Retinopathy of Prematurity and Multiple Postnatal Infections in Preterm Neonates: Delays in White Matter Development with Poorer Neurodevelopmental Outcomes.

by

Torin James Alexander Glass

A thesis submitted in conformity with the requirements for the Degree of Master of Science

Institute of Medical Science

University of Toronto

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Abstract

Preterm birth is a common cause of neurodevelopmental disorders in childhood. Little is known about the outcome of infants with severe retinopathy of prematurity (ROP) and multiple infections in the postnatal period. This thesis describes their associations with abnormal brain development using multi-modal MR imaging and standardized outcome assessments. Those infants with severe ROP had delayed brain maturation of the posterior white matter and optic radiations, with poorer 18 month cognitive and motor outcomes. Compared to fewer episodes of infection, three or more postnatal infections was associated with delayed maturation of the posterior limb of the internal capsule (PLIC), corpus callosum and the optic radiations, with poorer 36 month motor outcomes. Our findings support that severe ROP and multiple postnatal infections in very preterm newborns are associated with decreased brain maturation and poorer
neurodevelopmental outcomes, and that advancements in these disorders have the potential to improve outcomes.
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Contributions

The author was responsible for the writing and preparation of this original thesis. All of the work presented, including the planning, analysis and writing of the original research, was performed by the author, with the guidance and expertise of the individuals listed below. The following contributions to the work in this thesis are formally and inclusively acknowledged:

**Dr. Vann Chau:** Provided clinical assessments and data described in Chapters 2 – 3 as well as producing the DTI and MRSI database used in Chapters 2 and 3.

**Dr. Emma Duerden and Mr. Justin Foong:** Assisted in the development of the TBSS analysis method and provided the figures in Chapters 2 and 3.

**Dr. Jessie Guo:** Provided the brain segmentation volumes used in Chapter 3.

**Dr. Anne Synnes and Dr. Ruth Grunau:** Contributed to the overall planning and assessments for the follow-up and outcomes in Chapters 2 and 3.

**Dr. Jane Gardiner:** Provided the retinopathy of prematurity scoring in Chapters 2 and 3.

**Dr. Ken Poskitt:** Provided the MRI analysis and injury scoring in Chapters 2 and 3.

**Dr. Jillian Vinall:** Contributed DTI analysis to the study in Chapter 2.
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List of Abbreviations

ω3-PUFA – ω3 Polyunsaturated fatty acids
3D – Three-dimensional
ADHD – Attention-deficit hyperactivity disorder
BPD – Bronchopulmonary dysplasia
BSID-III – Bayley Scales of Infant and Toddler Development-III
CA – Corrected age
CHO – Glycerophosphocholine + phosphocholine
CI – Confidence interval
CNS – Central nervous system
CONS – Coagulase-negative staphylococcus aureus
CR – Creatine + phosphocreatine
CSF – Cerebrospinal fluid
DCD – Developmental coordination disorder
DHA - Docosahexaenoic acid
DNA – Deoxyribonucleic acid
DTI – Diffusion tensor imaging
DWI – Diffusion weighted imaging
EPO – Erythropoietin
FA – Fractional anisotropy
GA – Gestational age
GABA – Gamma-aminobutyric acid
GBS – Group B streptococcus
IGF-1 – Insulin-like growth factor 1
IL – Interleukin
INS – Myo-inositol
IQ – Intelligence quotient
IQR – Interquartile range
IVH – Intraventricular hemorrhage
LAC – Lactate
LMP – Last menstrual period
MD – Mean diffusivity
MEG - Magnetoencephalogram
MHz – Megahertz
MR – Magnetic resonance
MRI – Magnetic resonance imaging
MRSI – Magnetic resonance spectroscopic imaging
NAA – N-acetylaspartate + n-acetylaspartylglutamate
NEC – Necrotising enterocolitis
NICU – Neonatal intensive care unit
OCT – Optical coherence topography
OL – Oligodendrocyte
OR – Odds ratio
PDA – Patent ductus arteriosus
PDMS-2 – Peabody developmental motor scales 2
PLIC – Posterior limb of the internal capsule
PMA – Post-menstrual age
PPM – Parts per million
PRR – Protein recognition receptor
PVL – Periventricular leukomalacia
RF – Radiofrequency
RNFL – Retinal nerve fibre layer
ROI – Region of interest
ROP – Retinopathy of prematurity
ROS – Reactive oxygen species
RR – Risk ratio
SNAPPE-II – Score for neonatal acute physiology with perinatal extension-II
TBSS – Tract-based spatial statistics
TE – Echo time
TNF-α – Tumor necrosis factor alpha
TR – Repetition time
VEGF – Vascular endothelial growth factor
WMI – White matter injury
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Chapter 1

Literature Review
1.1 Introduction

Retinopathy of prematurity (ROP) and postnatal infection are important factors in children born preterm, which are often associated with poor neurodevelopmental outcomes. The pathophysiologies of ROP and infection share several characteristics with inflammation and oxygenation alterations likely leading to impairments of the developing brain. Infections, in particular postnatal bacteremia and fungal infections, are an independent risk factor in the development of severe ROP in extremely preterm infants (Manzoni et al, 2006; Tolsma et al, 2011). This association is possibly explained by the combined effects of systemic inflammation and hypoxia-ischemia in the susceptible preterm infant (Chen et al, 2011; Dammann, 2010), though the complete effects of each remains uncertain. Furthermore, infection has been shown to have effects on insulin-like growth factor 1 (IGF-1) and vascular endothelial growth factor (VEGF), growth factors known to have critical roles in the growth of normal and abnormal vascularization of the developing retina (Biswas et al, 2006; Heemskerk et al, 1999).

Structural MRI brain imaging of preterm infants has the potential to predict long-term outcomes, with abnormalities in motor pathway tracts shown to be associated with a higher likelihood of developing neurodevelopmental impairments (de Vries et al, 2011; Guo et al, 2017). Advanced MRI techniques, such as diffusion tensor imaging (DTI) to measure fractional anisotropy, have described delayed development of motor pathways in children with cerebral palsy (Thomas et al, 2005; Yoshida et al, 2010), further strengthening the role of MRI in predicting outcomes.
While much is known about postnatal infection and severe ROP, and their associated developmental outcomes, descriptions of the alterations in the brain of infants with these conditions are few. The research presented in this thesis examines the association of severe ROP and of multiple postnatal infections with brain development of preterm infants using multi-modal MRI methods, and describes the neurodevelopmental outcomes of children with these disorders.

1.2 Preterm Birth

1.2.1 Overview

Every year approximately 15 million infants world-wide are born preterm (before 37 completed weeks of gestation), with complications stemming from prematurity the number one cause of death in infants under 5 years of age (Blencowe et al, 2012). Surviving preterm infants have higher rates of cognitive dysfunction, motor impairments, attention-deficit hyperactivity disorder (ADHD) and autism than infants born at term (Bhatta et al, 2002; Johnson et al, 2010; Wood et al, 2000). This leads to an increased requirement for medical services resulting in a greater cost to the medical system than term-born infants (Petrou, 2005).

Infants born prematurely are roughly grouped into three categories based upon the completed weeks of gestation since the last menstrual period (LMP), approximated with antenatal ultrasound measurements, and referred to as their “gestational age” (GA). Infants born at <28 weeks GA are termed “extremely preterm”, 28 to <32 weeks GA as “very preterm”, and 32 to <37 weeks as “moderate to late preterm”. The GA at birth carries
a wealth of information about the potential risks of poor outcomes resulting from greater immaturity of the lungs, brain and other developmental systems. More medical interventions and an increased risk of their potential complications are consequences of a lower GA at birth (Leviton et al, 2005) with GA being more informative than birthweight for these risks.

Preterm birth often occurs as a result of several maternal (e.g. pre-eclampsia, diabetes), obstetric (e.g. short cervix, placenta previa, chorioamnionitis) and/or fetal (multiple gestations, genetic disorders) causes. Poorer outcomes in an infant are often linked to specific causes of preterm labour, with several perinatal factors associated with delivery, such as asphyxia, inflammation, fetal growth restriction and major birth defects all occurring more commonly in those infants with poorer outcomes (Delorme et al, 2016; Nelson and Blair, 2015).

There is significant epidemiological data describing the marked worldwide variations in outcomes of infants born preterm (Hossain et al, 2015; Shah et al, 2016). Current data supports the resuscitation of infants ≥23 weeks GA, considered by many experts in the field to be the limit of viability for the human infant. A “gray zone” still exists <23 weeks and <500 grams where infant survival to neonatal intensive care unit (NICU) discharge ranges between 0% and 50%, with wide variation between centres (Rysavy et al, 2015; Seri and Evans, 2008; Stoll et al, 2010). Neurodevelopmental outcomes of preterm-born infants have been shown to be significantly impaired compared to their peers born at term-age, with the greatest percentage of impairments seen within the extremely preterm group (Marlow et al, 2005; Stoll et al, 2010). However, a significant proportion of children born near the limits of viability at 23-24 weeks will perform within
the expected school norms at early school age, providing meaningful reassurance that resuscitation of these infants should be continued (Garfield et al, 2017; James et al, 2017).

In helping to understand the clinical factors that have the greatest impact upon the long-term development of the infant born preterm, large epidemiologic studies have been reported. One particular study of 5 year outcomes reported increased mortality and morbidity independently with each of brain injury, bronchopulmonary dysplasia (BPD) and retinopathy of prematurity (ROP) (Schmidt et al, 2015). They were also able to show that in those infants with a combination of morbidities the risk for poor outcomes increased significantly from 11% in those with none of the above morbidities to 62% in those with all three (Figure 1.1) (Schmidt et al, 2015). What causes such a large percentage of infants with these conditions to have poor outcomes remains an important area of research in the care of the preterm infant.
1.3 Brain injury in Preterm Infants

1.3.1 White Matter Injury

Severe white matter injury (WMI), known today as cystic periventricular leukomalacia (PVL), was first described by Banker and Laroche in 1962 based upon pathological specimens of infants who were found to have lesions consisting of necrosis and macrophage activity within the periventricular white matter (Banker and Laroche, 1962). The identified cause of PVL in the majority of these cases was severe anoxia, with...
WMI now seen as the major form of brain injury recognized in survivors of preterm birth, with the greatest risk to infants <32 weeks PMA (Volpe, 2009).

PVL is often caused by cerebral ischemia because of hemodynamic instability with hypoxia-ischemia or inflammation as a result of intrauterine or postnatal infections. During the development of the cerebral vascular bed in the preterm infant, vascular watershed regions develop. These areas are vulnerable to reductions in perfusion as they are furthest from the arterial supply and their blood and oxygen delivery supply is at the border of two vascular territories. In the preterm infant brain, the white matter surrounding the periventricular region is in a major watershed zone. Thus, PVL has historically been considered a form of watershed ischemic brain injury of the preterm infant.

During the initial phase of WMI the major cells thought to degenerate are the pre-oligodendrocyte (OL) lineage cells. Axonal injury can also occur and is a prominent feature of cystic WMI where necrosis is present, such as seen in classical cystic PVL. Though in a recent series, cystic PVL comprised only about 5% of the total burden of WMI (Haynes et al, 2008; Kinney and Back, 1998). Disturbances in the normal myelination pathway are initiated by the selective vulnerability of the late pre-OL in the most common form of WMI at 23 – 32 weeks GA (Back and Miller, 2014; Back et al, 2001).
Figure 1.2 Severity of WMI. MRI examples of (A) mild (B) moderate and (C) severe white matter injury on T1 1mm sagittal images of preterm infants. Scoring scale adapted from Miller et al (2005) *Journal of Pediatrics*. Arrows highlight the areas of T1 hyperintensity which are marked as areas of WMI.

Figure 1.3 WMI pathophysiology. Proposed pathogenic mechanisms with distinct differences in periventricular leukomalacia [PVL] (upper pathway) and diffuse WMI (lower pathway). More severe events of hypoxia-ischemia results in cystic PVL, with milder events resulting in selective pre-oligodendrocyte (OL) death and myelination failure.
The predominant form of WMI in the preterm infant is diffuse WMI, which is more commonly seen in those infants with necrotizing enterocolitis (NEC), infection, and hypoxia-ischemia, and which results in poorer outcomes than expected for a neonates' GA (Glass et al, 2008; Shah et al, 2008). Through hypoxia-ischemia and inflammation, glutamate-mediated injury occurs to the pre-OL cell leading to selective cell death and loss of maturation-dependent cellular processes (Khwaja and Volpe, 2008). It remains unknown the extent to which the individual and combined effects of hypoxia-ischemia or inflammation result in WMI, with white matter cellular injury described in animal models of both (Hagberg et al, 2002). There remains significant debate in the literature considering the effects of both inflammation and hypoxia-ischemia in WMI, with some experts suggesting it is predominantly a result of inflammation (Gilles et al, 2017) and others suggesting it’s predominantly from hypoxia-ischemia (Hagen et al, 2014). What seems most likely is that the developing cells are susceptible to the combined effects of both inflammation and hypoxia-ischemia, resulting in cellular injury (Back, 2006; Back and Rivkees, 2004; Khwaja and Volpe, 2008; Penn et al, 2016). In addition, inflammation can be induced by hypoxia-ischemia, resulting in a greater cellular impairment, while hypoxia-ischemia can occur from inflammation, such as from hypotension during an infection. The best description of these effects refers to the “upstream” mechanisms of hypoxia-ischemia and inflammation in activating brain microglia, followed by the “downstream” mechanisms of excitotoxicity with generation of free radicals resulting in the subsequent
death of the vulnerable pre-OL (Khwaja and Volpe, 2008). Regardless of cause, as we gain a greater understanding of the pathophysiology of WMI we will be better prepared to evaluate new strategies in its prevention and treatment.

The outcomes of children with classical PVL range from CP to milder motor impairments (Fazzi et al, 1994; Hamrick et al, 2004; Miller et al, 2000; Spittle et al, 2011). The outcomes of diffuse WMI are less robust in the literature with motor and cognitive impairments described (Woodward et al, 2006; Woodward et al, 2012). The outcomes of infants following diffuse WMI are best predicted by the location of the injury, with presence of frontal lobe WMI the strongest predictor of poor motor, language and cognitive outcomes (Guo et al, 2017). With the development of advanced MR techniques in the preterm infant we are gaining further information about white matter maturation (Hoon et al, 2009; Miller et al, 2002), connectivity (Smyser et al, 2013) and regional abnormalities (Pierson et al, 2007) seen in white matter injury of the preterm infant.

1.3.2 Intraventricular Hemorrhage

Intraventricular hemorrhage (IVH) is a condition in which there is hemorrhage in the ventricular system of the brain, or the sub ependymal zone immediately adjacent to it. IVH is especially common in preterm infants with a highest period of injury at <30 weeks due to the fragility of the germinal matrix at this time. The germinal matrix is a region of blood vessels immediately adjacent to the lateral ventricles which arises during fetal development and usually disappears before 35 weeks GA. When born prematurely, infants are at risk of rupturing the germinal matrix, particularly those infants requiring resuscitation with fluctuations in blood pressure (Ballabh, 2010). IVH is categorized into grades of severity with grade I considered mild, grade II moderate and grades III and IV
severe. IVH grades I and II have a low possibility of long-term damage as the blood does not cause excessive pressure or occlude the normal ventricular system. However, in grades III and IV there is frequently blood occluding the ventricular system resulting in a backflow of fluid, potentially irreversibly injuring the brain. Post-hemorrhagic ventricular dilatation often develops slowly in causing injury to the brain parenchyma, and while the optimal management of this condition remains uncertain, treatment with a CSF reservoir and/or ventricular-peritoneal shunt remains standard therapy. Improvements in antenatal care of the pregnancy at-risk for preterm birth has resulted in reduced rates of IVH for those infants exposed to antenatal steroids across all gestational ages (Wei et al, 2016), suggesting preventative therapies can effectively reduce brain injury in this population. The extent of the injury and the resultant neurodevelopmental impairments remains a function of the severity of the hemorrhage and the location of any parenchymal injury (McCrea and Ment, 2008). When accompanied by parenchymal injury there is a greater risk of cerebral palsy, low mental and motor scores and visual and hearing impairments (Vohr et al, 2003); though without associated parenchymal injury the risk of adverse outcomes is low (Han et al, 2002; Linsell et al, 2016; O'Shea et al, 1998).

1.3.3 Cerebellar Hemorrhage

The cerebellum is a compact and particularly important anatomical region involved in neurodevelopment of the preterm infant, encompassing more than 3.5 neurons for every neuron in the cerebrum (Herculano-Houzel, 2010). Its role in motor, language and cognitive development is evident. Injury to the cerebellum, such as a hemorrhage, is shown to result in significant adverse outcomes with greater rates of autism, motor impairments and an abnormal neurological examination (Tam et al, 2011; Tam et al,
2009; Ure et al, 2016; Wang et al, 2014). The size of cerebellar hemorrhage is an important predictor of outcomes, with small hemorrhages (<4mm on MRI) not seen to be associated with poorer outcomes (Steggerda et al, 2013). In addition, the location of the hemorrhage is also of importance, with injury to the vermis thought to confer a greater risk of impairments than injury to a hemisphere (Hashimoto et al, 1995). Delays in the cerebellar development with poorer neurodevelopmental outcomes are also seen following cerebellar hemorrhage (Lee et al, 2016; Messerschmidt et al, 2008). With the exploration and expansion of research into the cerebellum the medical community has learned that the cerebellum in the preterm infant is “rapidly developing, vulnerable and clinically important” (Volpe, 2009).

