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Pharmacokinetics during therapeutic hypothermia in neonates: from pathophysiology to translational knowledge and physiologically-based pharmacokinetic (PBPK) modeling

Karen Leys ^{a#}, Marina-Stefania Stroe ^{b#}, Pieter Annaert ^{a,c}, Steven Van Cruchten ^b, Sebastien Carpentier ^d, Karel Allegaert ^{e,f,g,h} and Anne Smits ^{e,h,i}

^aDrug Delivery and Disposition, Department of Pharmaceutical and Pharmacological Sciences KU Leuven, Leuven, Belgium; ^bComparative Perinatal Development, Department of Veterinary Sciences, University of Antwerp, Antwerp, Belgium; ^cBioNotus GCV, Niel, Belgium; ^dFacility for SYstems BIOlogy Mass Spectrometry, KU Leuven, Leuven, Belgium; ^eDepartment of Development and Regeneration, KU Leuven, Leuven, Belgium; ^fDepartment of Pharmaceutical and Pharmacological Sciences, KU Leuven, Leuven, Belgium; ^gDepartment of Hospital Pharmacy, Erasmus MC, GA, Rotterdam, The Netherlands; ^hChild and Youth Institute, KU Leuven, Leuven, Belgium; ⁱNeonatal Intensive Care Unit, University Hospitals Leuven, Leuven, Belgium

ABSTRACT

Introduction: Perinatal asphyxia (PA) still causes significant morbidity and mortality. Therapeutic hypothermia (TH) is the only effective therapy for neonates with moderate to severe hypoxic-ischemic encephalopathy after PA. These neonates need additional pharmacotherapy, and both PA and TH may impact physiology and, consequently, pharmacokinetics (PK) and pharmacodynamics (PD). **Areas covered:** This review provides an overview of the available knowledge in PubMed (until November 2022) on the pathophysiology of neonates with PA/TH. In vivo pig models for this setting enable distinguishing the effect of PA versus TH on PK and translating this effect to human neonates. Available asphyxia pig models and methodological considerations are described. A summary of human neonatal PK of supportive pharmacotherapy to improve neurodevelopmental outcomes is provided.

Expert opinion: To support drug development for this population, knowledge from clinical observations (PK data, real-world data on physiology), preclinical (in vitro and in vivo (minipig)) data, and molecular and cellular biology insights can be integrated into a predictive physiologically-based PK (PBPK) framework, as illustrated by the I-PREDICT project (Innovative physiology-based pharmacokinetic model to predict drug exposure in neonates undergoing cooling therapy). Current knowledge, challenges, and expert opinion on the future directions of this research topic are provided.

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1. Pathophysiology of the asphyxiated neonate receiving therapeutic hypothermia

1.1. Definitions and general aspects

Perinatal asphyxia (PA) is a medical condition at delivery caused by oxygen deprivation, potentially resulting in hypoxic-ischemic encephalopathy (HIE) [1]. Approximately 0.5% of all live-born neonates are affected by PA, and about 0.18% are diagnosed with HIE (mild, moderate, or severe). Perinatal asphyxia is still one of the most common causes of death in (near)term neonates [2]. Oxygen deprivation is reflected in the diagnostic clues for HIE, consisting of clinical findings (Apgar score and Thomson score), and findings based on either laboratory values (arterial blood base excess, lactate, and pH for acidosis) or monitoring (electroencephalographic (EEG) activity) [1,3]. Therapeutic hypothermia (TH; target core temperature of 33.5°C for 72 hours with subsequent slow rewarming at 0.5°C/hour) is effective in reducing mortality or impaired neurodevelopmental outcome in moderate to severe HIE (number needed to treat 7, outcome variable decreases from about 55% to 45%) [4,5].

1.2. The pathophysiology of asphyxia, modulated by therapeutic hypothermia

Asphyxia is a syndrome affecting multiple organs, including the central nervous system (CNS). The initial cardiovascular response to oxygen depletion results in organ-specific vasoconstriction. This subsequently results in perfusion and oxygen supply redistribution from non-vital organs to the heart and brain. Other organs, such as the kidneys, liver, and intestines, are in a low perfusion setting [6,7]. Therefore, it is essential to be aware that TH further modulates the 'natural' progression of the pathophysiology of asphyxia. As TH is only indicated in (near)term neonates with moderate or severe HIE, this mainly refers to (patho)physiological parameters on cardiac output (CO; stroke volume, myocardial strain, and heart rate), regional blood flow (pulmonary hypertension, cerebral, renal, and hepatic blood flow), renal function (glomerular filtration rate, GFR), plasma composition (albumin, hematocrit, and α -1 glycoprotein) and intestinal functions (gastric emptying, intestinal motility, and intestinal permeability). Using an exploratory search in PubMed on PA and TH in human neonates until November 2022, we have listed pathophysiological characteristics of system-

CONTACT Anne Smits  anne.smits@uzleuven.be  Neonatal Intensive Care Unit, University Hospitals Leuven, Herestraat 49, Leuven 3000, Belgium
Joint first authorship.

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Article highlights

- Therapeutic hypothermia (TH) represents the standard of care for neonates with moderate to severe perinatal asphyxia (PA) and hypoxic-ischemic encephalopathy (HIE). To support drug development for this population, knowledge from clinical observations (pharmacokinetic (PK) data and real-world data on physiology), preclinical (in vitro and in vivo animal model) data, and molecular and cellular biology insights can be incorporated into a physiologically-based PK (PBPK) model to serve as an integrative predictive modeling approach.
- Using an exploratory search in PubMed (until November 2022), pathophysiological characteristics of system-specific functions in asphyxiated neonates were summarized, mainly referring to cardiac output, regional blood flow, renal function, plasma composition, or intestinal functions.
- The current knowledge regarding cellular and molecular mechanisms of essential biochemical pathways affected by PA and/or TH is presented, with attention to pharmacologically relevant genes and proteins.
- Animal models can provide useful information on mechanisms in human diseases, but also on exploring (patho)physiological factors affecting PK, safety, and efficacy of drugs. Currently available in vivo asphyxia pig models and the methodological considerations are also covered in this review.
- By combining in vitro disposition research with in vivo animal and human data, the PBPK model development can be initiated using a multidisciplinary and translational approach. Physiologically-based PK modeling represents an established tool for drug development with a good track record, including regulatory acceptance. It represents an instrument for data integration, hypothesis testing, and knowledge generation. However, its use in neonatal pharmacotherapy is still limited.

specific functions in asphyxiated neonates undergoing TH or not (Table 1). This approach has the intention to provide the reader with a snapshot of system-specific observations, but is not complete, nor does it claim to be systematic.

We will further discuss the cardiovascular system, renal impairment, and acute kidney injury (AKI), and hepatic impairment, including drug-induced liver injury (DILI) diagnosis. All

these aspects can significantly affect pharmacokinetics (PK) and should therefore be considered in physiologically-based pharmacokinetic (PBPK) models. Furthermore, AKI and DILI are relevant to tailor pharmacovigilance in this population.

1.2.1. Cardiovascular system

The CO is affected by the disease (PA) and the treatment (TH), as there are significant reductions in cardiac biomarkers and less myocardial dysfunction on electrocardiography (ECG) and ultrasound in TH cases compared to non-TH asphyxia cases [9]. The myocardial function (strain rate about 50% slower) is mainly determined by asphyxia, while hypothermia reduces the heart rate [8]. Gebauer et al. documented in a paired study that CO during whole-body hypothermia (33°C) was significantly lower when compared to normothermia (37°C) (median and range: 169 (157–202) versus 254 (241–339) mL/kg/min) [20]. Right and left ventricular output in asphyxiated whole-body TH cases compared to normative values were 108 (–51%) and 107 (–52%) compared to 224 and 222 mL/kg/min, respectively [21]. There is also an even more pronounced decrease in intestinal and hepatic blood flow, reflected by an increased blood flow velocity via ultrasound, with a subsequent increase during and after rewarming [22].

1.2.2. Renal impairment and acute kidney injury

For drugs that are mainly eliminated by the kidneys (glomerular filtration (GF) + renal tubular processes), and used during PA/TH, reports consistently observed a significant decrease in clearance (–30 to –60%) compared to non-asphyxia control cases, with an increase in clearance over postnatal age (PNA). Interestingly, the highest reduction in clearance (40–60%) seems to be present during the first day of life, with less pronounced differences afterward (<30%). Clearance becomes similar to healthy controls in the second part of the first week of life [23]. Favié et al., classifying renally eliminated drugs as part of the high-clearance drugs, estimated a time-dependent

Table 1. System-specific observations on the impact of perinatal asphyxia on neonatal physiology, with or without therapeutic hypothermia.

