hours after start of lidocaine administration. Plasma concentrations of lidocaine and MEGX were determined using a validated liquid chromatography-tandem mass spectrometric assay (LC-MS/MS) at the Clinical Pharmaceutical and Toxicological Laboratory of the Department of Clinical Pharmacy of the University Medical Center Utrecht, the Netherlands.(36)

Population pharmacokinetic analyses

PK analyses were performed using non-linear mixed effects modelling NONMEM (version 7.3, Icon Development Solutions).(37) Lidocaine hydrochloride doses were converted to lidocaine base and consecutively, all units of dose and concentration for lidocaine and MEGX were converted to µmol and µmol/L, respectively for the purpose of the PK analysis. If the measured concentration for one of the compounds was below the lower limit of quantification (LLQ), it was fixed to LLQ/2; samples with measurements below LLQ for both lidocaine and MEGX were excluded from analysis. As the fraction (F) of lidocaine converted to MEGX was unknown, MEGX parameters were estimated relative to F. Based on the previous publications, a one-compartment model for lidocaine with a consecutive one-compartment model for MEGX were used as structural models. BW was used as a descriptor for body

size and was related to pharmacokinetic parameters using allometric relationships. GA, PNA, PMA and body temperature (TEMP) were tested as covariates on clearance. TEMP was tested as a continuous variable using a dynamic model as described previously.(35) Inclusion of covariates was guided by effect size, biological plausibility and statistical significance.

Interindividual variability (IIV) was modelled using a proportional model and tested on all parameters. To account for below LLQ data, both proportional and additive error models were used to model residual unexplained variability in which the additive error was fixed on LLQ/2 . Separate error models were used for lidocaine and MEGX. Parameter precision was assessed with sampling importance resampling.(38) Both graphical (e.g. goodness-of-fit plots) and statistical model evaluation procedures were used to assess model adequacy.

Dosing regimen evaluation

To evaluate the previously developed dosing regimen, a simulation dataset was created by replicating the patient characteristics of each neonate included in the original dataset seven times. The observed lidocaine plasma concentrations from neonates receiving the novel dosing regimen were compared to the predicted lidocaine plasma concentrations using this simulation dataset and the parameter estimates from the pharmacokinetic model as reported by Van den Broek et al.(32) Additionally, simulations were conducted using the final parameter estimates from the pharmacokinetic model developed in this study and novel dosing regimen. Mean peak lidocaine plasma concentrations < 9 mg/L were considered effective and safe. Additionally, accumulation of MEGX should be avoided to reduce the occurrence of adverse effects related to MEGX, however an upper limit cannot be established based on literature.

Anti-epileptic effectiveness and cardiac events

Anti-epileptic effectiveness and incidence of cardiac events were assessed for neonates in the PharmaCool study and in the second cohort for clinical care as these have been published previously for the other datasets (Table 2).(18,33) Lidocaine was considered effective for seizure control if no additional anti-epileptic drug was needed after lidocaine therapy. Incidence of cardiac toxicity such as bradycardia, tachycardia, arrhythmias or asystole was based on spontaneous reports by the local investigators or the treating physicians.

Results

Patient characteristics

In total, 159 neonates were included in this study (SHIVER n = 21, PharmaCool n = 22, clinical care cohort 1 n = 46, clinical care cohort 2 n = 70, Table 2). Fifty neonates (31.4%) were born prematurely (<36 weeks GA) and 49 (30.8%) received TH (GA 36 to 42 weeks). Patient characteristics are presented in Table 3. Lidocaine and/or MEGX plasma concentrations were determined in 444 samples.

Table 3: Patient characteristics

Parameter	**Patients (n = 159)**
Gestational age; wk, mean ± sd	37.0 ± 4.84
Prematurity*; n (%)	50 (31.4%)
Body weight; kg, mean ± sd	2.89 ± 1.05
Male; n (%)	86 (54.1%)
Treated with TH; n (%)	49 (30.8%)

*Neonates born with a gestational age < 36 weeks

TH = therapeutic hypothermia

Population pharmacokinetic analyses

Pharmacokinetic parameter estimates of the final model are shown in Table 4. GA but not PNA was identified as covariate for both lidocaine and MEGX clearance. However, combining GA and PNA into PMA as covariate on clearance for both compounds provided a better fit of the data. Average lidocaine clearance for a neonate of 3.5 kg at PMA 40 weeks was 1.77 L/h (95% CI 1.63 – 2.03) whereas average lidocaine clearance for a neonate of 1 kg at PMA 25 weeks was 0.191 L/h (PMA effect 0.69%/day, 95% CI 0.54% – 0.84%). During TH, lidocaine clearance was reduced by 21.8% compared to normothermia (7.26%/°C, 95% CI 1.63% – 11.2%). No effect of TH on clearance of MEGX could be identified. Model evaluation demonstrated that the final models adequately described the data. Goodness-of-fit plots of observed versus population and individual predicted concentrations showed no systematic deviation and the weighted residuals were homogeneously scattered for both lidocaine and MEGX (Figures S1-S4).

Dosing regimen evaluation

Figure 1 shows that the observed lidocaine plasma concentrations from 22 normothermic neonates receiving the novel dosing regimen were comparable to the predicted lidocaine plasma concentrations for normothermic neonates. Only one neonate received the novel dosing regimen while treated with TH. Therefore, comparing the observed lidocaine plasma concentrations to the predicted range during TH was not sensible. Lidocaine plasma concentrations achieved with the novel dosing regimen (Table 1) were predicted using a simulation dataset of 1113 patients and the final PK parameter estimates. Both with and without TH, mean lidocaine peak plasma concentration was well below 9 ml/L (Figure 2) and no accumulation of MEGX occurred. During normothermia, 20.0% of simulations reached a lidocaine plasma concentration above 9 mg/L (4.7% >11 mg/L). During TH, lidocaine plasma concentrations exceeding 9 mg/L were reached in 31.8% of simulations (12.8% > 11 mg/L) .

Table 3: Final model pharmacokinetic parameter estimates and SIR results

	Lidocaine		**MEGX**[§]	
Parameter	*Estimate*	*SIR* 95% CI*	*Estimate*	*SIR* 95% CI*
Cl, l/h[$]	1.77	1.63 – 2.03	1.51	1.37 – 1.73
V, l[$]	9.32	8.49 – 9.63	15.8	13.6 – 18.7
PMA on Cl, %/d	0.690	0.581 – 0.837	0.350	0.114 – 0.805
TEMP^ on Cl, %/°C	7.26	1.63 – 11.2	NA	NA
Interindividual variability				
Cl, variance (rsd)	0.231 (48.1%)	0.211 – 0.336	0.237 (48.7%)	0.148 – 0.368
V, variance (rsd)	0.0673 (25.9%)	0.0365 – 0.0956	0.478 (69.1%)	0.346 – 0.672
Residual variability				
Additional, mg/L	0.1 (fixed)	NA	0.1 (fixed)	NA (fixed)
Proportional, variance (rsd)	0.0379 (19.5%)	0.0274 – 0.0431	0.0550 (23.5%)	0.0445 – 0.0754

Final model:

$Cl_{LIDOCAINE} = 1.77 \times (BW/3.5)^{0.75} \times (1 + 0.00690 \times (PMA – 280)) \times (1 + 0.0726 \times (TEMP-36.5))$

$V_{LIDOCAINE} = 9.32 \times (BW/3.5)^{1}$

$Cl_{MEGX}/F_{MEGX} = 1.51 \times (BW/3.5)0.75 \times (1 + 0.00350 \times (PMA – 280))$

$V_{MEGX}/F_{MEGX} = 15.8 \times (BW/3.5)^{1}$

[§]MEGX estimates are relative to formation fraction F.

*Ten iterations; no. of samples 1000,1000,1000,1000,1000,1000,2000,2000,2000,2000; no. of resamples 200,200,400,400,500,500,1000,1000,1000,1000

[$]Estimates for neonate with a birth weight of 3.5 kg and PMA 40 weeks

^In neonates treated with TH, TEMP was set to 33.5°C during TH with rewarming at 0.4°C/h. Normothermia for all neonates was set to 36.5°C

Cl = clearance, V = volume of distribution, PMA = postmenstrual age, TEMP = body temperature, SIR = sampling importance resampling, NA = not applicable, rsd = relative standard deviation, TH = therapeutic hypothermia

Figure 1: Evaluation of the dosing regimen presented in Table 1 in 22 normothermic neonates. Black dots represent the observed lidocaine plasma concentrations. Solid line indicates the mean lidocaine plasma concentration and dark gray area represents the 95% prediction interval simulated with the final parameter estimates as reported by Van den Broek et al.(32)

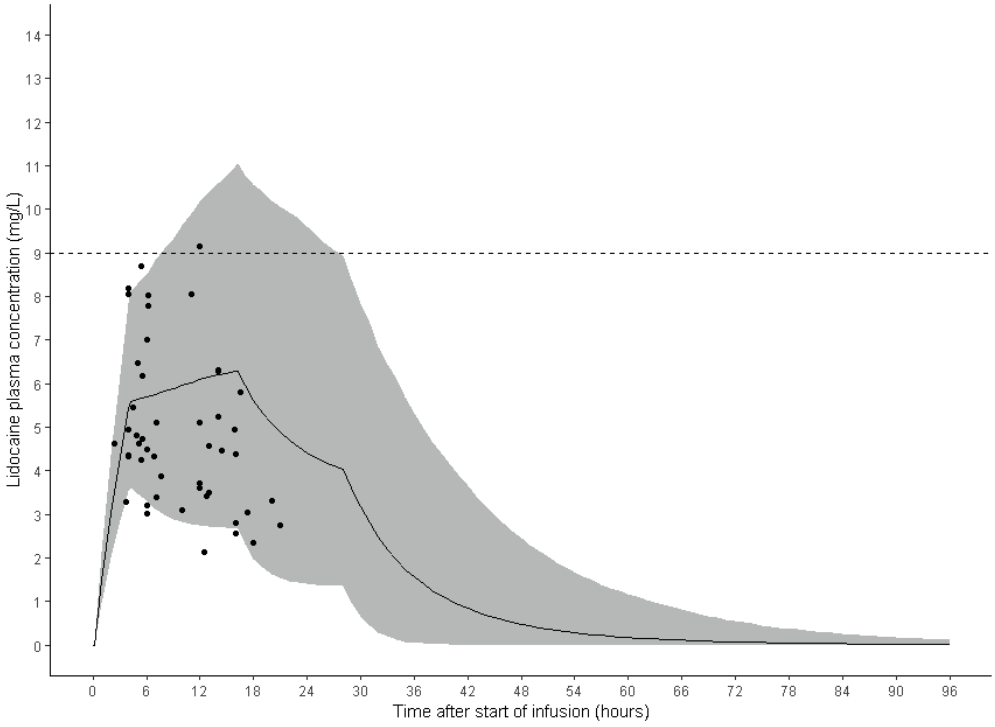
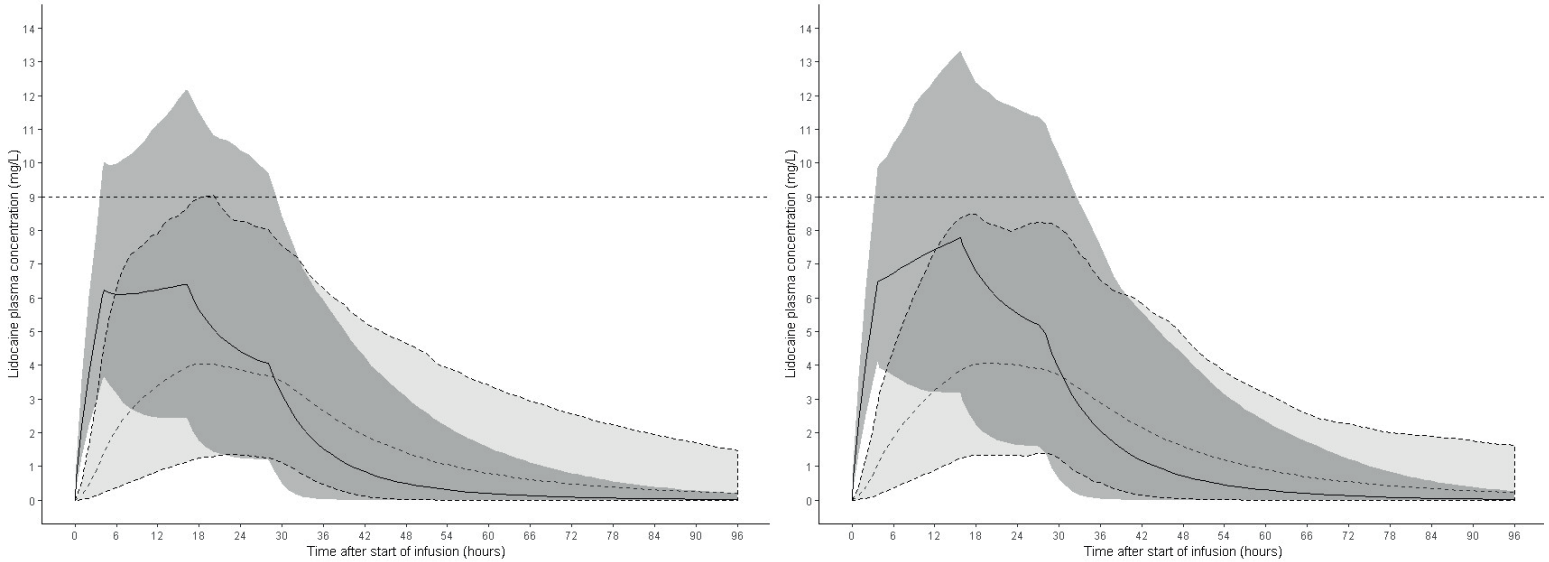


Figure 2: Simulated lidocaine and MEGX plasma concentrations for normothermic (left) and hypothermic (right) neonates with the final parameter estimates obtained in this study and dosing regimen presented in Table 1. Solid line indicates the mean lidocaine plasma concentration; dark gray area represents the 95% prediction interval. Dotted lines and light gray area indicate the mean MEGX plasma concentration and 95% prediction interval. Horizontal dotted line indicates the suggested upper limit for lidocaine of 9 mg/L.



Anti-epileptic effectiveness and cardiac events

Effectiveness and safety were assessed in 92 neonates. Overall, lidocaine was considered an effective anticonvulsant in 53.3% (49/92) of neonates. Of the 26 infants without TH who were unresponsive to lidocaine 13 had meningitis, and 7 a major haemorrhage. Other diagnoses included asphyxia (4), GNAO1 mutation (1), and tuberous sclerosis (1). Lidocaine appeared to be most effective in term neonates not treated with TH. The predicted lidocaine plasma concentrations at the end of the loading phase did not differ between effective and ineffective treatment (Figure 3). The novel dosing regimen was administered to 23 neonates (25.0%). Within this subgroup, effectiveness was 56.5% (13/23) and lidocaine effectiveness appeared to be greater in (near-)term neonates than in preterm neonates. Lidocaine effectiveness is summarised in Table 5. No cardiac adverse events were reported.

Figure 3: Predicted lidocaine plasma concentrations at the end of the loading phase in neonates where lidocaine was effective (left) and ineffective (right). Horizontal lines represent the median plasma concentration; blue boxes represent the interquartile ranges; vertical lines indicate the highest and lowest plasma concentration.

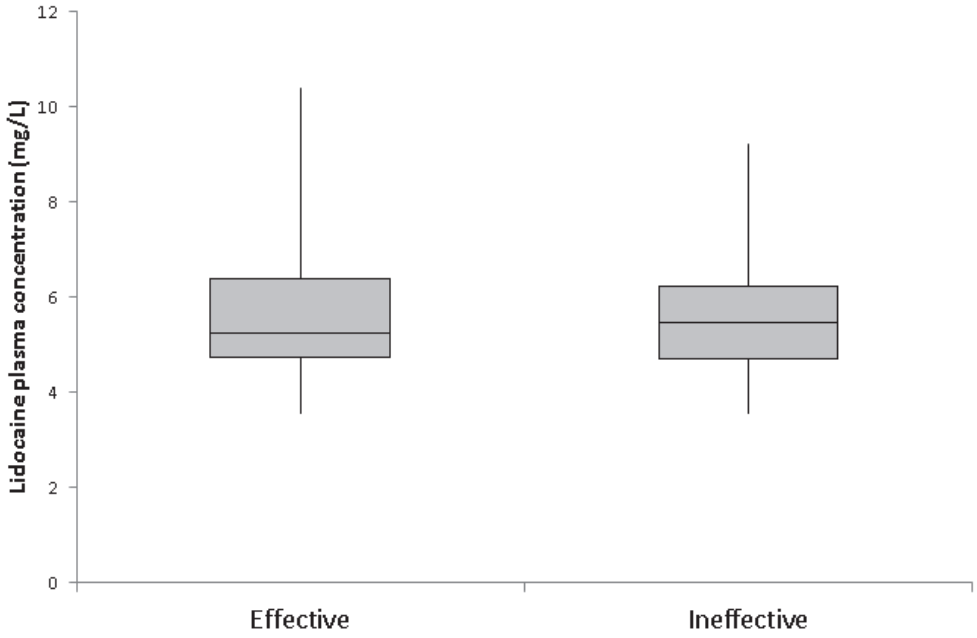


Table 5: Effectiveness of lidocaine as anti-epileptic drug

	Effective, n (%)	**Ineffective, n (%)**
All patients (n=92)	49 (53.3%)	43 (46.7 %)
GA < 36 weeks (n=28)	*11 (39.3%)*	*17 (60.7%)*
GA ≥ 36 weeks, NT (n=36)	*27 (75.0 %)*	*9 (25.0%)*
GA ≥ 36 weeks, TH (n=28)	*11 (39.3 %)*	*17 (50.0%)*
Novel dosing regimen (n=23)	13 (56.5%)	10 (44.5%)
GA < 36 weeks (n=6)	*3 (50%)*	*3 (50%)*
GA ≥ 36 weeks (n=17)	*10 (58.8%)*	*7 (41.2%)*

GA = gestational age, NT = normothermia, TH = therapeutic hypothermia

Discussion

This study successfully described lidocaine PK in a large dataset combining both preterm and term neonates with and without TH treatment. PMA and TH were identified as significant covariates on lidocaine clearance. The final parameter estimates for lidocaine clearance and volume of distribution obtained in this study are largely consistent with the previous findings, which confirms the previously identified PK parameters.(18,32,33) Evaluation of the novel dosing regimen showed that average lidocaine plasma concentrations are well below the potential toxic upper limit of 9 mg/L for all neonates. Lidocaine effectiveness did not differ between the novel and previous dosing regimens and no cardiac toxicity occurred.

Lidocaine is metabolised by the liver and its metabolites, including MEGX, are subsequently eliminated via the kidneys. PMA is identified as a covariate for maturation of both lidocaine and MEGX clearance. PMA was found to be a descriptor for maturation of morphine clearance, a hepatically cleared drug, by

Knøsgaard et al. and for glomerular filtration rate by Rhodin et al.(39,40) Both studies included data from neonates as well as older children and adults and incorporated PMA using a sigmoidal Hill equation. The TM_{50}, the PMA at which clearance is 50% of the mature value, was estimated in both studies to be around 55 weeks. Our study was comprised of only neonatal data with a minimum PMA of 25 weeks and maximum PMA of 42.7 weeks. In this age range, the sigmoidal Hill equations approach linearity. Therefore, we decided to include PMA as a linear effect in this study.

TH reduced lidocaine clearance by 21.8% compared to normothermia. This is consistent with our findings in a previous study.(33) Lidocaine is qualified as a drug with a high hepatic extraction ratio, with hepatic perfusion as rate limiting step for metabolism.(41) During hypothermia, hepatic blood flow is decreased due to a decrease in cardiac output and stroke volume. Therefore, we hypothesise that reduced blood flow during TH is primarily responsible for decreased lidocaine clearance.(28–31) However, an additional or synergistic effect of TH on activity of CYP enzymes cannot be excluded based on this data. Renal perfusion is also dependent on cardiac output, but an effect of TH on clearance of MEGX has not been identified. Although an effect of TH on other renally cleared drugs and metabolites has been noted, this finding is consistent with the current knowledge regarding MEGX clearance.(33,35,42,43)

Simulations performed with the final PK estimates from the present study shows that the novel dosing regimen as depicted in Table 1 leads to acceptable average lidocaine plasma concentrations for both normothermic preterm and term neonates as well as term neonates treated with TH. The large interpatient variability will lead to plasma concentrations exceeding the supposed threshold for cardiac toxicity of 9 mg/L in some neonates for an acceptable period of time. Additionally, the novel dosing regimen was validated by comparing the observed lidocaine plasma

concentrations from neonates receiving this regime to the predicted concentrations based on the original PK model.(32) In 22 normothermic neonates the observed plasma concentrations fall largely within the predicted range. Unfortunately, this validation could not be performed for neonates treated with TH due to insufficient data.

Anticonvulsant effectiveness for lidocaine was assessed in 92 neonates in whom effectiveness was not reported previously. No relationship between effectiveness and lidocaine plasma concentration at the end of the loading phase could be identified. A higher lidocaine success rate was noted in (near-)term neonates. This is consistent with a retrospective study with 413 term and preterm neonates where a significantly lower success rate was observed in preterm vs term neonates (55.3% vs 76.1%); overall anti-epileptic effectiveness of lidocaine was 71.4%.(19) Seizure control in our study was slightly lower but did not differ between the novel and the old dosing regimen. In neonates treated with TH, however, lidocaine responds rate was markedly lower in the present study compared to Van den Broek et al. (39.3% vs 91%).(33) The majority of data on neonates treated with TH comes from the PharmaCool study (22/28, 78.6%). In the PharmaCool study, data was only collected for five days after birth. As lidocaine was administered mostly as third line anticonvulsant after phenobarbital and midazolam in this population, only neonates who resorted to lidocaine treatment relatively early were included. It could be argued that these were neonates with more severe HIE unresponsive to any anti-epileptic treatment. Mortality for neonates receiving lidocaine in the PharmaCool study was 77.3% (17/22), confirming the highly moribund prognosis. Additionally, Van den Broek et al. defined effectiveness as a reduction of electrographic seizure burden of >80% within 4 hours of commencing lidocaine instead of no need for additional anticonvulsants after lidocaine therapy and thus a shorter time frame for detecting treatment failure.

Cardiac toxicity was not seen in any neonate included in this study. Although lidocaine associated cardiac toxicity has been reported, it is extremely rare and fatal incidents have only been described when lidocaine was used concomitantly with phenytoin.(20,23,44,45) Lidocaine cardiac toxicity has been associated with plasma concentrations exceeding 9 mg/L. However, the relationship between toxicity and plasma concentration is based on anecdotal evidence in adults with coronary disease and might not be valid for neonates.(46,47) Additionally, neonates undergoing TH might be even less susceptible to cardiac side effects. Lidocaine inhibits voltage-dependent sodium channels on the cardiomyocyte, which are only available during the systolic phase.(33,48) During TH, the heart frequency is reduced compared to normothermia, thereby reducing the potential for cardiotoxicity. However, to minimise the chance of cardiac toxicity, lidocaine should not be co-administered with phenytoin or to neonates with congenital heart disease.(22)

Conclusion

This study describes lidocaine PK across the entire neonatal age range, with PMA and TH as significant covariates on clearance. The previously developed novel dosing regimen leads to lidocaine plasma concentrations within the desired range with comparable effectiveness to the older regimens. No cardiac toxicity occurred. With the developed dosing regimen, lidocaine can be a safe and effective add-on anticonvulsant in preterm and term neonates with and without TH.

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Compliance with Ethical Standards

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Conflicts of Interest: The authors declare no conflicts of interest.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the SHIVER and PharmaCool studies.