1.4 Retinopathy of Prematurity

1.4.1 Background

Retinopathy of prematurity (ROP) is a vascular proliferation disorder of the developing retina, and is the leading cause of visual impairment and blindness in infants born preterm (Rivera et al, 2011). First described in the late 1940s, ROP appeared suddenly in those infants who were the first to receive supplemental oxygen in closed incubators. Initially called “retrolental fibroplasia,” meaning “proliferation of fibrous tissue behind the lens,” ROP had severe consequences with retinal detachment leading to blindness in the most severe cases (Hellstrom et al, 2013). As the shift away from unrestrained oxygen therapy has been made in NICUs throughout the developed world,
there remains a delicate balance of oxygenation between lung maturity and survival with poorer visual outcomes (Hellstrom et al, 2013).

ROP severity is scored clinically by a pediatric ophthalmologist via a dilated eye examination using an indirect ophthalmoscope with examinations starting in those infants born <31 weeks GA. The first examination is normally planned for around 4 weeks following delivery. Two components are utilized in scoring, - the zone and the grade. The location of the abnormal vessel growth, referred to as the “zone”, is determined by proximity to the central vision and optic nerve. Zone 1 includes abnormal vessel growth in the central retina, whereas zone 3 is when abnormal vessel growth occurs in the peripheral retina (Figure 1.4).

Figure 1.4 Retinopathy of Prematurity (ROP) scoring scale. Showing zone borders and clock hours used to describe the location and extent of ROP. Figure originally published in Pediatrics (2006), reproduced with permission from the American Academy of Pediatrics.

The second component, the “stage,” ranges from 1 to 5, with stages 1 and 2 considered mild, and stages 3 to 5 considered severe, on which treatment is usually
provided according to the Early Treatment for ROP trials (Early treatment of Retinopathy of prematurity cooperative group, 2003) (Figure 1.5).

Figure 1.5 Stages of Retinopathy of Prematurity. Stage 1 is characterized by a thin demarcation line between non-vascularized and vascularized retina, stage 2 by a ridge, stage 3 by extraretinal fibrovascular proliferation, stage 4 by part retinal detachment, and stage 5 by total retinal detachment. Figure originally published by Hellstrom et al (2013) in The Lancet; reproduced with permission from Elsevier.

The current mainstay of treatment is with laser photocoagulation therapy to stop the growth of the abnormal vessels and to prevent retinal detachment. Other approved therapies include the intravitreal injection of anti-VEGF agent Bevacizumab (Trade name Avastin) (Gunther and Altaweel, 2009) and cryotherapy in which reginal retinal destruction was performed through freezing of the area (Pearce et al, 1998). Other therapies of interest include scleral buckling, which is aimed to treat retinal detachments, propranolol, to reduce the progression of ROP, and recombinant humanized IGF-1 to prevent
excessive vessel formation through reducing VEGF (Hellstrom et al, 2003; Hellstrom et al, 2013). The neurodevelopmental outcomes following treatment for severe ROP are still not understood.

1.4.2 Pathophysiology

During embryogenesis, the retina, ciliary body, iris and optic nerves arise from the diencephalon, which goes on to form the caudal forebrain; thus the retina develops as part of the central nervous system (CNS). The retina includes layers of cells with three neural cells including the photoreceptor cells, bipolar cells and ganglion cells, and a fourth layer of pigmented epithelial cells. These layers are complete in their development by around 16 weeks PMA, though are not vascularized. Normal retinal vascularization continues until term age through the balance of growth factors of insulin-like growth factor (IGF-1), vascular endothelial growth factor (VEGF), erythropoietin (EPO) and ω3 polyunsaturated fatty acids (ω3 PUFA), among others. Following preterm birth, normal vascularization is inhibited and normal vascularization may continue if the optimal environment for retinal development is achieved. However, should there be a relative imbalance of growth factors with increasing metabolism, hyperoxia or hypoxia, the potential for abnormal retinal neovascularization occurs (Hellstrom et al, 2013).

Of particular importance to reducing the development of ROP is the prevention of hyperoxia with reduced oxygen supplementation in the care of the preterm infant. Because of improved NICU care and practices, ROP is relatively uncommon in those infants born >30 weeks PMA, though a significant proportion of infants <28 weeks will have at least some retinal vasoproliferation. Furthermore, infants who are small for gestational age, and those with hyperglycemia, hyperinsulinemia, and/or postnatal
infections, are at greater risk for ROP and are more likely to require treatment to preserve vision (Hellstrom et al, 2013).

Figure 1.6: Retinopathy of Prematurity Pathophysiology. (A) In-utero: normal vascular growth with low oxygen tension. (B) Phase 1: retinal vascularization is inhibited by hyperoxia and loss of growth-factors from the placenta. Blood-vessel growth stops and with retinal maturation hypoxia results. (C) Phase 2: hypoxic retina stimulates expression of oxygen-regulated factors such as erythropoietin (EPO) and vascular endothelial growth factor (VEGF) which in turn stimulate retinal neovascularization with Insulin-like growth factor 1 (IGF-1). (D) Resolution: retinopathy may be prevented or treated with prevention of phase 1 or inhibition of phase 2 with laser therapy or an antibody. ω3 PUFA= ω3 polyunsaturated fatty acids. Figure originally published by Hellstrom et al (2013) in The Lancet; reproduced with permission from Elsevier.

Low IGF-1 has a strong association with later ROP due to poor retinal vascular growth. This is likely due to its impacts upon VEGF, which is an important factor in normal vascularization (Hellstrom et al, 2013) (Figure 1.6). IGF-1 is also an important growth
factor for the fetus in-utero and remains an important regulator of glucose metabolism in postnatal development (Cheng et al, 2000). Low IGF-1 concentrations in the early postnatal period in babies born preterm correlates strongly with later ROP and other comorbidities, including a slower growth rate of brain volume, a marker of cerebral development (Hansen-Pupp et al, 2011; Hellstrom et al, 2003). Low IGF-1 concentrations are nutrition-dependent markers and can be reduced following starvation, infection and stress (Demendi et al, 2012). In addition, hyperglycemia within neonates, absence of ω3 PUFAs in diet and low levels of EPO are identified as other potential risks for increased ROP (Hellstrom et al, 2013).

1.4.3 Outcomes of Infants with ROP

In assessing the neurodevelopmental outcomes of children with ROP, Schmidt et al reported an odds ratio of 4 for death or disability at 5 years, greater than that of brain injury or BPD (Schmidt et al, 2015) with a 3 to 4 times greater risk for non-visual disabilities than those without ROP (Msall et al, 2000; Schmidt et al, 2014). While vision abnormalities are common in preterm infants (O'Connor et al, 2002), visual outcomes have improved in the CRYO-ROP (Cryotherapy for Retinopathy of Prematurity Group, 1996), early treatment for ROP (Early Treatment For Retinopathy Of Prematurity Cooperative, 2003) and Avastin trials (Martinez-Castellanos et al, 2013; Mintz-Hittner et al, 2011). Common visual disturbances seen in long-term follow-up of children with ROP include strabismus, amblyopia and refraction abnormalities (O'Connor et al, 2002). The impacts of these visual disturbances are thought to be minor, though when severe, early visual-motor coordination and vestibular system dysregulation can effect developmental mechanisms (Goyen et al, 2006; Prechtl et al, 2001). The association between severe
ROP and brain maturation and long-term neurodevelopmental outcomes remains an area in which little is known.

1.5 Infection in Preterm infants

1.5.1 Background

In many large epidemiological studies of preterm infants worldwide, infection has been determined to be a strong predictor of poorer outcomes compared to non-infected infants (Kiechl-Kohlendorfer et al, 2009; Mitha et al, 2013; Rand et al, 2016; Schlapbach et al, 2011; Stoll et al, 2004; Van der Ree et al, 2011). Rates of infection in preterm infants range between 20 – 65% within industrialized countries, with even greater rates reported in developing countries, where mortality rates are also significantly higher (Adams-Chapman and Stoll, 2006; Orsi et al, 2009; Stoll et al, 2004; Zaidi et al, 2005). It is for these reasons that infection remains an important determinant of childhood outcomes and prevention strategies remain a key initiative of the World Health Organization.

1.5.2 Classification

Traditionally, neonatal infections are divided into two categories based upon the timing of the infection in relation to birth; [1] early-onset infection, classified as presenting within the first 72 hours of life, and [2] late-onset infection, presenting past 72 hours of life. This division has been utilized to assist in the differentiation of the early-onset infections, which are thought to be a result of maternal risk factors or transmission and acquisition of the infection via the birth canal, in comparison to late-onset infections which
are more often related to acquisition in the hospital environment. It is because of these factors that they are also referred to as “pre-natal” and “postnatal” infections.

### 1.5.3 Pre-Natal Infection

Infections occurring in the first 72 hours of life appear to have a different pathophysiology than that of postnatal infections, evident by transmission via the maternal bloodstream or via direct exposure to the organism ascending to within the uterus or from delivery through the vaginal canal. Chorioamnionitis, the most common pre-natal infection, involves infection of the fetal membranes, placenta or amniotic fluid, and is commonly diagnosed in the third trimester with associated maternal fever, leukocytosis, uterine tenderness and foul-smelling amniotic fluid with fetal tachycardia. Current treatment of chorioamnionitis consists of appropriate intravenous antibiotic treatment early in the maternal illness and may include postnatal antibiotics following delivery. Pre-natal infection is a known cause for stillbirth as well as a common cause of preterm delivery (Goldenberg et al, 2008; McClure et al, 2010). Infants delivered following a pre-natal infection have a higher mortality rate and are commonly infected with organisms such as *Escherichia coli* and group B *Streptococcus* (GBS), which are less commonly seen in postnatal infections (Jiang et al, 2004; Stoll et al, 2005).

The literature with regards to whether pre-natal infection confers a greater risk of brain injury and poorer outcomes remains unclear with some studies reporting increased rates of cerebral palsy and cystic PVL (Dammann et al, 2002; Dammann and Leviton, 1998; Grether and Nelson, 1997; Mitha et al, 2013), while other studies report no significant difference in WMI, brain maturation or poorer outcomes (Chau et al, 2009; Grether et al, 2003). While the neurodevelopmental outcomes resulting from pre-natal
infection alone remain unclear, there is research supporting that the combined influence of pre-natal and postnatal infections together may confer a stronger risk for cerebral injury and cerebral palsy (Mitha et al, 2013; Yanni et al, 2017).

1.5.4 Postnatal Infection

Infections occurring after 72 hours of life are more common than pre-natal infections and have a far greater association with poorer outcomes, with a higher percentage of infected infants developing motor and/or cognitive impairments in follow-up compared to uninfected infants (Jiang et al, 2004; Stoll et al, 2004). Studies reporting long-term outcomes at school age continue to support the association of infection with greater frequency of cognitive and motor dysfunction, as well as ADHD and other mental health impairments (Mitha et al, 2013; Rand et al, 2016).

Published research of clinical cohorts exploring postnatal infection have investigated the different types of infections separately, with the hypothesis that sepsis and meningitis would be more likely to result in poorer outcomes. However, among all infants with postnatal infections there is little difference between those with “clinical infection alone” and those with sepsis and/or meningitis (Stoll et al, 2004). The same has been shown for necrotizing enterocolitis (NEC), thought to convey an increased inflammatory response, resulting in a greater risk of WMI and motor impairments (Shah et al, 2008; Stoll et al, 2004). In multiple cohorts of preterm infants, postnatal infection was associated with an increased risk of WMI, as well as worsened WMI seen on subsequent MRIs, highlighting it as an important risk factor for brain injury in the preterm newborn (Chau et al, 2009; Glass et al, 2008). In addition to increases in WMI, infants with postnatal infection have delayed brain development using measures of white matter
maturation with increased average diffusivity, decreased white matter fractional anisotropy and lower MRSI metrics of brain maturation (Chau et al, 2012). The link between multiple postnatal infections and development of the preterm brain has not been thoroughly investigated and remains an area of interest in the field.

Common organisms involved in postnatal infection include gram-negative and gram-positive bacteria, as well as fungal species, with coagulase-negative staphylococcus (CONS) species the most common (Orsi et al, 2009). Other organisms such as Klebsiella pneumoniae, Escherichia coli, Enterococcus species, Enterobacter species and Candida species are less commonly seen (Orsi et al, 2009). Among the organisms implicated in postnatal infections, it has been shown that infants are at risk of poorer outcomes as a result of any infectious organism (Stoll et al, 2004), suggesting that the impacts on the brain are not specific to one organism, but rather related to the inflammatory response that occurs during the infection and the impacts on the hemodynamic circuitry and immune system of the body.

Implementing tighter infection control measures within NICUs, such as increased hand hygiene by health care workers, reduces infection rates, particularly of hospital-acquired infections (Schelonka et al, 2006; Won et al, 2004). The implementation of improved infection control measures can result in reductions in the length of hospital stay and of other co-morbidities, and is even associated with improved outcomes at 2 years in one study (Davis et al, 2016). However, it is not fully understood whether these procedures, and other more costly measures, need to be put in place for an entire NICU unit, or whether the most vulnerable infants would benefit better through an individualized approach. Additionally, there is no testing of these personalized or “precision” infection
control measures specifically targeted at those infants at greatest risk of adverse outcomes.

### 1.5.5 Neonatal Inflammation

When an infectious pathogen makes contact with a mature host’s immune system, the innate immune system identifies and eliminates the invading pathogen through the activation of pattern recognition receptors (PRRs). Within the preterm infant, however, the innate immune system is not well developed, with a deficiency of PRRs, which results in an unbalanced and potentially harmful inflammatory response (Kan et al, 2016; Van der Poll et al, 2017). This heightened vulnerability of the preterm infant brain to infection was first illustrated and described by Gilles in 1976 when a bacterial lipopolysaccharide, injected systemically into newborn kittens, caused a leukoencephalopathy, though it resulted in no effect in the mature cat (Gilles et al, 1976). The activation of toll-like receptors within the human epithelium of the bloodstream, known to be activated by bacterial lipopolysaccharides, is believed to be one mechanism involved in the activation of the local host response on the blood brain barrier (Strunk et al, 2014). This results in the production of various inflammatory mediators, with cytokines, prostaglandins and reactive oxygen species (ROS) released into the CNS causing direct cytotoxicity on the brain; and may occur even without direct bacterial invasion into the CNS, such as in meningitis (Strunk et al, 2014).

Increased levels of cytokines tumor necrosis factor (TNF-α) and interleukins (IL-1β, IL-6, IL-8, IL-10, IL-12, IL-17 and IL-18) have been investigated in the fetal and neonatal responses to infection, with some shown to have a pro-inflammatory role within the developing immune system (Dammann and Leviton, 1997; Leviton et al, 2005; Van...
der Poll et al, 2017). In those infants with higher levels of these pro-inflammatory markers, cerebral injury is more commonly seen, with greater extent of white matter injury and PVL (Duggan et al, 2001; Viscardi et al, 2004). In animal models of fulminant infection, blocking some of these cytokines has been shown to result in increased survival and has been suggested as a potential therapeutic target against the hyper-immunity in sepsis (Flierl et al, 2008).

The impact of multiple inflammatory events has been investigated in what is referred to as the “two-hit” model, in which an initial inflammation sensitizes the vulnerable immune system to a subsequent inflammatory event. The second inflammation event is thought to have a threshold stimulus lower than is required for the initial event and results in the release of excitotoxic inflammatory markers, leading to cerebral injury and potential epigenetic alterations (Fleiss and Gressens, 2012; Fleiss et al, 2015; Yanni et al, 2017). Both pre-natal and postnatal stimuli have been shown to sensitize the immune system with increased levels of pro-inflammatory cytokines (Yanni et al, 2017). Pre-natal infections, and in particular chorioamnionitis, are often associated with a fetal inflammatory response in which pro-inflammatory markers are increased and thought to act as an initial trigger (Wang et al, 2007), with some studies reporting a greater incidence of WMI and cerebral palsy as a result (Leviton et al, 2010; Yanni et al, 2017). Postnatal events, such as prolonged mechanical ventilation, NEC and postnatal infection, often trigger similar inflammatory responses to those seen in pre-natal infections, resulting in a significant release of pro-inflammatory markers (Aden et al, 2010; Hagberg et al, 2015; Volpe, 2008). Furthermore, it has been shown that exposure of the developing brain to multiple inflammatory events leads to excitotoxicity, with greater mitochondrial
impairment and weakened vascular integrity potentially causing direct brain injury or interfering with normal CNS development (Boisse et al, 2004; Fleiss et al, 2015; Wang et al, 2009; Yanni et al, 2017), and potentially altering the epigenome (Fleiss and Gressens, 2012). Multiple inflammation events have the potential to result in an altered immune response leading to a chronic inflammatory condition.

