Pharmacokinetics of Melatonin As A Neuroprotectant In Preterm Infants

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For my husband Rakesh and my son Aaditya

Abstract

Background and purpose: Advances in perinatal care have increased survival rates of infants but long-term neurodisability and social consequences have remained unchanged over the last decade. Preterm infants are deprived of the normal intrauterine exposure to maternal melatonin and experimental studies suggest that melatonin has a neuroprotective effect on cerebral white matter injury. However, pharmacokinetic data on melatonin in preterm infants are lacking, which hinders potential therapeutic trials. The aims of this study were to determine the pharmacokinetics of melatonin in the relevant preterm population, assess the tolerability of melatonin and determine a dose regime that would allow replication of adult melatonin levels.

Methods: In a multi-centre, single dose escalation/de-escalation, open label study in preterm infants less than 31 weeks gestation, melatonin was administered to eighteen infants in doses ranging from 0.04-0.6 micrograms/kilograms, over 0.5-6 hours. Pharmacokinetic profiles were analysed individually and by population methods.

Results: Baseline melatonin was largely undetectable. At the highest and lowest doses half-life could not be calculated due to blood concentrations not reaching a consistent steady state, but infants receiving melatonin at 0.1 micrograms/kilogram/hour for 2 hours showed a median half-life of 15.82 hours and median maximum plasma concentration of 203.3 picograms/millilitre. Population pharmacokinetic analysis showed that clearance was 0.045 litre/hour, volume of

distribution 1.098 litres and elimination half-life 16.91 hours with gender ($p=0.047$) and race ($p<0.0001$) as significant covariates. Melatonin infusion appeared to be well tolerated in preterm infants.

Conclusions: The pharmacokinetic profile of melatonin in preterm infants differs from that of adults. Slow clearance makes replication of adult and thus fetal concentrations of melatonin problematic. Further studies are needed to confirm these findings.

Declaration of Originality

I declare that the work described in and the contents of this thesis are my own work, and that all else is appropriately referenced.

Acknowledgements

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List of Abbreviations

- **ADC- Apparent Diffusion Coefficient**
- **AMPA- alpha 3 amino hydroxyl 5- methyl 4- Isoxazole propionic acid**
- **aMT6s- 6 sulfatoxymelatonin**
- **AUC- Area under the plasma concentration time curve**
- **BDNF- Brain derived neuroprotective factor**
- **CL- Clearance**
- **CP- Cerebral palsy**
- **CWRES- Conditional weighted residuals**
- **DBM- deformation based morphometry**
- **DEHSI- Diffuse excessive high signal intensity**
- **DME- Drug metabolising enzymes**
- **DQ- developmental quotient**
- **DTI- Diffusion Tensor Imaging**
- **ELS- Extended least squares**
- **EPO- Erythropoeitin**
- **EU- European Union**
- **FA- Fractional anisotropy**
- **FDA- Food and Drug Administration**
- **FO- First order**
- **FOCE- First order conditional estimation**
- **GDNF- Glial derived neurotrophic factor**
- **GMH-IVH- Germinal matrix haemorrhage- intraventricular haemorrhage**

- **GW- Gestational week**
- **HIF1α- Hypoxic inducible factor 1 alpha**
- **HPI- Haemorrhagic parenchymal infarction**
- **IGF- Insulin like growth factor**
- **IGF-1- Insulin like growth factor 1**
- **IIV- Inter individual variability**
- **IZ- Intermediate zone**
- **KA- Kainate**
- **MHRA- Medicines and Healthcare products Regulatory Agency**
- **MDI- Mental Developmental index**
- **MIND- Melatonin Neuroprotection Dosage Study**
- **MINT- Melatonin Neuroprotection Trial Study**
- **MOFV- Minimum value of objective function**
- **MR- Magnetic resonance**
- **MZ- Marginal zone**
- **NAC- N-acetyl cysteine**
- **Na-K-ATP- Sodium Potassium Adenosine Triphosphate**
- **NIHR MCRN- National Institute for Health Research Medicines for Children Research Network**
- **NOAEL- No observed adverse effect level**
- **NOS- Nitric oxide synthase**
- **OFV- Objective function value**
- **OL- Oligodendrocytes**

- **OLS- Ordinary least squares**
- **PAMPS- pathogen associated molecular patterns**
- **PBPK- Physiologically based pharmacokinetic**
- **PD- pharmacodynamics**
- **PFC- Prefrontal cortex**
- **PIP- Paediatric Investigation Plan**
- **PK-PD- Pharmacokinetic –pharmacodynamic**
- **PK- pharmacokinetics**
- **PLIC- Posterior limb of internal capsule**
- **PP- Preplate**
- **PPK- Population pharmacokinetics**
- **PPV- population parameter variability**
- **PVL- Periventricular leucomalacia**
- **RIA- Radioimmunoassay**
- **RNS- Reactive nitrogen species**
- **ROS- Reactive oxygen species**
- **RUV- Residual unexplained variability**
- **SI- Signal intensity**
- **SMPC- Summary of product characteristics**
- **SP- Subplate**
- **SVZ- Subventricular zone**
- **t1/2- Half life**
- **TBSS- Tract based spatial statistics**

- **Tmax- Time to maximum concentration**
- **TNF- Tumour necrosis factor**
- **UK- United Kingdom**
- **US- United States**
- **V- Volume of distribution**
- **VEGF- vascular endothelial growth factor**
- **VLBW- very low birth weight**
- **VZ- Ventricular Zone**
- **WHO- World Health Organisation**

Statement of Publications

The work described in this thesis has been published in:

- **Merchant N**, Azzopardi D, Hawwa A, McEnlay J, Arendt J, Middleton B, Gressens P, Edwards AD. Pharmacokinetics of Melatonin in Preterm Infants- Br J Clin Pharmacol. 2013 Nov;76(5):725-33. doi: 10.1111/bcp.12092
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Conference Posters and Presentations

*The work described in this thesis has been presented at the following meetings***:**

Presentations

- **Merchant N**, Azzopardi D, Edwards AD. Pharmacokinetics of Melatonin in preterm infants. *European Society of Paediatric Research*, Newcastle, UK; 2011.

Posters

- **Merchant N**, Azzopardi D, Edwards AD. Pharmacokinetics of Melatonin in Preterm Infants. Pediatric Academic Societies, Denver, USA; 2011.
- **Merchant N**, Azzopardi D, Edwards AD. Melatonin as a novel neuroprotectant in preterm infants- MCRN conference Jan 2008.

1. INTRODUCTION AND AIMS

1.1. Need for neuroprotective therapies in preterm infants

It is estimated that 15 million preterm infants are born worldwide every year [1, 2]. In the United Kingdom (UK), 7-8% of all babies are born preterm [3]. Epidemiological studies have identified a number of factors that confer increased risk for preterm birth [4]. These include: multiple pregnancies [5], low socioeconomic status [6], maternal infection during pregnancy [7] and iatrogenic causes including induction/elective caesarean sections and in vitro fertilization [4, 8]. Despite public health and medical preventive measures that have been introduced, the incidence of preterm delivery has been increasing over last two to three decades [9].

1.1.1. Consequences of Preterm Birth

Preterm birth is the leading direct cause of neonatal death (27%) and more than one million preterm infants die every year [10, 11]. It also acts as a risk factor for many neonatal deaths due to other causes particularly infection [12]. Mortality and morbidity rates increase with decreasing gestational age and are highest among infants born at less than 32 weeks [10]. In the United States (US), 2% of neonates born at less than 32 weeks' gestation account for more than 50% of neonatal deaths, thus making this group a high risk population [13].

With advances in perinatal medicine such as use of antenatal steroids, advanced ventilation techniques, good intensive care and decreased sepsis, the survival rate of extreme premature infants has increased significantly [14]. In the US, survival rate

increased from 49% at 20 months in very low birth weight infants (VLBW) born in 1982–1989 to 68% and 71% in 1990–1999 and 2000–2002 respectively [15]. Data from the UK in 1997 [16] shows that 27% of infants born less than 26 weeks; 68% born at 26-28 weeks gestation; and 92% of those born at 28-32 weeks survived to discharge, respectively while the EPICure data of 1995 showed that 26% of babies born at 24 weeks survived to discharge, and among those born at 25 weeks, 44% survived to discharge [17]. The 2006 EPICure data showed that the survival at 24 weeks had increased to 40%, 66% at 25 weeks and 77% at 26 weeks gestation [18].

Preterm birth also leads to significant neonatal morbidities. Compared with infants born at term, preterm infants have greater rates of temperature instability, respiratory distress, infections, apnoea, hypoglycaemia, seizures, jaundice, feeding difficulties, necrotising enterocolitis, intraventricular haemorrhage, periventricular leucomalacia and rehospitalisation [19]. Long-term they have greater rate of cardiovascular, pulmonary, vision and hearing impairments and an increased neurodevelopmental and behavioural sequelae [20]. Preterm birth is also associated with increased perinatal and infant mortality, diminished long-term survival, and lower rates of reproduction [21].

1.1.2. Neurological Impairment in Preterm Infants

In recent decades, although the survival rate of very premature infants has increased significantly, the incidence of neurological impairment and disability has remained relatively constant or even increased [22, 23]. In addition to cerebral palsy, blindness, deafness and developmental delay, preterm children at school age have increased

rates of academic problems and cognitive deficits, including executive dysfunction, as well as behavioural disorders, inattention and hyperactivity [24-29]. In babies with a birth weight less than 1500 grams or less than 28 weeks gestation, approximately 10% later exhibit cerebral palsy, and 50%, cognitive and behavioural deficits [30-32]. In the UK EPICure Study Group of 1995 [17], half of all surviving infants born before 26 weeks gestation showed neurodevelopmental impairment at 30 months of age (Figure 1.1).

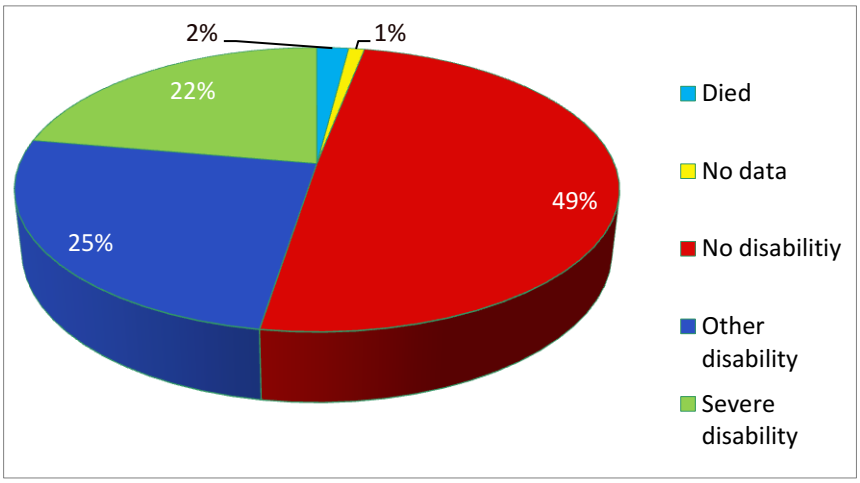


Figure 1.1: EPICure Study, UK half of all surviving infants < 26 weeks gestation showed neurodevelopmental impairment at 30 months of age. *(Modified from Marlow et al 2005 [17]).*

When assessed at a median age of 6 years and 4 months; along with full term infants as controls, cognitive impairment was present in 41% of the children born extremely preterm as compared with those for their classmates (Figure 1.2). The rates of severe, moderate, and mild disability were 22, 24 and 34% respectively. Among children with severe disability at 30 months of age, 86% still had moderate-to-severe disability at 6 years of age [17].

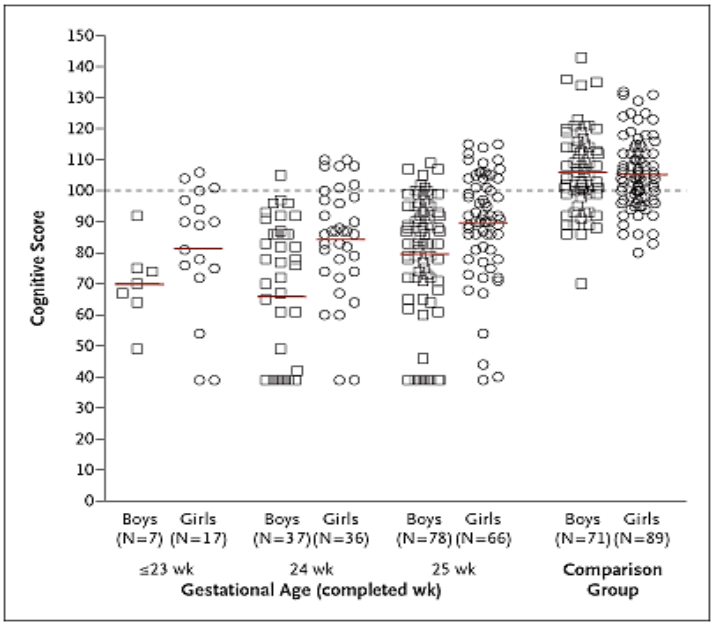


Figure 1.2: EPICure Study, UK Follow up at 6 years of age continued to show significant cognitive delay. *(Reproduced from Marlow et al 2005 [17]).*

The EPICure data from 2006 showed that 13% of extreme preterm had serious brain abnormalities and 74% were still dependent on oxygen at expected date of delivery [18]. This has remained unchanged from the 1995 EPICure data. The prevalence of neurological impairment was inversely related to the gestational age: 45% at 22-23 weeks, 30% at 24 weeks, 25% at 25 weeks and 20% at 26 weeks [33]. Overall mental developmental index scores ranged from 80 at 22-23 weeks to 87 at 24 weeks, 88 at 25 weeks and 91 at 26 weeks (Figure 1.3).

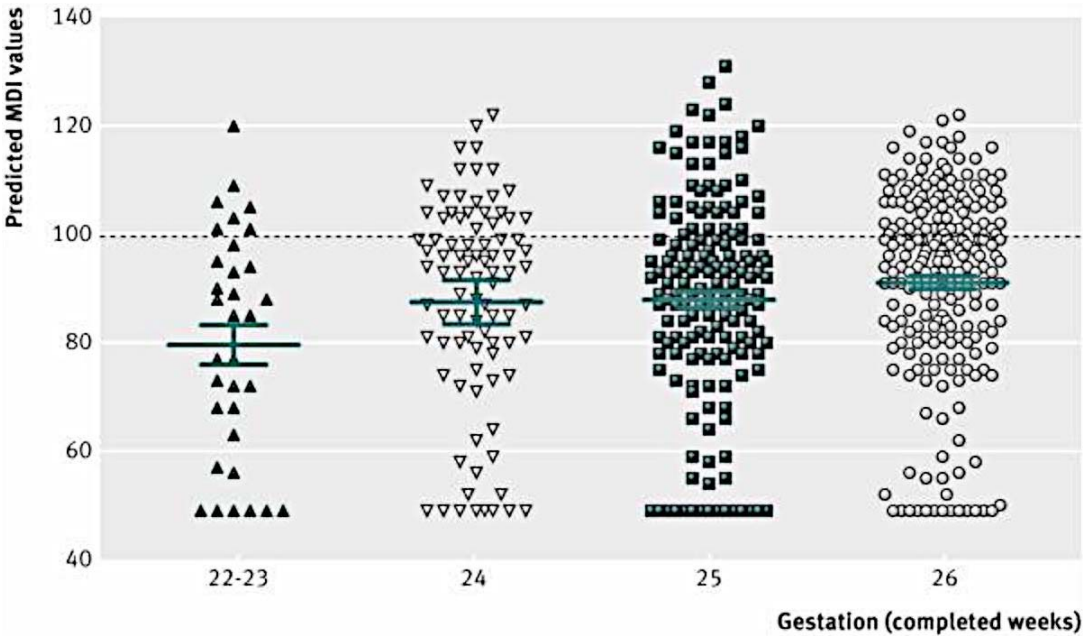


Figure 1.3: EPICure Study, 2006 Mean mental developmental index (MDI) scores along with confidence intervals at 30-36 months age (*reproduced from Moore T et al 2012 [33]).*

In the EPIPAGE cohort of infants less than 32 weeks gestation, the incidence rate of cerebral palsy was significantly increased, affecting 8.2% of the preterm infants at 2 years of age, and 9% at 5 years of age [34, 35]. From this cohort, 42% of children born at 24-28 weeks gestation and 31% born at 29-31 weeks gestation needed special health-care support owing to neurological sequelae compared with only 16% of those born at 39-40 weeks [36]. Data from the UK Millennium Cohort study showed that 66% of very preterm children (less than 32 weeks gestation) children do not reach a 'good level of overall achievement' at school age, predominantly due to difficulties in the cognitive domain. The relative risk for poor performance at school age was 1.32 (95% CI 1.10-1.58) for very preterm infants [37]. Data from the 2005 school census which linked data from more than 400,000 school aged children in Scotland showed

that children born at 28-32 and 24-27 weeks were 2.66 and 6.92 times more likely to have a special educational need [38]. The 1995 EPICURE cohort when assessed at 11 years of age showed a significantly lower score than their classmates for cognitive ability (-20 points, 95% CI -23 to -17), reading (-18 points, 95% CI-22 to -15) and mathematics (-27 points, 95%CI -31 to -23). Thirteen percent of the extreme preterm infants attended special school while 57% of those who attended mainstream school required SEN resource provision [39].

1.1.3. Economic Burden

The adverse consequences of preterm delivery are far-reaching and represent a significant psychological and economic burden not only to the individual and their family but also to health and education services [3, 40]. The true costs of prematurity and its consequences are poorly understood and grossly underestimated. The lifetime cost for a child with cerebral palsy has been approximated at up to €860,000 in Denmark with a large component of the costs being accounted by social care costs [41], while in the US it was estimated to be $921,000 in 2003 [42]. In the UK, the total hospital unit cost per annum is estimated at £12,500 for a child with hemiplegia, of which only 43% of this accounted for expenses directly related to the impairment [43]. The mean societal costs of care throughout childhood per preterm survivor less than 32 weeks gestation is £151,189-£113,160 per annum attributable to health, education, parental expenses and lost productivity costs while for a term infant it is estimated to be £51,394 [44]. The annual perinatal care costs related to prematurity exceeds $26billion every year in the US alone, with a dose dependant relationship between the gestational age at birth and cost per surviving child [45, 46].

1.2. Hypothesis and Aims

Currently preterm birth cannot be prevented, and although antenatal steroid therapy and possibly antenatal magnesium administration improve outcome [47] there are no specific postnatal neuroprotective therapies available apart from neonatal intensive care.

Melatonin, a naturally occurring hormone, has been shown to have neuroprotective properties in preclinical models, and preterm infants are deprived of the normal intrauterine exposure to maternal melatonin. Melatonin has favourable pharmacological and toxicological properties that make it an attractive candidate for clinical studies of neuroprotection, and studies in animals have raised the hypothesis that ***melatonin is an effective and safe neuroprotectant for use in human preterm infants***, either as a replacement hormonal therapy to replicate fetal physiological levels, or at a higher pharmacological dose.

However, the essential first step in addressing this hypothesis is to determine the pharmacokinetics of melatonin, to allow accurate replacement or pharmacological administration after birth. Preterm infants have major differences in multiple physiological variables including body composition, renal function and hepatic metabolism compared to adults. Hence prior to any study of the neuroprotective effects of melatonin PK data have to be obtained from preterm infants to establish dosing. Data from adult and animal studies together with knowledge of neonatal physiology provide data for estimation of a starting dose in such studies, but these estimations are sufficiently uncertain to make a dose ranging study essential.

Aims of this study

- To determine the pharmacokinetic profile of melatonin in preterm infants;
- To assess tolerability of melatonin in preterm infants;
- To determine whether a specific dosage scheme would allow replication of adult concentrations of melatonin.

1.3. Thesis Outline

The outline of this thesis is summarised below:

Chapter 2 discusses normal brain development and then the characteristic cerebral injuries seen in preterm infants, using experimental and histopathological data alongside information obtained *in vivo* using magnetic resonance (MR) imaging.

Chapter 3 describes the regulatory and practical environment for developing new treatments for newborn infants.

Chapter 4 explores potential neuroprotective therapies available for translation to clinical studies.

Chapter 5 discusses the current state of development of melatonin, the key gaps in knowledge and the proposed planning of the clinical trials of neuroprotection with melatonin.

Chapter 6 describes the approaches to determining the pharmacokinetic parameters including the population pharmacokinetic modeling method used in the study.

Chapter 7 presents the execution and results of a study of the pharmacokinetics of melatonin in preterm infants.

Chapter 8 discusses the implications of the pharmacokinetic study of melatonin in preterm infants.

Chapter 9 describes the proposed set up of a drug development pipeline for melatonin as a neuroprotectant in preterm infants. Future perspectives and challenges are also discussed in this chapter.

2. THE DEVELOPING BRAIN AND PRETERM BIRTH

2.1. Normal Brain Development

Brain development is a series of complex dynamic and adaptive processes and starts from the first few weeks of conception and continues right through early adulthood arguably throughout the lifespan [32, 48-52]. Both gene expression and environmental input, along with their interaction are important for normal brain development. Knowledge of specific events taking place during the ontogeny along with the timings of these events is useful in understanding the developmental disorders occurring at various points during brain development and interpretation of neurological findings at different ages. Although a detailed description of all of the processes involved in the formation and maturation of the human brain is outside the scope of this thesis, a broad overview summarised below (figure 2.1).

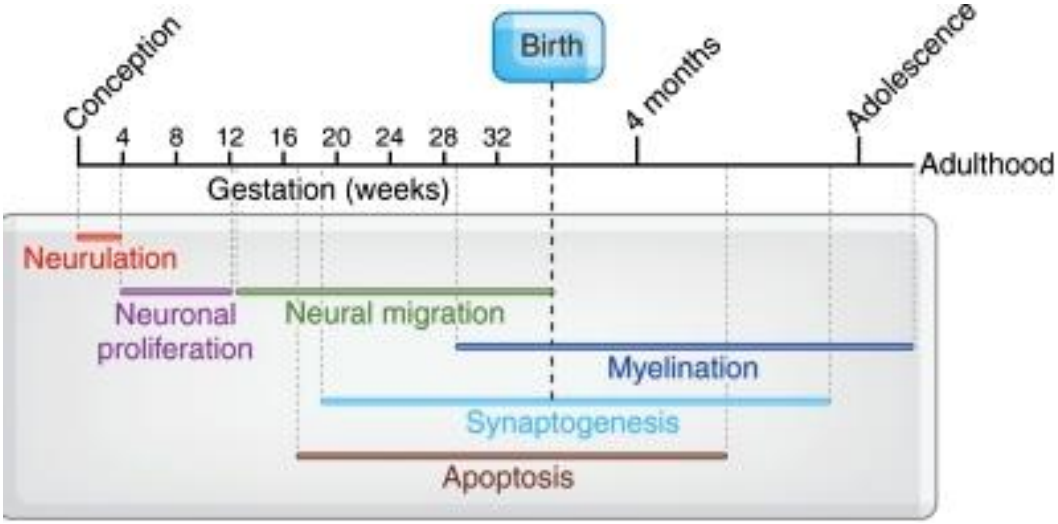


Figure 2.1: Timeline of major events in brain development. *(Figure reproduced from Tau GZ 2010 [53])*

2.1.1. Neurulation

The neuro-ontogenic process starts in the 2-3rd week post-conceptional age with folding and fusion of the ectodermal region containing the neural progenitor cells (neural plate) to form the neural tube, the primordium of the central nervous system [54]. As the neural progenitor cells are located in the region finally forming the ventricles, that area is called the ventricular zone (VZ) and later forms the germinal matrix. In the 4th week, the rostral part of the neural tube forms three primary vesicles which then forms the forebrain (prosencephalon), midbrain (mesencephalon), and the hindbrain (rhomboencephalon) [17, 55-59]. The vesicle forming the forebrain [56] subdivides into 2 secondary vesicles which finally becomes the telencephalon (cerebral cortex) and the diencephalon (thalamus, hypothalamus, and other structures). Failure of neurulation results in neural tube defects, such as spinal bifida and anencephaly.

2.1.2. Neuronal Proliferation

The human brain contains billions of neurons and migrating immature neurons before 18 months of age, but this proliferation subsides in older children and is nearly extinct by adulthood [60]. By the 5th-6th week, the neural progenitor cells, which are mitotic cells, are proliferating rapidly within the VZ lining the ventricles [61-75]. By week 6, the neural progenitor cells shift from a symmetrical mode of cell division where two identical neural progenitor cells are produced to an asymmetrical mode of cell division where two different cells are produced- one neural progenitor cell and one neuron [52, 76]. These are differentiated into either specific neuronal cells or

microglia, depending on their location in the germinal matrix. Neurons are post-mitotic cells; i.e. they cannot produce new cells once formed.

2.1.3. Neural Migration

The neurons migrate radially from the VZ to the developing neocortex to form cortical laminae in an 'inside–out' manner in which deeper cortical layers are formed before the superficial ones [71, 73, 74, 77, 78] (figure 2.2). Initially a somal translocation method is used where the neuron extends a cytoplasmic process and attaches to the pial surface, allowing the nucleus to move into the brain area [79]. Later, most neurons travel along radial glial cells, which act as scaffolding and guide the neurons to their final destination [80, 81]. A smaller group of neurons originates from the basal ganglia nuclei and migrate parallel to the outer cortical surface to the thalamus and the cerebral cortex and form the GABAergic neurons in the mature brain [67, 69, 82, 83]. Neuronal migration peaks between gestational week (GW) 12 and 20 and continues till 18 months of age [28, 60, 84]. During postnatal migration, not all new neurons reach the olfactory bulb as previously thought but there is a rostral migratory stream, which targets the prefrontal cortex. Postnatal neurogenesis may play an important role in learning and memory. It is hypothesised that these escape pathways from the VZ can provide interneurons to the different regions of the developing brain and may provide a mechanism for delayed postnatal plasticity thus acting as potential targets of neurological injuries affecting neonates [60].

Migrational errors can cause significant neurodevelopmental sequelae e.g. Lissencephaly, a disorder of neuronal migration disrupts the normal patterning of

sulci and gyri and can result in severe developmental delay [85]. Abnormal neuronal migration can lead to foci of ectopic cortical tissue in the white matter that can result in seizures [86, 87].

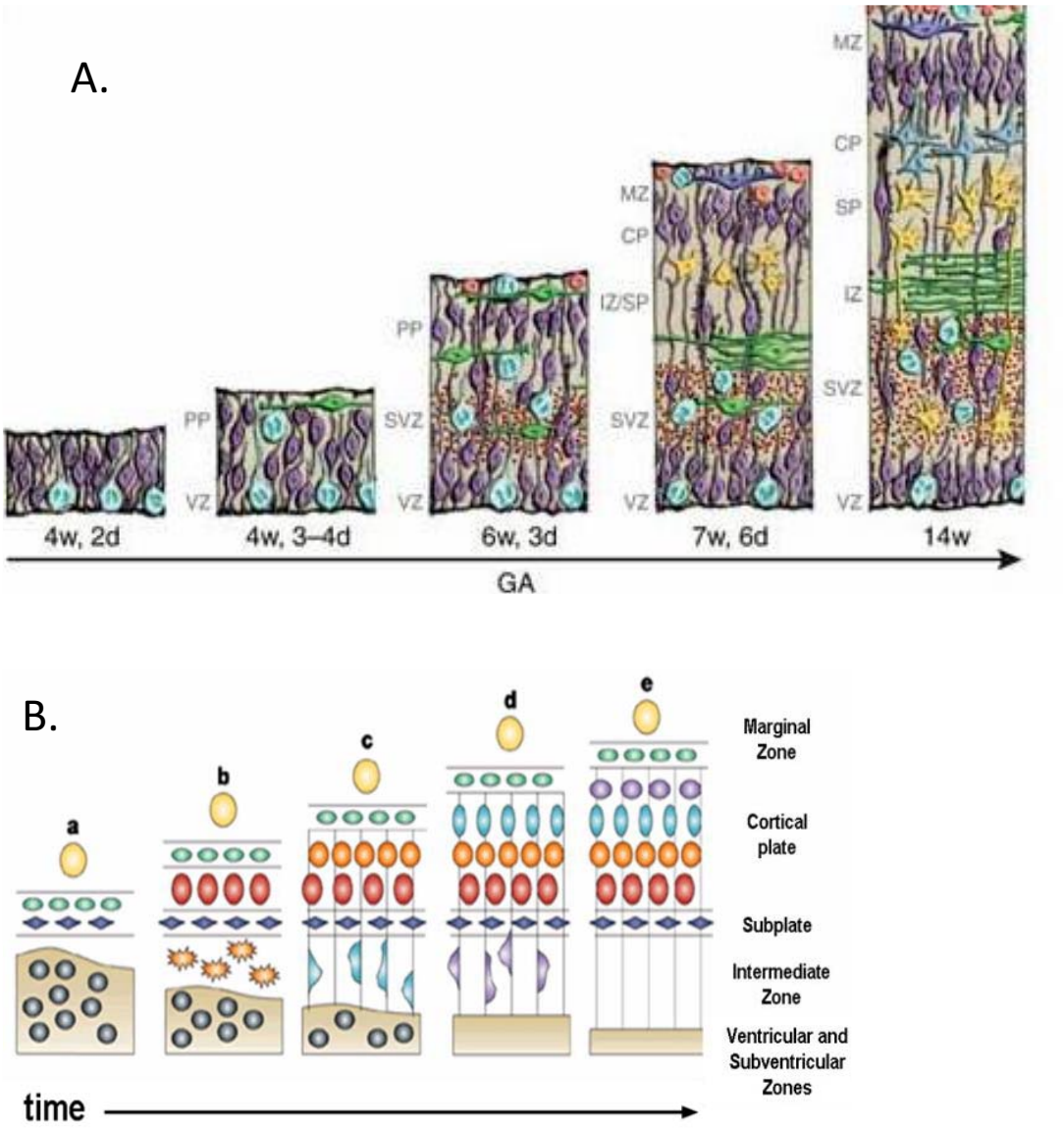


Figure 2.2: Embryonic layers of the developing human neocortex. (a) Cortical layer development timeline. (b) Neuronal migration seen during corticogenesis. (ventricular zone (VZ), preplate (PP), subventricular zone (SVZ), intermediate zone (IZ), subplate (SP), cortical plate (CP), marginal zone (MZ). *(Figure reproduced and adapted from Bystron et al 2008 [61], Tau et al 2010 [53])*

2.1.4. Synaptogenesis

Neurons form synaptic connections by developing neuronal processes on reaching their target region of cortex and these connections are continuously refined and modified and contribute to more stable connections that are seen in mature circuits [53]. Axons are the principal means of sending signals from the neurons while dendrites receive input from the neuron. At the tip of the axon there is a growth cone which is the site of elongation and extension [88]. The growth cone samples the local environment for guidance molecules that direct the axon toward its target [52]. On reaching the target, synapses form within the target cell. The earliest synaptic connections are formed in week 5 by the neurons in the first cortical layer (preplate or primordial plexiform layer) [89-94].

Subplate: The subplate is a transient though important embryonic cortical layer that forms within the preplate [61, 95]. Between GW 18-22, the subplate is rich with synapses and five times thicker than the cortical plate [96, 97]. Neurons in the subplate play an essential role in establishing pathways to and from the visual and somatosensory thalamus, cholinergic afferents from the basal forebrain, and monoaminergic afferents from the brainstem [93, 95, 98-102]. This shows that the major neurotransmitter systems are established early in development, although their function in early development is at present poorly understood. In the absence of subplate signaling, normal patterns of connections between the thalamocortical axons and layer 4 cortical neurons and similarly between the corticothalamic connections between layers 5 and 6 of the cortex and thalamus do not develop [52]. The coincident timing of subplate development and the highest incidence of major

brain pathologies associated with preterm birth indicates that during this period the subplate is specifically vulnerable to injury in these infants [103]. Exposure to drugs are thought to alter signaling, metabolism, or other elements of neurotransmitter physiology, which may affect the development of neural circuits.

Cortical Plate: The cortical plate is formed within the preplate splitting it into two layers: the subplate below and the marginal layer above. The cortical plate forms the laminae II–VI of the mature cerebral cortex and the marginal zone will ultimately form cortical layer I. Depending on the specific cortical region, cortical layer I is formed between GW 24 and 34, and layers III and IV appear between GW 32 and 34 [104]. Each layer contains a distinct array of cells types, the morphology, and laminar location determines the pattern of local and distant projections that each cell may send or receive and change in cortical cytoarchitecture can be detected with diffusion tensor imaging (DTI) [105]. From around 26 weeks, the thalamo-cortical afferents begin to synapse in the deep layers of the cortical plate, before progressing into layers III and IV and the subplate begins to disappear [97]. Dissolution of the subplate and the concurrent maturation of the cortical plate signal a transition in the maturation of cortical circuits.

Lamination is present first in the primary sensory and motor cortices as early as GW 25. By GW 32, the developing cortex has a full adult complement of distinct vertical lamina [104] containing afferents of all the major neurotransmitter systems [66] and a diversity of differentiated glia and neuronal cell types [106]. Laminar projections follow a characteristic pattern. Layer II extend cortical afferents more locally and layer III more distally while layer V and VI extend to subcortical areas with layer V

projecting to the brainstem, midbrain and basal ganglia and layer VI primarily to the thalamus. Excitatory cells usually project onto inhibitory neurons while inhibitory neurons may project on either excitatory or other inhibitory neurons. Thus it is the inhibitory neurons that regulate information transmission from one excitatory neuron to another.

The timing of synaptogenesis differs in the different cortical layers and in the different cortical regions. Synaptogenesis begins in the deeper layers first following the inside-out sequencing of cortical lamination [107]. Although many studies reported no differences in the rates of synaptogenesis in the different cortical regions [108-111], others have found that synaptogenesis begins in primary motor areas earlier followed by the prefrontal cortex (PFC) [112, 113]. Synaptogenesis peaks around GW 34 when almost 40000 new synapses are formed every second, a process that continues well into early postnatal life.

2.1.5. Apoptosis

Two key processes occur during neurodevelopment that results in substantial loss of neural elements. This includes the naturally occurring cell death that usually occurs in the prenatal period and the second process is the exuberant production and pruning of synaptic connections, which is mainly a postnatal event [52, 114]. The number of neurons in the human brain peaks at GA week 28. However as many as half of the neurons produced during neurogenesis die in the process of apoptosis, or programmed cell death, [115]. A number of reasons for cell death have been proposed. Cells that send processes to incorrect regions are eventually eliminated

thus establishing effective and functional neuronal circuits and normal pattern of brain organisation and connectivity [116-119]. The neurotrophic theory hypothesizes that neurons that have effective connections are able to obtain more neurotrophic factors which protect the neurons from apoptosis [120]. Apoptosis is also important in removing cell populations that have a transient role in brain development such as the subplate cells and ventricular zone cells [121, 122]. The apoptotic mechanisms resulting from excitotoxicity and mitochondrial dysfunction are also crucial during pathological events such as neonatal hypoxic ischaemic brain injury [123]. Synaptic exuberance and pruning although mainly takes place in the postnatal period, also supports the connectivity of the developing brain. Across the brain, the number of synapses are initially twice and slowly declines to normal adult levels during childhood and adolescence [113], (Figure 2.3).

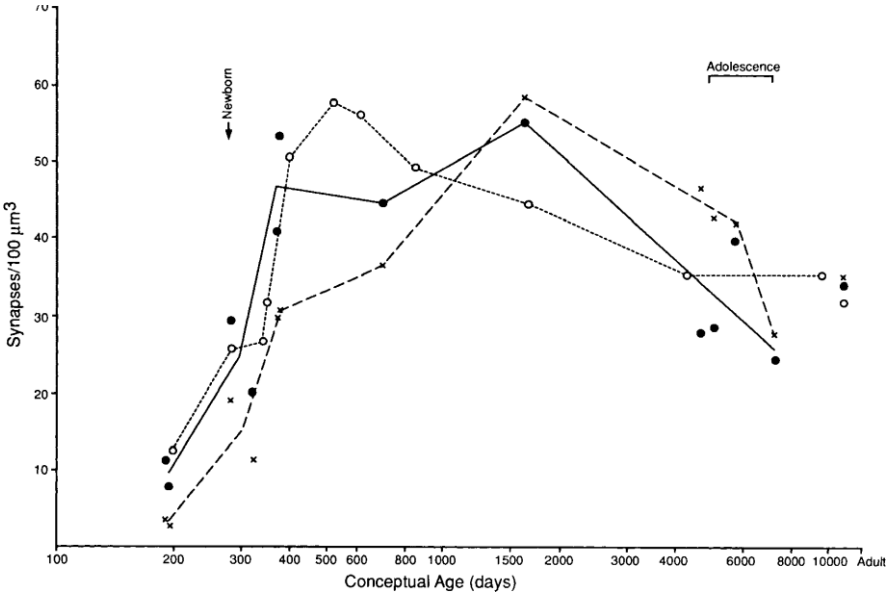


Figure 2.3: Synaptic connectivity in human cerebral cortex. Mean synaptic density per 100 µm3 as a function of pre and postnatal age in the auditory cortex (filled circles), visual cortex (open circles) and prefrontal cortex (x). *(Reproduced from*

Huttenlocher and Dabholkar 1997 [113])

2.1.6. Glial Cells and Myelination

Throughout the brain, glial cells surround and interact with neurons and are vital for brain development and function [124]. Microglia migrate into and colonise the brain from the haematopoietic system early in development, and are the resident macrophages of the central nervous system [125]. Macroglia, are derived from the same progenitor cells as neurons, and form astrocytes and oligodendrocytes (OL) [17, 124, 126]. Astrocytes are important in neuronal homeostasis, clearance of neurotransmitters, inflammatory responses, and modulation of synapses [124]. Immature oligodendrocytes proliferate and surround the developing axons of the white matter, and when matured through neuronal neuregulin expression, are responsible for the formation of the fatty myelin bilayer, which ensheathes the axons and enhances the efficacy of information transmission in the neuronal pathways [17, 126, 127]. Between GW 20 and 28, mature myelin is detected first in subcortical regions and later in cortical regions [53]. At GW 35, it is seen in the precentral and postcentral gyri and optic radiation, and at GW 40, it is present in the acoustic radiation [128]. Between GA weeks 36 and 40, the proportion of total brain volume that contains myelinated white matter increases from 1 to 5% [129]. Thus myelination is predominately a post-term process and increases in the first 2 years of postnatal life continuing even in adolescence [29, 130-132].

Premyelinating oligodendrocytes are particularly vulnerable to perinatal hypoxia or ischaemia, resulting in disruption of the white matter tracts in the frontal and

temporal lobe [133, 134]. Preterm birth is also thought to disrupt myelination and thereby predispose to poor cognitive, neurodevelopmental, and neuropsychiatric outcomes. Recent research also suggests that oligodendrocytes synthesize trophic factors that contribute to the integrity of axons and neuronal survival [135].

2.1.7. Maturation of the cerebral cortex

The brain initially begins as a smooth lissencephalic structure that gradually matures with the characteristic pattern of sulci and gyral folding. Early signs of gyrification are seen from GW 8 [136, 137]. Gyrification follows an orderly sequential pattern; with the interhemispheric fissure developing from 8 weeks GA; sylvian, cingulate, parieto-occipital and calcarine fissure between 14-16 weeks GA; central and superior temporal (GW 20-24); and superior frontal, precentral, inferior frontal, postcentral, and intraparietal (GW 25-26); secondary sulci between GW 30-35; formation of tertiary sulci begins during 36 weeks and extends well into the postnatal period [29, 136, 137]. The primary sulci start as shallow grooves that progressively become deeper [138-140]. This is followed by secondary sulci between 30-35 weeks gestation that emerge from the primary sulci and later develop tertiary sulci resulting in complex patterns of folding characteristically seen in the mature adult brain [29, 141]. This results in dramatic increase in the brain's surface area (rather than thickness) with an exponential growth trajectory in contrast to a linear increase in whole brain volume (Figure 2.4) [137, 142]. Human brain growth is seen to obey an allometric scaling law with an exponent of 2/3 explaining the relationship between the rapidly folding cortical surface area and the whole brain volume. The mechanistic forces generated by "visoelastic tension" through the developing connections of the

cortical fibres have been proposed to underlie the formation of the cortical gyri [137, 143]. Under this theory, areas of cortex which are densely connected to each other are "pulled together" by the tension exerted by the fibres, thereby leading to the protrusion of gyri as the brain continues to grow (with the sulci formed from early interconnected regions) [143]. This process also serves to maximise the efficiency of the brain's framework of intrinsic connectivity by shortening the distance between communicating regions [144].

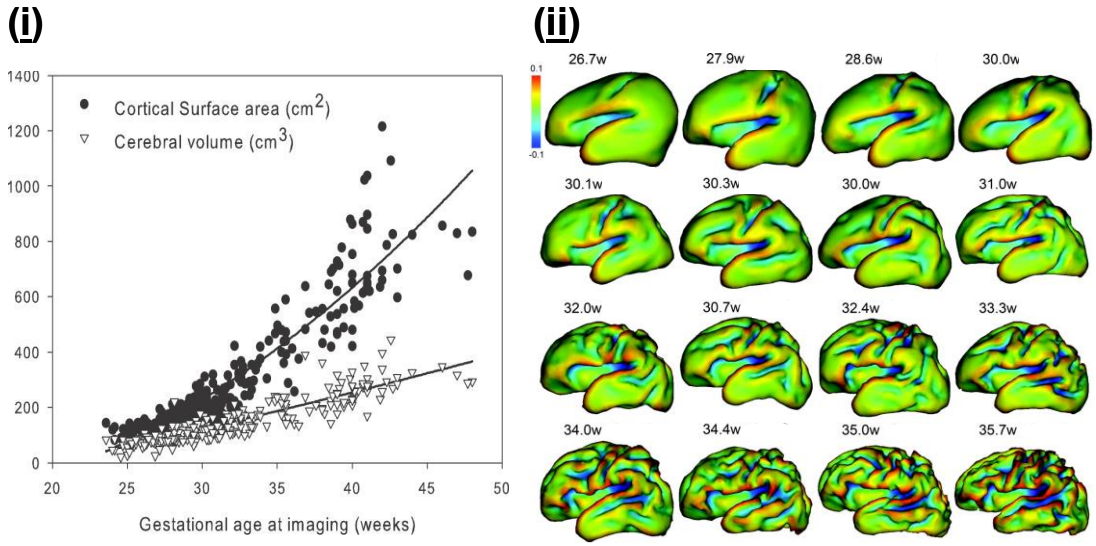


Figure 2.4: Brain growth (i) The surface area of the brain increases more rapidly than brain volume. *(Reproduced from Kapellou et al. 2006 [142]).* (ii) Development of cortical folding from 26 to 36 weeks post-menstrual age. At 26 weeks, the brain is relatively lissencephalic with the exception of the central sulci and sylvian fissures, in marked contrast to deeper and richer (but still highly immature) pattern of folding seen at 36 weeks. *(Reproduced and adapted from Dubois et al. 2008 [141]).*

2.2. Brain development studied *in vivo* by Magnetic Resonance Imaging

Although pathological studies have been useful in obtaining information about maturation and development of the brain, imaging has the added advantage of being able to study a live fetus or infant along with serial imaging allowing longitudinal assessment of brain growth and development both in-utero and ex-utero.

Magnetic Resonance (MR) imaging allows non-invasive acquisition of high-resolution 3D structural brain imaging in the absence of irradiation and other side effects. Because of the quantifiable nature of the acquired signal, detailed visualisation and accurate measurement of different tissue structures can be made. Many papers have now been published on the safe techniques of MR imaging ([51, 145, 146]. MR imaging is now used routinely clinically for diagnosis of various disorders. In research settings MR imaging has been used to study the physiology of early brain development, to recognise early patterns of disease, to identify which patients will respond to intervention and is being developed as a biomarker tool for prediction of response to treatment [147-151].

2.2.1. Cerebral tissue composition observed by MR

MR provides data on brain composition. Quantitative measurements of proton density and T1 and T2 relaxation times have shown that the immature brain has higher water content which decreases over time (Figure 2.5) [51].

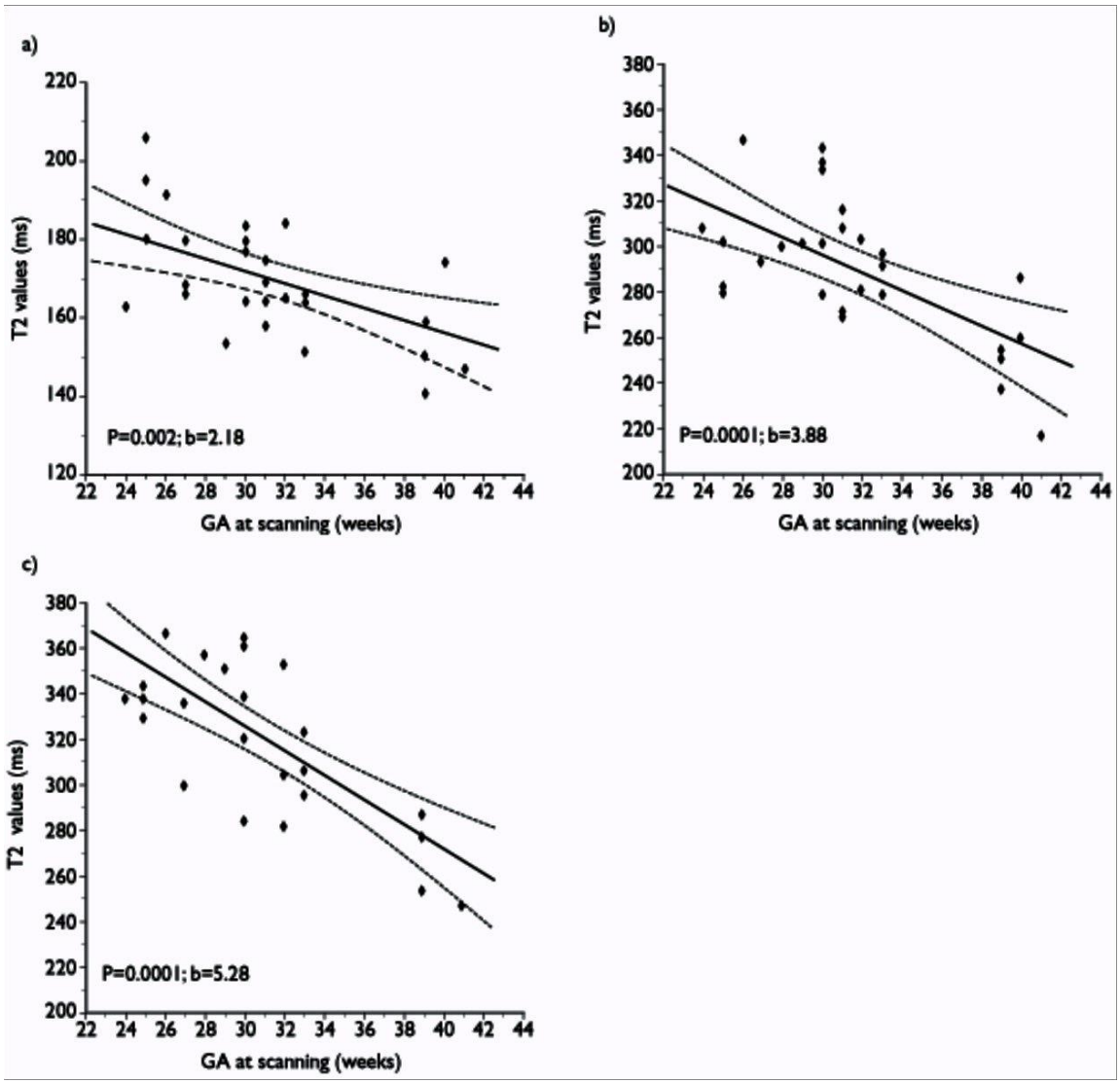


Figure 2.5: Decreasing T2 values with increasing age (a) the lentiform nuclei (b) the anterior white matter and (c) the posterior white matter at the level of the centrum semiovale. *(Taken from Battin, M. and Rutherford M.A., 2002 [51]).*

2.2.2. Cortical Folding

As described earlier, before 24 weeks the brain is relatively agyric with the exception of the Sylvian fissure. Subsequently there is progressive increase in the sulci and gyral pattern with increase depth, width and complexity of sulcation leading to development of nearly adult pattern by term [138, 152-154]. Using MRI, systematic

studies of sulcal and gyral developmental patterns have been reported and various developmental scores have been suggested. One such scoring system is the gyral developmental scoring used by Battin et al where a score of 1-5 is given for the different areas of the brain depending on the depth, width, the relationship to adjacent sulci, and the complexity of sulcation and gyration [139]. This shows that the increase in cortical folding relates to increasing developmental age (Figures 2.6, 2.7).