Supplementary materials

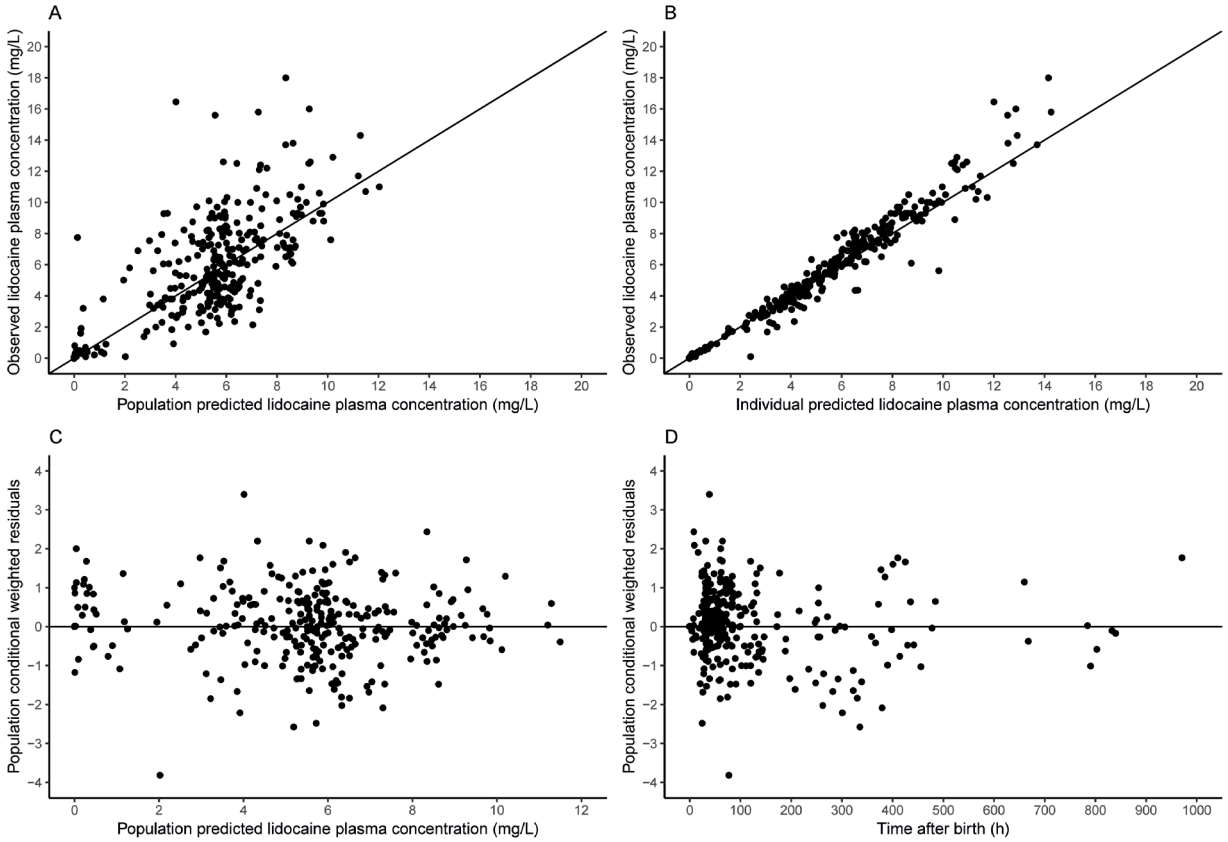

Figure S1: Lidocaine goodness-of-fit plots for normothermic neonates. A = observed vs population predicted plasma concentrations; B = observed vs individual predicted plasma concentrations; C = population conditional weighted residuals vs population predicted plasma concentrations; D = population conditional weighted residuals vs time after birth;

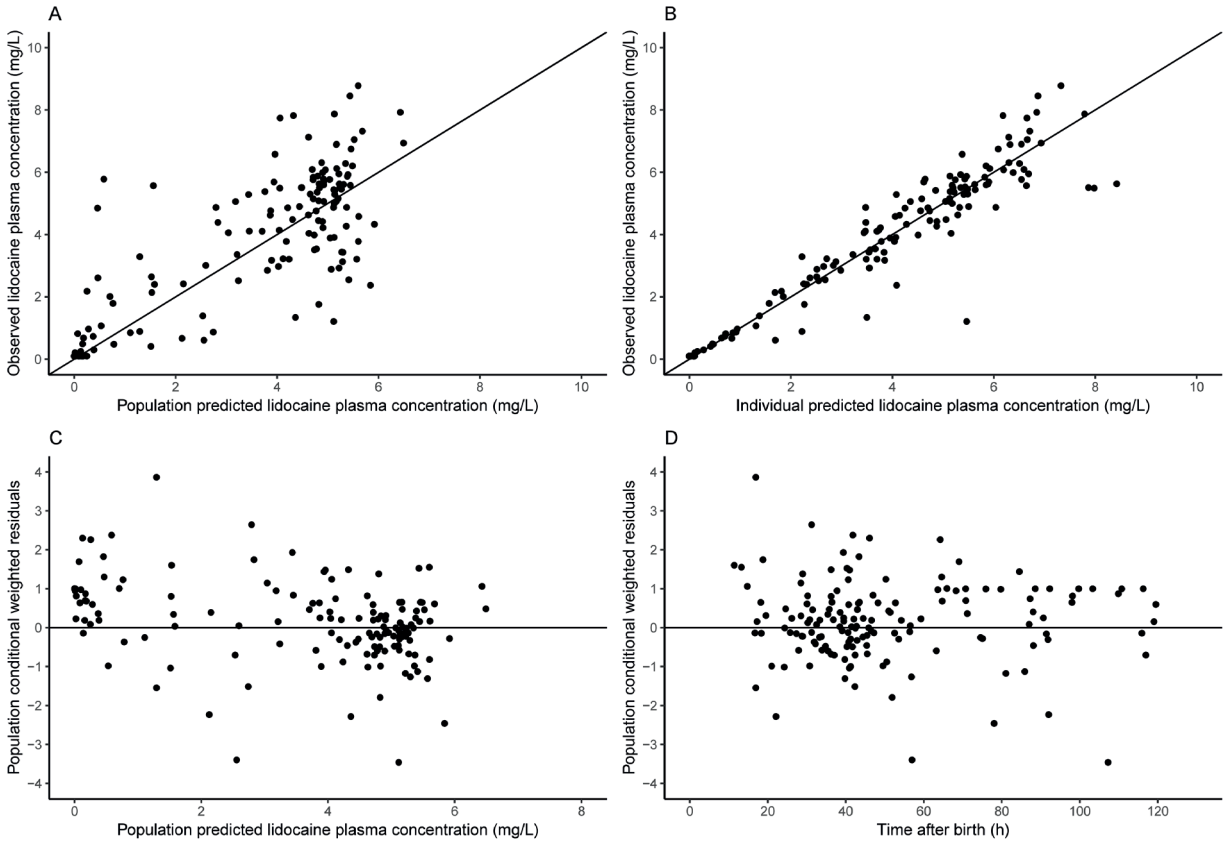

Figure S2: Lidocaine goodness-of-fit plots for hypothermic neonates. A = observed vs population predicted plasma concentrations; B = observed vs individual predicted plasma concentrations; C = population conditional weighted residuals vs population predicted plasma concentrations; D = population conditional weighted residuals vs time after birth;

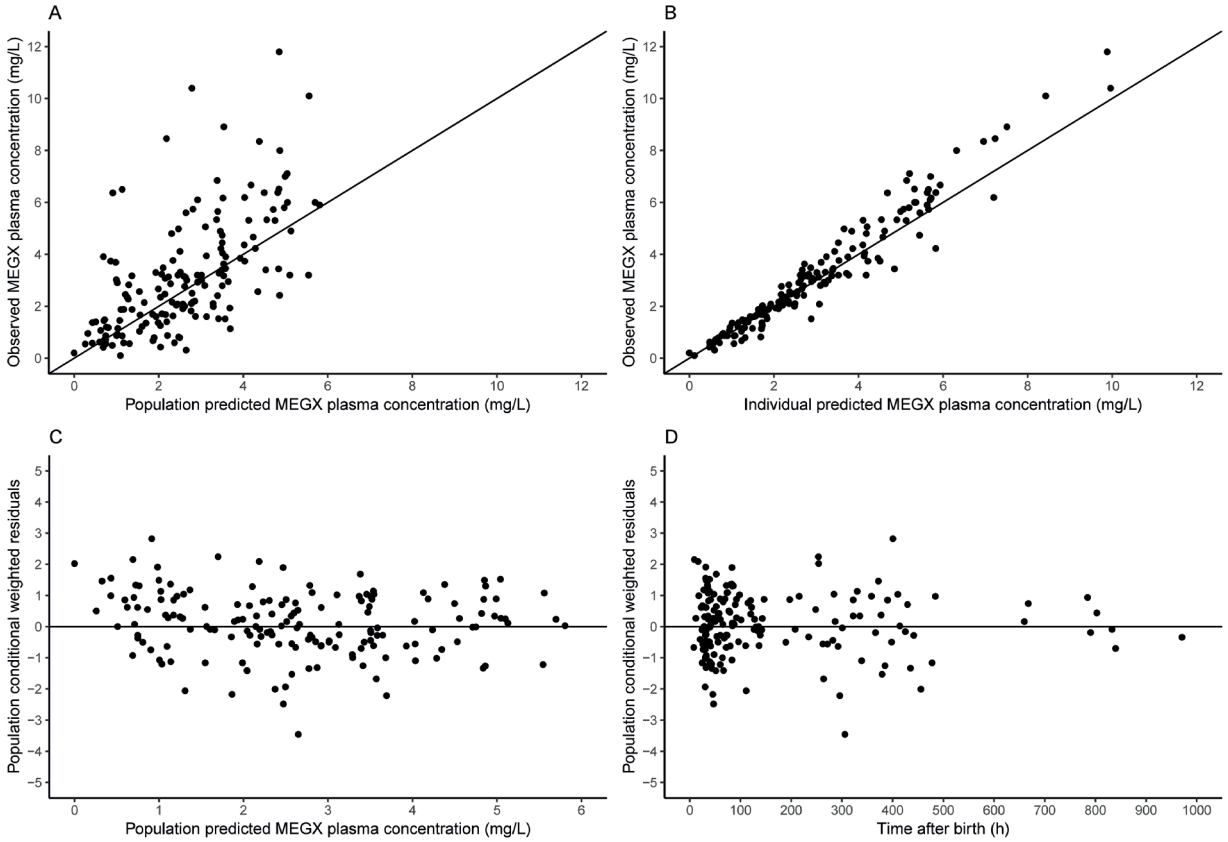

Figure S3: MEGX goodness-of-fit plots for normothermic neonates. A = observed vs population predicted plasma concentrations; B = observed vs individual predicted plasma concentrations; C = population conditional weighted residuals vs population predicted plasma concentrations; D = population conditional weighted residuals vs time after birth;

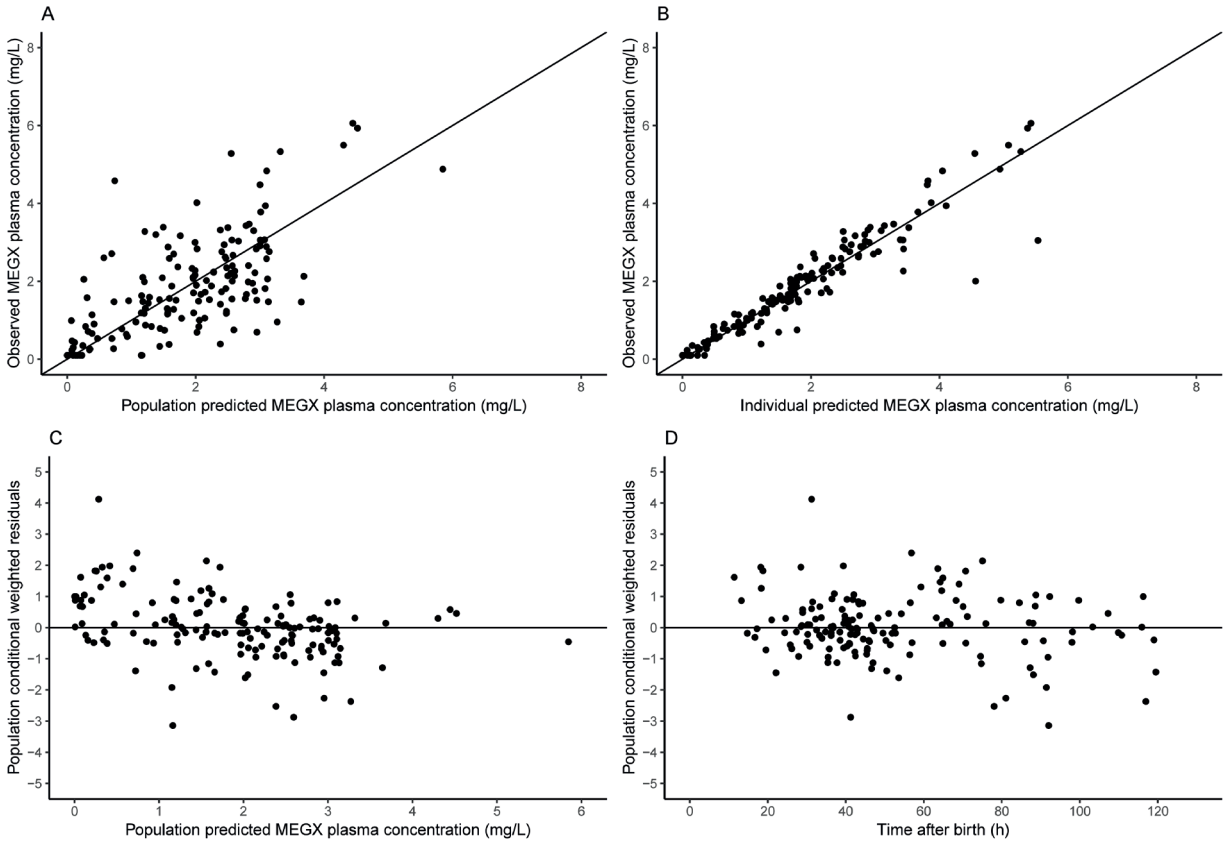

Figure S4: MEGX goodness-of-fit plots for hypothermic neonates. A = observed vs population predicted plasma concentrations; B = observed vs individual predicted plasma concentrations; C = population conditional weighted residuals vs population predicted plasma concentrations; D = population conditional weighted residuals vs time after birth; solid line indicates the linear regression line

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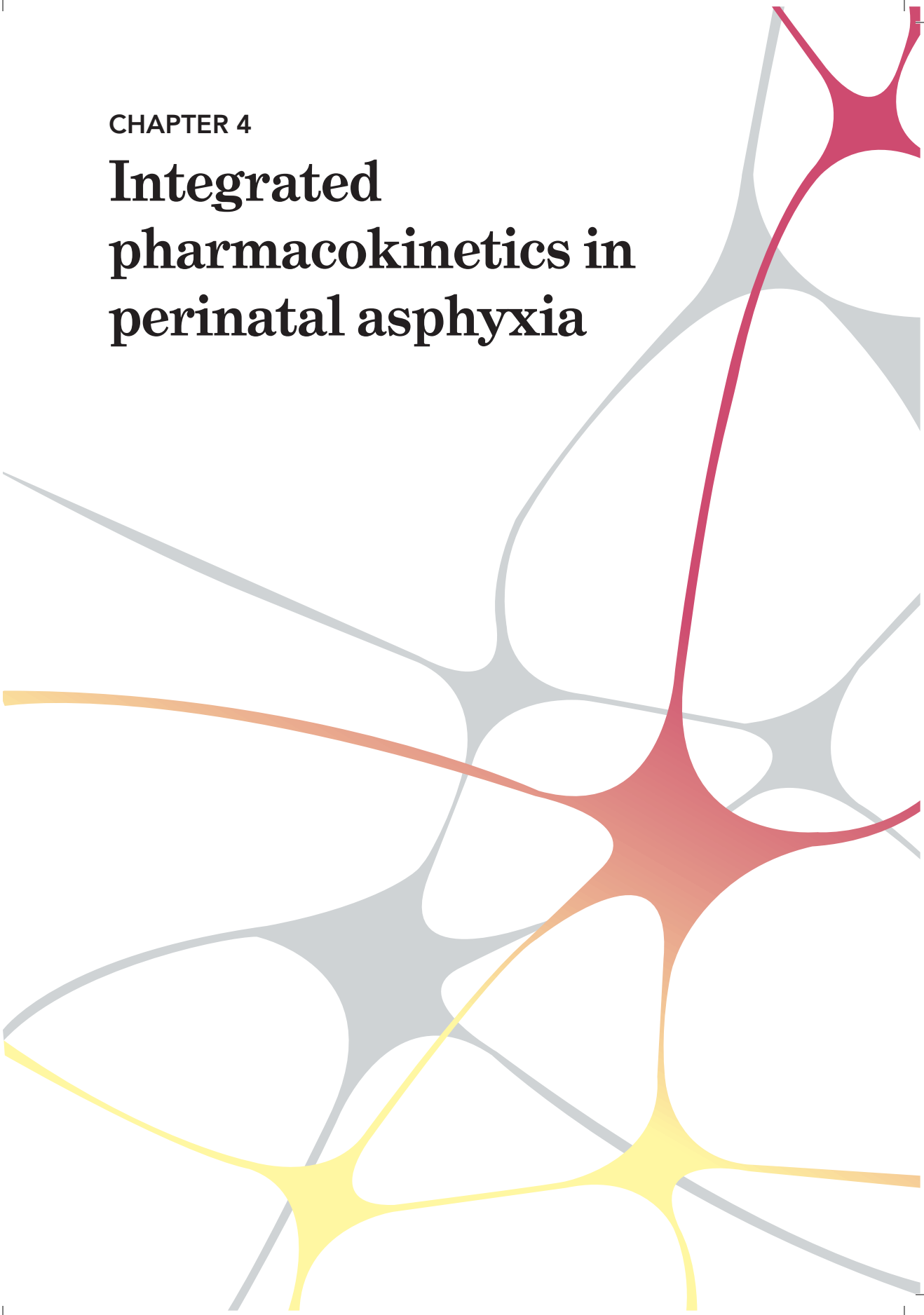
CHAPTER 4

Integrated pharmacokinetics in perinatal asphyxia



Chapter 4.1

An integrated population pharmacokinetic analysis of seven drugs and five metabolites in critically ill neonates with perinatal asphyxia treated with therapeutic hypothermia

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Abstract

Drug dosing in encephalopathic neonates treated with therapeutic hypothermia is challenging. Drug exposure is dependent on body size and maturation but can also be influenced by factors related to disease and treatment. Data from seven drugs administered to these neonates (n = 192) were combined to study these structural effects using NONMEM. An integrated population pharmacokinetic model was developed based on previously developed models for the individual drugs. For all compounds, body size was related to clearance using allometric relationships and maturation was described with gestational age in a fixed sigmoidal Hill equation. Organ recovery was incorporated using postnatal age. Clearance increased by 1.23%/h (95% CI 1.03 – 1.43) and by 0.54%/h (95% CI 0.371 – 0.750) for high and intermediate clearance compounds respectively. Therapeutic hypothermia reduced clearance of intermediate clearance compounds by 6.83%/˚C (95% CI 5.16%/˚C – 8.34%/˚C). This integrated model can be used to facilitate future PK studies in this population.

Introduction

Dosing of drugs in neonates is extremely challenging due to body size and composition related differences in pharmacokinetics (PK) and ongoing maturation of organ function.(1) For neonates admitted to a neonatal intensive care unit (NICU), finding the optimal dose is even more challenging due to variability associated with illness severity and recovery. In addition, these neonates are exposed to a multitude of drugs such as sedatives, analgesics, antibiotics, and anti-epileptic drugs (AED) that may kinetically and dynamically interact with each other.(2)

Perinatal asphyxia (i.e. severe interruption of circulation and oxygenation at birth) can lead to hypoxic-ischemic organ damage throughout the body, potentially resulting in multi organ failure.(3,4) Central nervous system dysfunction leading to hypoxic ischemic encephalopathy (HIE) is a major concern as the brain is least likely to have a quick or complete recovery.(5) Therefore, (near-)term neonates with moderate or severe HIE after perinatal asphyxia are routinely treated with therapeutic hypothermia (TH) to reduce the incidence of death and long term developmental disability.(6,7) According to the Dutch national clinical protocol, TH is intended for neonates with documented moderate or severe HIE and a gestational age (GA) of at least 36.0 weeks and has to be started within 6 hours after birth. During TH, the body temperature of the neonate is reduced to 33.5 °C for 72 hours, after which the neonate is gradually rewarmed.(8) Appropriate dosing of essential drugs in this extremely vulnerable population is challenging due to the multitude of complicating factors.

To investigate the PK of frequently used drugs in neonates with HIE during and after treatment with TH, the prospective multicenter observational cohort PharmaCool study has been conducted in 12 level III NICUs in the Netherlands and Belgium.(2) The resulting publications from the PharmaCool study group have described

the PK of antibiotics, sedatives and AEDs in this population and a population pharmacokinetic model was developed for each individual drug.(9–12) For all drugs, body size was related to clearance using an allometric relationship. However, the effects of TH, maturation and recovery of organ function after asphyxia varied between the different drugs and metabolites. This can partly be explained by the different number of patients available for each individual compound with sometimes relatively small numbers, albeit that this was the largest study performed thus far in this population.

It can be anticipated that the effects regarding body size, maturation, recovery of organ function and body temperature transcend individual drugs and reflect underlying physiological processes that can be applied to PK in general. Therefore, we hypothesized that for drugs with similar clinical pharmacological characteristics (e.g. hepatic or renal clearance) the impact of these effects on PK is similar. Therefore, in the current study all data available for analysis from the PharmaCool study were integrated to study the structural system specific effects of body size, maturation, recovery of organ function and temperature on the PK of commonly used drugs in HIE. Additionally, it was expected that the clearance of the different drugs and metabolites were correlated within one individual, especially between drugs eliminated via the same organ system.

Methods

Setting, study design and study population

The prospective multicenter observational cohort PharmaCool study (www.trialregister.nl, NTR2529) was conducted in twelve tertiary NICUs in the Netherlands and Belgium. Neonates undergoing TH for HIE were eligible for inclusion; full inclusion and exclusion criteria have been published previously.(2) Written parental informed consent was obtained from each included neonate. The PharmaCool study

was approved by the institutional review boards of all participating centers and was supported by a grant from the Netherlands Organization for Health Research and Development (ZonMw) Priority Medicines for Children project (grant number: 40-41500-98-9002).

Data collected from seven drugs in the PharmaCool study were included in this study: gentamicin, amoxicillin, benzylpenicillin, morphine, midazolam, phenobarbital and lidocaine. For none of the drugs, initial dosing or choice of therapy was influenced by the study protocol. Any dose adjustment was based on clinical care or therapeutic drug monitoring according to local clinical protocol. Full dosing information was recorded in the online Case Report Forms of the neonates participating in this study.

Pharmacokinetic sampling and bioanalyses

For the PK analyses, blood samples were obtained on days 2-5 after birth, both during and after TH. All plasma concentrations were determined using validated liquid chromatography-tandem mass spectrometric (LC-MS/MS) essays. Metabolites for morphine (morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G)), midazolam (1-hydroxymidazolam (OHM) and hydroxymidazolam glucuronide (HMG)) and lidocaine (monoethylglycinexylidide (MEGX)) were also included. The sampling schedule for each drug as well as details regarding the bioanalyses have been described previously.(2,9–13)

Population pharmacokinetic analysis

A population PK model was developed using the nonlinear mixed effect modelling program NONMEM (version 7.3, Icon Development Solutions) with R (version 3.4.1), Xpose (version 4) for data visualization and Piraña for run management. (14) Datasets from the individual drugs were restructured and combined so that all data could be fitted simultaneously. Each neonate was included once in the

final combined dataset but could contain data from multiple drugs. The previously developed structural models were used with regard to number of compartments and structural PK parameters estimated. For midazolam, OHM, HMG, lidocaine and MEGX, measurements below the lower limit of quantification (LLQ) were fixed to LLQ/2;(15) for the other compounds, no below LLQ data were present. To reduce model complexity, all volumes of distribution and the associated variability were fixed to the parameter estimates from the individual models. As a separate population PK model for lidocaine and MEGX from PharmaCool data was not yet available, the volume of distribution for lidocaine and MEGX were fixed to values obtained in a previous population PK study in the same population.(13)
The incorporation of body size, maturation, recovery of organ function after birth and body temperature in the population PK model is described in detail below.

Body size

All physiological processes are related to body size, and body weight (BW) is the most common descriptor for body size. It is well documented that many physiological processes and organ sizes exhibit an allometric relationship with BW, not only for humans but across different species.(16) Because PK parameters are dependent on these physiological processes, parameters such as clearance and volume of distribution can also be described using allometric equations relative to BW.(16,17) In this study, an exponent on BW of 0.75 for clearance and an exponent of 1 for volume of distribution were used.(18)

Maturation

In neonates, the function of organs responsible for drug clearance is immature and as a result, PK parameters in neonates differ from older children and adults. Neonatal renal function at birth is underdeveloped compared to adults and undergoes maturation over the first weeks to months of life until it reaches body size adjusted adult values between eight and twelve months after birth. Hepatic

clearance in neonates is also attenuated compared to adults and the enzymes responsible for drug metabolism such as the cytochrome P 450 (CYP450) and glucuronosyltransferase (UGT) mature at different rates.(1,19,20) Rhodin et al. showed that maturation of renal clearance across the entire pediatric population was well described using postmenstrual age (PMA) with a sigmoidal Hill equation. The TM50, the PMA at which clearance is 50% of the mature value, was estimated at 55.4 weeks and the Hill coefficient describing the slope of the sigmoidal curve at 3.33.(21) Recently, Knøsgaard et al. found that maturation of morphine, a hepatically cleared drug, was also related to PMA with a sigmoidal Hill equation and with similar values for TM_{50} and Hill coefficient (54.2 weeks and 3.92, respectively).(22)

In our study, PMA was almost fully determined by GA as data was only collected until five days after birth. GA was, therefore, introduced in our model as a covariate describing maturation using the following equation:

$$GA_i = GA_i^{HILL}/(GA_i^{HIL} + TM_{50}^{HILL})$$

Our population consisted of neonates with a GA between 36 and 42 weeks, which is at least 12 weeks before reaching the TM_{50} for both models describing maturation. Both published models on maturation behave similarly at these early time points after birth and, therefore, we choose to fix the Hill coefficient to 3.92 and TM_{50} to 54.2 weeks.

Recovery of organ function

In the individual models, a strong increase in clearance after birth was identified, which is much larger than can be explained by maturation or the influence of TH. During the hypoxic-ischemic event, both kidneys and liver are deprived of oxygen, resulting in possible functional nephron and hepatocyte damage.(23–27)

Additionally, cardiac output might also be hampered due to a loss in myocardial function.(28) After resuscitation and stabilisation at the NICU, gradual recovery of organ function after birth was anticipated. In our model, this was described using postnatal age (PNA) as a separate covariate on clearance. The effect was tested separately for the drugs and metabolites fully renally cleared (gentamicin, amoxicillin, benzylpenicillin, M3G, M6G, HMG and MEGX) versus hepatically cleared drugs and metabolites (morphine, phenobarbital, midazolam, OHM and lidocaine). Subsequently, it was tested whether differences in effect could be identified for hepatically cleared drugs with high clearance (lidocaine) vs. intermediate (morphine, midazolam, OHM) vs. low clearance drugs (phenobarbital). Because we hypothesized that recovery of organ function could differ between neonates due to differences in asphyxia severity, interindividual variability of the effect of PNA on clearance was also included.

Body temperature

Alterations in body temperature (TEMP) may be of influence for drug clearance. TH decreases heart rate and cardiac output which will subsequently reduce kidney and liver perfusion.(29,30) Unfortunately normal values for renal and hepatic flow in this population are unknown and are highly variable so the effect of flow change on PK cannot be estimated. Additionally, hepatic clearance might also be affected by altered activity of liver enzymes. Since most enzymatic processes exhibit temperature dependency, a lower body temperature might result in reduced enzyme activity and thus reduced clearance.(31,32) Upon rewarming, processes hampered by the hypothermic state can recover, resulting in an increase in drug clearance. TEMP was tested as a continuous variable using a dynamic model of temperature over time. For all neonates, the reported start and end times of TH were used to determine the period of TH treatment. TEMP during TH was set at 33.5˚C, with consecutive rewarming at 0.4˚C/hour (i.e. rewarming time 7.5h) until 36.5˚C after which body temperature was set to 36.5˚C for the remainder of the study time.

Similarly to the effect of recovery of organ function, the effect of TEMP on clearance was tested separately for the renally vs. hepatically cleared drugs.

Correlation in clearance

Interindividual variability in clearance not explained by BW, GA, PNA or TEMP was introduced using a log-normal distribution according to the following equation:

$$CL_{i,n} = TVCL_n * e^{\eta_{i,n}}$$

In which $CL_{i,n}$ is the clearance of compound n in individual i, $TVCL_n$ is the typical value of clearance of compound n and $\eta_{i,n}$ describes the interindividual variability assuming that all values of η_n have a normal distribution with mean 0 and standard deviation ω_n. In the first step a full OMEGA matrix was considered to study the correlation between interindividual variability of the different clearance components. However, the resulting OMEGA matrix contained 12 diagonal and 66 off-diagonal parameters to be estimated. Therefore, the model was simplified by introducing one common η value on all clearance parameters describing the correlation between the different components according to the following equations:

$$CL_{i,1} = TVCL_1 * e^{\eta_{i,1}+\theta_1*\eta_{i,common}}$$

$$CL_{i,2} = TVCL_2 * e^{\eta_{i,2}+\theta_2*\eta_{i,common}}$$

In which $CL_{i,1}$ and $CL_{i,2}$ denote the clearance of compound 1 and 2 for individual *i*, respectively. $TVCL_1$ and $TVCL_2$ denote the typical values of clearance for compound 1 and 2, respectively, $\eta_{i,1}$ and $\eta_{i,2}$ describe interindividual variability of compound 1 and 2, respectively. Correlation is described by ηi,common which is the part of interindividual variability which is common for both clearance components and

θ_1 and θ_2 denote the scaling factor for this common variability component. For morphine this scaling factor was fixed to 1 and all other scaling factors were, therefore, relative to morphine clearance. Interindividual variability in clearance and correlation between different clearance components were subsequently calculated using:

$$\omega_{1,total} = \sqrt{\omega_1^2 + (\theta_1 * \omega_{common})^2}$$

$$\omega_{2,total} = \sqrt{\omega_2^2 + (\theta_2 * \omega_{common})^2}$$

$$R_{1,2} = \frac{\theta_1 * \theta_2 * \omega_{common}^2}{\omega_{1,total} * \omega_{2,total}}$$

In which $\omega_{1,total}$ and $\omega_{2,total}$ denote the total interindividual variability of compound 1 and 2, respectively; ω_1^2 and ω_2^2 denote the compound-specific interindividual variability for compound 1 and 2, respectively, and ω_{common} denotes the common interindividual variability for all clearance components. $R_{1,2}$ describes the correlation in clearance between compound 1 and 2.