1.5.6 Neonatal Hypoxic-Ischemia Injury

The preterm infant brain is particularly susceptible to the effects of hypoxia-ischemia as reflected in the development of white matter injury and PVL. The susceptibility of the OL progenitor cell is a maturation-dependent phenomenon, which is represented by a greater resistance of the mature OL cells to the effects of oxidative stress than the immature pre-OL cell (Back et al, 1998). What results is a critical period of vulnerability for the preterm infant during which irreversible injury can occur to the OL cell, resulting in myelination dysfunction. The over-expression of the immune system during this period may contribute to the cerebral injury as a consequence of the pressure-passive and poor auto-regulatory system of the preterm infant (du Plessis, 2009; Khwaja and Volpe, 2008). We know that cardiovascular collapse commonly occurs during an acute infection (Healy et al, 2004), which when during a vulnerable period, may potentiate the hypoxic-ischemic injury (Khwaja and Volpe, 2008). Animal models have shown the combined effects of hypoxia-ischemia insults and infection can worsen neuronal injury, with particular periods in which there is greater susceptibility to injury (Eklind et al, 2005; Larouche et al, 2005).

There are various mechanisms which make the preterm infant brain vulnerable to the effects of hypoxia (Gopagondanahalli et al, 2016). Firstly, the preterm brain receives
a relatively reduced global and regional supply of blood compared to the term infant brain, which may result in a limited margin for reduced supply (Altman et al, 1988; Pichler et al, 2014). Additionally, arterial extension into the developing brain is incomplete resulting in poorly vascularized end-zone watershed regions, particularly of the periventricular white matter regions (Altman et al, 1988; Inage et al, 2000). Include these factors with the poor cardiac output commonly seen in the preterm infants (Kluckow and Evans, 2000), and the poor correlation between blood pressure monitors and cerebral blood flow (Weindling and Kissack, 2001), and it becomes clear that poor cerebral blood flow is likely underestimated in the clinical care of the preterm infant.

The pressure passive blood pressure system of the preterm infant brain is vulnerable to alterations in blood pressure, in which the greatest fluctuations are seen in the smallest infants (Soul et al, 2007). The regulation of blood pressure in the preterm infant has a poor reserve for fluctuations and is dysfunctional in response to hypotension (Munro et al, 2004). Treatment with inotropes and fluids, and other appropriate therapies for low blood pressure, can improve cerebral blood flow, reducing cerebral ischemia (Munro et al, 2004). Poor cerebral autoregulation is associated with a higher frequency of IVH and WMI, thus preserving a stable mean arterial blood pressure is paramount in reducing injury to the developing brain (O'Leary et al, 2009; Vesoulis and Mathur, 2017).

Further potentiating the blood pressure fluctuations in the brain are the effects of hyperoxygenation and hypocarbia, resulting in vasoconstriction of the blood vessel wall, with greater amounts of brain injury (Fujimoto et al, 1994; Khwaja and Volpe, 2008). Hypocarbia is also a strong factor in the development of severe ROP, with changes in the
oxygen targets and more awareness of hyperoxigenation and hypocarbia helping to reduce the severity and incidence of ROP (Sears et al, 2009).

1.6 Magnetic Resonance Imaging

1.6.1 Imaging the Preterm newborn

Brain imaging in the very preterm newborn has developed within the last two decades to be the gold standard assessment for brain injury, and assists in identification of those infants at high-risk for adverse outcomes in infancy and childhood (Anderson et al, 2017; Woodward et al, 2006). Magnetic resonance imaging (MRI) of the preterm newborn, in many instances without the need for sedation, is a safe and reliable technique with a greater sensitivity for white matter abnormalities than cranial ultrasound (Inder et al, 2003; Miller et al, 2003; Plaisier et al, 2012). Following the use of routine term-equivalent MRI in some centres, 25-33% of infants were found to have brain abnormalities, many of which were not detected with conventional cranial ultrasound (Kidokoro et al, 2014; Neubauer et al, 2017). The timing of the MRI is an important consideration in detecting injury in the preterm infant as WMI is more easily seen in the preterm period, and may be undetectable on subsequent imaging at term-equivalent age (Kersbergen et al, 2014; Martinez-Biarge et al, 2016). As a result of this, it is recommended that serial imaging of the preterm brain be done as this improves the sensitivity for WMI, providing a more definite prediction of childhood outcomes (Martinez-Biarge et al, 2016; Sarkar et al, 2015). However, MRI does have its limitations with the current clinical scanners unable to visualize microscopic WMI, the least severe form of
WMI, which remains a neuropathological diagnosis, and suggests that MRI is liable to underestimating the full extent of WMI (Back et al, 2012; Volpe, 2017). With the addition of advanced MR techniques, these milder injuries and delays in development may be more readily assessed allowing a further understanding of the long-term effects of prenatal and postnatal injuries (de Vries et al, 2011; Guo et al, 2017; Kwon et al, 2014; Rutherford et al, 2005; Thomas et al, 2005).

1.6.2 Overview of MRI Basics

MRI utilizes the nuclear magnetic resonance properties particularly of hydrogen protons, which when combined with radiofrequency (RF) waves, results in energy release which can be detected, interpreted and transformed into images. The images are formed as a result of the physical properties of the molecules and their response to the magnetic field. When first placed within a strong magnetic field, such as that provided by the main $B_0$ field, the hydrogen protons within the body polarize. The RF pulse is then used to kick spins off their axes, which results in excitation when applied at the correct resonance frequency, flipping the natural spins of the hydrogen atoms into the anti-parallel state. This is known as the “Larmor Frequency” and it depends on the field strength of the magnet (42.6 MHz/Tesla). As the spins return, or relax, from the high energy anti-parallel state back to their equilibrium they release energy in the form of an oscillation magnetic field at the Larmor frequency.

For visualization, the vector representation is used such that when excited by the RF pulse, the net storage of energy is represented by the tipping of the vector onto its side, away from the z-axis and into the x-y planes, the amount referred to as the ‘Flip angle’. Following this, the vector begins to precess around the z-axis slowly returning to
equilibrium through the relaxation phase. The relaxation of the protons can be divided into two processes: T1 and T2 relaxation, described below.

1.6.3 MRI sequences

T1 relaxation, also referred to as spin-lattice relaxation, refers to the exponential recovery of the protons to the z-axis. T2 relaxation, also known as spin-spin relaxation, refers to the loss of phase coherence among excited protons that leads to exponential decay of the component of the magnetization in the transverse plane. Variations in the relaxation rates of T1 and T2 signal occurs as a result of the diverse concentrations of molecules in the different tissues within the brain. Fat, which has tighter bonds to surrounding structures than water, releases energy quickly; while water results in a slower relaxation, resulting in water having a higher T1 and higher T2 signal than fat.

The signal received by the receiver coils during the relaxation phase, is acquired in a temporary image space, also referred to as ‘k-space’, in which the digitized MR signals are stored during data acquisition. The images are then produced using the mathematical conversion known as the “Fourier transform”. The k-space is expressed as a summation of overlapping sinusoids that are used to produce an anatomical image.

The repetition time (TR) and echo time (TE) play key roles in the contrast of a MR image due to the variations in the recovery and relaxation times of different tissues. The TR refers to the time, in milliseconds, between successive RF excitation pulses, with the TE reflecting the time, also in milliseconds, from the application of the RF excitation pulse and the peak signal induced by the coil. T1-weighted sequences maximize T1 contrast.
by utilizing a short TR and short TE, increasing tissue contract. T2-weighted sequences use a longer TE and long TR, minimizing the T1 contrast.

Two-dimensional multi-slice imaging is produced by sequentially exciting and collecting data from one slice region at a time. The thickness of the tissue excited in a single image slice of the MR image is determined by changes in the RF pulse and/or the gradient strength. These images are then stacked together to produce a combined 3D image of the brain. Three-dimensional (3D) volumetric imaging is produced by exciting a thick slab of tissue, which is then spatially encoded by traversing the 3D k-space, reconstructed in 3D and resliced for image review.

1.6.4 Diffusion Weighted Imaging and Diffusion Tensor Imaging

Diffusion weighted imaging (DWI) is a MR technique that measures free motion of water in tissue, based upon measuring the random Brownian motion of water molecules within a voxel of tissue. Unlike the free diffusion, or movement, of water inside a container, diffusion of water inside a voxel of the brain is hindered by cellular membrane boundaries in both the intracellular and extracellular compartments. Using these properties, DWI can be utilized in assessing micro-structural architecture and is sensitive to cell changes as a result of ischemia and those seen in highly cellular tumours. Images are produced by adding extra gradients to standard MR imaging sequences, such as a T2-weighted echo planar sequence, which sensitizes the sequences to molecular diffusion. The diffusion gradients are equal in magnitude and centered on a 180-degree refocusing radiofrequency pulse. DWI images are produced as static hydrogen protons are unaffected by the diffusion gradient and will retain their signal, whereas water molecules
moving in the axis of the gradient will accumulate phase by the first gradient, but not the second and hence will lose their signal.

Diffusion tensor imaging (DTI) is another quantitative MR method that uses the same properties of diffusion of water molecules in conventional DWI imaging, though does so using a Gaussian model of diffusion. The Gaussian distribution is defined by a 3x3 symmetric, positive definite matrix, with 3 orthogonal (mutually perpendicular) eigenvectors and 3 positive eigenvalues in each voxel. The direction of fastest diffusion, the principle eigenvector ($\lambda_1$), is referred to as axial diffusivity, with the other eigenvectors ($\lambda_2$ and $\lambda_3$) termed radial diffusivity. These eigenvectors, in combination with the 3 eigenvalues define an ellipsoid reflecting the diffusion probability within each voxel (Figure 1.7).

Figure 1.7 Representation of the 3D diffusion ellipsoid. Presented as an ellipsoid with three unit eigenvectors ($\varepsilon_1$, $\varepsilon_2$, and $\varepsilon_3$), with corresponding lengths ($\lambda_1$, $\lambda_2$, and $\lambda_3$), the eigenvalues.

DTI characterizes the 3D spatial distribution of water diffusion in each voxel of the MR image, providing an indirect measure of microstructure (Beaulieu, 2002; Miller et al,
Within each voxel it is possible to infer the axial diffusivity ($\lambda_1$), the radial diffusivity ($\lambda_2$ and $\lambda_3$), and mean diffusivity (MD), a measure of the average size of diffusion in each voxel, where $D_{xx}$, $D_{yy}$ and $D_{zz}$ are the diagonal terms of the diffusion tensor:

$$MD = \frac{\lambda_1 + \lambda_2 + \lambda_3}{3} = \frac{D_{xx} + D_{yy} + D_{zz}}{3} = \text{Trace}$$

Molecules diffuse differently within tissues depending on the type, integrity, architecture and the presence of barriers generating a quantitative anisotropy. Anisotropy refers to the diffusion of water which may be unrestricted in all directions, such as within CSF, termed “isotropic”, or restricted to a single plane, such as in the white matter pathways of the mature brain, characterized as “anisotropic” (Soares et al, 2013). Fractional anisotropy (FA), the normalized measure of the fraction of the tensor's magnitude due to anisotropic diffusion, corresponds to the degree of directionality and ranges from 0 (isotropic diffusion) to 1 (anisotropic diffusion), averaged between bilateral brain regions in mean FA analyses (Soares et al, 2013). FA increases with white matter maturation, and is thought to reflect the maturation of the oligodendrocyte lineage and early events of myelination within the preterm brain (Drobyshevsky et al, 2005; Miller et al, 2002). Using this technology, DTI can be displayed by condensing the tensor information into one number, referred to as a “scalar”. The tensor can also be represented using glyphs, a small 3D representation of the major eigenvector or whole tensor, or presented as 4 numbers in producing colour-coded FA map. The colour FA map is
produced with each colour representing the primary diffusion direction within each voxel of the image (Figure 1.8) (Soares et al, 2013).

![DTI image](image)

Figure 1.8 DTI colour map. DTI map of a preterm infant brain at 32 weeks PMA in the axial plane at the level of the superior white matter (left) and basal ganglia (right). Colour coding is reflected by red colour representing the left-to-right orientation within the image, green the posterior-to-anterior and blue the inferior-to-superior axis of diffusion.

Another way of viewing the DTI image includes estimations of the course of the white matter tracts through the brain, termed tractography. The most common approach, streamline tractography, involves following the path of successive principle eigenvectors from a given voxel origin, and is a form of “deterministic” tractography. “Probabilistic” tractography, another widely utilised method, relies upon connection probabilities between voxels and is more reliable at reconstructing crossing fibers than deterministic...
tractography (O'Donnell and Westin, 2011). Despite significant promise with tractography, both methods have their limitations and challenges with interpretation, in part as a result of the complexity of the crossing fibres within the brain, but also as a result of the large numbers of potential false positive or negative tracts that can result from the analysis.

Tract-based spatial statistics (TBSS; http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/TBSS), a semi-automated and 3D assessment of FA, provides a less user-dependent measure of FA than complementary ROI-based analyses. TBSS utilizes “voxel-based morphometry,” in which each subject’s FA image is registered to a standard space, following which voxelwise statistics are applied to find areas that correlate to the covariate of interest. Through TBSS, FA data can be projected onto a group mean FA tract skeleton which can be used to display the voxelwise cross-subject statistics as an assessment of white matter integrity differences (Smith et al, 2006). The normalization of each image to a standard space is a crucial step to the TBSS model, but can lead to misalignment and issues with the normalization of the data. This is especially true for the preterm infant brain which is undergoing rapid changes in size and shape within the normal fetal third trimester. The FA images are aligned using voxelwise nonlinear registration, which is driven by the FA images. Secondly, the mean of the aligned FA image is utilised to produce a skeletonised mean FA image. Following that, each subjects’ aligned FA image is projected on the skeleton filling the skeleton with FA values, on which cross-subject voxelwise statistics can be performed. In producing voxelwise statistical models with a correction for multiple comparisons, a Gaussian random field theory thresholding approach is often applied for analysis (Smith et al, 2006). If the spatial width of this Gaussian filter is not chosen appropriately then the signal-to-noise ratio will be reduced resulting in poorer power of
the model and more imperfections. Cluster-size thresholding, a permutation-based approach to thresholding, can help to reduce this without the use of a Gaussian filter, while still allowing the appropriate statistical test. By using a cluster size determined by 500 permutations of the cluster size using Randomise v.2.9 within the functional MRI of the Brain Software Library (http://sel.fmrib.ox.ac.uk/fsl/fslwiki/Randomise) the familywise error rate is lessened while maintaining the ability to search the entire FA skeleton for regions of significant difference (Smith et al, 2006). In showing cross-subject voxelwise significance, a threshold of P <0.05, equivalent to the 95th percentile of the distribution, is used for the clusters, and corrected for multiple comparisons across space. Using these techniques, a preterm infant model for assessing TBSS with the white matter skeleton separated into four categories based upon GA at scan was developed by Duerden et al (Duerden et al, 2015) (Figure 1.9).

Figure 1.9 Tract-based spatial statistic (TBSS) model. Age-specific models for preterm infants are shown here. Green regions reflect the white and grey matter skeleton template for the respective age group overlaid upon an axial T1-image.
1.6.5 Magnetic Resonance Spectroscopic Imaging

Proton magnetic resonance spectroscopic imaging (MRSI) uses the signals produced by the different metabolite molecules within the voxel to produce spectra of individual peaks. These peaks are based upon the concentrations of metabolites, represented by parts per million (ppm) on the spectroscopic map. The principle behind MRSI is that the distribution of electrons around hydrogen protons in the different metabolites results in slight alterations to the magnetic field. Because these metabolites have molecular concentrations 10,000 times lower than water, the signal produced is much lower, thus larger voxels are required and the water signal has to be suppressed (Posse et al, 2012). The most commonly used MRSI method involves using conventional phase-encoded imaging in which the phase encoding gradient amplitude is incremented once per TR. Phase encoding modulates the signal phase and amplitude of the MR signal before detection of the signal frequency, which ensures that subsequent phase and amplitude modification due to the chemical shift is independent of spatial encoding (Posse et al, 2012). Suppression of the water signal is then achieved by using either a chemical shift selective or inversion recovery technique, which when the Fourier transform is applied, separates the signal into individual frequencies. The greater the magnetic field strength, the greater the signal-to-noise ratio, which allows smaller voxels to be used in producing the metabolite spectra. Another technique increasingly used is echo-planar MRSI, which has strong localization performance and large volume coverage with improved spatial and temporal resolution (Posse et al, 2012). This process utilizes a zigzag trajectory in k-t-space creating a series of gradient echoes that are modulated by
the chemical shift evolution and relaxation. Through reformatting, the data format is equivalent to conventional phase-encoded MRSI.