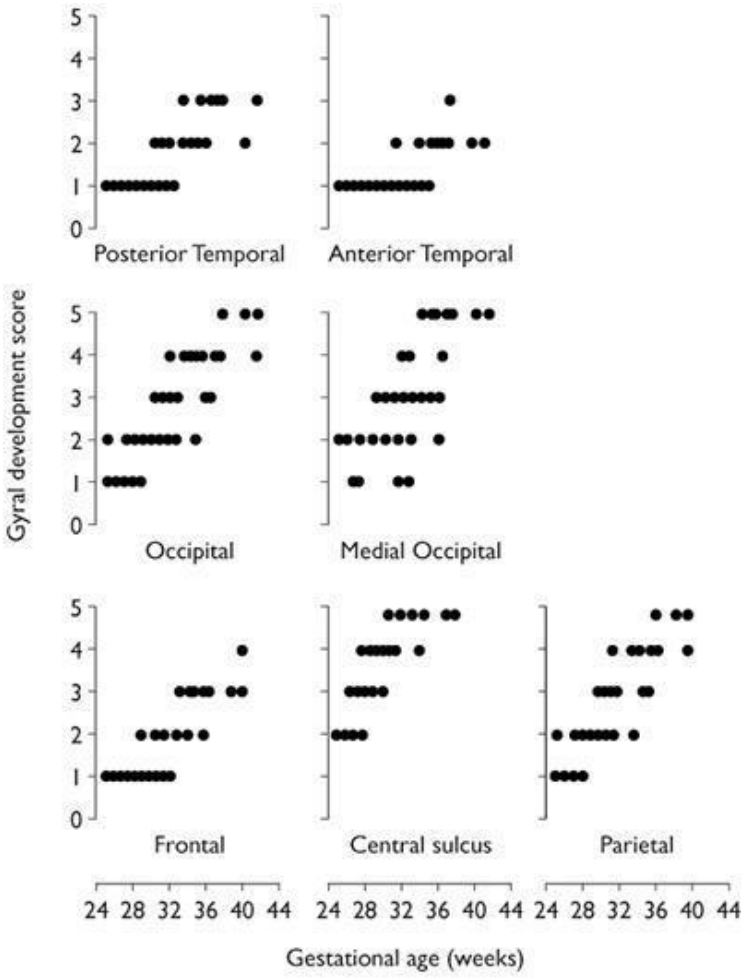
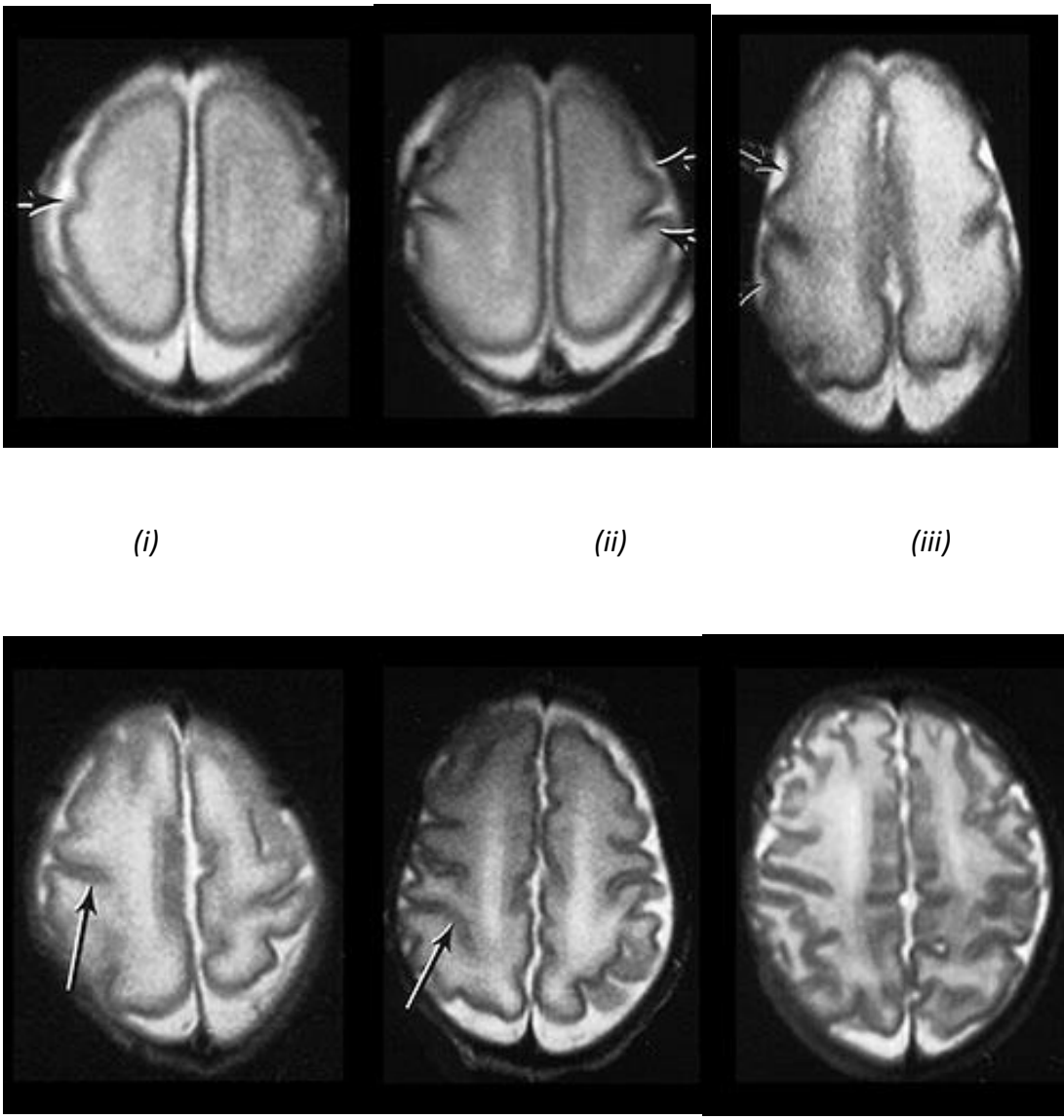


Figure 2.6: Gyral development score versus gestational age. The graphs demonstrate the different rates of cortical development in the different brain regions. *(Reproduced from Battin M et al, 1998 [139]).*



(i) *(ii)* *(iii)*

(iv) *(v)* *(vi)*

Figure 2.7: Cortical folding at increasing gestations. T2 weighted fast spin echo sequence in the transverse plane at supraventricular level (i) 24 weeks, the brain surface is smooth but the central sulcus is rudimentary (*arrow*); (ii) 26 weeks, there is a rudimentary precentral sulcus (*top arrow*); (iii) 28 weeks, further development of the central and precentral sulcus with signs of the postcentral sulcus forming posteriorly to the central sulcus (*arrowhead*); (iv) 30 weeks, central sulcus is deep and narrow but unbranched (*arrow*); (v) 32 weeks, the central sulcus is more complex

(*arrow*); (vi) 34 weeks, the sulci are complex *(Reproduced from Battin, M. and Rutherford M.A., 2002 [51]).*

2.2.3. White matter

As mentioned earlier, the white matter has a high water content resulting in long T1 and T2 relaxation time. The brain water content decreases between 24 weeks and term and further decreases throughout the first 2 years of life [51].

At around 24 weeks gestation, well defined bands of alternating signal intensity (SI) are seen in the white matter and are thought to relate to transient features of the developing cortex [97, 138, 139, 155]. During this time 4 layers are identified at the level of the centrum semiovale which represent the cortex (high SI on T1), subcortical white matter (low SI on T1), intermediate zone of migrating glial cells (high SI on T1) and periventricular zone (low SI on T1) [51]. The periventricular zone lies adjacent to the subependymal layer and represents the developing white matter. Within the white matter in the periventricular region distinct zones of high SI on T2-W images and low SI on T1-W images are seen in the form of caps adjacent to the anterior horns of the lateral ventral ventricles and arrowheads adjacent to the posterior horns of the lateral ventricles [51]. These are thought to relate to dense white matter fibres converging on different parts of the brain.

2.2.4. Myelination

Myelination is predominantly a post term process. On MR imaging, myelination is associated with a shortening of T1 and T2 and is seen as high SI on T1-W sequences

and low signal on T2-W imaging [51, 156]. Myelination in the preterm is better appreciated on T2-W imaging while in post term infants, myelination is observed better on T1-W imaging [51, 156]. By 25 weeks gestation, myelination is seen on MR sequences in the inferior cerebellar peduncles followed by a caudal-cranial progression with myelination in the inferior colliculi, posterior brain stem and ventro-lateral nuclei of thalamus by 29 weeks gestation. Subsequently there is relatively little new myelination till myelination is evident in the posterior limb of the internal capsule by 35 weeks (Figure 2.8) and in the centrum semiovale by 42 weeks [51, 129, 136]. Preterm infants show myelination at an earlier post menstrual age as compared to more mature infants [51]. Although myelin is not seen in structures such as the corpus callosum till 3 months postnatal age, structures may still be visible on MR which correlates with a premyelinating state secondary to tightly packed fibres [51, 129, 157-159].

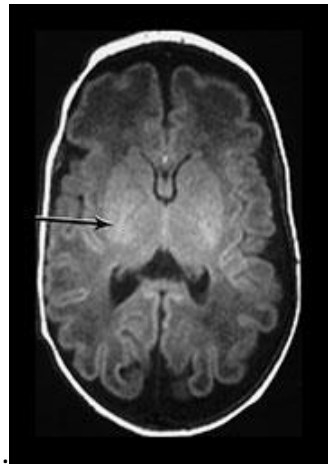
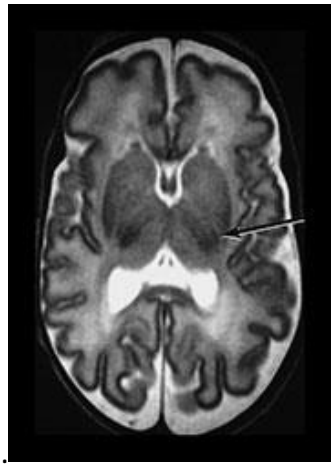
a.  b. 

Figure 2.8: Myelination in the PLIC in 30 week preterm infant imaged at 35 weeks (a) T2-W fast spin echo sequence and (b) T1-W spin echo. There is obvious myelin within the PLIC (arrow) (*Taken from Battin, M. and Rutherford M.A., 2002 [51]).*

2.2.5. Basal ganglia and Thalamus

The basal ganglia and thalami in very preterm infants are seen as high SI in T1-W images and low SI in T2-W images related to the increased cellular density which can be demonstrated histologically [51]. From 24 weeks until term the basal ganglia and thalami become more isointense on both T1 and T2 sequences. By 35 weeks' gestation there is only small foci high signal intensity in the thalamus on T1-W related to early myelination in the PLIC.

2.2.6. Germinal Matrix

The germinal matrix or subependymal layer is seen just at the surface of the lateral ventricles and is highly vascularised with endothelial-lined vessels. It is from this matrix that the neuroblasts and glial cells are produced between 10 and 20 weeks gestation resulting in an increase in its size; it then regresses although persists in the caudothalamic notch and the roof of the temporal horn [51]. This layer is seen as a high SI on T1-W images and a low SI on T2–W images [51, 146, 160]. Using MR imaging, it is possible to delineate the thicker rests of germinal matrix over the caudothalamic notch and temporal horn of the lateral ventricles along with the thinner cellular layers lining the ventricles. This thin layer is not usually detected on cranial ultrasound.

2.3. Preterm Brain Injury

Brain growth particularly in the cortex and deep grey matter are critical neuroanatomic correlates of poor outcome along with germinal matrix-

intraventricular haemorrhage (GMH-IVH), haemorrhagic parenchymal infarction (HPI) and periventricular leucomalacia (PVL) [32, 142]. These above lesions can result in significant long-term morbidity, developmental delay and poor cognitive and motor outcome. With advances in standard intensive care, the incidence of previously common forms of preterm brain injury i.e. cystic PVL and HPI have decreased significantly [161-165]. However neurodevelopmental abnormalities are still common suggesting a more global insult to the brain and hence preterm brain injury is now often called encephalopathy of prematurity [46, 103, 166].

2.3.1. Germinal Matrix- Intraventricular Haemorrhage (GM-IVH)

GMH-IVH is a common complication of preterm birth and occurs in 20-49% of infants born less than 30 weeks gestation [32, 167-169]. GMH-IVH is caused by rupture of the fragile capillary network in the subependymal matrix of the developing brain which is weakly supported by stromal tissue [170]. Furthermore the germinal matrix is probably a vascular end zone (watershed area) and thus vulnerable to ischemia. IVH is typically classified by grade (1-4) based on ultrasound appearances [171]. The haemorrhage when it evolves may be confined to the germinal matrix region (grade I IVH) or it may extend and rupture into the adjacent ventricular system (Grade II or III IVH depending on the extent of the blood) or extend into the white matter (Grade IV) [32, 172]. In the EPIPAGE cohort, the incidence of IVH without ventricular dilatation (grades I and II) was 20% of all infants between 22-32 weeks gestation affected, although the incidence was markedly higher in the most immature infants (40% of infants delivered at 23-24 weeks gestation). The incidences of IVH with ventricular dilatation (grade 3) and extension into the adjacent parenchyma (grade IV/HPI) are

significantly less affecting 3-5.7% of preterm population [164, 167] but 26% of infants less than 750g and 12% between 751-1000g still develop these severe forms of haemorrhage [173]. This is of particular relevance as more preterms are being born at the cutting edge of viability and long-term neurocognitive sequelae are more likely with severe haemorrhage. The aetiology is multifactorial and lack of cerebral autoregulation, changes in blood flow secondary to mechanical ventilation, hypoxia, hypoglycaemia, fluctuating blood pressure, along with clotting disturbances and immature vascular integrity are all thought to contribute to IVH [174-185]. Recent evidence suggests that intravascular responses may be modulated by inflammation or the administration of medications such as glucocorticoids [186, 187]. IVH can be complicated by HPI and /or hydrocephalus [32]. GM-IVH is seen within 24 hours after delivery in at least 50% of affected infants, and by 72 hours approximately 90% of the lesions can be identified [32, 188]. It is very rare after the first week. In 15–25% of infants with GMH on day 1 or 2 there is progression to an IVH and/ or a parenchymal lesion in the following days.

2.3.2. Periventricular White Matter Injury and Haemorrhagic Parenchymal Infarction

In 15% of GMH-IVH, periventricular white matter injury or infarction complicates the GMH-IVH. This parenchymal lesion is usually located at the same side of the GMH-IVH at the anterolateral angle of the lateral ventricle sometimes extending posteriorly. Neuropathological studies have shown that this parenchymal lesion is not an extension of the haemorrhage into the periventricular white matter as previously thought but a venous infarction related to obstruction to the venous drainage of the

white matter. The parenchymal lesion subsequently undergoes cystic degeneration and by term age there is a porencephalic cyst. There is often reduction in the myelination of the PLIC which is thought to be secondary Wallerian degeneration rather than direct injury [189]. Although the incidence is decreasing, 17% of infants less than 26 weeks still have HPI [167]. This is of particular importance as HPI is a well-recognised correlate for hemiplegic CP, neurocognitive delay, seizures and death [189-191].

2.3.3. Outcome of GMH-IVH

Preterm infants with GMH-IVH have a higher mortality compared to preterms without IVH [192]. A normal cranial ultrasound carries a risk of 5% (4-6%, 95% CI) of developing cerebral palsy [193]. Both grade III and IV IVH carry a significant risk of cerebral palsy, lower IQ and post haemorrhagic hydrocephalus [194]. 26% of infants with Grade III and 53% of infants with Grade IV develop cerebral palsy (CP) at 2 years of age [193, 195]. Vohr et al reported that 75% of infants with moderate to severe IVH required special education in school [196], although a much more recent study shows a slightly better functional outcome than previously thought [197]. Uncomplicated IVH (Grade I and II) can also cause motor and cognitive sequelae with a 9% (4-22%, 95% CI) risk of developing cerebral palsy [193, 198].

The mechanism of actual brain injury from GMH-IVH (also in cases where the haemorrhage does not directly involve brain tissue) probably relates to several factors, as suggested by Volpe [32] (Table 2.1).

Table 2.1: Hypothesised mechanisms of Brain Injury with GM-IVH

Preceding hypoxic-ischaemic injury
Destruction of glial precursors in germinal matrix causing deleterious effect on myelination
Destruction of periventricular white matter with subsequent impairment of cortical organisation
Periventricular white matter injury secondary to intraventricular, parenchymal or subarachnoid blood products (by vasoconstriction with decreased blood flow and by generation of free radicals)
Intracranial hypertension and impaired cerebral perfusion
Arterial vasospasm with focal brain ischaemia
Posthaemorrhagic hydrocephalus

2.3.4. Periventricular Leucomalacia (PVL) and white matter injury

Currently it is believed that apart from focal injury seen in HPI, all forms of WMI are a spectrum of PVL and this includes focal necrosis and a more common diffuse white matter brain injury [199] (figure 2.9). Histologically PVL is described as a combination of focal and diffuse abnormalities with reactive gliosis and microglial activation in the deep white matter around the necrotic foci. [200-202].

Cystic PVL, which is at one end of the spectrum, is characterised by focal macroscopic areas of necrosis in the periventricular white matter leading to cyst formation. These are commonly seen on cranial ultrasound [17]. The incidence of cystic PVL has decreased significantly over last few decades but is still seen in 3-9% of the very

preterm population [161, 167, 203-205]. It has a significant impact on neurodevelopmental outcome with a high incidence of diplegic CP, poor visual spatial skills and lower IQ scores [35, 204, 206-209]. More commonly, the focal lesions are microscopic in size and evolve to small glial scars, which are not easily detected on ultrasonography. Within weeks and months the cavities collapse due to continued proliferation of astrocytes and are often followed by ventricular enlargement.

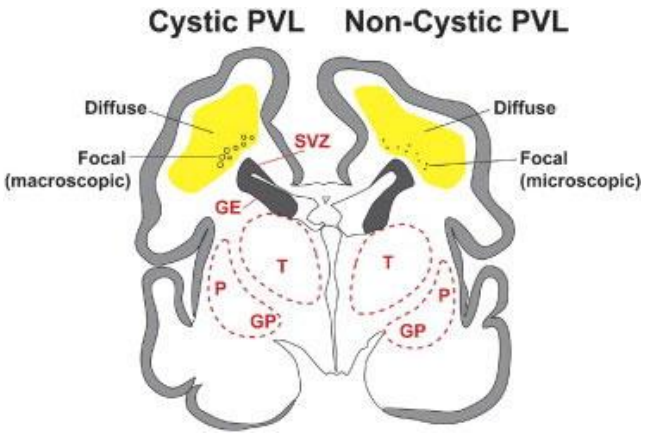


Figure 2.9: Schematic diagram of cystic and noncystic PVL. In cystic PVL (left) the focal necrotic lesions (circles) are macroscopic in size and evolve primarily to cysts, and in noncystic PVL, the focal necrotic lesions (dots) are microscopic in size and evolve primarily to glial scars. The diffuse component (yellow) is characterised by pre-OL injury, astrogliosis and microgliosis. Abbreviations: SVZ, subventricular zone; GE, ganglionic eminence; T, Thalamus; P, putamen; GP, globus pallidus. *(Adapted from Volpe JJ, 2009 [103])*

The current hypothesis of the pathogenesis of PVL (figure 2.10)

Myelination failure is the hallmark feature that distinguishes preterm brain injury from other forms of CP that mainly involve the gray matter [32]. Controversy exists regarding the mechanism of myelination failure and whether it involves axonopathy, arrested OL lineage progression or disturbances in myelination initiated by OLs. It is believed that pre-OLs are the key cellular target in preterm white matter injury. Pre-OLs consist of 04-positive, 01-negative pre-OLs and the 01-positive immature oligodendrocytes based on different labeling with monoclonal antibodies. The 04-positive, 01-negative pre-OLs account for 90% and 10% respectively of the total oligodendrocytes at 28 weeks gestation and are extremely vulnerable to hypoxic injury [133]. The vulnerability is maturation-dependent and not present in mature oligodendrocytes. Pre-OL injury may result in death or survival of pre-OLs with loss of cell processes or an apparent replenishment of pre-OLs but subsequent failure in differentiation [210-214]. As these cells do not seem to have the capacity for full differentiation to myelin-producing cells they are more vulnerable to subsequent hypoxic-ischemic insults [215].

PVL was originally thought to be due to ischaemia, but growing evidence now suggests that other factors (e.g., inflammatory processes and oxidative stress) play important roles. The pathogenesis of PVL is therefore characterised by an interplay between several factors: Vascular factors, intrinsic vulnerability of pre-OL, glutamate excitotoxicity and inflammation [32].

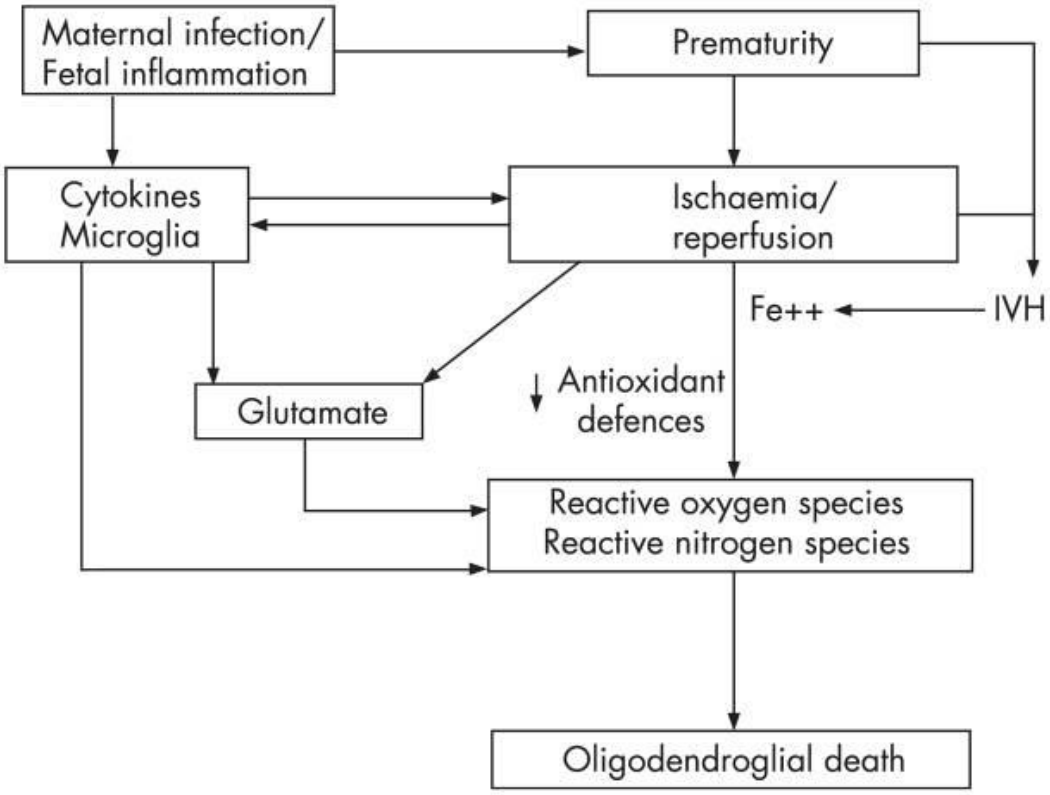


Figure 2.10: Pathogenesis of PVL. The two major upstream mechanisms are ischaemia and systemic infection/inflammation, activating three major downstream mechanisms, microglial activation, glutamate excitotoxicity and ultimately, free radical attack. *(Adapted from Volpe JJ, 2011 [216])*

Vascular factors: Cerebral blood flow regulation in a preterm is particularly vulnerable in the arterial end zones and border zones, i.e. the white matter, where the blood supply is immature. Furthermore, the blood flow to the white matter of the preterm brain is very low, and this provides a minimal margin of safety. Thus the preterm infant is vulnerable to low and fluctuating blood pressure [17, 217]. Carbon dioxide is a powerful vasodilator and important in cerebral blood flow regulation [218]. Hypocarbia has shown to be highly associated with white matter injury [219].

This is very important as hypocarbia often occurs as a consequence of ventilatory management in preterm infants [17].

Inflammation and systemic infection: Maternal, intrauterine and neonatal infection is seen in 65% of VLBW infants and can contribute significantly to the pathogenesis of pre-OL injury [32]. A number of studies have shown an association with PVL and infection/inflammation. [17, 178, 220-223]. Microglial activation appears to be the key event as discussed below [17, 211, 224].

Interaction of hypoxia-ischaemia and systemic inflammation: Neuropathological studies have shown that there is a generally increased gliosis when hypoxia-ischemia occurs in conjunction with systemic infection/inflammation [224] and systemic infection may further potentiate hypoxic ischaemic insults. [174, 225-231]. Experimental mice models have shown that prior exposure of the fetus to infection/inflammation (by giving LPS) can sensitise the preterm infant to low postnatal hypoxic ischemic insults resulting in increased PVL and white matter injury [228].

Specific vulnerability of pre-OL to free radical injury: Pre-OLs are thought to represent the major cellular target in PVL. Experimental models suggest that ischaemia and inflammation lead to cell death principally by free radical mechanisms [32]. In vivo and in vitro studies have shown evidence of oxidative stress in PVL [211, 215, 232] and it is the pre-OLs that are susceptible to free radical attack [17, 211, 233-237]. Free radicals accumulate in pre-OLs possibly because antioxidant defence mechanisms are insufficiently developed in these immature cells [233, 238-241].

Hydrogen peroxide accumulates and is converted to hydroxyl radical in the presence of ferrous ions (Fenton Reaction). This is supported by deposition of iron in the pre-OLs and in PVL [242-244]. IVH also results in large amounts of iron deposition and it has been shown that IVH can increase the incidence of PVL by 5-9 times [244]. Free radical production leads to cell membrane damage, excitotoxic energy depletion, cytosolic calcium accumulation, and activation of pro-apoptotic genes that cause damage to cellular components and result in cell death [245].

Vulnerability of Pre-OLs to Excitotoxicity: Excitotoxicity refers to excessive release of glutamate, which has been shown to contribute importantly to the pathophysiology of PVL. Failure of the sodium-potassium-adenosine triphosphate (Na-K-ATP) pump, which maintains the polarity of the neuronal membrane, is seen in ischaemia with subsequent loss of the Na^+ gradient across the membrane. This results in failure of sodium dependent glutamate transporters and excessive glutamate release [246-249]. The main glutamate transporter EEAT2 has high expression in pre-OLs during the peak period of PVL occurrence [250]. Excess glutamate is released into synapses and re-uptake is decreased during and after ischaemia [251]. Glutamate causes pre-OL injury and death by receptor and non-receptor mediated mechanisms.

A. Receptor mediated glutamate toxicity: The glutamate receptors, alpha-3-amino-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate (KA) are seen mainly in the cell body of the pre-OLs and when excessively activated results in Ca^{2+} influx, generation of reactive oxygen species and reactive nitrogen species (ROS/RNS) and subsequent cell death [237, 246, 252-263]. It has been shown in experimental preterm animal rat models that carotid ligation and ischaemia results in pre-OL death

and subsequent hypomyelination as seen in human PVL and AMPA receptor blocking drug such as topiramate can prevent this white matter injury [253]. It has also been shown in experimental work that it is the pre-OLs (and not the mature OLs) that are deficient in GluR2 subunit, which prevents Ca2+ influx [237, 254, 257, 264, 265]. The other glutamate receptor NMDA receptor is mainly present in the oligodendroglial processes of the pre-OLs and when excessively activated in ischaemia results in loss of processes but not death [246, 260, 266-271]. Activation of the NMDA receptor leads to massive influx of sodium and calcium and generation of ROS/RNS. Experimental rat model work has shown that memantine, a NMDA receptor blocker can decrease white matter injury [266, 270]. It is likely that NMDA receptor activation occurs earlier than in AMPA receptor activation as NMDA receptor has a higher affinity for glutamate receptor.

Loss of axonal degeneration has also been seen in PVL and possibly due to trophic support of the OL processes and myelin [272].

B. Nonreceptor-mediated glutamate toxicity (oxidative glutamate toxicity): Glutamate competes with cystine for glutamate/cystine antiporter. Cystine is reduced to cysteine, a precursor of glutathione within the cells and blockade of cystine transport by glutamate results in glutathione depletion, oxidative stress induced injury and necroptosis (non-apoptotic cell death) [273-275].

Vulnerability of pre-OLs to systemic infection and inflammation: Microglial activation appears to play a central role in diffuse PVL [211] but how systemic inflammation leads to cerebral microglial activation is unclear [17, 174]. The theories

include transfer of pathogen-associated molecular patterns (PAMPs) or pathogen-activated immune cells into brain, effect of cytokines on brain endothelial cells, or a combination of the above mechanisms [17, 231, 276-278]. Activation of microglia via toll-like receptors results in release of cytokines, ROS/RNS, these in turn can impair glutamate uptake and reduce the expression of glutamate transporters [224]. Lechpammer et al showed in experimental preterm rat model that minocycline, a drug which that inhibits microglial activation can decrease pre-OL injury and PVL [279]. Astrocytes are the main source of the cytokine interferon and pre-OLs are mainly targeted as they have an interferon receptor [239]. Tumour necrosis factor (TNF), another important cytokine has been shown to have independent toxic action and is essential for LPS induced toxicity.

3. DRUG DEVELOPMENT IN NEONATES

Although smallpox and rabies vaccination was discovered in the eighteenth and nineteenth centuries, most drug development in paediatrics and especially in neonates has taken place in the last century with the concept of the randomised clinical trial introduced from the latter half of the twentieth century. Several of the milestones that impacted on paediatrics have their origins in other disciplines (e.g. the discovery of antibiotics starting with sulphonamides in Germany).

3.1. Need for clinical drug studies in neonates

Good prescribing includes quality assurance (including an age-appropriate formulation), correct dose prescription, efficacy (knowing which medicines work under well controlled conditions) and sufficient information about safety and risks of the medicine for a clinician to make a reasonable judgment about the benefit–risk balance for the medicine in a particular baby [280]. The 'Make Medicines Child Size' campaign by WHO and UNICEF have highlighted the global need for more studies to obtain age-appropriate information for medicines [281]. The number of drugs currently being studied in neonates has remained very small with most studies being conducted in full term infants.

3.1.1. Off label drugs

Although the term 'therapeutic orphans' was coined for children [282], neonates are the real therapeutic orphans as most medicines given to neonates have not had a formal review under the licensing process. The few drug studies that are carried out

in this population have been in full term neonates while the population admitted on the neonatal intensive care is primarily preterm infants; as a consequence they are not labelled for use in this age group [283]. This is compounded by the frequent off label use of drugs that have been approved from adults. Up to 90% of neonates are receiving medications in an unlicensed and off-label manner [284]. Traditionally neonatal doses are empirically derived, with unknown safety and presumed efficacy [285, 286]. This on several occasions has been shown to have serious consequences. One such example is the toxicity of chloramphenicol in neonates recognised when three neonates died without explanation during treatment with chloramphenicol [287]. Subsequently a trial of prophylactic antibiotics in prolonged rupture of membranes showed that the group treated with chloramphenicol had mortality of 60% while mortality was 18% in the placebo group [288]. Later studies showed that elimination of chloramphenicol is reduced in preterm infants compared with older children and adults and its accumulation leads to the grey baby syndrome [289].

3.1.2. Toxicity

Limited repertoire of signs and symptoms in neonates can mask the recognition of adverse drug reactions. For example, toxicity produced by benzoic acid can easily be mistaken for sepsis [290]. Developmental changes highlighted below can increase adverse effects. In addition polypharmacy is common in neonates [291]. Also most of the adverse events frequently highlighted to support need for paediatric drug testing has occurred in the neonatal population [292].

3.1.3. Delayed consequences

Specific consequences of medical interventions may only become apparent long after exposure. For example, maternal treatment with diethylstilboestrol to prevent preterm birth was associated with the occurrence of adenocarcinoma of the vaginas in young women whose mothers received diethylstilboestrol during their pregnancies [293]. Unravelling possible long-term effects on skeletal, behavioural, cognitive, immune and pulmonary development and function requires studies in large number of newborns coupled with mechanistic experimental and clinical studies to demonstrate a cause- effect relationship. One of the biggest challenges is the difficulty of distinguishing between drug effects on growth and development and abnormalities related to prematurity [294].

3.1.4. Delayed implementation of proven therapies

Even when paediatric and neonatal clinical studies have shown benefit, implementation of proven therapies into clinical practice is often delayed. For example, the development of surfactant replacement therapy for respiratory distress syndrome in preterm newborn infants, spanned more than 50 years after Kurt von Neergaard in 1929 showed experimentally the importance of surfactant and its relevance to the first breath of a newborn baby. Clinical trials during the 1980s showed that surfactant replacement therapy almost halved the mortality from respiratory distress syndrome and is now used routinely in respiratory distress syndrome [295].

3.1.5. Formulations

Tablets and capsules are unsuitable for the neonatal population. Lack of liquid oral formulations has resulted in the practice of crushing or pulverizing tablets or opening capsules and mixing with formula or a solid food with usually no data documentation on the absorption of the desired dose [296, 297]. Although taste perception develops early in fetal life, palatability studies on drug formulations are usually not available. Potentially toxic excipients may be present in the formulations [298-302]. Propylene glycol, benzyl alcohol and polyethylene glycol have been implicated in neonatal toxicity. Some formulations require large volumes to be given and this is difficult on the neonatal intensive care where most neonates have restricted fluids.

3.2. Developmental pharmacology in neonates and implications for research

Although concepts of clinical pharmacology apply to all neonates, the characteristics of this population require a tailored approach and have implications for research. Improving our knowledge in developmental physiology and pharmacology can improve our approach to treating various disorders and diseases Studies guiding dosing adjustments which are often more complicated than simply scaling down adult doses on the basis of weight as human growth is not a linear process and age associated changes are dynamic [303, 304]. Evidence from neonatal studies is critical in understanding developmental variations in the pharmacokinetics and pharmacodynamics of a drug with improved understanding of the efficacy, tolerability and safety of a drug.

3.2.1. Pharmacokinetics and Ontogeny

Absorption: Intraluminal pH changes will not only affect the stability of a drug but also change the amount of drug available for absorption. As intragastric pH is relatively high in neonates, oral administration of acid labile drugs like penicillin G results in greater bioavailability in neonates while phenobarbital, a weak acid may have a lower bioavailability [305, 306].

Developmental changes in absorptive surfaces influence the bioavailability of a drug [307]. Delayed gastric emptying in neonates results in slower absorption of drugs and a longer time to achieve maximum plasma concentration. Developmental differences in the activity of intestinal enzymes and efflux transporters can affect bioavailability [308]. Usually in neonates the intravenous route is often preferred as there is a wide variability in absorption. Also most neonates requiring intensive care are invariably kept nil by mouth or on trophic feeds.

Distribution: The two important variables to take into account in paediatrics are the body composition and binding capacity related to circulating plasma proteins [309, 310]. Relative to body weight, the total body water content in neonates is markedly higher (80–85%) than in infants (60–65%) or adults (60%). Similarly, the body fat content is lower in neonates (10%) and increases to 15–20% in infants and 20–25% in adults. These changes will affect the distribution of lipophilic and hydrophilic drugs. For example, a lower dose of propofol (a lipophilic drug) is required to achieve plasma peak concentration in neonates similar to that of adults. In contrast, a higher dose of paracetamol is required in children to obtain plasma concentrations similar to those

of adults. Competitive binding at albumin sites between a drug and endogenous compounds such as bilirubin can result in increase in free bilirubin; high free bilirubin levels are cytotoxic and potentially result in kernicterus in neonates. Progressive gestation dependent increase in the ratios of brain to plasma phenobarbital suggest that the passive diffusion of drugs is age dependent although data is limited [311].

Metabolism: Cardiovascular collapse associated with grey baby syndrome in neonates administered chloramphenicol is probably the most common cited consequence of not recognising developmental immaturity [288]. The maturation of drug metabolising enzymes (DME) can contribute to extensive interindividual variability and increased risk of adverse reactions. Drug inhibition of a specific DME activity superimposed on an already developmentally decreased enzyme activity can have significant implications. For example, CYP3A4 activity is low at birth and increases to 50% adult levels by 1 year of age. Co-administration of erythromycin an inhibitor of CYP3A4 with midazolam a CYP3A4 substrate produces deeper and prolonged sedation in neonates compared to older infants and children [312]. CYP1A2 also has a low activity in the neonatal period and matures rapidly in the first year of life [313].

Elimination [314, 315]: At birth the glomerular filtration rate is 2–4 mL/min/m2 in term neonates but can be as low as 0.6–0.8 mL/min/m2 in preterm infants. Adult values of 6 L/hour/70 kg are achieved by 1 year of age. As renal clearance in neonates is dependent on glomerular filtration rate, there is a prolonged elimination half-life of drugs cleared predominantly by this route. Diminished renal function can increase the toxicity of excipients.

3.2.2. Developmental pharmacodynamics

Maturation of the biologic systems can impact on the potency, efficacy or therapeutic range of drugs. For example, neonates have more mu opioid receptors and hence are more sensitive to morphine, which can result in adverse effects such as an increased frequency of apnoeas [316]. GABA receptors that switch from an excitatory to inhibitory mode during early development help to explain paradoxical seizures experienced by infants after exposure to benzodiazepines [317].

Unfortunately, there are only limited data on the influence of growth and development on drug effects and further research is needed.

3.3. Difficulties of performing clinical trials in newborns

Ethical issues, lack of incentives for pharmaceutical sponsors, limited number of patients available, lack of appropriate study designs and uniqueness of neonatal conditions makes it difficult to perform clinical studies in neonates.

Neonatal studies are often challenging. Neonates are not just one group but actually include preterms at different gestation and term infants. Each group may have different dosing needs, different responses to medications and different adverse reaction profiles, and may need different dosage forms for safe and accurate administration. Also most of the clinical trials are required to be set up on the neonatal intensive care units, which often have limited resources or equipment for efficient conduct of the studies. For example, there may be little if any research nurse support for the clinical staff which makes errors and protocol violations more likely

and electronic data capture is often not available, so that data must be transcribed on to case record forms manually. Multiple trial sites and a longer period of recruitment are usually needed to achieve an adequate sample size, which increases complexity and makes neonatal trials expensive. In addition, the limited pool of patients available for clinical research fosters competition amongst studies. As a consequence neonatal studies tend to be smaller than adult studies, the findings are less likely to be confirmed in additional studies and therapies may be introduced into practice or rejected without a sufficient evidence base. A number of study design issues have to be taken into consideration in this vulnerable population. In some conditions there is no universally accepted definition e.g. apnoea of prematurity. Other important design problems are stratification by gestational age and postnatal age; identification, definition and relationship of endpoints and clinical surrogates to clinically relevant outcomes. Often there is a lack of a validated biomarker to measure outcome. For example testing efficacy of sedatives can be problematic because of a lack of a validated scale to measure to sedation. These further add to the cost and complexity of clinical trials in these populations, making neonatal trials unattractive for pharmaceutical companies. With a small market and lack of incentives, clinical trials in neonates are usually considered not cost-effective. The unforeseeable nature of some clinical responses and long-term effects, compounded with difficulty in predicting dose–response relationships by extrapolation of adult data further hinders neonatal clinical research.

There are specific ethical issues concerning clinical trials in neonatal populations. As a result of these factors many medications given to children today are off-label, often without adequate understanding of the appropriate dose, safety or efficacy.

3.4. Ethical Considerations for neonatal clinical drug trials

3.4.1. Risk Benefit Assessment

The most important ethical question is whether the benefit of the knowledge gained from research is reasonable in relation to the risk posed to the participating children. It is important that this vulnerable group is protected against the risks of research but at the same time this should not deny children the benefits of research and improve the treatments available to them. As highlighted above, there is a need to carry out clinical trials in children but a child's interests must always prevail over those of society and science.

A fundamental principle of paediatric research is the ethical principle of scientific necessity; that is, children should not be subjected to research that is not relevant to child health. A corollary is that children should not be enrolled in studies that are duplicative or unlikely to yield important knowledge applicable to children about the product or condition under investigation. The risks should be considered along with the severity of the condition or diseases, age of the child and the risks and benefits of alternative treatments. For example, in oncology trials, the adverse reactions tolerated would be higher than those for an antibiotic to treat a urinary tract infection (Table 3.1.).

Table 3.1: Determinations of research risks and potential benefits (From Field and Boat [318].

Are risks to research participants minimised by using procedures that are consistent with sound research design and that do not unnecessarily expose participants to risk and, whenever appropriate, by using procedures already being performed for diagnostic or treatment purposes? *Are risks to participants reasonable* in relation to anticipated benefits to participants and to the importance of the knowledge reasonably anticipated from the research? *Is the selection of research participants equitable*, taking into account the purposes of the research, its setting and the special problems of research involving vulnerable populations, such as children? *Are appropriate provisions for monitoring participant safety made?* *Are appropriate provisions for protecting privacy and confidentiality made?* *Does the research meet the regulatory criteria for studies involving children*, including those requiring parental permission and, as appropriate, child assent?

All paediatric research must meet certain ethical standards and this applies to all human research as covered by European Union (EU) and US legislation. The research ethics committees in the European states and the institutional review boards in the USA were created under regulations aimed at ensuring that research is conducted ethically and that the rights and welfare of research participants are protected thus conforming to the terms of Good Clinical Practice as specified by the International Committee on Harmonisation [319]. The guidance also discourages enrolment in multiple clinical trials, as may happen when a condition is rare in childhood. There is a conflict of interest policy to protect the integrity of research from bias arising from the financial relationships of the investigators. Regulatory groups Medicines and

Healthcare products Regulatory Agency (MHRA) in UK and Food and Drug Administration (FDA) in the US also conduct inspections and audits to ensure the scientific and ethical integrity of clinical trial data. Sponsors have a legal responsibility to select appropriate investigators and to monitor the conduct of the research studies.

Risks are grouped into three categories as shown in Table 3.2.

Table 3.2: Risk categories and examples [320]

Risk category	Procedures
Minimal risk	History, examination, blood pressure, ultrasound, single venepuncture
Minor risk over minimal risk	Multiple venepuncture, peripheral venous line, nasogastric tube
Greater than minor increase over minimal risk	Sedation, anaesthesia, surgery

Minimal risk is defined as 'a probability of harm or discomfort not greater than that ordinarily encountered in daily life or during the performance of routine physical or psychological testing' [320]. This risk can be quantified as similar to that of a single car trip across town during a rush hour, which is considered to pose approximately a one in 100,000 chance of death in a child.

Benefit can be defined as 'progress in treatment, diagnosis or prevention for the individual or group'. This could be seen as an increase in efficacy, a better safety profile or an alternative to an existing treatment such as a better route of administration, decreased frequency of dosing, reduced treatment duration, a more

relevant age-appropriate formulation or an improvement in relation to potential medication errors.

3.4.2. Consent and Parental Involvement

One of the key elements of clinical trials is that there is informed consent and this could be seen to be a significant impediment to clinical trials in children [321]. Informed consent can be challenging especially in emergencies and there may be limited time to obtain consent [322]. Consent is a dynamic and continuous process and there is a need for periodic checks and documentation at repeat visits as stated in the EU guidance [320]. The lack of legal ability to consent has implications for the design and analysis of clinical trials and the comparators used in trials. It is a requirement of ethics committees and regulatory authorities that parental permission for a child's participation in research is obtained. Financial inducements can influence parental consent; for example, there is a correlation between rates of consent, the offer of free medication and lower family income [323, 324]. Clinicians and parents may be reluctant to enrol a child in a trial, especially a placebo-controlled trial, when the trial medication is already approved for marketing to adults and thus available for off-label use. It is also good practice for the investigator to take consent rather than the treating physician unless it is an emergency situation.

Input of parents in the design of the research is important and gives perspective of families. The experience of the National Institute for Health Research Medicines for Children Research Network (NIHR MCRN) is that parent input to studies is invaluable.

3.4.3. International clinical trials

With globalisation of research and the increasing numbers of international studies, there have been concerns about the adequacy of the oversight of the international trial sites especially whether appropriate protections are in place for children and their families [325, 326]. Maintaining the quality of research data across all trial sites is critical for good quality international research, as discrepancies in data at different sites will bias results. There may be conflicts of interest and inappropriate inducements for parents to permit their children's participation in research, as these drugs may not be available without research. Furthermore, there is the risk that trial medications and medical care may stop when the trial is finished. In addition, there is concern that any new medications arising from the research will be limited to wealthier countries, as the new drugs will be unaffordable in the poorer countries. Further discussion about international trials ethics is given in the Nuffield Council on Bioethics [325]. It is recommended that in a multinational study each country should carry out its own research ethical review.

3.4.4. Trial design and methodology

Pain, fear, distress and parental separation are unique in paediatrics and need to be taken in consideration. Age-appropriate formulations are recommended to reduce the risk of adverse reactions and the risk of dosing errors because of inaccuracy. Parental consultation and patients from the age groups included in the trial are paramount. Ethics committees now have a parent advisory board member. Design methods are optimised to allow for the least number of patients to be recruited to

give a statistically and clinically significant result. Pharmacokinetic studies in neonates often require adaptation as there are specific guidelines for the amount of blood that can be taken in children [327]. This equates to 2.4 mL/kg for the 3% limit over a 4-week period, that is, less than 2 mL in a very low birth weight preterm. The burden of repeated blood sampling in PK studies may be overcome by the population PK approach as the statistical solution to sparse data [328]. This potentially decreases the number of patients that need to be recruited. Timing of samples should coordinate as far as possible with therapeutic sampling. Local anaesthetics and indwelling catheters should be used whenever possible.

Placebo-controlled trials: Placebo-controlled trials are usually regarded as the gold standard but this may mean that effective treatment may be withheld. EU guidance states that the placebo should not be used if effective treatment will be withheld. Off-label treatments can be considered for use as a comparator drug in a trial if they are considered to be standard of care. This may result in increased adverse events and should be highlighted. For neuroprotective studies in hypoxic ischaemic encephalopathy, therapeutic hypothermia is used as a comparator rather than placebo as cooling is now standard of treatment.

3.4.5. Safety monitoring

With evolving knowledge during the conduct of the trial clinical risk may change so it is essential that monitoring takes place. A review of paediatric randomised controlled trials from 1996 to 2002 showed that very few (13%) paediatric trials had a data safety monitoring committee [42]. EU and US guidance recommends that an

independent data safety monitoring committee with appropriate expertise is used. When not appropriate, for example, in certain small PK studies, this should be justified.

3.5. Regulation in Paediatric Drug Development

Although children have benefitted from regulatory laws in adults [329-331], drug testing in paediatrics came into focus only in the 1990s [332-337]. The shortage of paediatric drug studies and the delayed initiation and completion of paediatric studies compared to studies in adults has prompted many regulatory changes and guidelines over the last decade to promote further studies in paediatric populations.

The two US paediatric legislations, FDA Modernisation Act and Pediatric Research Equity Act, are often described as the 'carrot and the stick' as they combine mandatory requirements to carry out appropriate drug studies in children with a reward in the form of market exclusivity [333, 334, 338]. Similarly, The EU Regulation on Paediatric Medicines established a legislative framework to allow increased availability of medicines specifically adapted and licensed for use in the paediatric population and improved quality of research in children. Similar to the US legislation there were financial incentives and market exclusivity was given along with a system of requirements [336].