Separate proportional error models for all compounds were used to model residual unexplained variability. For midazolam, OHM, HMG, lidocaine and MEGX, an additive error fixed on LLQ/2 to account for below LLQ data was also included.

Parameter precision was assessed with sampling importance resampling (SIR). (33) Both graphical (e.g. goodness-of-fit plots) and statistical model evaluation procedures were used to assess model adequacy.

Results

Patient characteristics

In total, 192 neonates from the PharmaCool study were included in this analysis. Patient characteristics as well as number of patients and plasma samples per drug are displayed in Table 1.

Table 1: patient characteristics

Parameter	**Patients (n = 192)**	
Gestational age; wk, mean ± sd	39.7 ± 1.66	
Birth weight; kg, mean ± sd	3.38 ± 0.617	
Male, n (%)	118 (61.5%)	
Drug	*Patients (n)*	*Samples (n)*
Morphine	180	534
Amoxicillin	125	1280
Midazolam	118	376
Phenobarbital	113	378
Gentamicin	47	471
Benzylpenicillin	43	416
Lidocaine	28	77

Population pharmacokinetic analysis

The final structural models from the original publications were used as the structural model for this analysis.(9–13) PK estimates of the parameters related to clearance from final model are shown in Table 2. Full PK parameter estimates from the final model are included in Table S1.

Table 2: Pharmacokinetic parameter estimates and SIR results relating to clearance

Compound		Parameter		
		Cl, L/h[#]	PNA on Cl, %/h	TEMP on Cl, %/ ˚C
Morphine	Estimate	0.811	0.540	6.83
	*SIR 95% CI**	*0.707 – 0.937*	*0.371 – 0.750*	*5.16 – 8.34*
Midazolam	Estimate	0.511	0.540	6.83
	*SIR 95% CI**	*0.387 – 0.620*	*0.371 – 0.750*	*5.16 – 8.34*
OHM[§]	Estimate	1.72	0.540	6.83
	*SIR 95% CI**	*1.43 – 2.05*	*0.371 – 0.750*	*5.16 – 8.34*
M3G[§]	Estimate	0.241	1.23	NA
	*SIR 95% CI**	*0.220 – 0.269*	*1.03 – 1.43*	*NA*
M6G[§]	Estimate	0.765	1.23	NA
	*SIR 95% CI**	*0.697 – 0.854*	*1.03 – 1.43*	*NA*
HMG[§]	Estimate	0.111	1.23	NA
	*SIR 95% CI**	*0.0977 – 0.126*	*1.03 – 1.43*	*NA*
Amoxicillin	Estimate	0.178[^]	1.23	NA
	*SIR 95% CI**	*0.159 – 0.196*	*1.03 – 1.43*	*NA*
Benzylpenicillin	Estimate	0.359[^]	1.23	NA
	*SIR 95% CI**	*0.297 – 0.423*	*1.03 – 1.43*	*NA*
Gentamicin	Estimate	0.108[^]	1.23	NA
	*SIR 95% CI**	*0.0968 – 0.120*	*1.03 – 1.43*	*NA*
Lidocaine	Estimate	0.937	1.23	NA
	*SIR 95% CI**	*0.783 – 1.11*	*1.03 – 1.43*	*NA*
MEGX[§]	Estimate	1.51	1.23	NA
	*SIR 95% CI**	*0.991 – 2.06*	*1.03 – 1.43*	*NA*
Phenobarbital	Estimate	0.00930	NA	NA
	*SIR 95% CI**	*0.00785 – 0.0111*	*NA*	*NA*

[#]Estimates for a neonate with BW 3.5 kg, GA 280 days, PNA 0h and TEMP 36.5˚C

* Six iterations; no. of samples 4000, 4000, 4000, 4000, 4000, 4000; no. of resamples 250, 250, 250, 250, 250, 1000

[§]All metabolite estimates are relative to their formation fraction

[^]Estimated clearance in the central compartments. Peripheral compartment estimates are included in the Appendix

Cl = clearance, PNA = postnatal age, TEMP = body temperature, SIR = sampling importance resampling, CI = confidence interval, OHM = 1-hydroxymidazolam, M3G = morphine-3-glucuronide, M6G = morphine-6-glucuronide, HMG = hydroxylmidazolam glucuronide, MEGX = monoethylglycinexylidide, BW = body weight, GA = gestational age, NA = not applicable

Body size and maturation

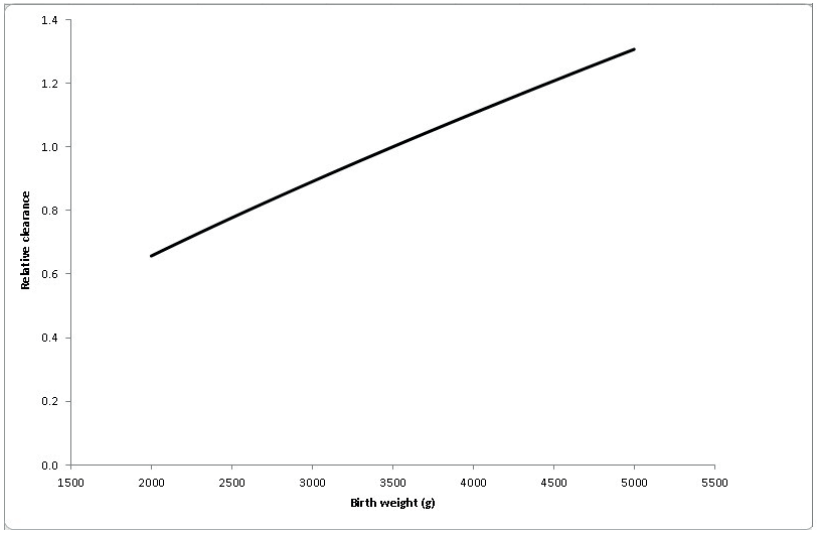
BW was used as a descriptor for body size in our population and was related to PK parameters using allometric relationships with an exponent of 0.75 on (intercompartmental) clearance and an exponent of 1 on volume of distribution. GA was used to describe maturation using a sigmoidal Hill equation with 54.2 weeks as TM_{50} and a Hill coefficient of 3.92. To test the validity of these assumptions, the population conditional weighted residuals for each compound were plotted against BW and GA. No systematic deviation was observed indicating the appropriateness of these assumptions (Supplementary Materials). The influence of BW and GA on drug clearance are depicted in Figure 1.

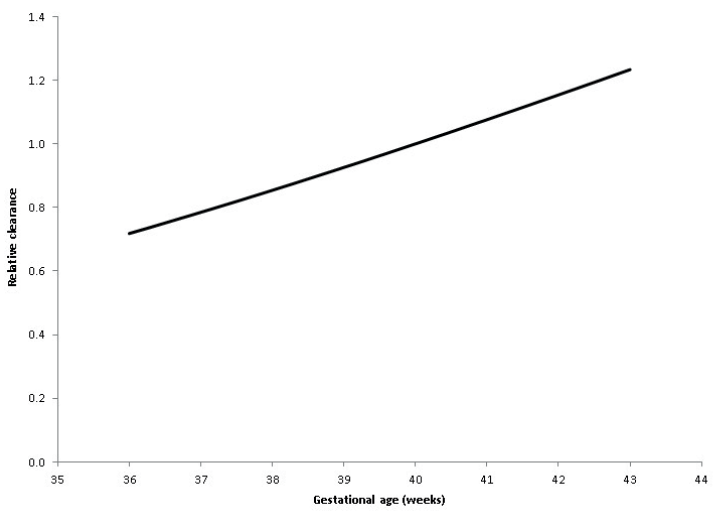
Recovery of organ function

PNA was identified as a covariate on clearance for all compounds except phenobarbital. After grouping these compounds into renal clearance (amoxicillin, benzylpenicillin, gentamicin, M3G, M6G, HMG, MEGX), hepatic high clearance (lidocaine) and hepatic intermediate clearance (morphine, midazolam and OHM), it was found that the effect of PNA on renally cleared compounds was similar to the effect of PNA on lidocaine clearance. Therefore, it was decided to separate the covariate PNA into two groups: high clearance compounds (renally cleared compounds and lidocaine) and intermediate clearance compounds (morphine, midazolam and OHM). In the high clearance group, the relative effect of PNA on clearance was 1.23%/h (95% CI 1.03 – 1.43); in the intermediate clearance group, this was 0.54%/h (95% CI 0.371 – 0.750). As expected, the effect of PNA by far exceeded the effects of maturation. According to the sigmoidal Hill equation used to describe maturation, clearance would increase with approximately 0.05%/h in the first five days after birth in this population.(22)

Furthermore, large interindividual variability was found for these effects: 71.6% for the high clearance compounds and 54.8% for the intermediate clearance

Figure 1: The influence of birth weight (left) and gestational age (right) on clearance of all drugs in the final pharmacokinetic model



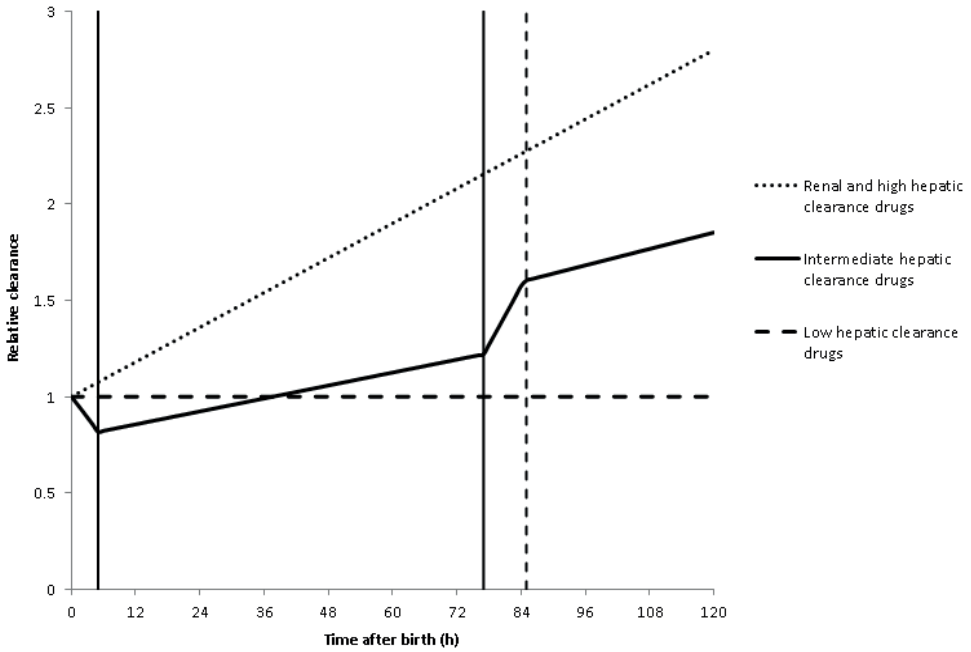


compounds. Interindividual variabilities in both effects showed a very high correlation, which was subsequently fixed to 100% to reduce model complexity (Table S1).

Body temperature

TEMP was identified as an independent covariate on clearance for all compounds except phenobarbital. However, after inclusion of PNA and maturation, the influence of TEMP on clearance was only significant for the intermediate clearance drugs (morphine, midazolam and OHM). During TH, clearance was decreased by 20.5% (6.83%/˚C, 95% CI 5.16%/˚C – 8.34%/˚C) compared to normothermia. The influence of both PNA and TEMP on the average clearance for the three identified drug groups are shown in Figure 2.

Figure 2: The influence of postnatal age and body temperature on the clearance for the three identified drug groups in the final pharmacokinetic model. Vertical solid lines indicate therapeutic hypothermia; vertical dashed line indicates return to normothermia



Correlation in clearance

Correlation in clearance was calculated between all compounds except phenobarbital, lidocaine and MEGX. Phenobarbital was not tested since its clearance visually did not correlate with any other compound. Correlation for lidocaine and MEGX could not be estimated due to the sparseness of the data (only 28 neonates and 77 samples). Correlation in clearance for the remaining compound is presented in Table 3. All correlations were positive. The highest correlation was between clearance of M3G and M6G (96.2%). Correlation in clearance in the hepatically cleared compounds was relatively high; the renally cleared compounds do not show a higher correlation within the group than compared to the hepatically cleared compounds.

Model evaluation demonstrated that the final model was adequate in describing the data. Goodness-of-fit plots of observed versus population and individual predicted concentrations showed no systematic deviation and the weighted residuals were homogeneously scattered versus predicted values and time for all compounds (Supplementary Materials).

Discussion

This study successfully combined clinical and pharmacological data collected for seven drugs (in total twelve compounds including metabolites) in the multicenter PharmaCool study into one PK model. Body size related effects were adequately captured with allometric scaling. Previously developed models for maturation were successfully implemented in this population using GA as descriptor. Subsequently, the model was extended to quantify the effects of body temperature and recovery of organ function. As drug dosing is highly challenging in neonates, this may be even more difficult in critically ill neonates with HIE treated with TH. Studies in this populations are extremely difficult to perform and, therefore, it is of importance to

Table 3: Correlation in clearance. Correlations < 40% are indicated in yellow; correlations 40-60% are indicated in orange; correlations < 60% are indicated in grean. Top left black box indicates correlation between the hepatically cleared compounds. Bottom right black box box indicates correlation between the renally cleared compounds

	Morphine	Midazolam	OHM	M3G	M6G	HMG	Amoxicillin	Benzylpenicillin	Gentamicin
Morphine									
Midazolam	62.2%								
OHM	63.3%	57.0%							
M3G	35.4%	31.9%	32.2%						
M6G	32.5%	29.3%	29.6%	94.8%					
HMG	62.6%	56.4%	57.0%	31.9%	29.3%				
Amoxicillin	42.1%	38.0%	38.3%	21.4%	19.7%	37.9%			
Benzylpenicillin	58.6%	52.8%	53.4%	31.9%	27.4%	52.8%	35.5%		
Gentamicin	45.9%	41.4%	41.8%	23.4%	21.5%	41.4%	27.9%	38.8%	

fully elucidate and quantify the processes that influence the PK of drugs used in this population.

BW was used as descriptor for body size with an allometric exponent of 0.75. This allometric relationship between BW and clearance is based on physiological observations both within and across species and is the most widely used method to describe size differences.(16–18,34,35) Although its validity has been debated over the years, especially in neonates,(36) we showed that allometry adequately described the relationship with body size in the model which also included the other structural effects including maturation.

PMA is acknowledged as the most reliable age-related factor to reflect the biology of clearance maturation.(37) Incorporating PMA with a sigmoid E_{max} model is believed to be the best empirical approach to describe maturation of clearance over time.(18) In our study population, PMA was determined by GA for more than 98% since data was collected for only a short period after birth. Therefore, we included GA as a descriptor for maturation. In the literature, maturation of both renal and hepatic clearance has been described using PMA with a sigmoid E_{max} model with similar values for Hill coefficient and TM_{50}.(21,22) We hypothesized that this function would adequately describe maturational differences in clearance for all compounds. Careful model evaluation supported this hypothesis. It should, however, be noted that full evaluation of maturation in this population is hampered by the small range in GA (no preterm neonates included) and a relatively short observation period.

After including BW and GA as fixed effects, clearance proved to be strongly dependent on PNA. This effect was much larger than can be expected from maturation alone.(38) Therefore, we hypothesize that this was caused by recovery of organ function after asphyxia. The effect of PNA was largest in the high clearance compounds. For these drugs and metabolites, clearance is closely linked to

organ perfusion. As damaged liver and kidney cells regenerate, organ perfusion and thereby clearance will increase rapidly. Clearance of intermediate clearance compounds is not only dependent on organ perfusion but also on enzyme capacity. Enzymes such as cytochrome P450 3A and UDP glucuronosyltransferase 2B7 might show slower recovery or may even be unaffected by asphyxia which could explain the smaller effect of PNA in this group compared to the high clearance compounds. No effect of PNA was identified on phenobarbital clearance. As phenobarbital is an extremely low clearance drug for which clearance is independent of hepatic perfusion, this finding was not unexpected. Interindividual variability on the effect of PNA on clearance was 71.6% and 54.8% for high and intermediate clearance compounds, respectively. As the severity of asphyxia differs between neonates with HIE, so will recovery of organ function. A much stronger recovery in less severely ill neonates versus no recovery at all in the most severely ill might explain this relatively high variability, which supports the interpretation that the large effect of PNA on clearance is most likely caused by recovery of organ function.

After inclusion of PNA, TEMP could only be identified as covariate on clearance of the intermediate clearance compounds. Although this is somewhat contradictory to the individual models, we believe that in the current model the relatively small decrease in clearance caused by a lower temperature is not identifiable due to the much more pronounced effect of PNA in the high clearance group. In the intermediate clearance group, the effect of TEMP on clearance could be identified separately from the smaller effect of PNA.

Positive correlation in clearance was identified for all compounds except phenobarbital, lidocaine and MEGX. The high correlation between M3G and M6G was expected given the structural similarities.(12) For all other compounds relatively high positive correlations were found, where the hepatically cleared compounds showed highest correlation.

This study is the first to integrate data from some of the most important and most

frequently used drug in neonates treated with TH for HIE into one integrated population PK model. Previously, the population PK model for amoxicillin was successfully applied to predict clearance of benzylpenicillin in the PharmaCool study population.(11) The present model included compounds with hepatic and renal elimination and has identified covariates on clearance that transcend individual drugs and routes of elimination. This integrated model can be used to predict clearance of other drugs in this population based on data from older children or adults. Levetiracetam is an AED which is used increasingly in neonates treated with TH for HIE.(39) Based on its clinical pharmacokinetic profile, levetiracetam can be grouped with the high clearance drugs and it can be expected that clearance increases strongly with PNA in this population. (40,41) The same prediction can be made for 2-iminobiotin and allopurinol, high clearance drugs that are currently being investigated for additional neuroprotection in combination with TH.(42–44) Although our model cannot be used to fully describe the PK of any (new) drug administered to neonates treated with TH for HIE, it can be used to predict changes in drug clearance. While individual PK studies will still be necessary, knowledge obtained from this integrated model can facilitate the design and might reduce the number of patient needed for those studies.

Conclusion

Data from seven different drugs (twelve compounds) administered to neonates treated with TH for HIE were successfully combined into one integrated population PK model. PNA was identified as a covariate on clearance of both high clearance and intermediate clearance compounds and TEMP was subsequently identified as covariate on intermediate clearance compounds. Individual clearance values were positively correlated for nine compounds. This integrated model can be used to facilitate future PK studies for other drugs in this population by predicting (changes in) drug clearance based on the clinical pharmacokinetic properties of that drug.

Supplementary Materials

Table S1: full final pharmacokinetic parameter estimates and SIR results

Structural PK parameters[#§]			
Compound		**Estimate**	**SIR 95% CI***
Morphine	Cl, l/h	0.811	*0.707 – 0.937*
	V, l	8.88 FIX	NA
Midazolam	Cl, l/h	0.511	*0.387 – 0.620*
	V, l	5.42 FIX	NA
OHM	Cl, l/h	1.72	*1.43 – 2.05*
	V, l	4.18 FIX	NA
M3G	Cl, l/h	0.241	*0.220 – 0.269*
	V, l	0.264 FIX	NA
M6G	Cl, l/h	0.765	*0.697 – 0.854*
	V, l	4.53 FIX	NA
HMG	Cl, l/h	0.111	*0.0977 – 0.126*
	V, l	1.06 FIX	NA
Amoxicillin	Cl, l/h	0.178	*0.159 – 0.196*
	V_c, l	1.21 FIX	NA
	Q, l/h	0.686	0.600 – 0.814
	V_p, l	1.21 FIX	NA
Benzylpenicillin	Cl, l/h	0.359	*0.297 – 0.423*
	V_c, l	2.08 FIX	NA
	Q, l/h	0.178	0.137 – 0.244
	V_p, l	3.55 FIX	NA
Gentamicin	Cl, l/h	0.108	*0.0968 – 0.120*
	V_c, l	1.63 FIX	NA
	Q, l/h	0.158	0.130 – 0.208
	V_p, l	1.52 FIX	NA
Lidocaine	Cl, l/h	0.937	*0.783 – 1.11*
	V, l	10.9 FIX	NA
MEGX	Cl, l/h	1.51	*0.991 – 2.06*
	V, l	4.59 FIX	NA
Phenobarbital	Cl, l/h	0.00930	*0.00785 – 0.0111*
	V, l	3.60 FIX	NA

Table S1 continued

Covariates	**Estimate**	**SIR 95% CI***
PNA intermediate clearance, %/h	0.540	*0.371 – 0.750*
PNA high clearance, %/h	1.23	*1.03 – 1.43*
TEMP intermediate clearance, %/ ˚C	6.83	5.16 – 8.34

OMEGA structure			
	Estimate	**SIR 95% CI***	
Common OMEGA on Cl, rsd	43.7	35.4 – 50.6	
Compound		**Estimate**	**SIR 95% CI***
Morphine	IIV Cl, rsd	29.0	21.5 – 34.8
	Common THETA on IIV Cl	1 FIX	NA
	IIV V, rsd	68.1 FIX	NA
Midazolam	IIV Cl, rsd	56.0	40.3 – 68.8
	Common THETA on IIV Cl	1.46	1.11 – 1.86
	IIV V, rsd	96.6 FIX	NA
OHM	IIV Cl, rsd	51.8	40.8 – 61.7
	Common THETA on IIV Cl	1.38	0.984 – 1.78
	IIV V, rsd	0 FIX	NA
M3G	IIV Cl, rsd	49.6	43.9 – 55.4
	Common THETA on IIV Cl	0.532	0.338 – 0.755
	IIV V, rsd	0 FIX	NA
M6G	IIV Cl, rsd	52.1	46.0 – 58.6
	Common THETA on IIV Cl	0.504	0.282 – 0.725
	IIV V, rsd	0 FIX	NA
HMG	IIV Cl, rsd	33.5	26.5 – 41.8
	Common THETA on IIV Cl	0.870	0.603 – 1.18
	IIV V, rsd	91.5 FIX	NA

Table S1 continued

Amoxicillin	IIV Cl_c, rsd	40.4	34.0 – 47.6
	Common THETA on IIV Cl_c	0.541	0.325 – 0.762
	IIV V_c, rsd	103 FIX	NA
	IIV Q, rsd	0 FIX	NA
	IIV V_p, rsd	0 FIX	NA
Benzylpenicillin	IIV Cl_c, rsd	37.4	25.6 – 48.8
	Common THETA on IIV Cl_c	0.847	0.449 – 1.25
	IIV V_c, rsd	81.9 FIX	NA
	IIV Cl_p, rsd	0 FIX	NA
	IIV Q, rsd	68.5 FIX	NA
Gentamicin	IIV Cl_c, rsd	21.6	16.0 – 28.6
	Common THETA on IIV Cl_c	0.327	0.184 – 0.526
	IIV V_c, rsd	79.9 FIX	NA
	IIV Q, rsd	0 FIX	NA
	IIV V_p, rsd	85.4 FIX	NA
Lidocaine	IIV Cl, rsd	21.2	12.6 – 28.2
	Common THETA on IIV Cl	NA	NA
	IIV V, rsd	72.5 FIX	NA
MEGX	IIV Cl, rsd	80.1	56.2 – 109
	Common THETA on IIV Cl	NA	NA
	IIV V, rsd	93.2 FIX	NA
Phenobarbital	IIV Cl, rsd	57.6	44.0 – 73.8
	Common THETA on IIV Cl	NA	NA
	IIV V, rsd	21.0 FIX	NA

IIV on PNA effect	**Estimate**	**SIR 95% CI***
IIV – high clearance, rsd	71.6	60.1 – 83.4
Scaling factor for intermediate clearance	0.765	0.660 – 0.925
Correlation of PNA effects, %	100	NA

Table S1 continued

SIGMA structure			
Compound		**Estimate**	**SIR 95% CI***
Morphine	Proportional, rsd	23.4	21.3 – 25.3
	Additive, mg/l	NA	NA
Midazolam	Proportional, rsd	36.7	33.5 – 41.0
	Additive, mg/l	0.01 FIX	NA
OHM	Proportional, rsd	29.8	26.9 – 33.6
	Additive, mg/l	0.01 FIX	NA
M3G	Proportional, rsd	19.2	18.0 – 21.1
	Additive, mg/l	NA	NA
M6G	Proportional, rsd	18.0	16.5 – 19.6
	Additive, mg/l	NA	NA
HMG	Proportional, rsd	27.2	24.7 – 29.7
	Additive, mg/l	0.01 FIX	NA
Amoxicillin	Proportional, rsd	23.1	21.9 – 24.6
	Additive, mg/l	NA	NA
Benzylpenicillin	Proportional, rsd	36.2	33.5 – 39.5
	Additive, mg/l	NA	NA
Gentamicin	Proportional, rsd	24.8	23.1 – 27.1
	Additive, mg/l	NA	NA
Lidocaine	Proportional, rsd	22.3	19.4 – 25.6
	Additive, mg/l	0.1 FIX	NA
MEGX	Proportional, rsd	25.2	22.0 – 29.2
	Additive, mg/l	0.1 FIX	NA
Phenobarbital	Proportional, rsd	9.25	8.46 – 10.0
	Additive, mg/l	NA	NA

#Estimates for a neonate with BW 3.5 kg, GA 280 days, PNA 0h and TEMP 36.5˚C

*Six iterations; no. of samples 4000, 4000, 4000, 4000, 4000, 4000; no. of resamples 250, 250, 250, 250, 250, 1000

§All estimates for OHM, M3G, M6G, HMG and MEGX are relative to their formation fraction

Final model:

$GA_i = GA_i^{3.92}/(GA_i^{3.92} + 54.2^{3.92})$

$GA_{st} = 40^{3.92}/(40^{3.92} + 54.2^{3.92})$

$Cl_{MORPHINE} = 0.811 \times (BW/3.5)^{0.75} \times GA_i/GA_{st} \times (1 + PNA \times 0.0054) \times (1 + 0.0683 \times (TEMP-36.5))$

$Cl_{MIDAZOLAM} = 0.511 \times (BW/3.5)^{0.75} \times GA_i/GA_{st} \times (1 + PNA \times 0.0054) \times (1 + 0.0683 \times (TEMP-36.5))$

$Cl_{OHM}/F_{OHM} = 1.72 \times (BW/3.5)^{0.75} \times GA_i/GA_{st} \times (1 + PNA \times 0.0054) \times (1 + 0.0683 \times (TEMP-36.5))$

$Cl_{M3G}/F_{M3G} = 0.241 \times (BW/3.5)^{0.75} \times GA_i/GA_{st} \times (1 + PNA \times 0.0123)$