As with conventional MR, the TE is an important factor in the information obtained. With a short TE of 30 msec, metabolites with both short and long T2 relaxation times are observed (Brandao and Domiques, 2003). A longer TE of 270 msec results in only metabolites with a long T2 to be seen. An intermediate length TE of 144 msec is often used as it allows the imaging of lactate as an inverted doublet at 1.3 ppm. MRSI metabolites of importance in the brain include N-acetyl-aspartate + N-acetylaspartylglutamate (NAA) at 2.0 ppm, lactate (LAC) at 1.3 ppm, glycerophosphocholine + phosphocholine (CHO) at 3.2 ppm, creatine + phosphocreatine (CR) at 3.0 ppm and myo-Inositol (INS) at 3.5 ppm (Card et al, 2013) (Figure 1.10). A long TE spectra at 270 msec produces a spectra of primarily NAA, CR and CHO, while the other metabolites are best seen with a short TE with a high signal to noise ratio. Other metabolites of interest include glutamate/glutamine and gamma-aminobutyric acid (GABA) [all at 2.2 – 2.4 ppm], which have been shown to correlate with white matter injury (Wisnowski et al, 2013). NAA is a neuronal marker, present in high concentrations in the brain, and synthesized in neurons (Moffett et al, 2007). CHO is a measure of increased cellular turnover or membrane breakdown, and is often elevated in tumours and inflammatory disorders, while CR provides a measure of brain metabolism and energy stores. INS participates in phospholipid metabolism and plays a role in cellular message transduction and a proposed marker for gliosis. Unfortunately, many notable metabolites are not represented in the MRSI spectra, with neurotransmitters acetylcholine, dopamine
and serotonin all absent, either as a result of low concentrations or molecules that don't respond to conventional MRSI techniques.

The metabolites of interest are commonly presented as ratios, which helps to control for changes in brain water content, the T1 and T2 relaxation times of water and possible CSF fluid contamination of the voxel in use (Card et al, 2013). Using absolute metabolite concentrations is becoming more common, though its use in vivo is difficult and requires intensive post-processing requirements. CHO is commonly used as a denominator in metabolite ratios due to the fact that its concentration reduces over time as the rapid brain growth of the neonate slows in infancy. During the preterm period dramatic increases in CR/CHO and NAA/CHO ratios are seen with decreases in INS/CR and INS/CHO ratios (Card et al, 2013). LAC/CR and LAC/CHO ratios have been shown to correlate with the severity of asphyxia at birth and likely reflects lactate as a marker of anaerobic metabolism seen in tissue infarction. White matter injury has previously been shown to result in lower NAA/CHO and NAA/CR ratios (Card et al, 2013; Chau et al, 2009).
Figure 1.10 Magnetic Resonance Spectroscopic Imaging (MRSI) example. Example of a preterm infant scanned at term with a 6mm voxel located in the left basal ganglia with a long echo time (TE) of 144 ms on a 3T magnet. The peaks of Cr2 (phosphocreatine), Cho (choline), Cr (creatine), and N-acetylaspartate (NAA) are shown at their respective parts per million position on the spectroscopy (ppm) on the x-axis with the MR signal peak amplitude on the y-axis.
1.7 Neurodevelopmental Outcomes

1.7.1 Background

Standardized developmental assessments are important in the early detection of developmental delays both for the eligibility requirements of early intervention programs, but also in the evaluation of therapies in the attempt to improve childhood developmental outcomes (Johnson and Marlow, 2006). While no assessment battery is perfect and several standardized assessments for infants are available, the Bayley Scales of Infant and Toddler Development and the Peabody Developmental Motor scales, along with their revisions, are validated and widely reported assessment methods (Anderson et al, 2010).

1.7.2 The Bayley Scales of Infant and Toddler Development

The Bayley Scales of Infant and Toddler Development, 3rd edition (BSID-III) are meant to examine all facets of a young child’s development with norm-referenced, standardised testing that can be done for infants 1 to 42 months of age (Bayley, 2005). Initially developed in 1969 as the first edition, changes were made in the second edition utilized from 1993 – 2006, with the 3rd edition used since 2006. Originally standardized to 1,700 infants, toddlers and pre-schoolers in the United States of America, the scoring scale reflects an assessment of healthy individuals and does not include at-risk or disabled populations (Bayley, 1993; Bayley, 2005). Qualified therapists, through the administration of a battery of developmentally appropriate tests of child interaction for infants, toddlers and pre-school age children, calculate an individual infant’s cognitive, language and motor composite scores. Social-emotional and adaptive behaviour assessments are also included and conducted via parent questionnaires. The scoring
scales for each composite have a standardized and normalized mean of 100 with standard deviation of 15, with scores ≤85 considered below average and <70 considered severely abnormal (Bayley, 2005). When assessing the scores across timelines and previous literature it is important to note that there is considered to be a large difference between the scales on the BSID-III and the previously used BSID, 2nd edition, particularly at the lower score values (Moore et al, 2016).

Cognitive scoring is done by assessing the way the child thinks, reacts and learns about the world around them through their interaction with familiar and unfamiliar objects. The language scale includes two components, with the receptive communication (RC) part assessing the ability for the child to recognize sounds and understand words and directions. Expressive communication (EC) assesses how well the child communicates with sounds, gestures or words. The motor scale also has two parts with fine motor (FM) assessing how well the child uses his or her fingers to manipulate objects, while the gross motor (GM) part looks at how well the child moves his or her body within their environment.

1.7.3 Peabody Developmental Motor Scales

The Peabody Developmental Motor Scales, 2nd edition (PDMS-2), was first published in 2000, and is a developmental assessment focused on the motor skills of children from birth through to age 5 years (Folio and Fewell, 2000). The PDMS-2 was validated on 2,003 children residing in 46 U.S. States and one Canadian province, and matched to a normative sample of children <5 years with regard to geographic region, sex, race, rural or urban residence, ethnicity, family income, parent education and disability (Folio and Fewell, 2000). The testing contains six subtests which are divided
into the following categories: reflexes, stationary, locomotion, object manipulation, grasping and visual-motor integration. All of the sub-tests allow the formation of a total motor quotient, considered the best estimate of overall motor abilities. The composite scores for each of gross motor and fine motor quotients are a combination of a number of the subsets. The PDMS-2 composite scores have high correlation with the BSID-III composite motor score, greatest in those infants >18 months of age (Connolly et al, 2012).

1.7.4 Cerebral Palsy

Cerebral palsy (CP) is the most common movement disorder in children, with an estimated incidence of 2.1 per 1,000 live infants (Oskoui et al, 2013). Some of the first descriptions of CP were made by Hippocrates in the 5th century BCE, with its first description in modern medicine in the 19th century by William John Little, and first termed “cerebral palsy” by William Osler (Panteliadis et al, 2013). CP is defined as “a group of permanent disorders of the development of movement and posture, causing activity limitation, that are attributed to non-progressive disturbances that occurred in the developing fetal or infant brain” (Rosenbaum et al, 2006). Often accompanied with the disorders of motor function are disturbances of sensation, perception, cognition, communication and behaviour, epilepsy and secondary musculoskeletal problems. The diagnosis of CP is made following an assessment by a pediatrician or neurologist familiar with the development of children. While many factors contribute to the risk of CP, preterm birth has a strong prevalence among children with CP. With many improvements in NICU care over the past several decades, the incidence has decreased significantly in those infants born >1000 grams (Sellier et al, 2016), though has remained stable in those infants born <28 weeks or <1000 grams, with rates as high as 112 (95% CI, 70-180) per 1000
live births (Conde-Agudelo and Romero, 2009; Oskoui et al, 2013; Robertson et al, 2007; van Haastert et al, 2011). As a measure of severe motor impairment, several NICU practices have been shown to reduce the likelihood of CP in the preterm infant with antenatal betamethasone (French et al, 2004), magnesium sulfate (Nelson and Grether, 1995; Rouse et al, 2008), postnatal caffeine therapy (Schmidt et al, 2007), increased caesarian section rate and antenatal antibiotics (van Haastert et al, 2011) all showing a reduced incidence. Despite these improvements in NICU care, CP remains a common lifelong condition that results in significant impairments. Postnatal infection and severe ROP are strong predictors of CP with double the risk in those infants with infection (Stoll et al, 2004), and a three-fold increase of motor impairments seen in those infants with severe ROP (Schmidt et al, 2014). Efforts to reduce the incidence of CP and its impact upon the developing child need to be continued.
1.8 Hypothesis, Major Goal and Specific Aims

1.8.1 Hypothesis

I hypothesize that retinopathy of prematurity (ROP) and multiple postnatal infections will be associated with delays in the brain maturation evident on multi-modal MR imaging with associated poorer neurodevelopmental outcomes.

1.8.2 Major Goal

My goal is to identify whether retinopathy of prematurity and/or multiple infections in the preterm infant are associated with poorer outcomes and/or delayed white matter maturation. Through this investigation I hope to assist in altering the care of the preterm infant to optimize developmental outcomes and provide insight into which infants are at greatest risk of poor outcomes and would benefit most from early intervention practices. These findings will expand the understanding of these factors in preterm infant development following complications of preterm birth and contribute to the understanding of these variables in the brain development of the preterm infant, while potentially supporting further research in the field.
1.8.3 Specific Aims

1. To characterize the brain maturation and neurodevelopmental outcomes of extremely preterm infants with severe ROP.

2. To describe the association of multiple postnatal infections with developmental outcomes and brain maturation of very preterm infants.
Chapter 2

Severe Retinopathy of Prematurity predicts delayed white matter maturation and poorer neurodevelopment at 18 months CA

This chapter is modified from work published in the Archives of Disease in Childhood - Fetal and Neonatal Edition: Glass TJA et al. Severe retinopathy of prematurity predicts delayed white matter maturation and poorer neurodevelopment Archives of Disease in Childhood - Fetal and Neonatal Edition Published Online First: 23 May 2017. doi:10.1136/archdischild-2016-312533

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A link to the published paper can be found at:

http://fn.bmj.com/content/early/2017/05/22/archdischild-2016-312533

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2.1 Introduction

Retinopathy of prematurity (ROP) is a proliferative disorder of retinal vascularisation that is most severe in extremely preterm neonates born at less than 28 weeks gestational age (GA). Primary therapeutic strategies for ROP include prevention of abnormal vessel development with tight control of supplemental oxygen therapy and, when severe, treatment with either retinal laser photocoagulation therapy or, more recently, intravitreal anti-vascular endothelial growth factor (VEGF) (Gunther and Altaweel, 2009; Lee and Dammann, 2012). Several large cohort studies of preterm neonates have shown that severe ROP is associated with lower cognitive and motor scores in early childhood (Bassler et al, 2009; Harrell and Brandon, 2007; Hellstrom et al, 2013; Schmidt et al, 2003). Despite favourable visual outcomes, severe ROP continues to strongly predict non-visual disabilities independent of brain injury (Schmidt et al, 2014).

Given this link between severe ROP and non-visual disabilities, we hypothesized that severe ROP would be associated with white matter maturational delay on advanced MRI. White matter maturation is well recognized as an important predictor of neurodevelopmental outcomes in infants born preterm (Chau et al, 2013).

The objectives of this prospective cohort study of extremely preterm neonates were to determine the association of severe ROP with: (1) early brain development as measured by MR diffusion-tensor imaging (DTI) and tract-based spatial statistics (TBSS); and (2) motor and cognitive outcomes at 18 months corrected age (CA). We tested the hypothesis that severe ROP would be associated with abnormalities in early brain
microstructural development and with worse cognitive and motor development at 18 months CA follow-up.

2.2 Material and Methods

2.2.1 Participants

This study was approved by the University of British Columbia/Children's and Women's Health Centre of British Columbia Research Ethics Board. As part of a larger prospective study of neonates born 24 to 32 weeks GA, informed consent was obtained from parents/guardians prior to recruitment of the neonates from April 2006 to September 2013 at British Columbia Women’s Hospital (BCWH), the major provincial tertiary-level neonatal referral center. Neonates were excluded if they had (1) clinical evidence of a congenital malformation or syndrome, (2) congenital infection, or (3) ultrasound evidence of a large parenchymal hemorrhagic infarction (>2 cm) (Papile et al, 1978). This cohort has been described previously addressing separate hypotheses (Adams et al, 2010; Brummelte et al, 2012; Chau et al, 2009; Chau et al, 2013; Duerden et al, 2015). Only extremely preterm neonates born ≤ 28 weeks GA were included in this sub-study as they are the subset at greatest risk of severe ROP.

2.2.2 Clinical Characteristics

Neonates were screened by a pediatric ophthalmologist at BCWH as per the International Classification of ROP and the maximal ROP severity in sequential assessments was included in the analysis (International Committee for the Classification of Retinopathy of Prematurity, 2005). Severe ROP was defined as ROP requiring
treatment as per the Early Treatment for Retinopathy of Prematurity (ETROP) study (Early treatment of Retinopathy of prematurity cooperative group, 2003). Intravitreal anti-VEGF treatment was not used at our institution at the time of the study. Clinical characteristics were collected systematically by chart review, as previously described; (Chau et al, 2012; Chau et al, 2009): with bronchopulmonary dysplasia (BPD) defined as oxygen therapy beyond 36 weeks PMA, hypotension defined as any treatment for low blood pressure, culture positive infection as any positive blood, urine, cerebrospinal fluid or respiratory culture, and necrotising enterocolitis (NEC) defined as stages 2 and 3 of Bell’s criteria (Bell et al, 1978).

2.2.3 MRI Studies

Neonates were scanned first when clinically stable in the preterm period and again at term-equivalent age. MRI studies were completed without pharmacological sedation on a Siemens 1.5 Tesla Avanto scanner with 3D coronal volumetric T1-weighted and axial fast-spin echo T2-weighted images. Neonates were scanned using an MR-compatible isolette (Lammers Medical Technology, Luebeck, Germany) and specialized neonatal head coil (Advanced Imaging Research, Cleveland, OH). An experienced neuroradiologist, blinded to the participant’s medical history, reviewed the images and recorded the WMI, IVH, ventriculomegaly and cerebellar hemorrhage severity according to scales previously described (Chau et al, 2009; Miller et al, 2005).

2.2.4 Diffusion Tensor Imaging

DTI reflects the water movement of an ellipsoid space with axial diffusion (λ1), the preferred movement of water along the white matter tracts, and radial diffusion (λ2 and
reflecting the orthogonal planes. DTI characterizes the 3-dimensional (3D) spatial distribution of water diffusion in each voxel of the MR image, providing an indirect measure of microstructural integrity (Beaulieu, 2002; Miller et al, 2002; Mukherjee et al, 2002). Mean FA, the average directionality of diffusion increases with white matter maturation, reflecting the maturation of the oligodendrocyte lineage and early events of myelination (Drobshevsky et al, 2005; Miller et al, 2002). Diffusion tensor imaging parameters of FA, $\lambda_1$, $\lambda_2$ and $\lambda_3$ were measured in seven manually placed white matter regions of interest (ROI) and acquired using a multi-repetition single-shot echo planar sequence (Chau et al, 2009). ROIs were excluded if significant motion artifact was present. The ROI areas were acquired in the anatomical regions of the calcarine, anterior, central and posterior white matter regions, optic radiations, splenium of the corpus callosum and posterior limb of the internal capsule (PLIC). These regions have been shown previously to correlate with motor, language and cognitive outcomes (Chau et al, 2009; Chau et al, 2013).

TBSS, a semi-automated and 3D DTI assessment of mean FA, provides a less user-dependent measure of FA than complementary ROI-based analyses. TBSS was performed using functional MRI of the Brain software library (FSL) and, after correction for eddy currents, each DTI volume was registered to a non-DTI volume for each subject (Smith et al, 2006). The TBSS FA data were projected onto a mean FA tract skeleton, which was used to apply the voxelwise cross-subject statistics (Smith et al, 2006). We used five age-based templates and were able to compare neonate groups with a standardised analysis and a calculated voxel significance threshold of $p < 0.05$ adjusted for PMA at scan (Duerden et al, 2015). White matter abnormalities detected with TBSS
at term-equivalent age have been shown to predict neurodevelopmental outcomes in preterm neonates, with increased FA at 2 years CA being associated with improved outcomes (Counsell et al, 2002; Duerden et al, 2015).

2.2.5 Developmental Follow-up

At 18 months CA, neurodevelopment was assessed with the Bayley Scales of Infant and Toddler Development-III (BSID-III) cognitive, language and motor composite scores with a mean of 100 and standard deviation of 15 (Bayley, 2005). Assessors were qualified therapists blinded to the imaging findings of the participants. Severe cerebral palsy (CP) was defined as any diagnosis by an experienced clinician prior to or at the 18 month assessment of CP. Hearing impairment was diagnosed when audiograms showed hearing threshold >70 dB. Visual acuity was assessed sequentially by the treating ophthalmologist using varied assessment techniques depending upon age at follow-up and collected with a retrospective chart review. Visual impairment was defined as best visual acuity <20/200. Socioeconomic status was assessed as number of years of maternal education.

2.2.6 Data Analysis

Statistical analysis was performed using Stata 14.1 (StataCorp, 2015). Clinical characteristics were compared using Fisher’s exact and the Kruskall–Wallis tests for categorical and continuous data, respectively, with a statistical significance of \( p < 0.05 \). The association of severe ROP and other clinical variables with WMI was tested with univariate logistic regression. The mean values of FA, averaged bilaterally, were compared between neonates with and without severe ROP, in a generalized least
squares regression model for repeated measures, adjusting for PMA at MRI scan and multiple ROIs with a p<0.05. We examined the relationship of ROP with FA modified by PMA at MRI scan, considering p<0.1 as significant due to the interaction term.