System	Observations
Cardiovascular System	Asphyxia was the primary determinant of impaired myocardial function (strain rate about 50% slower), improving from day 4 (after TH) [8]. In a meta-analysis, short-term outcome studies suggest cardioprotective effects (via cardiac biomarkers, blunted myocardial dysfunction on ECG, and cardiac ultrasound) of TH [9]. QTcs are prolonged during TH and normalize afterward [10].
Renal system	Median sCr values over PNA days 1, 2, 3, 5, and 7 were 0.96, 0.80, 0.64, 0.75, and 0.47 mg/dL in PA/TH cases. This significantly differs from healthy controls: 0.71, 0.77, 0.67, 0.46, and 0.42 mg/dL, reflecting poorer kidney function in PA/TH cases [11,12]. In addition, a meta-analysis showed a significantly lower incidence of AKI in neonates treated with TH than in cases without TH (relative risk = 0.81, TH is protective) [9].
Coagulation system and acid-base balance	Compared to term controls, hematocrit is lower in asphyxiated term neonates (0.41 to 0.45 on day 1 of PNA versus 0.5 to 0.53 on day 5 of PNA) [13]. Albuminemia is decreased (24 g/L) in the setting of asphyxia and relates to the asphyxia severity grade (29 g/L in mild cases; 24 g/L in moderate cases; and 20 g/L in severe cases) [14]. Acidosis is a critical finding in these patients (umbilical cord pH < 7 as one of the criteria). However, TH also affects blood gas parameters such as pH and PCO ₂ . At lower temperatures, the pH increases and PCO ₂ decreases [15]. Umbilical cord blood lactate levels are significantly higher among neonates with HIE (mean 8.72 ± 1.75 mmol/L, range 5.12–11.96 mmol/L) compared to those without HIE (mean 6.86 ± 1.33 mmol/L, range 4.74–10.30 mmol/L; <i>p</i> = 0.001) [16].
Digestive and hepatic system	The intestinal and hepatic blood flows are decreased in asphyxiated term neonates, reflected in increased (20–25%) regional flow velocity and reduced CO [13]. There is higher passive intestinal permeability (lactulose and renal lactulose/L-rhamnose test) up to at least day 9 of PNA in asphyxiated term neonates [17]. Hepatic injury, marked by raised liver enzyme levels, is common in PA; however, TH blunts the hepatic injury. The same holds for the inflammatory response (TH is associated with lower CRP) [14].
Central Nervous System	Neurovascular coupling is impaired in neonates with asphyxia [18]. Seizures commonly occur and are associated with an adverse neurodevelopmental outcome [19].

AKI = acute kidney injury; CO = cardiac output; CRP = C-reactive protein levels; ECG = electrocardiography; EEG = electroencephalography; HIE = hypoxic-ischemic encephalopathy; PCO₂ = partial pressure of carbon dioxide; PNA = postnatal age; QTc = Corrected QT Interval; sCr = serum creatinine; TH = therapeutic hypothermia.

maturation function of clearance (1.23%/hour), far exceeding the effects of maturation on clearance (estimated to be 0.05%/hour) [24]. These authors also stressed the large (71.6%) inter-individual variability in clearance, as these patterns are also reflected in datasets on serum creatinine (sCr) values in PA/TH cases compared to controls [24].

1.2.3. Hepatic metabolism and drug-induced liver injury

Favié et al. also estimated a maturation function for intermediate clearance drugs (0.54%/hour; liver metabolism mediated), which still exceeded the estimated maturation of ~0.05%/hour [24]. When quantified by temperature reduction (between 33.5°C and 37°C,) clearance changed by 6.83%/°C and was, therefore, 23.9% lower at 33.5°C compared to 37°C.

When screening for DILI, there are certain commonly used biomarkers. However, there are limitations when using these to screen for DILI in neonates, as (indirect) hyperbilirubinemia is commonly part of neonates' normal developmental physiology. Neonatal hyperbilirubinemia has a natural trend over time, with a peak on days 3 to 4 and a subsequent decrease over the first 10 to 14 days of PNA. However, the peak and mean total serum bilirubin levels are lower in asphyxia cases, either undergoing TH or not [25,26]. Among others, Muniraman et al. and Michniewicz et al. reported on liver enzyme patterns in asphyxia cases (Table 2) [3,14]. Liver-injury-related enzymes (alanine transaminase (ALT) and aspartate transaminase (AST)) are higher in asphyxia cases during hospital stays when compared to healthy neonates and are modulated by HIE severity.

1.2.4. The pathophysiological implications of hypoxic-ischemic encephalopathy and therapeutic hypothermia on the brain

Three main consecutive phases associated with neuronal cell damage occur in neonates with HIE; the primary phase and the latent phase, which consists of the secondary and tertiary phases. A first peak of neuronal cell damage occurs during the primary phase and is caused by primary energy failure during the hypoxic event at birth. A second peak results from subsequent reoxygenation and reperfusion after birth ('reperfusion injury;' latent phase). In the primary phase, the acute hypoxia shortly before or during delivery results in primary oxygen and energy deficiency, resulting in depolarization, potassium efflux, sodium and calcium influx, adenosine triphosphate (ATP) degradation, and finally resulting in cell necrosis if the event is sufficiently severe or prolonged. Subsequently, this results in the release of excitatory neurotransmitters, with glutamate as the most relevant compound (with subsequent

N-methyl-D-aspartate (NMDA) receptor activation). However, the neonatal brain releases proportionally more inhibitory neurotransmitters, which is likely to contribute to the remarkable tolerance of the neonatal brain to hypoxia and ischemia [1,27].

During the latent phase (from 6–8 hours onwards), the secondary phase further causes cell damage and induces an inflammatory response. The subsequent apoptotic activity and the downregulation of trophic factors further contribute to neuronal cell injury. This latent phase can last for days (such as mitochondrial dysfunction and inflammatory response; secondary phase) up to weeks/years (tertiary phase) [1,27]. The primary phase is, therefore, the time window relevant to initiate TH to blunt the secondary phase, while the secondary phase is the 'main target time interval' for most ongoing drug development programs, like allopurinol, 2-iminobiotin, or erythropoietin (EPO) [28–30]. Figure 1 illustrates the phases of injury, fundamental mechanisms, and potential drug targets to improve outcomes [1].

The following sections will first discuss the impact of hypothermia on biological processes, including gene expression and protein activity (section 2). This will be followed by a discussion of the current pharmacological strategies (section 3). We hereby distinguish between research lines for add-on pharmacotherapy during TH to improve neurodevelopmental outcomes and clinical pharmacology knowledge on drug classes used in the supportive care of neonates with PA/TH. Furthermore, comparative in vivo neonatal HIE pig models for drug development (section 4) and the relevance of real-world human neonatal data to improve pharmacotherapy (section 5) are summarized. Finally, the concept for developing a PBPK hypothermia model is covered in section 6. Besides an overview of currently available parameters in PBPK tools, the essential changes to modify these tools to a PA/TH setting are also discussed.

2. Impact of hypothermia on biological processes

This section will address current knowledge regarding cellular and molecular mechanisms of essential biochemical pathways affected by hypothermia. Attention was given to pathways (potentially) interfering with the function or expression of absorption, distribution, metabolism, and excretion (ADME) of relevant and/or pharmacological targets. The subsections will describe (2.1.) the modulation of hypoxia-associated events by TH; (2.2.) the fundamental knowledge regarding the influence of hypothermia on biological systems, with attention to pharmacologically relevant genes and proteins.

Table 2. Overview of median values and the range of liver enzymes (ALT = alanine transaminase; AST = aspartate transaminase; highest value observed during hospital stay) for the different grades (1–3) of hypoxic-ischemic encephalopathy (HIE) in two published cohorts [3,14].