$Cl_{M6G}/F_{M6G} = 0.765 \times (BW/3.5)^{0.75} \times GA_i/GA_{st} \times (1 + PNA \times 0.0123)$

$Cl_{HMG}/F_{HMG} = 0.111 \times (BW/3.5)^{0.75} \times GA_i/GA_{st} \times (1 + PNA \times 0.0123)$

$Cl_{AMOXICILLIN} = 0.178 \times (BW/3.5)^{0.75} \times GA_i/GA_{st} \times (1 + PNA \times 0.0123)$

$Q_{AMOXICILLIN} = 0.686 \times (BW/3.5)^{0.75}$

$Cl_{BENZYLPENICILLIN} = 0.359 \times (BW/3.5)^{0.75} \times GA_i/GA_{st} \times (1 + PNA \times 0.0123)$

$Q_{BENZYLPENICILLIN} = 0.178 \times (BW/3.5)^{0.75}$

$Cl_{GENTAMICIN} = 0.108 \times (BW/3.5)^{0.75} \times GA_i/GA_{st} \times (1 + PNA \times 0.0123)$

$Q_{GENTAMICIN} = 0.158 \times (BW/3.5)^{0.75}$

$Cl_{LIDOCAINE} = 0.937 \times (BW/3.5)^{0.75} \times GA_i/GA_{st} \times (1 + PNA \times 0.0123)$

$Cl_{MEGX}/F_{MEGX} = 1.51 \times (BW/3.5)^{0.75} \times GA_i/GA_{st} \times (1 + PNA \times 0.0123)$

$Cl_{PHENOBARBITAL} = 0.00930 \times (BW/3.5)^{0.75} \times GA_i/GA_{st}$

$IIV\ CL_{MORPHINE,\ TOTAL} = 29.0\% + 43.7\%$

$IIV\ CL_{MIDAZOLAM,\ TOTAL} = 56.0\% + (1.46 \times 43.7\%)$

$IIV\ CL_{OHM,\ TOTAL} = 51.8\% + (1.38 \times 43.7\%)$

$IIV\ CL_{M3G,\ TOTAL} = 49.6\% + (0.532 \times 43.7\%)$

$IIV\ CL_{M6G,\ TOTAL} = 52.1\% + (0.504 \times 43.7\%)$

$IIV\ CL_{HMG,\ TOTAL} = 33.5\% + (0.870 \times 43.7\%)$

$IIV\ CL_{AMOXICILLIN,\ TOTAL} = 40.4\% + (0.541 \times 43.7\%)$

$IIV\ CL_{BENZYLPENICILLIN,\ TOTAL} = 37.4\% + (0.847 \times 43.7\%)$

IIV $CL_{GENTAMICIN, TOTAL}$ = 21.6% + (0.327 x 43.7%)

IIV $CL_{LIDOCAINE, TOTAL}$ = 21.2%

IIV $CL_{MEGX, TOTAL}$ = 80.1%

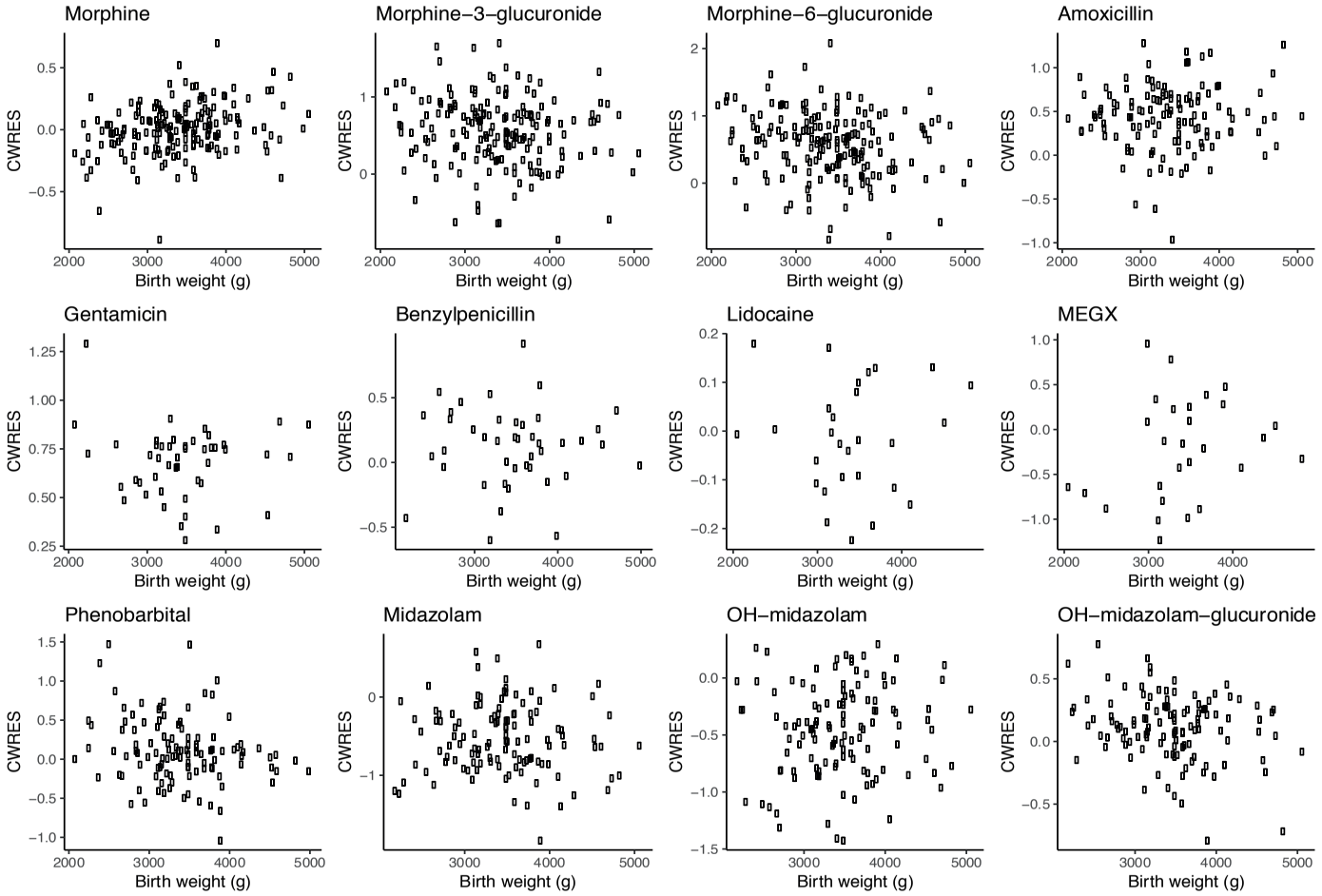
IIV $CL_{PHENOBARBITAL, TOTAL}$ = 57.6%

IIV $PNA_{HIGH\ CLEARANCE}$ = 71.6%

IIV $PNA_{INTREMEDIATE\ CLEARANCE}$ = 0.765 x 71.6%

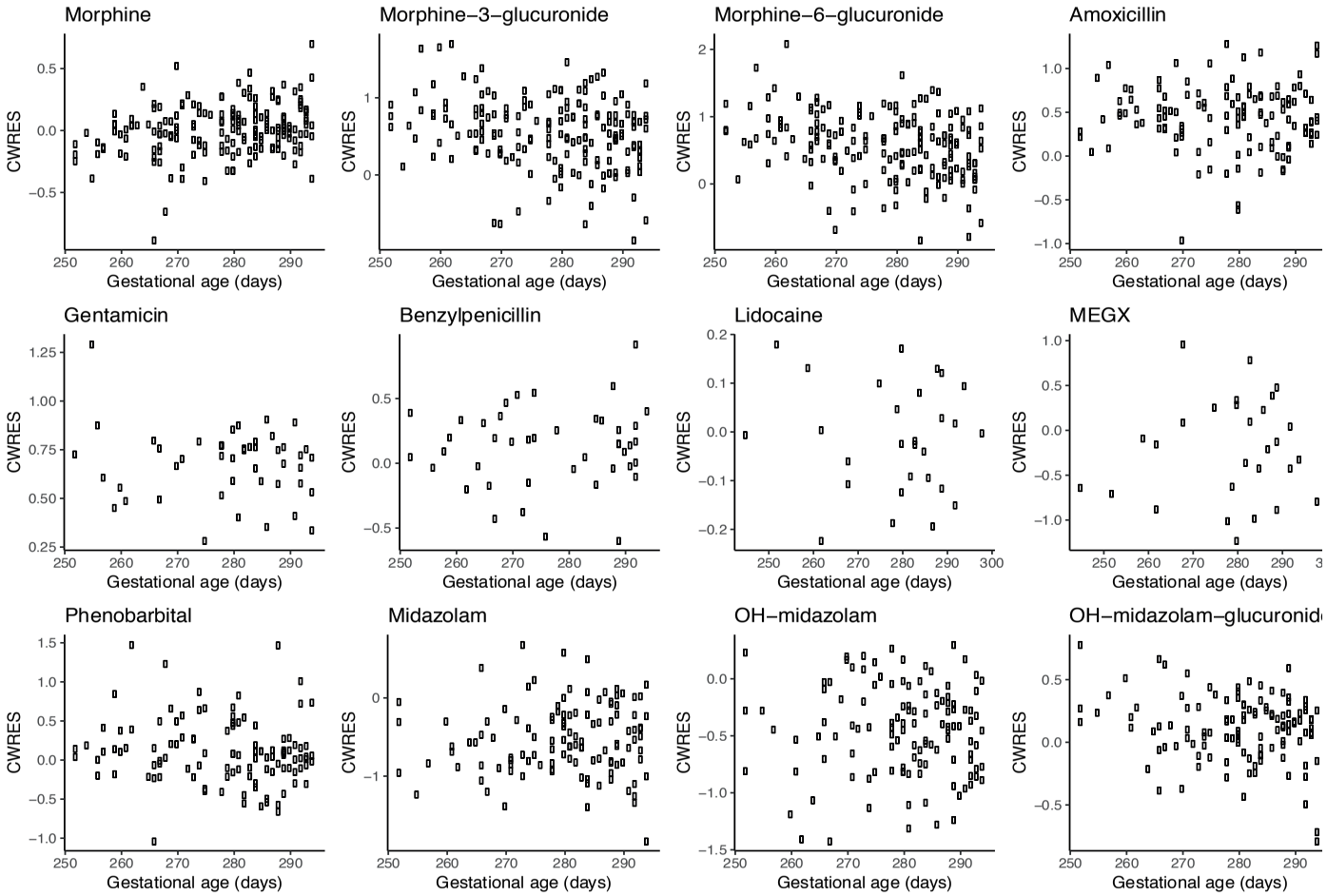
PK = pharmacokinetics, Cl = clearance, V = volume of distribution, Q = intercompartment clearance, CI = confidence interval, OHM = 1-hydroxymidazolam, M3G = morphine-3-glucuronide, M6G = morphine-6-glucuronide, HMG = hydroxylmidazolam glucuronide, MEGX = monoethylglycinexylidide, PNA = postnatal age, TEMP = body temperature, rsd = relative standard deviation, IIV = interindividual variability, SIR = sampling importance resampling, BW = body weight, GA = gestational age, NA = not applicable

Figure S1: population conditional weighted residuals vs birth weight for all compounds



CWRES = population conditional weighted residuals

Figure S2: population conditional weighted residuals vs birth weight for all compounds