3.5.1. Paediatric Investigation Plan

The paediatric investigation plan (PIP) is a development plan aimed at ensuring that the necessary data are obtained through studies in children, when it is safe to do so,

to support the authorisation of a medicine for children [336]. Pharmaceutical companies submit proposals for PIPs to the Paediatric Committee. The Paediatric Regulation requires these plans to be submitted to the Agency early wherever possible, in contrast to US requirements which require submissions at a much later date although the development plan can be modified at a later stage, as knowledge increases.

PIPs should include the following:

• A description of the studies and of the measures to adapt the medicine's formulation to make its use more acceptable in children, such as use of a liquid formulation.

• Cover of the needs of all paediatric age groups, from birth to adolescence.

• Examination of the timing of studies in children compared to adults.

In some cases, studies can be *deferred* until after the studies in adults have been conducted. This ensures that research in children is carried out only when it is safe and ethical to do so. Even when studies are deferred, the PIP should include details of the paediatric studies and their timelines

As some diseases do not affect children (e.g. Parkinson's disease), the development of medicines for these diseases should not be performed in children. In these cases, a PIP is not required and it will be *waived*. Key components of application for a PIP are summarised in Table 3.3.

Table 3.3: Key components of a Paediatric Investigation Plan [339]

Part A: Administrative and product information,	
Part B: Overall development of the medicinal product	
Part C: Applications for product specific waivers,	
Part D: Paediatric investigation plan	
D.1	Purpose of and existing data for the proposed paediatric development
D.1.1	Paediatric Investigation Plan indication
D.1.2	Selected paediatric subset(s)
D.1.3	Outline of the existing quality, non-clinical and clinical data
D.2	Quality aspects
D.2.1	Strategy in relation to quality aspects
D.2.2	Outline of each of the planned and/or ongoing, studies and steps in the pharmaceutical development
D.3	Non-clinical aspects
D.3.2	Strategy in relation to non-clinical aspects
D.3.2	Overall summary table of all planned and/or ongoing non-clinical studies
D.3.3	Synopsis/outline of protocol of each of the planned and/or ongoing non-clinical studies
D.4	Clinical aspects
D.4.1	Strategy in relation to clinical aspects
D.4.2	Overall summary table of all planned and/or ongoing clinical studies
D.4.3	Synopsis/outline of protocol of each of the planned and/or ongoing clinical studies
D.5	Timelines of measures in the paediatric investigation plan
Part E: Applications for deferrals,	
Part F: Annexes.	

3.5.2. Paediatric incentives

For new products and certain changes to the marketing authorisation for products still covered by patent protection, a 6-month extension of the supplementary protection certificate if information arising from a completed PIP is incorporated into the Summary of Product Characteristics.

For orphan medicinal products there is a 2-year extension of market exclusivity if information arising from a completed PIP is incorporated into the Summary of Product Characteristics.

For off-patent products a new category of marketing authorisation called the paediatric use-marketing authorisation will be associated with a 10-year period of data and market protection.

3.6. Planning Neonatal Drug Development Studies

High quality neonatal studies are important so as to expedite the drug pipeline and to reduce the risks involved in this vulnerable population [280, 339, 340]. **Efficacy** (does the drug work in research environment) needs to be established with documented safety before moving on to study **effectiveness** (how to use the medicine in real life). Phase 4 studies and registries are used to develop the drug 'life-cycle' further.

3.6.1. Strategy in relation to clinical aspects

Indication whether the therapeutic agent is being studied for intended diagnosis, prevention or treatment should be clear with justification of conducting the studies in this vulnerable population with strengths and limitations clearly outlined [335] . The group in which studies are conducted should be representative of the population in which the drug will be used. The study should be feasible and should avoid unnecessary repetitive work. If the disease has similar progression in neonates, children and/or adults, with comparable response to treatment expected, extrapolation of adult efficacy data may be appropriate. In this case, pharmacokinetic

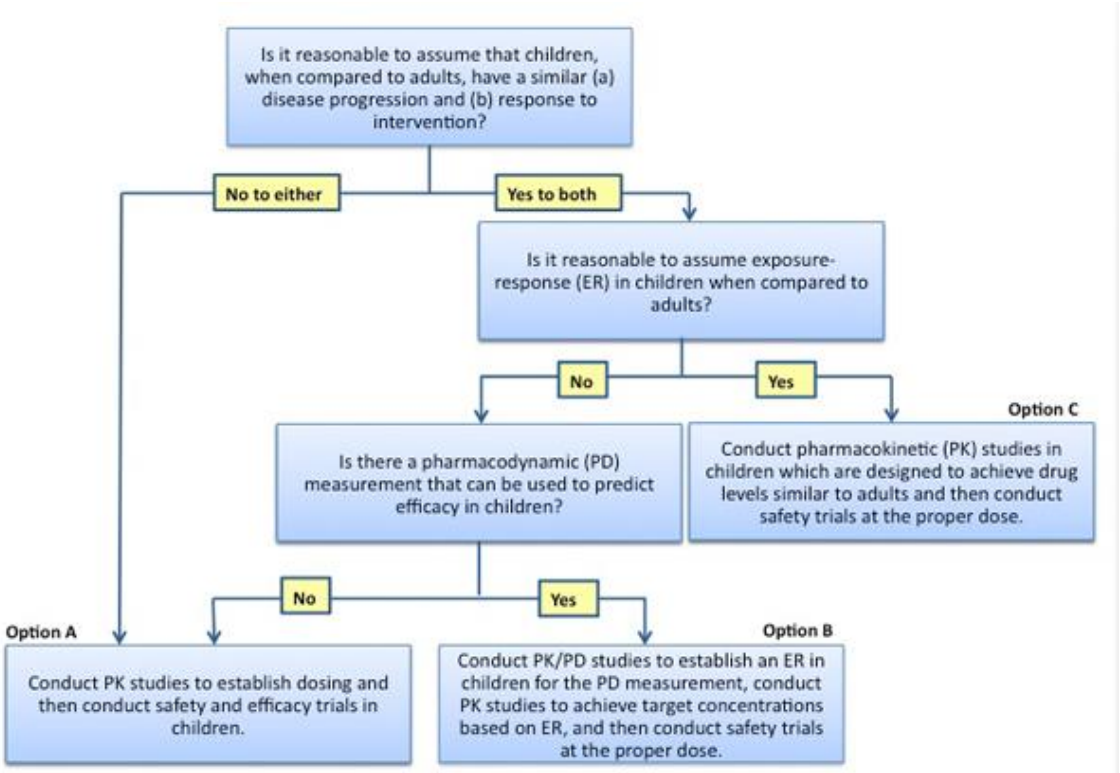
studies can be done to demonstrate the equivalent dose extrapolated from adult studies together with safety studies. An approach based on pharmacokinetics is likely to be insufficient for medicinal products where blood levels are known or expected not to correspond with efficacy or where there is concern that the concentration-response relationship may differ between the adult and paediatric population, then it is useful to use measurements of a pharmacodynamic effect related to clinical effectiveness to confirm the expectations of effectiveness and to define the dose and concentration needed to attain that pharmacodynamic effect (PK/PD approach) combined with safety studies.

Unfortunately some neonatal conditions are unique and may use the same or similar drug as in adults but for different indications. Here it is important to conduct dose-finding studies to first optimize the dose and then follow on with safety and efficacy studies. In all cases information on safety issues relevant to neonates is obtained along with the data required for long-term safety studies.

Tolerability refers to the demonstration that there are no gross problems that would prevent a medicine from being used in a population. This is always a concern when a medicine is first given to humans. Neonatal studies are rarely first in human studies however this is a highly vulnerable population requiring intensive care.

Figure 3.1. gives a decision tree for neonatal drug developmental studies. There needs to be a clear understanding about each stage of the drug development process with flexibility built in so that if there are unexpected results at any stage, changes can be made in the later studies.

Figure 3.1: Decision tree for neonatal drug developmental studies [341]



Adaptive Designs for Clinical Trials: Measures should be taken to protect the neonatal study population during development and adaptive designs are often used. An adaptive design clinical study is defined as a study that includes a prospectively planned opportunity for modification of one or more specified aspects of the study design and hypotheses based on analysis of data (usually interim data) from subjects in the study [342]. Adaptive designs have the advantage of making a clinical study more efficient by shortening the duration of the study or recruiting fewer patients. They can also provide improved understanding of PK-PD response (e.g. by providing broader dose-response information or subgroup effects), can lead to better quality studies (e.g. revising sample size based on event rates or discontinuation, thus

avoiding an underpowered study) and increases the likelihood of success of study objective. One of the weaknesses of an adaptive design is that it can introduce bias and result in an increased rate of false positive study results (increased Type 1 error). Bias can adversely affect development decisions, such as choice of dose, population or study endpoints in subsequent studies. The risk of bias can be greatly reduced if adaptations rely only on blinded analyses, but in exploratory trials this can be difficult. When the study design includes adaptations that, during the course of the study, change the nature or type of data used in the primary analysis (e.g., changing the endpoint), interpretation of the study results could become more difficult. This uncertainty can become increasingly problematic when multiple adaptations are made during conduct of the study.

Biomarkers and Surrogate Outcomes: World Health Organization (WHO) defined a biomarker as any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease [343]. Biomarkers need to validated (can measure what it is supposed to measure accurately and reproducibly) and qualified (does it predict outcome) [344]. Biomarkers are often required in neonatal clinical trials either to define inclusion or exclusion criteria or to define endpoints and are sometimes used as surrogate outcome measures [345]. Unfortunately few biomarkers used in neonates are validated and qualified. Biomarkers do not always necessarily correlate with a patient's experience and sense of wellbeing in contrast to clinical endpoints (how a patient feels, functions or survives) [346]. A validated and qualified biomarker can sometimes be used as outcomes in clinical trials and are considered as surrogate

endpoints or substitutes for clinically meaningful endpoints [346]. Use of surrogate endpoints can expedite clinical trials and the drug pipeline. Primary clinical endpoints such as death can occur infrequently so that their use in trials can be impractical or even unethical. Diagnosis of disability or delayed development in newborns may require to wait for 2 years at least and possibly for 6 years to make a definitive diagnosis. Biomarkers can provide interim evidence about safety and efficacy of treatment while clinical data is being collected. It also reduces the number of patients required in a clinical trial and protects patients from non-therapeutic effects and possible harmful effects of an experimental drug. Thus by reducing the time to approval of new medications, more efficient trials could speed the overall drug development process, allowing effective treatments to reach their target patient populations sooner, while conserving both material and human resources for other research projects, even other clinical trials.

For example the randomised controlled trials of therapeutic hypothermia in term infants included a meta-analysis of 767 patients using neurological outcome at 18 months as clinical outcome[347], while using conventional MR imaging 131 patients are needed [348] and only 20 patients are needed if computational MR imaging is used [349]. On the other hand biomarkers may be indirect signs of pathway that are not fundamental to the key disease processes and may be wrongly used. Also it is often assumed that surrogate markers for predicting effects of a given class of treatment on one clinical endpoint can be safely relied on acting as surrogates for evaluating other classes of treatments or for other clinical endpoints, this may not always be true [350].

Contemporary methodologies such as dried blood spots and population pharmacokinetic models allow neonatal drug trials to be more feasible and reduce the number of patients recruited in a study thus minimising the uncertainties that hinder drug development.

3.6.2. Strategy in relation to quality aspects

Chemical, pharmaceutical, biological and biopharmaceutical aspects related to the administration of the product should be taken into account while setting the studies. As highlighted previously it is important that formulation along with route of administration i.e. 'fitness for purpose' is decided before the clinical trials are embarked upon along with plans for bridging between formulations. Potential issues related to formulation such as excipients should be considered and anticipated.

3.6.3. Strategy in relation to non-clinical aspects

If human safety data and previous animal studies are considered insufficient for reassurance, juvenile animal studies should be considered. This may be especially important for long-term outcomes and late adverse events.

Mechanism based approach: This drug discovery process corresponds to the target based approach, which takes a rational and scientific approach to the drug discovery process by defining specific molecular mechanisms or mode of action to be targeted by the treatment based on biological and clinical findings [351]. Mechanistic studies often utilise in vitro techniques and can help in channelizing specific drug therapies in the clinical pipeline and improve our understanding, refining and implementing a

drug therapy. In addition it might become possible to identify subsets of patients on which treatment may be more effective i.e. a tailored approach to management. This can improve the chances of success for the drug. However it is important that along with target based studies, this should include studies of the whole organ or animal to validate any biological relevance [352].

3.6.4. Merits of repurposing existing medicines

It is estimated that the probability of a novel drug for reaching preclinical development was only 3% [353]. Each drug discovery takes 2-4 years and on an average 33 molecules have to be evaluated to identify one drug that can proceed into the preclinical development stage, that means at least 66 years of research are required to produce one successful drug discovery project [351]. This is not counting for the attrition rates caused by toxicological effects and lack of clinical effects. Hence there are advantages of using existing drugs for new indications. Drug re-profiling strategy allows novel uses for existing medicines whose safety and pharmacokinetic effects have been confirmed already in humans. It can also allow for development of safer drugs for example by identifying mechanisms underlying side effects of a drug, new medicines with similar target mechanisms but less side effects can be developed. This is highly important in the neonatal world where commercial pharmaceutical companies may not be willing to invest a lot of time and money for a specialised population. Repurposing existing drugs has been linked to the concept of ‘eco-medicine’ i.e. making drugs economically viable [354].

Summary

Neonatal drug development is especially challenging as it poses considerations and requirements over and above those required for drug development in children and adults. Developmental differences in neonates make it necessary to conduct clinical trials in this population. In addition, there are unique ethical dilemmas, such as the investigators' duty to safeguard the best interests of the child and the requirement of parental consent. Recent government directives and regulations represent significant and important steps forward in testing new and established off-label drugs for children. A systematic well thought process allows for neonatal drug development studies to proceed with difficulties. Consideration of pharmacoeconomics of medicines in neonates is important and repurposing existing medicines, using adaptive designs and biomarkers can expedite drug development in neonates.

4. STUDIES OF NEUROPROTECTIVE THERAPIES IN NEWBORN INFANTS

Currently, prematurity cannot be prevented. Standard neonatal intensive care is routine but this has not brought about a significant decrease in neurodevelopmental impairment and neurodisability. The consequences of perinatal brain injury will stay with an individual for their whole lifespan and yet there are no effective protective or restorative treatments currently in routine clinical use. This is clearly an unmet medical need.

The goals of a neuroprotective therapy would be to prevent injury progression, salvage and protect the cells that would otherwise be injured or die, repair the injured cells, enhance neurogenesis with the long-term goal of improving neurodevelopmental outcomes. A lot has been learnt recently from animal and adult studies on the mechanisms of brain injury and potential treatment strategies, yet little has been translated into clinical research. Few neonatal clinical trials of neuroprotective therapies have been conducted particularly in the preterm population. Nevertheless, the success of moderate hypothermia in term hypoxic ischaemic encephalopathy has shown that neuroprotection is possible in the developing brain.

There are 3 main issues in planning any neonatal neuroprotection study: the study population, outcome measures studied and the agent.

Study Population: The adult and neonatal brain does not respond to insults in the same manner due to differences in many factors, including gene regulation during hypoxia and disparate susceptibility to oxidative stress and excitotoxicity [355]. Hence

therapies that may look promising in adults may not work in the same way in neonates. Antenatal factors such as multiple pregnancy, chorioamnionitis, intrauterine growth restriction along with delivery complications: abruption, cord prolapse, need for resuscitation rather than stabilization and post natal factors such as prolonged ventilation, surgery, poor nutrition, infection can not only contribute to the heterogeneity of the outcome but makes this group of patients a high risk population. Planning studies can also be affected by reluctance to put vulnerable neonates into clinical trials. However, it can easily be argued that they also need to benefit from research specific to their needs.

Outcome measures*:* To measure neuroprotective effect using clinical assessment requires a large number of patients and 2-6 years follow-up without loss of patients. Recent work has shown that it is important to have a longer follow up and impairment seen at a younger age may not be present at a later age. Also as shown by the caffeine neuroprotection trial, neuroprotective effects of a drug may not be seen when examined in the older age group on follow up [356, 357]. This understandably causes a significant delay in moving the pipeline of drugs from bench to bedside. This has led to the search for qualified biomarkers, which can reduce the sample size along with the time to completion and cost. MR imaging methods offer appropriate outcome measures to allow appropriately powered small studies.

The agent*:* The neuroprotective therapy should not only combine all or some of the goals mentioned above along with ability to cross the blood brain barrier, but be stable, easy to administer and have a safe pharmacokinetic profile with few or no side effects. It is also important that the drug does not interfere with normal on-going

development and growth. The drug should not have adverse interactions with on-going treatment required for these sick infants. The timing, duration and dose of the drug can be critical in optimising benefit as neuronal apoptosis is prolonged after brain injury and that neurogenesis and angiogenesis play an important part in brain repair.

A number of therapies are in the preclinical phase or currently undergoing phase 2 clinical trials. They include molecules that target the different mechanisms of brain injury described above. Some of the potential neuroprotective therapies are discussed below.

4.1. Growth Factors

Growth factors have been known to be neuroprotective when administered after hypoxic-ischaemic injury for nearly three decades, when injection of insulin-like growth factor-1 (IGF-1) was shown to reduce brain injury. More recent studies have shown that preconditioning sublethal hypoxic stimulus also provides long lasting protection against subsequent insults [358-362] through stabilisation of the transcription factor Hypoxia-inducible factor 1α (HIF-1α) which generates production of neuroprotective growth factors, such as IGF-1 vascular endothelial growth factor (VEGF), and erythropoietin (EPO) [358, 363]. These factors offer attractive opportunities for neuroprotection.

Erythropoeitin (EPO): EPO is a red cell production regulator with immunomodulatory effects and vasogenic and proangiogenic effects through its interaction with VEGF [364-366]. There is increasing evidence that EPO has a neuroprotective effect. The

protective effects are a combination of direct neuronal receptor mediated interaction and indirect effects. It has been shown in many animal models that EPO decreases apoptosis [367], excitotoxicity [368], glutamate toxicity [369] and inflammation [370]. It stimulates and interacts with other protective factors such as brain derived neuroprotective factor (BDNF) and glial derived neutrophic factor (GDNF) [371]. EPO has angiogenic properties and promotes angiogenesis and repair along with VEGF [372]. EPO also promotes maturation and differentiation of oligodendrocytes that are critically vulnerable and important in white matter injury [373]. EPO has been shown to improve cognitive outcome [374-376] with evidence of neurogenesis [365, 377]. Delayed treatment 24 hours after the insult was also neuroprotective [378].

Translation to clinical studies: Although EPO for neuroprotection is not approved for use in neonates, it's safety and efficacy as a haemopoietic agent has been well explored in both adults and children and has been used for treatment of anaemia in preterm infants [379]. In a recent literature review of potential neuroprotective therapies [380] for neonatal hypoxic injury, EPO was regarded by the assembled panel of experts as the 2nd most attractive agent for therapeutic investigation.

Pharmacokinetics: EPO can easily be given intravenously or subcutaneously and crosses the blood brain barrier in higher doses [381-383]. No neurorotective effect has been established in human infants, and consequently the optimal neuroprotective dose, dosing interval, timing of administration and duration is unknown. Higher doses with more frequent dosing is required in neonates to achieve equivalent haemopoietic response [384]. Doses used to treat anaemia of prematurity (200-400 IU/kg) did not raise brain EPO concentrations. Also as the molecular size of

EPO is quite large (37kD) only 1-2% of EPO would cross the blood brain barrier. The dose required for neuroprotection is therefore likely to be much higher [385-387]. Multiple doses or a longer duration of treatment may provide more sustained neuroprotection as compared to a single dose strategy as seen in some animal studies [388]. This may be due to the fact that EPO decreases late apoptosis and may stimulate repair processes such as neurogenesis, angiogenesis and migration of regenerating neurons [389].

EPO has been tested for neuroprotection in adult and neonatal animal models of brain injury in a range of doses from 300 IU/kg/dose to 30,000 IU/kg/dose [368]. In rat pups with hypoxia-ischaemic insult, neuroprotection was achieved with a dose 5000 IU/kg, to achieve peak plasma concentrations between 6000 and 12000 mU/kg, with sustained levels in the 6000 mU/mL range for up to 12 hours [383].

Wu et al [390] conducted an open label phase I study to determine the safety and pharmacokinetics of EPO in 24 moderate to severe HIE term infants who were receiving therapeutic hypothermia as a standard. The doses ranged from 250-2500 U/kg given intravenously and each patient received 6 doses every 48 hours starting within 24 hours of age. They showed that although EPO followed nonlinear pharmacokinetics, excessive accumulation did not occur during multiple dosing. No deaths or serious adverse effects were seen. They concluded that EPO given at 1000 U/kg/dose IV in conjunction with hypothermia was well tolerated and achieved plasma concentrations that are neuroprotective in animals.

Juul et al [387] also showed in preterm infants that EPO followed non-linear pharmacokinetics. In an open label, randomised control safety and efficacy study of preterm infants less than 28 weeks gestation, both 1000 and 2500 U/kg produced peak serum EPO concentrations that were comparable to neuroprotective concentrations that are seen in experimental animals but only the 2500 U/kg dose provided sustained levels above 6000 IU/L. Steady state was achieved within 24 to 48 hours. No excess adverse events occurred in the infants given EPO as compared with the control.

Safety: EPO may be protective to other systems. EPO receptors are known to be present in the developing intestine and EPO may protect the gut from necrotising enterocolitis [391]. On the other hand, EPO was shown to have increased cardiovascular adverse events and death when used in cancer treatment [392]. A clinical trial in stroke was recently suspended [393]. Whilst acknowledging that higher doses are probably required for neuroprotection it is also important to remember that very high doses are likely to be harmful rather than beneficial [394]. There are also concerns that high doses of EPO can cause thrombotic and haematological complications [395, 396].

Clinical trials of EPO in neonatal neuroprotection: Five clinical trials looking at safety and efficacy of EPO as a potential neuroprotective therapy in neonates have been published: two in preterm infants and three in term infants [390, 397-400], however only 2 of these studies looked at outcome measures between 6-18 months. In both the clinical trials, the patient numbers were small and a decreased length of follow up A pilot trial using EPO in infants requiring cardiac surgery has also been done recently

did not show any neuroprotective effect but authors suggest the dose was small [401]. Further trials are ongoing or about to start- one in France and another in USA.

IGF-1: IGF-1 is important for growth and maturation of the fetal brain as well as differentiation of oligodendrocyte precursors [402] and has recently been shown to protect pre-OLS against TNF-alpha induced damage [403]. Neonatal trials have not been conducted and safety, pharmacokinetic profile and therapeutic window is not known.

4.2. Antioxidants

Free radical injury is thought to be an important mechanism for preterm brain injury. There are a number of mechanisms that produce free radicals in biological tissues. Radicals are produced normally as part of the bactericidal function in leucocytes, and thus are increased in inflammatory lesions. They are also generated as a result of hypoxia, and as part of the nitric oxide signalling cascade. Nitric oxide has several chemical states, including ones with unpaired electrons, which can generate harmful hydroxyl radicals.

Nitric oxide synthase inhibitors: Selective inhibition of neuronal or inducible nitric oxide synthase (NOS), which produces nitric oxide, has shown potential as a neuroprotective strategy [404] but nonspecific blockade of NOS has not shown to be protective [405]. No studies of nitric oxide synthase inhibitors have been carried out in newborn infants, and the strong effects of NO on vascular tone and normal neurological functioning make these compounds problematic for trials in preterm infants.

Allopurinol: Allopurinol is a xanthine oxidase and xanthine dehydrogenase inhibitor and decreases free radical production as xanthine oxidase is a ready source of superoxide and hydrogen peroxide. Animal studies have shown to decrease acute oedema and long-term infarct volume when given in high doses [406]. Short-term benefits were seen in neonates requiring cardiac surgery for hypoplastic left heart syndrome [407] although there was no improvement after severe birth asphyxia in the 3 neonatal trials [408-410]. The intravenous preparation is highly alkaline and it is likely that allopurinol needs to be administered early in the injury process to prevent free radical injury [380]. No studies have been carried out in preterm infants.

N-acetylcysteine (NAC): NAC is a glutathione precursor, an antioxidant and free radical scavenger. Animal studies suggest that NAC attenuates lipopolysaccharide-induced white matter injury, marked improvement in the redox state and inhibition of apoptosis [411-413]. NAC may have significant side effects and is associated with anaphylactoid reactions in some patients [414]. Recently it was seen in fetal sheep that it can exacerbate LPS induced hypoxaemia and hyptension [415]. Nevertheless, the relatively favourable adverse effects profile make this compound a possible candidate for neonatal trials.

4.3. Excitotoxicity

Memantine is a NMDA receptors antagonist [416]. Memantine attenuates acute white matter injury in P6 rats, resulting in long-term histological improvement [270, 417, 418]. Clinical studies have not been done.

Topiramate an antiepileptic is an AMPA-kainate receptor antagonist shown to be neuroprotective in animal rat models [419].

Magnesium sulphate has also shown to be neuroprotective in animal models [420-422] and one possible mechanism is the blockade of NMDA receptors [423]. In a multicenter trial of antenatal magnesium intervention for preterm delivery, no perinatal side effects were seen and there was some benefit in the neurodevelopment of survivors [424]. However, magnesium administered to term HIE infants did not result in improvements in aEEG patterns and when given in larger doses was associated with profound hypotension [425, 426].

4.4. Anti-inflammatory agents

Therapies that block microglial activation and cytokine release may be neuroprotective. Minocycline, a tetracycline derivative has anti inflammatory properties, decreases microglial activation and caspase 3 expression, lipid peroxidation increases anti apoptotic gene expression, protects the oligodendrocytes [427-432]. Animal studies have not shown consistent results and there appeared to be a transient protective effect in a neonatal stroke model [433] while in the mouse model minocycline worsened brain injury [434]. Neonatal trials have not been done due to the known adverse effects of this class of tetracycline like agents on bone and teeth development.

4.5. Combination Therapy

As there are multiple upstream and downstream mechanisms involved in perinatal brain injury, targeting just one mechanism may not be the answer and a combination therapy providing synergistic and or an additive effect may be required for a more long lasting neuroprotective effect. For comparison, the prototypical neuroprotective therapy for cerebral injury in term infants suffering hypoxic ischaemic encephalopathy, moderate hypothermia, provides only an 11% reduction in risk of death or disability from 58 to 47% suggesting that adjuvant therapies might be beneficial. [347].

5. MELATONIN

Melatonin is an endogenous mediator with neuroprotective and antioxidant properties, robust pre-clinical data and favourable pharmacological and toxicological properties making it a premier candidate for neuroprotective treatment in neonates [352, 380].

5.1. The Pineal Gland and Synthesis of Melatonin

Melatonin (N-acetyl-5-methoxytriptamine) is synthesised in the pineal gland, a small, unpaired, central, cone shaped structure, essentially an appendage of the brain. The main function of the pineal gland in all species is to transduce information concerning light-dark cycles to body physiology, particularly for organisation of body rhythms via its main hormone melatonin [435-437].

Melatonin is synthesised within pinealocytes from tryptophan (Figure 5.1). N-acetyl transferase and hydroxyindole-O-methyltransferase are the 2 most important enzymes required for the synthesis of melatonin. Most synthetic activity occurs during the dark phase. The rhythm of production is endogenous in that it is generated in the suprachiasmatic nuclei, the major central rhythm-generating system or "clock" in mammals. The rhythm is synchronised to 24 hours primarily by the light-dark cycle acting via the retina and the retinohypothalamic projection to the suprachiasmatic nuclei [438].

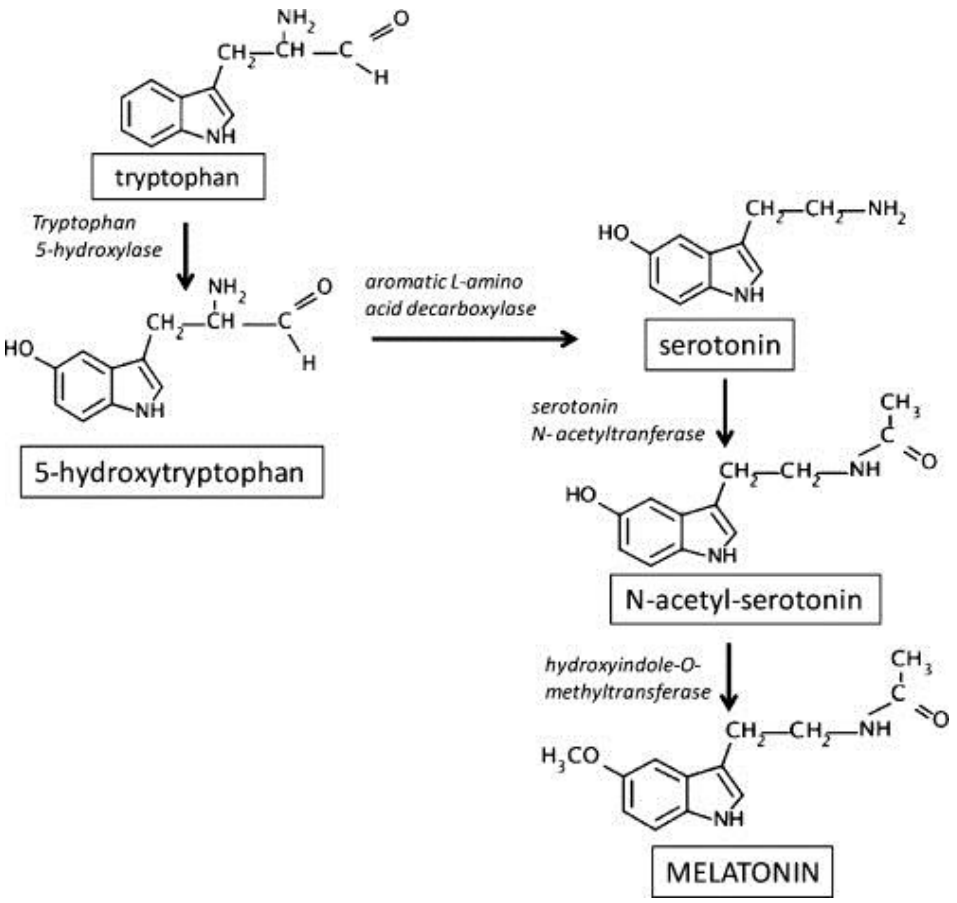


Figure 5.1: Melatonin Synthesis

5.2. Ontogeny of Melatonin Secretion

The fetus does not secrete melatonin but receives melatonin by rapid trans-placental transfer according to the maternal circadian secretion [439, 440]. In term infants, melatonin is secreted without diurnal variation in the first few weeks of postnatal life [441]. The production of melatonin is only 25% of adult levels at 24 weeks and 50% by one year of age [441]. Preterm infants even when corrected for gestation have a retarded melatonin rhythm development and rhythmic pineal hormone production is only apparent after 12 weeks of corrected gestational age [441]. The onset of pineal secretion is even more delayed when exposed to neurological insults [440]. Diurnal

variation appears by 6 months of life reaching maximum levels between 3-6 years of age [442]. During sexual maturation, melatonin secretion decreases and again by 40-50 years there is a decline in the melatonin concentrations with almost negligible levels by 70 years of age [443].

5.3. Current Knowledge of the Pharmacokinetics of Melatonin

General aspects of pharmacokinetics are discussed in chapter 6 along with population pharmacokinetics. In the subchapter, the pharmacokinetics of melatonin is discussed.

5.3.1. Melatonin Pharmacokinetics in Adults

In adults, the endogenous melatonin pharmacokinetic profile has been well defined [437, 444]. There is a circadian secretion of endogenous melatonin with a marked diurnal variation. Secretion usually starts by 9 pm and peaks at 3 am, then plasma concentrations decline to negligible levels by 9 am [437, 445]. The adult peak melatonin concentration is reported to be 44.3 pg/mL but can range from ≤ 8 - 275 pg/mL [445, 446].

The pharmacokinetic profile of melatonin administered to adults has also been well described. The pharmacokinetics of melatonin is constant in a given individual but there is marked variability among individuals.

Absorption: The oral bioavailability of melatonin is variable [447, 448] and 30-60% is immediately metabolised to 6-sulfatoxymelatonin (aMT6s) (first pass effect) [449]. Oral bioavailability is dependant on the dose and larger doses are likely to saturate first pass metabolism increasing the absorption [450]. In adults the reported absolute

bioavailability is about 15% when doses of 2 mg or 4 mg were given orally [451]. Inspite of variable bioavailability, melatonin is rapidly absorbed after oral formulation and time to maximum concentration (Tmax) after 3 mg oral ingestion of melatonin was found to be only 16 min [452].

Distribution: Once absorbed, melatonin is extremely lipophilic and is almost completely distributed in tissues and readily crosses the placenta and the blood brain barrier [439, 453, 454].

Okatani et al showed that there was no difference of mean melatonin concentrations in the maternal peripheral venous blood, umbilical arterial and umbilical venous blood after administration of a single oral dose of 3 mg to pregnant women pre-Caesarean section [439].

Plasma protein binding studies have not been extensively studied, although indirect evidence for plasma protein binding is provided by demonstration of differing levels of melatonin in blood and saliva as this fluid is devoid of albumin and globulins; the saliva/plasma ratio should reflect the free/bound ratio in blood. Salivary concentrations of melatonin represent an average of 23% of the total circulating melatonin level [455]. The physiological significance of the plasma binding of melatonin has not been seriously considered previously and is unlikely to affect the biological activity of the hormone as there is very low affinity binding measured (on the order of 1 mmol/L), which is many orders of magnitude lower than those of many other hormones. Thus, binding to proteins may have little real effect in buffering

transfer of melatonin across membranes or preventing binding to the high affinity melatonin receptors [455].

In adults, melatonin is excreted in breast milk with a diurnal variation of melatonin in human milk with a peak melatonin level in milk of 99 +/- 26 pmol/L [456]. In rat pups that were breast fed, there is no day-night difference in plasma melatonin [457]. No studies have been done looking at effect of melatonin in human milk and dosing studies.

In adults the distribution half-life, volume of distribution, time taken to reach steady state has been documented. After intravenous bolus injection of 5 mcg to healthy adults Iguchi et al showed that the distribution half-life was 3 minutes with an elimination half-life of 46.15 minutes and a mean apparent volume of distribution of 0.5 L/kg [458]. Mallo et al also showed a similar short distribution half-life and volume of distribution but a shorter elimination half-life of 28.4 minutes following a bolus dose of 5 mcg [459]. After intravenous infusion of 4 mcg/hr for 5 hours, the distribution half-life was similar of 3.27 minutes, an elimination half-life of 45.39 minutes and a volume of distribution of 0.88 L/kg [459].

In animal studies it has been shown that melatonin is readily taken up by the cerebrospinal fluid from the circulation with maximum levels attained within the cerebrospinal fluid, choroid plexus, hypothalamus and mesencephalon within few minutes after administration followed by rapid elimination of melatonin within 20 minutes [460-462].

Metabolism: Circulating melatonin is predominantly metabolised by hydroxylation at the C-6 position in the liver by hepatic P450 mono-oxygenases and then conjugated with sulfate to form aMT6s (70%) or to a lesser extent with glucuronide (6%) [463]. O-demethylation represents a relatively minor pathway to form N-acetylserotonin which is again excreted as sulphate and glucuronide conjugate [463]. Melatonin binds to human or rat liver microsomal cytochrome P-450 in the same manner as a type II substrate (Cyp1A > Cyp2B >Cyp2C) [464]. Studies with human tissue in vitro indicate that melatonin is almost exclusively metabolised by Cyp1A2 [465]. Ma et al showed that CYP1A1, CYP1A2 and CYP1B1 all 6- hydroxylated melatonin with CYPC19 playing a minor role. CYP1B1 is not expressed in the liver but has an extrahepatic distribution and may be important in regulating levels of melatonin in the intestine and cerebral cortex. It is likely that melatonin has extrahepatic metabolism as shown by elevated melatonin metabolite levels in the cerebral cortex. As there is minimal CYP1A2 in the brain, the metabolism of melatonin is possibly related to CYP1B1 [463].

The elimination half-life of melatonin in adults is 45 - 60 minutes [466].

Excretion: Less than 1% of circulating melatonin is excreted unchanged into urine [467, 468]. Urine melatonin concentrations in adults are variable but usually within the range of 38.2-179.1 ng in 24 hour samplings [469] and a mean of 29.7 ng (range 9-59.1 ng) in nocturnal samples [470]. Mean aMT6s excretion in adults was 11100 ng (range 4100-24200 ng) in a 16 hour period 6 pm -10 am [470, 471].

There is a strong correlation with the adult endogenous melatonin blood levels and aMT6s in the blood and urine [467, 471]. On oral administration of exogenous

melatonin, serum melatonin has shown to correlate well with urinary melatonin but not with aMT6s [447, 470]. This is probably related to first pass effect where 30-60% of melatonin is immediately metabolised to aMT6s and hence a certain portion of absorbed melatonin never appears in the systemic circulation or in the body tissues but is directly excreted as aMT6s in the urine.

5.3.2. Melatonin Pharmacokinetics in Infants and Children

There is no data on the pharmacokinetics of melatonin in neonates. There is limited data on the pharmacokinetics of exogenous melatonin in the paediatric population. In a study of the pharmacokinetics of intravenous melatonin, 9 prepubertal (mean age 8.4 years) and 8 pubertal children (mean age 12.9 years) received a single dose of melatonin, 0.5 μg/kg [472]. The elimination half-life was 0.67 hours in prepubertal and 0.78 hours in pubertal. This was comparable to that observed in adults in the same study of 0.79 hours. The authors concluded that there were differences in the pharmacokinetics that were dependent upon sexual maturity, specifically that there is a shorter half-life and a smaller AUC in prepubertal children than in adults. These differences were thought to be due to faster metabolism in children than in adults.

In a study of exogenous melatonin in four children (ages 1-4 years) with therapeutic cerebrospinal fluid drainage, oral melatonin dose of 5 mg was shown to produce an increase in cerebrospinal melatonin levels between 10 and 80 minutes after intake [473].

The adult pharmacokinetic data on melatonin demonstrates a very short distribution half-life, a short elimination half-life, a high systemic clearance, relatively low volume

of distribution compared to other hormones and presence of tissue binding –sites of high affinity and rapid clearance from the brain suggesting easy diffusion.

5.4. Pharmacogenetics

There is not much work done on the genetic makeup influencing an individual's response to melatonin. The cytochrome p450 enzymes play an important role in the metabolism of melatonin. CYP1A2 and CYP1B1 have shown to have interindividual and racial variability in metabolic activity due to single nucleotide polymorphisms [463, 474]. These differences can lead to significant changes in the melatonin levels especially in the target tissues.

5.5. Measurement of Melatonin

de Almeida et al have written an extensive review of the different methods and procedures used for measurement of melatonin and its metabolites [475]. Some of the methods used today are summarised in Table 5.1.

Melatonin and its metabolites are commonly measured by radioimmunoassay (RIA) [444, 471]. In this method, a known amount of radioactive melatonin (I^{125}-iodomelatonin or 3H-melatonin) is mixed with a fixed amount of antibody and increasing concentrations of unlabeled melatonin are added to the mixture, which will compete with labeled melatonin causing its displacement from the antibody. Free-labeled melatonin is then separated from remaining antibody-bound radioactive melatonin and radioactivity is measured. As the concentration of unlabeled melatonin increases in the mixture, competition for the antibodies also increases and

bound labeled melatonin decreases. A calibration curve constructed from known amounts of labeled and unlabeled melatonin allows the determination of unknown melatonin concentrations in biological samples.

Measurement of melatonin in urine is not widely used as only a small amount of melatonin appears unaltered in the urine and extraction from the urine can be tedious [467].

Table 5.1 Methods used for measurement of melatonin (HPLC- high performance liquid chromatography, RIA- radioimmunoassay, GCMS- gas chromatography mass spectrometry

Type	Repeatability (intra-assay variation)	Reproducibility (inter- assay variation)	Limit of Detection	Advantage	Disadvantage
Isocratic HPLC with flurometric detection [476] Liquid-Liquid extraction with dichoromethane on Chem-Elut catridges	3.24%	9.4%	0.5pg/ml	Does not need internal std Short run time Simple extraction procedure	Presence of several interfering substances makes it difficult to assay
HPLC with fluorimetric detection [477] Solid and liquid phase extraction		9.4%	5 pg/ml	Sensitive	Large dilution factor, Presence of several interfering substances makes it difficult to assay Recovery 70%
Reversed phase HPLC using electrochemical detection [478]	2.54%	7.05%	1 pg/ml	Short analysis time <10 min Simple procedure	Variable reproducibility

RIA [479]	7.2%	6.6%	0.5 pg/ml	Highly specific and sensitive Ease of measurement of large samples at the same time	Potential risk of cross reactivity with similar compounds
GCMS [475]				Most specific and sensitive and is used to validate other techniques	Time consuming Labour intensive
Immunoaffinity chromatography [480] Polyclonal antibodies characterised by enzyme-linked immunosorbent assay were used for preparation of immunoaffinity gel, followed by HPLC-MS	1.9%	5.4%	10 fmol	Single step processing	Potential risk of cross reactivity with similar compounds
Capillary Electrophoresis with UV or fluorescence or electrochemical detector [481]	3.73%	4.6%	9 ng/ml	Very small volumes Ease of sample taking	Not been validated

5.6. Experimental Evidence of Neuroprotective Effects of Melatonin

Melatonin has been shown to have significant neuroprotective effects in in-vitro and in-vivo preclinical models. It is sometimes difficult to assess if the model used is a preterm or term injury model, although melatonin appears to have a neuroprotective effect in both types of injury. Some of the important studies are highlighted below.

5.6.1. Neonatal Brain Injury Models

In the rat model for preterm brain injury both in vivo and vitro, melatonin has been shown to attenuate myelination defects, promote oligodendroglial maturation and decrease microglial activation leading to a normalisation of the myelination process [482]. Melatonin has also been studied in a fetal sheep model of preterm brain injury which demonstrated that melatonin attenuates the increase in activated microglia, suggesting decrease inflammatory response and a reduction of cell death (decrease in TUNEL positive cells) in the cerebral white matter [483]. Melatonin given to pregnant rats prevented oxidative mitochondrial damage in premature fetal rat brain post ischaemic insult [484]. This was also seen in the late gestation fetal sheep brain in response to umbilical cord occlusion [485]. In a recent study melatonin was found to reduce microglial activation, macrophage infiltration and apoptosis when administered to a spiny mouse model of birth asphyxia for 7 days at the end of the pregnancy prior to hypoxia–ischaemic insult [486]. In a mouse model of excitotoxic-induced periventricular white matter injury [487], melatonin when given with or prior to insult, produced significant protection against ibotenate (activating NMDA receptors) and S-bromo-willardiine (activating AMPA and kainite receptors) induced

white matter lesions but did not affect the cortical plate lesions (Figure 5.2i). Melatonin did not modify the lesion size in the first 24 hours but over the next 4 days induced dramatic regression in white matter lesion (upto 82% reduction) (Figure 5.2ii). When given after the excitotoxic challenge, neuroprotection was a function of time with protection from white matter injury being seen if melatonin was given within 4 hours of insult (Figure 5.2iii). In the same study, melatonin also induced axonal re-growth or sprouting thus promoting lesion repair, this is of particular relevance in preterm brain injury where growth failure is a significant component. In a follow-up study melatonin preserved learning abilities on water-maze testing [488].

In a recent study in the perinatal asphyxia piglet model, Robertson et al showed that the Lactate/Creatine and Lactate/N acetyl aspartate were significantly lower in the piglets who received melatonin along with moderate hypothermia [489].

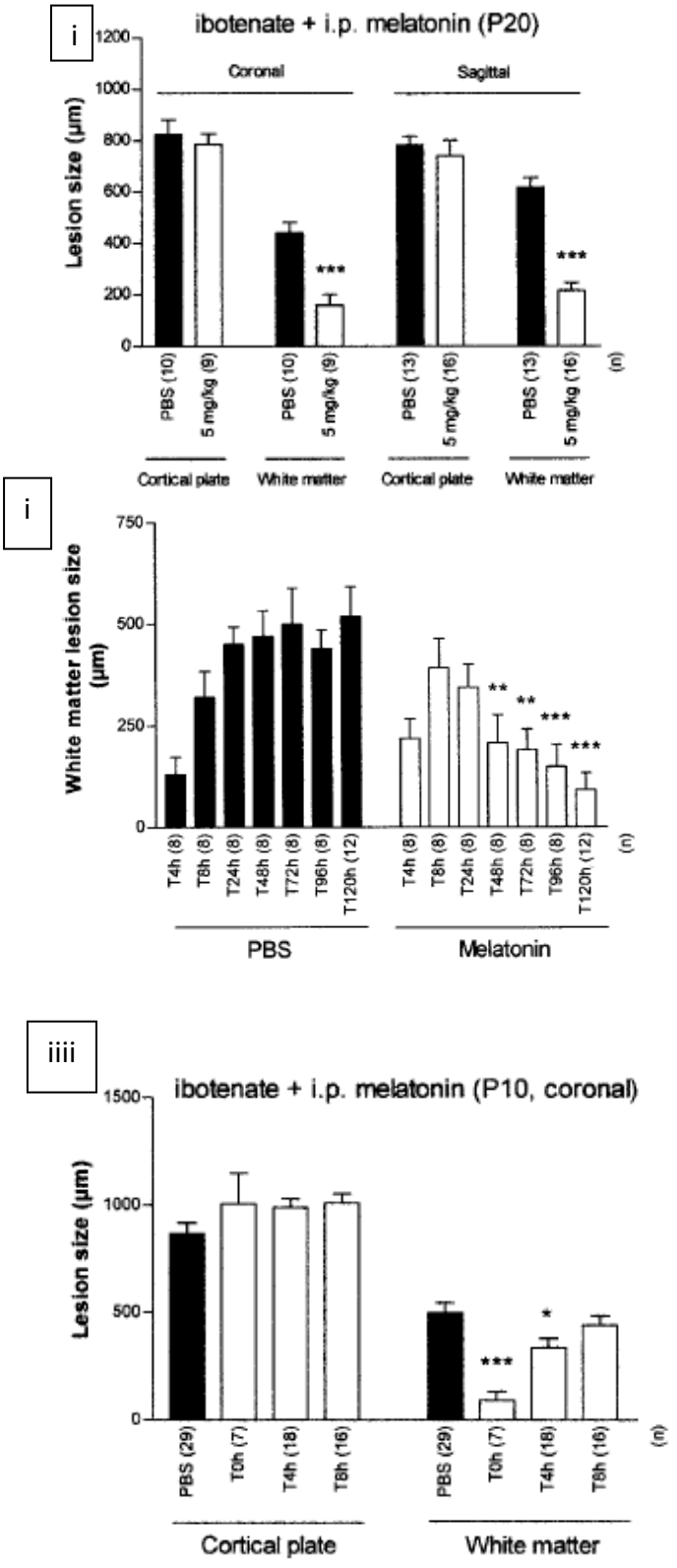


Figure 5.2: **Mouse model of preterm brain injury**, (i) melatonin produced significant protection against ibotenate induced white matter lesions but did not protect against cortical plate lesions; (ii) melatonin did not modify the lesion size in the first 24 hours

but over the next 4 days induced dramatic regression in white matter lesion; (iii) decrease in white matter lesion was seen if melatonin was given within 4 hours of insult *(Reproduced from Husson et al, 2002 [487]).*

5.6.2. Mature Brain Injury Models

In adult animal models of traumatic and focal ischaemic brain injury, melatonin decreases the size of injury, neuronal cell death, oxidative stress, DNA damage, mitochondrial injury [490-493] and also alters the enzyme activities that improve the total anti-oxidative defence capacity of the organism [494, 495]. It is also neuroprotective in mature animals via inhibition of caspase-3 and attenuation of apoptosis [496, 497]. Melatonin prevents kaïnate-induced neuronal cell death and DNA fragmentation; reduces microglial inflammation; maintains glutathione homeostasis and decreases reactive oxygen species mediated apoptotic neuronal death [498-501]. In mature melatonin-deficient rats, lesions induced by hypoxia-ischemia were larger than controls suggesting that endogenous melatonin is neuroprotective [502]. In stroke models, melatonin has been shown to decrease the infarct size if given before or up to 2 hours after the insult [503].