Enzyme	Mild HIE (grade 1)	Moderate HIE (grade 2)	Severe HIE (grade 3)	Reference
ALT	54 (8–656) not reported	90 (10–1796) 27 (8–571)	149 (3–1903) 108 (16–1195)	Muniraman et al. [14] Michniewicz et al. [3]
AST	118 (36–855) not reported	212 (18–4728) 109 (21–819)	465 (68–3150) 271 (66–3175)	Muniraman et al. [14] Michniewicz et al. [3]

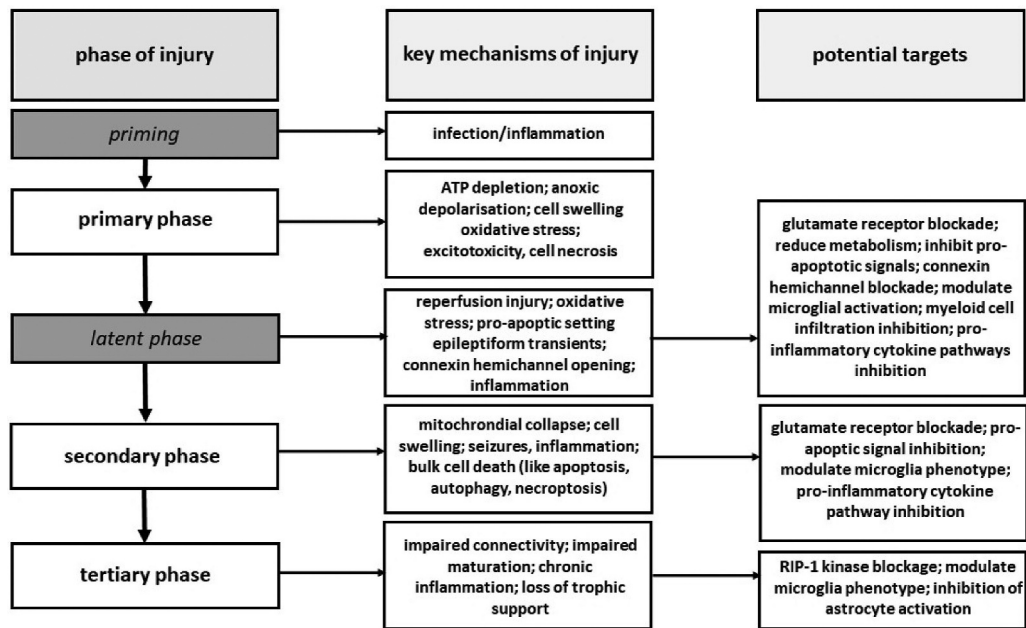


Figure 1. Flow chart on mechanisms involved in the consecutive phases of neonatal hypoxic-ischemic encephalopathy over postnatal age. Adapted from Davidson et al. and used with permission from Elsevier [1]. ATP = adenosine triphosphate; RIP-1 kinase = receptor-interacting serine/threonine-protein kinase 1.

2.1. Modulation of hypoxia-associated events by therapeutic hypothermia

Several reviews have addressed the mechanisms governing neuroprotection during hypothermia in acute neurological diseases characterized by hypoxia (such as HIE) [31–33].

Across acute and subacute ischemic phases, TH has been described to specifically modulate cellular pathways leading to (1) excitotoxicity; (2) apoptosis; (3) inflammation; (4) free radical production. Moreover, TH will modulate blood flow, slow overall cellular metabolism, and support blood-brain barrier integrity. The latter will be vital in determining CNS access of administered drugs. In the acute phase, hypothermia will cause a decrease in brain oxygen consumption and glucose metabolism of about 5%/°C. Hypothermia thus preserves ATP levels and pH, eventually preventing (excessive) lactate formation and acidosis. Notably, at least theoretically, the maintenance of ATP levels is expected to play an important role in keeping ATP-binding cassette (ABC) transporters (many are drug efflux transporters) functional. However, intrinsic protein function (independent of ATP supply) will also determine drug transporter activity at reduced temperatures. Based on *in vitro* transport experiments in cells transfected with multidrug resistance 1 (MDR1, also known as P-glycoprotein or P-gp), Jin et al. demonstrated a decrease of transport of the P-gp substrate digoxin up to 50% at 32°C compared to 37°C [34]. However, the effect was limited to a 20% reduction for the P-gp substrate quinidine. At the same time, the impact of mild hypothermia (32–35°C) on passive diffusion (via trans- or paracellular pathways) appeared negligible.

The effects of TH on the blood flow in damaged brain tissue during reperfusion are characterized by a blunted overshoot as TH prevents the release of excitatory amino acids (e.g. glutamate). This is typically explained in terms of better maintenance of ion homeostasis, thus preventing necrosis.

Three early molecular/cellular events associated with ischemia, and which are likely affected by hypothermia include (1) immediate early gene (IEG) expression; (2) cellular stress response; (3) micro-ribonucleic acid (miRNA)-mediated signaling. TH induces the expression of so-called IEGs, which are known to encode transcription factors. Kamme et al. described the induction of the IEGs in the primary (1–2 hours after reperfusion) and secondary phase (12–36 hours after reperfusion) [35]. Hypothermia accelerated the expression of some IEGs, which was interpreted as a more rapid recovery of intracellular signaling of neurons after ischemic insult. The neuroprotective effect of hypothermia via IEG modulation has been confirmed in animals [36]. Global expression profiling efforts consistently revealed remarkable temperature-induced changes in the expression of neuroprotection factors in rat brains [37].

MiRNA is a type of non-coding RNA playing a role in cellular signaling pathways and has been shown to exhibit increased expression within 2 hours of ischemic insults. At the same time, hypothermia can modulate the expression of specific miRNAs [38,39]. In recent years, several independent studies have elucidated particular miRNA profiles associated with HIE and TH, thereby opening the door to using umbilical cord blood levels of specific miRNAs as prognostic biomarkers for the early diagnosis and the grading of HIE injury [40].

Winkler et al. discovered that 48 hours after birth, miR-145-5p is significantly increased in neonates with a poor neurodevelopmental outcome after PA compared to those with a good neurodevelopmental outcome and moderate to severe HIE [41]. However, TH was a potential confounder in this study, as all neonates had already been treated with whole-body TH [41]. O'Sullivan et al. validated the decrease of 3 miRNAs (miR-374a, miR-376c, and miR-181b) as biomarkers in umbilical cord blood of neonates with PA and HIE. Three other miRNAs

(miR199a, miR-376a, and miR-410) were not validated as significantly altered in both cohorts [42].

2.2. Fundamental knowledge regarding the influence of hypothermia on biological systems

2.2.1. Mechanisms governing the impact of hypothermia on gene expression

Both hypo- and hyperthermia can induce a cellular stress response, a cold shock response (CSR) and a heat shock response (HSR), respectively. Cold reduces the rates of many enzymatic reactions, slows down diffusion, and since membrane fluidity is heavily dependent on temperature, membrane transport and signaling are also affected. Low temperatures cause alterations in the properties of the lipid bilayer and might even change the fatty acid composition [43,44]. Denaturation of proteins is not only caused by heat but can also be caused by cold. Depending on the intensity and the time of exposure, low temperatures can trigger a cellular stress response (e.g. CSR), activate the apoptotic program, or lead to necrosis [44].

The effect of TH on the cellular stress response has been studied primarily by evaluating the expression of heat shock proteins (Hsp), particularly the Hsp70 family. The Hsp70 family is upregulated during HSR and consists of anti-apoptotic proteins that help with, e.g. the folding and refolding of misfolded/aggregated proteins. An example of such a cellular stress condition is reflected in a recent study by Seo et al., which confirmed the association between Hsp70 (and Hsp47) expression and HIE severity [45]. However, inconsistent findings have been reported on whether the hypothermia-altered cellular stress response is neuroprotective [46]. For CSR, Matz et al. determined that Hsps could be neuroprotective in mice, while Kumar et al. found no neuroprotective effect of Hsp72 during hypothermia in gerbils after ischemia [47,48].

Another class of temperature-induced proteins, namely cold shock proteins (Csps), mitigate the detrimental effects of cold shock. Unlike Hsps, Csps are multifunctional RNA/DNA binding proteins [49]. Csps are highly conserved across almost all species and are characterized by at least one cold shock domain involved in RNA and DNA binding [49,50]. The varied biological activities of Csps include transcription regulation, translation, splicing, and orchestrating the exosomal RNA content [49].

