Multimodal Characterisation of the Infant Response to Retinopathy of Prematurity Screening and Treatment

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Great are the works of the LORD

They are studied by all who delight in them.

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This thesis is dedicated to my daughter Antonia, before whom all paths lie open, and for whom I pray every step will be crowned with blessing.

Thesis Contribution

Recruitment

I screened the Neonatal Intensive Care Unit inpatients weekly for eligibility for recruitment into the studies presented in this thesis. I approached parents of eligible infants and offered a Patient Information Leaflet if they were interested. I reapproached parents who had demonstrated interest in order to receive informed consent for participation in the study. I maintained records of all patients screened for the study and the outcome of every approach.

Data collection

I oversaw the study occasion for each participant. Each study involved attaching an electroencephalography montage, cardiac monitoring, and a saturations probe to the participant. A facial video was recorded prior to the ophthalmological procedure. During the procedure, the data recording computers were actively monitored and events marked in the recording trace. A second video was recorded after the procedure, and once data collection was complete, all study monitoring leads were removed. For each study occasion I was assisted by two other members of the research group. We were flexible in our roles for each study, and therefore over the course of the project I participated in all activities related to the study occasion.

Data analysis

I devised the experimental design together with my co-supervisor, and discussed my approaches to data analysis with my supervisory team. I adapted source code from three group members (Caroline Hartley, Kirubin Pillay, Tricia Adjei) and the R HRV Project, in order to carry out the analysis. I pre-processed and analysed the data for each study participant. I calculated the clinical pain scores (Premature Infant Pain Profile - Revised), characterised the trends in heart rate and oxygen saturations, and calculated the changes in heart rate variability and brain activity for all participants.

Journal Articles

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Presentations

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Contents

Abstract

Retinopathy of prematurity (ROP) is a condition which affects premature infants and is a cause of childhood blindness. Screening is performed repeatedly during the preterm period, using binocular indirect ophthalmoscopy (BIO), to identify disease at a treatable stage; unfortunately screening and treatment are considered to be painful and stressful for infants. Pain and stress during the preterm period can lead to negative consequences for infant development. Reduction of pain and stress during ROP procedures has therefore been attempted using pharmacological and non-pharmacological strategies. However, it is challenging to evaluate the effectiveness of such interventions, due to limitations in the accurate measurement of infant pain and stress.

In this thesis, novel approaches to quantifying infant pain and stress evoked by ROP procedures are presented. Infant brain activity was characterised using quantitative electroencephalography (EEG) analysis to test the hypothesis that ROP screening evokes noxious-related changes in infant brain activity. The results of this study suggest BIO ROP screening evokes a significant increase in higher frequency brain activity (12 - 30 Hz), and that increase in relative beta power may be a measure of nociception in preterm infants.

Infant cardiac autonomic reactivity was characterised using heart rate variability (HRV) analysis to test the hypothesis that ROP screening evokes stress-related autonomic changes. The results of this study suggest BIO ROP screening evokes significant reduction in HRV measures of parasympathetic nervous system activity, indicating a physiological stress response in preterm infants.

An approach to characterising the infant response to non-contact ultra-widefield photography (Optos screening) and ROP treatment was also demonstrated. Recruitment of subjects was curtailed by the outbreak of COVID-19, therefore the investigations are presented as an example of approaches which could be performed in a larger sample size.

In summary, the research described in this thesis aims to contribute to understanding of the infant experience of ROP procedures; to characterise changes in noxious-related brain activity and stress-related cardiac reactivity evoked by BIO ROP screening, and to use these measures to investigate the infant response to an alternative screening method and to ROP treatments. Improved understanding of the infant experience of ROP screening and treatment may allow clinicians to better identify and treat infant pain and stress during essential clinical procedures.

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Nomenclature

BIO	Binocular indirect ophthalmoscopy
bpm	Beats per minute
ECG	Electrocardiography
EEG	Electroencephalography
ERP	Event-related potential
HRV	Heart rate variability
LQ	Lower quartile
NICU	Neonatal intensive care unit
NN	Normal-to-normal interval (beat to beat interval after artefact removal)
PIPP-R	Premature infant pain profile - revised
pNN50	Number of interval differences greater than 50ms / Total number of NN intervals
ROP	Retinopathy of prematurity
SDANN	Standard deviation of the average NN interval over 5 minutes
SDNN	Standard deviation of the NN interval
SDNN index	Average of the standard deviation of the NN interval over 5 minutes
SDSD	Standard deviation of successive differences between NN intervals
UQ	Upper quartile
VEGF	Vascular endothelial growth factor

Chapter 1

Introduction

1.1 Thesis Overview

Birth before 37 completed weeks of gestation, termed preterm birth (World Health Organisation, 1977), affects 14.8 million live births per year globally (Chawanpaiboon *et al*, 2019). In economically developed countries, infants born prematurely are supported in a clinical environment termed the Neonatal Intensive Care Unit (NICU). The NICU provides essential care to this fragile population, such as airway and breathing support, maintenance of body temperature, and identification and treatment of infection. However, the NICU differs significantly from the uterine environment (Als and McAnulty, 2011), and may present challenges to preterm infant neurodevelopment (Grunau, 2013).