CWRES = population conditional weighted residuals

Figure S3: Morphine goodness-of-fit plots

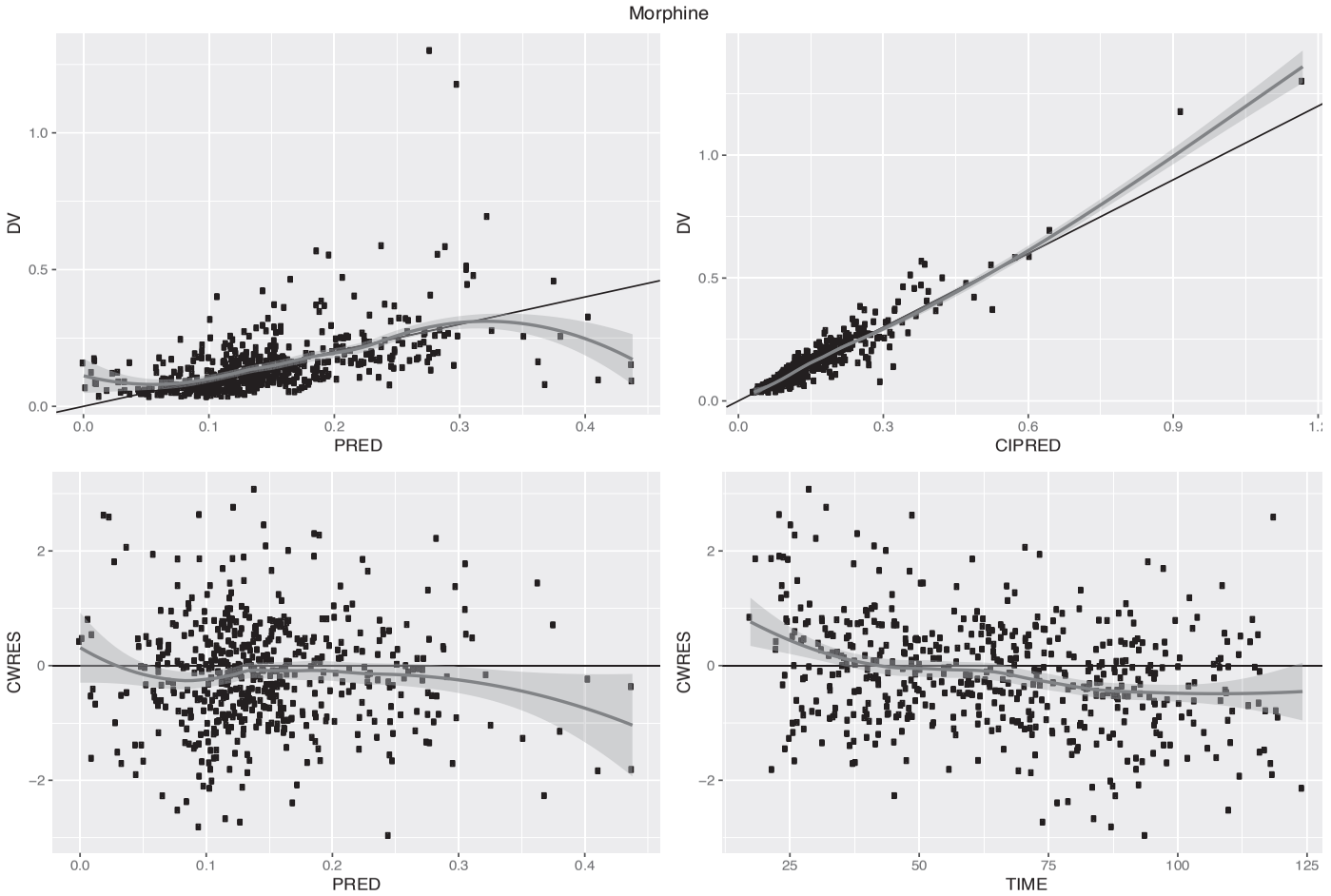


Figure S4: Morphine-3-glucuronide goodness-of-fit plots

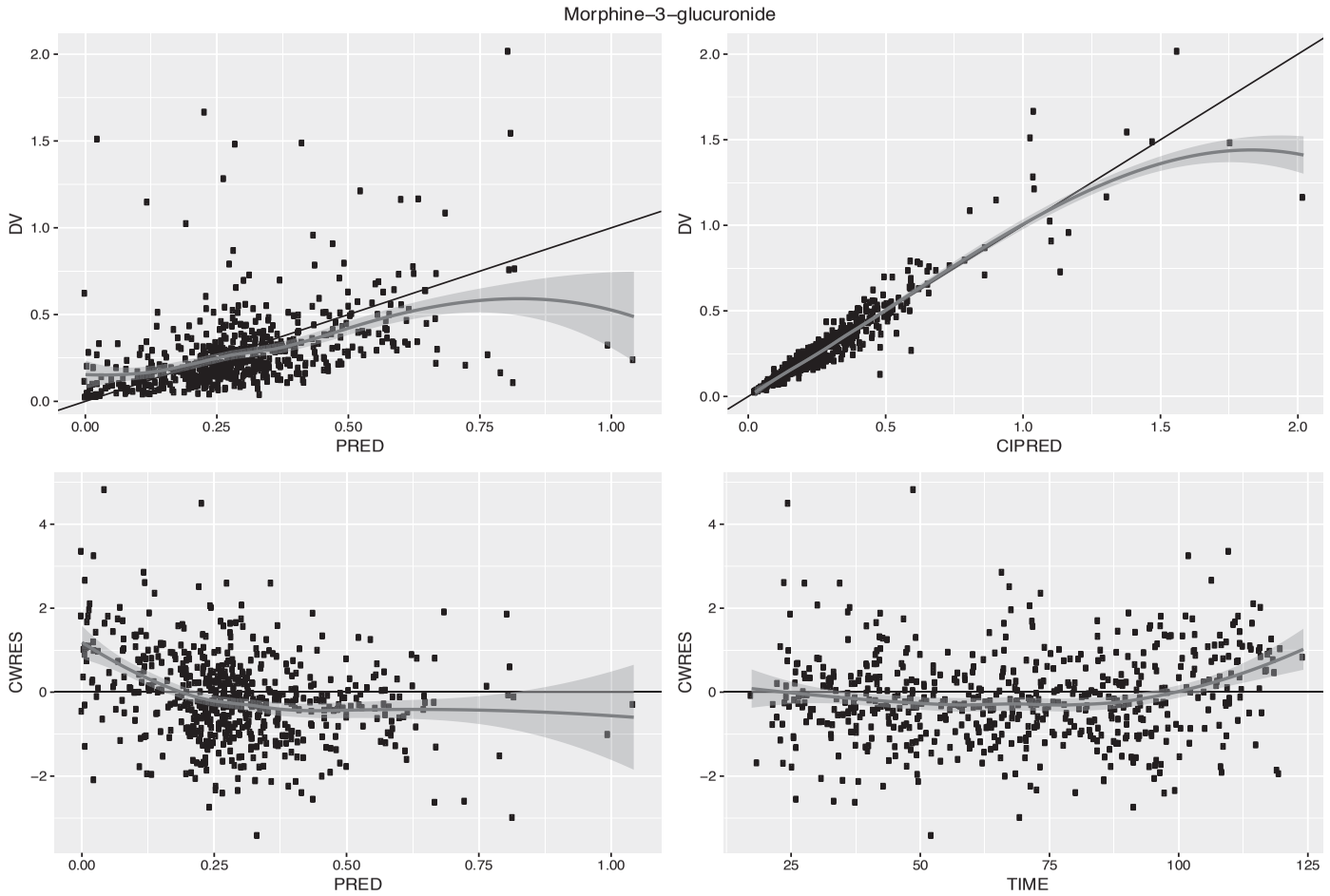


Figure S5: Morphine-6-glucuronide goodness-of-fit plots

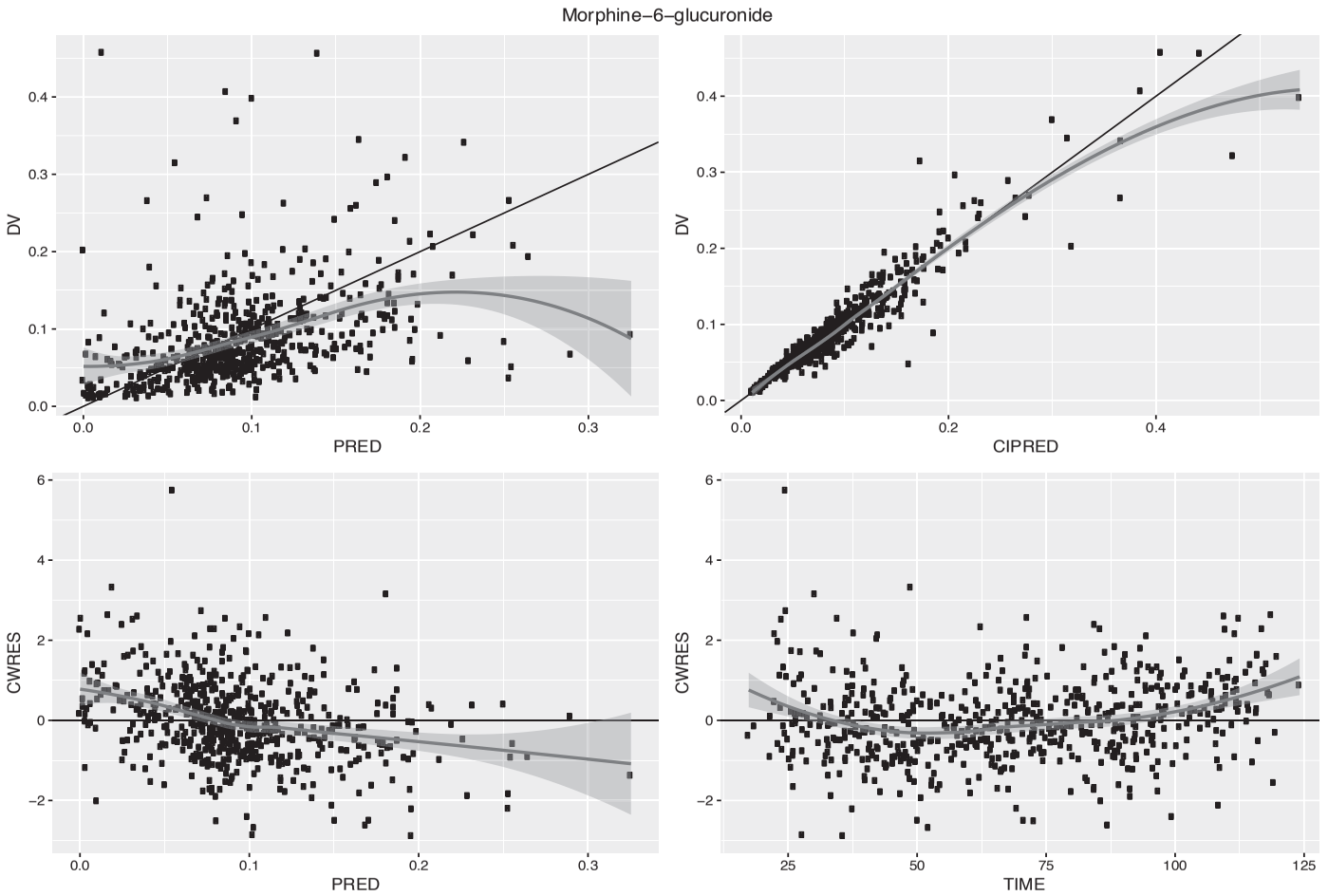


Figure S6: Amoxicillin goodness-of-fit plots

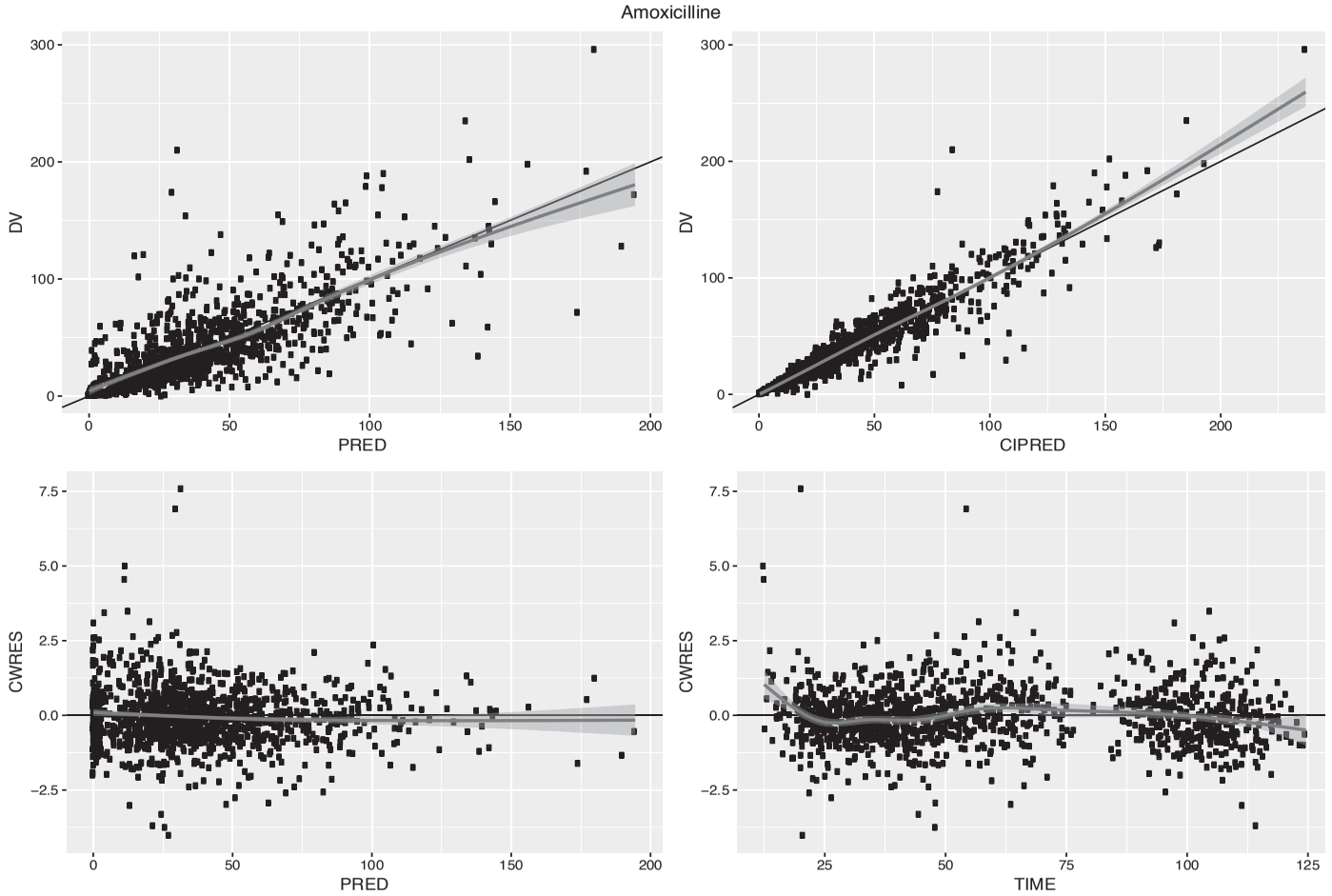


Figure S7: Gentamicin goodness-of-fit plots

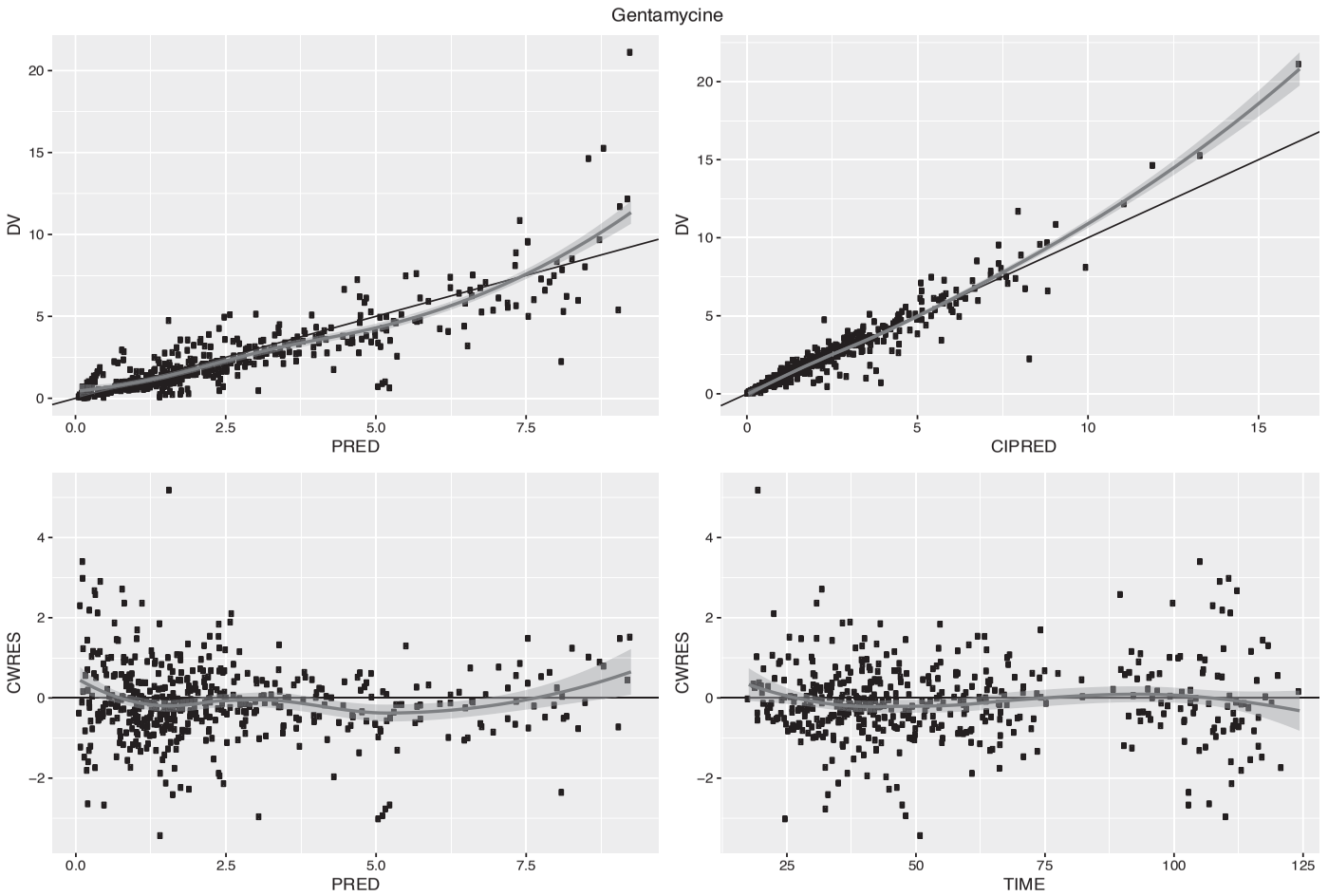


Figure S8: Benzylpenicillin goodness-of-fit plots

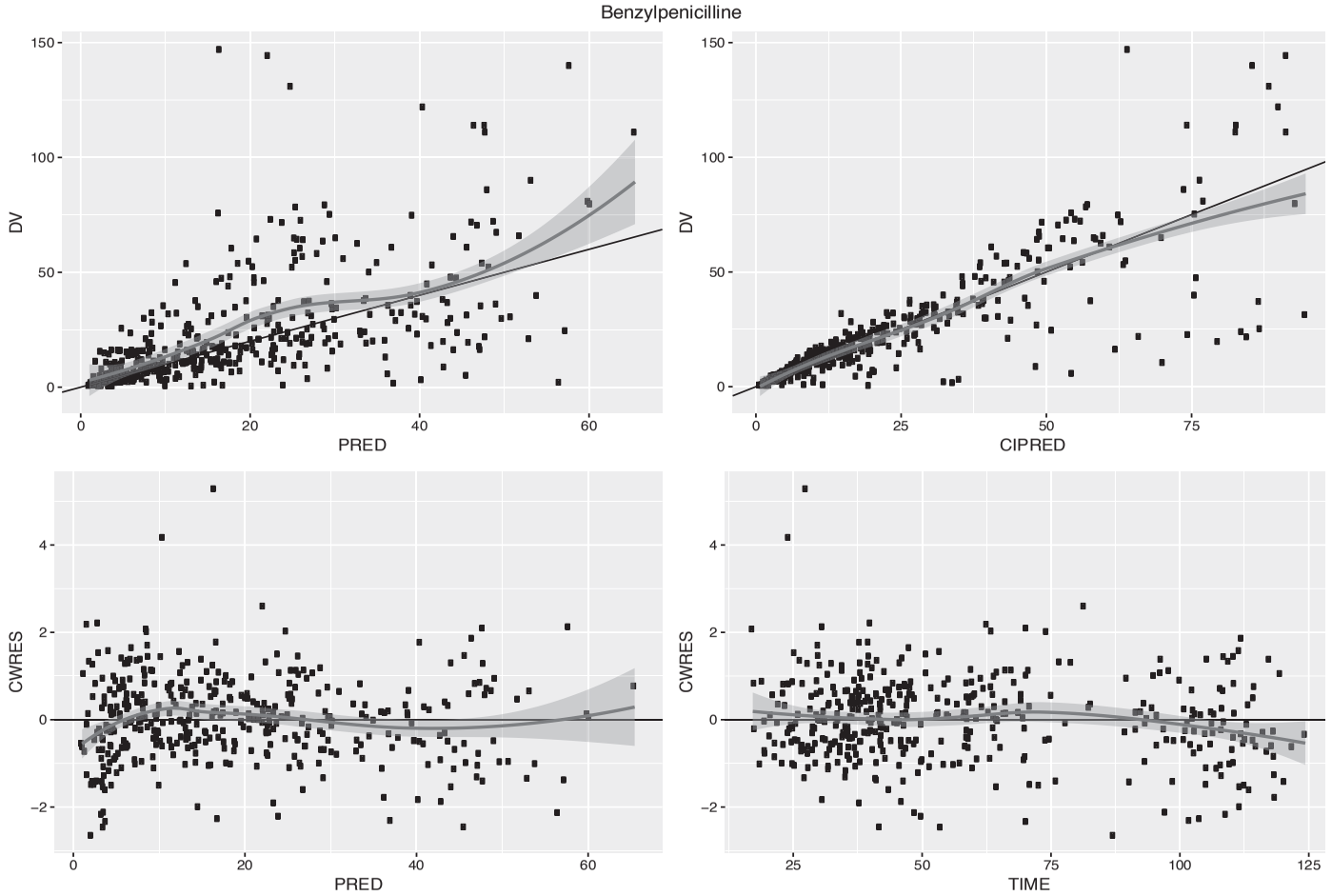


Figure S9: Lidocaine goodness-of-fit plots

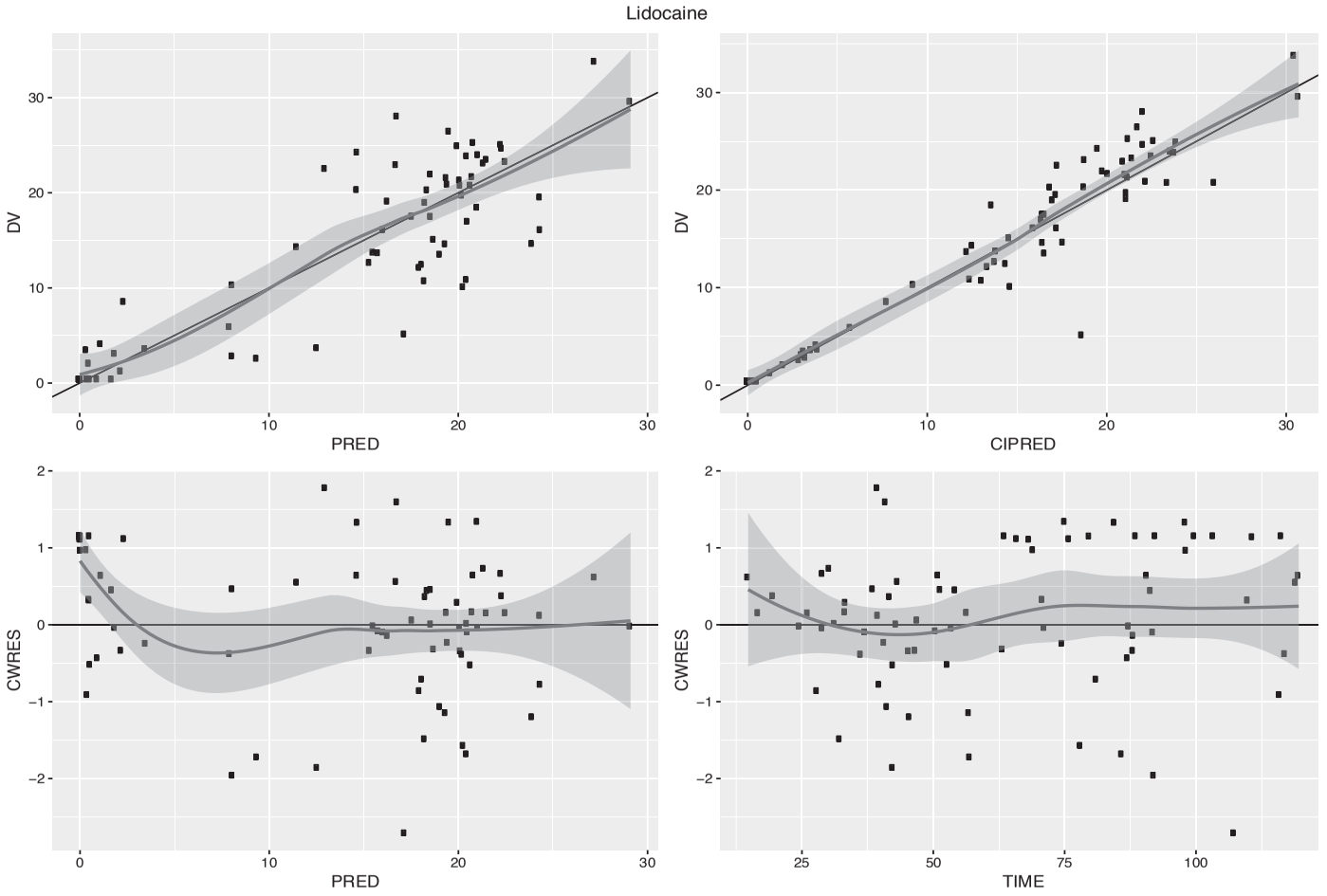


Figure S10: Monoethylglycinexylidine goodness-of-fit plots

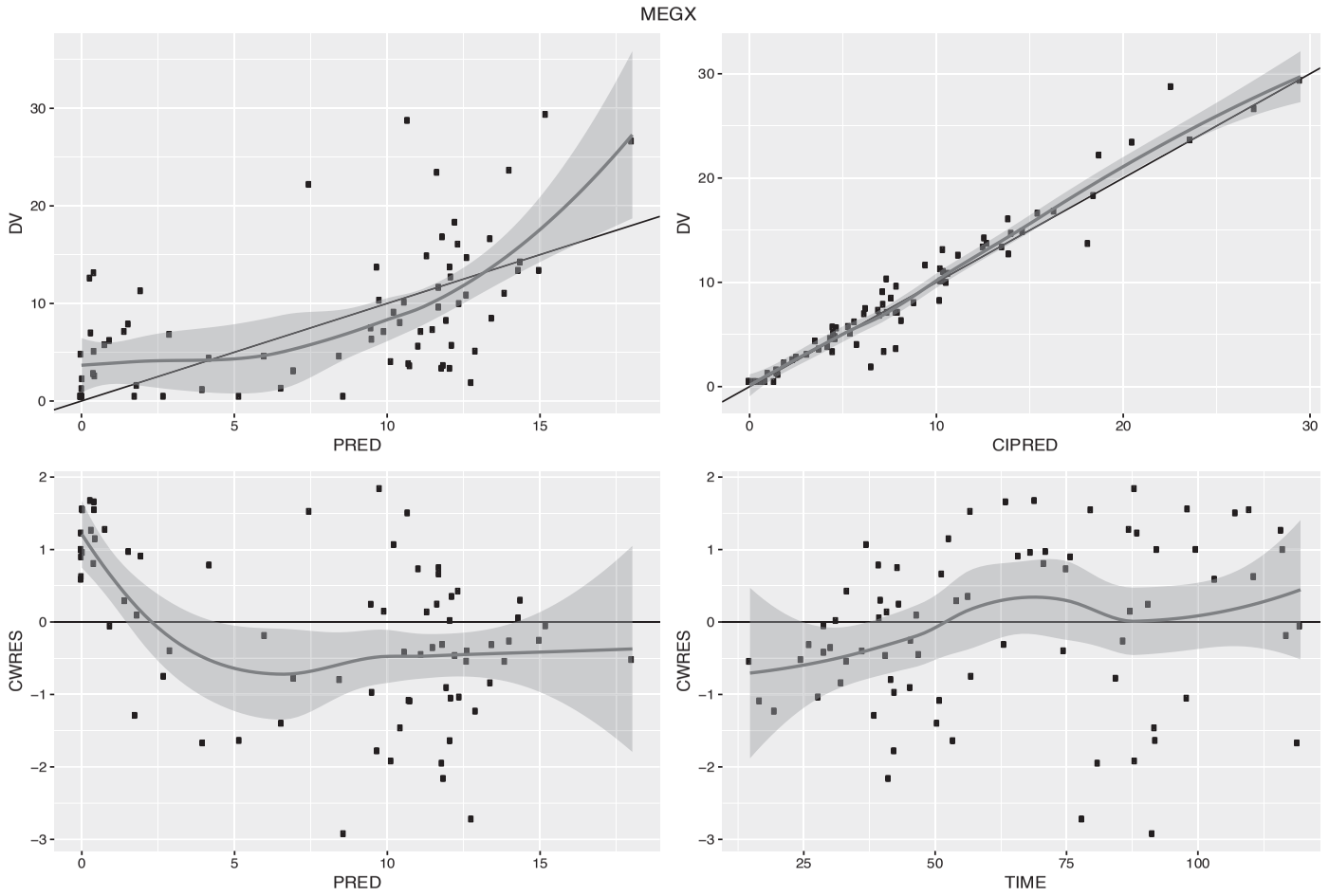


Figure S11: Phenobarbital goodness-of-fit plots

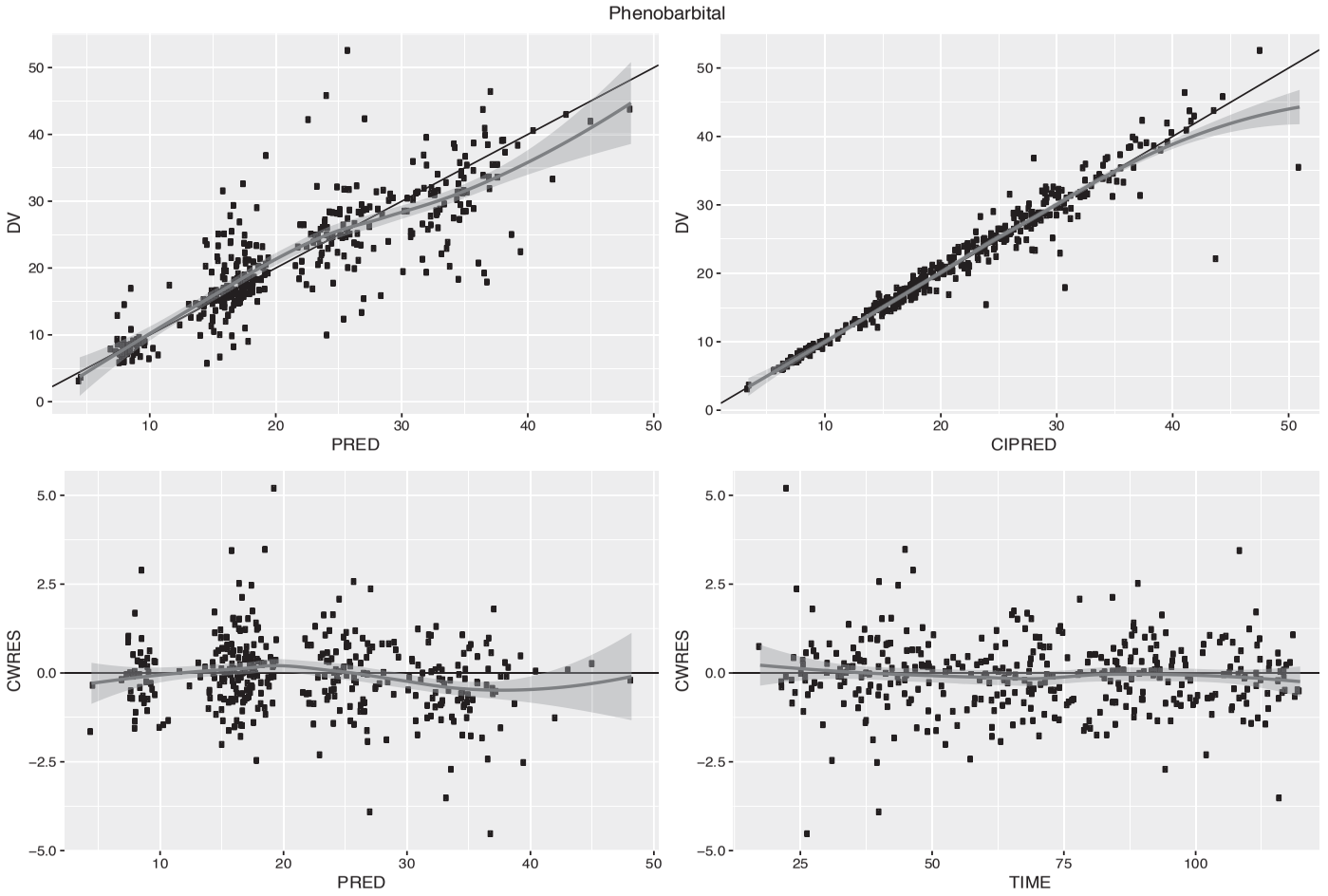


Figure S12: Midazolam goodness-of-fit plots

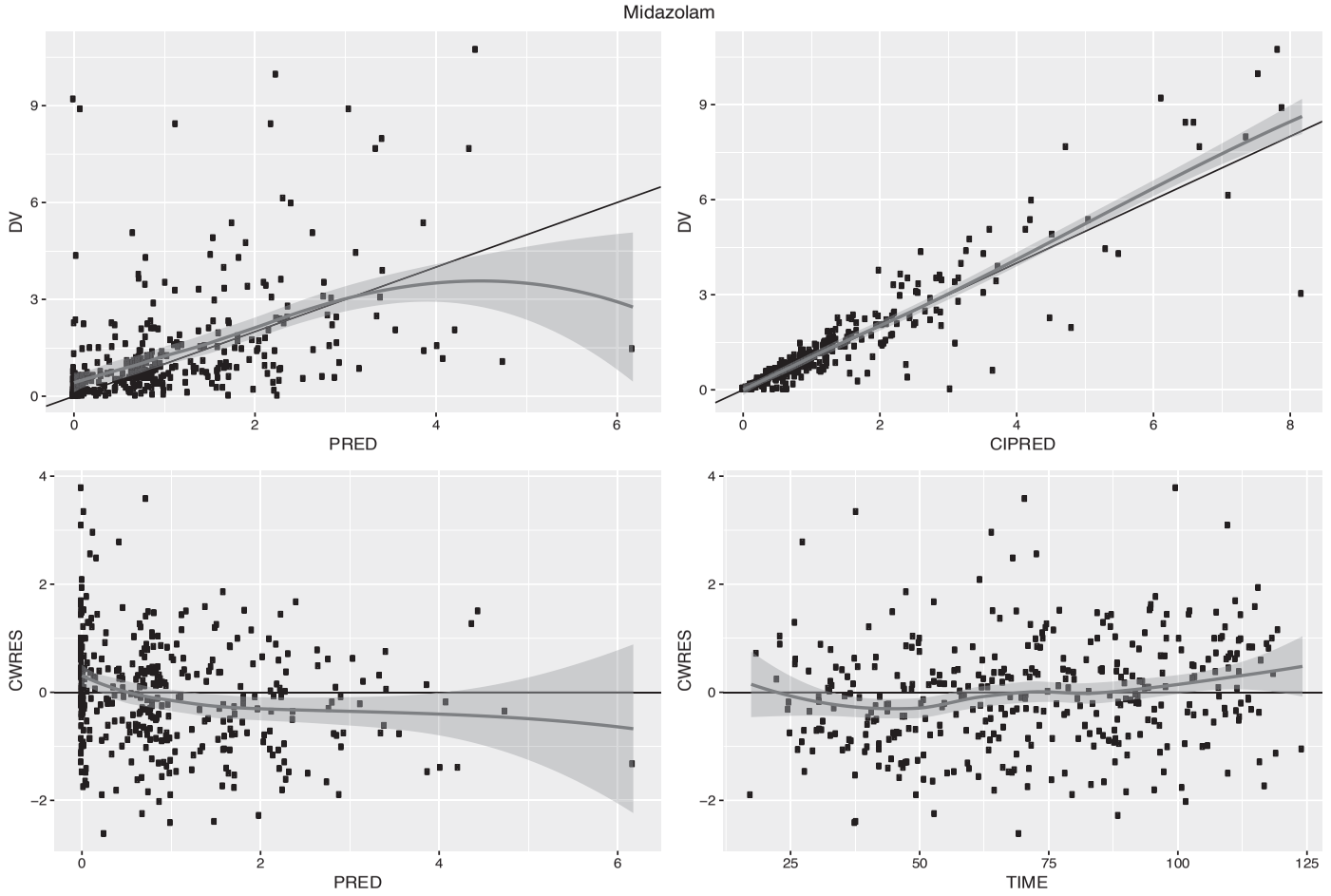


Figure S13: 1-hydroxymidazolam goodness-of-fit plots

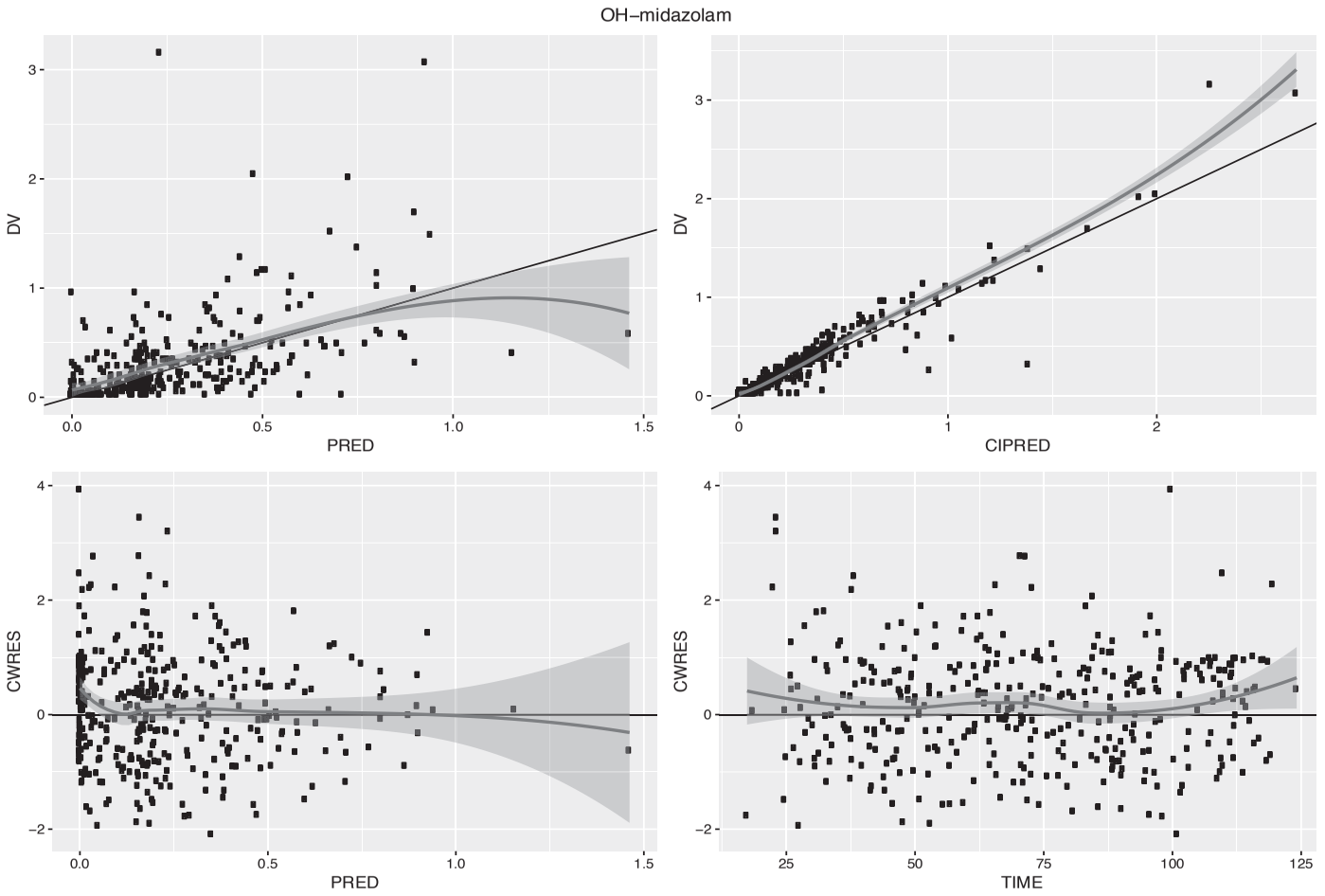
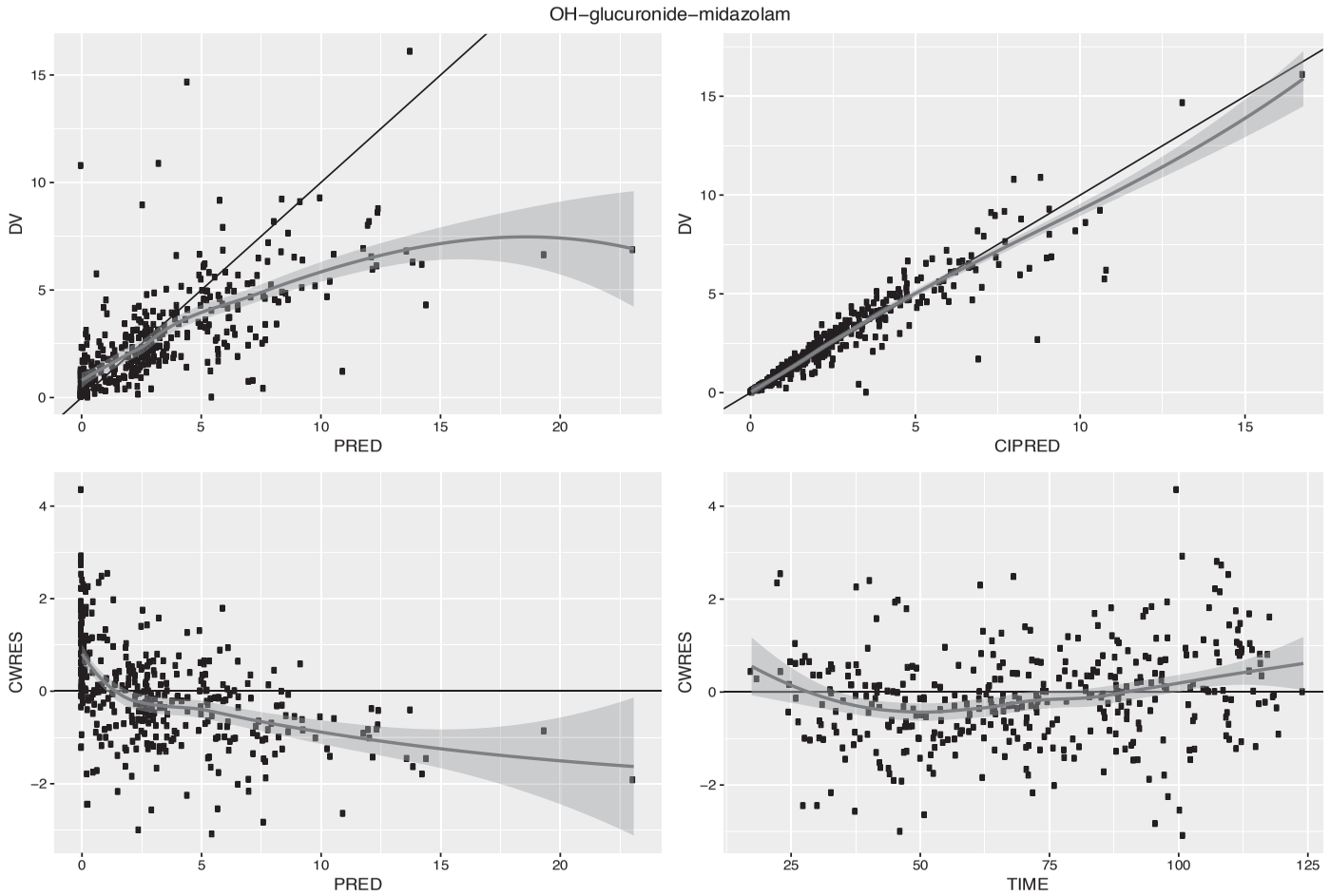


Figure S14: hydroxymidazolam glucuronide goodness-of-fit plots



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CHAPTER 5

General discussion

General discussion

Perinatal asphyxia, deprivation of oxygen around the time of birth, is one of the most predominant childbirth complications worldwide.(1,2) It can lead to widespread hypoxic-ischaemic damage to organ systems such as heart, lungs, liver and kidney, but central nervous system dysfunction is the biggest concern, as the brain is least likely to have a quick or complete recovery.(3,4) Brain damage as a result of perinatal asphyxia is classified as hypoxic-ischaemic encephalopathy (HIE). In countries with advanced economies, HIE followed by therapeutic hypothermia (TH) has a mortality rate of approximately 30%, while another 15% develop severe neurodevelopmental disabilities such as epilepsy, cerebral palsy, visual and auditory handicaps and developmental delay.(5–7)

TH in the early postnatal period is a proven neuroprotective strategy for moderate to severe HIE. Applied as whole body hypothermia, TH consists of lowering the neonates' core body temperature to 33.5 °C for 72 hours. In order to be effective, TH needs to be started within six hours of birth.(6) Although TH has reduced the incidence of a combined adverse outcome from 60% to 45%, it has not been able to reduce these percentages further.(5–7) Therefore, additional therapies complementary to TH, to provide neuroprotection but also neuroregeneration, are needed to further improve the outcome of neonates with HIE.(8–10)
The pharmacokinetics (PK) of drugs administered to neonates is dependent on maturation of organ function, predominantly liver and kidney function. In critically ill neonates admitted to a neonatal intensive care unit (NICU), additional factors related to the disease (e.g. hypoxic-ischaemic damage of these organs) and the treatment (e.g. TH) can also influence PK and thereby increase the complexity of pharmacotherapy in this population.