5.6.3. Mechanism of Neuroprotective Action of Melatonin

The mechanisms underlying the neuroprotective benefits of melatonin are not yet fully elucidated. Melatonin's protective actions are believed to stem from the interaction of its receptors MT1 and MT2 with its coupling to G proteins [504] and its direct free radical scavenging (receptor-independent actions) [505] and because of yet-undefined functions. MT1 and MT2 are members of the 7-transmembrane G-

protein-coupled receptor family and are expressed by microglia, astrocytes and oligodendrocytes. Both receptors are involved in signalling mainly through inhibition of cAMP and protein kinase A activity [506]. Luzindozole a melatonin receptor blocker appears to abolish the neuroprotective effects of melatonin [482]. It is likely that melatonin in smaller doses acts via the melatonin receptors, which are potential targets for various disorders such as circadian sleep disorders, depression, cardiovascular diseases and immune dysfunction [507] and higher doses are required to obtain an antioxidant effect [487]. Indeed Husson et al showed that melatonin at lower doses decreases white matter lesions but not cortical lesions while antioxidants like N acetyl cysteine appears to protect both lesions [487]. The third melatonin receptor MT3, an enzyme quinone reductase, is unlikely to be involved in neuroprotection as blockade of the MT3 receptor by prazosin did not appear to have any effect on the neuroprotective effect of melatonin [487]. There is some evidence of circadian variation in the melatonin receptor expression [508].

5.7. Other Factors Contributing to the Neuroprotective Benefits of Melatonin

Brain growth may be vulnerable to immunological, nutritional or pharmacological factors associated with premature exposure to the extra uterine environment. The beneficial effect of melatonin on multiple systems may explain its potent neuroprotective effect.

5.7.1. Immune system

Melatonin decreases serum inflammation markers in septic neonates and improved clinical outcome for these babies [509]. A growing body of evidence suggests that

melatonin acts an immunomodulator. It can promote not only the proliferation of peripheral blood mononuclear cells, but also the proliferation of neonatal cord blood mononuclear cells. This suggests that melatonin could be involved in the regulation of the newborn immune system. This is also of particular relevance as pre-OLs are highly vulnerable to inflammation [217, 510].

5.7.2. Respiratory system

Melatonin decreases cytokine levels in preterm respiratory distress syndrome and chronic lung disease. The total duration of ventilation and maximum Fi02 used was decreased in preterm babies given melatonin [511, 512].

5.7.3. Surgery

Melatonin decreases oxidative stress post-surgery in neonates, although clinical benefits of this have not been clearly defined [513].

5.8. Other Uses of Melatonin

Melatonin is believed to have a direct impact on sleep. It is used to prevent jet lag, sleep disorders and as a premedication [514-518]. Melatonin is increasingly prescribed for children with learning disabilities and Attention Deficit Hyperactivity Disorder to help them sleep [519].

5.9. Safety Profile of Melatonin in Pharmacological Use

5.9.1. Animal studies

Adverse effects were only observed when very high doses of melatonin were administered (100-300 mg/kg). Some of the studies are summarised below.

Toxicology: Male and female rats behaved similarly after a single administration.

Single Dose Toxicity: Melatonin might be described as only slightly, or practically not acutely toxic. Nonclinical data in the literature indicate that melatonin has hypnotic properties. In terms of potency, melatonin is similarly hypnotic to thiopental and propofol but is less able to abolish the righting reflex in rats following intravenous administration [520]. Single dose toxicity of melatonin was observed with doses in excess of 250 mg/kg in the rat, 100 mg/kg in the dog and 125 mg/kg in the rabbit (Communication with Alliance, MENDS IMPD).

Repeat Dose Toxicity (Communication with Alliance, MENDS, IMPD)**:** Melatonin might be described as not toxic after repeated administration. In non-pivotal studies, toxicity of melatonin was in excess of 250 mg/kg in the rat, 50 mg/kg in the dog and 125 mg/kg in the rabbit. Histopathological changes were limited to the liver of rats that had received 1000 or 1500 mg/kg/day. They were of the adaptive (enzyme induction) type and were accompanied by hypertrophic and hyperplastic changes in the thyroid. The rat is particularly sensitive to such changes in the thyroid gland. In the dog at termination, higher than expected liver weights were encountered that

probably also indicate adaptive change. Adverse changes in the rabbit were non-specific.

In pivotal studies; in the 13-week rat study, animals were dosed at 100, 300 and 1000 mg/kg/day. The main observations were transient post-dose clinical signs, darkened faeces (melatonin degradation products) and initial reductions in bodyweight gain and food consumption. Increases in thyroid gland, liver and kidney weights were seen with correlating hypertrophy in the liver and thyroids. Darkened thyroids were seen at all dose levels macroscopically with pigmentation only being recorded microscopically at 1000 mg/kg/day. The no observed adverse effect level (NOAEL) for rat males was 300 mg/kg/day and for females 100 mg/kg/day. In the 13-week dog study doses of 2, 12 and 72 mg/kg/day were well tolerated. Dark/black faeces (melatonin degradation products) were noted in all animals given 72 mg/kg/day. Fluctuating food consumption and, in females, corresponding low individual bodyweight gains were noted in two animals given 72 mg/kg/day. Although these findings were confined to a small number of animals, they were considered to be potentially adverse. There were no other toxicologically significant effects of treatment. The NOAEL for dogs for 13 weeks was therefore considered to be 12 mg/kg/day.

Genotoxicity: There is no evidence of genotoxicity in literature [521] and melatonin is neither toxic nor mutagenic in the Ames test or mouse lymphoma test (Communication with Alliance, MENDS, IMPD). In-vivo testing on doses upto maximum tolerated dose of 700 mg/kg/d for mouse did not induce micronuclei in this test system (Communication with Alliance, MENDS, IMPD).

Carcinogenicity: Melatonin is not classifiable with respect to carcinogenic risk for humans. There is no evidence of carcinogenicity, attributable to melatonin, in literature. In fact there is considerable evidence to suggest that melatonin may have a protective or slowing effect on carcinogenesis [522].

In vitro studies have shown that uptake of melatonin into hepatoma results in anticancer activity in vitro [523]; inhibition of growth of diethylstilbestrol-induced prolactin producing pituitary cell tumour [524] on treatment with melatonin; pinealectomy or a constant light regime results in mammary carcinoma in rat [521]. In vivo studies have shown better survival in non small cell (lung) tumour patients treated with chemotherapy and melatonin [525].

Reproductive and Developmental Toxicity: There are some reports of melatonin having an effect to sperm production and function. One report was of possible involvement in mediation of testicular regression in aging hamsters [526]and a possibility of reduction of mobility of rat sperm in vitro [527]. In the frog, melatonin seems to have an inhibitory action on spermatogonial proliferation, possibly by a direct local action on the Leydig cells [528].

In a double blind crossover study in man, extended daily treatment with 3 mg resulted in reduction of sperm count and motility in two out of eight subjects [529].

Fertility and Early Embryonic Development to Implantation: Low dose melatonin has been shown to restore full sexual activity in impotent male rats [530]. No effect was seen on embryonic development in pinealectomised rats [531].

In the rat fertility and early embryonic study commissioned by the Alliance Pharmaceuticals (Communication with Alliance, MENDS IMPD), animals were orally dosed with 100, 300 or 1000 mg/kg/day melatonin prior to mating, and for the females, for up to 6 days of gestation. A number clinical observations such as decreased activity, flat posture, rapid breathing, partially closed eyes, unsteady gait and in the males, red scrota that were dose related but which resolved within the first few days of dosing. Black faeces (melatonin degradation products) were seen throughout the study. At the high dose, body weight and food consumption was lower. In addition, there were more acyclic females and consequently few oestrus cycles. The high dose had no effect on mating activity, but there were fewer live embryos per female because of a lower number of corpora lutea per female. There was no dose related effect on pre or post-implantation loss. The NOAEL for the following parameters were considered to be: toxicity: 300 mg/kg/day (male), 300 mg/kg/day (female); fertility and mating performance: 1000 mg/kg/day (male), 300 mg/kg/day (female); pregnancy and early embryonic development: 300 mg/kg/day.

In the developmental range finding studies, (Communication with Alliance, MENDS, IMPD), reduced rat litter size and fetal weight was observed at 1000 mg/kg/day. Maternal body weight loses were observed at 1000 mg/kg/day and reductions in body weight gain at 250 and 500 mg/kg/day. The rat NOAEL fetal was taken as 1000 mg/kg/day and the maternal 500 mg/kg/day.

In the rabbit, post implantation losses were higher at 250 mg/kg/day, though there were no maternal effects. Maternal NOAEL was 125 mg/kg/day, and fetal 250 mg/kg/day.

In the rat general reproductive range finding study, weight loss was seen at various times with 1000 mg/kg/day. At 1000 mg/kg/day (maternal), pup bodyweight gain was lower over days 1 to 7 of lactation. The parental NOAEL was 1000 mg/kg/day, neonatal 500 mg/kg/day. In the rat and rabbit developmental toxicity studies at 300 and 750 mg/kg/day, dose-related bodyweight loss was recorded for 1 day; these doses were also associated with lower bodyweight gain and lower food consumption lower. At these doses, there appears to be developmental delay. The fetal NOAEL was 300 mg/kg/day, and maternal 300 mg/kg/day.

Pre- and Post- Natal Development and Maternal Function: Melatonin is said to have a role in the maintenance of gestation in the Indian Palm Squirrel rodent [532]. Studies conducted by Alliance Pharmaceuticals (MENDS, IMPD), showed no significant toxicity in rat pre-natal and post-natal development studies. The maternal NOAEL was taken as 300mg/kg/day and the postnatal NOAEL for offspring was taken as 300 mg/kg/day pre-weaning and 750 mg/kg/day post-weaning.

Cardiotoxicity: In vitro HERG study done by Alliance Pharmaceuticals (MENDS, IMPD) did not produce a statistically significant inhibitory effect on HERG tail current and concluded that melatonin did not predict prolonged cardiac action potential and hence QT interval in man. In the in-vivo dog telemetry study conducted by the same group, it was observed that oral administration of 2, 12 and 72 mg/kg/day melatonin did not have any statistically significant effect on arterial blood pressure, heart rate or lead II ECG parameters in the 20 hours following dosing.

Robertson et al noted that there was a significant fall in blood pressure when piglets were given melatonin at 10 mg/kg/hr (total dose of 60 mg/kg) but 5 mg/kg/hr over 6 hours was well tolerated [489].

Phototoxicity and Photosensitivity: Moderate phototoxicity has been predicted from in vitro studies [533].

5.9.2. Human Studies

Adults: Exogenous melatonin has been used in various conditions including jet lag, shift worker sleep disorder, delayed sleep phase, advanced sleep phase, sleep wake disorder, the blind, non-24 hour sleep wake disorder, as well as in the elderly population where both sleep disorders and impaired melatonin production are common. Two meta-analysis which included 27 trials with 873 participants showed no evidence of adverse effects of melatonin with short term use (three months or less) in primary or secondary sleep disorders and sleep disorders occurring with sleep restriction [534, 535]. The most commonly reported adverse events were headache, dizziness, nausea, and drowsiness. The occurrence of these outcomes did not differ significantly for melatonin versus placebo. Melatonin administration has been associated with mild-moderate cooling which may be part of the physiological action [536].

Melatonin is not licensed in the EU. Circadian, 2 mg prolonged release melatonin tablet is licensed in adults over 55 years for short-term treatment of insomnia. Table 5.2 gives the adverse events listed in the summary of product characteristics (SMPC).

Table 5.2: Adverse events listed in the SMPC of Circadian

Within each frequency grouping, undesirable effects are presented in order of decreasing seriousness: very common ≥1/10); common (≥1/100 to <1/10); uncommon (≥1/1,000 to <1/100); Rare (≥1/10,000 to <1/1,000); Very rare (<1/10,000); Not known (cannot be established from the available data).

There were no very common, common or very rare adverse events listed in the SMPC

System Organ Class	Uncommon	Rare	Not known
Infections and infestations		Herpes zoster	
Blood and lymphatic system disorders		Leukopenia, thrombocytopenia	
Immune system disorders			Hypersensitivity reaction
Metabolism and nutrition disorders		Hypertriglyceridaemia, hypocalcaemia, hyponatraemia	
Psychiatric disorders	Irritability, nervousness, restlessness, insomnia, abnormal dreams, nightmares, anxiety	Mood altered, aggression, agitation, crying, stress symptoms, disorientation, early morning awakening, libido increased, depressed mood, depression	
Nervous system disorders	Migraine, headache, lethargy, psychomotor hyperactivity, dizziness, somnolence	Syncope, memory impairment, disturbance in attention, dreamy state, restless legs syndrome, poor quality sleep, paraesthesia	
Eye disorders		Visual acuity reduced, vision blurred, lacrimation increased	

Ear and labyrinth disorders		Vertigo positional, vertigo	
Cardiac disorders		Angina pectoris, palpitations	
Vascular disorders	Hypertension	Hot flush	
Gastrointestinal disorders	Abdominal pain, abdominal pain upper, dyspepsia, mouth ulceration, dry mouth, nausea	Gastro-oesophageal reflux disease, gastrointestinal disorder, oral mucosal blistering, tongue ulceration, gastrointestinal upset, vomiting, bowel sounds abnormal, flatulence, salivary hypersecretion, halitosis, abdominal discomfort, gastric disorder, gastritis	
Hepatobiliary disorders	Hyperbilirubinaemia		
Skin and subcutaneous tissue disorders	Dermatitis, night sweats, pruritus, rash, pruritus generalised, dry skin	Eczema, erythema, hand dermatitis, psoriasis, rash generalised, rash pruritic, nail disorder	Angioedema
Musculoskeletal and connective tissue disorders	Pain in extremity	Arthritis, muscle spasms, neck pain, night cramps	
Renal and urinary disorders	Glycosuria, proteinuria	Polyuria, haematuria, nocturia	
Reproductive system and breast disorders	Menopausal symptoms	Priapism, prostatitis	Galactorrhoea
General disorders and administration site conditions	Asthenia, chest pain	Fatigue, pain, thirst	
Investigations	Liver function test abnormal, weight increased	Hepatic enzyme increased, blood electrolytes abnormal, laboratory test abnormal	

Children: A review of the literature shows that melatonin has been used in an estimated 3000 patients aged less than 18 years (some of whom were epileptics). The doses given varied between 0.1mg and 500mg and were taken for varying lengths of time, from two 10mg doses one hour apart to 6 years. It is difficult to estimate the incidence of adverse events reported in this sample population, but it is less than 1%, with the most common being changes in mood and mild headache. In an anonymous survey done to examine the prescribing practices of paediatricians in UK, adverse events were reported by 27 out of the 148 respondents and included new onset seizure activity (n=2), increased seizure frequency (n=3), hyperactivity (n=5), behaviour changes (n=6), worsening sleep pattern (n=6), nightmares (n=2) and constipation (n=2) [537]. Melatonin had been thought to have proconvulsant properties [538] but this has now been disputed and it has been clearly shown that it is an anticonvulsant [539]. Other misconceptions are treatment with melatonin can cause precocious puberty but only very large doses of melatonin may minimally delay the onset of puberty [540]. Melatonin therapy does not appear to have any adverse effects on cognition or memory and is not thought to be addictive [541, 542].

Neonates: Although few studies have been published in literature, these studies show that melatonin has a safe profile with minimal adverse events documented. Significantly high doses of melatonin have failed to show any potential serious adverse effects. See Table 5.3 for further information on the neonatal studies of melatonin. Melatonin supplementation does not suppress endogenous secretion of melatonin but is known to aid establishment of appropriate circadian rhythms [440].

Long-term complications: Significant complications with long-term melatonin therapy in children and adults have not been reported [543, 544]. Palm et al [543] gave oral melatonin to 8 children and young adults who were functionally blind and had delayed development in an open study between 1-6 years. No side effects were noted during this therapy. One patient reverted back to previous sleep disturbance pattern. Carr et al [544] prospectively followed up 44 children with neurodevelopmental disabilities who participated in a placebo controlled, double blind crossover trial of sustained-release melatonin. Over 3.8 years of follow up there were no adverse reactions to melatonin therapy and development of tolerance was not evident.

Endogenous melatonin: Melatonin supplementation does not suppress endogenous secretion of melatonin but is known to aid establishment of appropriate circadian rhythms [440].

Drug Interactions (relevant to neonatal practice)

Caffeine– Caffeine and melatonin compete for metabolic capacity and inhibit production of 6-hydroxymelatonin by inhibiting mainly, CYP1A2 [545]. Caffeine is routinely prescribed to preterm infants in the neonatal intensive care. Co-administration of caffeine may increase the blood level of melatonin.

Table 5.3: Neonatal clinical trials with melatonin

Study	Trial Design	Population	No in melatonin group	No in non-melatonin group	Adverse Events documented	Dose, Route
Gitto 2004 [513]	Open comparative	Term neonates with surgical malformations	10	10- surgical 10-healthy	No mortality	10mg/kg/dose as IV infusion over 2hrs 10 doses over 72hrs
Gitto 2001 [509]	Open comparative	Term neonates with sepsis	10	10-septic 10- healthy	No mortality in treated group, 3 deaths in untreated group	10mg- 2 doses orally
Gitto 2004 [511]	Randomised double blinded placebo controlled	Preterm infants less than or equal to 32 weeks gestation	60	60	No difference in respiratory, cardiovascular, gastrointestinal, septic, retinopathy of prematurity complications in the study group as compared to the control group 2 deaths in control group, none in study group No untoward effect of melatonin.	10mg/kg/dose as IV infusion over 2hrs -10 doses over 72hrs
Fulia 2001[546]	Randomised blinded placebo controlled	Neonates with perinatal asphyxia	10	10	3 deaths in control group, none in study group	10mg every 2hrs, 8 doses oral
Gitto 2004 [547]	Randomised	Preterm infants	40	34	None reported	10mg/kg/dose as IV infusion over 2hrs -10 doses over 72hrs
Gitto, 2005 [512]	Randomised double blind, placebo controlled	Preterm infants less than or equal to 32 weeks gestation	55	55	Incidence of complications was higher in the placebo group	10mg/kg/dose as IV infusion over 2hrs 10 doses over 72 hrs

5.10. Doses of Melatonin Used and Exposure-Response Relationship

Doses required to achieve specific plasma levels in preterm infants have not been defined. Although various doses of melatonin ranging from 5-20 mg/kg/hr have been used in animal studies, few have looked at exposure response relationship. In the mouse model of ibotenate induced brain injury, doses ranging from 0.0005-5mg/kg were used and there was a dose dependent reduction of periventricular white matter injury [487]. Doses from 0.005 mg/kg were found to significantly decrease white matter injury while a lower dose of 0.0005 mg/kg showed a reduction in the white matter injury; this was not a significant reduction (figure 5.3). In the same study Husson et al showed that the melatonin doses of 5 mg/kg or less decreased white matter injury but showed no difference in the lesion size in the cortical plate in contrast to an antioxidant drug such as N-acetylcysteine which reduced both cortical and white matter lesions. This may be due to a dose dependant effect of melatonin where a lower dose may act on the MT1 and MT2 receptors while higher doses may target oxidative stress and may even desensitise the melatonin receptors.

Pozo et al showed that the inhibitory effect of melatonin on rat cerebellar nitric oxide synthase activity was dose dependant and significant inhibition of enzyme activity was seen at 1 nM melatonin, which is in the range of the physiological serum concentration at night [548].

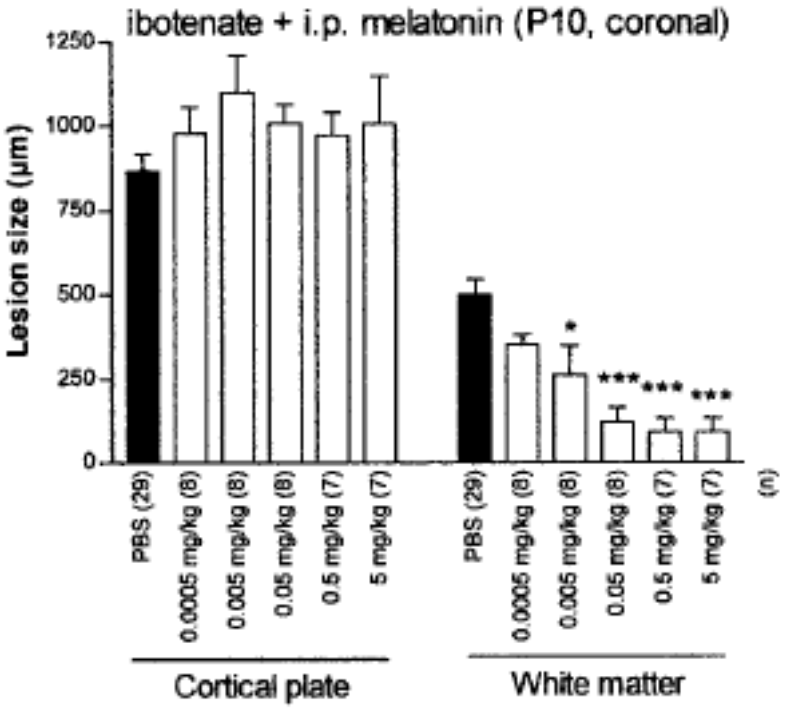


Figure 5.3: Dose dependant reductions in white matter injury in mouse model of ibotenate induced brain injury (Adapted from Husson et al, 2002, [487])

5.11. Implications of existing data and strategies for designing and planning drug development studies for melatonin as a neuroprotectant agent

5.11.1. Implications of existing clinical data and key knowledge gaps

Preterm infants are at an increased risk of neurological impairment and disability with significant psychological and economic impact both to the families and society. Currently, preterm birth cannot be prevented and apart from neonatal intensive care there are no specific neuroprotective therapies available.

Melatonin along with the natural circadian rhythm has been shown in experimental models to be important for normal neurodevelopment and embryonic growth and preterm infants are deprived of the normal intrauterine exposure to maternal melatonin. Studies in animals have raised the hypothesis that melatonin is an effective and safe neuroprotectant for use in human preterm infants, either as a replacement hormonal therapy to replicate fetal physiological levels, or at a higher pharmacological dose. Comparative pharmacodynamics data in adults is difficult as brain injury in preterm is unique in this age group. Also adults secrete melatonin in contrast to neonates and have circadian rhythm.

Melatonin has favourable pharmacological and toxicological properties shown by adult and animal data but pharmacokinetic studies have not been conducted in preterm infants.

Preterm infants have major differences in multiple physiological variables including body composition, renal function and hepatic metabolism compared to children and

adults. This functional immaturity of physiological processes and organ function can lead to potential inefficacy or reduced safety of melatonin in preterm infants.

5.11.2. Implications of existing quality data and key knowledge gaps

Currently there are no approved formulations available for melatonin in neonates. Currently the only approved formulations available for melatonin in adults is a prolonged release tablet 'circadian' for treatment of insomnia, which is not suitable for neonates. Oral solution of melatonin is available as off label drug for paediatric use. There are currently no approved injectable forms of melatonin available in any age group.

Need to develop injectable and oral formulation for neonatal use: In view of variable oral bioavailability and that preterm infants are usually on minimal trophic feeds or not fed in the first week of life and have intravenous access, an injectable form of melatonin is the most suitable formulation required in the first week of life. Later on it would be preferable to have an oral formulation with ease of administration as by then most preterm infants are fully enterally fed and do not have intravenous access. Most animal studies have ethanol or propylene glycol as an excipient. There is a need to develop a formulation that will be safe for use in neonates.

5.11.3. Information on existing non-clinical data and key knowledge gaps

Data from animal models has shown that melatonin has a strong safety profile although long-term data is limited. The developmental toxicity no adverse effect level (NOAEL) is considered to be ≥ 200 mg/kg/day. Animal models have used a range of

doses to achieve neuroprotection although data on physiological replacement doses of melatonin is extremely limited.

5.11.4. Approach and rationale for development of clinical studies

Referring to the decision tree in drug development in figure 3.1, we know that neonates do not have similar disease progression and/or response to intervention as adults. Hence it is important to first conduct PK studies in this population. Information on PK is important to support adequate dosing in this specialised population and assessing potential adverse effects. However, PK alone is of limited value and safety and efficacy studies will be needed and PK-PD studies which can follow on from the initial PK studies.

A population PK approach described in the next chapter is preferable due to the importance of finding covariates related to dose-individualisation between individuals and over time in the maturating individual [549]. The analysis can be made on rich and/or sparse data depending on the number of patients available and the possibility of developing highly sensitive analytical methods where very small sample volumes could be used.

6. PHARMACOKINETICS

Different populations vary significantly in their response to drugs and therefore require individualised doses. There are many sources of variability but mainly they can be divided into two major phases of drug action- pharmacokinetics (what the body does to the drug) and pharmacodynamics (what the drug does to the body).

Pharmacokinetics provides a mathematical basis to assess the time course of drug concentration in the body and the effects of the drug in the body. The pharmacokinetic processes – absorption, distribution, metabolism and excretion, often referred to as ADME, determine the drug concentration in the body and the effectiveness of a dose regimen is determined by the drug concentration in the body.

Traditionally, pharmacokinetics was based on abstract mathematical modelling of the time course of drugs in drug concentrations in theoretical compartments, which were not directly related to physical, or physiological body compartments [550]. Rate constants describe the transfer of molecules between the compartments and out of compartments (elimination). The approach relies on nonlinear regression analysis to fit an exponential equation to the data. By contrast, the non-compartmental method is based on the area under concentration versus time curve and can be applied practically to any PK data. Non-compartmental analysis requires fewer assumptions to be made than for compartmental analysis in modeling concentration versus time data. Physiologically based pharmacokinetic (PBPK) modeling is a mathematical modeling technique for predicting the absorption, distribution, metabolism and excretion (ADME) of synthetic or natural chemical substances in humans and other

animal species. PBPK are usually multi-compartmental models, with compartments corresponding to predefined organs or tissues. Besides the advantage of allowing the recruitment of *a priori* information about parameter values, these models also facilitate inter-species transpositions or extrapolation from one mode of administration to another.

The fundamental PK parameters reviewed below can be calculated by the compartmental as well as non-compartmental approach.

6.1. Clearance

Clearance (CL) describes the efficiency of irreversible elimination of a drug from the systemic circulation. Clearance is defined as the volume of blood cleared of drug per unit time' and the units are thus volume per time, usually litres per hour (L/hr) or (mL/min). Clearance is determined by the blood flow to an eliminating organ such as the liver and the efficacy of irreversible extraction of the drug from the blood by the organ.

Clearance is also the constant relating the plasma concentration of drug (C) to the rate at which the drug is eliminated from the body.

$$\textbf{Elimination rate } (\textbf{mg/hr}) = \textbf{Clearance}(\textbf{L/hr}) * \textbf{Plasma drug concentration } (\textbf{mg/L})$$

Equation 6.1

Clearance determines the maintenance dose rate required to achieve a target plasma concentration at steady state i.e. where the rate of drug administration is equal to the rate of drug elimination.

$$\textbf{Maintenance dose rate } (\textbf{mg/hr}) = \textbf{Clearance } (\textbf{L/hr}) * \textbf{Steady state drug concentration } (\textbf{mg/L})$$

Equation 6.2

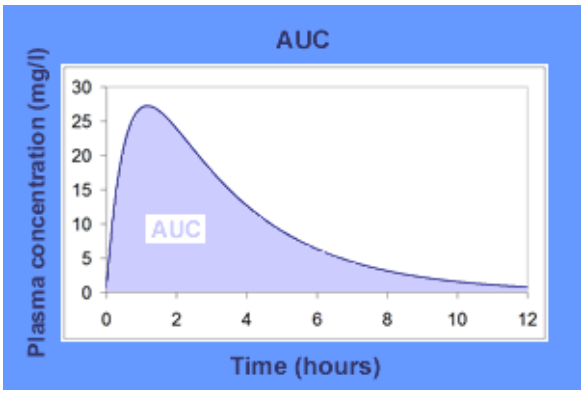
Thus for a given dose rate the plasma drug concentration is inversely proportional to clearance. For example, if the clearance is reduced by half the steady state concentration will double.

Alternatively, for a single intravenous dose, clearance can be determined by the ratio of the dose to the total area under the plasma concentration time curve (AUC) (figure 6.1).

$$\textbf{Dose } (\textbf{mg}) = \textbf{Clearance} (\textbf{L/hr}) * \textbf{Area under the curve } (\textbf{mg} * \textbf{hr/L})$$

Equation 6.3

A



B

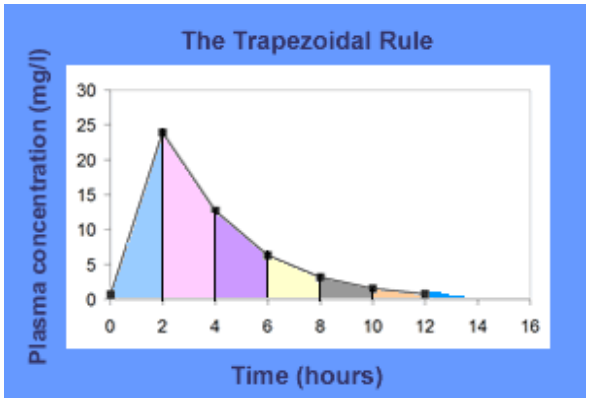


Figure 6.1: Measurement of clearance after a single dose.

Area under the curve (AUC) is measured by assuming each pair of drug concentration time observations form a trapezoid. The area of each trapezoid is calculated and then summed.

6.2. Volume of Distribution

Volume of distribution (V) is the other major independent pharmacokinetic parameter that relates the concentration of the drug in the plasma to the total amount of drug in the body. V is not a real volume.

$$\mathbf{V} = \frac{\textbf{total amount of drug in the body}}{\textbf{Plasma drug concentration}}$$

Equation 6.4

The major determinant of V is the relative strength of binding of the drug to tissue components as compared with plasma proteins. If the drug is tightly bound by the tissues and not by blood, most of the drug will be in the tissues and little in the plasma, so the drug will appear to be dissolved in a large volume and V will be large e.g. imipramine and chlorpromazine. Conversely if drug is tightly bound to plasma proteins, V can be very close to blood volume. Factors such as lipid solubility can play an important role in volume distribution. Highly lipid soluble drugs can readily pass through the lipid component of the membranes, hence can diffuse to different parts of the body easily. They can also get concentrated in the fat tissues as compared to lipid soluble substances.

V can be determined by extrapolating the plasma concentration to zero time (C_0) after administration (Figure 6.2).

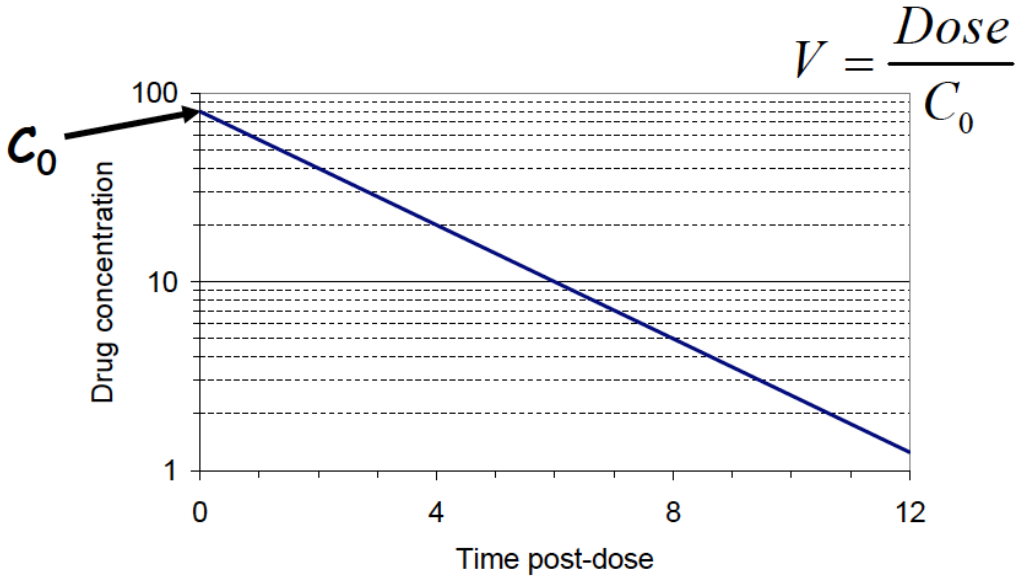


Figure 6.2: Measurement of volume of distribution. An intravenous bolus dose is given and plasma drug concentrations are plotted on a logarithmic scale against time and extrapolated to find C_0 (plasma concentration at zero time) to calculate the volume of distribution. *(Reproduced from Birkett DJ, 2010 [550]*).

Using the same principle, V can be used to calculate the loading dose.

$$\textbf{Loading dose} = \textbf{V} * \textbf{target plasma concentration}$$

Equation 6.5

6.3. Half-Life

Half-life ($t_{1/2}$) is the time taken for the amount of drug in the body (or plasma concentration) to fall by half. The elimination of a drug is usually an exponential (logarithmic) process so that a constant proportion of the drug is eliminated per unit time. This is called first order elimination (figure 6.3).

A. B.

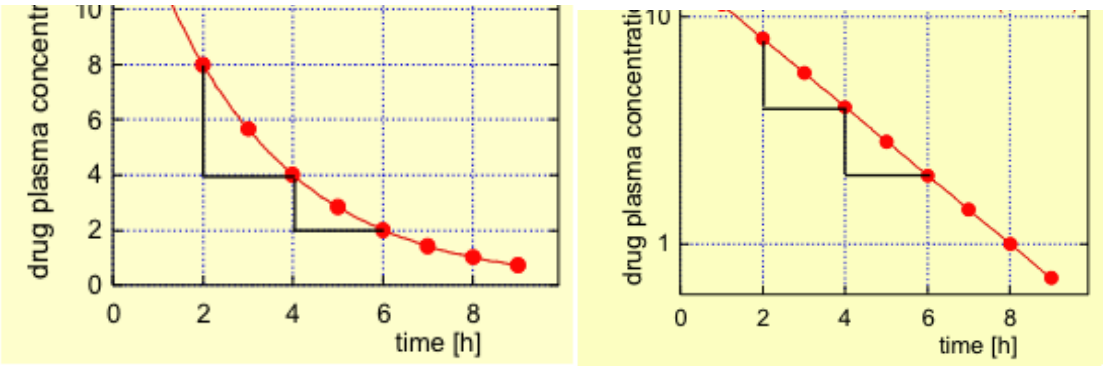


Figure 6.3: First order elimination of a drug plotted on linear (A) and logarithmic (B) scale. The plasma concentration falls by half each half-life *(reproduced from Birkett DJ, 2010* [550]).

Thus the fall in plasma drug concentration after a single dose is an exponential function of the time after dose and described by the equation below where Ct is the concentration at various times (t) after the dose, C_0 is the initial concentration at time zero and k is the elimination rate constant

$$C_t = C_0 * e^{-kt}$$

Equation 6.6

When the plasma concentration is falls by half, $C_t = 0.5*C_0$, resulting in the below equation where logarithm of 2=0.693

$$k = \frac{0.693}{t_{1/2}}$$

Equation 6.7

Half-life is the composite pharmacokinetic parameter that is determined by clearance and volume of distribution.

$$t_{1/2} = \frac{0.693 * V}{Cl}$$

Equation 6.8

Thus half-life is increased by a decrease in clearance or an increase in volume of distribution. A decrease in the efficiency of elimination will increase the time taken for drug elimination. The larger the volume of distribution, the more drug is concentrated in the tissues compared to the blood. As the drug in the blood is exposed to clearance, if the volume of distribution is large there is less or slower elimination.

Half-life determines:

i. The duration of action after a single dose. But it is important to remember that the duration of action is usually a logarithmic and not a linear function of dose.
ii. The time required to reach steady state with chronic dosing. It takes 3-5 half-lives to reach target plasma concentration.
iii. The dosing frequency required to avoid large fluctuations in plasma concentrations during the dosing interval.

6.4. Population Pharmacokinetics

Population pharmacokinetics (PPK) is the study of the sources and correlates of variability in drug concentrations [551, 552]. PPK seeks to obtain the relevant pharmacokinetic information from individuals who represent the target patient population receiving clinically relevant doses of a drug of interest; recognises that variability is an important feature that should be identified and measured during drug development and evaluation; helps in explaining variability by identifying factors of demographic, pathophysiological, environmental or drug related origin that alter the pharmacokinetic behaviour of a drug and seeks to quantitatively estimate the magnitude of the unexplained variability (random) in the target population [553].

6.4.1 Advantages of PPK Methods

PPK helps in improving our understanding of the relationships between drug input patterns, patient characteristics and drug disposition and helps in identifying factors that affect drug behaviour or explain variability in a target population [554]. It allows development of optimum dose strategy for individuals; subgroup or population based on patient specific covariates and increases the safety and efficacy of a drug. This is especially important if there is a heterogeneous population or a narrow target concentration window.

PPK allows analysis not only from sparse data but also analysis of data from unbalanced designs (different samples/subjects) or pooled data studies. This is especially important in the special populations (paediatric and geriatric) and phase IV post surveillance studies, where it is not possible to do the usual pharmacokinetic

analysis with multiple points to plot a dose concentration curve [555]. Also focusing on a single variable (e.g. renal function) in a classical PK study makes it difficult to look at interactions among variables.

6.4.2. Disadvantages of PPK Methods

PPK method depends on the model used and complex computational work done to achieve the final model, which can be time consuming, expensive and requires expertise. Four main disadvantages have been identified by Nedelman [556]. The first one- deliberate construction of data e.g. not selecting and prespecifying times at which samples are collected can lead to imprecision in the data collected resulting in inaccuracy in the model and its robustness. Specifying time windows can improve the statistical efficacy of the model with better fit of the data to the model. The second important aspect is correlations among predictor variables (collinearity) can reduce statistical efficacy and result in large standard errors of parameter estimates. The third important aspect is the reliability of the data. It is assumed that data is correct and deviations such as noncompliance or incorrect recorded sampling times or dose taken can result in wrong selection of a model. The fourth important aspect is unobserved confounding variables which may bias inferences.

6.4.3. Population Models

PPK parameters can be estimated by namely 3 methods [557]:

Naïve Pooled Data Approach: PPK parameters are estimated either by fitting all individual's data together as though there were no individual kinetic differences.

Simulations indicate that the pooled data approach fails to estimate variabilities and produces imprecise estimates of mean kinetics.

Two-stage Approach: PPK parameters are first estimated by fitting each individual's data separately using nonlinear regression, and then in the second stage, combining the individual parameter estimates obtained in the first stage to calculate the descriptive summary statistics (mean parameter estimates, variance and covariance of the individual parameter estimates). The two-stage approach can be used in 'data –rich' situations and gives good estimates of mean kinetics (fixed effects), but biased and overestimation of variance and covariance (random effects) [558-561].

Nonlinear Mixed Effect Modeling Approach: A third approach, which can be used in situations where extensive measurements are not made (data sparse situations) takes a middle course between the above two approaches. It uses the individual pharmacokinetic data, which may be sparse, unbalanced and fragmentary in addition to conventional pharmacokinetic data from traditional pharmacokinetic studies. It is called "mixed-effect" because the model has a fixed structure and a random block. The fixed structure contains what we know, or suspect, of the models behaviour and interaction with other parameters. For example, if a drug is mainly cleared by the renal elimination, renal clearance might be included as a scaling parameter for the observed concentration of drug. The random block describes the residual variance. This includes the interindividual and intraindividual variability and will always be present, e.g. daily biological changes in a person, diet, compliance and any possible errors in the sampling procedure. The common factor is that they cannot be predicted in advance. Together, these fixed effects and random effects are called

mixed-effects [562]. Nonlinear mixed effects modeling allows pharmacokinetic studies with less rigid designs than needed for the two-stage approach method. It produces accurate and precise estimates of all parameters even with sparse data, and also reasonable confidence intervals for them along with sometimes a pharmacodynamics parameter [554, 563]. As in all paediatric studies where the data is sparse and estimates of individual parameters are a priori, nonlinear mixed effect modeling is used in our study. Various statistical programs and software packages are used such as NONMEM (UCSF, Globomax), SAS (SAS Institute Inc), Splus (Insightful Corporation) or R (Free), WinBUGS (MRC Biostatistics, Free), ADAPT II (USC, Free), WinNonLin/WinNonMix (Pharsight), Trial Simulator (Pharsight). We have used NONMEM for our study, which is described in further detail in the methods section of Chapter 7.

6.4.4. Study Design and execution of a PPK approach [564-568]

It is important that the objective of the study is designed so that the objectives can be achieved and the study can be designed appropriately. Preliminary pharmacokinetic information and the drug's major elimination pathway must be known before embarking on a PPK study. This is important, as sparse data collected during population PK studies may not give adequate information for discriminating among PK models. Correct evaluation of PK data depends on the accuracy of the analytical data obtained, which in turn depends on the validity of the assays and assay methods used to measure the drug and its clinically relevant metabolites. Design limitations such as sampling times, number of samples per subject and number of subjects (as in paediatric population) should be considered at the outset [555]. Simulation studies

allow anticipation of certain fatal designs. Designs should include enough patients in important subgroups to ensure accurate parameter estimation and in detection of any subgroup differences.

Sampling Designs: Various sampling designs are used in PK studies such as single-trough, multiple trough and full population PK designs are used and there are advantages and disadvantages of each method [552].

In the single-trough sampling design, a single blood sample is obtained from each patient at, or close to, the trough of drug concentrations [569]. It assumes that there is a large sample size, assay and sampling errors are small and the dose regimen and sampling times are same for every patient. This type of design can give information on the optimal concentration range of a given drug and apparent clearance but not half-life and apparent volume of distribution. Also interindividual and residual variability cannot be separated. In multiple-trough sampling design, two or more blood samples are obtained near the trough of steady state concentrations; thus allowing separation of interindividual and residual variability. This also requires a large sample size.

In full PPK sampling design, blood samples are drawn at various points following drug administration to describe a PK profile [560]. This allows an estimation of the PK parameters and variability using the NONMEM approach. This approach allows the relationship between the drug PK parameters and target population characteristics (demographics and pathophysiological features) to be explored in detail. Sampling the same individual on more than one occasion allows exploration of intraindividual

variability and interoccasion variability (variability of observations due to variability of PK model from occasion to occasion) [570, 571].

Study Execution: PK studies should be conducted according to current good clinical practice and good laboratory practice standards taking appropriate measures allowing accurate information on dosing and timing of samples relative to dosing history. Errors in recording sampling times could result in biased and imprecise parameter estimates [568]. Adequate resources should be available to ensure compliance with medication, optimal sample preparation, storage and transport of samples prior to analysis [572]. It is important that the steps taken to develop a population model are clearly outlined so as to allow reproducibility of the analysis [573-575].

Error Modeling: The nomenclature used to describe error in models can be confusing and some of the terms are explained with respect to modeling. Variance refers to the spread or the average squared difference of a value from its mean, while variability is the difference of a value from its mean. It is often described by the standard deviation (i.e. square root of the variance) or coefficient of variation (standard deviation divided by the mean). The variability of a parameter such as clearance in a population is called population parameter variability (PPV). Residual error describes what is left over after all other sources of variability have been accounted for thus it is the difference between an observation and predicted values and the variability is called residual unexplained variability (RUV).

Error modeling is used to describe the variability in how well the data is described by a parameter. Residual error models aim to minimize the differences between the observed and predicted values. Three types of error models are used: additive, poisson and proportional. Poisson models are rarely used as they can be only used if standard deviation =mean. Most error models use both additive and proportional errors.

7. PHARMACOKINETICS OF MELATONIN IN PRETERM INFANTS

7.1. Introduction

Experimental work has raised the hypothesis that melatonin is an effective and safe neuroprotective agent for preterm infants. This chapter reports the first steps required for the testing of this hypothesis in human infants. This clinical study aimed:

- To determine the pharmacokinetic profile of melatonin in preterm infants;
- To assess tolerability of melatonin in preterm infants;
- To determine whether a specific dosage scheme would allow replication of adult and thus fetal concentrations of melatonin.

7.2. Patients and Methods

7.2.1. Ethics

The study had approval from Hammersmith and Chelsea Research Ethics Committee and the UK Medicines and Healthcare Products Regulatory Agency and was registered in the International Standard Randomized Controlled Trial Number Register (ISRCTN 01115788, EUDRACT no 2007-007156-33). All procedures in the study were in accordance with the Helsinki Declaration of 1975 (as revised in 1983). Written parental consent for participation was obtained for all trial participants.

7.2.2. Patients

We conducted an open label dose ranging study between May 2010 and December 2010 in 3 neonatal intensive care units in the UK. Infants born less than 31 weeks gestation and less

than 7 days old were eligible for the study, although those with: major congenital malformation; or cystic periventricular leucomalacia or haemorrhagic parenchymal infarcts on cranial ultrasound were excluded. It was intended to recruit 4 infants at each dosing concentration and another 2-4 to confirm the dosing concentration. Groups were identified by each dosing concentration.

Indications to stop the infusion of melatonin were: withdrawal of parental consent; or any suspected unexpected serious adverse reaction, defined an adverse reaction that is both unexpected and also meets the definition of a Serious Adverse Event/Reaction. The reference safety information was included in the investigator's brochure and dossier.

7.2.3. Timing and Length of Duration of Treatment

As there is no documented safety profile for melatonin in neonates, for the pharmacokinetic study, one dose of melatonin was given to each participating preterm infant, anytime in the first seven days after birth.

7.2.4. Proposed Dosing Regime

It is not known what plasma melatonin levels are needed in preterm infants to achieve neuroprotective effect. Two approaches were considered- one using animal data and two using adult PK data.

Calculation of the starting dose using animal data: The lowest effective neuroprotective dose in the mouse model used was 0.005 mg/kg. The human equivalent dose (HED) can be calculated by using the formula

HED = animal dose in mg/kg x (animal weight in kg/human weight in kg)$^{0.33}$ [576].

Taking the animal weight as 0.01 kg (as per discussion with Professor Gressens who conducted the mouse model study) and using human weight as 1 kg (preterm infant) the HED is 1.09 mcg/kg.

Calculation of the starting dose using adult PK data: It is known as fetus' do not produce their own melatonin but actually get it transplacentally from their mothers and if born prematurely, do not produce their own melatonin for quite some time. Therefore, it makes sense to start with a 'physiological' dose i.e. dose which would reach the same plasma maternal levels which the fetus is exposed to before moving on to a higher i.e. 'pharmacological dose'.

Using adult pharmacokinetic data and equation 6.2, based on a clearance of 0.05 L/min/kg and a concentration of 58 pg/mL the estimated starting rate of infusion of melatonin will be 0.174 mcg/kg/hr i.e. 1.04 mcg/kg over 6 hours. As circadian rhythm is important in neurodevelopment, it may be important to give melatonin over a similar period mimicking adult profile. Hence after taking advice with Professor Boobis (Pharmacology Professor at Imperial College) it was decided to start with a starting dose of was 0.1 mcg/kg/hr for 6 hours (0.6 mcg/kg over 6 hours). This dose is likely to be a tolerable and a safe dose along with the possibility of achieving neuroprotection.

Comparing starting doses from the above 2 approaches showed that although the dose obtained from both approaches were similar. Hence it was decided to use this dose of 0.1 mcg/kg/hr as a 6-hour infusion as a starting point.

An adaptive design approach was used where successive groups received varying doses depending on the melatonin PK results and mean adult physiological concentrations were achieved (approximately 46-58 pg/mL). The dose regimens given were 0.1 mcg/kg/hr intravenously for 6 hours, 0.1 mcg/kg/hr for 2 hours, 0.02 mcg/kg/hr for 2 hours, 0.01 mcg/kg/hr for 2 hours and 0.04 mcg/kg over 30 minutes.

7.2.5. Formulation

SAFC Pharma via Alliance Pharmaceuticals supplied recombinant human melatonin substance for this study. There is no licensed intravenous formulation available in the UK. Consequently a melatonin injection 0.1 microgram (mcg) in 1 millilitre (mL) was formulated (Stockport Pharmaceuticals, Stockport UK). The formulation used consisted of melatonin (0.1 mcg), sodium chloride (9 mg) and water for injection as solvent (to 1 mL). The product was terminally sterilised using a standard pharmacopoeia cycle 121^{o}C for 18 minutes and manufactured in clear glass ampoules.