Further, hypothermia can impact gene expression. In a model of hemorrhagic rats, Alam et al. observed mechanisms for changes in gene expression due to severe hypothermia (15°C) [51]. Of 23 000 genes tested, 571 were affected, with 382 being up-regulated and 187 being down-regulated (Supplementary Table A). Hypothermia generally induces the up-regulation of pro-survival pathways and causes the down-regulation of several metabolic pathways, with the most significant impact on the liver [51]. Research into other cellular stressors in rodents suggests inhibition of protein synthesis [52,53]. As hypothermia is a cellular stressor, it might also inhibit protein synthesis.

Published hypothermia studies describe a limited number of human cells (e.g. skeletal muscles, sperm, embryos, fibroblastic cells, chondrocytes, THP-1 cells, and stem cells); as

a consequence, only limited data is available about the effect of temperature on, e.g. gene expression and epigenetic regulation in humans [54–60]. Sonna et al. suggest a significant difference in response between CSR and HSR [54]. Hypothermia can alter gene expression through (1) responses of cells to systemic signals; (2) a direct effect on cells; (3) modulating the effect of other stimuli [54]. More generally, hypothermia suppresses the immune system by inducing changes in immune modulators; it increases phosphorylation and ubiquitination [52,54,61–64]. Finally, the literature does not agree on whether hypothermia reduces or increases inflammation [65–67].

2.2.2. Mechanisms governing the impact of hypothermia on protein function/activity

As mentioned above, temperature affects protein activity (e.g. enzymatic rates), and the effect on proteins depends on the temperature preference of the organism.

The first possible effect of temperature is on protein folding during synthesis. Indeed, even small temperature changes can impact the protein conformation, while more extreme temperature changes (species- and protein-dependent) can affect protein stability. Heat shock proteins can stabilize proteins at high and low temperatures by stabilizing unfolding or refolding misfolded/aggregated proteins (e.g. by cold denaturation) [68]. However, current data do not suggest Hsp upregulation during CSR (section 2.2.1.), and as such, only existing Hsps can help counteract problems with folding. Folded proteins are generally highly sensitive to environmental conditions such as temperature, pH, and osmolyte concentration. Even relatively small changes in these environmental conditions can cause protein denaturation, leading to loss of protein function [69]. The temperature sensitivity of proteins is due to their low net free energies of stabilization, which allows for structural flexibility [70]. This structural flexibility is required to facilitate catalytic reaction by allowing necessary conformational changes [69]. Different proteins undergo cold denaturation at different temperatures, and these temperatures are also dependent on other environmental conditions. Therefore, it is crucial to study the behavior of biomolecules as a function of the solution temperature to assess their biological activity and function [71]. Additionally, Almeida et al. suggest that the optimal temperature range of a protein is not an intrinsic property as is reported but instead a direct consequence of the enzymatic degradation at specific experimental conditions [72]. The denaturation rate is also influenced when the experimental conditions change, including enzyme concentration and assay time. Therefore, a protein's optimal temperature range should not be considered an intrinsic property [72,73]. Almeida et al. highlighted that measuring the heat denaturation rates is especially important at temperatures close to or above an enzyme's melting transition (T_m) [72]. However, the actual temperature at which these effects do or do not occur varies between enzymes.

Secondly, as enzymes are energy-dependent processes, they are temperature-sensitive due to the association between heat capacity and enzyme catalysis changes in relation to different temperatures. The kinetic energy of protein substrates increases when the temperature is raised; when the

kinetic energy is higher, the substrates have a higher chance of colliding and overcoming the activation energy. Generally, a reaction rate doubles with each temperature increase of 10°C, with a linear rate between a protein's degradation temperatures [74]. At certain temperatures, there is little to no temperature-driven protein degradation. However, at other temperatures, the protein denaturation (which causes a decrease in enzyme activity) can be overcome by the temperature-driven increased reaction rate [72]. The temperature range in which a protein does not denature from heat or cold is organism and protein-dependent [74].

Cytochrome P450s (CYPs), for example, are important enzymes for the metabolism of xenobiotics and medicines, and their activity is reduced during TH [75]. CYPs can be divided into three classes, depending on their preferred ligand path [76]. Preclinical studies also suggest that CYPs are affected differently by a change in temperature as their active sites have different volumes [77]. CYPs with large active sites/binding pockets are CYP2C5, CYP2C8, CYP2C9, and CYP3A4. CYPs with medium active sites are CYP2B4 and CYP2D6, and CYP2A6 has a small active site. Therapeutic hypothermia affects CYPs with a narrow active site more than CYPs with a large(r) active site [76,78].

Thirdly, ligand binding is highly temperature sensitive as well due to its reliance on three events which can easily be disrupted (1) the geometry of the binding site in the protein; (2) the noncovalent bond establishment between ligand and binding site; (3) protein conformation change during ligand binding (applicable to many enzymes) [79]. The geometry of the protein binding site depends on the stability of the protein at a certain temperature. Proteins are only slightly stable at physiological temperatures. When the temperature increases, a subtle structural change occurs in the protein, which nevertheless has a physiologically important impact

on the enzyme conformation [70]. Proteins need to be able to retain their 3D structure to maintain functionality. Yet high flexibility is required due to a need for rapid, precise, and reversible changes in protein assembly and conformation [70,79]. Regarding hypothermia, a conformational change in the binding site of an enzyme can reduce the enzyme activity. For CYPs, the temperature dependence might not be equal among the protein family. There are several differences in structures, both in the preferred paths of the ligands and the volume of the active site of these proteins [76]. Otyepka et al. and Cojocararu et al. have published models of CYPs and their structural features, such as channels and active sites [76,80]. However, current data suggest that drug-protein binding is not significantly altered during mild to moderate hypothermia (30°C–35°C), unlike drug elimination [77].

Table 3 presents half-lives ($t_{1/2}$) of important human enzymes and a clear variability in the estimated $t_{1/2}$ of specific CYPs is observed, depending on which method was used to assess the $t_{1/2}$ [97]. This difference in $t_{1/2}$ might mean that some proteins are more affected by a change in temperature than others. For example, paused protein synthesis at lower temperatures might have a more significant impact on proteins with a shorter $t_{1/2}$.

Neonatal ontogeny (not further discussed in this review) is also relevant, as neonates have lower expression levels of CYPs and have several different isoforms than adults, such as CYP3A7 instead of CYP3A4. As discussed previously, neonates undergo rapid physiological changes in early life. This makes studying liver metabolism and the impact of PA/TH on liver metabolism challenging [98].

Values in brackets show the range, and \pm shows the standard deviation. * $t_{1/2} = 0.693/k_{deg}$. In vitro methods: (1) radiolabeling of the enzyme ('Pulse-chase' method); (2) degradation of enzyme in cultured hepatocytes or liver slices. In vivo

Table 3. Half-lives ($t_{1/2}$) of several human cytochrome P450 (CYP) enzymes and the method used to calculate their half-life. Table adapted from Yang et al. [97].

CYP	$t_{1/2}$ (hours)	Method $t_{1/2}$ estimation	Reference
CYP1A2	51	In vitro (1)	Diaz et al. [81]
	36 (8–58)	In vitro (2)	Renwick et al. [82]
	39 (27–54)	In vivo (3)	Faber et al. [83]
	105	In vivo (5)	Magnusson et al. [84]
	26 (19–37)	In vitro (2)	Renwick et al. [82]
CYP2A6	32	In vitro (2)	
CYP2B6	23 (8–41)	In vitro (2)	
CYP2C8	104	In vitro (2)	
CYP2C9	26 (7–50)	In vitro (2)	
CYP2C19	70	In vitro (2)	Renwick et al. [82]
CYPD26	51	In vivo (4)	Liston et al. [85]; Venkatakrisnan et al. [86]
	27 (7–40)	In vitro (2)	Renwick et al. [82]
CYP2E1	60	In vivo (3)	Lucas et al. [87]
	50 \pm 19	In vivo (4)	Emery et al. [88]
	44	In vitro (1)	Pichard et al. [89]
CYP3A4	79	In vitro (2)	Renwick et al. [82]
	96 \pm 38 (53–154)	In vivo (5)	Levy et al. [90]
	72 (20–146)	In vivo (3)	Lai et al. [91]
	(85–806)	In vivo (3)	Warren et al. [92]
	(36–50)	In vivo (3)	Fromm et al. [93]
	94 (62–205)	In vivo (5)	Rostami-Hodjegan et al. [94]
	70	In vivo (5)	Magnusson et al. [84]
	85 \pm 61	In vivo (5)	Hsu et al. [95]
	140 (48–284)	In vivo (5)	von Bahr et al. [96]
	36 (15–70)	In vitro (2)	Renwick et al. [82]
CYP3A5			

methods: (3) recovery of enzyme activity after enzyme induction; (4) recovery of enzyme activity after mechanism-based inhibition (MBI); (5) PK modeling of auto-induction.