The specific objectives of this thesis were to explore promising novel neuroprotective agents for HIE additional to TH, to optimise current pharmacotherapy of sedatives and AEDs in neonates with HIE and TH and identify structural effects relating to PK of all drugs in this population.

In this thesis, PK and safety of two promising novel agents for neuroprotection in addition to TH were investigated: ventilation with the noble gas argon in a piglet model for HIE and the selective inhibitor of neuronal and inducible nitric oxide synthase (nNOS/iNOS) 2-iminobiotin in twelve neonates treated with TH for HIE. Safety of ventilation with up to 80% argon in normothermic non-asphyxiated piglets and 50% in asphyxiated piglets with and without TH was confirmed. Also, increasing argon blood concentrations could be detected with increasing dose. The use of 2-iminobiotin in addition to TH in human neonates with HIE appeared safe; based on the PK parameters obtained in this study, a dose for future clinical trials is proposed. Seizures are a component of moderate to severe HIE, either apparent or subclinical, and adequate seizure control is considered neuroprotective.(11–13) Although a wide variety of novel anti-epileptic drugs (AEDs) have become available for adults and older children over the past decades, neonatal seizures are still predominantly treated with older drugs: phenobarbital, midazolam and lidocaine. In neonates with HIE and TH, the PK of these and other drugs, such as morphine, which is administered to provide sedation and comfort during TH, has only been sparsely investigated. In this thesis, PK and clinical effectiveness of phenobarbital and midazolam in neonates with HIE were investigated as part of the prospective multicentre PharmaCool study (NTR2529). PK for both drugs were unaffected by TH and effectiveness was comparable to previous reports. Based on the PK analysis, a phenobarbital dose of 30 mg/kg is recommended. Additionally, a drug-drug interaction between phenobarbital and midazolam was identified. Morphine PK was investigated in a combined dataset with neonates from the SHIVER

and PharmaCool studies; morphine clearance was reduced by 20.7% during TH and a loading dose of 50 µg/kg followed by continuous infusion of 5 µg/kg/h was postulated as starting dose. Lidocaine PK was studied in a dataset encompassing both preterm and term neonates. Lidocaine clearance increased with increasing postmenstrual age (PMA) and was reduced by 21.8% in neonates receiving TH. This study also concluded that a previously developed dosing regimen can be utilised for safe and effective lidocaine use across the entire neonatal age range. Finally, data from all drugs investigated in the PharmaCool study were combined in order to identify structural effects of body size, maturation, recovery of organ function and TH on PK in this population. In the combined PK model, common descriptors for body size and maturation were included for all drugs and metabolites. For high clearance drugs (clearance predominantly dependent on organ perfusion), clearance strongly increased with postnatal age (PNA) as a marker for organ recovery, but no effect of TH could be identified. For intermediate clearance drugs (clearance dependent on organ perfusion and enzyme activity), the increase in clearance with PNA was less pronounced and was hampered by TH. The knowledge derived from this model can facilitate future PK studies for other drugs in this population.

In this final chapter, the results of the individual studies presented in this thesis will be placed in broader perspective focussing on three main topics:

- Neuroprotection and neuroregeneration
- Treatment of neonatal seizures
- Drug research in paediatrics

Neuroprotection and neuroregeneration

In the following section, several promising therapies for neuroprotection (prevention of further brain damage) and neuroregeneration (repair of cells damaged by the hypoxic-ischaemic event) additional to TH are being discussed.

Following perinatal asphyxia and resuscitation, two distinct phases of neuronal cell death can be identified.(9) The first phase is called the primary energy failure, which sets in immediately after birth as a direct result of the actual hypoxic-ischaemic event is often accompanied seizures.(14–16) After a latent phase of six to fifteen hours, a secondary phase of energy failure commences.(17) This phase is associated with encephalopathy and increased seizure activity, and accounts for a significant proportion of the final cell loss. (16) Pathways of cell death during the secondary energy failure may differ from those during primary energy failure. Figure 1 summarises the destructive pathways during TH as well as the potential neuroprotective and neuroregenerative strategies mentioned below.

Allopurinol

Allopurinol is a xanthine oxidase inhibitor predominantly used to treat gout by lowering plasma uric acid concentrations. Especially in high dosages, allopurinol is hypothesised to be a free-iron chelator and free radical scavenger.(18) Free radicals peak early after hypoxic-ischaemic injury, indicating a short therapeutic time window for allopurinol as a potential neuroprotective agent.(9) Preclinical studies in various animal models have shown neuroprotective effects when used alone or in addition to TH.(19–21) Several small clinical studies administrating allopurinol in the early postnatal period showed a beneficial effect on short-term cerebral biomarkers and even on long-term outcome.(22–24) Currently, a phase III study investigating allopurinol in addition to TH is underway. In this study, the first dose of allopurinol is administered within 45 minutes of birth, with a follow-up dose twelve hours later.

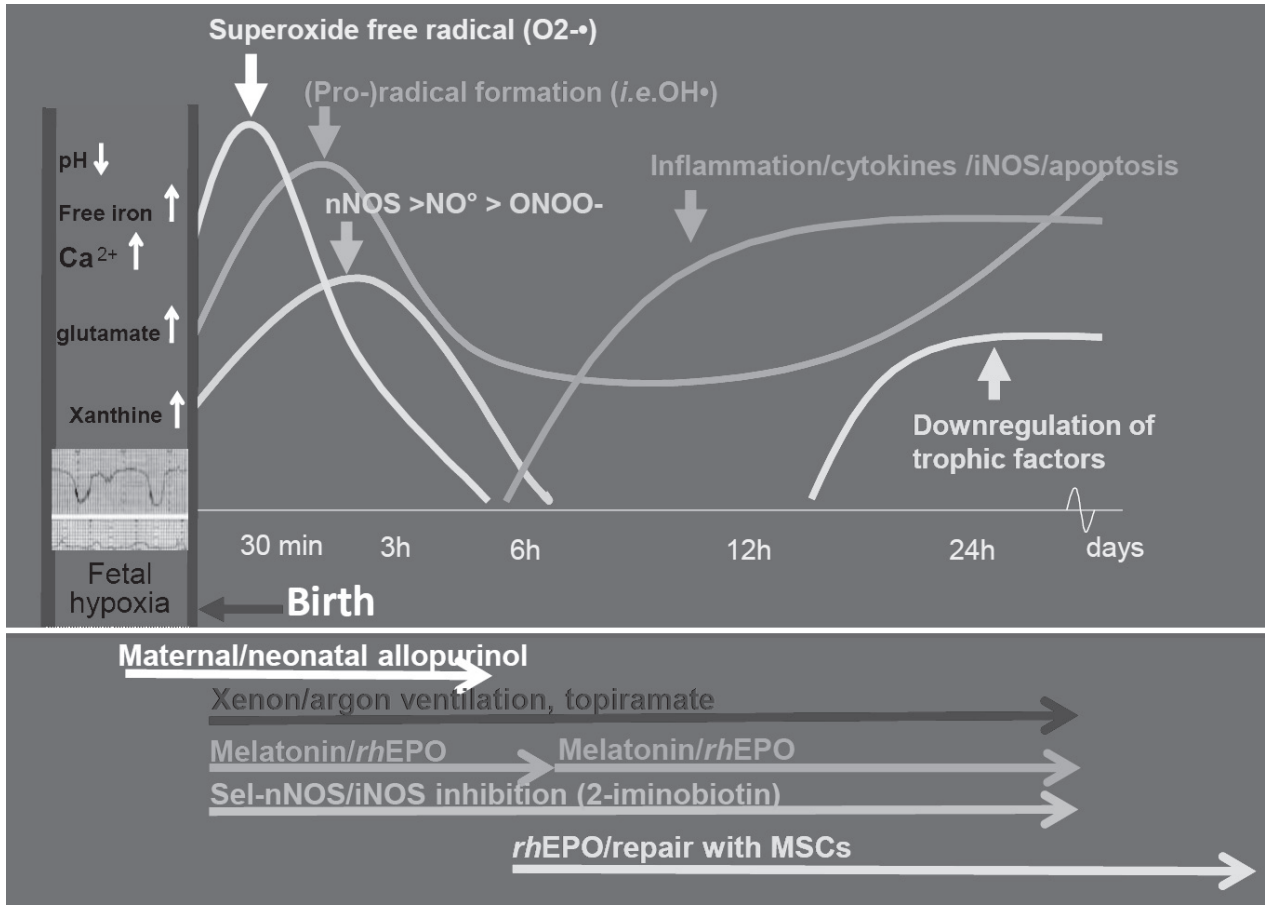

Figure 1: Time profile of the destructive pathways leading to neuronal damage following perinatal asphyxia (top panel) and the potential targets for neuroprotective and neuroregenerative therapy (bottom panel). Adapted from (9)

Primary outcome is death or severe neurodevelopmental impairment at the age of 2 years (ALBINO study, NCT03162653).

Topiramate

Topiramate is an anticonvulsant approved for the treatment of several epilepsy syndromes in both adults and children. Topiramate blocks the voltage-dependent sodium and calcium channels and also inhibits the excitatory glutamate pathway while enhancing the inhibitory effects of gamma-aminobutyric acid. (10) In neonates with HIE, a dual application both as an anticonvulsant and as a neuroprotective agent has been suggested.(25,26) Neuroprotection has been demonstrated in animal models with and without TH.(25,26) To date, studies in humans have confirmed safety in neonates in addition to TH but have failed to reduce the combined frequency of mortality and severe neurological disability. (27) A placebo-controlled phase II study with topiramate as adjuvant to TH is presently enrolling. Primary outcome is the occurrence of seizures at discharge from the NICU; secondary outcomes include normalisation of amplitude-integrated electroencephalogram at discharge, magnetic resonance imaging (MRI) score at day 5-7 and developmental outcome at 9, 18 and 27 months (NCT01765218). A possible limitation for topiramate use is the unavailability of an intravenous dosage form.

Melatonin

Melatonin is an endogenous neuroendocrine hormone secreted by the pineal gland and well known for its role in modulating the circadian rhythm. Medically, it is used for treatment of sleeping disorders. Besides this, melatonin has several other mechanisms that suggest an important role in recovery and repair from brain injury. (10,28–30) In a piglet model for HIE, melatonin used in addition to hypothermia decreased injury, which was determined via magnetic resonance spectroscopy. (31) In a pilot study in neonates, melatonin in combination with TH resulted in fewer

seizures, less evidence of white matter injury on MRI, and a higher rate of survival without developmental or neurological abnormalities compared to TH alone.(32) A follow-up open label dose escalation study is currently recruiting (NCT02621944).

Erythropoietin and darbepoietin

Erythropoietin (EPO) is an endogenous protein, synthesized in the liver, which has an impact on multiple critical pathways. Besides stimulating erythropoiesis, EPO is a cytokine that influences the body's immune response and has anti-oxidant, as well as anti-inflammatory properties.(33–35) EPO interacts with the EPO receptor which is widely expressed throughout the central nervous system, sparking an interest in EPO for neuroprotection. Numerous studies in different animal models for HIE showed its neuroprotective properties with respect to brain damage, reducing apoptotic and excitotoxic cell injury.(36–38) In a clinical trial in human neonates without TH, EPO decreased the incidence of disabilities at 18 months of age.(39) Exploratory trials with EPO in addition to TH confirmed safety, even at relatively high daily doses, and resulted in less MRI brain injury and potential for improved short-term motor outcomes compared to placebo. This prompted a large phase III randomised placebo-controlled clinical trial with death and disability at the age of 2 years as primary outcome measure (PAEAN study, NCT03079167).(40,41) Besides neuroprotection EPO is also believed to exhibit neurotrophic properties, possibly stimulating neuroregeneration in a later stage.(42) Darbepoietin is a long acting synthetic EPO analogue. Where EPO requires daily administration, darbepoietin offers the benefit of once weekly administration. No safety concerns arose in combination with TH and a larger placebo controlled feasibility trial with neurodevelopmental delay as primary outcome is currently running (MEND study, NCT03071861) as well as a placebo-controlled trial in neonatal stroke (DINOSAUR study, NCT03171818).(43)

Xenon and argon

A direct result of the hypoxic-ischaemic event is the activation of the N-methyl D-aspartate (NMDA) receptor through glutamate release from adjacent brain cells. Xenon inhibits NMDA signaling and thus may have potential in reducing the acute cell injury.(9) Studies in a piglet model for HIE combining xenon ventilation with TH showed promising results. A feasibility study in 14 neonates combined TH with up to 50% xenon ventilation for 18 hours, and demonstrated no adverse effects. (44,45) However, a proof-of-concept clinical trial in 92 neonates concluded that ventilation with 30% xenon, started concomitantly with TH, is unlikely to enhance the neuroprotective effects of TH. The authors hypothesize that a higher dose started sooner after birth might be necessary for xenon to show neuroprotective properties. (46) Furthermore, xenon is a very expensive noble gas requiring a specialised delivery system.(47) Therefore, researchers were prompted to investigate other more abundant noble gases such as argon.(9,10) Argon exhibits neuroprotective properties most likely via interaction with toll-like receptors.(48) Neuroprotection was achieved with argon ventilation in animal models for HIE, both with and without TH.(49,50) Unlike xenon, which is also used as an inhaled anaesthetic, argon had no application in humans. The lack of medical grade argon gas licensed for human use and regulatory and financial challenges in preparing a ventilator to include argon has prevented research groups from performing clinical trials in neonates to date.

Mesenchymal stem cells

A very promising lead in the field of neuroprotection and neuroregeneration is the use of mesenchymal stem cells. Stem cell therapy could have protective effects by acting on inflammation, apoptosis and oxidative stress, but may also enhance regeneration by secreting trophic and immunomodulatory factors that help in repair from brain injury over longer periods of time.(10,51) A meta-analysis of animal studies demonstrated a significant positive effect on neurobehavioral outcome following hypoxic-ischaemic injury.(52) While mesenchymal stem cells are promising

in its potential, well designed trials are required to prove safety and efficacy. Also, the preferred route of administration has not been elucidated. Intravenous administration might seem the obvious choice, but intranasal application might provide improvement over systemic routes as mesenchymal stem cells are targeted directly to the brain, bypassing peripheral organs.(51)

Future perspective: combination therapy

The molecular pathways responsible for brain damage following perinatal asphyxia are diverse and, in some cases, limited to a very distinctive time frame, so it is unrealistic to assume that a single intervention will suffice for additional neuroprotection. Ideally, agents selected for combined neuroprotective therapy additional to TH should possess the following qualities:

- Distinctive or synergistic mechanisms of action
- Easy and timely administration with adequate bioavailability
- Proven efficacy additional to TH
- Acceptable safety profile during and/or after TH

The window of opportunity for neuroprotection opens immediately after birth. Therefore, neuroprotective interventions should be started as soon as possible. In Chapter 2.1, 2-iminobiotin was identified as the selective nNOS/iNOS inhibitor with the greatest potential to progress to human clinical trials, and in Chapter 2.2, safety in neonates during TH is confirmed and a dose for future clinical (efficacy) trials is proposed. 2-iminobiotin should be administered as soon as possible after birth, but has potential benefit for up to 48 hours. Allopurinol targets a separate pathway and could be synergistic to 2-iminobiotin in the early phase; clinical feasibility of timely administration is currently being investigated. Topiramate and melatonin could also potentially be used in combination with
2-iminobiotin and/or allopurinol, although their mechanism of action is less well defined.

Chapter 2.3 shows that ventilation with argon concentrations of up to 80% is safe in newborn piglets and others have demonstrated neuroprotection in the same species. However, the lack of argon availability suitable for ventilation of neonates does not bode well for human clinical trials in the near future. Also, the disappointing results for xenon as an additional neuroprotective agent has diminished the interest in noble gases in general.

Although neuroprotective interventions targeting the destructive pathways occurring in the first hours to days after birth are essential to prevent further brain damage, they will not cure damage caused by the hypoxic-ischaemic event itself. Erythropoietin and darbepoietin could potentially bridge the gap between neuroprotection and neuroregeneration. For neuroprotection, combination with TH seems feasible, and repeated administration beyond the hypothermic phase might stimulate neuroregeneration. Mesenchymal stem cells focused on repair could potentially make all other interventions obsolete, but the current lack of data regarding safety and the preferred route of administration stand between a promising concept and routine clinical application.

To date, none of the interventions mentioned above have proven neuroprotective or neuroregenerative efficacy and no trials have been conducted combining two or more of these compounds. Neuroprotective efficacy in addition to TH will need to be proven for each compound separately before the focus can shift to combination therapy. However, instead of organising separate trials for each compound, the use of master protocols in which several agents can be investigated in parallel substudies using a common design can increase operational efficiency and reduce costs.(53) Over the next few years, clinicians and researchers are challenged with the task of identifying the right compound at the right time but also with combining their research efforts in (inter)national consortia for optimal use of knowledge and resources. A viable example of such a consortium is the Netherlands Neonatology

Research Network (N3, (https://neonatologynetwork.eu/). This collaboration of NICUs in the Netherlands and Belgium has facilitated four multicentre trials including the PharmaCool study. On a pan-European level, the Conect4Children project (https://conect4children.org/) involves partners from the academic, public and private sectors, and aims to facilitate high quality multicentre drug research for all paediatric age groups.

Treatment of neonatal seizures

In this section, the evidence regarding both efficacy and safety of the currently available AEDS for treatment of neonatal seizures is addressed, including the PK based dosing guidelines derived from this thesis.

Neonatal epileptic seizures are almost always provoked by an underlying condition, such as HIE, cerebral haemorrhage, hypoglycaemia, trauma, metabolic disorders including electrolyte imbalance, congenital malformations or infections.(11) Irrespective of the origin, it is generally accepted that recurrent seizures during the neonatal period increase the risk of neurological disabilities later on in life and that adequate treatment is important.(12,54–57) Benefit of treatment for neonatal seizures must be weighed against the potential adverse effects. Acute side effects of commonly used AEDs include hypotension, respiratory depression and arrhythmias, possibly requiring respiratory and/or inotropic support or causing prolonged hospitalisation. Chronic use is associated with cognitive and memory difficulties. While none of the potential negative effects have been systematically studied in humans, the quest for safe and effective alternatives continues.

Phenobarbital

To date, phenobarbital is the most commonly prescribed drug to treat seizures in both term and preterm neonates in all areas of the world.(58–60) Phenobarbital is

a barbiturate originally developed as a broad central nervous system depressant. It exhibits anti-epileptic properties through agonism of the inhibitory GABA-A receptor. Concerns have been raised about potential long-term consequences of phenobarbital use. In several neonatal animal models, exposure to phenobarbital has led to acute neuronal apoptosis in several important brain regions, as well as longer term changes with measurable effect on learning and memory.(61–67) In humans, neonatal phenobarbital use has been associated with long term cognitive and motor impairment.(68)

As described in Chapter 3.2, phenobarbital effectiveness in our study was 65.5%, comparable to previous reports.(69–74) Simulations based on the final PK model show that a dose of 30 mg/kg leads to desired plasma concentrations. Therefore, our advice to clinicians is to administer a dose of 30 mg/kg. Phenobarbital is a strong inducer of CYP3A, which should be taken into account in the choice of second line therapy if treatment with phenobarbital is ineffective.
Due to its long half-life of approximately one week in neonates phenobarbital can be administered as a bolus infusion without the need for continuous administration. Therefore, it is feasible to start treatment in a referring hospital before transfer to a level III NICU for continued care.

Midazolam

Midazolam is a benzodiazepine with a wide variety of clinical applications in humans.(61) In neonates, it is utilized for sedation and as second line anti-epileptic drug. The most predominant short-term side effect of midazolam is hypotension requiring inotropic support. Especially in neonates suffering from hypoxic-ischaemic injury, additional hypoperfusion of brain tissue as a result of hypotension is undesirable.(59,75) There is also concern for neurotoxicity. Animal models have demonstrated that midazolam can induce both apoptosis and necrosis and has negative long-term consequences on learning.(76–78)

In Chapter 3.2, effectiveness of midazolam in our population was 37.1% as second line therapy after phenobarbital. Like phenobarbital, midazolam acts as a GABA agonist. This overlap in mechanism of action is a possible explanation for the relatively low effectiveness as add-on anti-epileptic drug in neonates refractory to phenobarbital. Chapter 3.2 also shows a prominent drug-drug interaction between midazolam and phenobarbital identified through PK modelling, most likely based on CYP3A induction. Its side effect profile coupled with limited effectiveness and drug-drug interaction makes midazolam a less ideal choice as second line anti-epileptic drug following phenobarbital.

Lidocaine

Lidocaine is predominantly used as a local anaesthetic and as a class 1b antiarrhythmic drug in adults. It blocks fast voltage-gated sodium channels in the cardiac conduction system but also in the central nervous system. Reports of lidocaine use as treatment for neonatal seizures originate in the 1970s.(79) Since then, several studies have reported on clinical effectiveness of lidocaine as second or third line anti-epileptic drug. Effectiveness varies between 55.3% for preterm neonates to 91% for term neonates treated with TH.(80–83) Several studies have also demonstrated that lidocaine has similar or greater effectivity than midazolam for add on seizure control.(80,83–85). Cardiac arrhythmias might be a serious and potentially life threatening side effect of lidocaine and have been associated with plasma concentrations above 9 mg/L.(82,86)

Chapter 3.3 describes lidocaine use in a heterogeneous population consisting of preterm and term neonates with and without TH treatment, and evaluates a previously developed lidocaine dosing regimen with increasing mg/kg doses for increasing weight. Lidocaine clearance was found to be dependent on PMA and TH. The dosing regimen, with body weight as a descriptor for PMA and a reduced dose during TH, leads to comparable lidocaine plasma concentrations across the entire

neonatal spectrum. In our study of 159 neonates, no cardiac events occurred. Other recent studies also indicate that cardiac toxicity following lidocaine administration is exceedingly rare.(80,82,83,87). Several studies have also demonstrated that lidocaine has similar or greater effectivity than midazolam for add on seizure control.(80,83–85) We believe that lidocaine is preferable to midazolam as second line anti-epileptic drug and can be administered safely when adhering to the proposed dosing regimen, which also accounts for the reduced lidocaine clearance during TH.

Phenytoin

Phenytoin is a classic anticonvulsant with widely established use in both adults and older children. Its mechanism of action is comparable to lidocaine and involves blockade of voltage-gated sodium channels. Phenytoin has an unfavourable side effects and interaction profile. Neonates receiving phenytoin are at risk for hypotension and cardiac arrhythmias; concomitant use of phenytoin and lidocaine is not recommended. Phenytoin is a strong inducer of many liver enzymes such as CYP3A, P-gp and UGT, giving it a high potential for drug-drug interactions. Furthermore, phenytoin has a narrow therapeutic window and exhibits nonlinear PK, necessitating therapeutic drug monitoring, and has been associated with neurotoxicity in animal models.(59,60,64,88) We are convinced that phenytoin has no additional benefit over the currently used AEDs in neonates. This is reflected in the current clinical practice in the Netherlands.