Stability and Shelf Life: Stability was assessed by the West Midlands Regional Quality Control Laboratory (Birmingham, UK) and Stockport Pharmaceuticals. The data was generated over a period of time and cumulated to give a subsequent and final shelf life of 1 year. Twenty- five ampoules of 0.1 microgram/ml of product were autoclaved and 25 were left unautoclaved. Both were assayed for % melatonin at 5 ^{0}C, 25 ^{0}C and 40^{0}C at various time points as stated in Table 7.1**.**

Table 7.1: Melatonin Assay at various timepoints for unautoclaved and autoclaved ampoules.

A. Unautoclaved ampoules			
Time Point	**Melatonin Assay, % of stated**		
	5 ^{0}C	**25 ^{0}C**	**40 ^{0}C**
Initial	110.0	110.0	110.0
77 days	110.5	109.8	108.9
3 months	116.9	114.0	113.2
Repeat of 3 months	113.1	112.1	110.9
6 months	107.2	107.4	106.5
7 months	115.3	111.8	113.3
12 months	116.7	-	-

B. Autoclaved ampoules			
Time point	**Melatonin Assay, % of stated**		
	25 ^{0}C	**40 ^{0}C**	**Room temperature in light**
Initial	101.9	101.9	101.9
77 days	99.5	98.8	86.2
3months	109.6	93.7	89.3
Repeat of 3 months	106.0	90.7	85.7
6 months	100.8	98.7	75.2
7 months	108.9	103.7	74.1
12 months	100.2	95.1	56.8

Key Points on the Stability Data: The stored samples of unautoclaved melatonin samples were stable at 5 and 25 ^{0}C with no degradation peaks. Melatonin did not demonstrate potency loss (percentage of melatonin over time) on autoclaving. At room temperature exposed to light, there was significant loss of melatonin concentration with significant degradant peaks on chromatography. The peaks added up to 55% of the melatonin peaks. All the solutions remained clear with no visible particles seen. The pH varied slightly (5-7) between all the ampoules in autoclaved and unautoclaved conditions with no significant trend.

The test batch has an assay of 110 % of expected concentration. There is a difference of 8 % between the initial unautoclaved and autoclaved samples. The assay difference was not accompanied by any other chromatographic anomalies, such as degrading peaks. In order to demonstrate whether the autoclave process caused degradation or other loss of active melatonin, test samples from the unautoclaved 5 ^{0}C were retested following 1 and 2 autoclave cycles. Melatonin assay when autoclaved once was 112.4 % of expected concentration and when autoclaved twice was 111.1 % of expected concentration. This data suggests that the autoclave process did not significantly influence the percentage of concentration) of the sample. The difference between the original unautoclaved and autoclaved ampoules therefore could not be explained due to autoclaving. Inorder to explain the high assay of the unautoclaved samples, we went back to the pharmaceutical starting material and checked with the laboratory reference sample. The starting material was 101.1 % with reference to the laboratory reference material; again we were not able to explain the high assay of the unautoclaved samples. It was concluded that the high initial assay of the unautoclaved samples might have been related to the weighing and sequential

dilution of the stock solution. The autoclaved samples were also the first samples to pass through the in-line filter. Drug absorption onto the filter may have caused a loss from solution, which would then give a low assay result. A filtration study was carried out where samples were tested for melatonin content before filtration and after several volumes of solution had run through the 0.2 um Sartobran 300 capsule filter. Table 7.2 shows that the melatonin concentration decreases by 35% on initial filtration. The concentration increases as more solution is run through the filter but does not return to the initial concentration of 99.11%. Using the Baxa 0.2 um filter 50 mm diameter and running the same procedure resulted in only 4% loss after 500ml of solution running through the filter. Hence for the final product the Baxa filter was used.

Table 7.2. Filtration of melatonin samples

Sample	**Volume, ml**	**Melatonin, µg/ml**	**Melatonin, %s/s**
Pre-filtration	0	0.0988	99.11
F1	250	0.0640	63.98
F2	525	0.0752	75.18
F3	800	0.0824	82.40
F4	1075	0.0806	80.58
F5	1350	0.0836	83.55
F6	2875	0.0881	88.13
F7	3400	0.0875	87.45

7.2.6. Investigations

Capillary, venous or arterial blood was collected for melatonin levels at various time points. The samples were centrifuged within 30 minutes at 3000 rpm for 10 min and frozen to -20 °C until assay. Urine samples were collected as 2 hourly samples and frozen to -20°C until assay. Melatonin and its primary metabolite 6-sulfatoxymelatonin (aMT6s) were analysed by specific radioimmunoassay (RIA) at University of Surrey, Guildford, UK based on a method described by Arendt and coworkers [444, 471]. All plasma samples were assayed at 1:5 dilution (100ul volume) then diluted 1:10 whenever possible and checked twice at both dilutions and the mean value was taken. The urine samples were assayed at 1:250 dilutions and some at 1:25 dilution. The detection limit of the plasma melatonin assay was 3 pg/mL; that of urine aMT6s was 0.2 ng/mL and of urine melatonin was 0.03 ng/mL.

7.2.7. Pharmacokinetic Modeling

Noncompartmental analysis was used for the initial data analysis. The elimination half-life, CL, V and AUC was calculated for each individual using STATA 11 (Stata Corporation, College Station Tx) and equations mentioned in chapter 6.

Population modeling was carried out in collaboration with the Clinical and Practice Research Group, School of Pharmacy, Queen's University, Belfast (Professor J McEnlay and Dr A Hawwa) by means of nonlinear mixed-effect modeling using NONMEM (NONMEM version VII, level 2.0, ICON Development Solutions, Ellicott City, MD) which is a computer program designed to help construct models utilising the nonlinear mixed effects approach explained in Chapter 4 [577]. To achieve linearisation it uses a first order (FO) Taylor series expansion in respect to the random effect variables or first order conditional estimation (FOCE) or

Laplacian methods. FOCE is a FO expansion about conditional estimates (empirical Bayesian estimates) of the random effects. The Laplacian method uses second order expansions about the conditional estimates of the random effects [578]. We used FOCE in our study.

As with all other nonlinear mixed effect models, NONMEM takes into consideration both fixed effects and random effect. It considers 2 main types of variability: interindividual variability and residual variability. Residual variability can be thought of as random unexplained variability. Random error is always present and is unpredictable. One of the aims of modeling is to reduce the residual unexplained variability by describing as much of IIV as possible.

Step 1. Determination of the basic pharmacokinetic model:

a. Combined data from all subjects was used to develop the population pharmacokinetic model that describes the influence of patient variables on melatonin disposition. Potential models considered were classical linear 1- and 2-compartment models. Inter individual variability (IIV) in the estimated parameters - Clearance (CL) and volume (V) was modeled using an exponential error model, as their distribution is often right skewed [579]. The covariance of CL and V was also incorporated into the model.
b. Subjects were allocated in two subgroups depending on the dosages used and which had least variable data (0.1 mcg/kg/hr versus 0.01-0.02 mcg/kg/hr). PK parameters in each group were estimated and compared. When each group is analysed separately, IIV was combined with the residual variability.
c. Proportional and additive components of residual variability were estimated.

d. It is possible to combine the above steps in a mixed equation (Equation 7.1) where C_{ij} is the measured and C_{predij} is the model predicted value of melatonin concentration of the i^{th} individual at the j^{th} sampling time and ε_{ij} is the residual error term, which is a random variable with zero mean and variance of σ^2 and A is the residual variance component. However, simplification was considered during the model building by deleting the additive residual variance component as its value was close to zero.

$$C_{ij} = C_{predij} \times (1 + \in_{pij}) + \in_{Aij} \quad \text{Equation 7.1}$$

e. Body weight was included as *a priori* covariate on CL and V and was scaled to the median weight of the studied population as shown in Equation 7.2 and 7.3 [443, 444], where CL_i and V_i are the CL and V of the i^{th} individual with weight (WT_i) and θ_{CL} and θ_V are the mean population estimates of CL and V in a standard individual with median weight (WT_{median}).

$$CL_i = \theta_{CL} \times \left(\frac{WT_i}{WT_{median}}\right)^{0.75} \quad \text{Equation 7.2}$$

$$V_i = \theta_V \times \left(\frac{WT_i}{WT_{median}}\right) \quad \text{Equation 7.3}$$

Step 2: Selection of the influential covariates*:*

Various covariates can be tested for inclusion into the model. The following covariates were tested for inclusion into the model: gestational age, postnatal age, gender, race, serum creatinine, bilirubin and urea concentrations. Visual examination of scatter plots (or box-and-whisker plots in the case of categorical covariates) of empirical Bayesian estimates and IIV obtained for each pharmacokinetic parameter from Step 1 versus each covariate were

used to help identify whether the pharmacokinetic parameter might be significantly related to the covariate. Direct covariate testing was then performed to see if this relationship was significant.

Step 3: Development of the final covariate population pharmacokinetic model: The final model was established using the forward inclusion–backward elimination method using an objective function value [580].

Objective Function Value (OFV) describes how good a model is at fitting the observed data. These are statistical criterions applied to non-linear regression models as an objective measure of the differences between the observed and predicted values of parameters and dependant variable. The concept of 'a minimum objective value is better' means that the aim is to find a parameter estimate that minimises the objective function. However the absolute value is meaningless and it is the reduction in objective function that is important. A reduction of 3.84 corresponds to a p value of < 0.05 (from chi squared distribution with one degree of freedom) and a reduction of >10.83 corresponds to a p value of <0.001. Only covariates that showed a significant contribution were kept in the final model.

In our model, forward inclusion of a covariate required a reduction in the minimum value of the objective function (MOFV) of at least 3.84 (p<0.05, df=1). However, the level of significance for retaining a covariate in the model during backward elimination was set at a more stringent ΔMOFV of >10.83 (p<0.001, df=1). Only covariates that showed a significant contribution were kept in the final model. Graphical inspection of goodness-of-fit was used throughout model building and evaluation [574] by examining the residuals (RES),

conditional weighted residuals (CWRES) and measured melatonin concentrations plotted separately against predicted concentrations.

7.3. Results

7.3.1. Demographic and Tolerability Data

Eighteen preterm infants (9 male, 9 female) born before 31 weeks gestation and less than 7 days of postnatal age were enrolled into the study (Table 7.3). The median (range) birth weight was 867.5 grams (g) (610 - 1430 g) and gestation at birth was 26.63 weeks (24.71 - 30.28 weeks). The median age at melatonin infusion was 2 days (range 1 - 6 days). All infants were receiving some human donor or maternal milk, and none received artificial milk formula. All the preterm infants were treated with caffeine (12.5 mg/kg loading dose followed by 6 mg/kg/dose once a day) and 16 out of 18 were receiving total parenteral nutrition. Six of 18 infants were receiving intravenous co-amoxiclav but none had confirmed sepsis. No infant was withdrawn from the study or required the infusion to be stopped early. None of the infants required inotropic support or required to be reventilated during the infusion. No adverse events related to the melatonin infusion occurred.

Table 7.3: Patient Characteristics

Patient Characteristics (n=18)	**Median (Range)**
Post menstrual age at birth/weeks	26.63 (24.71-30.28)
Birth weight/g	867.5 (610-1430)
Post natal age/days	2 (1-6)
Sex (M:F)	1:1
Ethinicity	6 white, 5 Asian, 5 Black, 2 mixed

7.3.2. Individual Pharmacokinetic Data

An adaptive design was used. For the first dose of 0.1 mcg/kg/hr over 6 hours, blood samples were collected at 0, 1, 3 and 6 hours. At the starting dose of 0.1 mcg/kg/hr for 6 hours, the median (range) plasma melatonin concentrations at the end of the 6 hour infusion were 393.3 pg/mL (339.3 -554.5 pg/mL). As steady state concentrations were not reached and higher than expected plasma concentrations were achieved, the sample times were changed to 0, 2, 6 and 24 hours along with the reduction in the duration of infusion to 2 hours. Melatonin concentrations achieved with a 2 hour infusion of 0.1 mcg/kg/hr were median (range) of 203.3 pg/mL (160 - 220 pg/mL). At this infusion dose, the median (range) half-life was 15.82 hours (11.3-21.0 hours) and area under the curve (AUC) was 1665.08 pg*hr/mL (1392.06 -1992.14) while the CL was 0.06 L/hr/kg. As the plasma concentrations were still on the higher range of adult values, the dose was decreased further to 0.02 mcg/kg/hr. In the first patient at this dose (patient 9 in table 7.5), sample times were kept the same at 0, 2, 6 and 24 hours, which although had plasma melatonin in the range we wanted, PK parameters could not be calculated as the plasma melatonin levels were undetectable at 6 and 24 hours. Hence we changed the times to 2, 4, 5 and 6 hours for the next 3 patients (patient nos 10, 11 and 12) and also gave one patient 0.01 mcg/kg/hr for 2 hours (patient 15). Again it was not possible to calculate the PK parameters and so for patients 13, 14 and 16, blood samples were collected at 2, 6, 24 and 48 hours. Further 2 patients were given doses of 0.04 mcg/kg over 30 minutes. Lower doses of melatonin infusion (0.02 mcg/kg/hr for 2 hours, 0.01 mcg/kg/hr for 2 hours and 0.04 mcg/kg over 30 minutes) gave highly variable plasma melatonin concentrations and unreliable estimates of pharmacokinetics in individuals.

Tables 7.4, 7.5, 7.6, 7.7 and Figures 7.1, 7.2 give the measured concentrations of melatonin in plasma and urine, and urinary aMT6s along with the individual half-life and AUC. Pre-infusion median plasma melatonin concentrations were not detectable except one infant who had a pre-infusion concentration measured as 38.5 pg/mL. Urine melatonin and urine aMT6s concentrations were negligible at all doses.

Figure 7.1: Dosing concentrations used in the different groups along with the number of infants in each group

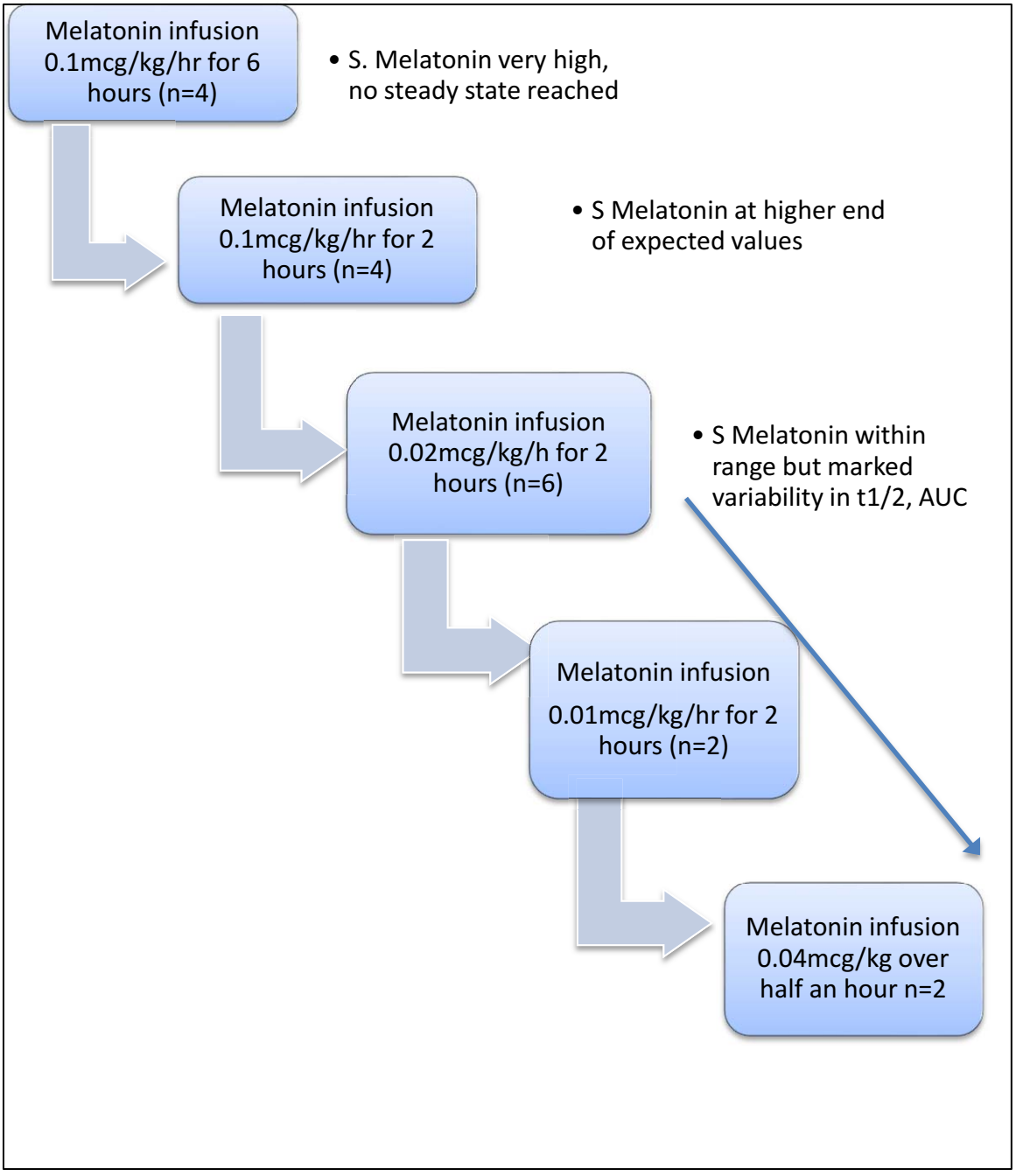


Table 7.4: Plasma Melatonin Concentrations at Different Doses

Dose (No of Patients)	Time Hours	Median Plasma Melatonin (range) pg/mL
0.1 mcg/kg/hr for 6 hours (n=4)	0	0 (0-38.5)
	1	31 (0-77)
	3	153.3 (145.5-317.5)
	6	393.3 (339.3-554.5)
0.1 mcg/kg/hr for 2 hours (n=4)	0	0 (0-0)
	2	203.3 (160-220)
	6	145.4 (110.8-156.8)
	24	71.6 (51.8-79)
0.02 mcg/kg/hr for 2 hours (n=6)	0	0 (0)
	2	48.7 (38.8-71)
	4	52 (41-65)
	5	64 (49.5-67)
	6	63 (0-71)
	24	0 (0-53.5)
0.01 mcg/kg/hr for 2 hours (n=2)	2	54.8 (43.5-66)
	4	110 (110)
	5	103.8 (103.8)
	6	97.5 (97.5)
	24	28 (28)
	48	38.8 (38.8)
0.04 mcg/kg for ½ hr (n=2)	1	58.5 (42-75)
	72	120.3 (110.5-130)
	96	108 (97-118.5)
	336	74.8 (66.5-83)

Not-detectable concentrations were recorded as 0 pg/mL

Table 7.5: Individual melatonin concentrations with sample timings

subject	dose	time	Conc pg/ml
1	0.1 mcg/kg/hr for 6 hours	0	0.00
		1	14.50
		3	145.50
		6	339.30
2	0.1 mcg/kg/hr for 6 hours	0	0.00
		1	0.00
		3	154.80
		6	414.29
3	0.1 mcg/kg/hr for 6 hours	0	0.00
		1	77.01
		3	317.49
		6	554.50
4	0.1 mcg/kg/hr for 6 hours	0	38.49
		1	47.49
		3	151.81
		6	372.30
5	0.1 mcg/kg/hr for 2 hours	0	0.00
		2	220.00
		6	142.51
		24	72.00
6	0.1 mcg/kg/hr for 2 hours	0	0.00
		2	160.00
		6	110.79
		24	71.30
7	0.1 mcg/kg/hr for 2 hours	0	0.00
		2	199.30
		6	156.80
		24	51.81
8	0.1 mcg/kg/hr for 2 hours	0	0.00
		2	207.31
		6	148.31
		24	79.00
9	0.02 mcg/kg/hr for 2 hours	0	0.00
		2	52.00
		6	0.00
		24	0.00
10	0.02 mcg/kg/hr for 2 hours	2	38.79
		4	65.01
		5	63.99
		6	71.00

subject	dose	time	Conc pg/ml
10	0.02 mcg/kg/hr for 2 hours	2	38.79
		4	65.01
		5	63.99
		6	71.00
11	0.02 mcg/kg/hr for 2 hours	2	71.00
		4	41.00
		5	49.49
		6	59.00
12	0.02 mcg/kg/hr for 2 hours	2	48.49
		4	52.00
		5	67.01
		6	67.01
13	0.02 mcg/kg/hr for 2 hours	2	49.00
		24	53.50
		48	40.00
14	0.02 mcg/kg/hr for 2 hours	2	46.01
		24	44.50
		48	42.51
15	0.01 mcg/kg/hr for 2 hours	2	66.01
		4	110.00
		5	103.81
		6	97.49
16	0.01 mcg/kg/hr for 2 hours	2	43.50
		6	40.00
		24	28.00
		48	38.80
17	0.04 mcg/kg for ½ hr	1	75.0
		72	110.5
		96	97
		336	66.5
18	0.04 mcg/kg for ½ hr	1	42
		72	130
		96	118.5
		336	83

Not-detectable concentrations were recorded as 0 pg/mL

Table 7.6: Individual data on half-life and Area under the curve (AUC)

Patient no	Dose	AUC (0, Tmax) Pg*hr/ml	Half-Life hours
1	0.1 mcg/kg/hr for 6 hours	886.03	NC
2		984.27	NC
3		1777.96	NC
4		997.25	NC
5	0.1 mcg/kg/hr for 2 hours	1486.93	14.8
6		1392.06	21.01
7		1992.14	11.3
8		1843.23	16.85
9	0.02 mcg/kg/hr for 2 hours	13304.17	NC
10		280.66	NC
11		302.67	NC
12		3181.75	153
13		285.16	NC
14		2873.76	401.90
15	0.01 mcg/kg/hr for 2 hours	462.32	11.49
16		1454.75	NC
17	0.04 mcg/kg for ½ hr	6699.13	1965
18		13304.17	28

NC= not able to calculate

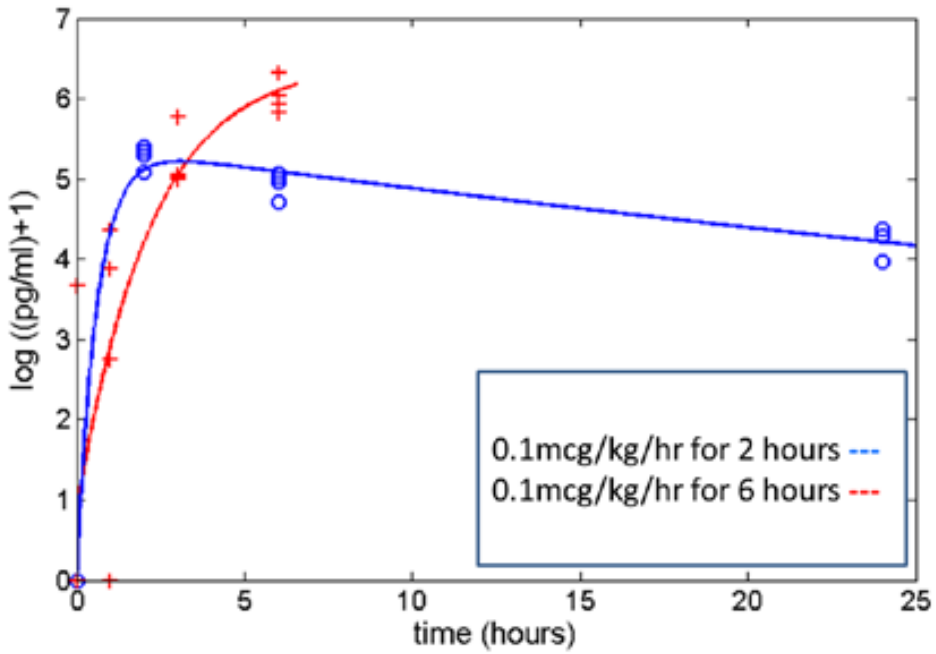
Table 7.7: Urine melatonin and its metabolite aMT6s Levels at different doses.

The detection limit of urine aMT6s was 0.2 ng/ml and urine melatonin was 0.03ng/ml. (Not detected levels were taken as 0 ng/ml). NE-not estimated as previous samples were negligible.

Dose	Time (Hours)	Median aMT6s (ng/ml)	Median Urine Melatonin (ng/ml)
0.1 mcg/kg/hr for 6 hours	0	0.8 (0-0.8)	0 (0-0)
	2	2.1 (0-3.7)	0.01 (0-0.08)
	3.5	0.6 (0-1.1)	0 (0-0.03)
	6	0.4 (0-0.8)	0 (0-0.16)
0.1 mcg/kg/hr for 2 hours	0	0.9 (0.8-1.0)	0 (0-0)
	2	0.8 (0.8-0.8)	0.02(0.02)
	4	0.6 (0.6-0.6)	0.18(0.18)
	6	1.3 (0.7-2.4)	0.04 (0-0.06)
0.02 mcg/kg/hr for 2 hours	2	1.2 (0.7-1.7)	0 (0-0.01)
	4	0.25 (0-0.5)	0 (0-0.01)
	6	0.4 (0-0.8)	0 (0-0)
	26	1.6 (0-2.2)	0 (0-0)
	50	0(0-0)	NE
0.01mcg/kg/hr for 2 hours	2	0.5 (0.5)	NE
	4	0.4 (0.4)	NE
	6	0.4 (0.4)	NE
	26	0.4 (0-0.7)	NE
	50	0 (0)	NE
0.04mcg/kg for ½ hr	72	0 (0-0)	NE
	96	0 (0-0)	NE
	336	0 (0)	NE

Figure 7.2: Plasma (log) melatonin concentrations versus time curve.

Data fitting was performed using a robust non-linear fitting algorithm as implemented in the curve fitting toolbox in MATLAB (2011, The Mathworks, Natick, MA, USA). Blue circles show the data points for the dose of melatonin @ 0.1mcg/kg/hr for 2 hours. Red crosses show the data points for the dose of melatonin @ 0.1 mcg/kg/hr for 6 hours. On giving melatonin as a continuous infusion of 0.1 mcg/kg/hr for 6 hours, steady state concentrations were not reached. On giving melatonin at the dose of 0.1 mcg/kg/hr for 2 hours there was a time dependant decrease in plasma melatonin levels suggesting first order kinetics. The graph also demonstrates that melatonin has a long half-life in preterm infants as compared to adults again highlighting the fact that the pharmacokinetic profile in preterm infants differs significantly from adults. As the dose is the same for the first 2 hours, data was used in both to make the model fit more accurately, although data points have been plotted separately.



7.3.3. Population Pharmacokinetic Modeling

Data from 16 infants were used in the population pharmacokinetic modeling. Two infants who were given infusion rates of 0.04 mcg/kg over 30 minutes were not included as the results were highly variable.

Base Model: The one-compartment model with first order elimination adequately described the disposition of melatonin in plasma. The resulting estimates of CL and V when the base model was fitted to each group of preterm neonates receiving 0.1 mcg/kg/hr, 0.02 mcg/kg/hr or 0.01 mcg/kg/hr melatonin dose are shown in Table 7.8.

Table 7.8: Pharmacokinetic parameter estimates of melatonin

Obtained from fitting the base model to two subsets of patients receiving the 0.1 mcg/kg/hr (over 2 hours and 6 hours) or the lower doses of melatonin infusion (0.02 mcg/kg/hr for 2 hours, 0.01 mcg/kg/hr for 2 hours). Pharmacokinetic parameter estimates are presented as mean (RSE%) and are scaled to a median weight of 0.867 kg. Variance terms are presented as CV% of the estimate (%RSE), where %RSE= standard error of the estimate/Mean estimate

Parameter estimates	0.1 mcg/kg/hr	0.02 or 0.01 mcg/kg/hr
Observations	23	23
Number of patients	8	8
CL (L/hr/0.867kg)	0.045 (7.7%)	0.012 (15.4%)
V (L/0.867kg)	1.098 (11.5%)	0.364 (32.1%)
$t_{1/2}$ (hr)	16.91	21.02
Residual variability (CV%)	30.24 (43.0%)	62.93 (17.5%)

In the combined dataset, the covariance matrix showed high correlation (ρ = 0.99) between CL and V random variables and, as a result, the model was reduced to a shared variance model:

$CL_i = \theta_{CL} \text{ X } \exp(\eta_{i,CL})$ and

$V_i = \theta_V \text{ X } \exp(\theta_{corr}. \eta_{i,CL})$

Where $\eta_{i,CL}$ is a random variable (zero mean and a variance of ω_{CL}^2) that distinguish the *i*th individual's parameter (CL_i) from the population mean values. θ_{corr} is the shared variance between CL and V as described by the following equation: $\theta_{corr} = \omega_V/\omega_{CL}$

Covariate screening and selection of the final model: During forward selection of covariates, inclusion of gender as a covariate affecting V in the model, as shown below, resulted in significant decrease in the MOFV (3.92 unit; p = 0.047). Inclusion of race as a covariate affecting both CL and V using an exponential model resulted in further 17.1 unit decrease in the MOFV ($p<1x10^{-4}$) and significant improvement in goodness-of-fit and was therefore included in the final model, Table 7.9.

Table 7.9: Stages of the model-building process to reach the final pharmacokinetic model of melatonin in the combined dataset.

Parameters are presented as mean parameter estimate (CV% in the estimate). MOFV: Minimum value of objective function; ΔMOFV indicates a change in MOFV relative to the previous model; *ΔMOFV of -3.84 is significant at P < 0.05 (1 df). Weight (WT) is scaled to a median of 0.867 kg; θ_{race}: fractional difference from θ_{CL} and θ_{V} at a certain value of Race; θ_{gender}: fractional difference from θ_{V} at a certain gender value; ω:Inter-individual variability; $Corr_{CL,V}$: correlation between CL and V variability parameters; θ_{Corr}: shared variance between CL and V; σ: residual variability.

Parameter	Base Model (WT-power allometric; full covariance matrix)	Reduced Base Model (shared variance model)	Intermediate Model (Gender-prop on V)	Final model (Gender-prop on V; Race-exp on CL and V)
Observations	46	46	46	46
Patients	16	16	16	16
MOFV	381.97	381.97	378.05	360.91
p-value	--	--	0.047*	3.48×10^{-5}*
CL (L/hr)	0.02	0.020 (0.39)	0.021 (0.37)	0.021 (0.63)
V (L)	0.75	0.75 (0.14)	0.64 (0.14)	0.74 (0.08)
$t_{1/2}$ (hr)	26.10	26.10	21.10	24.30
θ_{gender}	--	--	0.47 (0.67)	1.73 (0.20)
θ_{race}	--	--	--	1.09 (0.13)
ωCL(CV%)	111.80	111.90 (0.47)	103.0 (0.48)	111.90 (0.75)
$Corr_{CL,V}$	0.999	--	--	--
ωV (CV%)	51.70	--	--	--
θ_{Corr}	--	0.46 (0.26)	0.47 (0.26)	0.215 (0.57)
σ_{prop} (CV%)	24.90	24.90 (0.29)	24.40 (0.30)	22.90 (0.29)

In the backward elimination step, the effect of both covariates was confirmed since the MOFV increased by more than 10.83 units ($p < 0.01$, df = 1) when either of them was removed from the model. None of the other tested covariates, including serum creatinine, urea and bilirubin levels resulted in any significant improvement of the model predictions. The final model for melatonin was, therefore, as follows:

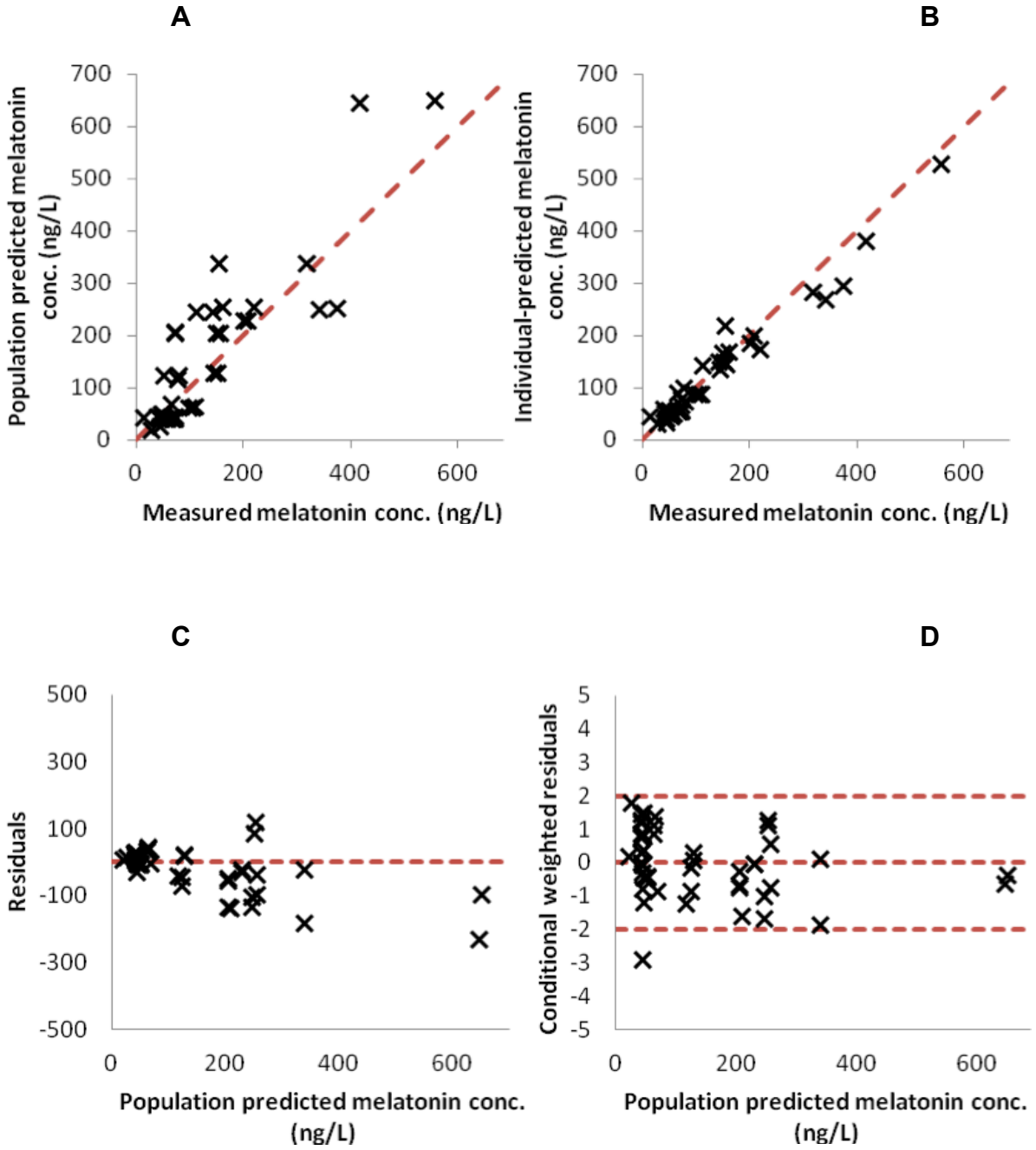
$$\mathrm{CL}(\mathrm{Lh}^{-1}) = \theta_{\mathrm{CL}} \times \left(\frac{\mathrm{WT}}{0.867}\right)^{0.75} \times \mathrm{Exp}(-\theta_{race} \times \mathrm{Race})$$

$$\mathrm{V}(\mathrm{l}) = \theta_{\mathrm{V}} \times \left(\frac{\mathrm{WT}}{0.867}\right) \times \left(1 + \theta_{gender}\right) \times \mathrm{Exp}(-\theta_{race} \times \mathrm{Race})$$

where θ_{CL} and θ_{V} are the population estimates for CL and V, respectively, standardised to a median weight of 0.867 kg using allometric models. θ_{race} is a parameter estimating the fractional difference from θ_{CL} and θ_{V} at a certain value of Race. θ_{gender} describes the fractional difference from θ_{V} at a certain gender value.

According to this final model, the estimates for CL and V in a typical individual who weighs 867 g, are 0.021 L/hr and 0.74 L, respectively, resulting in an elimination half-life of 24.3 hr. The IIV associated with the shared variance between CL and V was reduced from 46.2% (base model) to 20.5% (final model), i.e. a reduction of 55.6% of the initial value. The residual variability (CV %) in final model was 29.8%. Graphical Evaluation is shown in Figure 7.3.

Figure 7.3: Plots of measured versus population predicted (A) and individual predicted (B) melatonin concentrations from the final model as well as plots of residuals (C) and conditional weighted residuals (D) versus population predicted melatonin concentrations.



7.4. Summary of the Results

It was possible to determine the pharmacokinetic profile of melatonin in preterm infants based on individual pharmacokinetic analysis and population modeling. Reliable pharmacokinetic data and melatonin closest to physiological adult concentrations were achieved with a 2 hour infusion of 0.1 mg/kg/hr with a time dependant decrease in plasma melatonin concentrations. Lower doses of melatonin infusion gave unreliable estimates of pharmacokinetics in individuals. The population pharmacokinetic one compartment model with first order elimination adequately described the disposition of melatonin in plasma. Incorporating race and gender as covariates resulted in a reducing the shared variance of CL and V by 57%.

There were no gross problems during the administration of melatonin in this preterm population and none of the patients required the infusion to be stopped early, demonstrating tolerability of melatonin in preterm infants.

Slow clearance and a long half-life of melatonin in preterm infants make it difficult to allow replication of adult and thus fetal concentrations of melatonin.

8. DISCUSSION

Although melatonin has been administered to newborn infants in a variety of doses [509, 511-513, 546, 547, 581], a systematic analysis of the pharmacokinetic profile in preterm infants has not previously been available. Conducting PK studies of melatonin is important in this age group, as brain injury in this age group is unique in this population. Data from adult and animal studies together with knowledge of neonatal physiology provide data for estimation of a starting dose in such studies, but these estimations are sufficiently uncertain. Preterm infants require intensive care and have co-morbidity, hence it is important to conduct a tolerability study along with the PK study before moving on to safety and efficacy trials of neuroprotection.

8.1. Pharmacokinetics

Compared to adults and older children, in preterm infants melatonin half-life and clearance were prolonged and volume of distribution decreased.

In adults, the melatonin pharmacokinetic profile has been well defined [437, 444]. There is a circadian secretion of endogenous melatonin with a marked diurnal variation. Secretion usually starts by 9 pm and peaks at 3 am, then plasma concentrations decline to negligible levels by 9 am [437, 445]. The adult peak melatonin concentration is reported to be 44.3 pg/mL but can range from ≤8 - 275 pg/mL [445, 446]. Circulating melatonin is predominantly metabolised by hydroxylation at the C-6 position in the liver and then conjugated with sulfate to form aMT6s or to a lesser extent with glucuronide (6%). Less than 1% of circulating melatonin is excreted unchanged into urine [467, 468]. The elimination half-life of melatonin in adults is 45 - 60 minutes [466]. Urine melatonin concentrations in adults are

variable but usually within the range of 38.2 - 179.1 (ng) in 24 hour samplings [469] and, for example a reported mean excretion between 6pm –10am of 29.7 ng (range 9-59.1 ng) [470]. Mean aMT6s excretion in adults was 11100 ng (range 4100 - 24200) in a 16 h period 6 pm - 10 am [446, 546]. Endogenous plasma melatonin concentrations generally correlate well with urinary melatonin and aMT6s [470].

Clearance: In the present study, urine aMT6s and melatonin concentrations were very low, suggesting that preterm infants do not excrete melatonin as rapidly as expected from adult studies. This should not have been unexpected as physiological differences between children and adults, results in age related differences in pharmacokinetics. In neonates especially in preterm infants, decreased weight adjusted doses are required because of decreased protein binding, renal excretion and metabolism. CYP1A2 and CYP1C19 along with other cytochrome p450 have decreased activity in neonates and can result in marked decrease clearance. Another commonly used drug in neonatal practice, caffeine, is known to be cleared by CYP1A2 and has a half-life of 72-96 hours in neonates compared to adults and older children where the half-life is 5 hours. As renal clearance in neonates is dependent on glomerular filtration rate, there is a prolonged elimination half-life of drugs cleared predominantly by this route. The type of milk is also known to affect the clearance of a drug. Neonates who were breast-fed have been shown to have lower clearance of caffeine as compared to formula fed infants. As melatonin is also metabolised by CYP1A2 similar to caffeine, this may be of importance. In our study all the preterm infants were on breast milk.

Volume of distribution: Melatonin is extremely lipophilic and the lower body fat content in preterm infants (10%) as compared to adults and children (20-25% and 15-20%) can result in

the low volume of distribution obtained. This may be relevant for future studies as the target organ for neuroprotection is the brain and a low volume of distribution can result in lower brain concentrations of melatonin.

Half- life: Half-life of drug is directly related to clearance and inversely related to the volume of distribution. Thus half-life is increased by a decrease in clearance or an increase in volume of distribution. In our study the half-life of melatonin was very prolonged in contrast to adult melatonin half-life, although this is not surprising given the slow clearance.

Plasma concentrations: The decreased clearance and low volume of distribution explains the higher plasma concentrations obtained in preterm infants in this study. Some drug interactions can also increase melatonin concentrations. Caffeine is known to increase the concentrations of melatonin in the blood by competing for the same metabolic pathway in adults and by acting as a substrate for CYP1A2 [582]. Caffeine improves outcomes for preterm infants and is routine in neonatal care [583]; all infants in this study received caffeine. Plasma melatonin concentrations assayed may be higher due to heparin in the circulation, but in our study venous or capillary blood samples were obtained in all except one infant where samples were obtained from a heparinised arterial line. Plasma melatonin may also be higher if there is a delay in extraction of the plasma resulting in drug diffusing out of the erythrocytes into the plasma. In our study, all the blood samples were sent to the local laboratory to be centrifuged within an hour of collection and plasma was frozen till analysis. Cold chain was maintained throughout transportation to Guildford where the assays were analysed.

There were no significant differences in the blood melatonin and urine aMT6s concentrations at different gestations, although the numbers in the study were small to definitely confirm this.

Previous studies have suggested that preterm infants do not secrete melatonin until at least 52 weeks post conception [441] and in the present study melatonin was undetectable before administration in all infants except one. All subjects received maternal or human donor milk which was a potential source of exogenous melatonin, being present in human milk with a peak concentration of 23 +/- 6 pg/mL [456]. The volume of milk feeding was small in this group but melatonin present in the milk can account for the detectable melatonin in this single baseline sample. The high melatonin concentration in this one infant on day 2, prior to the infusion could also reflect maternal melatonin and given the prolonged clearance there may have been some transplacental melatonin but his twin had undetectable melatonin concentrations making it difficult to explain this finding.

Implications of long half-life and decreased clearance: The long half-life of melatonin in the preterm infants is unlikely to reflect the normal fetus where transplacental equilibration is likely to control blood concentrations more than renal or hepatic drug handling. It may thus be difficult for experimentalists to recreate the fetal circadian rhythm in preterm infants.

8.2. Dose, route of administration and timing of melatonin infusion

Initial dosing regime: We considered 2 approaches for the dosing regime - one using animal data and two using adult PK data. Using the lowest effective neuroprotective dose in the mouse model of 0.005 mg/kg, the HED was calculated as 0.18 mcg/kg/hr. Using adult PK data and aiming to achieve adult plasma melatonin levels the dose was calculated as 0.17

mcg/kg/hr. As both dose regimes were similar, for the present PK study we decided to use a slightly lower dose of 0.1 mcg/kg/hr as the initial starting dose, as tolerability was also an important issue. Knowing from experimental models that circadian rhythm is likely to be important in neurodevelopment in preterm infants, it was decided to give the initial infusion over 6 hours. As melatonin has a very short half-life in adults, it was thought that steady state would be achieved by 6 hours at least if not earlier. In our study we showed that plasma melatonin concentrations were higher than predicted and steady state was not achieved at 6 hours following the 6-hour infusion.

Modifications in later dosing regimes: Reliable pharmacokinetic data and melatonin concentrations closest to physiological adult concentrations were achieved on individual data analysis with a 2 hour infusion of 0.1 mcg/kg/hr. Lower doses of melatonin infusion (0.02 mcg/kg/hr for 2 hours, 0.01 mcg/kg/hr for 2 hours and 0.04 mcg/kg over 30 minutes) resulted in wide ranges of plasma melatonin levels making it difficult to calculate the pharmacokinetic parameters. This wide range in the plasma melatonin levels may have been because of the minute amounts of melatonin used, difficulties in setting up an infusion with small volumes, flushes through the intravenous line at the end of the infusion. Errors in the assays of melatonin may have also resulted in the varied plasma melatonin levels. This was again checked with the laboratory and some of the samples were rechecked giving the same results.

Sample Timings and modifications: Timed samples were taken which were adapted depending on the results. It was not possible to estimate PK parameters on a number of our patients. We deliberately continued to take samples before the start of the melatonin infusion, as we felt there was not enough literature review of levels of melatonin in the first

week of life especially in preterm infants. With the limitations of bloodletting set by the European Directive for clinical trials, this could possibly have been avoided and another sample point could have been taken instead allowing for better profiling of the time concentration curves. Sample timings could have been spread out better which would have resulted in better PK profiling and improved estimates of PK parameters.

Timing of melatonin infusion: In the present study, it was decided to give melatonin once in the first seven days after birth. It is likely that a neuroprotective agent will be needed for a much longer period of time based on the pathogenesis of brain injury as discussed earlier, however as this study also looked at the tolerability of melatonin, it was decided to administer the dose of melatonin only once to each patient during the first week after birth. Also it is during this time, preterm infants have intravenous access, hence ethically feasible to conduct this study.

Route of administration: We administered melatonin by intravenous infusion because previous studies suggest that oral melatonin has variable bioavailability [447, 448] and 30-60% is immediately metabolised to aMT6s [456].

Infusion versus bolus: Although melatonin has a high safety profile, there is not much published data in preterm infants or even neonates. Hence tolerability is not known. As per verbal advice and discussion with MHRA, it was decided that minimum infusion rate would be at least 30 minutes. This allowed us to stop the infusion if there were any adverse events.

8.3. Tolerability of melatonin in preterm infants

Adverse events: There were no drop-outs in the study. There were no serious adverse events or SUSARS which had to be notified to the sponsor or MHRA/Ethics committee. None of the patients had low temperatures documented. As most of these infants were in servo-controlled incubators, small changes in temperature may have been undetected. Hypotension has been noted to be an issue in some of the animal studies. None of patients were on inotropes before the onset of the infusion and none had to be started on inotropes after the initiation of melatonin infusion. It is likely this dose of melatonin was too small and further studies should monitor blood pressure effect of melatonin. We did not follow these babies long term so the developmental effects are not known although it is unlikely that one dose of melatonin to have an effect either positive or negative.

8.4. Compartmental modeling

The PPK one-compartment model with first order elimination adequately described the disposition of melatonin in plasma. The results shown in Figure 8.3 C, D indicate that the assumption of random effects was appropriate because both residuals and conditional weighted residuals were evenly distributed around 0 and almost all conditional weighted residuals were contained within ± 2 units of the null ordinate indicating absence of any influential observations. The limited number of samples collected shortly after melatonin administration made it difficult to accurately evaluate the distribution phase and hence the 2-compartment model would not have provided a better fit to our data.

8.5. PPK model and covariates

The model revealed considerable variation in CL and V which was reduced when race and gender were incorporated as covariates, reducing the shared variance of CL and V by over 50%. A black female is likely to have higher clearance in this population. A plausible explanation for the effect of race on melatonin CL could be the variation between races in the allele frequency of polymorphisms affecting melatonin 6-hydroxylation by CYP1A2 and CYP1B1 enzymes [463, 584]. We did not look for genetic and polymorphisms in our study which would have been very useful to confirm the influence of the covariates.

Allometric size adjustment, with fixed exponents of 0.75 for CL and 1 for V, was used for *a priori* inclusion of weight. Investigation of models where the power values were not fixed but included as additional thetas (θs), did not result in any significant improvement in model fit.