Lastly, close to the physiological temperature range, biological membranes also have a T_m [99]. Transmembrane proteins such as CYPs might therefore be affected due to differences in the membrane at different temperatures. For example, CYP3A4 interacts with the membrane through specific lipid-protein interactions. This binding to the membrane significantly impacts the dynamic and structure of its globular domain [100]. Therefore, a lower temperature might also influence protein activity due to the possible closing of access sites or increased rigidity and slower conformation changes. However, studies into the homeoviscous adaptation of the membrane at different temperatures suggest a total or partially self-correcting system that changes the membrane lipid composition. The composition of a membrane changes to become more rigid when exposed to higher temperatures and more flexible at lower temperatures to maintain membrane functions such as action potential [101–103]. However, the homeoviscous adaptation does not seem possible at lower and higher temperatures for all cells [104].

3. Pharmacological strategies for asphyxiated neonates receiving therapeutic hypothermia

3.1. Disease mechanisms and pharmacological targets

Despite the positive effect of TH, PA still accounts for a relevant portion of neonatal mortality and morbidity in (near)term neonates. Therefore, interventions to further improve outcomes are urgently needed. From a drug development aspect, PA qualifies as an orphan disease. The Committee for Orphan Medicinal Products estimates that

about 15 000 neonates (0.3/10 000 EU inhabitants) are affected annually.

The specific setting to conduct clinical trials in this population makes PA/TH studies difficult. Active research is currently mainly driven by repurposing approaches like EPO, allopurinol, 2-aminobiotin, cannabidiol, melatonin, magnesium sulfate, sildenafil, vitamin C, E4, RLS-0071, or stem cells. The Helping to End Addiction Long-term Initiative (HEAL) consortium recently reported that EPO administration to neonates undergoing TH for HIE did not result in a lower risk of death or neurodevelopmental impairment than placebo and was associated with a higher rate of serious adverse events [30]. Based on a search within the European Medicines Agency databank on orphan designations (April 6, 2023), we provide an overview of the current programs in the European Union (Table 4).

In the reviewed study protocols, phase 3 studies showed a consistency in the 'intact' survival without neurodevelopmental impairment as a primary outcome variable for neonates with PA/TH between 18–24 months. In contrast, short-term outcome variables showed more heterogeneity. Short-term outcome variables are biomarkers expected to be predictive for this primary outcome and useful in preceding phase 2 studies. Biomarkers include advanced EEG analyses over time throughout or after TH, Magnetic Resonance Imaging (MRI) in early neonatal life (first week) with scoring systems, or biochemical markers in blood or cerebrospinal fluid with neuron-specific enolase or S100B as the most commonly used brain-specific proteins [105,106]. Besides EPO, allopurinol also progressed toward a clinical phase 3 randomized controlled trial (RCT; Albino trial [28]). Victor et al. summarized the status of the add-on compounds for neuroprotection in neonatal HIE, which are currently under investigation [107]. Overall, therapies that further improve outcomes are highly needed and might have neuroprotective

Table 4. Overview of retrieved orphan designation decision on Perinatal asphyxia (PA)/hypoxic-ischemic encephalopathy (HIE) from the Community Register of orphan medicinal products.

Orphan Designation Decision	Company	Number	Disease
2-aminobiotin	Neurophyxia B.V., The Netherlands	EU/3/09/701	PA
Allopurinol sodium	ACE Pharm, The Netherlands	EU/3/15/1493	PA
	Pharmathen S.A., Greece	EU/3/12/1076	PA
Argon	Air Liquide Santé, France	EU/3/18/2031	PA
Autologous mononuclear human cord blood cells	BrainRepair, Germany	EU/3/16/1743	HIE
Cannabidiol	GW Pharma Ltd, United Kingdom	EU/3/15/1520	PA
Estretol (E4)	Mithra, Belgium	EU/3/17/1865	HIE
Melatonin	Therapicon, Italy	EU/3/18/2127	PA
	ESPL Regulatory Consulting Limited, United Kingdom	EU/3/18/1996	HIE
	Chiesi Farmaceutici S.p.A., Italy	EU/3/23/2757	PA
PIC1-dPEG24 (Ile-Ala-Leu-Ile-Leu-Glu-Pro-Ile-Cys-Cys-Gln-Glu-Arg-Ala-Ala-(discrete-polyethylene glycol)24)	Clinipace, Germany	EU/3/12/978	PA
VH-N439	Vect-Horus, France	EU/3/20/2283	HIE
Xenon	Vect-Horus, France	EU/3/16/1824	PA
	Neuroprotexon Ltd, United Kingdom	EU/3/15/1483	PA

opportunities in low- and middle-income settings without access to TH.

3.2. Impact of therapeutic hypothermia on supportive pharmacotherapy

The modulation of the pathophysiology by TH in asphyxiated neonates is relevant for dosing of the commonly used drugs (antibiotics, analgesodatives, anti-epileptics, inotropes). A review by Lutz et al. analyzed 36 studies using 15 compounds. The impact of PA/TH on drug clearance was dependent on the route of elimination and was most pronounced for renal elimination during TH (no difference for phenobarbital, –25% for 1-hydroxymidazolam, –24% for lidocaine; –21% to –47% for morphine) [108]. Mannitol and amikacin clearance (–40% to –60%) were estimated in the first 24–48 hours of life [109,110]. Based on gentamicin disposition (sampling days 2–5) in PA/TH cases, clearance was initially decreased (25–35%), with a subsequent increase observed after normothermia (96–120 hours) [111]. Consequently, prolonging the dosing interval resulted in better target attainment for amikacin and gentamicin, characterized by renal elimination [110,112]. Population PK model-based dosing for neonates with PA receiving TH has been published for some commonly used drugs, like amikacin [110]. Nevertheless, therapeutic drug monitoring of compounds with a small therapeutic window (e.g. aminoglycosides) is still recommended in these critically ill neonates to obtain safe and effective therapy.

For drugs undergoing metabolic elimination, the decreased clearance may reflect overall lower enzymatic activity with decreasing temperature or reduced hepatic blood flow. However, the underlying pathophysiological mechanisms still need further exploration and research [113]. Besides opioids, alpha-2-adrenergic receptor agonists like dexmedetomidine are increasingly used for sedation and their opioid-sparing effect in neonates. The drug undergoes hydroxylation (mainly by CYP2A6) and direct N-glucuronidation in adults [114]. The efficacy and short-term safety of dexmedetomidine were described in 19 neonates receiving TH [115]. Despite an association with lower heart rate, Elliott et al. concluded that the compound can be used successfully in neonates with HIE receiving TH [116]. In 2020, a phase 1 study was published in 7 neonates with PA/TH receiving dexmedetomidine [117]. Clearance was comparable to slightly lower, and the volume of distribution was larger compared to a non-HIE, non-TH neonatal cohort but not statistically significant [117]. Compared to fentanyl, dexmedetomidine appears to provide comparable control of agitation, reducing the need for additional sedatives, shorter time to extubation, and discontinuation of sedatives [118]. Although the compounds seem promising, further research on optimal analgesodative pharmacotherapy in the setting of TH is requested and ongoing [119,120]. Besides clinical studies, preclinical research is paramount to gaining insights into this field. The following section discusses available *in vivo* pig models and methodological challenges.

4. Comparative *in vivo* neonatal hypoxic-ischemic encephalopathy pig models for drug development

4.1. Currently available *in vivo* pig asphyxia models

Much of the current understanding of PA in humans has been derived from animal studies, and several species have been used for this purpose [121]. Among those animal models, the piglet asphyxia model is a well-established and relevant model for studying cerebral blood flow and cerebral metabolism [122]. Term neonatal piglets also show a development similar to (near)term human neonates (36 to 38 weeks old) with comparable body systems and size at birth (1.5–2 kg). This represents an advantage over other species, such as rodents and even dogs and non-human primates, since this larger body size at birth allows for sampling at early stages without hampering the animal's physiology and facilitates the adaptation of neonatal intensive care unit equipment for pigs. Furthermore, the hypoxic insult and the resulting hemodynamic derangements can be managed in the pig model, in contrast to smaller animal models where the adaptation of monitoring equipment is not feasible [121]. As the piglet asphyxia model is well-characterized, this opens opportunities for pharmacological interventions and procedures applied in a clinical setting, such as TH. The following section will discuss the procedures for establishing the PA pig model.