2.3 Results

2.3.1 Clinical Characteristics

Of the 234 neonates born 24-32 weeks GA, 126 were born at 24-28 weeks GA. Ninety-eight (79%) extremely preterm neonates were assessed for ROP at BCWH and were included in the analysis; infants not assessed were older at birth but had no difference in other demographics. Neonates were born at a median GA of 26.0 weeks (interquartile range [IQR], 25.0–26.9 weeks). Early MRI scans were completed in the 98 neonates assessed for ROP at median 32.4 weeks (IQR, 30.3-35.0 weeks) and term-equivalent MRI was performed in 87 (89%) at median PMA 39.8 weeks (IQR, 38.3-41.6 weeks) (Figure 2.1).
Figure 2.1 Study flow chart. Flow chart of study enrollment, ROP treatment, MRI scans and follow-up. Ages presented are median, with corrected age used for Bayley-III Assessment.

2.3.2 Retinopathy of Prematurity

Of the 98 neonates in this cohort, 67 (68%) had any stage of ROP (stage 1 = 7, stage 2 = 47, stage 3 = 13, stage 4 = 0, stage 5 = 0), and 19 (19%) neonates required treatment for severe ROP as per criteria defined in the ETROP study at a median PMA of 37.9 weeks (IQR, 36.1 – 39.3 weeks). Neonatal parameters associated with severe ROP included GA at birth, birth weight, birth length, head circumference at birth, BPD,
NEC and hypotension (Table 2.1). Culture positive infection, histologic chorioamnionitis and multiple gestation were not significantly different in those with and without severe ROP.

Table 2.1 Demographic, clinical characteristics and imaging findings of neonates 24-28 weeks GA with and without severe ROP treated with retinal laser therapy. Number (%) or median (Interquartile range)

<table>
<thead>
<tr>
<th></th>
<th>No Severe ROP n=79</th>
<th>Severe ROP n=19</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>38 (48%)</td>
<td>14 (74%)</td>
<td>0.07</td>
</tr>
<tr>
<td>Multiple gestation</td>
<td>5 (6%)</td>
<td>4 (21%)</td>
<td>0.07</td>
</tr>
<tr>
<td>Age at birth (weeks)</td>
<td>26.1 (25.3-27.1)</td>
<td>25.4 (24.7-25.9)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Weight at birth (grams)</td>
<td>845 (745-991)</td>
<td>700 (630-795)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Length at birth (cm)</td>
<td>34 (32-36)</td>
<td>32 (31-34)</td>
<td>0.02</td>
</tr>
<tr>
<td>Head Circumference at birth (cm)</td>
<td>24 (22.5-25)</td>
<td>22.5 (22-23)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Conventional ventilation (days)</td>
<td>16 (5-33)</td>
<td>51 (37-59)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Histologic chorioamnionitis</td>
<td>39 (51%)</td>
<td>9 (53%)</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Hypotension</td>
<td>37/79 (47%)</td>
<td>15/19 (79%)</td>
<td>0.02</td>
</tr>
<tr>
<td>Bronchopulmonary dysplasia (BPD)</td>
<td>22/79 (28%)</td>
<td>11/19 (58%)</td>
<td>0.02</td>
</tr>
<tr>
<td>Necrotizing enterocolitis (NEC)</td>
<td>3/79 (4%)</td>
<td>4/19 (21%)</td>
<td>0.03</td>
</tr>
<tr>
<td>Culture positive infection</td>
<td>46/79 (58%)</td>
<td>15/19 (79%)</td>
<td>0.12</td>
</tr>
<tr>
<td>Intraventricular hemorrhage (IVH)</td>
<td>43/78 (55%)</td>
<td>10/19 (53%)</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>White matter injury (WMI)</td>
<td>24/78 (31%)</td>
<td>8/19 (42%)</td>
<td>0.42</td>
</tr>
<tr>
<td>Cerebellar hemorrhage</td>
<td>16/78 (21%)</td>
<td>2/19 (11%)</td>
<td>0.51</td>
</tr>
<tr>
<td>Severe WMI and/or IVH</td>
<td>12/78 (15%)</td>
<td>3/19 (16%)</td>
<td>&gt;0.99</td>
</tr>
</tbody>
</table>
2.3.3 Brain Injury

Ninety-eight early and 87 term-equivalent scans were scored. Findings included: WMI in 30 (35%) neonates, severe WMI in 9 (11%), IVH in 47 (55%) neonates, severe IVH in 5 (6%), ventriculomegaly in 27 (32%), and cerebellar haemorrhage in 16 (19%). Severe ROP was not associated with an increased risk of WMI, IVH, ventriculomegaly or cerebellar haemorrhage even when comparing the most severely affected forms of WMI, IVH, and ventriculomegaly separately (all p>0.05) (Table 2.1).

2.3.4 White Matter Maturation

Mean FA in neonates with severe ROP was significantly lower for the posterior white matter (effect size, -0.02; 95% confidence interval (CI), -0.04 to -0.004; p=0.02) which was more pronounced over time on interaction analysis (p=0.08) (Figure 2.2). A trend was seen in the FA of the optic radiations (effect size, -0.02; 95% CI, -0.39 to 0.001; p=0.07) (Figure 2.2).
Figure 2.2 Mean Fractional Anisotropy (FA) values. Mean FA in those with and without severe retinopathy of prematurity (ROP) by post menstrual age at scan in (A) posterior white matter [p=0.02], (B) optic radiations [p=0.07], (C) splenium of the corpus callosum [p=0.50], and (D) calcarine white matter [p=0.78]. (E) Diffusion tensor imaging region of interest model map at the level of the high centrum semiovale and (F) basal ganglia: (AWM) anterior white matter, (CWM) central white matter, (PWM) posterior white matter, (PLIC) posterior limb of the internal capsule, (SCC) splenium of the corpus callosum, (OR) optic radiations, (CWM) calcarine white matter.
Using voxelwise regression analysis in TBSS, white matter regions with significantly lower FA in severe ROP included the optic radiations, PLIC and external capsule on scans at 34-37 weeks (n=6/30) and 42+ weeks GA (n=3/24) (Figure 2.3).

Figure 2.3 Tract based spatial statistics (TBSS) model. TBSS using semi-automated preterm and term neonate templates. White matter regions where neonates with severe retinopathy of prematurity (ROP) differ from those with no severe ROP [p<0.05], corrected for age at scan, shown in blue overlaid on the FA white matter skeleton in yellow. The number of subjects in each sample with ROP is reflected by the numerator, and total subjects in the sample, the denominator. R = right, L = left, P = posterior, A=anterior.

FA on ROI analysis in the anterior white matter (effect size, 0.001; p=0.93), central white matter (effect size, -0.002; p=0.84), splenium of the corpus callosum (effect size, -
0.010; \( p=0.50 \), PLIC (effect size, -0.01; \( p=0.19 \)) and calcarine white matter (effect size, -0.002; \( p=0.78 \)) did not differ significantly in neonates with and without ROP. In the posterior white matter and optic radiations, there was no difference in the relationship with FA in neonates with severe ROP in the radial axes (\( \lambda_2 \) and \( \lambda_3 \)) and the axial diffusion axis (\( \lambda_1 \)).

### 2.3.5 Developmental Outcomes

A total of 83 (85%) infants were assessed at 18 months CA follow-up (Table 2); there were no significant differences in the 7 who withdrew from the study after at least one MRI scan or the 8 who died or were lost to follow-up.

Table 2.2 18 month corrected age (CA) follow-up assessments of neonates 24-28 weeks GA with and without severe ROP treated with retinal laser therapy. Number (%) or median (Interquartile range)

<table>
<thead>
<tr>
<th></th>
<th>No Severe ROP ( n=67 )</th>
<th>Severe ROP ( n=16 )</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSID-III Cognitive score</td>
<td>105 (100-115)</td>
<td>95 (88-105)</td>
<td>0.02</td>
</tr>
<tr>
<td>BSID-III Motor score</td>
<td>94 (85-110)</td>
<td>85 (75-93)</td>
<td>0.01</td>
</tr>
<tr>
<td>BSID-III Language score</td>
<td>96 (83-112)</td>
<td>89 (83-103)</td>
<td>0.32</td>
</tr>
<tr>
<td>Severe cerebral palsy</td>
<td>6/67 (9%)</td>
<td>1/16 (6%)</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Visual impairment</td>
<td>1/67 (1%)</td>
<td>0/16 (0%)</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Hearing impairment</td>
<td>2/67 (3%)</td>
<td>0/16 (0%)</td>
<td>&gt;0.99</td>
</tr>
</tbody>
</table>

Infants with severe ROP had lower BSID-III motor and cognitive scores, with no difference in language scoring relative to infants without severe ROP. There was no difference in years of maternal education between groups (\( p=0.91 \)). In a multivariate
model, the relationship between severe ROP and motor and cognitive scores remained significant when adjusting for GA at birth and severe WMI and/or IVH (Table 2.3).

Table 2.3 Multivariate linear regression analysis. 18-month corrected age Bayley Composite scoring adjusted for GA at birth and severe white matter injury and/or intraventricular hemorrhage.

<table>
<thead>
<tr>
<th></th>
<th>Effect size (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSID-III Cognitive Composite</td>
<td>-9.22 (-17.69 - -0.75)</td>
<td>0.03</td>
</tr>
<tr>
<td>BSID-III Motor Composite</td>
<td>-10.77 (-19.87 - -1.66)</td>
<td>0.02</td>
</tr>
<tr>
<td>BSID-III Language Composite</td>
<td>-3.45 (-13.80 – 6.89)</td>
<td>0.51</td>
</tr>
</tbody>
</table>

One infant in the “no severe ROP” group was unable to complete the BSID-III assessment due to severe CP and cortical visual impairment; when assigned a value of 2 standard deviations below the mean, there was no meaningful difference in the findings. There was no difference in the rate of cerebral palsy, hearing or visual impairment in infants with and without severe ROP (Table 2.2). Visual acuity assessments were available in 12 (63%) newborns with severe ROP at a median age of 3.9 years (IQR, 3.1-5.0 years). Median visual acuity was 20/30 OD and 20/40 OS in infants with ROP; no infants met guidelines for severe visual impairment.

2.4 Discussion

In a prospective longitudinal cohort of extremely premature neonates, we found a significant maturational delay of the posterior white matter regions with delay on TBSS
analysis in the optic radiations, PLIC and external capsule in those neonates with severe ROP, which to our knowledge has not been previously reported. These novel findings occurred independently of severe white matter injury or intraventricular hemorrhage and likely indicate similar vital mechanisms in the development of severe ROP with poor maturation of the white matter. It is well known that circulating levels of insulin-growth factor 1 (IGF-1) are important modifiers in the circulatory levels of VEGF and the development of severe ROP (Harrell and Brandon, 2007). Within the brain, IGF-1 has been shown to have a significant role in the augmentation and utilisation of glucose across all neural cell lineages (Cheng et al, 2000; Dercole and Ye, 2008). Furthermore, mean IGF-1 concentrations between birth and 35 weeks PMA in preterm neonates correlate with total brain, white matter, grey matter, and cerebellar brain volumes, with poorer cognitive development seen in those infants with the slowest rate of increase (Hansen-Pupp et al, 2013; Hansen-Pupp et al, 2011). These factors support severe ROP as a marker of adverse brain development, independent of visual outcomes, and are highlighted by our 18 month CA follow-up assessments showing lower cognitive and motor developmental scores in those with severe ROP, consistent with larger cohorts (Bassler et al, 2009; Schmidt et al, 2003; Schmidt et al, 2015). Our study emphasizes how adverse motor and cognitive developmental outcomes are associated with maturational delays on DTI and TBSS independent of the traditional markers of brain injury in the preterm neonate.

Maturational delays in the optic radiations are consistent with previous studies using DTI and TBSS in preterm neonates that showed a relationship between severe ROP and delayed white matter microstructural development in the optic radiations at
term-equivalent age, independent of white matter injury (Bassi et al, 2008; Thompson et al, 2014). Further research at 7 years of age showed delayed optic radiation microstructure in children with a history of severe ROP compared to those with milder ROP (Thompson et al, 2014). We have shown that these delays are present in the late preterm period and involve non-visual pathways, highlighting an early link of severe ROP with poor white matter maturation in preterm neonates.

When considering the importance of a retinal disorder on the white matter development of the preterm neonate, retinal nerve fibre layer (RNFL) thickness using optical coherence tomography (OCT) in preterm neonates can be examined. In a study comparing RNFL thickness with 18-24 CA developmental scores, thinner RNFL thickness across the papillomacular bundle correlated with lower cognitive and motor scores (Rothman et al, 2015). These findings provide an understanding of the associations between the retina and the underlying white matter pathways, and offer a “window into the brain” of the connections that the retinal ganglion and photoreceptor cells share with the white matter pathways. The maturational delays in the motor, visual and visual-association pathways of the developing brain detected in neonates with severe ROP suggests a mechanism by which these factors act on neurodevelopment, potentially due to an imbalance of growth factors.

2.4.1 Limitations

Although we were able to compare a large number of extremely preterm neonates with serial MRI scans, the timing of the follow-up MRI scans may have affected the imaging results due to the development of ROP over time as a progressively acquired disorder. We were unable to control for the timing of the ROP retinal laser therapy and its
possible confounding of the imaging. Visual acuity follow-up was not done in conjunction with the 18-month CA neurodevelopmental assessments and was not standardised across the cohort.

2.5 Conclusions

Severe ROP requiring laser photocoagulation therapy is associated with delayed maturation in the motor, visual and visual-association pathways. Infants with severe ROP had lower motor and cognitive functioning at 18 months CA, independent of severe brain injury and GA at birth. More research is needed to determine which potential mechanisms in severe ROP prevent optimal neurodevelopment, and the impacts on brain maturation of early and effective treatment of severe ROP.
Chapter 3

Multiple Postnatal Infections in Preterm Newborns is associated with delayed Maturation of Motor Pathways at Term-equivalent age and Poorer Motor Outcomes at 3 years
3.1 Introduction

Preterm newborns born less than 32 weeks gestational age (GA) are at a substantial risk of postnatal infection with 20–65% of newborns suffering from at least a single infection during this period of significant brain development (Adams-Chapman and Stoll, 2006; Orsi et al, 2009; Stoll et al, 2004). In a large epidemiologic study, postnatal infection was found to double the risk of motor impairment and cerebral palsy, while also greatly increasing the risk of cognitive impairment (Stoll et al, 2004). Similarly, a study of very preterm infants followed to 9 years of age showed that infants who had postnatal infection were more likely to have poorer motor development, cognitive delays, school delays, attention-deficit hyperactivity disorder and other mental health disorders (Rand et al, 2016). While white matter injury (WMI) is a known complication of postnatal infection, the majority of infants with postnatal infection do not have punctate WMI on clinical neuroimaging (Chau et al, 2012; Chau et al, 2009; Glass et al, 2008; Stoll et al, 2004). Experimental models of WMI with hypoxia-ischemia show inflammation to have additive effects on the injury present (Eklind et al, 2001). Microscopic WMI, present on neuropathological studies, has been shown to be more widespread in the brain of infants with macroscopic WMI, suggesting a more diffuse brain injury may be present, but below the resolution of current clinical MRI techniques (Buser et al, 2012). Chau et al. reported that very preterm newborns exposed to infection had reduced measures of white matter development on MRI, even when adjusting for WMI, which was most prominent in brain regions important for motor and cognitive development (Chau et al, 2012). This impairment in the development of the white matter may reflect injury to the pre-OL lineage cells following hypoxic-ischemic and/or inflammatory events in which the premature
infants’ immune system, and brain, may be primed for further events, increasing their vulnerability to injury (Hagberg and Mallard, 2005; Wang et al, 2012; Yanni et al, 2017).

Our aim was to investigate the association of multiple postnatal infections with the white matter development and outcomes of very preterm newborns with the hypothesis that a greater number of infections would be associated with delayed white matter development and poorer motor outcomes at 36 months corrected age (CA) compared to non-infected neonates.

3.2 Material and Methods

3.2.1 Study Population

The study was approved by the University of British Columbia/Children’s and Women’s Health Centre of British Columbia Research Ethics Board and informed consent was obtained from parents/guardians prior to recruitment. Preterm neonates 24-32 weeks gestational age (GA) were recruited into a prospective longitudinal cohort study at British Columbia Women’s Hospital from April 2006 to September 2013. Infants were excluded if they had clinical evidence of a congenital malformation or syndrome, congenital infection, or ultrasound evidence of a large parenchymal hemorrhagic infarction (>2cm), as these conditions are strongly predictive of neurodevelopmental impairments or early mortality. This cohort has been described previously to address different hypotheses (Adams et al, 2010; Brummelte et al, 2012; Chau et al, 2012; Chau et al, 2013; Duerden et al, 2015; Glass et al, 2017).
3.2.2 Clinical Characteristics

Infection characteristics were collected by systematic chart review. Cultures that had multiple growths with organisms consistent with contamination were excluded. Culture positive infection was defined as any positive blood, urine, tracheal aspirate and/or cerebrospinal fluid culture treated with ≥5 days of antibiotics with “clinical-only infection” defined as any instance in which there was a clinical concern for infection with negative cultures in which the antibiotic treatment duration was ≥5 days. A positive culture with the same organism in each of two separate locations or cultures during a continued antibiotic course was considered a single infection. Tracheal aspirates required a positive culture and ≥4 white blood cells per field to be considered a positive culture. An “early infection” was defined as any infection occurring at <72 hours of postnatal age, and “postnatal infection” as any infection ≥72 hours after birth, with infections included up to 40 weeks post menstrual age (PMA). Other clinical characteristics were collected via chart review: histologic chorioamnionitis as confirmed by clinical pathology assessment, hypotension as any treatment for low blood pressure, patent ductus arteriosus (PDA) as any requiring pharmacological or surgical treatment, BPD as oxygen therapy beyond 36 weeks PMA, and NEC as stages 2 and 3 of Bell’s criteria (Bell et al, 1978).