8.6. Problems and limitations of the study

The main limitations of the study were the small sample size, limited samples collected during the distribution phase for each patient and expectation that the PK parameters would be similar to adults.

An adaptive design was used for this study based on the analysis of the data. Using an adaptive design allowed us to decrease the duration of the study and recruit less patients. One of the weaknesses of an adaptive design is that it can introduce bias especially in unblinded study like the present study. This can adversely affect development decisions, such as choice of dose, population or study endpoints in subsequent studies. Multiple adaptations as in our study can also make the interpretation of the study more difficult.

As melatonin has a very short half-life in adults, it was thought that steady state would be achieved by 6 hours at least if not earlier. In our study we showed that plasma melatonin concentrations were higher than predicted and steady state was not achieved at 6 hours following the 6-hour infusion. This was an important problem that we could have easily predicted before setting up this study as melatonin is cleared by cytochrome p450 enzymes and CYP1A2 and CYP1C19 activities are highly reduced in preterm infants. Taking more samples during the distribution phase would have allowed us to give a better profile of the pharmacokinetics of melatonin in preterm infants. We deliberately continued to take samples before the start of the melatonin infusion. With the limitations of blood letting set by the European Directive for clinical trials, this could possibly have been avoided and another sample point could have been taken instead allowing for better profiling of the time concentration curves. Sample timings could have been spread out better which would have resulted in better PK profiling and improved estimates of PK parameters. A one compartmental model was used in our study. Given the lipophilic property of melatonin it might have been better to use 2 or more compartments although there were limitations imposed by the distribution of the samples and timings.

We unfortunately due to blood volume constraints did not measure melatonin metabolites in the serum but it is likely that they would have been low. We also did not examine melatonin levels in the maternal milk, which would have been useful. Although laboratory measures were made in multiples, we did not send the assays to another laboratory to confirm our study which would have been useful, as it would have discovered if there were any errors related to the laboratory assay. We also did not examine any CYP1A2

polymorphisms in our study. This is relatively easy to do with buccal genetic sampling and it would not have been necessary to take extra blood sampling.

All the infusions were given during daytime. This made it difficult to detect if there was any melatonin secreted at night-time. Use of bolus injection would have been easier to give and less challenging as these vulnerable infants are on multiple infusions and medications. Infusions are more complex and depends not only on available intravenous access for a long duration, but also dependant on infusion pumps, availability of nursing staff and increased chances of loss of intravenous access or extravasation. Administering oral melatonin rather than an intravenous preparation would have been even better allowing administration in the later weeks after birth when these preterm infants are more stable. Special time consuming formulation as required for intravenous formulation used in our study would have not been necessary as oral melatonin, although not licensed is available through off label use.

Comparison of the changes in heart rate, respiratory rate and blood pressure with a comparative preterm population would have allowed us to confirm safety and tolerability of melatonin in this age group better. All neonatal intensive care units have the ability to download this data from their electronic monitors and this could have been easily done in this study.

8.7. Alternatives to the above study

One of the alternatives to this study would have been to review melatonin concentrations in the umbilical cord i.e. reflecting mother's plasma concentrations along with neonatal melatonin plasma and urine concentrations. This would allow us to establish PK profile

without giving melatonin. This study although has the advantage of not dosing preterm infants with a new drug, has the disadvantage of getting maternal consent and it may not be ethically and logistically possible with a limited time frame available. Also with the network structure in the UK, many of our patients are transferred in –utero in labour or ex-utero, making it difficult to achieve this alternative. Another alternative would be to use many more sampling times and this can be achieved by developing blood spot testing for melatonin assays.

8.8. Conclusions

Melatonin infusion is safe and well tolerated in preterm infants. Melatonin has slow clearance and a long half-life in preterm infants. Slow clearance makes replication of adult and thus fetal concentrations of melatonin problematic. Further studies are needed to confirm this.

9. PROPOSED DRUG DEVELOPMENT PIPELINE FOR CLINICAL TRIALS OF MELATONIN IN PRETERM INFANTS

A clinical trial for any therapy is based and justified on a backbone of evidence provided in a range of animal models, clinically feasible administration route and formulation, recognised pharmacokinetics and a favourable safety profile. The cycle of moving from animal models to human work and using this information to further refine and understand pathophysiology and pharmacology using animal models is very important in the success of drug. Repurposing existing drugs such as melatonin allows us explore novel uses for the drug where safety and pharmacokinetic effects have already been confirmed. These drugs when used in neonates, although have the advantage of not requiring basic safety testing and toxicity animal work still have to undergo formulation work and PK studies before moving on to safety and efficacy studies. This is especially important as disease progression may be very different as compared to adults or completely unique to neonates, basic physiology varies dramatically and most of the time little is known about the impact of a drug across organ systems in the developing animal. The pharmacokinetic work described in the thesis allows us to move forward in setting up studies on neuroprotection with melatonin and assessing safety and efficacy of melatonin in a systematic manner.

9.1. Future work in animal studies

The importance of preclinical safety studies especially in juvenile animal models has been emphasised both by the FDA and EMEA with special consideration for organ systems that undergo postnatal development. Dosing studies for melatonin in different groups of neonatal animal models with their pharmacokinetics can help establish human equivalent

doses for neonatal clinical trials. Gressens et al have shown that the in the mouse model of ibotenate induced brain injury, there was a dose dependent reduction of periventricular white matter injury. Further work can be done in animal models to see if the concentrations achieved are neuroprotective in animal models. Multiple dosing, delayed treatment, therapeutic window, routes of administration along with assessment of outcome should be explored in much further detail in animal studies. Improving physiological, psychomotor and cognitive testing in neonatal animal models allows for better understanding of effectiveness and toxicity of melatonin [352]. Developing a pharmacodynamic – pharmacokinetic marker will help expedite clinical trials of neuroprotection. MR imaging has now been used along with histopathological outcome in some animal models e.g. in HIE piglet model, MR spectroscopy has now been used to show correlation with histopathological outcome. This can then be replicated in neonatal studies. These imaging markers need to be developed further.

9.2 Future work in neonatal studies

Table 9.1 suggests some of the future work in neonatal clinical studies with alternatives suggested. Using specialised MR imaging as surrogate endpoints, we can now complete a phase II exploratory trial in a much shorter timeframe and enrolling fewer patients in the trials. This allows us to move in a few different directions. If there is a significant difference in the treatment group then we can perform a larger phase III randomised clinical trial

If there is no significant difference in the phase II trial using this physiological dose, it is possible that we need to give a higher or a pharmacological dose for neuroprotection. This brings into question: safety, feasibility and dose required. Melatonin has been given in much

higher doses in neonates and children with no adverse events, although relatively small numbers of studies have been done in neonates. Melatonin is an extremely lipophilic drug and does not dissolve easily in saline or water. For the small dose of 0.1 microgram in 1 ml as used in the current study, it was easy to dissolve melatonin in normal saline but a higher dose will require a suitable solvent such as propylene glycol or ethanol. Given that both these excipients can potentially have adverse effects on the developing brain, it will require further analytical and stability work before a pharmacological dose can be used.

9.2.1. Lessons learnt from the pharmacokinetic studies to be incorporated in future studies

The sample numbers in the distribution phase were limited in the PK study and future studies should look at taking more samples during the distribution phase.

Although one compartmental model adequately described the data in the PK study, 2 or more compartmental models should be looked at, which may describe the pharmacokinetics better.

Race and gender were important covariates in the PK study and future studies should incorporate genetic and polymorphism testing.

Electronic clinical data including blood pressure monitoring should be incorporated in the case report form. This will further document the safety of melatonin in the preterm population.

9.3. Modelling and simulation

The term PK/PD modeling refers to a data (pharmacokinetic and pharmacodynamic) driven exploratory analysis based on a mathematical / statistical model [585]. PK-PD models and simulations assist in formulating how the next step in development is to be performed and can be used to predict trial outcomes. Modeling can help in predicting clinical efficacy estimates; can provide dose range guidance to be tested in clinical trials, optimal sampling guidance along with margins of safety based on target efficacy concentrations. PK-PD modeling has potential to decrease cost and time in early preclinical and clinical trials. It can also help to refine data obtained from initial PK dosing studies.

9.4. Future Challenges

Many other questions need to be addressed: what are the optimum indications for melatonin treatment, what should be the duration of melatonin treatment and how early should we start treatment? Also we have shown in our pharmacokinetic study that it will be difficult to replicate fetal concentrations and diurnal circadian given the long half-life. Further work will be needed looking at circadian rhythm and neuroprotection.

Table 9.1: Outline of each planned study and steps in pharmaceutical development is proposed (Study 1 is taken as the pharmacokinetic study as outlined in section 7)

Study	2	3	4
Objective	Melatonin will reduce preterm brain injury Safety and efficacy study	Melatonin will reduce preterm brain injury Safety and efficacy study	Pharmacological dose of Melatonin will reduce preterm brain injury Development of an injectable formulation of melatonin of higher strengths Safety and efficacy study
Design	Phase 2 - Double blinded 2 arm placebo controlled trial in preterm infants starting within 48 hours after birth for 7 days	Phase 3 - Double blinded 2 arm placebo controlled trial in preterm infants starting within 48 hours after birth for 7 days	Phase 2 - Double blinded 2 arm placebo controlled trial in preterm infants less than 31 weeks gestation starting within 48 hours after birth for 7 days
Design Alternatives	Give for longer duration Start antenatally	Give for longer duration Start antenatally	multiple arm study using different doses
Dose	Dose derived from study 1	Dose derived from study 1	Dose derived from animal data
Dose alternatives			10 times previous dose (log doses)
Endpoints	MRI at term corrected age	MRI at term corrected age	MRI at term corrected age
Alternative Endpoints	Neurodevelopmental assessment at term, 2 years and 6 years of age.	Neurodevelopmental assessment at term, 2 years and 6 years of age.	Neurodevelopmental assessment at term, 2 years and 6 years of age.
Analyses	Fractional anisotropy measured by tract based spatial statistics on MR imaging carried out at term corrected age Pharmacokinetics	Fractional anisotropy measured by tract based spatial statistics on MR imaging carried out at term corrected age Pharmacokinetics	Fractional anisotropy measured by tract based spatial statistics on MR imaging carried out at term corrected age Pharmacokinetics
Number of patients	40-60	300	60-120

APPENDIX

The pharmacokinetic study described in the above thesis can be used to set up early exploratory trials of neuroprotection with melatonin. For maximum efficiency in a preliminary study MR biomarkers can be used as surrogate endpoints. Appendix A describes the set up and design of the phase 2 exploratory study of neuroprotection trial in preterm infants with melatonin which is currently ongoing and appendix B describes the MR imaging and the new MR techniques which can be used as surrogate endpoints.

A1. Setup of the MINT Trial (Melatonin Neuroprotection Trial)

Study Design: This study was set up as a phase 2 exploratory; multi-centre double-blinded randomised placebo controlled 2-arm trial evaluating melatonin as a neuroprotective agent in addition to standard intensive care.

The 2 arms of the trial were Melatonin (active treatment) and normal saline (placebo). The study drug was given an intravenous infusion every day for 7 days starting by 48 hours after birth. Melatonin injection was supplied in 5ml ampoules and consisted of 0.1 microgram/ml of melatonin (same as the pharmacokinetic study). The dose of melatonin was taken as 0.1 mcg/kg/hr (1ml/hr) given as an intravenous infusion for 2 hours. The placebo was an equal volume of normal saline and was supplied in similar 5 ml ampoules. All infants received the present standard of intensive care. Both drugs were packaged and labelled by the Stockport Pharmaceuticals (Stepping Hill Hospital, Poplar Grove Stockport Cheshire SK2 7JE) and were kept in each of the Hospital Pharmacy and neonatal unit in strict accordance with the local pharmacy governance guidelines.

Randomisation: To ensure equality of groups randomisation was taken in blocks of four infants stratified by gestational age, grouped as less than 27 weeks and 27 to 30 weeks gestation [586]. Analysis will be by intention to treat.

Sample size: A total of 60 preterm infants were estimated to be required for this study (30 in each arm). This was estimated from a simulation model based on data of fractional anisotropy (FA) analysed by tract based spatial statistics (TBSS) in our centre (table A2.1). This model indicates that a study with 30 babies in each arm and a treatment effect of 5% change in FA will detect a significant change ($p<0.05$) in approximately 35% of voxels, using TBSS.

Patient recruitment: Full ethical approval for this study was obtained from North West London Research Ethics Committee (REC ref: 11/LO/0839). Preterm infants were recruited from four tertiary level neonatal intensive care units of London (Queen Charlottes and Chelsea Hospital, London, UK; St Mary's Hospital, London, UK; St Thomas' Hospital, London, UK and Medway Maritime Hospital, Kent, UK). Mothers who were admitted in preterm labour were given information regarding the study antenatally.

Endpoint: MRI at term corrected age (37 - 44 weeks) or after discharge from local hospital was taken to be the endpoint for the study. MR images were obtained with the 3T MR facility on the neonatal unit at Queen Charlotte and Chelsea Hospital. Volumetric, conventional and diffusion-weighted images were obtained at term corrected age using published protocols by our group to provide imaging endpoints.

Table A1.1 Percentage of significant voxels detectable by TBSS in groups of preterm infants at term

% FA Increase	Group Size									
	5	**7**	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**45**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	1.81	0.10	0.00	0.00	0.71	0.00	1.73	3.50
4	0.00	1.17	2.89	5.16	3.18	0.17	9.37	2.81	28.97	27.20
5	0.00	3.72	5.25	18.32	15.43	7.69	36.37	32.32	63.28	64.72
6	0.00	5.92	19.81	44.39	38.12	39.99	64.36	54.31	75.31	79.03
7	0.00	7.92	41.31	53.17	58.49	63.73	74.86	73.46	81.27	83.44
8	0.01	14.31	47.07	60.26	71.84	73.73	81.09	80.67	85.87	87.13
9	0.27	28.71	54.54	65.73	76.77	80.31	84.56	85.52	88.14	89.76
10	0.47	36.22	62.03	74.28	79.56	83.51	87.68	87.79	90.28	91.46
11	1.27	47.10	66.85	77.15	84.04	86.66	89.17	90.16	91.77	92.19
12	2.47	59.29	71.80	79.96	86.99	88.63	90.61	91.03	92.47	92.86
13	5.22	62.91	74.85	82.38	88.30	89.61	91.55	92.09	93.09	93.12
14	7.02	65.96	78.15	84.42	89.78	90.60	92.26	92.53	93.26	93.23
15	14.52	71.14	82.71	87.44	90.42	91.75	92.54	92.78	93.36	93.54
16	20.59	73.06	83.74	88.65	91.80	92.15	92.78	93.22	93.45	93.64
17	25.93	74.96	85.11	89.62	92.11	92.39	93.17	93.39	93.58	93.70
18	26.44	77.70	86.62	90.43	92.43	92.86	93.27	93.44	93.62	93.72
19	32.72	81.43	89.84	91.28	92.69	93.05	93.36	93.54	93.69	93.82
20	39.46	82.65	90.31	91.96	92.84	93.25	93.43	93.62	93.76	94.17

A2. Imaging in Preterm Brain Injury

Conventional MR Imaging

MRI has been used to describe the different aspects of preterm brain injury, sequelae of preterm brain injury and to predict long-term neurodevelopmental outcome.

GMH-IVH is seen as a low SI on T2-W scans and high SI on T1- W imaging and is differentiated from the normal germinal layer by its irregular appearance and it is slightly more hypointense on T2- W scans [587]. This low SI may persist for several months due to presence of haemosiderin. HPI is characteristically seen initially as a fan shaped structure due to the obstructed medullary veins and later on as a porencephalic cyst.

MRI has also identified 'new' abnormalities such as punctate white matter lesions [588] and so-called diffuse excessive high signal intensity (DEHSI) [146]. The aetiology of these abnormalities remains obscure, although it has generally been assumed that they represent milder forms of PVL and DEHSI may be the MR correlate of diffuse PVL) [31, 146, 587]. This, however, has not been proven. Noncystic WMI in preterm infants is associated with reduced total brain volumes, reduced cerebellar volume, reduced cortical and deep gray matter volume at near term [221, 589-591].

MRI done at term equivalent age has prognostic significance and correlates with outcome. In a small study of 51 preterm infants, it was shown that the presence of parenchymal lesions at term equivalent age had a sensitivity of 100% and specificity of 79% for motor abnormality [592]. In a study of 167 preterm infants less than 30 weeks gestation, white matter changes (cystic or diffuse) was predictive of cognitive delay (OR, 3.6; 95% CI, 1.5-

8.7), motor delay (OR, 10.3; 95% CI, 3.5-30.8), cerebral palsy (OR, 9.6; 95% CI, 3.2-28.3), and neurosensory impairment (OR, 4.2; 95% CI, 1.6-11.3) at 2 years of age [593]. Lesions involving the posterior limb of internal capsule (PLIC) have been shown to correlate with motor outcome. Infants with HPI who had asymmetry myelination in the PLIC at term corrected age all developed hemiplegia at 12 to 24 months, while infants with normal symmetrical PLIC myelination had normal outcomes [189]. It is likely that PLIC injury seen on MRI reflects the effects of Wallerian degeneration of the corticospinal motor system as a consequence of grey or white matter injury reflecting established or widespread injury signal changes in the PLIC may reflect established and more widespread injury [594, 595]. The clinical significance of subtle lesions such as DEHSI remains controversial. Some studies have shown a correlation with lower developmental quotients at 18-24 months [596, 597] while punctate lesions did not show any correlation with long-term neurological outcome.

Early MRI i.e. performed before term equivalent age has been evaluated with mixed reports. Miller et all showed that moderate-severe white matter changes on early MRI is associated with abnormal neurodevelopmental outcome at 18 months [598]. On the other hand, Dyet et al reported that punctate lesions seem to resolve over time and along with haemorrhage alone or cerebellar haemorrhage did not predict long-term neurodevelopmental outcome [596]. Further studies are needed in early MRIs to predict accurately and reliably long term outcome.

Newer MR approaches

Severe abnormalities on conventional MRI are only moderately predictive of motor impairment and even less predictive of cognitive abnormalities [593, 599]. Newer MR based

techniques such as diffusion tensor imaging (DTI), Tract Based Spatial Statistics (TBSS), functional connectivity MRI, volumetric analysis, and deformation based morphometry (DBM) are now providing further insight into alterations in structural and functional brain maturation and can also provide early proof of tissue injury. These imaging techniques provide objective assessments rather than visual assessment and hence can offer surrogate outcome measures for proof of concept intervention studies.

Diffusion tensor imaging (DTI) is an MRI technique that characterises the diffusion properties of water molecules in tissue. In cerebral white matter water diffuses preferentially along the direction of axons and is relatively restricted perpendicular to axons, a phenomenon known as anisotropy. Quantitative measures derived from DTI provide objective and reproducible assessment of white matter and have provided insights into neonatal brain development and injury [158]. These quantitative measures include the apparent diffusion coefficient (ADC), a measure of the overall magnitude of water diffusion, fractional anisotropy (FA), the fraction of diffusion attributed to anisotropic diffusion, axial diffusion, the magnitude of diffusion parallel to fibres and radial diffusion, diffusion perpendicular to white matter tracts [79].

Abnormal diffusion imaging parameters at term equivalent age have been associated with poor neurodevelopmental outcome. Measurement of ADC is thought to provide a quantification of tissue characteristics that on visual assessment of T2 weighted scans appear as DEHSI. The finding of DEHSI by conventional MRI is associated with high ADC [600, 601]. In the presence of a normal conventional MRI, an isolated increase in ADC values in central white matter in preterm infants at term equivalent age correlated with a lower developmental quotient at 2 years corrected age [602, 603]. In the white matter, the

principal direction of diffusion represents the primary orientation of fibres. This information can be used for "tract tracing" to infer neural connections [604]. Low FA values in the PLIC in preterm infants at term equivalent age have been shown to correlate with cerebral palsy in the absence of focal lesions [605].

Tract based Spatial Statistics (TBSS) is an automated observer independent method of aligning FA images from multiple subjects to make non-biased assessments of localised changes in the major white matter tracts [606, 607]. Using TBSS our group has shown reduced FA in specific areas namely the centrum semiovale, frontal white matter and the genu of the corpus callosum in preterm infants [601]. TBSS is a sensitive tool for detecting global group wise differences in FA in the preterm population [608].

Volumetric measurements through MR Imaging

Volumetric measurements have provided valuable insights into impact of prematurity on the developing brain (Figure A1.1).

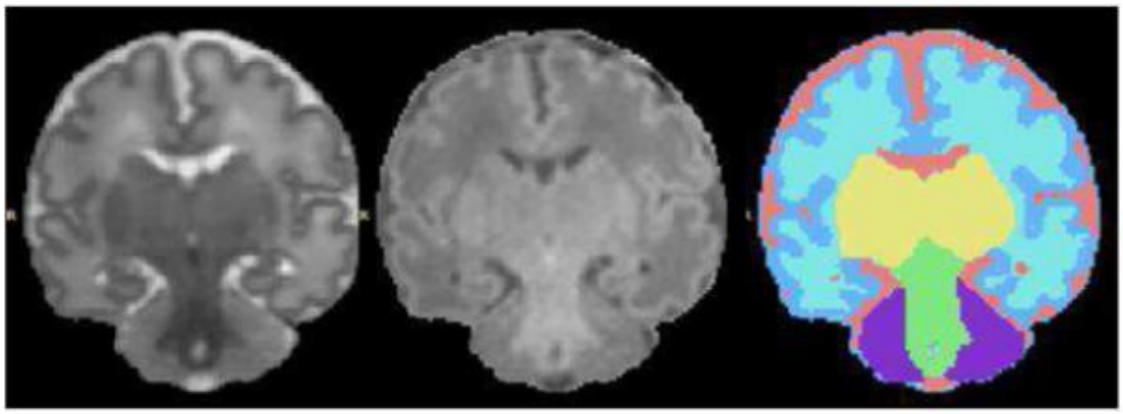


Figure A1.1: Image segmentation for quantitative volumetric analysis. L-R: coronal T2-weighted image, T1-weighted image, segmentation map derived from these MR images. Map components identified CSF (pink) unmyelinated white matter (taupe), cortical grey

matter (blue), basal ganglia (yellow), myelinated white matter (green) and cerebellum (purple). *(Reproduced from Mathur A et al, 2010 [609]).*

Prematurity and white matter injury are associated with decreased total brain volume, decreased white matter volume and increased cerebrospinal volume, which persist in adolescence [590, 591, 610, 611]. This in turn correlates with decreased full scale, verbal and performance IQ scores [612]. Our group has shown that the rate of cerebral cortical growth between 24 and 44 weeks postmenstrual age predicted global ability in later childhood, particularly complex cognitive functions but not motor functions [613] (Figure A1.2).

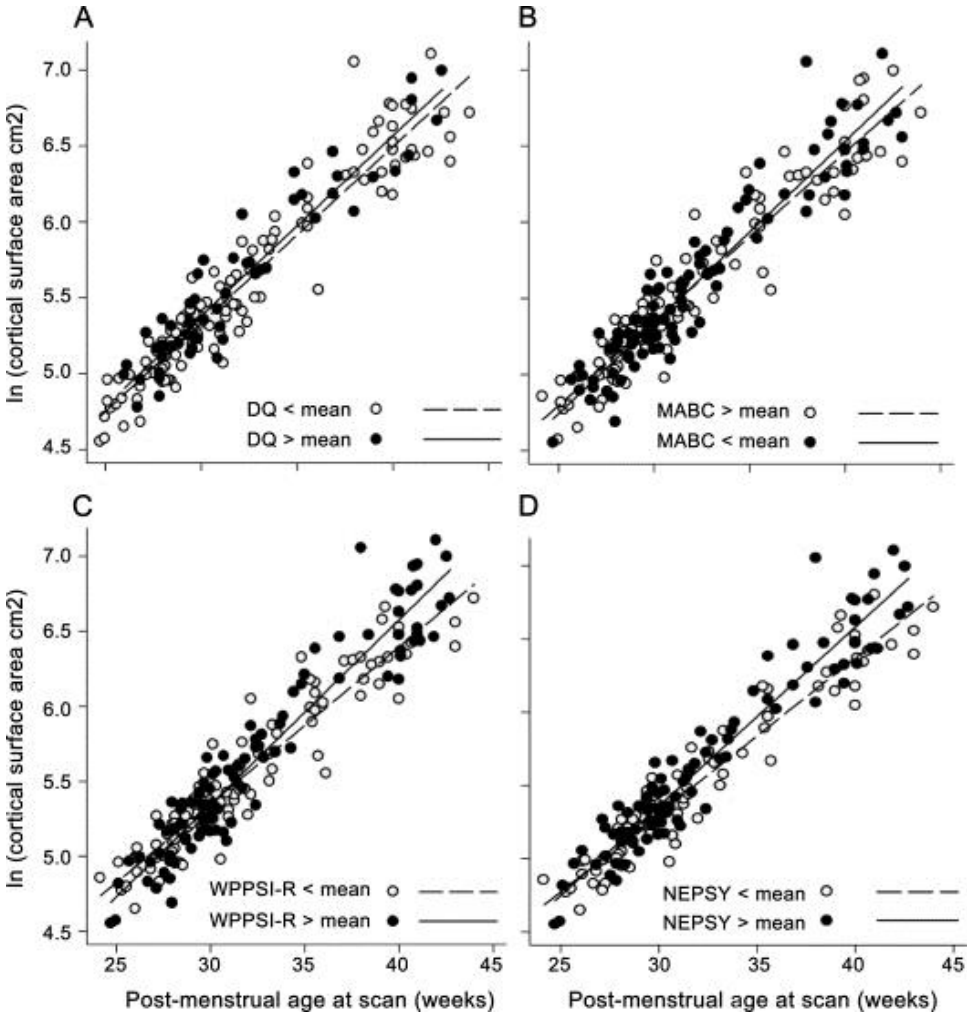


Figure A1.2: Relation between cortical surface area and postmenstrual age at scan. Data is divided into groups greater or less than mean values for the Griffiths Developmental Quotient (DQ; A), Movement Assessment Battery for Children (MABC; B), Wechsler Preschool and Primary Scale of Intelligence-Revised (WPPSI-R; C) and Developmental Neuropsychological Assessment (NEPSY; D) summary scores *(Reproduced from Rathbone R et al 2011 [614]).*

Peterson et al showed that in preterm infants, cortical volumes are smaller in specific regions mainly in the sensorimotor, premotor, mid-temporal and parieto-occipital regions. In preterm infants with evidence of PVL, there is a marked reduction in cortical grey matter

along with marked reduction in total white matter volume [615]. Inder *et al* [590] studied 119 infants using signal intensity based segmentation of grey and white matter, and found that reduced cortical grey matter volume predicted adverse neurodevelopmental outcome at 1 year of age and defects in working memory at 2 years of age . While volumetric MR imaging has a lot of potential for research, integration of this approach is currently limited because of it's complexity [609].

Deformation-based morphometry (DBM) allows non-subjective unbiased assessment of local brain volume differences between groups. At term corrected age, preterm infants show tissue volume reduction in the dorsomedial nucleus of the thalamus, the globus pallidus, periventricular white matter, the corona radiata and within the central region of the centrum semiovale [589, 616].

REFERENCES

1. Beck, S., et al., *The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity.* Bull World Health Organ, 2010. **88**(1): p. 31-38.

2. Blencowe, H., et al., *National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications.* Lancet, 2012. **379**(9832): p. 2162-72.

3. Petrou, S., O. Eddama, and L. Mangham, *A structured review of the recent literature on the economic consequences of preterm birth.* Arch Dis Child Fetal Neonatal Ed, 2011. **96**(3): p. F225-32.

4. Simmons, L.E., et al., *Preventing preterm birth and neonatal mortality: exploring the epidemiology, causes, and interventions.* Semin Perinatol, 2010. **34**(6): p. 408-15.

5. Blondel, B. and M. Kaminski, *Trends in the occurrence, determinants, and consequences of multiple births.* Semin Perinatol, 2002. **26**(4): p. 239-49.

6. Olsen, P., et al., *Epidemiology of preterm delivery in two birth cohorts with an interval of 20 years.* Am J Epidemiol, 1995. **142**(11): p. 1184-93.

7. Romero, R., et al., *The role of infection in preterm labour and delivery.* Paediatr Perinat Epidemiol, 2001. **15 Suppl 2**: p. 41-56.

8. Joseph, K.S., K. Demissie, and M.S. Kramer, *Obstetric intervention, stillbirth, and preterm birth.* Semin Perinatol, 2002. **26**(4): p. 250-9.

9. Goldenberg, R.L., et al., *Epidemiology and causes of preterm birth.* Lancet, 2008. **371**(9606): p. 75-84.

10. Lawn, J.E., et al., *Global report on preterm birth and stillbirth (1 of 7): definitions, description of the burden and opportunities to improve data.* BMC Pregnancy Childbirth, 2010. **10 Suppl 1**: p. S1.

11. Liu, L., et al., *Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000.* Lancet, 2012. **379**(9832): p. 2151-61.

12. Lawn, J.E., S. Cousens, and J. Zupan, *4 million neonatal deaths: when? Where? Why?* Lancet, 2005. **365**(9462): p. 891-900.

13. Behrman, R.E., *Preterm Birth: Causes, Consequences, and Prevention* C.o.U.P.B.a.A.H.O. Institute of Medicine, Editor 2007, National Academies Press: Washington, DC.

14. Field, D., et al., *Rates of very preterm birth in Europe and neonatal mortality rates.* Arch Dis Child Fetal Neonatal Ed, 2009. **94**(4): p. F253-6.

15. Wilson-Costello, D., et al., *Improved survival rates with increased neurodevelopmental disability for extremely low birth weight infants in the 1990s.* Pediatrics, 2005. **115**(4): p. 997-1003.

16. Draper, E.S., et al., *Mortality patterns among very preterm babies: a comparative analysis of two European regions in France and England.* Arch Dis Child Fetal Neonatal Ed, 2007. **92**(5): p. F356-60.

17. Marlow, N., et al., *Neurologic and developmental disability at six years of age after extremely preterm birth.* N Engl J Med, 2005. **352**(1): p. 9-19.

18. Costeloe, K.L., et al., *Short term outcomes after extreme preterm birth in England: comparison of two birth cohorts in 1995 and 2006 (the EPICure studies).* BMJ, 2012. **345**: p. e7976.

19. Saigal, S. and L.W. Doyle, *An overview of mortality and sequelae of preterm birth from infancy to adulthood.* Lancet, 2008. **371**(9608): p. 261-9.

20. Petrou, S., T. Sach, and L. Davidson, *The long-term costs of preterm birth and low birth weight: results of a systematic review.* Child Care Health Dev, 2001. **27**(2): p. 97-115.

21. Swamy, G.K., T. Ostbye, and R. Skjaerven, *Association of preterm birth with long-term survival, reproduction, and next-generation preterm birth.* JAMA, 2008. **299**(12): p. 1429-36.

22. Claas, M.J., et al., *Two-year neurodevelopmental outcome of preterm born children </= 750 g at birth.* Arch Dis Child Fetal Neonatal Ed, 2011. **96**(3): p. F169-77.

23. Hintz, S.R., et al., *Early-childhood neurodevelopmental outcomes are not improving for infants born at <25 weeks' gestational age.* Pediatrics, 2011. **127**(1): p. 62-70.

24. Anderson, P. and L.W. Doyle, *Neurobehavioral outcomes of school-age children born extremely low birth weight or very preterm in the 1990s.* JAMA, 2003. **289**(24): p. 3264-72.

25. Anderson, P.J. and L.W. Doyle, *Executive functioning in school-aged children who were born very preterm or with extremely low birth weight in the 1990s.* Pediatrics, 2004. **114**(1): p. 50-7.

26. Botting, N., et al., *Attention deficit hyperactivity disorders and other psychiatric outcomes in very low birthweight children at 12 years.* J Child Psychol Psychiatry, 1997. **38**(8): p. 931-41.

27. O'Callaghan, M.J. and J.M. Harvey, *Biological predictors and co-morbidity of attention deficit and hyperactivity disorder in extremely low birthweight infants at school.* J Paediatr Child Health, 1997. **33**(6): p. 491-6.

28. Delobel-Ayoub, M., et al., *Behavioral problems and cognitive performance at 5 years of age after very preterm birth: the EPIPAGE Study.* Pediatrics, 2009. **123**(6): p. 1485-92.

29. Bhutta, A.T., et al., *Cognitive and behavioral outcomes of school-aged children who were born preterm: a meta-analysis.* JAMA, 2002. **288**(6): p. 728-37.

30. Doyle, L.W., G. Roberts, and P.J. Anderson, *Outcomes at age 2 years of infants < 28 weeks' gestational age born in Victoria in 2005.* J Pediatr, 2010. **156**(1): p. 49-53 e1.

31. Volpe, J.J., *Cerebral white matter injury of the premature infant-more common than you think.* Pediatrics, 2003. **112**(1 Pt 1): p. 176-80.

32. Volpe, J.J., *Neurology of the Newborn*. 5th edition ed. 2008, Philadelphia PA, USA: Saunders Elsevier.

33. Moore, T., et al., *Neurological and developmental outcome in extremely preterm children born in England in 1995 and 2006: the EPICure studies.* BMJ, 2012. **345**: p. e7961.

34. Beaino, G., et al., *Predictors of cerebral palsy in very preterm infants: the EPIPAGE prospective population-based cohort study.* Dev Med Child Neurol, 2010. **52**(6): p. e119-25.

35. Ancel, P.Y., et al., *Cerebral palsy among very preterm children in relation to gestational age and neonatal ultrasound abnormalities: the EPIPAGE cohort study.* Pediatrics, 2006. **117**(3): p. 828-35.

36. Larroque, B., et al., *Neurodevelopmental disabilities and special care of 5-year-old children born before 33 weeks of gestation (the EPIPAGE study): a longitudinal cohort study.* Lancet, 2008. **371**(9615): p. 813-20.

37. Quigley, M.A., et al., *Early term and late preterm birth are associated with poorer school performance at age 5 years: a cohort study.* Arch Dis Child Fetal Neonatal Ed, 2012. **97**(3): p. F167-73.

38. MacKay, D.F., et al., *Gestational age at delivery and special educational need: retrospective cohort study of 407,503 schoolchildren.* PLoS Med, 2010. **7**(6): p. e1000289.

39. Johnson, S., et al., *Academic attainment and special educational needs in extremely preterm children at 11 years of age: the EPICure study.* Arch Dis Child Fetal Neonatal Ed, 2009. **94**(4): p. F283-9.

40. Mathiasen, R., et al., *Socio-economic achievements of individuals born very preterm at the age of 27 to 29 years: a nationwide cohort study.* Dev Med Child Neurol, 2009. **51**(11): p. 901-8.

41. Kruse, M., et al., *Lifetime costs of cerebral palsy.* Dev Med Child Neurol, 2009. **51**(8): p. 622-8.

42. *Economic costs associated with mental retardation, cerebral palsy, hearing loss, and vision impairment--United States, 2003.* MMWR Morb Mortal Wkly Rep, 2004. **53**(3): p. 57-9.

43. Beecham, J., T. O'Neill, and R. Goodman, *Supporting young adults with hemiplegia: services and costs.* Health Soc Care Community, 2001. **9**(1): p. 51-9.

44. Petrou, S. and K. Khan, *Economic costs associated with moderate and late preterm birth: primary and secondary evidence.* Semin Fetal Neonatal Med, 2012. **17**(3): p. 170-8.

45. McCormick, M.C. and R.E. Behrman, *The quiet epidemic of premature birth: commentary on a recent Institute of Medicine report.* Ambul Pediatr, 2007. **7**(1): p. 8-9.

46. Allen, M.C., *Neurodevelopmental outcomes of preterm infants.* Curr Opin Neurol, 2008. **21**(2): p. 123-8.

47. Chandiramani, M. and A. Shennan, *Preterm labour: update on prediction and prevention strategies.* Curr Opin Obstet Gynecol, 2006. **18**(6): p. 618-24.

48. Lagercrantz, H., et al., *The newborn brain. Neuroscience and clinical applications*. 2002, Cambridge Cambridge University Press.

49. Volpe, J.J., *Neurology of the newborn*. 4th ed ed. 2001, Philadelphia: WB Saunders Comp.

50. de Graaf-Peters, V.B. and M. Hadders-Algra, *Ontogeny of the human central nervous system: what is happening when?* Early Hum Dev, 2006. **82**(4): p. 257-66.

51. Battin, M. and M.A. Rutherford, *Magnetic resonance imaging of the brain in preterm infants: 24 weeks' gestation to term* in *MRI of the Neonatal Brain.*, M.A. Rutherford, Editor. 2002, WB Saunders: London.

52. Stiles, J. and T.L. Jernigan, *The basics of brain development.* Neuropsychol Rev, 2010. **20**(4): p. 327-48.

53. Tau, G.Z. and B.S. Peterson, *Normal development of brain circuits.* Neuropsychopharmacology. **35**(1): p. 147-68.

54. Ladher, R. and G.C. Schoenwolf, *Making a neural tube: neural tube induction and neurrulation.* , in *Developmental Neurobiology*, S.R. Mahendra and M. Jacobson, Editors. 2005, Kluwer Academic/Plenum Publishing: New York. p. pp 1-20.

55. Jessell, T.M. and J.R. Sanes, *Development. The decade of the developing brain.* Current opinion in neurobiology, 2000. **10**(5): p. 599-611.

56. Rash, B.G. and E.A. Grove, *Area and layer patterning in the developing cerebral cortex.* Current opinion in neurobiology, 2006. **16**(1): p. 25-34.

57. Rhinn, M., A. Picker, and M. Brand, *Global and local mechanisms of forebrain and midbrain patterning.* Current opinion in neurobiology, 2006. **16**(1): p. 5-12.

58. Volpe, P., et al., *Disorders of prosencephalic development.* Prenat Diagn, 2009. **29**(4): p. 340-54.

59. Stern, C.D. and S.E. Fraser, *Tracing the lineage of tracing cell lineages.* Nat Cell Biol, 2001. **3**(9): p. E216-8.

60. Sanai, N., et al., *Corridors of migrating neurons in the human brain and their decline during infancy.* Nature, 2011. **478**(7369): p. 382-6.

61. Bystron, I., C. Blakemore, and P. Rakic, *Development of the human cerebral cortex: Boulder Committee revisited.* Nat Rev Neurosci, 2008. **9**(2): p. 110-22.

62. Ghashghaei, H.T., et al., *Reinduction of ErbB2 in astrocytes promotes radial glial progenitor identity in adult cerebral cortex.* Genes Dev, 2007. **21**(24): p. 3258-71.

63. Hatten, M.E., *The role of migration in central nervous system neuronal development.* Current opinion in neurobiology, 1993. **3**(1): p. 38-44.

64. Jessell, T.M., Sanes J.R., *Development. The decade of the developing brain.* Curr Opin Neurobiol, 2000. **10**

p. 599-611.

65. Kornack, D.R. and P. Rakic, *Radial and horizontal deployment of clonally related cells in the primate neocortex: relationship to distinct mitotic lineages.* Neuron, 1995. **15**(2): p. 311-21.

66. Levitt, P., *Structural and functional maturation of the developing primate brain.* J Pediatr, 2003. **143**(4 Suppl): p. S35-45.

67. McManus, M.F., et al., *Axon mediated interneuron migration.* J Neuropathol Exp Neurol, 2004. **63**(9): p. 932-41.

68. Molliver, M.E., I. Kostovic, and H. van der Loos, *The development of synapses in cerebral cortex of the human fetus.* Brain Res, 1973. **50**(2): p. 403-7.

69. Monk, C.S., S.J. Webb, and C.A. Nelson, *Prenatal neurobiological development: molecular mechanisms and anatomical change.* Dev Neuropsychol, 2001. **19**(2): p. 211-36.

70. Pencea, V., et al., *Neurogenesis in the subventricular zone and rostral migratory stream of the neonatal and adult primate forebrain.* Exp Neurol, 2001. **172**(1): p. 1-16.

71. Rakic, P., *Neuronal migration and contact guidance in the primate telencephalon.* Postgrad Med J, 1978. **54 Suppl 1**: p. 25-40.

72. Rakic, P., *Early developmental events: cell lineages, acquisition of neuronal positions, and areal and laminar development.* Neurosci Res Program Bull, 1982. **20**(4): p. 439-51.

73. Rakic, P., *Specification of cerebral cortical areas.* Science, 1988. **241**(4862): p. 170-6.

74. Rakic, P., *A small step for the cell, a giant leap for mankind: a hypothesis of neocortical expansion during evolution.* Trends Neurosci, 1995. **18**(9): p. 383-8.

75. Rakic, P., *Developmental and evolutionary adaptations of cortical radial glia.* Cereb Cortex, 2003. **13**(6): p. 541-9.

76. Wodarz, A. and W.B. Huttner, *Asymmetric cell division during neurogenesis in Drosophila and vertebrates.* Mech Dev, 2003. **120**(11): p. 1297-309.

77. Wilson, C.J., et al., *Mitochondrial DNA point mutation T9176C in Leigh syndrome.* J Child Neurol, 2000. **15**(12): p. 830-3.

78. de Silva, H.R., N.L. Khan, and N.W. Wood, *The genetics of Parkinson's disease.* Curr Opin Genet Dev, 2000. **10**(3): p. 292-8.

79. Nadarajah, B. and J.G. Parnavelas, *Modes of neuronal migration in the developing cerebral cortex.* Nat Rev Neurosci, 2002. **3**(6): p. 423-32.

80. Rakic, P., *Mode of cell migration to the superficial layers of fetal monkey neocortex.* J Comp Neurol, 1972. **145**(1): p. 61-83.

81. Rakic, P., J.P. Bourgeois, and P.S. Goldman-Rakic, *Synaptic development of the cerebral cortex: implications for learning, memory, and mental illness.* Prog Brain Res, 1994. **102**: p. 227-43.

82. O'Rourke, N.A., et al., *Diverse migratory pathways in the developing cerebral cortex.* Science, 1992. **258**(5080): p. 299-302.

83. Van Eden, C.G., et al., *Prenatal development of GABA-ergic neurons in the neocortex of the rat.* J Comp Neurol, 1989. **289**(2): p. 213-27.

84. Gupta, R.K., et al., *Diffusion tensor imaging of the developing human cerebrum.* J Neurosci Res, 2005. **81**(2): p. 172-8.

85. Olson, E.C. and C.A. Walsh, *Smooth, rough and upside-down neocortical development.* Curr Opin Genet Dev, 2002. **12**(3): p. 320-7.

86. Gomez, M.R., Sampson, J.R.,Whittemore , V.H., *Tuberous Sclerosis*. 3rd ed. 1999, New York: Oxford University Press.

87. Uhlmann, E.J., et al., *Astrocyte-specific TSC1 conditional knockout mice exhibit abnormal neuronal organization and seizures.* Ann Neurol, 2002. **52**(3): p. 285-96.

88. Brown, M. and R. Keynes, *The developing brain*. 2001, Oxford: Oxford University Press.

89. Marin-Padilla, M., *Early prenatal ontogenesis of the cerebral cortex (neocortex) of the cat (Felis domestica). A Golgi study. I. The primordial neocortical organization.* Z Anat Entwicklungsgesch, 1971. **134**(2): p. 117-45.

90. Raedler, E. and A. Raedler, *Autoradiographic study of early neurogenesis in rat neocortex.* Anat Embryol (Berl), 1978. **154**(3): p. 267-84.

91. Rickmann, M., B.M. Chronwall, and J.R. Wolff, *On the development of non-pyramidal neurons and axons outside the cortical plate: the early marginal zone as a pallial anlage.* Anat Embryol (Berl), 1977. **151**(3): p. 285-307.

92. Stewart, G.R. and A.L. Pearlman, *Fibronectin-like immunoreactivity in the developing cerebral cortex.* J Neurosci, 1987. **7**(10): p. 3325-33.

93. Super, H., E. Soriano, and H.B. Uylings, *The functions of the preplate in development and evolution of the neocortex and hippocampus.* Brain Res Brain Res Rev, 1998. **27**(1): p. 40-64.

94. Wood, J.G., S. Martin, and D.J. Price, *Evidence that the earliest generated cells of the murine cerebral cortex form a transient population in the subplate and marginal zone.* Brain Res Dev Brain Res, 1992. **66**(1): p. 137-40.

95. Allendoerfer, K.L. and C.J. Shatz, *The subplate, a transient neocortical structure: its role in the development of connections between thalamus and cortex.* Annu Rev Neurosci, 1994. **17**: p. 185-218.

96. Kostovic, I. and P. Rakic, *Developmental history of the transient subplate zone in the visual and somatosensory cortex of the macaque monkey and human brain.* J Comp Neurol, 1990. **297**(3): p. 441-70.

97. Kostovic, I., et al., *Laminar organization of the human fetal cerebrum revealed by histochemical markers and magnetic resonance imaging.* Cereb Cortex, 2002. **12**(5): p. 536-44.

98. de Graaf-Peters, V.B., Hadders-Algra, M., *Ontogeny of the human central nervous system:what is happening when?* Early Hum Dev, 2006. **82**: p. 257-266.

99. Ghosh, A., et al., *Requirement for subplate neurons in the formation of thalamocortical connections.* Nature, 1990. **347**(6289): p. 179-81.

100. Ghosh, A. and C.J. Shatz, *Involvement of subplate neurons in the formation of ocular dominance columns.* Science, 1992. **255**(5050): p. 1441-3.

101. Kostovic, I. and P. Rakic, *Development of prestriate visual projections in the monkey and human fetal cerebrum revealed by transient cholinesterase staining.* J Neurosci, 1984. **4**(1): p. 25-42.

102. McConnell, S.K., A. Ghosh, and C.J. Shatz, *Subplate neurons pioneer the first axon pathway from the cerebral cortex.* Science, 1989. **245**(4921): p. 978-82.

103. Volpe, J.J., *The encephalopathy of prematurity--brain injury and impaired brain development inextricably intertwined.* Semin Pediatr Neurol, 2009. **16**(4): p. 167-78.

104. Kostovic, I., et al., *Ontogenesis of goal-directed behavior: anatomo-functional considerations.* Int J Psychophysiol, 1995. **19**(2): p. 85-102.

105. McKinstry, R.C., et al., *A prospective, longitudinal diffusion tensor imaging study of brain injury in newborns.* Neurology, 2002. **59**(6): p. 824-33.

106. Lund, J.S. and D.A. Lewis, *Local circuit neurons of developing and mature macaque prefrontal cortex: Golgi and immunocytochemical characteristics.* J Comp Neurol, 1993. **328**(2): p. 282-312.

107. Huttenlocher, P.R., *Morphometric study of human cerebral cortex development.* Neuropsychologia, 1990. **28**(6): p. 517-27.

108. Bourgeois, J.P., P.S. Goldman-Rakic, and P. Rakic, *Synaptogenesis in the prefrontal cortex of rhesus monkeys.* Cereb Cortex, 1994. **4**(1): p. 78-96.

109. Rakic, P., et al., *Concurrent overproduction of synapses in diverse regions of the primate cerebral cortex.* Science, 1986. **232**(4747): p. 232-5.

110. Zecevic, N., *Synaptogenesis in layer I of the human cerebral cortex in the first half of gestation.* Cereb Cortex, 1998. **8**(3): p. 245-52.

111. Zecevic, N., J.P. Bourgeois, and P. Rakic, *Changes in synaptic density in motor cortex of rhesus monkey during fetal and postnatal life.* Brain Res Dev Brain Res, 1989. **50**(1): p. 11-32.

112. Huttenlocher, P.R., *Synapse elimination and plasticity in developing human cerebral cortex.* Am J Ment Defic, 1984. **88**(5): p. 488-96.

113. Huttenlocher, P.R. and A.S. Dabholkar, *Regional differences in synaptogenesis in human cerebral cortex.* J Comp Neurol, 1997. **387**(2): p. 167-78.

114. Rakic, S. and N. Zecevic, *Programmed cell death in the developing human telencephalon.* Eur J Neurosci, 2000. **12**(8): p. 2721-34.

115. Lossi, L. and A. Merighi, *In vivo cellular and molecular mechanisms of neuronal apoptosis in the mammalian CNS.* Prog Neurobiol, 2003. **69**(5): p. 287-312.