Levetiracetam

Levetiracetam is a relatively new anti-epileptic drug which became available at the start of the 21st century. Of all the AEDS that have gained marketing authorisation in the past two decades, it is one of very few that can be administered intravenously. Its mechanism of action has not been fully unravelled, but it is thought to have inhibitory action via binding to the synaptic vesicle protein SV2a, which controls

neurotransmitter release and vesicle transport in neurons. It may also inhibit calcium- and potassium-gated channels.(89) Several smaller studies have reported effective seizure control when levetiracetam was used in neonates with seizures refractory to lidocaine with only mild short term side effects.(90–94). In a small subset of patients receiving levetiracetam as first line therapy, effectiveness was comparable to phenobarbital (64% vs 65%), and a recent review suggests that levetiracetam is as least as effective as phenobarbital as treatment for neonatal seizures.(95,96) Compared to phenobarbital, levetiracetam was not associated with neurotoxicity in animal models.(63,65)

Levetiracetam has linear PK, low protein binding, no hepatic metabolism and very little documented drug-drug interactions and side effects, making it an attractive choice of therapy in neonates (89,97,98). Levetiracetam does not necessitate continuous infusion, allowing for start of therapy in referring hospitals. Treatment of neonatal seizures with levetiracetam has soared over the past few years, even though there are no large randomised controlled trials or population cohort studies investigating the use of levetiracetam in neonates, and no consensus about dose and dosing frequency has been reached.(60,99).

Future perspectives

In contrast to adults and older children, no new drugs have been licensed for treatment of neonatal seizures over the last 50 years.(100) Drugs such as phenobarbital, midazolam and lidocaine currently remain the drugs of choice, even though they have been developed for different indications and have only been sparsely investigated in neonates.(101) The only way to change this status quo is to conduct well-designed studies assessing safety, effectiveness and PK in both preterm and term neonates with and without TH. Based on the current evidence, a study randomising between phenobarbital and levetiracetam as first line treatment is advised and necessary to solidify the role of levetiracetam as (first line) treatment

option for neonatal seizures. In neonates with HIE, levetiracetam clearance is likely impaired compared to non-asphyxiated neonates and a lower starting dose might be sufficient to achieve a similar exposure. Based on Chapter 4.1, it can also be expected that levetiracetam clearance increases strongly after birth. Lidocaine should be considered as second line AED and can be safely administered with the dosing regimen described in Chapter 3.3. Due to limited effectiveness and potential adverse effects, midazolam should be reserved as third or fourth line AED.

Drug research in paediatrics

In this section, several options to facilitate drug research in children and especially neonates are discussed.

For the better part of the 20th century, scientific research in paediatric patients was scarcely performed since it was considered unethical and too risky. As a result, drug use in children was often restricted to off label use of older drugs, based on extrapolations from adults with the same or similar disease, which has led to disasters like chloramphenicol-induced gray baby syndrome.(102,103) Over the past two decades, changes in legislation and the opinion of researchers, physicians and the general public have increased the opportunity for drug research in children.(104) However, several restrictions still apply. Aside from the ethical principles outlined by the Declaration of Helsinki, which applies to all participants in medical research, children can only participate in clinical trials if it offers them potential benefit or if they are exposed to minor increase over minimal risk.(105) Whereas minimal risk is classified as “the probability and magnitude of harm or discomfort anticipated in the research are not greater in and of themselves than those ordinarily encountered in daily life or during the performance of routine physical or psychological examinations or tests”, minor increase over minimal risk is less well defined. As a result, it is interpreted differently between countries and even between institutional review

boards (IRB) within countries.(106) It is perhaps impossible to solidly define ‘minor increase’ as this is subject to a continued scientific and public debate. It will be up to IRBs to decide for each study protocol if the patients involved are exposed to an acceptable risk. Legislative authorities and governing bodies should provide and evaluate the frameworks to aid both clinical researchers and IRBs in drafting and judging research protocols.

Parental informed consent

Parental informed consent is essential in performing research in neonates, but obtaining parental informed consent in this specific population is even more challenging than in older children. Parents are often unexpectedly faced with a critically ill child requiring specialised care. The acute situation and associated psychological impact may compromise the ability of parents to give informed consent, especially when time is limited because of an urgent need for intervention.(107) In the 2-STEP study, described in Chapter 2.2, participation rate was only around 30%. In this study, administration of 2-iminobiotin had to be started within twelve hours after birth. In future studies with 2-iminobiotin or other neuroprotective agents, this time window will likely become even shorter. For the observation PharmaCool study described in Chapters 3 and 4, participation rate was relatively high at 80%. The first blood sample for this study was required at day 2 after birth, allowing more time for parental informed consent. It has been postulated that a few hours constitutes as sufficient time for prospective informed consent, but that the information provided to parents can be improved.(107,108) Adding a short (e.g. one page), accessible parental informed consent form highlighting the most important information to the current informed consent procedure may be helpful for both the parents’ understanding and trial participation rates. (107,109,110)

When there is even less time, between 30 minutes and a few hours, normal informed consent becomes extremely difficult. In this situation, parents are likely

struggling to understand even basic information about the trial. The anxiety surrounding their child's condition and the need for urgent intervention could push them to make rapid, poorly informed decisions.(111) Nevertheless, even in a time-limited situation, parents need to be involved in decisions about their child's research participation.(112) Sometimes it is possible to provide anticipatory information to parents who are 'at risk' for being asked to include a child into a trial, for instance pregnant woman at risk for complications. However, this is a time consuming activity which could also lead to unnecessary consternation amongst the 'at risk' parents.(113) Another option is the use of oral consent, where the most important information regarding the trial is given verbally.(114) After oral consent, only the study activities that must be performed without delay can take place. As soon as possible, a second informed consent conversation takes place for the remainder of the study activities. This option provides parents with an opportunity for reflection and aides researchers in designing trials despite the limited timeframe.

Deferred consent

In emergency settings, other strategies may provide an alternative to the traditional informed consent procedure. With deferred consent, no or a minimal amount of information is given to the parents in advance. This can be viable when a delay in implementing the investigational treatment would endanger the child or reduce its chances of benefit from the trial, or family members are too overwhelmed by physical or emotional trauma to give informed consent.(115) The Declaration of Helsinki also mentions the possibility of exception from informed consent in emergency situations.(116) Trials eligible for deferred consent are those investigating treatments for immediately life-threatening conditions in which the available standard of care is unsatisfactory. Additionally, seeking prospective consent must be unfeasible and the research question can only be answered in this specific population and in the emergency setting. Under deferred consent, only the study activities that cannot be performed without delay should take place

and parental informed consent should be sought an soon as possible thereafter. (107) Consent in such circumstances involves asking the parents to decide about continuation or withdrawal from the trial. When study-related activities have ceased before consent could be asked, consent will only concern the use of the gathered data.(117) Trials in neonatal emergency settings are important to determine the optimal treatment of this vulnerable population. Standard informed consent could introduce selective recruitment if the more severely affected neonates are less likely to be recruited. This undermines the generalisability of the results to the target population.(118) Although deferred consent helps to avoid these difficulties, clinicians might feel uncomfortable conducting study activities before consent has been sought, especially in randomised controlled trials with an intervention and a control group.(119) The reason for deferred consent should be clearly stated in the research protocol and weighed by the IRB, possibly after external consultation with experts in the field: researchers, ethicists, clinicians and parents. Institutions that have approved trials with deferred consent should also consider providing information on their website about these trials, to provide transparency and to increase public awareness about the necessity of this type of research.

Broad consent for data sharing

Large amounts of real world data are being generated on NICUs every day. Although this data is used to evaluate and direct the clinical care for each individual patient, the true potential that this data holds is not yet unlocked.(120) What we consider the standard-of-care is often based on limited evidence and single centre or even single country observational studies produce (too) small cohorts resulting in underpowered analyses and contradicting findings. All NICUs worldwide or at least in developed countries should be incorporated into one observational research cohort. (121) Broad consent by parents for structured, anonymised data collection and storage should be standard procedure upon admission of their child to a NICU. This broad consent should include the automatic storage of residual blood samples

taken for clinical care into a neonatal biobank. Ownership of both the database and the biobank should be vested with national or international governing bodies such as the FDA and the EMA and shared with the contributing institutions after submission and appraisal of a specific research objective. Research output obtained with this database should be part of the Open Science initiative to maximise exposure and re-use of data.(122)

In the current era of rapid technological advancement in healthcare and electronic data registration, health care professionals will all agree that we must utilise this data to improve the care of our future patients. Continuous data on heart rate, blood pressure, body temperature, cerebral and transcutaneous oxygenation and amplitude-integrated electroencephalography together with laboratory values can provide valuable pharmacodynamic (PD) endpoints. Residual blood samples can be used to facilitate PK/PD research, but also to identify biomarkers for a better understanding of disease progression or drug effect. Ultimately, big data initiatives like these can facilitate personalized medicine for each neonate.(123) Although many organisational, financial and legislational hurdles need to be overcome before this can be implemented, we are ethically obliged to learn from exposing neonates to therapies that have been insufficiently studied and to continue to learn to improve the current standard of care.

Implementing investigator-initiated drug research in clinical practice

Off-label prescribing is still common practice in NICUs and numerous publications over the past two decades have highlighted this problem repeatedly. Both the USA and the European Union have since introduced legislation to stimulate drug research in paediatrics, but the resulting increase in licensing has been somewhat disappointing.(124,125) Furthermore, this legislation only incites pharmaceutical companies to conduct trials for new drugs to prolong market exclusivity. Paediatric and neonatal drug research should also be encouraged for generic drugs. This has

been recognised by the EMA, who have issued a “priority list for studies into off-patent paediatric medicinal products”.(126)

Knowledge, however, does not always end with the information mentioned in the label. Especially for generic drugs, investigator-initiated research has provided valuable information beyond the official Summary of Product Characteristics (SmPC). The Dutch National Children’s Formulary is an excellent example of combining on label and off label knowledge into evidence based dosing guidelines across the entire paediatric age range.(127) Recently, the Neodose project has resulted in updated and expanded dosing information for neonates specifically.(128) The Dutch National Children’s Formulary is instrumental in providing evidence based drug dosing and harmonisation between individual doctors and hospitals. Although currently only available in the Dutch language, it is currently being translated into other languages and could possibly be made available on a European level in the future. Furthermore, a platform such as the International Neonatal Consortium should be stimulated to provide international consensus in treatment protocols and dosing guidelines.(129) Additionally, legislative authorities must be mandated to expand label indications based on investigator-initiated research. Currently, FDA and EMA can only enforce restrictions in the SmPC due to safety concerns, whereas only a drug manufacturer can initiate adding an additional indication or expanding the current indication to a different age group. By changing these regulations, SmPCs for frequently used generic drugs in neonates can finally be a just reflection of all the available evidence.

Conclusions

Perinatal asphyxia remains one of the leading causes of neonatal death and disability, despite the current treatment with TH. Interventions aimed at enhancing the neuroprotective properties of TH or stimulating neuroregeneration will need

to be developed over the next few years to reduce the incidence of death and neurodevelopmental disabilities. A promising novel neuroprotective agent is 2-iminobiotin, which has shown encouraging results in animal studies. In this thesis, 2-iminobiotin administration concomitant with TH to neonates with HIE appeared safe and a dose for future efficacy studies has been proposed.

Neonates with HIE are exposed to a multitude of drugs, such as antibiotics, sedatives and AEDs. PK of these drugs is influenced by maturational changes in hepatic and renal clearance, but also by factors associated with the disease and TH treatment. In this thesis, studies were conducted to develop PK models for morphine, phenobarbital, midazolam and lidocaine in order to optimise pharmacotherapy in this vulnerable population. Clearance of morphine and lidocaine was found to be reduced during TH compared to normothermia and PK based dosing guidelines were developed for morphine and phenobarbital, while a previously developed dosing regimen for lidocaine was evaluated. Data from seven different drugs investigated in the PharmaCool study were combined into an integrated PK model incorporating structural effects of body size, maturation, organ recovery and TH that supersede individual drugs. Potential rapid changes in drug clearance should be anticipated in future PK trials in this population.

Drug research in paediatric patients and especially in neonates is still lacking compared to adults. International collaboration between researchers and clinicians, innovative clinical trial designs including informed consent procedure and optimal use of (big) data generated for clinical care must all be utilised to bring paediatric drug research into the 21st century. These can be applied to both novel drugs as well as for optimising existing pharmacotherapy.

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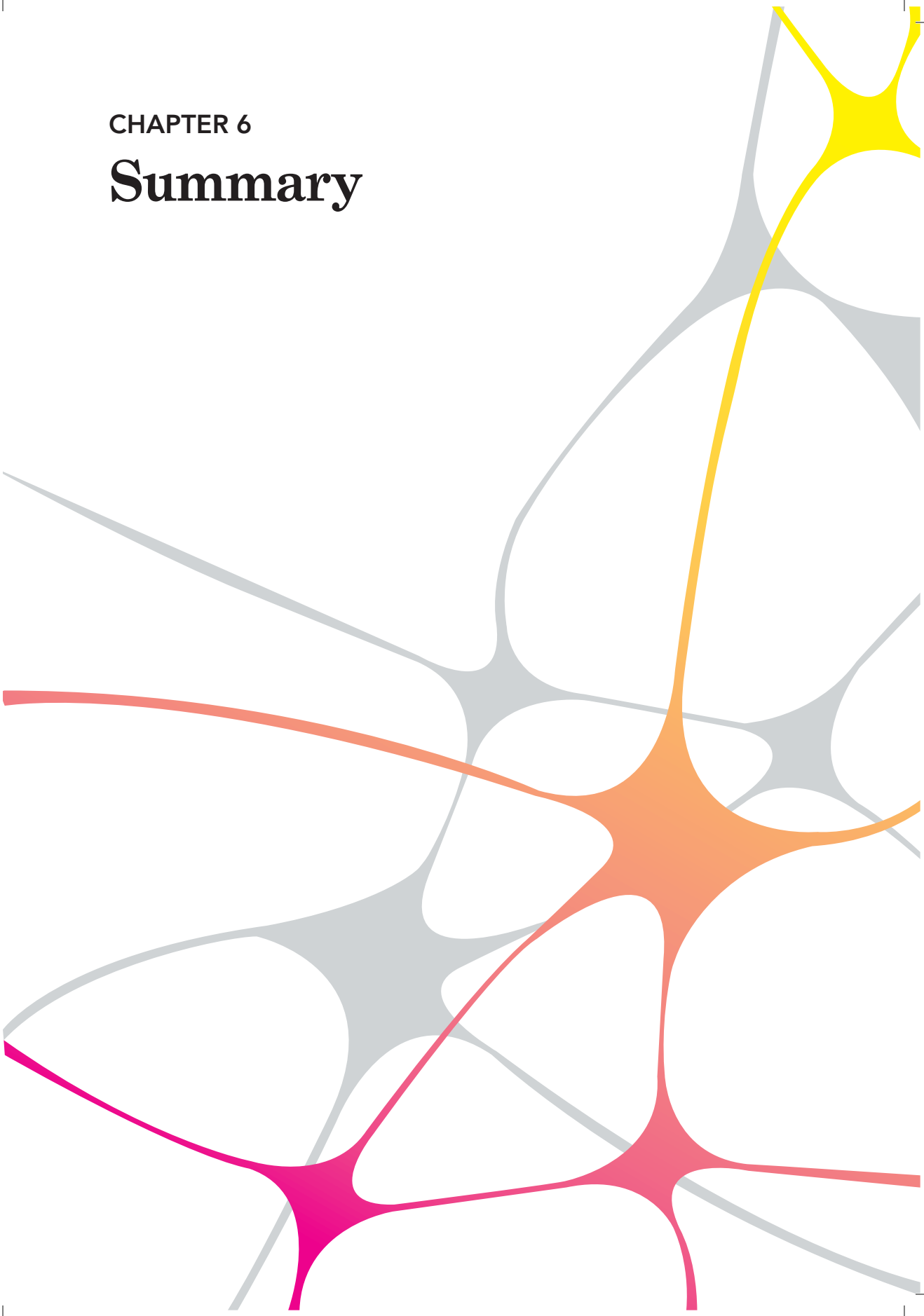
CHAPTER 6

Summary



Summary

Perinatal asphyxia, deprivation of oxygen around the time of birth, is one of the most frequent childbirth complications worldwide, affecting between four and nine million neonates each year. The causes of perinatal asphyxia can be various and include infection, placental abruption and umbilical cord entanglement, but often remains unknown. The diagnosis is based on a combination of the clinical condition of the neonate and laboratory values confirming the presence of a metabolic acidosis. Perinatal asphyxia can lead to widespread hypoxic-ischaemic damage to organ systems such as heart, lungs, liver and kidney, but central nervous system dysfunction is the biggest concern. Brain damage as a result of perinatal asphyxia is classified as hypoxic-ischaemic encephalopathy (HIE). In countries with advanced economies, HIE has a mortality rate of approximately 30%, while another 15% develop severe neurodevelopmental disabilities such as epilepsy, cerebral palsy, visual and auditory handicaps and developmental delay.

Neuronal damage related to HIE occurs in two phases. The actual hypoxic-ischemic period around birth is responsible for direct cell damage and production of pro-radicals and accumulation of xanthine. This first phase is called the primary energy failure. After resuscitation and return of systemic circulation, a latent period follows during which cerebral energy metabolism initially returns to normal. However, between six and fifteen hours after birth, a second phase of neuronal cell death commences. During this phase, pro-radicals and xanthine are responsible for the formation of hydroxyl free radicals and peroxynitrate. Both are considered extremely toxic, setting a pre-apoptotic pathway in motion resulting in further (delayed) brain damage. This phase of secondary energy failure accounts for a significant proportion of the final cell loss.

Currently, therapeutic hypothermia (TH) in the early postnatal period is the only intervention with proven neuroprotective efficacy. Applied as whole body hypothermia, the neonates' core body temperature is reduced to 33.5°C for 72 hours, after which the neonate is gradually rewarmed to normothermia. TH is indicated for neonates with a gestational age (GA) of at least 36.0 weeks suffering from moderate to severe HIE. In order to exhibit its neuroprotective properties, TH needs to be started as soon as possible, but preferably within six hours after birth. Although TH has reduced the incidence of a combined adverse outcome of death and disability from 60% to 45%, it has not been able to reduce this percentage further. Therefore, novel neuroprotective agents complementary to TH are needed to further improve the outcome of neonates with HIE.

Neonates with HIE are treated with a multitude of drugs, such as sedatives and anti-epileptic drugs (AEDs). Morphine is commonly prescribed conjoint with TH to provide analgesia and prevent stress, to ensure that the desired core temperature of 33.5°C can be maintained. Neonates with HIE often exhibit seizures as a result of the hypoxic-ischaemic brain damage. As seizures are detrimental for the developing brain, adequate treatment with AEDs is important. Over the last few decades, an abundance of new AEDs have become available for adults and older children. None of these drugs, however, are investigated in neonates. Therefore, older drugs such as phenobarbital, midazolam and lidocaine are still the drugs of first, second and third choice, based on extensive clinical experience.

Pharmacotherapy and adequate dosing is challenging in neonates in general and even more so in this population of critically ill neonates. The pharmacokinetics (PK) of drugs administered to neonates are dynamic due to maturational changes in organ function, predominantly the liver and the kidneys. In neonates with HIE, factors related to the disease, e.g. hypoxic-ischaemic damage to liver and kidneys and recovery of those organs, can also play a part. Additionally, treatment with

TH has the potential to influence PK. TH decreases heart rate and cardiac output which will subsequently reduce perfusion of the liver and kidneys and possibly drug clearance. Furthermore, activity of liver enzymes responsible for drug metabolism might be hampered by a lower body temperature. All these factors will have to be taken into account in order to optimise pharmacotherapy in neonates with HIE both during and after TH.

In **Chapter 1**, the Introduction of this thesis, mechanisms of brain damage as a result of HIE are discussed. Strategies targeting those mechanisms such as TH but also novel neuroprotective agents additional to TH are described. Additionally, the current knowledge regarding the management of sedation and seizures in neonates with HIE treated with TH is addressed as well as the challenges in optimising pharmacotherapy in this population. Finally, the objectives of the thesis and the content of the performed studies are outlined.

In **Chapter 2**, novel neuroprotective agents additional to TH are investigated. **Chapter 2.1** reviews the neuroprotective potential of nitric oxide synthase (NOS) inhibition based on animal models translational for HIE. Combined inhibition of neuronal NOS (nNOS) and inducible NOS (iNOS) is identified as the most promising strategy. **Chapter 2.2** describes the PK and short term safety of the combined nNOS/iNOS inhibitor 2-iminobiotin in twelve neonates with HIE in addition to TH. In group A, six neonates were treated with four doses of 0.16 mg/kg at six hour intervals. However, exposure in this group was almost double compared to the predefined target. Therefore, in group B the dose was reduced to 0.08 mg/kg every six hours. As 2-iminobiotin is believed to be of potential neuroprotective benefit for up to 48 hours after birth, the number of doses for group B was extended to eight. No short term safety concerns were observed. Based on these results, a placebo-controlled efficacy trial with 2-iminobiotin is deemed feasible. In **Chapter 2.3**, the safety of ventilation with the noble gas argon is studied in newborn piglets. Argon

is hypothesised to exhibit neuroprotective properties after perinatal asphyxia. Two animals were ventilated with argon concentrations increasing to 80%. Two animals were subjected to a hypoxic event and were subsequently ventilated with 50% argon and two animals received hypothermia following hypoxia as well as 50% argon ventilation. Argon ventilation did not affect heart rate, blood pressure, cerebral saturation and electrocortical brain activity in any setting. Also, increasing argon blood concentrations could be detected with increasing argon concentrations in the ventilation setup. Based on this study, a safety study with argon ventilation in humans seems justified.

Chapter 3 describes the PK of sedatives and AEDs in neonates with HIE both during and after TH in order to optimise the current pharmacotherapy. In **Chapter 3.1**, PK of morphine is investigated in 244 neonates included in the multicentre prospective cohort studies SHIVER and PharmaCool. The SHIVER study was performed in two neonatal intensive care units (NICUs) in the Netherlands; the PharmaCool study was performed in all ten NICUs in the Netherlands and two NICUs in Belgium. Both included neonates with HIE who were treated with TH. Data was collected regarding drug use and dosing and plasma concentrations of these drugs were determined in blood samples drawn both during and after TH. A population PK model was developed using non-linear mixed effects modelling (NONMEM) in which morphine clearance was dependent on GA and postnatal age (PNA). During TH, morphine clearance was reduced by 20.7% compared to normothermia. Based on simulations performed with the final population PK model, a starting dose of 50 µg/kg followed by a continuous infusion of 5 µg/kg/h is advised, although a higher dose might be necessary in individual patients to achieve the desired effect. **Chapter 3.2** describes the PK and clinical effectiveness of phenobarbital and midazolam as first and second line AEDs in neonates included in the PharmaCool study. Phenobarbital PK was investigated in 113 neonates and midazolam PK in 118 neonates using NONMEM. As phenobarbital is a known

inducer of the cytochrome P450 3A (CYP3A) enzyme and midazolam is primarily metabolised by CYP3A, a possible drug-drug interaction was also investigated. Clearance of neither drug was influenced by TH, but midazolam clearance in neonates who also received phenobarbital (n = 68) was increased by a factor 2.3. Clinical effectiveness as AED was 65.5% for phenobarbital. When midazolam was used as an additional anticonvulsant in neonates refractory to phenobarbital treatment, effectiveness was 37.1%. Based on simulations performed with the final population PK model, a phenobarbital dose of 30 mg/kg is advised to reach the desired plasma concentration. Levetiracetam is currently investigated as an alternative first line AED. Contrary to phenobarbital, levetiracetam does not induce CYP3A. Should phenobarbital in the future be replaced by levetiracetam, a lower midazolam dose as add-on AED might be necessary to avoid overexposure.
In **Chapter 3.3**, lidocaine as treatment for seizures across the entire neonatal population is evaluated. Lidocaine is considered an effective third line AED but is also associated with cardiac toxicity at higher plasma concentrations. Preterm and term neonates from two clinical care cohorts were combined with neonates included in the SHIVER and PharmaCool studies. Lidocaine PK was investigated using NONMEM in 159 neonates of which 50 were born premature and 49 were treated with TH for HIE. Lidocaine clearance increased with increasing postmenstrual age (PMA) and TH reduced lidocaine clearance by 21.8% compared to normothermia. Simulations showed that the previously developed lidocaine dosing regimen with body weight (BW) as a descriptor for PMA and a dose reduction during TH leads to acceptable lidocaine plasma concentrations. No cardiac toxicity was reported. Overall anticonvulsant effectiveness was 53.3% and appeared to be greater in normothermic term neonates. With the developed dosing regimen, lidocaine can be a safe and effective add-on AED in preterm and term neonates with and without TH.

In **Chapter 4**, structural effects influencing PK in neonates with HIE are investigated. **Chapter 4.1** combines data from seven different drugs administered to neonates

included in the PharmaCool study. Twelve compounds (seven drugs and five metabolites) were joined into one integrated population PK model to identify structural effects of body size, maturation, recovery of organ function and TH on PK in this population. For all compounds, BW was used as a descriptor for body size and related to clearance using allometric relationships and maturation was described with GA in a fixed sigmoidal Hill equation. Recovery of organ function was incorporated using PNA. For high clearance compounds (renally cleared drugs and metabolites and drugs with a high hepatic extraction ratio), clearance increased by 1.23%/h after birth. For intermediate clearance compounds (drugs and metabolites with an intermediate hepatic extraction ratio), clearance increased by 0.54%/h after birth. In this last group, clearance during TH was reduced by 20.5% compared to normothermia. This integrated model can be used to facilitate future PK studies for other drugs in this population by predicting (changes in) drug clearance based on the properties of that drug.

Chapter 5, the General Discussion, places the findings of this thesis in a broader perspective both in relation to clinical practice as well as paediatric drug research. In addition, future perspectives for clinical practice and research are provided.

CHAPTER 7

Samenvatting

Samenvatting

Perinatale asfyxie, zuurstoftekort rondom de geboorte, is wereldwijd een van de meest voorkomende complicaties van de bevalling en treft jaarlijks tussen de vier en negen miljoen pasgeborenen. Perinatale asfyxie kan talrijke oorzaken hebben zoals infectie, het loslaten van de placenta en afknelling van de navelstreng maar de oorzaak blijft meestal onbekend. De diagnose wordt gesteld door een combinatie van de klinische toestand van de pasgeborene en meetwaarden in het bloed.

Perinatale asfyxie kan door het zuurstofgebrek leiden tot schade aan organen zoals het hart, de longen, de lever en de nieren, maar schade aan de hersenen is het grootste probleem. Hersenschade na perinatale asfyxie wordt hypoxisch-ischemische encefalopathie genoemd. In ontwikkelde landen overlijdt 30% van de pasgeboren baby's met hypoxisch-ischemische encefalopathie en ontwikkelt 15% later in het leven blijvende handicaps zoals epilepsie, spierspasmen, gezichts- en gehoorschade en ontwikkelingsachterstand.