3.2.3 MR Brain Imaging

MRI scans were performed early in life when neonates were clinically stable, and again at term-equivalent age, all without pharmacological sedation. At both time points MRIs were carried out on a Siemens 1.5 Tesla Avanto scanner using an MR-compatible isolette (Lammers Medical Technology, Luebeck, Germany) and specialized neonatal
head coil (Advanced Imaging Research, Cleveland, OH). 3D coronal volumetric T1-weighted and axial fast-spin echo T2-weighted images were performed. An experienced neuroradiologist (K.J.P.), blinded to the participant’s medical history, reviewed the images and recorded the severity of WMI, IVH, ventriculomegaly and cerebellar hemorrhage according to previously described scales (Chau et al, 2009; Miller et al, 2005). The most severe injury score seen on the preterm or term scan was used in the structural MRI analysis as the greatest severity of injury and most likely to adversely impact developmental outcomes and to be associated with greater amounts of microscopic WMI, not seen on MRI. WMI volumes were calculated on the T1-weighted images with voxels of abnormal T1 shortening identified as WMI, reviewed by two neonatal neurologists (V.C. and S.P.M.), then manually segmented with simultaneous coronal, sagittal and axial views of the brain using Display software (http://www.bic.mni.mcgill.ca/software/Display) as previously reported (Guo et al, 2017).

3.2.4 Magnetic Resonance Spectroscopic Imaging

MR spectroscopic imaging (MRSI) was used as a measure of neuronal maturation using quantitative metabolite ratios with bilaterally placed 4mm ROI voxels in six anatomical regions: the anterior, central and posterior white matter, the caudate, lentiform nucleus (globus pallidus and putamen), and thalamus. To reflect the overall metabolism of the regions, we analyzed an average of the three white matter regions, and the three basal ganglia regions, the caudate, lentiform nucleus, and thalamus regions.

3.2.5 Diffusion Tensor Imaging
DTI is a measure of water diffusion in an ellipsoid space within each 3D voxel of the MR image. Mean FA, the average directionality of diffusion, increases with white matter maturation reflecting the maturation of the oligodendrocyte lineage and early myelination (Drobyshevsky et al, 2005; Miller et al, 2002). DTI parameters of FA, $\lambda_1$, $\lambda_2$ and $\lambda_3$ were acquired using a multi-repetition single-shot echo planar sequence, and excluded if significant motion artifact was present (Chau et al, 2009). ROI analyses were manually placed in seven white matter anatomical regions (anterior, central and posterior white matter regions, optic radiations, splenium of the corpus callosum, genu of the corpus callosum and PLIC) and three deep grey matter regions (caudate, lentiform nucleus and thalamus).

TBSS was performed using functional MRI of the brain software library (FSL; https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FSL) as previously described (Duerden et al, 2015; Glass et al, 2017). A diffusion tensor model was fit to the data at each voxel to calculate the voxelwise FA with the spatial location of alterations in diffusion measures done using the TBSS pipeline (Smith et al, 2006). The TBSS data were projected onto a mean FA tract skeleton using age appropriate templates (preterm scans: 27-29 weeks, 30-33 weeks; 34-36 weeks; term scans: 37-41 weeks, $\geq$42 weeks), which were then used to apply voxelwise regression cross-subject analysis. Cluster-size thresholding was applied to the data in which the size of the cluster was determined by 500 permutations by using Randomise v.2.9 within FSL. A threshold of $P<0.05$ (95% percentile of the distribution) was set for the clusters and corrected for multiple comparisons across space in determining the association of FA and $\geq$3 infections across subjects within each of the age-based templates and projected upon the white matter skeleton.
3.2.6 Developmental Follow-up

At 36 months CA, neurodevelopment was assessed with the Bayley Scales of Infant and Toddler Development-III (BSID-III) cognitive, language and motor composite scores and Peabody Developmental Motor scales 2nd edition (PDMS-2) all with a mean of 100 and standard deviation of 15 (Bayley, 2005; Folio and Fewell, 2000). In addition to the BSID-III motor composite, the PDMS-2 total, gross and fine motor quotient values were used in the analyses as a more robust assessment of motor impairment at 36 months CA (Folio and Fewell, 2000). Assessments were carried out by qualified therapists blinded to the imaging findings of the participants. Socioeconomic status was classified by the self-reported number of years of maternal education. Cerebral palsy was defined as a confirmed diagnosis made by an experienced pediatrician at the 36 month assessment.

3.2.7 Data Analysis

Statistical analysis was performed using Stata V.14.2 (StataCorp, 2015). Clinical and imaging characteristics were compared using Fisher’s exact test for categorical and the Kruskall–Wallis test for continuous data with a statistical significance threshold of P<0.05. The association between postnatal infections and other clinical variables with neurodevelopmental outcomes was tested with univariate logistic regression.

The mean values, averaged bilaterally, of FA and NAA/Choline were compared between neonates with and without postnatal infection, in a generalized least squares
regression model for repeated measures, adjusting for PMA at MRI scan and multiple ROIs with a P<0.05. A log-transformed outcome variable for the NAA/choline was used to determine the percentage differences of the MRS measures (Chau et al, 2012). An interaction term was examined describing the imaging relationships of postnatal infection modified by PMA at MRI scan, and considered P<0.1 as significant. The 36 month outcomes were compared using univariate analysis for multiple infections. An unadjusted risk ratio and adjusted odds ratio were calculated in assessing the 36 month outcomes association with the groups of infections.

3.3 Results

3.3.1 Clinical Characteristics

Of the 234 neonates born 24-32 weeks gestation recruited in the cohort, 219 (94%) completed at least one MRI with a median birth GA of 27.9 weeks (interquartile range [IQR], 26.0 – 29.7 weeks). Early MRI scans were completed at a median 32.1 weeks (IQR 30.4 – 34 weeks) with term-equivalent scans in 184 (84%) at median 40.2 weeks (IQR 38.7 – 42.0 weeks) (Figure 3.1).
Figure 3.1 Flow chart. Flow chart of study enrollment, MRI scans and follow-up. Ages presented are median, with corrected age used for *Bayley-III and PDMS-2 Assessments*.

### 3.3.2 Infection Characteristics

Of the 219 infants, 110 (50%) had one or more instances of postnatal infection and 109 (50%) had no infections in the postnatal period. Due to the small number of the “clinical-only infection” group we grouped them with “culture-positive infection” for the analysis and thus classified each infection event as a “postnatal infection”. There were 54 (25%) infants with one postnatal infection, 29 (13%) had two infections, 19 (9%) had three infections, 7 (3%) had four infections and 1 (0.5%) had five infections. Infants were
grouped according to the number of infections, with three or more infections included together in the analysis due to the small numbers in the four and five infections groups. One and two infections were presented together in the results section as there were few differences between the groups on clinical characteristics, neuroimaging and motor outcomes (Table 3.1).

There were 46 (21%) infants with early infection of whom 21 had no other infection, and 2 who had positive cultures (1 sepsis, 1 lower respiratory culture), which were included in the septicemia and lower respiratory analyses respectively. In total there were 202 distinct episodes of postnatal infection among the total of 110 infants. There was a greater rate of septicemia and lower respiratory infections in the three or more infections group as well as a greater rate of infection with coagulase-negative *Staphylococcus* (CONS) and *Enterobacter* species organisms compared to the one or two infection group (Table 3.2). Neonatal characteristics more common among those with higher numbers of infections included lower GA at birth, lower birth weight, lower birth length, smaller head circumference at birth, hypotension, PDA, BPD and NEC stage ≥2 (Table 3.1).
Table 3.1 Demographics table. Demographics, clinical characteristics and MRI findings of newborns 24-32 weeks GA classified by the number of postnatal infections. Number [%] or median (IQR). Early infection = any infection <72 hours of life; NEC = necrotizing enterocolitis; IVH = intra-ventricular hemorrhage; WMI = white matter injury

<table>
<thead>
<tr>
<th></th>
<th>No Infection N=109</th>
<th>One Infection N=54</th>
<th>Two Infections N=29</th>
<th>Three or more Infections N=27</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>54 [50]</td>
<td>30 [56]</td>
<td>10 [35]</td>
<td>19 [70]</td>
<td>0.12</td>
</tr>
<tr>
<td>Gestational age at birth (weeks)</td>
<td>29.4 (27.7-31.1)</td>
<td>27.3 (25.9-28.6)</td>
<td>25.6 (25.0-27.1)</td>
<td>25.7 (24.9-26.6)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Birth weight (grams)</td>
<td>1190 (1020-1376)</td>
<td>909 (789-1190)</td>
<td>835 (663-950)</td>
<td>755 (630-896)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Birth length (cm)</td>
<td>38 (35-40)</td>
<td>35 (34-39)</td>
<td>33 (31-35)</td>
<td>33 (31-35)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Birth head circumference (cm)</td>
<td>27 (26-28)</td>
<td>25 (23-27)</td>
<td>24 (22-25)</td>
<td>24 (22-25)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Twin birth</td>
<td>41 [38]</td>
<td>19 [35]</td>
<td>7 [24]</td>
<td>9 [33]</td>
<td>0.69</td>
</tr>
<tr>
<td>Histologic chorioamnionitis</td>
<td>33 [31]</td>
<td>22 [42]</td>
<td>14 [48]</td>
<td>8 [31]</td>
<td>0.18</td>
</tr>
<tr>
<td>IVH grades 2-4</td>
<td>34 [32]</td>
<td>12 [34]</td>
<td>13 [54]</td>
<td>9 [35]</td>
<td>0.67</td>
</tr>
<tr>
<td>WMI</td>
<td>38 [35]</td>
<td>14 [26]</td>
<td>8 [28]</td>
<td>8 [31]</td>
<td>0.49</td>
</tr>
<tr>
<td>WMI volume (mm)</td>
<td>35.8 (16.3-272.4)</td>
<td>41 (11-275)</td>
<td>53 (5-892)</td>
<td>17.9 (7.6-83.6)</td>
<td>0.60</td>
</tr>
<tr>
<td>Cerebellar hemorrhage</td>
<td>5 [5]</td>
<td>6 [17]</td>
<td>7 [29]</td>
<td>7 [27]</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Table 3.2 Infection characteristics. Infection location and organism characteristics divided by the number of infection groups. Number [%]

<table>
<thead>
<tr>
<th></th>
<th>One Infection N=54</th>
<th>Two Infections N=29</th>
<th>Three or more Infections N=27</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical-only infection</td>
<td>7 [13]</td>
<td>5 [17]</td>
<td>8 [30]</td>
<td>0.21</td>
</tr>
<tr>
<td>Septicemia</td>
<td>16 [30]</td>
<td>17 [59]</td>
<td>17 [63]</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Urinary tract infection</td>
<td>24 [44]</td>
<td>17 [59]</td>
<td>15 [56]</td>
<td>0.41</td>
</tr>
<tr>
<td>Lower respiratory infection</td>
<td>6 [11]</td>
<td>10 [34]</td>
<td>16 [59]</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Coagulase-negative staphylococcus (CONS)</td>
<td>23 [43]</td>
<td>17 [59]</td>
<td>21 [78]</td>
<td>0.01</td>
</tr>
<tr>
<td>Escherichia coli (E.coli)</td>
<td>5 [9]</td>
<td>2 [7]</td>
<td>6 [22]</td>
<td>0.17</td>
</tr>
<tr>
<td>Enterobacter species</td>
<td>1 [2]</td>
<td>5 [17]</td>
<td>9 [33]</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Other pathogens</td>
<td>7 [13]</td>
<td>11 [38]</td>
<td>9 [33]</td>
<td>0.02</td>
</tr>
</tbody>
</table>

3.3.3 MR Imaging

WMI was present on the first MRI in 31% of infants (33 mild, 23 moderate, 12 severe) with 6 new instances of WMI on the second scans (4 mild, 1 moderate, 1 severe). At least one infection occurred before the first MRI in 99 infants (90%). There was no increase in WMI seen, either mild or moderate/severe, with increased numbers of infection, with no increase in WMI when a separate analysis was done for the different types of infection and organisms (all P>0.05). Similarly, IVH severity and ventriculomegaly were not associated with infection (all P>0.05) (Table 3.1). Cerebellar hemorrhage was more common among those infants with postnatal infection (Table 3.1).
3.3.4 MRSI and DTI Imaging

The NAA/Cho ratio was lower over time in those infants with ≥3 infections in the white matter (coefficient, -1.4%/week; 95% CI, -2.3% to -0.5%; P<0.01) (Figure 3.2) and basal ganglia (coefficient, -0.7%/week; 95% CI, -2.5% to -0.1%; P=0.03) (Figure 3.2), after adjustment for WMI.
Those with ≥3 infections had lower mean FA over time in the PLIC (coefficient, -0.005; 95% CI, -0.002 to -0.008, P<0.01). There were no other regions with significant differences in the FA over time on the ROI analyses (all P≥0.10). The TBSS analysis of the preterm MRIs 30-34 weeks PMA revealed only delayed FA located within the posterior corpus callosum. In contrast, on the term scans at 37-42 weeks PMA lower FA was more widespread and involved the complete corpus callosum, the optic radiations and PLIC (Figure 3.3).
Figure 3.3 TBSS model for multiple infections. Shown in the pre-term (30-34 weeks GA) and term (37-42 weeks GA) model for infants with ≥3 infections compared to no infection group on a white matter skeleton map. The number of infants with ≥3 infections are indicated by the ‘N=’ with the denominator reflecting the total number of infants. R = right, L = left.

### 3.3.5 Developmental Outcomes

Thirty-six month corrected age outcome scores were available in 175 (82% of survivors) infants (median 35 months, IQR 34 – 37 months). There were no differences in the rates of WMI, IVH grade ≥3 or neonatal clinical factors (all P>0.05) in the follow-up and missed follow-up groups. Three infants were unable to complete the testing due to severe impairment thus were assigned scores of 49 (3.3 standard deviations from the
mean). On univariate analysis a greater number of infections in the neonatal period was significantly associated with poorer BSID-III motor composite score and PDMS-2 total, gross and fine motor scores, but not language and cognitive scores (Figure 3.4)(Table 3.3).