116. Buss, R.R., W. Sun, and R.W. Oppenheim, *Adaptive roles of programmed cell death during nervous system development.* Annu Rev Neurosci, 2006. **29**: p. 1-35.

117. Buss, R.R. and R.W. Oppenheim, *Role of programmed cell death in normal neuronal development and function.* Anat Sci Int, 2004. **79**(4): p. 191-7.

118. Eyre, J.A., et al., *Evidence of activity-dependent withdrawal of corticospinal projections during human development.* Neurology, 2001. **57**(9): p. 1543-54.

119. Goda, Y. and G.W. Davis, *Mechanisms of synapse assembly and disassembly.* Neuron, 2003. **40**(2): p. 243-64.

120. Oppenheim, R.W., *The neurotrophic theory and naturally occurring motoneuron death.* Trends Neurosci, 1989. **12**(7): p. 252-5.

121. de la Rosa, E.J. and F. de Pablo, *Cell death in early neural development: beyond the neurotrophic theory.* Trends Neurosci, 2000. **23**(10): p. 454-8.

122. Yeo, W. and J. Gautier, *Early neural cell death: dying to become neurons.* Dev Biol, 2004. **274**(2): p. 233-44.

123. Thornton, C., Rousset, C.I.,Kichev, A., Miyakuni, Y.,Vontell, R.,Baburamani, A.A.Fleiss, B.,Gressens, P.,Hagberg, H., *Molecular mechanisms of neonatal brain injury.* Neurology Research International, 2012.

124. Parpura, V., et al., *Glial cells in (patho)physiology.* J Neurochem. **121**(1): p. 4-27.

125. Pont-Lezica, L., et al., *Physiological roles of microglia during development.* J Neurochem. **119**(5): p. 901-8.

126. Miller, R.H., *Regulation of oligodendrocyte development in the vertebrate CNS.* Prog Neurobiol, 2002. **67**(6): p. 451-67.

127. Vartanian, G., Corfas, G., Li, Y. , Fischbach, G.D., Stefansson, K. *A role for acetylcholine receptor-inducing protein ARIA in oligodendrocyte development.* . in *Proceedings of the National Academy of Sciences* 1994. USA.

128. Iai, M., T. Yamamura, and S. Takashima, *Early expression of proteolipid protein in human fetal and infantile cerebri.* Pediatr Neurol, 1997. **17**(3): p. 235-9.

129. Huppi, P.S., et al., *Quantitative magnetic resonance imaging of brain development in premature and mature newborns.* Ann Neurol, 1998. **43**(2): p. 224-35.

130. Kinney, H.C., et al., *Sequence of central nervous system myelination in human infancy. II. Patterns of myelination in autopsied infants.* J Neuropathol Exp Neurol, 1988. **47**(3): p. 217-34.

131. Weidenheim, K.M., et al., *Temporal and spatial expression of major myelin proteins in the human fetal spinal cord during the second trimester.* J Neuropathol Exp Neurol, 1996. **55**(6): p. 734-45.

132. Deoni, S.C., et al., *Mapping infant brain myelination with magnetic resonance imaging.* J Neurosci. **31**(2): p. 784-91.

133. Back, S.A., et al., *Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury.* J Neurosci, 2001. **21**(4): p. 1302-12.

134. Ness, J.K., et al., *Perinatal hypoxia-ischemia induces apoptotic and excitotoxic death of periventricular white matter oligodendrocyte progenitors.* Developmental neuroscience, 2001. **23**(3): p. 203-8.

135. McTigue, D.M. and R.B. Tripathi, *The life, death, and replacement of oligodendrocytes in the adult CNS.* J Neurochem, 2008. **107**(1): p. 1-19.

136. Chi, J.G., E.C. Dooling, and F.H. Gilles, *Gyral development of the human brain.* Ann Neurol, 1977. **1**(1): p. 86-93.

137. White, T., et al., *The development of gyrification in childhood and adolescence.* Brain Cogn. **72**(1): p. 36-45.

138. van der Knaap, M.S., et al., *Normal gyration and sulcation in preterm and term neonates: appearance on MR images.* Radiology, 1996. **200**(2): p. 389-96.

139. Battin, M.R., et al., *Magnetic resonance imaging of the brain in very preterm infants: visualization of the germinal matrix, early myelination, and cortical folding.* Pediatrics, 1998. **101**(6): p. 957-62.

140. Habas, P.A., et al., *Early folding patterns and asymmetries of the normal human brain detected from in utero MRI.* Cereb Cortex. **22**(1): p. 13-25.

141. Dubois, J., et al., *Mapping the early cortical folding process in the preterm newborn brain.* Cereb Cortex, 2008. **18**(6): p. 1444-54.

142. Kapellou, O., et al., *Abnormal cortical development after premature birth shown by altered allometric scaling of brain growth.* PLoS Med, 2006. **3**(8): p. e265.

143. Van Essen, D.C., *A tension-based theory of morphogenesis and compact wiring in the central nervous system.* Nature, 1997

385: p. 313-8.

144. Bullmore, E. and O. Sporns, *The economy of brain network organization.* Nat Rev Neurosci. **13**(5): p. 336-49.

145. Merchant, N., et al., *A patient care system for early 3.0 Tesla magnetic resonance imaging of very low birth weight infants.* Early Hum Dev, 2009. **85**(12): p. 779-83.

146. Maalouf, E.F., et al., *Magnetic resonance imaging of the brain in a cohort of extremely preterm infants.* J Pediatr, 1999. **135**(3): p. 351-7.

147. Miller, D.H., *Biomarkers and surrogate outcomes in neurodegenerative disease: lessons from multiple sclerosis.* NeuroRx, 2004. **1**(2): p. 284-94.

148. Limperopoulos, C., *Advanced neuroimaging techniques: their role in the development of future fetal and neonatal neuroprotection.* Semin Perinatol. **34**(1): p. 93-101.

149. Ment, L.R., D. Hirtz, and P.S. Huppi, *Imaging biomarkers of outcome in the developing preterm brain.* Lancet Neurol, 2009. **8**(11): p. 1042-55.

150. Thayyil, S., et al., *Cerebral magnetic resonance biomarkers in neonatal encephalopathy: a meta-analysis.* Pediatrics. **125**(2): p. e382-95.

151. Matthews, P.M., I. Rabiner, and R. Gunn, *Non-invasive imaging in experimental medicine for drug development.* Curr Opin Pharmacol. **11**(5): p. 501-7.

152. McArdle, C.B., et al., *Developmental features of the neonatal brain: MR imaging. Part I. Gray-white matter differentiation and myelination.* Radiology, 1987. **162**(1 Pt 1): p. 223-9.

153. Barkovich, A.J., *MR of the normal neonatal brain: assessment of deep structures.* AJNR. American journal of neuroradiology, 1998. **19**(8): p. 1397-403.

154. Childs, A.M., et al., *Cerebral maturation in premature infants: quantitative assessment using MR imaging.* AJNR. American journal of neuroradiology, 2001. **22**(8): p. 1577-82.

155. Kostovic, I. and N. Jovanov-Milosevic, *The development of cerebral connections during the first 20-45 weeks' gestation.* Semin Fetal Neonatal Med, 2006. **11**(6): p. 415-22.

156. Arthur, R., *Magnetic resonance imaging in preterm infants.* Pediatr Radiol, 2006. **36**(7): p. 593-607.

157. Dudink, J., et al., *Connecting the developing preterm brain.* Early Hum Dev, 2008. **84**(12): p. 777-82.

158. Neil, J.J., et al., *Normal brain in human newborns: apparent diffusion coefficient and diffusion anisotropy measured by using diffusion tensor MR imaging.* Radiology, 1998. **209**(1): p. 57-66.

159. Wimberger, D.M., et al., *Identification of "premyelination" by diffusion-weighted MRI.* J Comput Assist Tomogr, 1995. **19**(1): p. 28-33.

160. Girard, N., C. Raybaud, and M. Poncet, *In vivo MR study of brain maturation in normal fetuses.* AJNR. American journal of neuroradiology, 1995. **16**(2): p. 407-13.

161. Platt, M.J., et al., *Trends in cerebral palsy among infants of very low birthweight (<1500 g) or born prematurely (<32 weeks) in 16 European centres: a database study.* Lancet, 2007. **369**(9555): p. 43-50.

162. Surman, G., et al., *Children with cerebral palsy: severity and trends over time.* Paediatr Perinat Epidemiol, 2009. **23**(6): p. 513-21.

163. Robertson, C.M., M.J. Watt, and Y. Yasui, *Changes in the prevalence of cerebral palsy for children born very prematurely within a population-based program over 30 years.* JAMA, 2007. **297**(24): p. 2733-40.

164. Groenendaal, F., et al., *Complications affecting preterm neonates from 1991 to 2006: what have we gained?* Acta Paediatr, 2010. **99**(3): p. 354-8.

165. van Haastert, I.C., et al., *Decreasing incidence and severity of cerebral palsy in prematurely born children.* J Pediatr, 2011. **159**(1): p. 86-91 e1.

166. Volpe, J.J., *Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances.* Lancet Neurol, 2009. **8**(1): p. 110-24.

167. Larroque, B., et al., *White matter damage and intraventricular hemorrhage in very preterm infants: the EPIPAGE study.* J Pediatr, 2003. **143**(4): p. 477-83.

168. Kadri, H., A.A. Mawla, and J. Kazah, *The incidence, timing, and predisposing factors of germinal matrix and intraventricular hemorrhage (GMH/IVH) in preterm neonates.* Childs Nerv Syst, 2006. **22**(9): p. 1086-90.

169. Sheth, R.D., *Trends in incidence and severity of intraventricular hemorrhage.* J Child Neurol, 1998. **13**(6): p. 261-4.

170. Levine, M.I. and L.S. de Vries, *Neonatal intracranial hemorrhage.* , in *Fetal and Neonatal Neurology and Neurosurgery*, M.I. Levine, F.A. Chervenak, and M. Whittle, Editors. 2001, Churchill Livingstone. p. 339–371.

171. Levene, M.I. and L.C. de Crespigny, *Classification of intraventricular hemorrhage.* Lancet, 1983. **1**(8325): p. 643.

172. Perlman, J.M. and N. Rollins, *Surveillance protocol for the detection of intracranial abnormalities in premature neonates.* Arch Pediatr Adolesc Med, 2000. **154**(8): p. 822-6.

173. Lemons, J.A., et al., *Very low birth weight outcomes of the National Institute of Child health and human development neonatal research network, January 1995 through December 1996. NICHD Neonatal Research Network.* Pediatrics, 2001. **107**(1): p. E1.

174. Volpe, J.J., *Neonatal encephalitis and white matter injury: more than just inflammation?* Ann Neurol, 2008. **64**(3): p. 232-6.

175. Perlman, J.M., J.B. McMenamin, and J.J. Volpe, *Fluctuating cerebral blood-flow velocity in respiratory-distress syndrome. Relation to the development of intraventricular hemorrhage.* N Engl J Med, 1983. **309**(4): p. 204-9.

176. Amato, M., J.C. Fauchere, and U. Hermann, Jr., *Coagulation abnormalities in low birth weight infants with peri-intraventricular hemorrhage.* Neuropediatrics, 1988. **19**(3): p. 154-7.

177. Soul, J.S., et al., *Fluctuating pressure-passivity is common in the cerebral circulation of sick premature infants.* Pediatr Res, 2007. **61**(4): p. 467-73.

178. Volpe, J.J., *Postnatal sepsis, necrotizing entercolitis, and the critical role of systemic inflammation in white matter injury in premature infants.* J Pediatr, 2008. **153**(2): p. 160-3.

179. Takashima, S., M. Itoh, and A. Oka, *A history of our understanding of cerebral vascular development and pathogenesis of perinatal brain damage over the past 30 years.* Semin Pediatr Neurol, 2009. **16**(4): p. 226-36.

180. Funato, M., et al., *Clinical events in association with timing of intraventricular hemorrhage in preterm infants.* J Pediatr, 1992. **121**(4): p. 614-9.

181. Meek, J.H., et al., *Low cerebral blood flow is a risk factor for severe intraventricular haemorrhage.* Arch Dis Child Fetal Neonatal Ed, 1999. **81**(1): p. F15-8.

182. Lundstrom, K., O. Pryds, and G. Greisen, *The haemodynamic effects of dopamine and volume expansion in sick preterm infants.* Early Hum Dev, 2000. **57**(2): p. 157-63.

183. Hunt, R.W., et al., *Low superior vena cava flow and neurodevelopment at 3 years in very preterm infants.* J Pediatr, 2004. **145**(5): p. 588-92.

184. Kluckow, M. and N. Evans, *Low superior vena cava flow and intraventricular haemorrhage in preterm infants.* Arch Dis Child Fetal Neonatal Ed, 2000. **82**(3): p. F188-94.

185. Osborn, D.A., N. Evans, and M. Kluckow, *Hemodynamic and antecedent risk factors of early and late periventricular/intraventricular hemorrhage in premature infants.* Pediatrics, 2003. **112**(1 Pt 1): p. 33-9.

186. Yanowitz, T.D., et al., *Variability in cerebral oxygen delivery is reduced in premature neonates exposed to chorioamnionitis.* Pediatr Res, 2006. **59**(2): p. 299-304.

187. Salhab, W.A., et al., *Necrotizing enterocolitis and neurodevelopmental outcome in extremely low birth weight infants <1000 g.* J Perinatol, 2004. **24**(9): p. 534-40.

188. Vallabh, P., *Intraventricular hemorrhage in premature infants: mechanism of disease, Pediatr Res., 2010 Jan, 67 (1): 1-8.* Pediatr Res, 2010. **67**(1): p. 1-8.

189. De Vries, L.S., et al., *Asymmetrical myelination of the posterior limb of the internal capsule in infants with periventricular haemorrhagic infarction: an early predictor of hemiplegia.* Neuropediatrics, 1999. **30**(6): p. 314-9.

190. Rademaker, K.J., et al., *Unilateral haemorrhagic parenchymal lesions in the preterm infant: shape, site and prognosis.* Acta Paediatr, 1994. **83**(6): p. 602-8.

191. Amess, P.N., et al., *Epilepsy in very preterm infants: neonatal cranial ultrasound reveals a high-risk subcategory.* Dev Med Child Neurol, 1998. **40**(11): p. 724-30.

192. Whitelaw, A., *Intraventricular haemorrhage and posthaemorrhagic hydrocephalus: pathogenesis, prevention and future interventions.* Semin Neonatol, 2001. **6**(2): p. 135-46.

193. Nongena, P., et al., *Confidence in the prediction of neurodevelopmental outcome by cranial ultrasound and MRI in preterm infants.* Arch Dis Child Fetal Neonatal Ed, 2010. **95**(6): p. F388-90.

194. Murphy, B.P., et al., *Posthaemorrhagic ventricular dilatation in the premature infant: natural history and predictors of outcome.* Arch Dis Child Fetal Neonatal Ed, 2002. **87**(1): p. F37-41.

195. Brouwer, A., et al., *Neurodevelopmental outcome of preterm infants with severe intraventricular hemorrhage and therapy for post-hemorrhagic ventricular dilatation.* J Pediatr, 2008. **152**(5): p. 648-54.

196. Vohr, B.R., et al., *School-age outcomes of very low birth weight infants in the indomethacin intraventricular hemorrhage prevention trial.* Pediatrics, 2003. **111**(4 Pt 1): p. e340-6.

197. Roze, E., et al., *Functional outcome at school age of preterm infants with periventricular hemorrhagic infarction.* Pediatrics, 2009. **123**(6): p. 1493-500.

198. Patra, K., et al., *Grades I-II intraventricular hemorrhage in extremely low birth weight infants: effects on neurodevelopment.* J Pediatr, 2006. **149**(2): p. 169-73.

199. Volpe, J.J., *Systemic inflammation, oligodendroglial maturation, and the encephalopathy of prematurity.* Ann Neurol. **70**(4): p. 525-9.

200. Deguchi, K., K. Oguchi, and S. Takashima, *Characteristic neuropathology of leukomalacia in extremely low birth weight infants.* Pediatr Neurol, 1997. **16**(4): p. 296-300.

201. Kinney HC, A.D., *Perinatal Neuropathology*, in *Neuropathology*, L.P. Graham DI, eds., Editor. 2002, GS Arnold Publishers: London,UK. p. 519-606.

202. Kinney HC, H.R., Folkerth RD, *White matter lesions in the perinatal period.*, in *Developmental Neuropathology. Pathology and Genetics.*, H.B. Golden JA, eds., Editor. 2004, ISN Neuropath Press: Basel, Switzerland. p. 156-70.

203. Hamrick, S.E., et al., *Trends in severe brain injury and neurodevelopmental outcome in premature newborn infants: the role of cystic periventricular leukomalacia.* J Pediatr, 2004. **145**(5): p. 593-9.

204. van Haastert, I.C., et al., *Decreasing incidence and severity of cerebral palsy in prematurely born children.* J Pediatr. **159**(1): p. 86-91 e1.

205. Sugiura, T., et al., *Periventricular leukomalacia is decreasing in Japan.* Pediatr Neurol. **47**(1): p. 35-9.

206. Roelants-van Rijn, A.M., et al., *Parenchymal brain injury in the preterm infant: comparison of cranial ultrasound, MRI and neurodevelopmental outcome.* Neuropediatrics, 2001. **32**(2): p. 80-9.

207. Jacobson, L., et al., *Visual and perceptual characteristics, ocular motility and strabismus in children with periventricular leukomalacia.* Strabismus, 2002. **10**(2): p. 179-83.

208. Ricci, D., et al., *Thalamic atrophy in infants with PVL and cerebral visual impairment.* Early Hum Dev, 2006. **82**(9): p. 591-5.

209. De Vries, L.S., et al., *Ultrasound abnormalities preceding cerebral palsy in high-risk preterm infants.* J Pediatr, 2004. **144**(6): p. 815-20.

210. Billiards, S.S., et al., *Myelin abnormalities without oligodendrocyte loss in periventricular leukomalacia.* Brain pathology, 2008. **18**(2): p. 153-63.

211. Haynes, R.L., et al., *Nitrosative and oxidative injury to premyelinating oligodendrocytes in periventricular leukomalacia.* J Neuropathol Exp Neurol, 2003. **62**(5): p. 441-50.

212. Bell, J.E., et al., *Brain damage and axonal injury in a Scottish cohort of neonatal deaths.* Brain : a journal of neurology, 2005. **128**(Pt 5): p. 1070-81.

213. Back, S.A., et al., *Hyaluronan accumulates in demyelinated lesions and inhibits oligodendrocyte progenitor maturation.* Nat Med, 2005. **11**(9): p. 966-72.

214. Robinson, S., et al., *Neonatal loss of gamma-aminobutyric acid pathway expression after human perinatal brain injury.* J Neurosurg, 2006. **104**(6 Suppl): p. 396-408.

215. Back, S.A., et al., *Selective vulnerability of preterm white matter to oxidative damage defined by F2-isoprostanes.* Ann Neurol, 2005. **58**(1): p. 108-20.

216. Volpe, J.J., et al., *Reprint of "The developing oligodendrocyte: key cellular target in brain injury in the premature infant".* Int J Dev Neurosci, 2011. **29**(6): p. 565-82.

217. Khwaja, O. and J.J. Volpe, *Pathogenesis of cerebral white matter injury of prematurity.* Arch Dis Child Fetal Neonatal Ed, 2008. **93**(2): p. F153-61.

218. Greisen, G. and W. Trojaborg, *Cerebral blood flow, PaCO2 changes, and visual evoked potentials in mechanically ventilated, preterm infants.* Acta Paediatr Scand, 1987. **76**(3): p. 394-400.

219. Shankaran, S., et al., *Cumulative index of exposure to hypocarbia and hyperoxia as risk factors for periventricular leukomalacia in low birth weight infants.* Pediatrics, 2006. **118**(4): p. 1654-9.

220. Wu, Y.W. and J.M. Colford, Jr., *Chorioamnionitis as a risk factor for cerebral palsy: A meta-analysis.* JAMA, 2000. **284**(11): p. 1417-24.

221. Shah, D.K., et al., *Adverse neurodevelopment in preterm infants with postnatal sepsis or necrotizing enterocolitis is mediated by white matter abnormalities on magnetic resonance imaging at term.* J Pediatr, 2008. **153**(2): p. 170-5, 175 e1.

222. Chau, V., et al., *Effect of chorioamnionitis on brain development and injury in premature newborns.* Ann Neurol, 2009. **66**(2): p. 155-64.

223. Leviton, A., et al., *Microbiologic and histologic characteristics of the extremely preterm infant's placenta predict white matter damage and later cerebral palsy. the ELGAN study.* Pediatr Res. **67**(1): p. 95-101.

224. Kadhim, H., et al., *Inflammatory cytokines in the pathogenesis of periventricular leukomalacia.* Neurology, 2001. **56**(10): p. 1278-84.

225. Eklind, S., et al., *Bacterial endotoxin sensitizes the immature brain to hypoxic--ischaemic injury.* Eur J Neurosci, 2001. **13**(6): p. 1101-6.

226. Lehnardt, S., et al., *Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway.* Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8514-9.

227. Ikeda, T., et al., *Combination treatment of neonatal rats with hypoxia-ischemia and endotoxin induces long-lasting memory and learning impairment that is associated with extended cerebral damage.* Am J Obstet Gynecol, 2004. **191**(6): p. 2132-41.

228. Wang, X., et al., *Dual role of intrauterine immune challenge on neonatal and adult brain vulnerability to hypoxia-ischemia.* J Neuropathol Exp Neurol, 2007. **66**(6): p. 552-61.

229. Wang, X., et al., *Lipopolysaccharide sensitizes neonatal hypoxic-ischemic brain injury in a MyD88-dependent manner.* J Immunol, 2009. **183**(11): p. 7471-7.

230. Wang, L.W., et al., *Low-dose lipopolysaccharide selectively sensitizes hypoxic ischemia-induced white matter injury in the immature brain.* Pediatr Res. **68**(1): p. 41-7.

231. Degos, V., et al., *Inflammation processes in perinatal brain damage.* J Neural Transm. **117**(8): p. 1009-17.

232. Kakita, H., et al., *High postnatal oxidative stress in neonatal cystic periventricular leukomalacia.* Brain Dev, 2009. **31**(9): p. 641-8.

233. Back, S.A., et al., *Maturation-dependent vulnerability of oligodendrocytes to oxidative stress-induced death caused by glutathione depletion.* J Neurosci, 1998. **18**(16): p. 6241-53.

234. Oka, A., et al., *Vulnerability of oligodendroglia to glutamate: pharmacology, mechanisms, and prevention.* J Neurosci, 1993. **13**(4): p. 1441-53.

235. Yonezawa, M., et al., *Cystine deprivation induces oligodendroglial death: rescue by free radical scavengers and by a diffusible glial factor.* J Neurochem, 1996. **67**(2): p. 566-73.

236. Li, J., et al., *Novel role of vitamin k in preventing oxidative injury to developing oligodendrocytes and neurons.* J Neurosci, 2003. **23**(13): p. 5816-26.

237. Deng, W., et al., *Calcium-permeable AMPA/kainate receptors mediate toxicity and preconditioning by oxygen-glucose deprivation in oligodendrocyte precursors.* Proc Natl Acad Sci U S A, 2003. **100**(11): p. 6801-6.

238. Volpe, J.J., *Neurobiology of periventricular leukomalacia in the premature infant.* Pediatr Res, 2001. **50**(5): p. 553-62.

239. Folkerth, R.D., et al., *Developmental lag in superoxide dismutases relative to other antioxidant enzymes in premyelinated human telencephalic white matter.* J Neuropathol Exp Neurol, 2004. **63**(9): p. 990-9.

240. Baud, O., et al., *Glutathione peroxidase-catalase cooperativity is required for resistance to hydrogen peroxide by mature rat oligodendrocytes.* J Neurosci, 2004. **24**(7): p. 1531-40.

241. Baud, O., et al., *Developmental up-regulation of MnSOD in rat oligodendrocytes confers protection against oxidative injury.* Eur J Neurosci, 2004. **20**(1): p. 29-40.

242. Savman, K., et al., *Non-protein-bound iron in brain interstitium of newborn pigs after hypoxia.* Developmental neuroscience, 2005. **27**(2-4): p. 176-84.

243. Connor, J.R. and S.L. Menzies, *Relationship of iron to oligodendrocytes and myelination.* Glia, 1996. **17**(2): p. 83-93.

244. Kuban, K., et al., *White matter disorders of prematurity: association with intraventricular hemorrhage and ventriculomegaly. The Developmental Epidemiology Network.* J Pediatr, 1999. **134**(5): p. 539-46.

245. Taylor, D.L., A.D. Edwards, and H. Mehmet, *Oxidative metabolism, apoptosis and perinatal brain injury.* Brain pathology, 1999. **9**(1): p. 93-117.

246. Matute, C., M. Domercq, and M.V. Sanchez-Gomez, *Glutamate-mediated glial injury: mechanisms and clinical importance.* Glia, 2006. **53**(2): p. 212-24.

247. Matute, C., et al., *Excitotoxic damage to white matter.* J Anat, 2007. **210**(6): p. 693-702.

248. Tekkok, S.B., Z. Ye, and B.R. Ransom, *Excitotoxic mechanisms of ischemic injury in myelinated white matter.* J Cereb Blood Flow Metab, 2007. **27**(9): p. 1540-52.

249. Benarroch, E.E., *Glutamate transporters: diversity, function, and involvement in neurologic disease.* Neurology. **74**(3): p. 259-64.

250. Desilva, T.M., et al., *The glutamate transporter EAAT2 is transiently expressed in developing human cerebral white matter.* J Comp Neurol, 2007. **501**(6): p. 879-90.

251. Loeliger, M., et al., *Extracellular glutamate levels and neuropathology in cerebral white matter following repeated umbilical cord occlusion in the near term fetal sheep.* Neuroscience, 2003. **116**(3): p. 705-14.

252. McDonald, J.W., et al., *Oligodendrocytes from forebrain are highly vulnerable to AMPA/kainate receptor-mediated excitotoxicity.* Nat Med, 1998. **4**(3): p. 291-7.

253. Follett, P.L., et al., *NBQX attenuates excitotoxic injury in developing white matter.* J Neurosci, 2000. **20**(24): p. 9235-41.

254. Follett, P.L., et al., *Glutamate receptor-mediated oligodendrocyte toxicity in periventricular leukomalacia: a protective role for topiramate.* J Neurosci, 2004. **24**(18): p. 4412-20.

255. Tekkok, S.B. and M.P. Goldberg, *Ampa/kainate receptor activation mediates hypoxic oligodendrocyte death and axonal injury in cerebral white matter.* J Neurosci, 2001. **21**(12): p. 4237-48.

256. Yoshioka, A., et al., *Non-N-methyl-D-aspartate glutamate receptors mediate oxygen--glucose deprivation-induced oligodendroglial injury.* Brain Res, 2000. **854**(1-2): p. 207-15.

257. Itoh, T., et al., *AMPA glutamate receptor-mediated calcium signaling is transiently enhanced during development of oligodendrocytes.* J Neurochem, 2002. **81**(2): p. 390-402.

258. Rosenberg, P.A., et al., *Mature myelin basic protein-expressing oligodendrocytes are insensitive to kainate toxicity.* J Neurosci Res, 2003. **71**(2): p. 237-45.

259. Sanchez-Gomez, M.V., et al., *Caspase-dependent and caspase-independent oligodendrocyte death mediated by AMPA and kainate receptors.* J Neurosci, 2003. **23**(29): p. 9519-28.

260. Karadottir, R. and D. Attwell, *Neurotransmitter receptors in the life and death of oligodendrocytes.* Neuroscience, 2007. **145**(4): p. 1426-38.

261. Fern, R. and T. Moller, *Rapid ischemic cell death in immature oligodendrocytes: a fatal glutamate release feedback loop.* J Neurosci, 2000. **20**(1): p. 34-42.

262. Alberdi, E., et al., *Ca(2+) influx through AMPA or kainate receptors alone is sufficient to initiate excitotoxicity in cultured oligodendrocytes.* Neurobiology of disease, 2002. **9**(2): p. 234-43.

263. Liu, H.N., et al., *AMPA receptor-mediated toxicity in oligodendrocyte progenitors involves free radical generation and activation of JNK, calpain and caspase 3.* J Neurochem, 2002. **82**(2): p. 398-409.

264. Talos, D.M., et al., *Developmental regulation of alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor subunit expression in forebrain and relationship to regional susceptibility to hypoxic/ischemic injury. I. Rodent cerebral white matter and cortex.* J Comp Neurol, 2006. **497**(1): p. 42-60.

265. Talos, D.M., et al., *Developmental regulation of alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor subunit expression in forebrain and relationship to regional susceptibility to hypoxic/ischemic injury. II. Human cerebral white matter and cortex.* J Comp Neurol, 2006. **497**(1): p. 61-77.

266. Volpe, J.J., et al., *The developing oligodendrocyte: key cellular target in brain injury in the premature infant.* Int J Dev Neurosci. **29**(4): p. 423-40.

267. Salter, M.G. and R. Fern, *NMDA receptors are expressed in developing oligodendrocyte processes and mediate injury.* Nature, 2005. **438**(7071): p. 1167-71.

268. Karadottir, R., et al., *NMDA receptors are expressed in oligodendrocytes and activated in ischaemia.* Nature, 2005. **438**(7071): p. 1162-6.

269. Micu, I., et al., *NMDA receptors mediate calcium accumulation in myelin during chemical ischaemia.* Nature, 2006. **439**(7079): p. 988-92.

270. Manning, S.M., et al., *NMDA receptor blockade with memantine attenuates white matter injury in a rat model of periventricular leukomalacia.* J Neurosci, 2008. **28**(26): p. 6670-8.

271. Bakiri, Y., et al., *Glutamatergic signaling in the brain's white matter.* Neuroscience, 2009. **158**(1): p. 266-74.

272. Haynes, R.L., et al., *Diffuse axonal injury in periventricular leukomalacia as determined by apoptotic marker fractin.* Pediatr Res, 2008. **63**(6): p. 656-61.

273. Murphy, T.H., et al., *Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress.* Neuron, 1989. **2**(6): p. 1547-58.

274. Hitomi, J., et al., *Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway.* Cell, 2008. **135**(7): p. 1311-23.

275. Degterev, A. and J. Yuan, *Expansion and evolution of cell death programmes.* Nat Rev Mol Cell Biol, 2008. **9**(5): p. 378-90.

276. D'Mello, C., T. Le, and M.G. Swain, *Cerebral microglia recruit monocytes into the brain in response to tumor necrosis factoralpha signaling during peripheral organ inflammation.* J Neurosci, 2009. **29**(7): p. 2089-102.

277. Owens, T., I. Bechmann, and B. Engelhardt, *Perivascular spaces and the two steps to neuroinflammation.* J Neuropathol Exp Neurol, 2008. **67**(12): p. 1113-21.

278. Ransohoff, R.M., *Microgliosis: the questions shape the answers.* Nat Neurosci, 2007. **10**(12): p. 1507-9.

279. Lechpammer, M., et al., *Minocycline treatment following hypoxic/ischaemic injury attenuates white matter injury in a rodent model of periventricular leucomalacia.* Neuropathol Appl Neurobiol, 2008. **34**(4): p. 379-93.

280. Turner, M.A., *Neonatal drug development.* Early Hum Dev, 2011. **87**(11): p. 763-8.

281. Organisation, W.H., *Make medicines child size*, 2010.

282. Shirkey, H., *Therapeutic orphans.* J Pediatr, 1968. **72**(1): p. 119-20.

283. O'Donnell, C.P., R.J. Stone, and C.J. Morley, *Unlicensed and off-label drug use in an Australian neonatal intensive care unit.* Pediatrics, 2002. **110**(5): p. e52.

284. Conroy, S., J. McIntyre, and I. Choonara, *Unlicensed and off label drug use in neonates.* Arch Dis Child Fetal Neonatal Ed, 1999. **80**(2): p. F142-4; discussion F144-5.

285. Lass, J., et al., *Drug utilisation pattern and off-label use of medicines in Estonian neonatal units.* Eur J Clin Pharmacol, 2011. **67**(12): p. 1263-71.

286. Turner, M.A., et al., *Prioritising neonatal medicines research: UK Medicines for Children Research Network scoping survey.* BMC Pediatr, 2009. **9**: p. 50.

287. Sutherland, J.M., *Fatal cardiovascular collapse of infants receiving large amounts of chloramphenicol.* AMA J Dis Child, 1959. **97**(6): p. 761-7.

288. Burns, L.E., J.E. Hodgman, and A.B. Cass, *Fatal circulatory collapse in premature infants receiving chloramphenicol.* N Engl J Med, 1959. **261**: p. 1318-21.

289. Weiss, C.F., A.J. Glazko, and J.K. Weston, *Chloramphenicol in the newborn infant. A physiologic explanation of its toxicity when given in excessive doses.* N Engl J Med, 1960. **262**: p. 787-94.

290. Menon, P.A., et al., *Benzyl alcohol toxicity in a neonatal intensive care unit. Incidence, symptomatology, and mortality.* Am J Perinatol, 1984. **1**(4): p. 288-92.

291. Lesko, S.M., M.F. Epstein, and A.A. Mitchell, *Recent patterns of drug use in newborn intensive care.* J Pediatr, 1990. **116**(6): p. 985-90.

292. Mitchell, A.A., et al., *Drug utilization and reported adverse reactions in hospitalized children.* Am J Epidemiol, 1979. **110**(2): p. 196-204.

293. Herbst, A.L., H. Ulfelder, and D.C. Poskanzer, *Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women.* N Engl J Med, 1971. **284**(15): p. 878-81.

294. Giacoia, G.P. and D.R. Mattison, *Newborns and drug studies: the NICHD/FDA newborn drug development initiative.* Clin Ther, 2005. **27**(6): p. 796-813.

295. Halliday, H.L., *Overview of clinical trials comparing natural and synthetic surfactants.* Biol Neonate, 1995. **67 Suppl 1**: p. 32-47.

296. Nahata, M.C., *Drug Formulations*, in *Paediatric Drug Formulations*, M.C. Nahata and V.B. Pai, Editors. 2004, Harvey Whitney Books.

297. Horn, N.A., et al., *Coagulation assessment in healthy pigs undergoing single xenon anaesthesia and combinations with isoflurane and sevoflurane.* Acta Anaesthesiol Scand, 2001. **45**(5): p. 634-8.

298. Glasgow, A.M., et al., *Hyperosmolality in small infants due to propylene glycol.* Pediatrics, 1983. **72**(3): p. 353-5.

299. Peleg, O., B. Bar-Oz, and I. Arad, *Coma in a premature infant associated with the transdermal absorption of propylene glycol.* Acta Paediatr, 1998. **87**(11): p. 1195-6.

300. Gershanik, J., et al., *The gasping syndrome and benzyl alcohol poisoning.* N Engl J Med, 1982. **307**(22): p. 1384-8.

301. Lopez-Herce, J., et al., *Benzyl alcohol poisoning following diazepam intravenous infusion.* Ann Pharmacother, 1995. **29**(6): p. 632.

302. Brown, W.J., et al., *Fatal benzyl alcohol poisoning in a neonatal intensive care unit.* Lancet, 1982. **1**(8283): p. 1250.

303. Kearns, G.L., et al., *Developmental pharmacology--drug disposition, action, and therapy in infants and children.* N Engl J Med, 2003. **349**(12): p. 1157-67.

304. Kearns, G.L., *Impact of developmental pharmacology on pediatric study design: overcoming the challenges.* J Allergy Clin Immunol, 2000. **106**(3 Suppl): p. S128-38.

305. Huang, N.N. and R.H. High, *Comparison of serum levels following the administration of oral and parenteral preparations of penicillin to infants and children of various age groups.* J Pediatr, 1953. **42**(6): p. 657-8.

306. Morselli, P.L.A.d.I.M.P., ed. Drug disposition during development. New York: Spectrum, 1977:311-60., *Antiepileptic drugs.*, in *Drug disposition during development*, P.L. Morselli, Editor. 1977, Spectrum: New York. p. 311-60.

307. Radde, I.C., *Mechanisms of drug absorption and their development.*, in *Textbook of Paediatric and Clinical Pharmacology*, S.M. Madeod and I.C. Radde, Editors. 1985, PSG Publishing: Litteton, Mass. p. 17-43.

308. Hall, S.D., et al., *Molecular and physical mechanisms of first-pass extraction.* Drug Metab Dispos, 1999. **27**(2): p. 161-6.

309. Widdowson, E.M., *Growth and compostion of the fetus and newborn*, in *Biology of Gestation*, N.S. Assali, Editor. 1968, Academic Press: New York. p. 1-49.

310. Ehrnebo, M., et al., *Age differences in drug binding by plasma proteins: studies on human foetuses, neonates and adults.* Eur J Clin Pharmacol, 1971. **3**(4): p. 189-93.

311. Painter, M.J., et al., *Phenobarbital and phenytoin in neonatal seizures: metabolism and tissue distribution.* Neurology, 1981. **31**(9): p. 1107-12.

312. de Wildt, S.N., et al., *Population pharmacokinetics and metabolism of midazolam in pediatric intensive care patients.* Crit Care Med, 2003. **31**(7): p. 1952-8.

313. Mulberg, A.E., S.A. Silber, and J.N. van den Anker, *Pediatric Drug Development: Concepts and Applications*. 2009, New Jersey: Wiley Blackwell.

314. van den Anker, J.N., et al., *Ceftazidime pharmacokinetics in preterm infants: effects of renal function and gestational age.* Clin Pharmacol Ther, 1995. **58**(6): p. 650-9.

315. van den Anker, J.N., et al., *Effects of prenatal exposure to betamethasone and indomethacin on the glomerular filtration rate in the preterm infant.* Pediatr Res, 1994. **36**(5): p. 578-81.

316. Smits, A. and K. Allegaert, *Perinatal pharmacology: Applications for neonatal neurology.* Eur J Paediatr Neurol, 2011. **15**(6): p. 478-86.

317. Andersen, D.L., et al., *GABA(A) receptor sites in the developing human foetus.* Brain Res Dev Brain Res, 2002. **139**(2): p. 107-19.

318. Field, M.J. and T.F. Boat, *Safe and effective medicines for children: pediatric studies conducted under the Best Pharmaceuticals for Children Act and the Pediatric Research Equity Act*, 2012, The National Academies Press: Washington DC.

319. ICH, *Guideline for Good Clinical Practice E6(R1)*, 1996.

320. Sammons, H., *Ethical issues of clinical trials in children: a European perspective.* Arch Dis Child, 2009. **94**(6): p. 474-7.

321. Barfield, R.C. and C. Church, *Informed consent in pediatric clinical trials.* Curr Opin Pediatr, 2005. **17**(1): p. 20-4.

322. Stenson, B.J., J.C. Becher, and N. McIntosh, *Neonatal research: the parental perspective.* Arch Dis Child Fetal Neonatal Ed, 2004. **89**(4): p. F321-3.

323. Rothmier, J.D., M.V. Lasley, and G.G. Shapiro, *Factors influencing parental consent in pediatric clinical research.* Pediatrics, 2003. **111**(5 Pt 1): p. 1037-41.

324. Sammons, H.M., et al., *What motivates British parents to consent for research? A questionnaire study.* BMC Pediatr, 2007. **7**: p. 12.

325. NBA, C.-N., *Ethical and policy issues in international research: clinical trials in developing countries*, 2001, National Bioethics Advisory Commission: Bethesda, MD.

326. Glickman, S.W., et al., *Ethical and scientific implications of the globalization of clinical research.* N Engl J Med, 2009. **360**(8): p. 816-23.

327. *Ethical considerations for clinical trials on medicinal products conducted with the paediatric population. Recommendations of the ad hoc group for the development of implementing guidelines for Directive 2001/20/EC relating to good clinical practice in the conduct of clinical trials on medicinal products for human use*, 2008, EU Legislation - Eudralex.

328. Offringa, M. and H. van der Lee, *Small sample approach, and statistical and epidemiological aspects.* Handb Exp Pharmacol, 2011. **205**: p. 181-202.

329. Junod, S.W., *Biologics centennial: 100 years of biologics regulation. Update from the food and drug law institute.*, 2002: Silver Spring, MD.

330. Nasr, A., T.J. Lauterio, and M.W. Davis, *Unapproved drugs in the United States and the Food and Drug Administration.* Adv Ther, 2011. **28**(10): p. 842-56.

331. Kuehn, B.M., *Frances Kelsey honored for FDA legacy: award notes her work on thalidomide, clinical trials.* JAMA, 2010. **304**(19): p. 2109-10, 2112.

332. EMEA. *Ethical considerations for clinical trials performed inchildren*. 2006 6th January 2013]; Available from: http://ec.europa.eu/enterprise/pharmaceuticals/paediatrics/docs/paeds_ethics_consultation20060929.pdf.

333. FDA, *Best Pharmaceuticals for Children Act.* , 2002, FDA: Silver Spring MD.

334. FDA, *Food and Drug Administration Modernization Act (FDAMA) of 1997*, 1997.

335. EMEA, *Note for guidance on clinical investigation of medicinal products in the paediatric population' (ICH Topic E11)*, 2002.

336. Agency, E.M., *REGULATION (EC) No 1901/2006 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL*

of 12 December 2006 on medicinal products for paediatric use and amending Regulation (EEC) No 1768/92, Directive 2001/20/EC, Directive 2001/83/EC and Regulation (EC) No 726/2004, 2006, Official Journal of the European Union.

337. Merchant, N. and D. Azzopardi, *Drug Development in Paediatrics and Neonatology*, in *The Textbook of Pharmaceutical Medicine*, J.P. Griffin, J. Posner, and G.R. Barker, Editors. 2013, Wiley Blackwell.

338. FDA. *Pediatric Research Equity Act of 2003*. 2003 6th January 2013]; Available from: http://www.fda.gov/downloads/Drugs/DevelopmentApprovalProcess/DevelopmentResources/UCM077853.pdf.

339. *Guideline on the format and content of applications for agreement or modification of a paediatric investigation plan and requests for waivers or deferrals and concerning the operation of the compliance check and on criteria for assessing significant studies (2008/C 243/01)*. 2008 6th January 2013]; Available from: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:C:2008:243:0001:0012:EN:PDF

340. Pritchard, J.F., et al., *Making better drugs: Decision gates in non-clinical drug development.* Nat Rev Drug Discov, 2003. **2**(7): p. 542-53.

341. FDA. *FDA Pediatric Study Decision Tree*. Available from: http://www.fda.gov/ScienceResearch/SpecialTopics/PediatricTherapeuticsResearch/ucm106614.htm.

342. FDA, *Adaptive Design Clinical Trials for Drugs and Biologics*, F. US Department of Health and Human Services, Editor Feb 2010.

343. Assessment, W.I.P.o.C.S.B.i.R. *Validity and Validation* 2001; Available from: http://www.inchem.org/documents/ehc/ehc/ehc222.htm.

344. Strimbu, K. and J.A. Tavel, *What are biomarkers?* Curr Opin HIV AIDS, 2010. **5**(6): p. 463-6.

345. Bleavins, M.B., et al., *Biomakers in drug development: a handbook of practice, applications and strategy,* . 2010, Hoboken, New Jersey, USA: John Wiley.

346. Biomarkers Definitions Working, G., *Biomarkers and surrogate endpoints: preferred definitions and conceptual framework.* Clin Pharmacol Ther, 2001. **69**(3): p. 89-95.

347. Edwards, A.D., et al., *Neurological outcomes at 18 months of age after moderate hypothermia for perinatal hypoxic ischaemic encephalopathy: synthesis and meta-analysis of trial data.* BMJ, 2010. **340**: p. c363.

348. Rutherford, M., et al., *Assessment of brain tissue injury after moderate hypothermia in neonates with hypoxic-ischaemic encephalopathy: a nested substudy of a randomised controlled trial.* Lancet Neurol, 2010. **9**(1): p. 39-45.

349. Porter, E.J., et al., *Tract-based spatial statistics of magnetic resonance images to assess disease and treatment effects in perinatal asphyxial encephalopathy.* Pediatr Res, 2010. **68**(3): p. 205-9.

350. Fleming, T.R. and D.L. DeMets, *Surrogate end points in clinical trials: are we being misled?* Ann Intern Med, 1996. **125**(7): p. 605-13.

351. Sams-Dodd, F., *Drug discovery: selecting the optimal approach.* Drug Discov Today, 2006. **11**(9-10): p. 465-72.

352. Ramanantsoa, N., et al., *Bench to cribside: the path for developing a neuroprotectant.* Transl Stroke Res, 2013. **4**(2): p. 258-77.

353. Haak, V.d., *Industry Success Rates 2004*, 2004, CMR Report 04-234R.

354. Mizushima, T., *Drug discovery and development focusing on existing medicines: drug re-profiling strategy.* J Biochem, 2011. **149**(5): p. 499-505.

355. McQuillen, P.S. and D.M. Ferriero, *Selective vulnerability in the developing central nervous system.* Pediatr Neurol, 2004. **30**(4): p. 227-35.

356. Hack, M., et al., *Poor predictive validity of the Bayley Scales of Infant Development for cognitive function of extremely low birth weight children at school age.* Pediatrics, 2005. **116**(2): p. 333-41.

357. Schmidt, B., et al., *Survival without disability to age 5 years after neonatal caffeine therapy for apnea of prematurity.* JAMA, 2012. **307**(3): p. 275-82.

358. Bergeron, M., et al., *Role of hypoxia-inducible factor-1 in hypoxia-induced ischemic tolerance in neonatal rat brain.* Ann Neurol, 2000. **48**(3): p. 285-96.

359. Sheldon, R.A., et al., *Hypoxic preconditioning reverses protection after neonatal hypoxia-ischemia in glutathione peroxidase transgenic murine brain.* Pediatr Res, 2007. **61**(6): p. 666-70.

360. Gidday, J.M., et al., *Neuroprotection from ischemic brain injury by hypoxic preconditioning in the neonatal rat.* Neurosci Lett, 1994. **168**(1-2): p. 221-4.

361. Vannucci, R.C., J. Towfighi, and S.J. Vannucci, *Hypoxic preconditioning and hypoxic-ischemic brain damage in the immature rat: pathologic and metabolic correlates.* J Neurochem, 1998. **71**(3): p. 1215-20.

362. Gustavsson, M., et al., *Hypoxic preconditioning confers long-term reduction of brain injury and improvement of neurological ability in immature rats.* Pediatr Res, 2005. **57**(2): p. 305-9.

363. Ran, R., et al., *Hypoxia preconditioning in the brain.* Developmental neuroscience, 2005. **27**(2-4): p. 87-92.

364. Villa, P., et al., *Erythropoietin selectively attenuates cytokine production and inflammation in cerebral ischemia by targeting neuronal apoptosis.* J Exp Med, 2003. **198**(6): p. 971-5.

365. Wang, L., et al., *Treatment of stroke with erythropoietin enhances neurogenesis and angiogenesis and improves neurological function in rats.* Stroke; a journal of cerebral circulation, 2004. **35**(7): p. 1732-7.

366. Chong, Z.Z., J.Q. Kang, and K. Maiese, *Angiogenesis and plasticity: role of erythropoietin in vascular systems.* J Hematother Stem Cell Res, 2002. **11**(6): p. 863-71.

367. Digicaylioglu, M. and S.A. Lipton, *Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades.* Nature, 2001. **412**(6847): p. 641-7.

368. Kellert, B.A., R.J. McPherson, and S.E. Juul, *A comparison of high-dose recombinant erythropoietin treatment regimens in brain-injured neonatal rats.* Pediatr Res, 2007. **61**(4): p. 451-5.

369. Kawakami, M., et al., *Erythropoietin inhibits calcium-induced neurotransmitter release from clonal neuronal cells.* Biochem Biophys Res Commun, 2000. **279**(1): p. 293-7.

370. Sun, Y., J.W. Calvert, and J.H. Zhang, *Neonatal hypoxia/ischemia is associated with decreased inflammatory mediators after erythropoietin administration.* Stroke, 2005. **36**(8): p. 1672-8.

371. Dzietko, M., et al., *Erythropoietin protects the developing brain against N-methyl-D-aspartate receptor antagonist neurotoxicity.* Neurobiol Dis, 2004. **15**(2): p. 177-87.