Fetal and early postnatal (<24 hours after birth) procedures to induce PA in piglets are reported [123]. Fetal procedures offer the advantage that the piglets have not been exposed yet to the external environment and may better mimic PA. However, the induced asphyxia by umbilical cord clamping appears to be not as severe as expected in human neonates, and the outcome is also very variable in piglets [123]. Therefore, early postnatal procedures (<24 hours after birth) are more commonly used, and several protocols are reported. Cheung et al. described an experimental protocol of severe hypoxemia induced via normocapnic alveolar hypoxia [124]. The neonatal piglets are surgically instrumented in a non-survival experiment, which allows the establishment of mechanical ventilation, arterial and central venous access, and the placement of catheters and flow probes to continuously monitor intra-vascular pressure and blood flow across different arteries. This protocol achieves severe hypoxemia by decreasing the inspired oxygen concentration to 10–15% and increasing the inhaled nitrogen gas concentration for two hours, aiming for 30–40% arterial oxygen saturation. This degree of hypoxemia produces clinical asphyxia, with severe metabolic acidosis, systemic hypotension, and cardiogenic shock with hypoperfusion of the vital organs [124]. Others have described a similar piglet model for PA via whole-body hypoxia-asphyxia [125]. This was induced by decreasing the inhaled oxygen to 10% for 45 minutes and the occlusion of the endotracheal tube for seven minutes [125]. Global hypoxia-ischemia was established by Kyng et al., combining ischemia through hypotension with low inspiratory oxygen fraction (FiO₂) via nitrogen gas and a flat trace amplitude-integrated electroencephalogram (aEEG), indicative of cerebral hypoxia [126]. Survival was improved by adjusting oxygenation according to the aEEG response and arterial blood pressure. A different approach for inducing transient hypoxia-ischemia was established by remote occlusion of both common

carotid arteries, using inflatable vascular occluders at the level of the fourth cervical vertebra, with a low FiO_2 of 9% [127]. Recently, a neonatal Göttingen minipig model for dose precision in PA has been developed, as this is the commonly used pig strain in drug development by pharmaceutical companies [128]. This provides opportunities for the development of new drugs in PA.

4.2. Further methodological considerations in pig asphyxia models

When developing a piglet asphyxia model, the choice of medication, the method of controlling the insult duration and severity, post-insult resuscitation and care, and outcome evaluation should be carefully considered and monitored [126]. In expected clinical asphyxia, systemic hypotension and hypoperfusion may also occur in the piglet. In hypotension, dobutamine, norepinephrine, and crystalloids can be used to maintain mean arterial blood pressure (MAP) above 45 mmHg, the lower limit of autoregulation reported in neonatal swine [125]. A MAP above 40 mmHg can be sustained by administering bolus infusions of 0.9% saline and continuous infusions of dopamine, dobutamine, adrenaline, or noradrenaline [127]. Neuromuscular blocking agents, such as vecuronium (0.2 mg/kg/h), are often used to prevent shivering and to provide consistent anesthesia [125]. Regarding the duration of the hypoxic insult, literature data is variable, ranging from 12.5 minutes to 2 hours, as this duration depends on the method used (see section 4.1) [124,126]. The invasiveness of the procedures before the insult (which may result in pain, hypothermia, and potential blood loss) and the piglets' age and weight may also impact this duration. Still, the variable duration of the insult in the different protocols is acceptable, as magnetic resonance spectroscopy analysis, EEG, brain histology, blood pressure, and/or blood gas analysis are used to assess the insult [124,126,127,129].

Nevertheless, there are also some limitations of the swine model for PA. The side effects of anesthesia and surgical trauma stress limit this model regarding survival length and the number of invasive procedures performed. The survival length may be increased with an adequate stabilizing period, appropriate use of anesthetic medications, and refined surgical techniques [124]. Regarding the latter, skilled personnel with experience in surgery and neonatal piglet anesthesia are essential for these experiments. Other limitations include the logistical aspects of getting neonatal piglets in the research facility when no (pregnant) sows are housed on-site. As transporting neonatal piglets is prohibited, transporting pregnant sows to the facility will be needed. This results in many piglets that must be instrumented quickly (<24 hours) when the sow delivers. Using older piglets (two or more days of age) risks weakening the clinical relevance of the piglet model, as the model should mimic human PA [130].

5. Benefits and challenges of real-world neonatal data: general reflections

5.1. Closing the gaps of special neonatal populations

Real-world data (RWD) obtained from clinical care can be used in research to generate real-world evidence [131]. Sources of RWD are electronic health records, patient registries, databases, or patient-generated data from mobile or wearable

devices. These data can be used to close gaps in PK and pharmacodynamics (PD) in special populations, including the safety, short-term, and long-term effects of pharmacotherapy in neonates and children [132]. In addition, RWD can be applied to improve current neonatal dosing recommendations or study designs and to support the approval of drugs for neonatal use by regulatory authorities. Recent examples include a proof-of-concept study to provide real-world (PK/PD) evidence for azlocillin in neonates and optimized neonatal dosing for teicoplanin based on PK modeling and simulation using RWD [133,134].

Physiologically-based PK model development can benefit from real-world datasets covering the physiology of the population of interest. Neonatal physiology drastically changes during the first weeks after birth. The predictive performance of PBPK models will increase if the neonatal population's time-dependent (PNA-dependent) physiology is well captured in the model [132]. As Zhou et al. highlighted the predictive performance of PBPK models for drugs undergoing metabolism by CYPs and renal clearance is reasonable in children one month and older [135,136]. For neonates, overall PBPK predictive performance needs improvement. Our research group currently works on a PBPK platform to predict drug disposition in asphyxiated neonates treated with TH (I-PREDICT project, Innovative physiology-based pharmacokinetic model to predict drug exposure in neonates undergoing cooling therapy, Figure 2) [113,128]. Real-world data of sCr, as a biomarker for the renal function of neonates with PA/TH, were pooled to generate sCr PNA profiles over time. Overall, sCr patterns over PNA were significantly higher in neonates with PA/TH than in controls [11]. These data can reflect renal physiology and be used to estimate GFR in a PA PBPK platform to improve the prediction of renally cleared drug exposure.

5.2. Challenges of real-world data research initiatives

Overall, the awareness and acceptance of real-world evidence are increasing among stakeholders (e.g. industry, regulators, clinicians, and patients). Real-world data has a promising role in improving pharmacotherapy. However, some challenges must be considered; compared to data collected during RCTs, RWD is usually unstructured and variable, requiring more advanced analytical methods/modeling and statistics for data analysis. Furthermore, concerns about the quality of the obtained data, privacy, and confidentiality may arise [137]. Patients' and parents' perspectives are hereby of relevance. Recently, Sawtell et al. reported on the view of parents, adults born preterm, and professionals on the linkage of routinely collected RWD of preterm babies [138]. It was considered a feasible and cost-efficient approach to gain insights into long-term outcomes and evaluate interventions in the neonatal period. The stakeholders all support data linkage, especially with opt-out consent. A minority, which is initially not supportive, becomes supportive if additional information is provided [138]. Overall, we consider RWD an essential data source for developing PBPK models. Section 6 describes some consecutive steps in constructing a PBPK model for the neonatal population with PA/TH.