Table 3.3 BSID-III and PDMS-2 outcomes at 36 months CA. Divided by number of infection groups. Median (IQR) or Number [%]

<table>
<thead>
<tr>
<th></th>
<th>No Infection N=93</th>
<th>One Infection N=42</th>
<th>Two Infections N=24</th>
<th>Three or more Infections N=22</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSID-III Motor composite score</td>
<td>103 (97 – 115)</td>
<td>103 (94 – 110)</td>
<td>100 (91 – 107)</td>
<td>93 (76 – 107)</td>
<td>0.02</td>
</tr>
<tr>
<td>BSID-III Language composite score</td>
<td>109 (103 – 118)</td>
<td>112 (103 -115)</td>
<td>106 (83 – 115)</td>
<td>100 (94 – 112)</td>
<td>0.18</td>
</tr>
<tr>
<td>BSID-III Cognitive composite score</td>
<td>100 (95 – 110)</td>
<td>105 (95 – 105)</td>
<td>100 (95 – 105)</td>
<td>100 (90 – 110)</td>
<td>0.57</td>
</tr>
<tr>
<td>PDMS-2 Total motor</td>
<td>96 (90 – 102)</td>
<td>94 (88 – 97)</td>
<td>93 (88 – 97)</td>
<td>87 (74 – 94)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PDMS-2 Gross motor</td>
<td>96 (89 – 102)</td>
<td>94 (87 – 98)</td>
<td>91 (85 – 96)</td>
<td>84 (72 – 94)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PDMS-2 Fine motor</td>
<td>100 (94 – 103)</td>
<td>94 (91 – 97)</td>
<td>97 (88 – 103)</td>
<td>93 (85 – 97)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Figure 3.4 BSID-III outcomes at 36 months CA. Divided by infection groups with motor, language and cognitive composite scores reflected by the shades indicated in the legend. The middle line and box reflect the median and IQR respectively, with the 1.5 IQR reflected by the whiskers and open circles as any outliers. ** p<0.05 (all others p >0.05)

Unadjusted risk ratio (RR) and adjusted odds ratio (OR) assessments of BSID-III scores ≤85 and PDMS-2 ≤80, considered clinically significant impairments, are shown (Table 3.4). These illustrate that a greater number of infections was significantly associated with lower Bayley-III motor composite scores and poorer performance on the PDMS-2 total and gross motor testing when adjusted for potential confounding factors, with less impairment in fine motor scores (Table 3.4). There was no significant increase
in the incidence of cerebral palsy at 36 months CA with multiple infections (P>0.05) (Table 3.3)

Table 3.4 RR and ORs. Unadjusted RR and adjusted OR values for poor BSID-III and PDMS-2 scores in infants with three or more postnatal infections. Odds (95% CI)

<table>
<thead>
<tr>
<th>BSID-III Motor composite &lt;85</th>
<th>Unadjusted RR</th>
<th>P-value</th>
<th>Adjusted OR*</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSID-III Language composite &lt;85</td>
<td>2.53 (1.10 – 5.85)</td>
<td>0.02</td>
<td>1.99 (1.2 – 3.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>BSID-III Cognitive composite &lt;85</td>
<td>5.86 (1.34 – 25.6)</td>
<td>&lt;0.01</td>
<td>1.68 (0.94 – 3.00)</td>
<td>0.08</td>
</tr>
<tr>
<td>PDMS-2 Total Motor &lt;80</td>
<td>0.55 (0.14 – 2.11)</td>
<td>0.37</td>
<td>0.55 (0.16 – 1.83)</td>
<td>0.33</td>
</tr>
<tr>
<td>PDMS-2 Gross Motor &lt;80</td>
<td>3.06 (1.27 – 7.4)</td>
<td>&lt;0.01</td>
<td>1.93 (1.21 – 3.09)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PDMS-2 Fine Motor &lt;80</td>
<td>2.07 (1.06 – 4.03)</td>
<td>0.03</td>
<td>1.84 (1.18 – 2.85)</td>
<td>&lt;0.01</td>
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* Adjusted for Gestational age, maternal education, cerebellar hemorrhage and WMI volume

3.4 Discussion

Using multimodal MR imaging methods and follow-up assessments in a cohort of very preterm newborns our results showed that three or more postnatal infections are associated with delays in brain maturation, particularly within subcortical nuclei and white matter implicated in motor function, and with poorer motor outcomes at 36 months CA. Findings were associated with alterations in brain maturation over time, with differences most prevalent on the term-equivalent MRI scans reflected by involvement of the PLIC, corpus callosum and optic radiations. This is in agreement with previous research suggesting that poorer motor outcomes and cerebral palsy are more likely in infants with reduced FA in the PLIC, corpus callosum and white matter in cohorts of preterm infants, implicating these important regions in motor development and highlighting the utility of

Very preterm newborns are at-risk of multiple postnatal infections due to many factors. The innate and adaptive immune systems that respond to infectious antigens are significantly impaired in the preterm newborn, leading the infant to be more prone to infections (Cuenca et al, 2013; Lavoie et al, 2010). Preterm infants also have reduced trans-placental maternal antibody transfer and immature protein recognition receptors important for bacterial antigen presentation and antimicrobial immune recognition (Kan et al, 2016; Wynn et al, 2008). The consequence of this is an inappropriate inflammatory response to infections with activation of toll-like receptors in the innate immune system, that when associated with hypoxia-ischemia, can result in neural injury (Lehnardt et al, 2003). This may also lead to priming of the immune system for subsequent events manifested by greater concentrations of inflammatory markers and free radicals in the cerebrospinal fluid (CSF), as seen in infants with WMI (Ellison et al, 2005; Inder et al, 2002). Neuropathology analysis of infants with severe WMI has showed that tumour necrosis factor α (TNF-α) and interleukin-1β (IL-1β) levels are elevated in those infants with infection, with greater levels than what is seen with hypoxia-ischemia alone, further supporting their combined effects (Kadhim et al, 2001).

In a large epidemiologic study of over 6000 infants born at extremely low birth weight (less than 1kg), all types of infection, not just sepsis or meningitis, increased the risk of severe cognitive and motor impairments (Stoll et al, 2004). We also found no major difference in the outcomes between the different types or organisms of infection. We did not observe an increased risk for severe impairments with increasing numbers
of infections, with a low rate of cerebral palsy overall. Rand et al. showed that long-term neurodevelopmental outcomes at 9 years were more likely to be delayed in infants with postnatal infection, but with no difference in severe motor outcomes (Rand et al, 2016). Furthermore, in a small subset of infants, Rand et al. also reported that two or more infections increased the risk for motor impairments two-fold (RR 5.7 vs 2.6) in addition to increasing the risk of cognitive impairment (RR 2.1 vs 1.3) (Rand et al, 2016). This is supported by the literature on the additive effects of subsequent events, as described by Khwaja and Volpe, in which the preterm infant brain is sensitized following an initial event of infection or hypoxia-ischemia and has at a greater likelihood to be injured following subsequent events of inflammation and/or hypoxia-ischemia by a stimulus with a lower threshold than was necessary for the initial response (Eklind et al, 2001; Hagberg et al, 2012; Khwaja and Volpe, 2008; Leviton et al, 2013; Yanni et al, 2017).

The brain of the preterm newborn also has an immature blood brain barrier which makes it especially vulnerable to hypoxia-ischemia injury, particularly of the pre-OL, the prominent cell injured in WMI of the preterm infant (Back et al, 2001; Back et al, 2002; Gilles et al, 1976; Wang et al, 2012). In hypoxia-ischemia models of preterm brain injury a significant increase in pro-inflammatory markers is seen, suggesting that even in the absence of infection, a significant immune system response occurs (Albertsson et al, 2014; Fleiss et al, 2015). Of the infants in the three or more infection group, 78% had a CONS infection, which is not known to cause direct cerebral inflammation, but frequently occurs in conjunction with hypotension (Mallard and Wang, 2012). In our group of infants with three or more infections 85% had at least a single event of hypotension, opposed to 45% and 19% in those with one or two and no infections.
respectively. Previous work in rats using a lipopolysaccharide injection with hypoxia-ischemia injury model showed a greater duration of hypoxia was required to induce injury in older rats than younger rats, indicating the importance of the developmental window on the systemic response and the vulnerability of the preterm brain to injury (Eklind et al, 2005). How hypoxia-ischemia and systemic inflammation interact in the genesis of neonatal brain injury and dysmaturation requires further study (Back and Miller, 2014; Fleiss and Gressens, 2012).

Another potential contributor to brain dysmaturation includes injury resulting from hyperoxia with hypocarbia. BPD is a known complication of chronic ventilation in the preterm baby, with hyperoxia a well-established causative factor in the development of BPD (Buczynski et al, 2013). Of those infants with three or more infections in our cohort, 65% required long-term oxygen therapy compared to 5% for the no infection group. Hyperoxia and hypocarbia are potent vasoconstrictors important in the pressure-passive autoregulatory system of the preterm brain, and are independent risk factors in the development of WMI (Shankaran et al, 2006). The impacts with which these factors also contribute to the poorer outcomes seen in infants with multiple infection also needs further study.

Multiple postnatal infections have been shown to be associated with progressive WMI on subsequent MRI scans (Chau et al, 2009; Glass et al, 2008). Within our cohort we found no difference in the rate or volume of MRI-detected WMI, ventriculomegaly or IVH and the numbers or types of infections. There was an increased rate of cerebellar hemorrhage among those infants with three or more infections, which may reflect their lower gestational age at birth, making them more vulnerable to cerebellar hemorrhage.
This has potential implications on the outcomes analysis due to the role that cerebellar hemorrhage has in association with poor neurological outcomes (Tam et al, 2011). Adams et al, in a smaller subset of the current cohort, showed that postnatal infection was associated with slower increase in corticospinal FA over time than non-infected newborns (Adams et al, 2010). These findings were expanded upon within our study to show the maturational delays in those infants with three or more infections included the corpus callosum and white matter regions including the PLIC on both tradition DTI and TBSS analyses with supportive findings on MRSI analyses (Chau et al, 2012).

Follow-up at 36 months CA revealed that those infants with three or more infections had poorer motor development than those infants with fewer infections. In comparison to other large cohorts of preterm newborns with infection, we did not see an increased risk of cognitive impairment in those infants with higher numbers of infections (Rand et al, 2016; Stoll et al, 2004). Some of the differences seen from previous cohorts may be due to improvements in clinical practices and NICU care in recent decades. In addition, the majority of infants in this cohort are from high-resource settings which are known to impact cognitive and language outcomes greater than motor development (Cusson, 2003; Gross et al, 2001; Howard et al, 2011). The differences from previous literature, with a predisposition for motor tracts and impairments seen in our study, may be a result of the changes seen in the distribution of WMI, from PVL in older studies, to more diffuse WMI in contemporary studies, with diffuse WMI resulting in less widespread axonal changes (Buser et al, 2012). As the motor tracts are some of the first areas to myelinate, they are also the most susceptible to injury in the preterm brain (Sie et al, 1997; Welker and Patton, 2012).
Limitations

As described previously in Chau et al (Chau et al, 2012), many of the infants who had multiple infections would have had later MRI scans due to taking longer to become clinically stable, which would result in differences in postnatal age at the MRI between the infection groups. This would, however, favor the delayed images appearing more mature in the multiple infection groups underestimating the extent of the injury and time interaction. It should also be considered that the “clinical-only” infections could be the result of a virus, which would also have implications on the white matter development, immune system development and pro-inflammatory markers. However, we did not find neuroimaging abnormalities consistent with known descriptions of viral infections, nor were there any positive viral cultures. Overall, our findings support the need for further monitoring of infants with postnatal infections and continued assessment of how to improve the care of these infants.

Conclusions

Three or more postnatal infections are associated with delays in brain maturation, particularly in areas of motor function, with poorer motor outcomes at 36 months CA. These results highlight the vulnerability of the preterm brain to multiple postnatal infections and supports the potential for combined detrimental effects of inflammation and hypoxia-ischemia within the NICU. Furthermore, it suggests the need for a personalized approach to infection control in those infants with one or two infections. Preventing further infections in this vulnerable group of preterm newborns may have the potential to improve outcomes. More research is needed on the function and role of the developing immune system and the impact of hypoxia-ischemia and
infection on pro-inflammatory markers, as well as investigating potential therapies to correct this inflammatory imbalance within the preterm newborn.
Chapter 4

Summary of Main Findings

and

Future Directions
4.1 Conclusions

Through the use of multi-modal MR and outcome assessments I have shown that severe ROP is associated with delays in brain maturation with poorer outcomes at 18 months. This work supports the need for research that investigates the potential for recovery in the most severe ROP cases through balancing of growth factors and preservation of vision, and points to the significance of severe ROP as a marker of outcomes. Moreover, with similar methods of assessment, we were able to show impairments in brain maturation in widespread brain regions for those infants with greater numbers of postnatal infections resulting in poorer motor outcomes at 3 years, thereby stressing the strong association of infection with poorer brain maturation. This work supports several basic science and clinical studies on impairment of brain maturation of the preterm infant and provides clinicians and families with the knowledge to make informed decisions about likely outcomes and areas in which outcomes can be improved. Intervventional follow-up programs remain an important monitor for those children with developmental impairments, though their implementation varies by country, location, and therapist with wide differences in care and follow-up practices. While much is known about the potential risk factors and diseases that increase the likelihood of poorer outcomes in preterm infants, many parents and clinicians are left with more questions than answers at a critical period in development. The work presented in this thesis supports further research in postnatal infection and severe ROP and highlights each as a factor which may adversely influence long term outcomes.
4.2 Future Directions

In considering the future potential for this research, several areas of this study warrant expanded analysis and research, and this work may enhance the development of other research disciplines.

4.2.1 Retinopathy of Prematurity and Neurodevelopment

In the care of the infant with ROP, the optimal treatment window for this disorder remains unknown. Current therapies involve serial observations and monitoring, with treatments provided after the development of “threshold ROP” when it is believed treatment is needed in order to preserve vision. It is unknown at present whether even earlier intervention, with treatment on lower stages of ROP than what is currently provided, improves visual outcomes, neurodevelopmental outcomes and brain development. Furthermore, the optimal therapy for the treatment of severe ROP remains unknown. Several methods of treatment are currently in use, or in development, with laser photocoagulation therapy still considered one of the gold standard therapies, though is starting to fall out of favour. Intravitreous bevacizumab (Avastin), an anti-VEGF monoclonal antibody, has been used more recently for severe ROP. Treatment failure (Patel et al, 2012) and neurodevelopmental outcomes (Morin et al, 2016) have been shown in one study to be worse among those treated with intravitreous bevacizumab compared to laser therapy, which raises concerns of the impact of bevacizumab on the brain. Another therapy of interest includes oral propranolol which was shown in a clinical trial to reduce the progression of ROP to higher stages that require treatment, however significant safety issues with bradycardia and hypotension may limit its use (Filippi et al,
Several other monoclonal antibodies are also currently under investigation as potential ROP treatments, including ranibizumab (*Lucentis*), afibercept (*Eylea*) and pegaptanib (*Macugen*). Which method of treatment will be the preferred therapy, or whether each will be used in a more personalized approach to the stage and location of the eye disease present, will be determined as each method is validated and compared through clinical trials.

Visual outcomes of preterm children with ROP are improving, though preterm children continue to have poorer visual outcomes than their peers without ROP (Al-Otaibi et al, 2012; Cryotherapy for Retinopathy of Prematurity Group, 2002; Pearce et al, 1998). While it has been shown, that through quality improvement measures, severe ROP can be reduced (Lee et al, 2014), severe ROP continues to be a commonly observed disorder among preterm infants < 28 weeks GA. Prevention of ROP altogether remains an elusive goal given the complex mechanisms involved in its development, with infection, chronic oxygen requirements and other complications of preterm birth keys to its pathophysiology. In each of these conditions, the implications of various growth factors in modulating the effects on preterm brain development are largely unknown. While there remains ongoing research into the impact of IGF-1 and VEGF as potential treatments for ROP, there is also a field of research in using these treatments to improve brain development (Hansen-Pupp et al, 2011). The use of IGF-1 levels as a biomarker of delayed brain maturation in the preterm infant has been reported, with investigations underway currently using recombinant humanized IGF-1 to see if it assists in accelerating postnatal brain development (Hansen-Pupp et al, 2013), and whether it improves outcomes. There are also several ongoing clinical trials using IGF-1 therapy in an attempt to improve
neurodevelopment and outcomes in Autism (Riikonen, 2016), Rett’s syndrome (Khwaja et al, 2014), and Duchenne’s muscular dystrophy (Malik et al, 2012), with overall positive effects presented thus far. The effects of IGF-1 treatment in preterm infants remains unknown at present, but with ongoing research and further investigation it may become a staple therapy for the preterm infant in treating ROP and supporting brain growth. Animal research of IGF-1 supports its use in neuroprotection with reduced cytokine and tumour necrosis factor production (Sukhanov et al, 2007) and a reduced infarct volume in animal models of stroke (DeGeyter et al, 2016). Other populations that appear worth further investigation into whether IGF-1 can provide neuroprotection or improve brain growth and neurodevelopmental outcomes, include infants with neonatal infarcts or strokes, intra-uterine growth restriction (IUGR), and congenital heart disease (CHD).

While my analysis supports severe ROP as resulting in poorer outcomes, we need further longitudinal assessment to confirm its implications into childhood and beyond. Assessments at 18 months CA have been shown to have poor correlation with childhood outcomes and may overestimate cognitive impairments (Hack et al, 2005), hence follow-up at 3 years and 4.5 years will be important in determining whether these delays persist into early childhood. Furthermore, there are significant differences in the language abilities of infants at 18 months (Lung et al, 2009), many of which are considered within normal range, making the interpretation of language outcomes at this age difficult, potentially over or underestimating language impairments. It continues to be unknown which is the best assessment method for predicting poor childhood outcomes, and when is the optimal timing for assessing infants to accurately predict normal development in childhood and beyond. Furthermore, significant alterations in the developmental
trajectories of an infant can occur given the wide variation in childhood experiences and educational supports available to infants, making more accurate predictions of outcome difficult. With more accurate socioeconomic analysis techniques and calculating the effectiveness of individual early intervention approaches, we may better understand the post-NICU factors that are the most important in promoting childhood development.

Further characterization of the impairments of infants with severe ROP is needed, with developmental visual follow-up assessments. While visual outcomes are much improved compared to previous decades, infants with severe ROP continue to have mild visual disturbances compared to their unaffected peers (O’Connor et al, 2002). Visual-motor integration and visual perception scores have been shown to be highly correlated with fine motor scores in preterm infants (Goyen et al, 2008). The extent to which these visual-motor impairments are present in severe ROP, and the impact of these impairments on infants’ brain development, remains unknown. Similarly, visual impairments are also common in infants with brain injury (Pike et al, 2008), and it is unclear to what extent that visual dysfunction impacts motor integration and performance.

Developmental coordination disorder (DCD) is a clinical childhood disorder with severe implications for childhood cognitive and motor performance, and is commonly associated with behavioural problems. DCD is hard to detect early in infants and remains a poorly recognized disorder by clinicians. Despite this, DCD is a common disorder in very preterm children at school age and is seen frequently in those infants with ROP (Zwicker et al, 2013). Visual disturbances with poor ocular alignment, binocular vision and refractive errors have all been shown to occur in a high prevalence in children with DCD (Creavin et al, 2014). This further suggests that mild impairments to the visual system
have the potential to adversely affect the motor and cognitive control of the developing brain. To better understand the pathophysiology for these impairments, more research is needed into the visual outcome monitoring of very preterm children in order to properly assess the impacts of these visual outcomes on development, and to assess their true prevalence.