Schade aan de hersencellen als gevolg van hypoxisch-ischemische encefalopathie gebeurt in twee fases. De daadwerkelijke periode van zuurstofgebrek bij de geboorte zorgt voor directe celschade, productie van pro-radicalen en opstapeling van xanthine. Deze periode wordt de primaire fase genoemd. Nadat de pasgeborene gereanimeerd is en de bloedsomloop is hersteld, volgt een rustperiode waarin de hersenen ogenschijnlijk weer normaal functioneren. Tussen de zes en vijftien uur na geboorte wordt echter een tweede periode van schade in gang gezet. Gedurende deze fase worden pro-radicalen en xanthine omgevormd tot vrije waterstofradicalen en peroxynitriet. Beide stoffen zijn extreem schadelijk voor de hersencellen en het afsterven van hersencellen zorgt voor verdere (vertraagde) hersenschade. Deze secundaire fase is verantwoordelijk voor het grootste gedeelte van de totale schade.

Therapeutische hypothermie vlak na de geboorte is tot op heden de enige behandeling waarvan neuroprotectie, een beschermend effect op de hersenen, is bewezen. Tijdens deze behandeling wordt het hele lichaam van de pasgeborene gekoeld om de inwendige temperatuur te verlagen tot 33,5°C. Na 72 uur wordt de pasgeborene langzaam weer opgewarmd. Therapeutische hypothermie wordt toegepast bij pasgeboren baby's met een zwangerschapsduur van 36 weken of langer die matige of ernstige HIE hebben. Om de hersenen te beschermen moet therapeutische hypothermie zo snel mogelijk gestart worden, maar in ieder geval binnen zes uur na de geboorte.

Hoewel therapeutische hypothermie het totaal aan negatieve gevolgen van overlijden en handicaps heeft weten terug te brengen van 60% tot 45%, dalen deze percentages niet verder. Om die reden zijn nieuwe neuroprotectieve stoffen die kunnen worden toegevoegd aan therapeutische hypothermie hard nodig om de kansen van pasgeborenen met hypoxisch-ischemische encefalopathie te verbeteren.

Pasgeboren baby's met hypoxisch-ischemische encefalopathie worden behandeld met vele verschillende geneesmiddelen, zoals middelen om in slaap te komen (sedativa) en middelen tegen epilepsie (anti-epileptica). Morfine wordt veel voorgeschreven tijdens therapeutische hypothermie voor pijnstilling en om stress te verminderen, zodat de inwendige temperatuur van 33.5°C stabiel blijft. Pasgeboren baby's met hypoxisch-ischemische encefalopathie hebben vaak epileptische aanvallen door de hersenschade. Omdat epileptische aanvallen ook schadelijk zijn voor het ontwikkelende brein is goede behandeling met anti-epileptica belangrijk. De laatste jaren zijn er veel nieuwe anti-epileptica bijgekomen voor volwassenen en oudere kinderen. Geen van deze middelen zijn echter onderzocht bij pasgeborenen. Om die reden worden oudere geneesmiddelen zoals fenobarbital, midazolam,

en lidocaine nog steeds gebruikt als eerste, tweede en derde keuze middelen, gebaseerd op jarenlange ervaring.

Farmacotherapie, behandeling van aandoeningen met geneesmiddelen, en het vinden van de juiste dosis is een uitdaging bij pasgeborenen in het algemeen en bij pasgeboren baby's met hypoxisch-ischemische encefalopathie in het bijzonder. De farmacokinetiek van geneesmiddelen bij pasgeborenen is variabel door veranderingen ten gevolge van rijping van orgaanfuncties, met name in de lever en de nieren. Bij pasgeboren baby's met hypoxisch-ischemische encefalopathie kunnen factoren die gerelateerd zijn aan de ziekte, zoals schade door zuurstofgebrek aan lever en nieren en herstel van deze organen, ook een rol spelen. Behandeling met therapeutische hypothermie kan de farmacokinetiek ook beïnvloeden. Door therapeutische hypothermie verlaagt de hartslag en vermindert de hoeveelheid bloed die het hart uitpomp, waardoor ook de doorbloeding van lever en nieren en mogelijk de afbraak en uitscheiding van geneesmiddelen afneemt. Daarnaast kan de lagere lichaamstemperatuur ervoor zorgen dat de leverenzymen die verantwoordelijk zijn voor de afbraak van geneesmiddelen minder actief zijn. Met al deze factoren moet rekening worden gehouden om de farmacotherapie bij pasgeboren baby's met hypoxisch-ischemische encefalopathie zowel tijdens als na therapeutische hypothermie te optimaliseren.

In **Hoofdstuk 1**, de introductie van dit proefschrift, worden mechanismen van hersenschade door hypoxisch-ischemische encefalopathie beschreven. Behandelingen die aangrijpen op die mechanismen zoals therapeutische hypothermie maar ook nieuwe neuroprotectieve stoffen die kunnen worden gecombineerd met therapeutische hypothermie worden beschreven. Daarnaast wordt de huidige kennis van zaken over sedatie en behandeling van epileptische aanvallen besproken evenals de uitdagingen bij het optimaliseren van de farmacotherapie bij pasgeboren baby's met hypoxisch-ischemische encefalopathie.

In **Hoofdstuk 2** worden nieuwe neuroprotectieve stoffen die kunnen worden toegevoegd aan therapeutische hypothermie beschreven. **Hoofdstuk 2.1** is een overzicht van de tot op heden gepubliceerde literatuur over de neuroprotectieve werking van remming van het enzym stikstofmonoxide synthase (NOS) in proefdiermodellen die lijken op hypoxisch-ischemische encefalopathie bij pasgeboren baby's. Gecombineerde remming van neuronaal NOS (nNOS) en induceerbaar NOS (iNOS) lijkt het meest veelbelovende aangrijpingspunt te zijn. **Hoofdstuk 2.2** beschrijft de farmacokinetiek en de korte termijn veiligheid van de gecombineerde nNOS/iNOS remmer 2-iminobiotin in combinatie met therapeutische hypothermie in twaalf pasgeborenen met hypoxisch-ischemische encefalopathie. In het eerste deel van het onderzoek werden zes pasgeborenen behandeld met vier keer een dosis van 0,16 mg/kg, met tussenpozen van zes uur. De blootstelling in deze groep was echter bijna het dubbele van de vooraf vastgestelde streefwaarde. Om die reden werd de dosis voor het tweede deel van het onderzoek verlaagd tot 0,08 mg/kg. Omdat 2-iminobiotin potentieel tot 48 uur na de geboorte effectief kan zijn, werd het aantal toedieningen verlengd tot acht.

Er waren geen aanwijzingen dat 2-iminobiotin op de korte termijn niet veilig zou zijn. Gebaseerd op deze resultaten zou een placebo-gecontroleerd onderzoek naar de effectiviteit van 2-iminobiotin mogelijk moeten zijn. In **Hoofdstuk 2.3** wordt de veiligheid van beademing met het edelgas argon bestudeerd in pasgeboren biggen. Argon heeft mogelijk neuroprotectieve eigenschappen na perinatale asfyxie. Twee dieren werden beademd met argon in concentraties oplopend tot 80%. Twee dieren kregen een periode van zuurstofgebrek en werden vervolgens beademd met 50% argon en twee dieren werden na een periode van zuurstofgebrek behandeld met therapeutische hypothermie en beademd met 50% argon. Beademing met argon had geen effect op hartslag, bloeddruk, zuurstofgebruik door de hersenen of hersenactiviteit. Toenemende concentraties argon in het bloed konden worden

gemeten bij toenemende concentraties van beademing met argon. Gebaseerd op dit onderzoek lijkt een onderzoek naar de veiligheid van beademing met argon bij mensen gerechtvaardigd.

Hoofdstuk 3 beschrijft de farmacokinetiek van sedativa en anti-epileptica bij pasgeborenen met hypoxisch-ischemische encefalopathie, zowel tijdens als na therapeutische hypothermie, om de huidige farmacotherapie met deze middelen te optimaliseren. In **Hoofdstuk 3.1** wordt de farmacokinetiek van morfine onderzocht in 244 pasgeboren baby's die meegedaan hebben aan de multicentrum prospectieve cohort studies SHIVER en PharmaCool. De SHIVER studie werd uitgevoerd op twee neonatale intensive care units van ziekenhuizen in Nederland; de PharmaCool studie werd uitgevoerd in alle tien neonatale intensive care units in Nederland en twee neonatale intensive care units in België. Aan beide studies deden pasgeboren baby's met hypoxisch-ischemische encefalopathie mee die werden behandeld met therapeutische hypothermie. Er werden data verzameld over het gebruik van geneesmiddelen en concentraties van deze geneesmiddelen werden gemeten in het bloed, zowel tijdens als na therapeutische hypothermie. Een farmacokinetisch model werd ontwikkeld met behulp van het computerprogramma NONMEM.
De uitscheiding van morfine bleek afhankelijk te zijn van zwangerschapsduur en leeftijd na geboorte; tijdens therapeutische hypothermie was de afbraak van morfine 20,7% lager in vergelijking met normale lichaamstemperatuur. Gebaseerd op simulaties uitgevoerd met het ontwikkelde farmacokinetsche model wordt een startdosis van 50 µg/kg gevolgd door een continu infuus van 5 µg/kg/h geadviseerd, hoewel voor sommige patiënten een hogere dosis nodig kan zijn om het gewenste effect te bereiken. **Hoofdstuk 3.2** beschrijft de farmacokinetiek en de effectiviteit van fenobarbital en midazolam als eerste en tweede keuze anti-epilepticum bij pasgeboren baby's die meededen aan de PharmaCool studie. De farmacokinetiek van fenobarbital werd onderzocht bij 113 pasgeborenen en de farmacokinetiek van midazolam bij 118 pasgeborenen, beide met behulp van NONMEM. Omdat van

fenobarbital bekend is dat het kan zorgen voor meer aanmaak van het leverenzym cytochroom P450 3A (CYP3A) en midazolam voornamelijk door CYP3A wordt afgebroken, werd ook de mogelijke wisselwerking tussen deze geneesmiddelen onderzocht. De afbraak van geen van beide geneesmiddelen werd beïnvloed door therapeutische hypothermie, maar de afbraak van midazolam bij 68 pasgeboren baby's die ook fenobarbital hadden gekregen was 2,3 keer sneller dan die van pasgeboren baby's zonder fenobarbital. Fenobarbital was effectief voor de behandeling van epileptische aanvallen bij 65,5% van de pasgeborenen. Midazolam als tweede keuze middel bij pasgeborenen die niet reageerden op fenobarbital was effectief bij 37,1%. Gebaseerd op simulaties uitgevoerd met het ontwikkelde farmacokinetische model wordt een dosis fenobarbital van 30 mg/kg geadviseerd om de gewenste concentratie in het bloed te bereiken. Levetiracetam wordt op dit moment onderzocht als alternatieve eerste keuze anti-epilepticum. In tegenstelling tot fenobarbital zorgt levetiracetam niet voor meer aanmaak van CYP3A. Mocht fenobarbital in de toekomst vervangen worden door levetiracetam als eerste keuze middel, dan zou van midazolam als tweede keuze middel een lagere dosis gegeven kunnen worden om overdosering te voorkomen. In **Hoofdstuk 3.3** wordt lidocaine als behandeling voor epileptische aanvallen in de gehele populatie van pasgeboren baby's onderzocht. Lidocaine wordt beschouwd als een effectief anti-epilepticum maar wordt ook in verband gebracht met hartritmestoornissen, voornamelijk bij hogere concentraties in het bloed. Informatie van premature pasgeborenen (geboren na een zwangerschapsduur van minder dan 36 weken) en à terme pasgeborenen (geboren na een zwangerschapsduur van 36 weken of meer), verzameld voor zorg in de dagelijkse praktijk, werden gecombineerd met pasgeboren baby's die meededen aan de SHIVER en PharmaCool studies. Lidocaine farmacokinetiek werd onderzocht in 159 pasgeborenen van wie 50 premature pasgeborenen en 49 à terme pasgeborenen die behandeld werden met therapeutische hypothermie voor hypoxisch-ischemische encefalopathie. Afbraak van lidocaine nam toe met toenemende leeftijd na de verwekking (een combinatie van zwangerschapsduur en

leeftijd na geboorte) en therapeutische hypothermie verminderde de afbraak van lidocaine met 21,8% ten opzichte van normale lichaamstemperatuur. Simulaties toonden aan dat het eerder ontwikkelde doseerschema, waarin de dosering lidocaine wordt aangepast aan het lichaamsgewicht en verlaagd wordt tijdens therapeutische hypothermie, tot acceptabele concentraties in het bloed leidt. Er werden geen hartritmestoornissen gemeld. De effectiviteit van lidocaine als anti-epilepticum voor alle pasgeborenen was 53,3% en leek beter te zijn bij à terme pasgeborenen met een normale lichaamstemperatuur. Met het ontwikkelde doseerschema is lidocaine een veilig en effectief derde keuze anti-epilepticum voor pasgeborenen van alle zwangerschapsduren zowel met als zonder behandeling met therapeutische hypothermie.

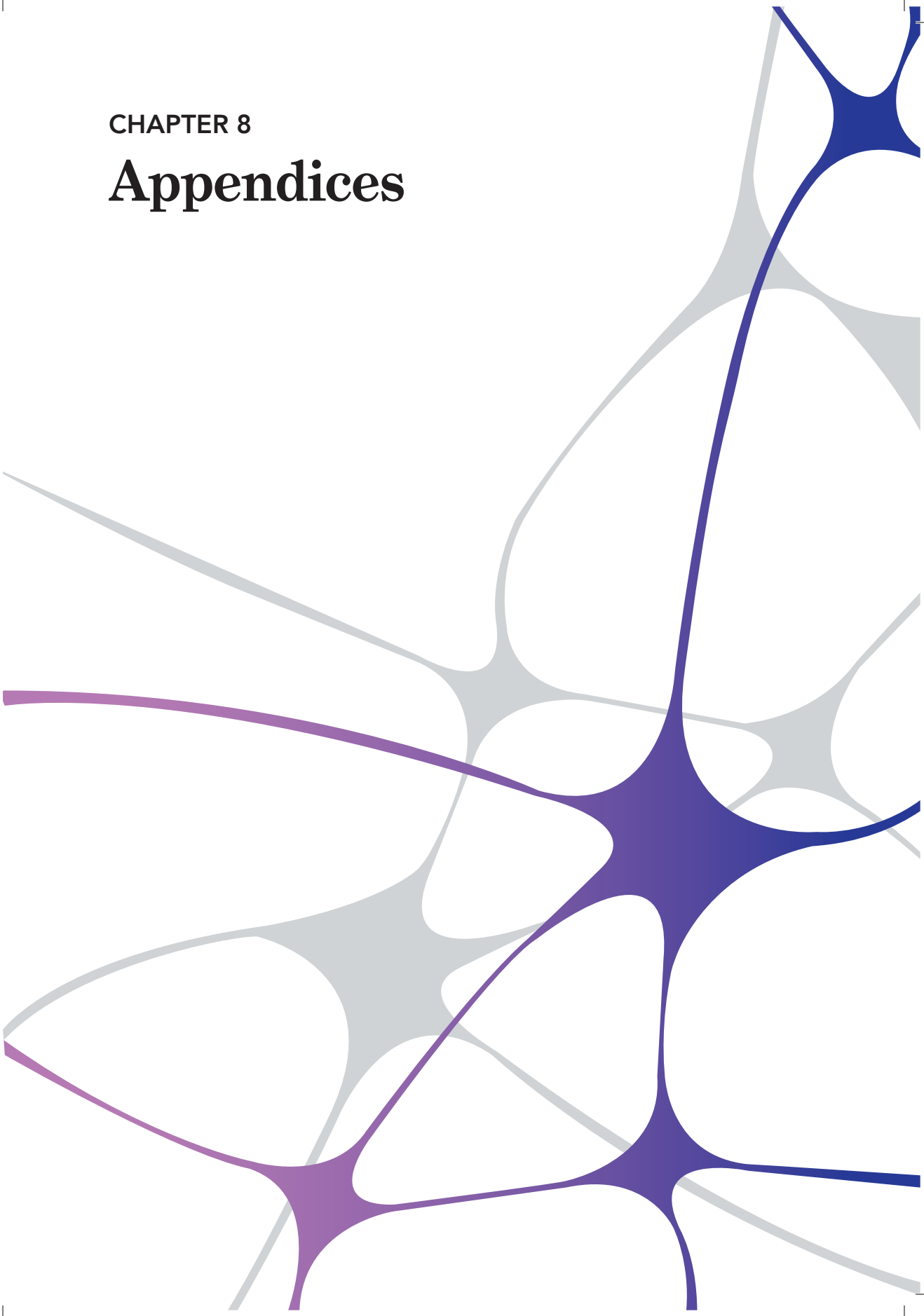
In **Hoofdstuk 4** worden structurele effecten onderzocht die van invloed kunnen zijn op de farmacokinetiek bij pasgeboren baby's met hypoxisch-ischemische encefalopathie. **Hoofdstuk 4.1** combineert data van zeven verschillende geneesmiddelen waarmee pasgeborenen die meededen aan de PharmaCool studie werden behandeld. Twaalf stoffen, zeven geneesmiddelen en vijf metabolieten (afbraakproducten) van deze geneesmiddelen, werden samengevoegd in één geïntegreerd farmacokinetisch model om de structurele effecten van lichaamsgrootte, rijping van orgaanfunctie, herstel van orgaanfunctie na zuurstofgebrek en therapeutische hypothermie op de farmacokinetiek van geneesmiddelen te onderzoeken. Voor alle stoffen werd lichaamsgewicht gebruikt als maat voor lichaamsgrootte en werd zwangerschapsduur gebruikt om rijping van orgaanfunctie te beschrijven volgens een wiskundige formule. Leeftijd na geboorte werd gebruikt om herstel van orgaanfunctie na zuurstofgebrek te beschrijven. Voor stoffen met een hoge uitscheiding (geneesmiddelen en metabolieten die via de nieren uitgescheiden worden of die voor uitscheiding voornamelijk afhankelijk zijn van doorbloeding van de lever) bleek de uitscheiding na de geboorte toe te nemen met 1,23%/uur. Voor stoffen met een gemiddelde uitscheiding

(geneesmiddelen en metabolieten die voor de uitscheiding niet alleen afhankelijk zijn van doorbloeding van de lever maar ook van activiteit van leverenzymen) nam de uitscheiding na geboorte toe met 0,54%/uur. In deze laatste groep was de uitscheiding tijdens therapeutische hypothermie 20,5% lager ten opzichte van normale lichaamstemperatuur. Dit geïntegreerde model kan gebruikt worden bij toekomstig geneesmiddelonderzoek bij pasgeborenen met hypoxisch-ischemische encefalopathie, door de (verandering in) uitscheiding te voorspellen aan de hand van de eigenschappen van het geneesmiddel.

Hoofdstuk 5, de algemene discussie, plaatst de uitkomsten van dit proefschrift in breder perspectief, zowel voor de zorg in de dagelijkse praktijk als voor geneesmiddelonderzoek bij kinderen. Ook worden er aanwijzingen gegeven voor de dagelijkse zorg voor pasgeborenen met hypoxisch-ischemische encefalopathie en geneesmiddelonderzoek in de toekomst.

CHAPTER 8

Appendices



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List of publications related to this thesis

Neuroprotection by Argon Ventilation after Perinatal Asphyxia: A Safety Study in Newborn Piglets

Thomas Alderliesten, Laurent M.A. Favié, Robert W. Neijzen, Volker Auwärter, Cora H.A. Nijboer, Roland E.J. Marges, Carin M.A. Rademaker, Jürgen Kempf, Frank van Bel, Floris Groenendaal

PLoS One; 2014 Dec 2; 9(12):e113575

Nitric oxide synthase inhibition as a neuroprotective strategy following hypoxic-ischemic encephalopathy: evidence from animal studies

Laurent M.A. Favié , Arlette R. Cox, Agnes van den Hoogen, Cora H.A. Nijboer, Cacha Peeters-Scholte, Frank van Bel, Toine C.G. Egberts, Carin M.A. Rademaker, Floris Groenendaal

Frontiers in Neurology; April 2018, Volume 9, Article 258

Pharmacokinetics of morphine in encephalopathic neonates treated with therapeutic hypothermia

Laurent M.A. Favié, Floris Groenendaal, Marcel P.H. van den Broek, Carin M.A. Rademaker, Timo R. de Haan, Henrica L. M. van Straaten, Peter H. Dijk, Arno van Heijst, Jeroen Dudink, Koen P. Dijkman, Monique Rijken, Inge A. Zonnenberg, Filip Cools, Alexandra Zecic, Johanna H. van der Lee, Debbie H.G.M. Nuytemans, Frank van Bel, Toine C.G. Egberts, Alwin D.R. Huitema

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Phenobarbital, midazolam pharmacokinetics, effectiveness and drug-drug interaction in asphyxiated neonates undergoing therapeutic hypothermia

Laurent M.A. Favié, Floris Groenendaal, Marcel P.H. van den Broek, Carin M.A. Rademaker, Timo R. de Haan, Henrica L. M. van Straaten, Peter H. Dijk, Arno van Heijst, Sinno H.P. Simons, Koen P. Dijkman, Monique Rijken, Inge A. Zonnenberg, Filip Cools, Alexandra Zecic, Johanna H. van der Lee, Debbie H.G.M. Nuytemans, Frank van Bel, Toine C.G. Egberts, Alwin D.R. Huitema, on behalf of the PharmaCool study group

Neonatology. 2019 Jun 28:1-9

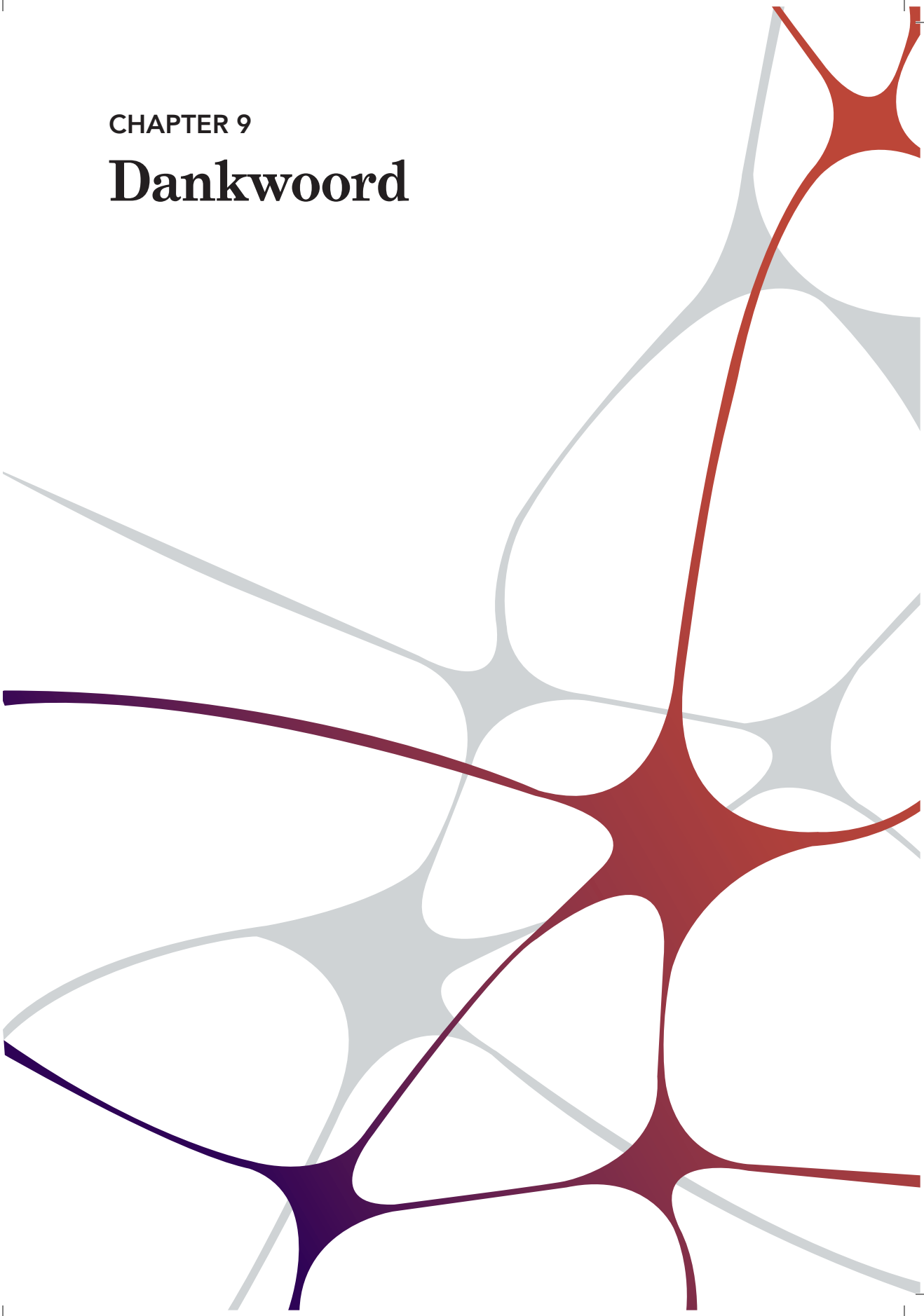
Curriculum Vitae

Laurent Favié was born on the 28th of February 1985 in Spanbroek, the Netherlands, where he grew up as the eldest of three siblings. After graduating from the grammar school of the Werenfridus College in Hoorn, he enrolled in the Pharmaceutical Sciences program at Utrecht University in 2004. During his study years, he performed his research internship titled 'Clinical setting influences off-label and unlicensed prescribing in a paediatric teaching hospital' at Curtin University and the Princess Margaret Hospital for children in Perth, Australia under the supervision of dr. Petra Czarniak and prof. dr. Bruce Sunderland. Furthermore, for a full year he acted as a board member of the Royal Dutch Pharmaceutical Student Association.

After obtaining his master's degree in 2012, Laurent started his career in hospital pharmacy at the Vlietland hospital in Schiedam. In 2013, he was accepted into the hospital pharmacy residency program at the University Medical Center Utrecht (supervisors dr. Karin Rademaker and dr. Ingeborg Wilting), in combination with a PhD research project. After completing his residency in 2017, he continued with his PhD research project at the Department of Clinical Pharmacy (prof. dr. Toine Egberts and dr. Karin Rademaker), in close collaboration with the Department of Neonatology (prof. dr. Frank van Bel and dr. Floris Groenendaal). During his PhD, he worked on the prospective multicentre observational cohort PharmaCool study, a joint project with the Amsterdam University Medical Center. Additionally, he started his training as a clinical pharmacologist, under the supervision of prof. dr. Alwin Huitema, dr. Douwe Dekker and dr. Wilma Knol.

CHAPTER 9

Dankwoord



Dankwoord

Het zit erop! Met het schrijven van dit dankwoord komt een einde aan een reis die bijna 6,5 jaar geleden begon. Een reis die ontzettend leerzaam is geweest, soms stressvol maar dankzij de steun van een groot aantal mensen onvergetelijk. Ik wil iedereen bedanken die ik in de afgelopen jaren heb ontmoet, met wie ik heb samengewerkt of die op een andere manier belangrijk zijn geweest voor mij of voor het tot stand komen van dit proefschrift. Ook wil ik alle ouders bedanken die de moeilijke en moedige beslissing hebben genomen om hun pasgeborene deel te laten nemen aan wetenschappelijk onderzoek. Zonder hen zou dit proefschrift niet mogelijk zijn geweest. Een aantal mensen wil ik in het bijzonder bedanken.

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“Mischief managed.”