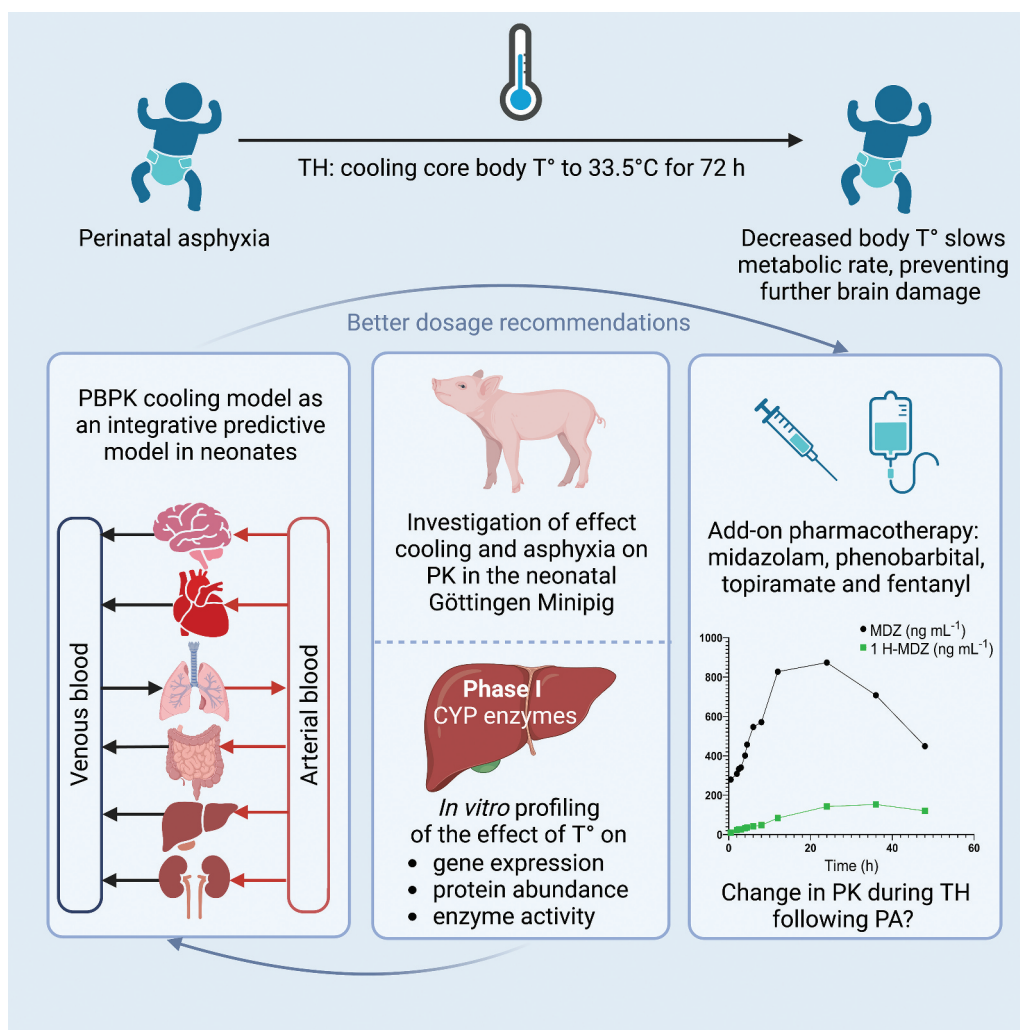


Figure 2. Visual presentation of the I-PREDICT project, aiming to improve dosage recommendation for drugs in neonates with perinatal asphyxia treated with therapeutic hypothermia by developing a physiologically-based pharmacokinetic model, combining preclinical (in vitro and animal data) and clinical data. Pharmacokinetic predictions of four model drugs (midazolam, fentanyl, phenobarbital, and topiramate) are investigated. Created with BioRender.com.

1-HMDZ = 1-hydroxymidazolam; CYP = cytochrome P450; MDZ = midazolam; PA = perinatal asphyxia; PBPK = physiologically-based pharmacokinetics; PK = pharmacokinetic; T° = temperature; TH = therapeutic hypothermia.

6. Physiologically-based pharmacokinetics (PBPK) as an integrative predictive model

6.1. Concept for development of a PBPK hypothermia model (I-PREDICT project)

As mentioned above, PBPK models are used for bottom-up (or middle-out) prediction of the PK of drugs and their metabolites in a predefined population [139,140]. Therefore, PBPK models are established with drug-dependent and system-dependent parameters on the relevant (patho)physiology. Drug-specific data include (1) physicochemical characteristics of the drug and its metabolites (e.g. lipophilicity, ionization behavior, and aqueous solubility); (2) parameters describing drug disposition pathways at the cellular and molecular level (e.g. membrane permeability, hepatic enzyme-mediated clearance, and plasma protein binding). Input data on the physiology includes organ weights, composition (water/lipids/proteins), subcompartmentalization (intracellular/interstitial/vascular), blood perfusion rates, and fluid exchange rates

between vascular and interstitial/intracellular volumes. In addition, information on the specific pathology (e.g. diabetes, asphyxia, renal impairment) and/or treatment conditions can also be specified at the population level. Importantly, inter-individual differences in these (patho)physiological data can also be included, allowing us to account for real-world inter-subject differences. Rapid and significant changes in ontogeny make PBPK models for the neonatal population challenging. Data on the impact of PA/TH (see previous sections) can then be integrated into such a PBPK model. Specifically for the neonatal population, adequate ‘scaling’ of physiological variables is crucial and essentially requires knowledge about the ontogeny of physiology and drug-metabolizing enzymes in the human neonatal population. This type of ‘rational’ extrapolation of adult to pediatric PBPK models has previously been illustrated for fentanyl and lorazepam [141,142]. The PBPK modeling study with fentanyl also covers the approach to be taken when multiple compounds are co-administered, i.e. when drug-drug interactions apply.

Recently, we reported on ongoing efforts to exploit PBPK models for predicting the impact of PA ('disease') and/or TH ('treatment') on the PK of model drugs in this vulnerable population [113]. As clinical research in this setting is hampered by ethical, statistical, and technical challenges, the I-PREDICT project proposes a multidisciplinary approach to generate the input data required for establishing a PBPK framework for this setting (Figure 2). In vivo studies in Göttingen minipigs with relevant model drugs (phenobarbital, midazolam, fentanyl, and topiramate) have been conducted to disentangle the impact of PA/TH on PK in a controlled in vivo setting (Figure 2) [128]. In addition, in vitro experiments are ongoing to study the impact of reduced temperatures on enzyme kinetics, protein abundance and gene expression in the liver. The combined insights obtained with these non-clinical approaches will be incorporated into a conceptual PBPK framework for bottom-up prediction of in vivo PK under PA/TH conditions.

The following section will summarize the physiological parameters currently used to build human neonatal virtual subjects for PBPK modeling. Next, parameters likely influenced by temperature will be proposed, i.e. to inform a strategy for generating PBPK models for neonates with PA/TH.

6.2. Physiological parameters in available PBPK tools

The open-source PBPK platform PK-Sim and its available database were used to generate a structured overview of the anatomical and physiological parameters informing PBPK model building (version 10 – build 257). As described in the documentation of the Open systems Pharmacology platform PK-Sim, the 'Individual' building block specifies a virtual subject's anatomical and physiological properties. This can be human or animal species [143]. In the case of animal species, age-dependent scaling is performed proportionally for all organs in a linear manner based on total body weight. Human virtual subjects are composed of a literature database describing the impact of age, body weight, body mass index, and sex on anatomical and physiological features such as organ weights and blood perfusion rates. The database is used to build virtual subjects for starting values of age, weight, and height. This is achieved by modifying organ volumes, volume fractions (database yields global scale factor), and other parameters (e.g. hematocrit) for which (population) distribution data are available. For the human species, PK-Sim offers the choice between 8 different subpopulations (Asian, Japanese, Black, White and Mexican American, European, preterm, and pregnant populations). Male and female human growth models for the preterm population specify body weight in the function of PNA for gestational ages between 24 and 37 weeks. These preterm growth models merge with the growth curves of term infants at two years of PNA.

Of note, capillary surface areas (SA) are estimated based on two relatively simple approaches (1) based on the fraction of vascular space, the organ volume, and a proportionality constant, assuming similar vascular tree morphologies across organs; (2) based on organ blood flow. Supplementary Table B provides a structured summary of the most important parameters used to build PBPK models for male neonates six hours

after birth, i.e. the 'starting' population in the context of PA/TH but excluding PA/TH itself. These data were obtained from the PK-Sim platform (version 10) and MoBi, specifying a male human subject 'European (ICRP, 2002)' and setting the age to 0.25 days (yielding a neonate of 3.50 kg, 51 cm). Reference data for this population have been reported previously [144].