372. Wang, L., et al., *Neural progenitor cells treated with EPO induce angiogenesis through the production of VEGF.* J Cereb Blood Flow Metab, 2008. **28**(7): p. 1361-8.

373. Sugawa, M., et al., *Effects of erythropoietin on glial cell development; oligodendrocyte maturation and astrocyte proliferation.* Neurosci Res, 2002. **44**(4): p. 391-403.

374. Sola, A., et al., *Potential for protection and repair following injury to the developing brain: a role for erythropoietin?* Pediatr Res, 2005. **57**(5 Pt 2): p. 110R-117R.

375. Kumral, A., et al., *Erythropoietin improves long-term spatial memory deficits and brain injury following neonatal hypoxia-ischemia in rats.* Behav Brain Res, 2004. **153**(1): p. 77-86.

376. Sola, A., et al., *Erythropoietin after focal cerebral ischemia activates the Janus kinase-signal transducer and activator of transcription signaling pathway and improves brain injury in postnatal day 7 rats.* Pediatr Res, 2005. **57**(4): p. 481-7.

377. Plane, J.M., et al., *Neonatal hypoxic-ischemic injury increases forebrain subventricular zone neurogenesis in the mouse.* Neurobiology of disease, 2004. **16**(3): p. 585-95.

378. Sun, Y., J.W. Calvert, and J.H. Zhang, *Neonatal hypoxia/ischemia is associated with decreased inflammatory mediators after erythropoietin administration.* Stroke; a journal of cerebral circulation, 2005. **36**(8): p. 1672-8.

379. Aher, S. and A. Ohlsson, *Late erythropoietin for preventing red blood cell transfusion in preterm and/or low birth weight infants.* Cochrane Database Syst Rev, 2006(3): p. CD004868.

380. Robertson, N.J., et al., *Which neuroprotective agents are ready for bench to bedside translation in the newborn infant?* J Pediatr, 2012. **160**(4): p. 544-552 e4.

381. Brines, M.L., et al., *Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury.* Proc Natl Acad Sci U S A, 2000. **97**(19): p. 10526-31.

382. Juul, S.E., et al., *Erytropoietin concentrations in cerebrospinal fluid of nonhuman primates and fetal sheep following high-dose recombinant erythropoietin.* Biol Neonate, 2004. **85**(2): p. 138-44.

383. Statler, P.A., et al., *Pharmacokinetics of high-dose recombinant erythropoietin in plasma and brain of neonatal rats.* Pediatr Res, 2007. **61**(6): p. 671-5.

384. Edwards, A.D., D. Azzopardi, and A.J. Gunn, *Neonatal Neural Rescue: A Clinical Guide*. 1 ed. 2013, U.K.: Cambridge University Press. 242.

385. Demers, E.J., R.J. McPherson, and S.E. Juul, *Erythropoietin protects dopaminergic neurons and improves neurobehavioral outcomes in juvenile rats after neonatal hypoxia-ischemia.* Pediatr Res, 2005. **58**(2): p. 297-301.

386. McPherson, R.J., E.J. Demers, and S.E. Juul, *Safety of high-dose recombinant erythropoietin in a neonatal rat model.* Neonatology, 2007. **91**(1): p. 36-43.

387. Juul, S.E., et al., *A phase I/II trial of high-dose erythropoietin in extremely low birth weight infants: pharmacokinetics and safety.* Pediatrics, 2008. **122**(2): p. 383-91.

388. Gonzalez, F.F., et al., *Erythropoietin sustains cognitive function and brain volume after neonatal stroke.* Dev Neurosci, 2009. **31**(5): p. 403-11.

389. Tsai, P.T., et al., *A critical role of erythropoietin receptor in neurogenesis and post-stroke recovery.* J Neurosci, 2006. **26**(4): p. 1269-74.

390. Wu, Y.W., et al., *Erythropoietin for neuroprotection in neonatal encephalopathy: safety and pharmacokinetics.* Pediatrics, 2012. **130**(4): p. 683-91.

391. Juul, S.E., et al., *Erythropoietin acts as a trophic factor in neonatal rat intestine.* Gut, 2001. **49**(2): p. 182-9.

392. Fishbane, S. and A.R. Nissenson, *The new FDA label for erythropoietin treatment: how does it affect hemoglobin target?* Kidney Int, 2007. **72**(7): p. 806-13.

393. Carmichael, S.T., *Translating the frontiers of brain repair to treatments: starting not to break the rules.* Neurobiol Dis, 2010. **37**(2): p. 237-42.

394. Weber, A., et al., *Neuronal damage after moderate hypoxia and erythropoietin.* Neurobiol Dis, 2005. **20**(2): p. 594-600.

395. Cariou, A., et al., *Early high-dose erythropoietin therapy and hypothermia after out-of-hospital cardiac arrest: a matched control study.* Resuscitation, 2008. **76**(3): p. 397-404.

396. Ehrenreich, H., et al., *Recombinant human erythropoietin in the treatment of acute ischemic stroke.* Stroke; a journal of cerebral circulation, 2009. **40**(12): p. e647-56.

397. Fauchere, J.C., et al., *An approach to using recombinant erythropoietin for neuroprotection in very preterm infants.* Pediatrics, 2008. **122**(2): p. 375-82.

398. Juul, S.E., et al., *Recombinant erythropoietin is neuroprotective in a novel mouse oxidative injury model.* Dev Neurosci, 2008. **30**(4): p. 231-42.

399. Zhu, C., et al., *Erythropoietin improved neurologic outcomes in newborns with hypoxic-ischemic encephalopathy.* Pediatrics, 2009. **124**(2): p. e218-26.

400. Elmahdy, M.F., et al., *Value of duplex scanning in differentiating embolic from thrombotic arterial occlusion in acute limb ischemia.* Cardiovasc Revasc Med, 2010. **11**(4): p. 223-6.

401. Lakic, N., et al., *Importance of erythropoietin in brain protection after cardiac surgery: a pilot study.* Heart Surg Forum, 2010. **13**(3): p. E185-9.

402. D'Ercole, A.J., et al., *The role of the insulin-like growth factors in the central nervous system.* Mol Neurobiol, 1996. **13**(3): p. 227-55.

403. Pang, Y., et al., *IGF-1 protects oligodendrocyte progenitors against TNFalpha-induced damage by activation of PI3K/Akt and interruption of the mitochondrial apoptotic pathway.* Glia, 2007. **55**(11): p. 1099-107.

404. van den Tweel, E.R., et al., *Long-term neuroprotection with 2-iminobiotin, an inhibitor of neuronal and inducible nitric oxide synthase, after cerebral hypoxia-ischemia in neonatal rats.* J Cereb Blood Flow Metab, 2005. **25**(1): p. 67-74.

405. Marks, K.A., et al., *Nitric oxide synthase inhibition attenuates delayed vasodilation and increases injury after cerebral ischemia in fetal sheep.* Pediatr Res, 1996. **40**(2): p. 185-91.

406. Palmer, C., et al., *Allopurinol administered after inducing hypoxia-ischemia reduces brain injury in 7-day-old rats.* Pediatr Res, 1993. **33**(4 Pt 1): p. 405-11.

407. Clancy, R.R., et al., *Allopurinol neurocardiac protection trial in infants undergoing heart surgery using deep hypothermic circulatory arrest.* Pediatrics, 2001. **108**(1): p. 61-70.

408. Benders, M.J., et al., *Early postnatal allopurinol does not improve short term outcome after severe birth asphyxia.* Arch Dis Child Fetal Neonatal Ed, 2006. **91**(3): p. F163-5.

409. Van Bel, F., et al., *Effect of allopurinol on postasphyxial free radical formation, cerebral hemodynamics, and electrical brain activity.* Pediatrics, 1998. **101**(2): p. 185-93.

410. Gunes, T., et al., *Effect of allopurinol supplementation on nitric oxide levels in asphyxiated newborns.* Pediatr Neurol, 2007. **36**(1): p. 17-24.

411. Aruoma, O.I., et al., *The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid.* Free Radic Biol Med, 1989. **6**(6): p. 593-7.

412. Plaisant, F., et al., *Recombinant peroxiredoxin 5 protects against excitotoxic brain lesions in newborn mice.* Free Radic Biol Med, 2003. **34**(7): p. 862-72.

413. Paintlia, M.K., et al., *N-acetylcysteine prevents endotoxin-induced degeneration of oligodendrocyte progenitors and hypomyelination in developing rat brain.* J Neurosci Res, 2004. **78**(3): p. 347-61.

414. Heard, K.J., *Acetylcysteine for acetaminophen poisoning.* N Engl J Med, 2008. **359**(3): p. 285-92.

415. Probyn, M.E., et al., *The anti-inflammatory agent N-acetyl cysteine exacerbates endotoxin-induced hypoxemia and hypotension and induces polycythemia in the ovine fetus.* Neonatology, 2010. **98**(2): p. 118-27.

416. Chen, H.S. and S.A. Lipton, *The chemical biology of clinically tolerated NMDA receptor antagonists.* J Neurochem, 2006. **97**(6): p. 1611-26.

417. Chen, H.S., et al., *Neuroprotective concentrations of the N-methyl-D-aspartate open-channel blocker memantine are effective without cytoplasmic vacuolation following post-ischemic administration and do not block maze learning or long-term potentiation.* Neuroscience, 1998. **86**(4): p. 1121-32.

418. Volbracht, C., et al., *Neuroprotective properties of memantine in different in vitro and in vivo models of excitotoxicity.* Eur J Neurosci, 2006. **23**(10): p. 2611-22.

419. Sfaello, I., et al., *Topiramate prevents excitotoxic damage in the newborn rodent brain.* Neurobiology of disease, 2005. **20**(3): p. 837-48.

420. Turkyilmaz, C., et al., *Magnesium pre-treatment reduces neuronal apoptosis in newborn rats in hypoxia-ischemia.* Brain Res, 2002. **955**(1-2): p. 133-7.

421. Marret, S., et al., *Prevention by magnesium of excitotoxic neuronal death in the developing brain: an animal model for clinical intervention studies.* Dev Med Child Neurol, 1995. **37**(6): p. 473-84.

422. Spandou, E., et al., *Neuroprotective effect of long-term MgSO4 administration after cerebral hypoxia-ischemia in newborn rats is related to the severity of brain damage.* Reprod Sci, 2007. **14**(7): p. 667-77.

423. Khashaba, M.T., et al., *Excitatory amino acids and magnesium sulfate in neonatal asphyxia.* Brain Dev, 2006. **28**(6): p. 375-9.

424. Crowther, C.A., et al., *Effect of magnesium sulfate given for neuroprotection before preterm birth: a randomized controlled trial.* JAMA, 2003. **290**(20): p. 2669-76.

425. Groenendaal, F., et al., *Effects of magnesium sulphate on amplitude-integrated continuous EEG in asphyxiated term neonates.* Acta Paediatr, 2002. **91**(10): p. 1073-7.

426. Levene, M., et al., *Acute effects of two different doses of magnesium sulphate in infants with birth asphyxia.* Arch Dis Child Fetal Neonatal Ed, 1995. **73**(3): p. F174-7.

427. Stirling, D.P., et al., *Minocycline as a neuroprotective agent.* Neuroscientist, 2005. **11**(4): p. 308-22.

428. Chen, M., et al., *Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease.* Nat Med, 2000. **6**(7): p. 797-801.

429. Zhu, S., et al., *Minocycline inhibits cytochrome c release and delays progression of amyotrophic lateral sclerosis in mice.* Nature, 2002. **417**(6884): p. 74-8.

430. Pruzanski, W., et al., *Inhibition of enzymatic activity of phospholipases A2 by minocycline and doxycycline.* Biochem Pharmacol, 1992. **44**(6): p. 1165-70.

431. Wang, J., et al., *Minocycline up-regulates Bcl-2 and protects against cell death in mitochondria.* J Biol Chem, 2004. **279**(19): p. 19948-54.

432. Cai, Z., et al., *Minocycline alleviates hypoxic-ischemic injury to developing oligodendrocytes in the neonatal rat brain.* Neuroscience, 2006. **137**(2): p. 425-35.

433. Fox, C., et al., *Minocycline confers early but transient protection in the immature brain following focal cerebral ischemia-reperfusion.* J Cereb Blood Flow Metab, 2005. **25**(9): p. 1138-49.

434. Tsuji, M., et al., *Minocycline worsens hypoxic-ischemic brain injury in a neonatal mouse model.* Exp Neurol, 2004. **189**(1): p. 58-65.

435. Klein, D.C., et al., *The melatonin rhythm-generating enzyme: molecular regulation of serotonin N-acetyltransferase in the pineal gland.* Recent Prog Horm Res, 1997. **52**: p. 307-57; discussion 357-8.

436. Korf, H.W., C. Schomerus, and J.H. Stehle, *The pineal organ, its hormone melatonin, and the photoneuroendocrine system.* Adv Anat Embryol Cell Biol, 1998. **146**: p. 1-100.

437. Arendt, J., *Melatonin and the Mammalian Pineal Gland*. First ed. 1995, London: Chapman & Hall.

438. Reiter, R.J., *Pineal melatonin: cell biology of its synthesis and of its physiological interactions.* Endocr Rev, 1991. **12**(2): p. 151-80.

439. Okatani, Y., et al., *Maternal-fetal transfer of melatonin in pregnant women near term.* J Pineal Res, 1998. **25**(3): p. 129-34.

440. Jan, J.E., et al., *Evidence supporting the use of melatonin in short gestation infants.* J Pineal Res, 2007. **42**(1): p. 22-7.

441. Kennaway, D.J., G.E. Stamp, and F.C. Goble, *Development of melatonin production in infants and the impact of prematurity.* J Clin Endocrinol Metab, 1992. **75**(2): p. 367-9.

442. Carpentieri, A., et al., *New perspectives in melatonin uses.* Pharmacol Res. **65**(4): p. 437-44.

443. Dziegiel, P., M. Podhorska-Okolow, and M. Zabel, *Melatonin: adjuvant therapy of malignant tumors.* Med Sci Monit, 2008. **14**(5): p. RA64-70.

444. Bojkowski, C.J. and J. Arendt, *Factors influencing urinary 6-sulphatoxymelatonin, a major melatonin metabolite, in normal human subjects.* Clin Endocrinol (Oxf), 1990. **33**(4): p. 435-44.

445. **Arendt, J.**, ***Mammalian pineal rhythms.*** Pineal Res. Rev., 1985. **3**: p. 161-213.

446. Arendt, J., *Melatonin.* Clin Endocrinol (Oxf), 1988. **29**(2): p. 205-29.

447. Waldhauser, F., et al., *Bioavailability of oral melatonin in humans.* Neuroendocrinology, 1984. **39**(4): p. 307-13.

448. Aldhous, M., et al., *Plasma concentrations of melatonin in man following oral absorption of different preparations.* Br J Clin Pharmacol, 1985. **19**(4): p. 517-21.

449. Lane, E.A. and H.B. Moss, *Pharmacokinetics of melatonin in man: first pass hepatic metabolism.* J Clin Endocrinol Metab, 1985. **61**(6): p. 1214-6.

450. Zawilska, J.B. and J.Z. Nowak, *Melatonin: from biochemistry to therapeutic applications.* Pol J Pharmacol, 1999. **51**(1): p. 3-23.

451. DeMuro, R.L., et al., *The absolute bioavailability of oral melatonin.* J Clin Pharmacol, 2000. **40**(7): p. 781-4.

452. Mistraletti, G., et al., *Pharmacokinetics of orally administered melatonin in critically ill patients.* J Pineal Res, 2010. **48**(2): p. 142-7.

453. Reppert, S.M., et al., *Maternal-fetal transfer of melatonin in the non-human primate.* Pediatr Res, 1979. **13**(6): p. 788-91.

454. Vitte, P.A., et al., *Plasma, cerebrospinal fluid, and brain distribution of 14C-melatonin in rat: a biochemical and autoradiographic study.* J Pineal Res, 1988. **5**(5): p. 437-53.

455. Kennaway, D.J. and A. Voultsios, *Circadian rhythm of free melatonin in human plasma.* J Clin Endocrinol Metab, 1998. **83**(3): p. 1013-5.

456. Illnerova, H., M. Buresova, and J. Presl, *Melatonin rhythm in human milk.* J Clin Endocrinol Metab, 1993. **77**(3): p. 838-41.

457. Rowe, S.A. and D.J. Kennaway, *Melatonin in rat milk and the likelihood of its role in postnatal maternal entrainment of rhythms.* Am J Physiol Regul Integr Comp Physiol, 2002. **282**(3): p. R797-804.

458. Iguchi, H., K.I. Kato, and H. Ibayashi, *Melatonin serum levels and metabolic clearance rate in patients with liver cirrhosis.* J Clin Endocrinol Metab, 1982. **54**(5): p. 1025-7.

459. Mallo, C., et al., *Pharmacokinetics of melatonin in man after intravenous infusion and bolus injection.* Eur J Clin Pharmacol, 1990. **38**(3): p. 297-301.

460. Anton-Tay, F. and R.J. Wurtman, *Regional uptake of 3H-melatonin from blood or cerebrospinal fluid by rat brain.* Nature, 1969. **221**(5179): p. 474-5.

461. Cardinali, D.P., M.T. Hyyppa, and R.J. Wurtman, *Fate of intracisternally injected melatonin in the rat brain.* Neuroendocrinology, 1973. **12**(1): p. 30-40.

462. Ferreira, S.A., M.D. Rollag, and J.D. Glass, *Pharmacokinetics of extracellular melatonin in Siberian hamster forebrain.* Brain Res, 1996. **733**(2): p. 318-20.

463. Ma, X., et al., *Metabolism of melatonin by human cytochromes p450.* Drug Metab Dispos, 2005. **33**(4): p. 489-94.

464. Klinger, W., et al., *Interactions of melatonin with the liver microsomal cytochrome P450 system of rats and humans in vitro and effects on the P450 system and the antioxidative status in rat liver after acute treatment.* Eur J Drug Metab Pharmacokinet, 2001. **26**(1-2): p. 31-5.

465. Skene, D.J., et al., *Contribution of CYP1A2 in the hepatic metabolism of melatonin: studies with isolated microsomal preparations and liver slices.* J Pineal Res, 2001. **31**(4): p. 333-42.

466. Fourtillan, J.B., et al., *Melatonin secretion occurs at a constant rate in both young and older men and women.* American journal of physiology. Endocrinology and metabolism, 2001. **280**(1): p. E11-22.

467. Paakkonen, T., et al., *Urinary melatonin: a noninvasive method to follow human pineal function as studied in three experimental conditions.* J Pineal Res, 2006. **40**(2): p. 110-5.

468. Vaughan, G.M., *Melatonin in humans*, in *Pineal research reviews*, R.J. Reiter, Editor. 1984, Liss: New York. p. 141-201.

469. Lang, U., et al., *Radioimmunological determination of urinary melatonin in humans: correlation with plasma levels and typical 24-hour rhythmicity.* J Clin Endocrinol Metab, 1981. **53**(3): p. 645-50.

470. Kovacs, J., et al., *Measurement of urinary melatonin: a useful tool for monitoring serum melatonin after its oral administration.* J Clin Endocrinol Metab, 2000. **85**(2): p. 666-70.

471. Arendt, J., et al., *Immunoassay of 6-hydroxymelatonin sulfate in human plasma and urine: abolition of the urinary 24-hour rhythm with atenolol.* J Clin Endocrinol Metab, 1985. **60**(6): p. 1166-73.

472. Cavallo, A. and W.A. Ritschel, *Pharmacokinetics of melatonin in human sexual maturation.* J Clin Endocrinol Metab, 1996. **81**(5): p. 1882-6.

473. Debus, O.M., et al., *Spontaneous central melatonin secretion and resorption kinetics of exogenous melatonin: a ventricular CSF study.* J Pineal Res, 2002. **33**(4): p. 213-7.

474. Dobrinas, M., J. Cornuz, and C.B. Eap, *Pharmacogenetics of CYP1A2 activity and inducibility in smokers and exsmokers.* Pharmacogenet Genomics, 2013. **23**(5): p. 286-92.

475. de Almeida, E.A., et al., *Measurement of melatonin in body fluids: standards, protocols and procedures.* Childs Nerv Syst, 2011. **27**(6): p. 879-91.

476. Rizzo, V., et al., *Determination of free and total (free plus protein-bound) melatonin in plasma and cerebrospinal fluid by high-performance liquid chromatography with fluorescence detection.* J Chromatogr B Analyt Technol Biomed Life Sci, 2002. **774**(1): p. 17-24.

477. Peniston-Bird, J.F., et al., *HPLC assay of melatonin in plasma with fluorescence detection.* Clin Chem, 1993. **39**(11 Pt 1): p. 2242-7.

478. Vieira, R., et al., *Pineal and plasma melatonin as determined by high-performance liquid chromatography with electrochemical detection.* Anal Biochem, 1992. **205**(2): p. 300-5.

479. Fraser, S., et al., *Direct radioimmunoassay for melatonin in plasma.* Clin Chem, 1983. **29**(2): p. 396-7.

480. Rolcik, J., et al., *Isolation of melatonin by immunoaffinity chromatography.* J Chromatogr B Analyt Technol Biomed Life Sci, 2002. **775**(1): p. 9-15.

481. Wu, X., et al., *Micellar electrokinetic capillary chromatography for fast separation and sensitive determination of melatonin and related indoleamines using end-column amperometric detection.* Electrophoresis, 2006. **27**(21): p. 4230-9.

482. Olivier, P., et al., *Melatonin promotes oligodendroglial maturation of injured white matter in neonatal rats.* PLoS One, 2009. **4**(9): p. e7128.

483. Welin, A.K., et al., *Melatonin reduces inflammation and cell death in white matter in the mid-gestation fetal sheep following umbilical cord occlusion.* Pediatr Res, 2007. **61**(2): p. 153-8.

484. Watanabe, K., et al., *Maternally administered melatonin protects against ischemia and reperfusion-induced oxidative mitochondrial damage in premature fetal rat brain.* J Pineal Res, 2004. **37**(4): p. 276-80.

485. Miller, S.L., et al., *Melatonin provides neuroprotection in the late-gestation fetal sheep brain in response to umbilical cord occlusion.* Developmental neuroscience, 2005. **27**(2-4): p. 200-10.

486. Hutton, L.C., et al., *Neuroprotective properties of melatonin in a model of birth asphyxia in the spiny mouse (Acomys cahirinus).* Developmental neuroscience, 2009. **31**(5): p. 437-51.

487. Husson, I., et al., *Melatoninergic neuroprotection of the murine periventricular white matter against neonatal excitotoxic challenge.* Ann Neurol, 2002. **51**(1): p. 82-92.

488. Bouslama, M., et al., *Melatonin prevents learning disorders in brain-lesioned newborn mice.* Neuroscience, 2007. **150**(3): p. 712-9.

489. Robertson, N., et al., *Melatonin Augments Hypothermic Neuroprotection in a Perinatal Asphyxia Piglet Model*, in *Pediatric Academic Societies*2012: Boston 2012.

490. Sun, F.Y., et al., *Neuroprotection by melatonin against ischemic neuronal injury associated with modulation of DNA damage and repair in the rat following a transient cerebral ischemia.* J Pineal Res, 2002. **33**(1): p. 48-56.

491. Beni, S.M., et al., *Melatonin-induced neuroprotection after closed head injury is associated with increased brain antioxidants and attenuated late-phase activation of NF-kappaB and AP-1.* FASEB J, 2004. **18**(1): p. 149-51.

492. Kilic, U., et al., *Signal transduction pathways involved in melatonin-induced neuroprotection after focal cerebral ischemia in mice.* J Pineal Res, 2005. **38**(1): p. 67-71.

493. Macleod, M.R., et al., *Systematic review and meta-analysis of the efficacy of melatonin in experimental stroke.* J Pineal Res, 2005. **38**(1): p. 35-41.

494. Barlow-Walden, L.R., et al., *Melatonin stimulates brain glutathione peroxidase activity.* Neurochem Int, 1995. **26**(5): p. 497-502.

495. Antolin, I., et al., *Neurohormone melatonin prevents cell damage: effect on gene expression for antioxidant enzymes.* FASEB J, 1996. **10**(8): p. 882-90.

496. Kilic, E., et al., *Melatonin reduces disseminate neuronal death after mild focal ischemia in mice via inhibition of caspase-3 and is suitable as an add-on treatment to tissue-plasminogen activator.* J Pineal Res, 2004. **36**(3): p. 171-6.

497. Cheung, R.T., *The utility of melatonin in reducing cerebral damage resulting from ischemia and reperfusion.* J Pineal Res, 2003. **34**(3): p. 153-60.

498. Giusti, P., et al., *In vitro and in vivo protection against kainate-induced excitotoxicity by melatonin.* J Pineal Res, 1996. **20**(4): p. 226-31.

499. Floreani, M., et al., *Melatonin maintains glutathione homeostasis in kainic acid-exposed rat brain tissues.* FASEB J, 1997. **11**(14): p. 1309-15.

500. Chung, S.Y. and S.H. Han, *Melatonin attenuates kainic acid-induced hippocampal neurodegeneration and oxidative stress through microglial inhibition.* J Pineal Res, 2003. **34**(2): p. 95-102.

501. Chang, C.F., et al., *Melatonin attenuates kainic acid-induced neurotoxicity in mouse hippocampus via inhibition of autophagy and alpha-synuclein aggregation.* J Pineal Res. **52**(3): p. 312-21.

502. Manev, H., et al., *Increased brain damage after stroke or excitotoxic seizures in melatonin-deficient rats.* FASEB J, 1996. **10**(13): p. 1546-51.

503. Pei, Z., S.F. Pang, and R.T. Cheung, *Pretreatment with melatonin reduces volume of cerebral infarction in a rat middle cerebral artery occlusion stroke model.* J Pineal Res, 2002. **32**(3): p. 168-72.

504. Dubocovich, M.L., et al., *International Union of Basic and Clinical Pharmacology. LXXV. Nomenclature, classification, and pharmacology of G protein-coupled melatonin receptors.* Pharmacol Rev, 2010. **62**(3): p. 343-80.

505. Tan, D.X., et al., *Chemical and physical properties and potential mechanisms: melatonin as a broad spectrum antioxidant and free radical scavenger.* Curr Top Med Chem, 2002. **2**(2): p. 181-97.

506. Mathes, A.M., *Hepatoprotective actions of melatonin: possible mediation by melatonin receptors.* World J Gastroenterol, 2010. **16**(48): p. 6087-97.

507. Altun A, U.-A.B., *Melatonin: therapeutic and clinical utilization.* Int J Clin Pract, 2007. **61**(5): p. 835-45.

508. Park, C.B., et al., *Tissue-specific suppression of estrogen, androgen and glucocorticoid receptor gene expression in feral vitellogenic male Mozambique tilapia.* Chemosphere, 2007. **69**(1): p. 32-40.

509. Gitto, E., et al., *Effects of melatonin treatment in septic newborns.* Pediatr Res, 2001. **50**(6): p. 756-60.

510. Tahraoui, S.L., et al., *Central role of microglia in neonatal excitotoxic lesions of the murine periventricular white matter.* Brain pathology, 2001. **11**(1): p. 56-71.

511. Gitto, E., et al., *Early indicators of chronic lung disease in preterm infants with respiratory distress syndrome and their inhibition by melatonin.* J Pineal Res, 2004. **36**(4): p. 250-5.

512. Gitto, E., et al., *Correlation among cytokines, bronchopulmonary dysplasia and modality of ventilation in preterm newborns: improvement with melatonin treatment.* J Pineal Res, 2005. **39**(3): p. 287-93.

513. Gitto, E., et al., *Melatonin reduces oxidative stress in surgical neonates.* J Pediatr Surg, 2004. **39**(2): p. 184-9; discussion 184-9.

514. Herxheimer, A. and K.J. Petrie, *Melatonin for the prevention and treatment of jet lag.* Cochrane Database Syst Rev, 2002(2): p. CD001520.

515. Waterhouse, J., et al., *Jet lag: trends and coping strategies.* Lancet, 2007. **369**(9567): p. 1117-29.

516. Penev, P.D. and P.C. Zee, *Melatonin: a clinical perspective.* Ann Neurol, 1997. **42**(4): p. 545-53.

517. Bersani, G. and A. Garavini, *Melatonin add-on in manic patients with treatment resistant insomnia.* Prog Neuropsychopharmacol Biol Psychiatry, 2000. **24**(2): p. 185-91.

518. Naguib, M. and A.H. Samarkandi, *The comparative dose-response effects of melatonin and midazolam for premedication of adult patients: a double-blinded, placebo-controlled study.* Anesth Analg, 2000. **91**(2): p. 473-9.

519. Jan, M.M., *Melatonin for the treatment of handicapped children with severe sleep disorders.* Pediatr Neurol, 2000. **23**(3): p. 229-32.

520. Naguib, M., et al., *Pharmacological effects of intravenous melatonin: comparative studies with thiopental and propofol.* Br J Anaesth, 2003. **90**(4): p. 504-7.

521. Anisimov, V.N., *Effects of exogenous melatonin--a review.* Toxicol Pathol, 2003. **31**(6): p. 589-603.

522. Malhotra, S., G. Sawhney, and P. Pandhi, *The therapeutic potential of melatonin: a review of the science.* MedGenMed, 2004. **6**(2): p. 46.

523. Blask, D.E., et al., *Melatonin uptake and growth prevention in rat hepatoma 7288CTC in response to dietary melatonin: melatonin receptor-mediated inhibition of tumor linoleic acid metabolism to the growth signaling molecule 13-hydroxyoctadecadienoic acid and the potential role of phytomelatonin.* Carcinogenesis, 2004. **25**(6): p. 951-60.

524. Karasek, M., et al., *Melatonin inhibits growth of diethylstilbestrol-induced prolactin-secreting pituitary tumor in vitro: possible involvement of nuclear RZR/ROR receptors.* J Pineal Res, 2003. **34**(4): p. 294-6.

525. Lissoni, P., et al., *Five years survival in metastatic non-small cell lung cancer patients treated with chemotherapy alone or chemotherapy and melatonin: a randomized trial.* J Pineal Res, 2003. **35**(1): p. 12-5.

526. Horton, T.H. and S.M. Yellon, *Aging, reproduction, and the melatonin rhythm in the Siberian hamster.* J Biol Rhythms, 2001. **16**(3): p. 243-53.

527. Gwayi, N. and R.T. Bernard, *The effects of melatonin on sperm motility in vitro in Wistar rats.* Andrologia, 2002. **34**(6): p. 391-6.

528. d'Istria, M., et al., *Effects of melatonin treatment on Leydig cell activity in the testis of the frog Rana esculenta.* Zygote, 2004. **12**(4): p. 293-9.

529. Luboshitzky, R., et al., *Melatonin administration alters semen quality in healthy men.* J Androl, 2002. **23**(4): p. 572-8.

530. Drago, F. and L. Busa, *Acute low doses of melatonin restore full sexual activity in impotent male rats.* Brain Res, 2000. **878**(1-2): p. 98-104.

531. Takayama, H., et al., *Pineal gland (melatonin) affects the parturition time, but not luteal function and fetal growth, in pregnant rats.* Endocr J, 2003. **50**(1): p. 37-43.

532. Bishnupuri, K.S. and C. Haldar, *Profile of organ weights and plasma concentrations of melatonin, estradiol and progesterone during gestation and post-parturition periods in female Indian palm squirrel Funambulus pennanti.* Indian J Exp Biol, 2000. **38**(10): p. 974-81.

533. Kim, Y.O., et al., *Phototoxicity of melatonin.* Arch Pharm Res, 1999. **22**(2): p. 143-50.

534. Buscemi, N., et al., *Efficacy and safety of exogenous melatonin for secondary sleep disorders and sleep disorders accompanying sleep restriction: meta-analysis.* BMJ, 2006. **332**(7538): p. 385-93.

535. Buscemi, N., et al., *The efficacy and safety of exogenous melatonin for primary sleep disorders. A meta-analysis.* J Gen Intern Med, 2005. **20**(12): p. 1151-8.

536. Cagnacci, A., J.A. Elliott, and S.S. Yen, *Melatonin: a major regulator of the circadian rhythm of core temperature in humans.* J Clin Endocrinol Metab, 1992. **75**(2): p. 447-52.

537. Waldron, D.L., D. Bramble, and P. Gringras, *Melatonin: prescribing practices and adverse events.* Arch Dis Child, 2005. **90**(11): p. 1206-7.

538. Sheldon, S.H., *Pro-convulsant effects of oral melatonin in neurologically disabled children.* Lancet, 1998. **351**(9111): p. 1254.

539. Peled, N., et al., *Melatonin effect on seizures in children with severe neurologic deficit disorders.* Epilepsia, 2001. **42**(9): p. 1208-10.

540. Dickerman, R.D., et al., *Precocious puberty associated with a pineal cyst: is it disinhibition of the hypothalamic-pituitary axis?* Neuro Endocrinol Lett, 2004. **25**(3): p. 173-5.

541. Jan, J.E., H. Espezel, and R.E. Appleton, *The treatment of sleep disorders with melatonin.* Dev Med Child Neurol, 1994. **36**(2): p. 97-107.

542. Leppamaki, S., et al., *Effect of controlled-release melatonin on sleep quality, mood, and quality of life in subjects with seasonal or weather-associated changes in mood and behaviour.* Eur Neuropsychopharmacol, 2003. **13**(3): p. 137-45.

543. Palm, L., G. Blennow, and L. Wetterberg, *Long-term melatonin treatment in blind children and young adults with circadian sleep-wake disturbances.* Dev Med Child Neurol, 1997. **39**(5): p. 319-25.

544. Carr, R., et al., *Long-term effectiveness outcome of melatonin therapy in children with treatment-resistant circadian rhythm sleep disorders.* J Pineal Res, 2007. **43**(4): p. 351-9.

545. Wang, B., et al., *Rapid and sensitive detection of severe acute respiratory syndrome coronavirus by rolling circle amplification.* J Clin Microbiol, 2005. **43**(5): p. 2339-44.

546. Fulia, F., et al., *Increased levels of malondialdehyde and nitrite/nitrate in the blood of asphyxiated newborns: reduction by melatonin.* J Pineal Res, 2001. **31**(4): p. 343-9.

547. Gitto, E., et al., *Oxidative and inflammatory parameters in respiratory distress syndrome of preterm newborns: beneficial effects of melatonin.* Am J Perinatol, 2004. **21**(4): p. 209-16.

548. Pozo, D., et al., *Physiological concentrations of melatonin inhibit nitric oxide synthase in rat cerebellum.* Life Sci, 1994. **55**(24): p. PL455-60.

549. EMEA, *Guideline on the investigation of medicinal products in the term and preterm neonate*, C.f.M.P.f.H.U.C.a.P.C. (PDCO), Editor 2009, EMEA: London.

550. Birkett, D.J., *Pharmacokinetics made easy (Australian Prescriber)*. 2nd Edition ed. 2010, Australia: McGraw-Hill Australia Pty Ltd.

551. Aarons, L., *Population pharmacokinetics: theory and practice.* Br J Clin Pharmacol, 1991. **32**(6): p. 669-70.

552. FDA, *Population Pharmacokinetics- Guidance for Industry*, F.a.D.A. U.S. Department of Health and Human Services, Editor 1999: Rockville, MD.

553. Steimer, J.L., S. Vozeh, and A. Racine-Poon, *The Population Approach: Rationale, Methods and Applications in Clinical Pharmacology and Drug Development*, in *Pharmacokinetics of Drugs (Handbook of Experimental Pharmacology)*, P.G. Welling and L.P. Balant, Editors. 1994, Springer-Verlag: Berlin-Heidelberg. p. 404-451.

554. Sheiner, L.B., *Learning versus confirming in clinical drug development.* Clin Pharmacol Ther, 1997. **61**(3): p. 275-91.

555. Jones, C.D., H. Sun, and E.I. Ette, *Designing Cross-Sectional Pharmacokinetic Studies-Implications for Pediatric and Animal Studies.* Clin Res Regul Affairs, 1996. **13**: p. 133-165.

556. Nedelman, J.R., *On some "disadvantages" of the population approach.* AAPS J, 2005. **7**(2): p. E374-82.

557. Ette, E.I., R. Miller, and W.R. Gillespie, *The Population Approach: FDA experience*, in *The Population Approach: Measuring and Managing Variability in Response, Concentration and Dose, tion in the field of Scientifi*, L.P. Balant and L. Aarons, Editors. 1997, Commision of the European Communities, European Cooperation in the field of Scientific and Technical Research: Brussels.

558. Sheiner, L.B. and S.L. Beal, *Evaluation of methods for estimating population pharmacokinetics parameters. I. Michaelis-Menten model: routine clinical pharmacokinetic data.* J Pharmacokinet Biopharm, 1980. **8**(6): p. 553-71.

559. Sheiner, L.B. and S.L. Beal, *Evaluation of methods for estimating population pharmacokinetic parameters. II. Biexponential model and experimental pharmacokinetic data.* J Pharmacokinet Biopharm, 1981. **9**(5): p. 635-51.

560. Sheiner, L.B. and S.L. Beal, *Evaluation of methods for estimating population pharmacokinetic parameters. III. Monoexponential model: routine clinical pharmacokinetic data.* J Pharmacokinet Biopharm, 1983. **11**(3): p. 303-19.

561. Steimer, J.L., et al., *Alternative approaches to estimation of population pharmacokinetic parameters: comparison with the nonlinear mixed-effect model.* Drug Metab Rev, 1984. **15**(1-2): p. 265-92.

562. Fisher, D. and S.S. Fisher. *Pharmacokinetic and Pharmacodynamic Analysis with NONMEM-Basic Concepts*. in *NONMEM Workshop*. 2007. Ghent, Belgium.

563. Beal, S.L. and L.B. Sheiner, *Estimating population kinetics.* Crit Rev Biomed Eng, 1982. **8**(3): p. 195-222.

564. Ette, E.I., H. Sun, and T.M. Ludden, *Design of Population Pharmacokinetic Studies.* Proc Am Stat Assoc (Biopharmaceutics Section), 1994: p. 487-492.

565. Fadiran, E.O., C.D. Jones, and E.I. Ette, *Designing population pharmacokinetic studies: performance of mixed designs.* Eur J Drug Metab Pharmacokinet, 2000. **25**(3-4): p. 231-9.

566. Hashimoto, Y. and L.B. Sheiner, *Designs for population pharmacodynamics: value of pharmacokinetic data and population analysis.* J Pharmacokinet Biopharm, 1991. **19**(3): p. 333-53.

567. Jonsson, E.N., J.R. Wade, and M.O. Karlsson, *Comparison of some practical sampling strategies for population pharmacokinetic studies.* J Pharmacokinet Biopharm, 1996. **24**(2): p. 245-63.

568. Sun, H., E.I. Ette, and T.M. Ludden, *On the recording of sample times and parameter estimation from repeated measures pharmacokinetic data.* J Pharmacokinet Biopharm, 1996. **24**(6): p. 637-50.

569. Group, I.E.W., *Studies in Support of Special Populations: Geriatrics, E7*, 1993.

570. Hossain, M., et al., *Nonlinear Mixed Effects Modeling of a Single Dose and Multiple Dose for an Immediate Release and a Controlled Release Dosage Form of Alprazolam.* Pharm Res, 1997. **14**: p. 309-315.

571. Wade, J.R., et al., *A novel statistical approach for the comparison of analytical methods to measure angiotensin converting enzyme activity, enalaprilat, and benazeprilat.* J Pharm Sci, 1993. **82**(5): p. 471-4.

572. Aarons, L., et al., *Practical experience and issues in designing and performing population pharmacokinetic/pharmacodynamic studies.* Eur J Clin Pharmacol, 1996. **49**(4): p. 251-4.

573. Ette, E.I. and T.M. Ludden, *Population pharmacokinetic modeling: the importance of informative graphics.* Pharm Res, 1995. **12**(12): p. 1845-55.

574. Mandema, J.W., D. Verotta, and L.B. Sheiner, *Building population pharmacokinetic--pharmacodynamic models. I. Models for covariate effects.* J Pharmacokinet Biopharm, 1992. **20**(5): p. 511-28.

575. Mandema, J.W., D. Verotta, and L.B. Sheiner, *Building Population Pharmacokinetic-Pharmacodynamic Models*, in *Advanced Pharmacokinetic and Pharmacodynamic System Analysis*, D.Z. D'Argenio, Editor. 1995, Plenum Press: New York. p. 69-86.

576. FDA, *Guidance for Industry and Reviewers- estimating the safe starting dose in clinical trials for therapeutics in adult healthy volunteers.*, 2002, US Department of Health and Human Services, Food and Drug Adminstration.

577. Beal, S.L., L.B. Sheiner, and A.J. Boeckmann, *NONMEM Users Guides*. 1989 -2006, Ellicott City, MD: Icon Development Solutions.

578. Ette, E.I. and P.J. Williams, *Population pharmacokinetics II: estimation methods.* Ann Pharmacother, 2004. **38**(11): p. 1907-15.

579. Bonate, P.L., *Pharmacokinetic-Pharmacodynamic Modeling and Simulation*. 2nd ed. 2006, New York: Springer.

580. Meibohm, B., et al., *Population pharmacokinetic studies in pediatrics: issues in design and analysis.* AAPS J, 2005. **7**(2): p. E475-87.

581. Gitto, E., et al., *Oxidative stress of the newborn in the pre- and postnatal period and the clinical utility of melatonin.* J Pineal Res, 2009. **46**(2): p. 128-39.

582. Ursing, C., et al., *Does hepatic metabolism of melatonin affect the endogenous serum melatonin level in man?* J Endocrinol Invest, 2002. **25**(5): p. 459-62.

583. Schmidt, B., et al., *Long-term effects of caffeine therapy for apnea of prematurity.* N Engl J Med, 2007. **357**(19): p. 1893-902.

584. CDC. *U.S. Genome Variation Estimates. CYP1B1 Allele and Genotype Frequencies. Available polymorphism(s).* [cited 2012 27 September]; Available from: Available at: http://www.cdc.gov/genomics/population/genvar/frequencies/CYP1B1.htm.

585. MHRA. *Could Alternative Methodologies be applied to reduce time and costs of clinical development without compromising safety and efficacy?* 2006; Available from: http://www.mhra.gov.uk/home/groups/es-policy/documents/websiteresources/con2030479.pdf.

586. Greenland, S. and H. Morgenstern, *Confounding in health research.* Annu Rev Public Health, 2001. **22**: p. 189-212.

587. Counsell, S.J., et al., *Magnetic resonance imaging of preterm brain injury.* Arch Dis Child Fetal Neonatal Ed, 2003. **88**(4): p. F269-74.

588. Cornette, L.G., et al., *Magnetic resonance imaging of the infant brain: anatomical characteristics and clinical significance of punctate lesions.* Arch Dis Child Fetal Neonatal Ed, 2002. **86**(3): p. F171-7.

589. Boardman, J.P., et al., *Abnormal deep grey matter development following preterm birth detected using deformation-based morphometry.* Neuroimage, 2006. **32**(1): p. 70-8.

590. Inder, T.E., et al., *Abnormal cerebral structure is present at term in premature infants.* Pediatrics, 2005. **115**(2): p. 286-94.

591. Thompson, D.K., et al., *Perinatal risk factors altering regional brain structure in the preterm infant.* Brain, 2007. **130**(Pt 3): p. 667-77.

592. Valkama, A.M., et al., *Magnetic resonance imaging at term and neuromotor outcome in preterm infants.* Acta Paediatr, 2000. **89**(3): p. 348-55.

593. Woodward, L.J., et al., *Neonatal MRI to predict neurodevelopmental outcomes in preterm infants.* N Engl J Med, 2006. **355**(7): p. 685-94.

594. Mazumdar, A., et al., *Diffusion-weighted imaging of acute corticospinal tract injury preceding Wallerian degeneration in the maturing human brain.* AJNR. American journal of neuroradiology, 2003. **24**(6): p. 1057-66.

595. Neil, J.J. and T.E. Inder, *Detection of wallerian degeneration in a newborn by diffusion magnetic resonance imaging (MRI).* J Child Neurol, 2006. **21**(2): p. 115-8.

596. Dyet, L.E., et al., *Natural history of brain lesions in extremely preterm infants studied with serial magnetic resonance imaging from birth and neurodevelopmental assessment.* Pediatrics, 2006. **118**(2): p. 536-48.

597. Domizio, S., et al., *Excessively high magnetic resonance signal in preterm infants and neuropsychobehavioural follow-up at 2 years.* Int J Immunopathol Pharmacol, 2005. **18**(2): p. 365-75.

598. Miller, S.P., et al., *Early brain injury in premature newborns detected with magnetic resonance imaging is associated with adverse early neurodevelopmental outcome.* J Pediatr, 2005. **147**(5): p. 609-16.

599. Cooke, R.W. and L.J. Abernethy, *Cranial magnetic resonance imaging and school performance in very low birth weight infants in adolescence.* Arch Dis Child Fetal Neonatal Ed, 1999. **81**(2): p. F116-21.

600. Miller, S.P., et al., *Serial quantitative diffusion tensor MRI of the premature brain: development in newborns with and without injury.* J Magn Reson Imaging, 2002. **16**(6): p. 621-32.

601. Anjari, M., et al., *Diffusion tensor imaging with tract-based spatial statistics reveals local white matter abnormalities in preterm infants.* Neuroimage, 2007. **35**(3): p. 1021-7.

602. Counsell, S.J., et al., *Specific relations between neurodevelopmental abilities and white matter microstructure in children born preterm.* Brain : a journal of neurology, 2008. **131**(Pt 12): p. 3201-8.

603. Krishnan, M.L., et al., *Relationship between white matter apparent diffusion coefficients in preterm infants at term-equivalent age and developmental outcome at 2 years.* Pediatrics, 2007. **120**(3): p. e604-9.

604. Mori, S. and P.C. van Zijl, *Fiber tracking: principles and strategies - a technical review.* NMR Biomed, 2002. **15**(7-8): p. 468-80.

605. Rose, J., et al., *Neonatal microstructural development of the internal capsule on diffusion tensor imaging correlates with severity of gait and motor deficits.* Dev Med Child Neurol, 2007. **49**(10): p. 745-50.

606. Smith, S.M., et al., *Tract-based spatial statistics: voxelwise analysis of multi-subject diffusion data.* Neuroimage, 2006. **31**(4): p. 1487-505.

607. Smith, S.M., et al., *Acquisition and voxelwise analysis of multi-subject diffusion data with tract-based spatial statistics.* Nat Protoc, 2007. **2**(3): p. 499-503.

608. Ball, G., et al., *Testing the sensitivity of tract-based spatial statistics to simulated treatment effects in preterm neonates.* PLoS One, 2013. **8**(7): p. e67706.

609. Mathur, A.M., J.J. Neil, and T.E. Inder, *Understanding brain injury and neurodevelopmental disabilities in the preterm infant: the evolving role of advanced magnetic resonance imaging.* Semin Perinatol, 2010. **34**(1): p. 57-66.

610. Nosarti, C., et al., *Adolescents who were born very preterm have decreased brain volumes.* Brain, 2002. **125**(Pt 7): p. 1616-23.

611. Panigrahy, A., et al., *Volumetric brain differences in children with periventricular T2-signal hyperintensities: a grouping by gestational age at birth.* AJR Am J Roentgenol, 2001. **177**(3): p. 695-702.

612. Peterson, B.S., et al., *Regional brain volume abnormalities and long-term cognitive outcome in preterm infants.* JAMA, 2000. **284**(15): p. 1939-47.

613. Rathbone, R., et al., *Perinatal cortical growth and childhood neurocognitive abilities.* Neurology, 2011. **77**(16): p. 1510-7.

614. Rathbone, R., *New section on standard method performance requirements.* J AOAC Int, 2011. **94**(4): p. 45A.

615. Inder, T.E., et al., *Periventricular white matter injury in the premature infant is followed by reduced cerebral cortical gray matter volume at term.* Ann Neurol, 1999. **46**(5): p. 755-60.

616. Boardman, J.P., et al., *A common neonatal image phenotype predicts adverse neurodevelopmental outcome in children born preterm.* Neuroimage, 2010. **52**(2): p. 409-14.