Follow-up MRIs in childhood or adolescence of infants born very preterm are also needed to monitor whether slower maturation on imaging methods DTI and TBSS continues over subsequent analysis. This is an important step in providing information on long-term outcomes from severe ROP, and will allow correlation over time of preterm and term-equivalent studies. These studies will also be of importance in determining which factors associated with childhood result in the greatest improvements in brain development into childhood, as we continue to search for additional interventions to improve outcomes and maximize each child’s potential.

In addition to the techniques utilized in this thesis, investigating severe ROP with other measures of brain development such as MRSI and probabilistic tractography may provide more information about the nature of the maturational deficits present. Tractography and TBSS methods have been shown to correlate with visual outcome scores in preterm infants, with delays in the optic radiations (Bassi et al, 2008), though the link with severe ROP was not explored in this group. Emerging techniques, such as diffusion kurtosis imaging, magnetization transfer ratio, myelin water fraction and quantitative susceptibility mapping, have all been shown to reflect features of white matter microstructure (Groeschel et al, 2016), and are techniques of interest in determining the method best predictive of outcomes in preterm infants. Resting state functional
connectivity MRI at term-equivalent age has previously shown that infants with WMI have aberrant connectivity, with the degree of variation determined by the severity of injury (Smyser et al, 2013). The use of functional MRI in those infants with severe ROP could be explored in this population to determine whether the maturational delays correlate with not only alterations in the resting state networks, but also in the visual-motor integration pathways, if utilized in older individuals. As MR methods continue to advance, it is hopeful that we will be able to visualize better the cortex and its development. Current techniques with volumetric analysis have shown global cerebral cortex volumes to be reduced in preterm infants at term-equivalent age (Inder et al, 2005) and in WMI (Inder et al, 1999), with cortical volumes reduced in cortical lobes in late childhood (Kesler et al, 2004), though these techniques have been unable to assess regional cortical volumes in sensitive areas in early infancy. Furthermore, alterations to the cortical volumes from severe ROP, particularly of visual regions in the primary visual cortex and surrounding regions, is another way in which neuroimaging can help us to understand the pathophysiology of these maturational delays, and assist in identifying ways in which we can improve outcomes.

4.2.2 Multiple postnatal infections and neurodevelopment

In the care of the infant with postnatal infection, infection control measures are largely provided universally and across units in attempts to reduce the incidence of infections. Despite this, a large number of preterm infants develop nosocomial infections during their NICU course. Improvements in NICU infection control have been shown to reduce infection rates and improve outcomes (Davis et al, 2016), though the specific features of a sepsis quality improvement project that result in lowered infections is
unknown. Increased hand hygiene results in significant decreases in nosocomial infections, shown in several studies (Lam et al, 2004; Won et al, 2004). A large-scale structured quality improvement project that included hand hygiene with stringent catheter-insertion policies and early advancement of enteral feeds was shown to reduce infections from 17% to 15% (Wirtschafter et al, 2011). While this is a significant improvement, the high rate of infants with infection is still concerning. In identifying which factors are the most important to reducing infection rates, and which can improve outcomes, the relatively high cost of implementing these procedures can be reduced. Of further interest is whether these procedures can be implemented in lower income countries where nosocomial infections contribute to a high rate of morbidity and mortality (Zaidi et al, 2005).

Providing personalized infection control measures to those infants most susceptible to the adverse effects of infection, is an intriguing concept of care that the results of this thesis supports as a potential intervention to improve outcomes. Further investigation is needed into whether, for those infants with one or two infections, outcomes are improved by more individualized infection control measures, such as reduced contact, more stringent guidelines for hand washing and line insertion, or other measures. A personalized care package, such as that implemented to reduce the incidence of intraventricular hemorrhage, has the potential to improve outcomes (Schmid et al, 2013). How these measures can be applied, while also allowing for the other features also important for the growth of the infant, are considerations that would need to be taken into account.
What also remains an important consideration in reducing the impact of infection is determining whether there exists a specific period of vulnerability in which postnatal infections are more likely to impact brain development. With the immature immune system of the preterm newborn, particularly before 28 weeks, it would seem that this is a period of particular importance. In addition, considering what we currently understand about the pre-OL cell and its maximal period of susceptibility, between 23 to 32 weeks GA (Back et al, 2001), it would seem reasonable to consider the period of maximal impact from infection during this period as well. Despite this, it is not known whether the timing of the infections is an important factor, and whether each infection has an equally cumulative impact on the white matter development. This highlights the question whether all infections are of equal importance in brain development and long term outcomes, and stresses what factors need more attention when improving the care provided.

As has been discussed previously in this thesis, inflammation and hypoxia-ischemia appear to have independent, yet contributory roles on the influence of infection on the brain. Some of this is seen with the impact of the early physiological alterations calculated via the Score for Neonatal Acute Physiology-II (SNAP-II), a measure of illness severity in the first 12 hours of NICU admission which calculates a morbidity and mortality risk score, and includes early measures of mean blood pressure, temperature, oxygenation, serum pH among other clinical factors. A higher SNAP-II has been shown to correlate with greater mortality (Harsha and Archana, 2015), delays in the corticospinal tracts (Zwicker et al, 2013), and poorer cognitive, behavioural, social, educational, and neurological outcomes at 10 years (Logan et al, 2017). Recent research in the fields of inflammation and hypoxia-ischemia injury has improved our understanding of the
contribution of those factors on the brain, and despite some divergence of these fields in clinical research, there is considerable research to support their joint impact upon the developing brain (Albertsson et al, 2014; Girard et al, 2008; Khwaja and Volpe, 2008). In developing and assessing neuroprotective treatments and mechanisms in the preterm brain, a greater understanding is needed of the numerous factors involved in the pathophysiology with which infection, inflammation and hypoxia-ischemia impact the brain. Investigating the effects of pro-inflammatory biomarkers on preterm brain development and white matter maturation is another area of interest, in addition to exploring potential therapies to mitigate these effects. Furthermore, consideration should be given to whether inflammation and hypoxia-ischemia contribute to other clinical disorders in the preterm newborn, such as ROP, NEC and BPD.

Another growing area of research includes exploring the effects of many of the drugs that are used in the treatment of the sick neonate. The impact of antibiotics, commonly prescribed to infants with infections, is of increasing concern with reports of poorer outcomes among those exposed, particularly early in life (Greenwood et al, 2014; Kuppala et al, 2011). Postnatal exposures to hydrocortisone or dexamethasone have been shown to be related to impaired cerebellar growth, though antenatal betamethasone did not (Tam et al, 2011). Midazolam, a commonly prescribed drug used for sedation and analgesia in the NICU, has been shown to result in decreased hippocampus growth, as well as poorer outcomes (Duerden et al, 2016). Morphine, another analgesic agent, has been shown to be associated with poorer cerebellar growth (Zwicker et al, 2016), cognitive and motor outcomes (Grunau et al, 2009) as well as a lower IQ (de Graaf et al, 2011). As a result of these findings, many NICUs are adapting to encourage non-
pharmacological interventions for analgesia with non-nutritive sucking, swaddling, music, kangaroo care, swaddling and facilitated tucking all used in attempting to reduce the effect of analgesia on the infant. While unequivocal evidence to support their use is not yet available, there are encouraging reports of their analgesic abilities (Cignacco et al, 2007). Sucrose is an agent that has been used extensively with non-nutritive sucking during painful procedures and has shown favourable results in reducing the effects of pain (Stevens et al, 2008). As we learn more about the effects of various medications commonly used in the NICU, we will gain more insight into the areas with which we can further improve the care provided.

One such area of research that has resulted in significant awareness and changes in practice has been in the field of neonatal pain. With growth of awareness in the ability for the preterm neonate to experience pain, we have learned much about how the preterm infant responds to pain, and how these painful experiences can adversely impact brain development (Brummelte et al, 2012; Ranger et al, 2013) as well as long term cognitive IQ scores (Vinall et al, 2014). Increased pain scores in the preterm period results in a slower increase in the FA of the corticospinal tract, even after adjusting for postnatal risk factors and gestational age (Zwicker et al, 2013). Neonatal pain has also been shown to impact the visual-perceptual abilities, using magnetoencephalography (MEG), in school age children who had greater skin breaking procedures in the NICU period (Doesburg et al, 2013). Similarly, neonatal pain results in alterations of the clinical responses to pain (Grunau et al, 2001) and to the hypothalamic-pituitary-adrenal axis with lower cortisol responses to stress (Grunau et al, 2005). The link between pain and abnormal brain growth is seen in long term follow-up with decreased cortical thickness of the frontal and
parietal lobes at 8 years of age in those children who had greater number of NICU painful procedures as very preterm infants (Ranger et al, 2013). While it is clear that pain is an important factor in the development of the preterm infant, little is known about whether there are other factors associated with infection that potentiate these impacts. Considering the significant number of procedures that are part of the management and monitoring of those infants with infection, it is also unclear how much of the impacts of multiple infections can be attributed to the increased number of painful procedures.

The microbiome of the preterm infant, which represents all the bacteria living in or on the host, is a field with which there is growing interest in how to maximize outcomes through investigating, what has been termed, the “microbiota-gut-brain axis” (DiBartolomeo and Claud, 2016). Research into the microbiome of meconium in preterm infants has shown that abnormal gut flora, likely introduced through the swallowing of amniotic fluid, correlates with a higher likelihood of preterm birth and may contribute to the early inflammatory response (Ardissone et al, 2014). Investigation into the healthy microbiota, in a mouse model of behavioural abnormalities and autism, showed improvements in the behaviours exhibited in those mice treated with probiotics (Hsiao et al, 2013). This has coincided with an expansion into the investigation of the role of probiotics in the preterm population, with studies reporting improvements with reduced death and incidence of NEC (Lin et al, 2008), though without an impact upon sepsis (Mihatsch et al, 2012) or early neurodevelopmental outcomes (Chou et al, 2010). As we learn more about the healthy microbiota of the preterm newborn, we will be able to further explore the impact of infection on this important system, and how treatments, such as with probiotics, may help improve outcomes in this vulnerable population.
Nutritional requirements and treatments is another field in which significant research exists, yet the metabolic requirements and optimal nutritional support of the infant with infection are not known. Increased brain growth in the NICU of the very preterm infant, catch-up growth in particular, and increased brain volumes are associated with improved outcomes at discharge and follow-up (Cheong et al, 2016; Franz et al, 2009). Conversely, brain atrophy, seen on repeat imaging, is associated with poorer cognitive, motor and behavioural scores at 3 year follow-up (Horsch et al, 2007). Early brain growth has a direct relationship with early nutrition with improved growth seen with higher protein intake (Cormack and Bloomfield, 2013), higher amino acid intake (Poindexter et al, 2006), higher energy and lipid intake (Beauport et al, 2017), as well as better growth with earlier enteral feeding (Dinerstein et al, 2006). In addition, the composition of the lipids is an important factor in development with increased levels of docosahexaenoic acid (DHA) and reduced levels of linoleic acid levels associated with decreased odds of IVH, increased brain microstructural development and improved developmental scores on follow-up (Tam et al, 2016). Suboptimal nutrition in the preterm period has also been shown to be predictive of childhood intelligent quotient (IQ) scores and a higher frequency of cerebral palsy in late childhood (Lucas et al, 1998). Similarly, increased intake of breast milk in the first 4 weeks of life in very preterm infants showed significant improvements in deep grey matter volumes at term (Belfort et al, 2016). These changes did not persist on follow-up imaging at 7 years, but subjects with greater breast milk intake showed better performance on IQ scores, mathematics, working memory, and motor functioning (Belfort et al, 2016). Using advanced MRI techniques, higher energy and fat intake has been shown to correlate with improved brain growth, particularly of the basal ganglia and
cerebellum, as well as a positive correlation with FA in the PLIC and higher cognitive and motor scores at 2 years CA (Coviello et al, 2017). Moreover, early establishment of full enteral breast feeding in very preterm infants has been shown to be a protective factor in the development of postnatal sepsis, suggesting a potentially protective effect from the early breast milk (Ronnestad et al, 2005). Reduced rates of infection have also been observed to be greater in breast milk fed infants than formula fed infants, further supporting the potential immune modifying benefits of breast milk (Hylander et al, 1998). While it is clear that early and adequate nutrition, preferably with breast milk, are important factors in the growth of the preterm brain, we do not have good evidence on the effects of infection upon the nutritional intake of the preterm infant. Furthermore, in the clinical treatment of the sick infant, feeds are often held or stopped in the acute management of infection, and switched to parenteral nutrition. While this treatment may be necessary in the evaluation and workup for NEC, the reduced enteral feeds provided during this time may adversely affect the brain growth during a time of particular vulnerability, and is a potential contributing source of maturational delays and poor development in those infants with multiple events of infection. More research is needed to further understand this relationship.

As noted in chapter 3, a significant number of infants in the multiple infection group had cerebellar hemorrhage. We know that cerebellar hemorrhage is an important factor in the development of infants, and is associated with several long term outcome developmental disorders, including motor and cognitive disabilities (Limperopoulos et al, 2007), autism (Wang et al, 2014) and cerebral palsy (Johnsen et al, 2005). What is also well known is that there are close connections between the cerebrum and cerebellum
with reduced volumes of the cerebellum seen in those infants with unilateral cerebral injury (Limperopoulos et al, 2005), with impacts seen in the pons of the brainstem in those infants with large cerebellar hemorrhages (Parodi et al, 2016). Yet, it is not well known what is the impact of cerebellar hemorrhage on the growth and development of the brain in the infant with infection, who may have a greater vulnerability to injury. Conversely, it remains undetermined how cerebellar hemorrhage impacts the outcomes of the child with infection and whether it factors into the poorer outcomes described.

The study of the epigenetics is a field of significant interest in predicting long term outcomes in preterm infants. Epigenetics is a field that involves the study of the impacts of environmental factors upon genetic expression. Through the processes of DNA methylation and acetylation, genetic signaling can be reduced or enhanced, potentially resulting in alterations in disease expression. Epigenetic factors have been shown to be altered in other populations of children with neurodevelopmental disabilities, such as Rett’s and Fragile X syndromes (Collins et al, 2004; Gu et al, 1996). Various other at-risk groups of preterm infants have been shown to have methylation alterations with poorer attention, self-regulation and quality of movements (Lester et al, 2015), traits which are commonly observed in children born preterm. Bacterial organisms themselves have been shown to produce “epimutations” in the epigenome with alterations that can potentially result in disruptions to host cell immune function and pathogen identification (Bierne et al, 2012). Furthermore, in a mouse model of prenatal infection, stable alterations in the DNA methylation within the cells of the prefrontal cortex and nucleus accumbens have been described (Richetto et al, 2017). Those alterations were seen in several regions important for neural function, WNT signaling, and GABA differentiation (Richetto et al, 2017). ROS
have been shown to impact DNA methylation through the formation of oxidized DNA lesions which are structurally similar to the methylated DNA cytosines (Lewandowska and Bartoszek, 2011). In addition, ROS can impact the histone-modifying enzymes involved in acetylation and methylation with direct effects upon the epigenetic fluctuations in the cell (Simpson et al., 2012). The impact of multiple infections on the epigenome of the preterm infant, and what epigenome alterations correspond with outcomes, is a field that is largely unexplored.

In considering alterations to epigenetic factors, the "epigenetic clock" is a feature of DNA methylation that has been developed as a biomarker of aging (Horvath, 2013). A preterm infant epigenetic clock using DNA methylation has been shown to be a strong predictor of GA in two separate birth cohorts, comparable to antenatal ultrasound methods estimates (Bohlin et al., 2016; Knight et al., 2016). In utilizing this epigenetic clock, higher maternal socioeconomic status and higher birthweight percentiles were associated with an accelerated biological age in preterm infants (Knight et al., 2016). Age acceleration using the epigenetic clock in adults has been shown to be associated with obesity (Horvath et al., 2014), HIV infection (Horvath and Levine, 2015), Down’s syndrome (Horvath et al., 2015), Alzheimer’s disease (Levine et al., 2015) and Parkinson’s disease (Horvath and Ritz, 2015), and is said to predict cancer, cardiovascular and all-cause mortality (Christiansen et al., 2016; Marioni et al., 2015; Perna et al., 2016). The preterm epigenetic clock has been used to show that age acceleration is associated with advanced maternal age, pre-eclampsia, previous fetal demise, lower 1-minute APGAR score, antenatal betamethasone and female sex; and that age deceleration is associated with insulin-treated gestational diabetes mellitus in a previous pregnancy (Girchenko et
al, 2017). The impact of many post-natal events upon the epigenetic age of the preterm infant remains unknown, yet this area has the potential to provide important information on alterations to genetic expression, and may aid in the understanding of the pathophysiology of the many neurodevelopmental impairments of the preterm infant.

There is much to be explored and investigated in the preterm infant in improving outcomes and contributing to the literature of pathophysiology of diseases that impact infants and children, while also assisting in the expansion of our understanding of brain development and diseases.


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