6.3. Pathophysiological changes to be explored during the development of PBPK models for perinatal asphyxia and therapeutic hypothermia

The underlined parameters in Supplementary Table B can be considered for modification when building a PBPK model for the neonatal PA/TH population. Such parameters include flow rates, GFR, and organ composition. Sensitivity analyses may inform prioritization for collecting pathophysiological data currently lacking for this specific condition/treatment. In addition, as described in more detail by Smits et al., the Göttingen minipig-based in vivo animal model for PA/TH is expected to further inform on non-maturational covariates [113,128]. Translational integration of these animal data into the human PBPK model will benefit from establishing a minipig PBPK model. Such a strategy has for instance already been applied to predict the PK of oseltamivir in a pediatric population [145]. A particular challenge associated with this is the much more limited physiological database for minipigs compared to humans. On the other hand, the availability of in vivo PK data at higher resolution, is expected to at least support the development and evaluation of mechanistic hypotheses regarding the (separate) impact of PA and TH on PK. When building the human PBPK model, information obtained from the PA/TH animal model will be evaluated in combination with real world human data (e.g. renal function, albumin).

Importantly, the PA/TH PBPK modeling framework should also account for changes in intrinsic ADME properties related to disease and reduced temperature. In vitro studies exploring the impact of temperature on enzyme activity and gene expression of enzymes are currently ongoing [146,147]. Translational integration of these in vitro data in the PBPK models will need to consider adequate scaling factors for consistent in vitro to in vivo extrapolation. Quantitative proteomics measurements on the in vitro systems as well as on in vivo (liver) samples are ongoing and expected to support this translation endeavor. As also proposed in Smits et al., exploration (and possible determination) of biomarkers (e.g. 4 β -hydroxycholesterol/6 β -hydroxycortisol as surrogate for CYP3A activity) represents another opportunity to establish a representative PA/TH population in terms of hepatic protein abundance [113]. In summary, carefully integrating the impact of temperature and/or PA on the physiological parameters and temperature-dependent intrinsic clearance is expected to enable a successful PBPK-based prediction strategy. A detailed workflow proposal has been described by Smits et al. [113].

7. Discussion

Since the introduction of TH as a standard therapy, knowledge of PK in this patient population is exponentially increasing.

However, knowledge gaps remain, e.g. underlying mechanisms of the impact of PA/TH on neonatal drug metabolism. To make progress in this research field, animal models are relevant since they allow uncoupling of the ‘phenotype = asphyxia +TH.’ In this preclinical space, the rodent (Rici-Vanuucci carotid ligation model) and the neonatal pig model are most commonly used [121,148]. However, we should be aware of the limitations of approaches in simulating clinical hypoxia. For example, the rodent model does not have multi-organ failure compared to the pig model, so interspecies extrapolations need additional attention (section 4). As mentioned in Section 6.3, building a PBPK model for the Göttingen minipig with PA and/or TH is expected to be instrumental in this respect.

The 2-iminobiotin PK study by Favié et al. illustrates that extrapolation from the rat is insufficient in integrating the pathophysiology of the asphyxiated neonate undergoing TH (0.08 instead of the predicted 0.16 mg/kg every six hours results in targeted exposure). Therefore, this extrapolation may result in the overexposure of 2-iminobiotin in the subsequent phase 2 studies [29]. Physiologically-based PK models are likely an effective tool to better integrate these different pieces of knowledge [113]. It also allows learning from pre-clinical data and integrating preclinical and clinical observations to improve the predictive performance of PBPK models. Victor et al. concluded that in the search for new neuroprotective therapies in neonatal HIE and to progress these possible new therapies toward the clinical trial level, optimal preclinical (animal/juvenile) models, as well as industry support and dose-ranging studies with PK/PD modeling, are needed [107]. Finally, optimal clinical trials for this orphan condition require well-designed protocols and multicenter collaboration to reach a sufficient sample size, avoid harmful exposures, and abandon therapies [107].

8. Conclusion

This review summarizes the current knowledge of pathophysiology and the cellular and molecular mechanisms of the biochemical pathways affected by PA and TH. It identifies several knowledge gaps in this field. First, the available asphyxia pig models and their methodological considerations were covered since animal models are valuable tools for neonatal and pediatric drug development. To enable precision dosing in neonates with PA/TH, we proposed a multidisciplinary and translational PBPK approach using clinical and preclinical observations for model development and evaluation. Finally, the applicability and challenges in developing such a PBPK model and recommendations for future directions were provided.

9. Expert opinion

Although this review provides a detailed overview of currently available data in both the preclinical and clinical fields of relevance for PBPK development in the setting of PA/TH, we want to share some reflections for further research. First, from a molecular point of view, we found no information regarding the effect of Hsp expression on ADME properties. However,

there is evidence that miRNA expression affects ADME-relevant protein expression (e.g. Rieger et al. [149]). There is limited or no information on IEG expression and ADME-relevant protein expression and function. Additionally, fewer data and published studies are available regarding epigenetics in humans and other animals [150]. This area of expertise is still largely unexplored and might be interesting to investigate further. Second, although this review mainly focuses on PK, data on PD in this population remains limited. This is not only the case in the setting of PA/TH, in general the collection of high-quality neonatal PD data is challenging. Advances in bedside neuromonitoring, neuroimaging tools, and laboratory biomarkers are promising to close this gap and link PK and PD observations [105,106]. Third, as neonatal clinical care evolved during the last decade, the clinical setting of TH at present differs from the setting during the first years after TH introduction. The ‘do’s’ and ‘don’ts’ for managing HIE during TH, as proposed in a recent care practice bundle by Wintermark et al., illustrate how guidance can be provided to healthcare providers in this setting [151]. However, the effect of using such a care bundle on outcomes needs to be demonstrated in the future. At present, the application of less invasive respiratory support, tailored analgosedation resulting in more reactive patients, involvement of parents/family in daily care, dedicated nurses experienced in using the bedside equipment needed in this setting (e.g. cooling device, neuromonitoring expertise), palliative care, and specialized multidisciplinary follow-up after discharge are currently implemented in many centers. Researchers need to be aware of this evolution in clinical care and, consequently, the change of target population over time to reliably compare study results, including PK/PD findings.

A disease-specific PBPK model for asphyxiated newborns undergoing TH is currently unavailable but would be instrumental in streamlining drug dose and exposure. However, this is just one of the many challenges. As part of the PBPK framework under construction in the I-PREDICT project, the utility of promising biomarkers for the prediction of (hepatic) drug disposition is explored [113]. Biomarker quantification (including 4 β -hydroxycholesterol and 6 β -hydroxycortisol as surrogate markers for CYP3A4 activity) in Göttingen minipigs and humans will be used to build additional confidence in bridging findings in Göttingen minipigs to humans. Moreover, the collection of such ADME-relevant biomarkers carries the promise to contribute to novel data regarding interindividual variability, for instance in metabolic activity in a very specific pediatric population. Subsequent refinement of the neonatal framework should also consider the impact of drug-drug interactions as well as pharmacogenetics as additional covariates [152,153]. However, the influence of a rapidly evolving ontogeny and associated developmental changes may overwhelm some of these other non-maturational covariates with a high impact in the adult population.

The specific setting to conduct clinical trials in this population (an orphan disease diagnosed very shortly after birth, unanticipated and no clustered care at diagnosis, the very short timelines to complete the consent process, uncertainties on relevant biomarkers for severity and outcome, uncertainties on how to model target exposure, the

challenges, and limitations of the ‘commonly used’ RCTs), make these studies extremely challenging.

Consequently, to facilitate ongoing research, stimulate new drug development programs, and improve the impact of these activities, multistakeholder collaboration is warranted to streamline study flow and improve study design. This will bring together academia, clinical research networks, patients/parents, industry, and authorities, as has been recently reported for other settings like pediatric inflammatory bowel disease or pediatric oncology [154,155].

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ORCID

Karen Leys  <http://orcid.org/0000-0002-4931-6562>
 Marina-Stefania Stroe  <http://orcid.org/0000-0003-4690-9458>
 Pieter Annaert  <http://orcid.org/0000-0003-3525-7351>
 Steven Van Cruichten  <http://orcid.org/0000-0002-3144-1840>
 Sebastien Carpentier  <http://orcid.org/0000-0002-7389-6405>
 Karel Allegaert  <http://orcid.org/0000-0001-9921-5105>
 Anne Smits  <http://orcid.org/0000-0002-0710-6698>

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