During normal gestation, certain external stimuli are processed by the developing brain. For example, the visual, auditory and the somatosensory cortical regions receive afferent input from physiologically-occurring stimuli before birth (Fabrizi *et al*, 2016). However after preterm birth, the neonate is exposed to greater levels of light, sound and tactile stimulation in the NICU environment than would occur *in utero*, which may influence the developing neural pathways (Blackburn, 1998; Als and McAnulty, 2011).

Noxious stimulation received in the NICU is of particular concern for infant neurodevelopment (Walker, 2019; Williams and Lascelles, 2020). Premature infants may undergo up to 17 clinically-necessary painful procedures daily while admitted to the NICU (Carbajal *et al*, 2008; Cruz *et al*, 2016). These include venepuncture, heel lance blood sampling, endotracheal suction, chest drain insertion, lumbar puncture, and eye screening.

Noxious input is not a feature of normal gestation, and nociceptive pathways do not receive physiological afferent input during brain development (Verriotis *et al*, 2016). Exposure to early life pain may adversely influence neurodevelopment in premature infants, including altered brain structure and function, reduced intelligence quotient, and altered pain perception (Brummelte *et al*, 2012; Ranger *et al*, 2013; Doesburg *et al,* 2013; Vinall and Grunau, 2014; Vinall *et al*, 2014).

It is therefore critical to identify and treat infant pain. However, both diagnosing pain and providing effective pain-relief strategies are challenging in the infant population (Simons *et al*, 2003). The International Association for the Study of Pain (IASP) defines pain as 'an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage' (IASP, 2020). Pain, therefore, is a subjective experience which cannot be fully communicated in non-verbal subjects including infants.

Certain approaches have been used as surrogate measures of pain in infants. These include measurements of physiological parameters, such as pain-associated changes in heart rate, heart rate variability, and oxygen saturation (Stevens and Johnston, 1994; De Jonckheere *et al*, 2011; Stevens *et al*, 2016), and characterisation of behavioural changes, such as facial expression, body movement, and cry (Grunau and Craig, 1987; Rushforth and Levene, 1994). Pain associated with clinically-necessary procedures such as heel lancing, chest drain insertion, and eye screening has been estimated in preterm infants using these methods (Boyle *et al*, 2006; Stevens *et al*, 2014; Buyuktiryaki *et al*, 2018).

Brain activity has also been used as a surrogate measure of the pain experience in infants. Using electroencephalography (EEG), a characteristic pattern of brain activity has been identified following acute painful procedures such as heel lancing and vaccination (Slater *et al*, 2010c; Fabrizi *et al*, 2011a; Verriotis *et al*, 2015). This central measure of noxious-related brain activity has been demonstrated to encode stimulus intensity (Hartley *et al*, 2015), and is modulated by pain-relieving interventions such as gentle stroking, paracetamol, and topical anaesthesia (Hartley *et al*, 2017; Gursul *et al*, 2018; Cobo *et al*, 2021).

A combination of measures may provide the best estimate of the infant pain experience (Van der Vaart *et al*, 2019). Acute noxious clinical procedures such as heel lancing and inoculation have been characterised using a multimodal approach (Slater *et al*, 2010; Hartley *et al*, 2015; Hartley *et al*, 2017; Gursul *et al*, 2018; Cobo *et al*, 2021). However, longer duration, complex procedures such as lumbar puncture, eye screening, and intubation have not been assessed in this manner.

In this thesis, screening and treatment for retinopathy of prematurity (ROP) are selected as the central focus for novel research into the infant pain experience. ROP is a disease of the retinal vasculature, and is a cause of childhood blindness (Blencowe *et al*, 2013; Tan *et al*, 2015; Norman *et al*, 2019). In many countries, a programme of screening exists in order to detect ROP at a sufficiently early stage at which treatment is possible (Royal College of Ophthalmologists, 2008; Fierson, 2013). Unfortunately screening and treatments for ROP are considered to be painful and stressful for infants (Belda *et al*, 2004; Gal *et al*, 2005; Grabska *et al*, 2005; Rush *et al*, 2005; O'Sullivan *et al*, 2010; Sun *et al*, 2010; Kandasamy *et al*, 2011; Mandel *et al*, 2012; Hartley *et al*, 2018; Sethi *et al*, 2020).

Screening for ROP using binocular indirect ophthalmoscopy (BIO) involves examining the retina with bright illumination, while the infant is gently restrained and the eyelids held open using an infant eyelid speculum. During the examination, the eye is stabilised using a scleral depressor. The screening examination takes

place repeatedly during the neonatal period, until the retinal vasculature is satisfactory. Alternative screening methods include RetCam screening, a method of fundal photography that requires contact with the ocular surface, and Optos screening, a ultra-widefield method of fundal photography which requires holding the infant to the camera aperture.

Treatment for ROP is performed either by applying laser photocoagulation to the retina, or by intravitreal injection of anti-vascular endothelial growth factor (anti-VEGF). In this centre, laser treatment is performed to awake, sedated infants, and intravitreal injection is performed to awake, alert infants in the NICU setting.

In this thesis, screening and treatment for ROP were selected in order to investigate whether multimodal pain assessment can be used to assess longer duration, complex painful procedures performed in preterm infants in the NICU. Behavioural, physiological and electrophysiological measures are used to characterise the infant nociceptive response to BIO ROP screening, Optos ROP screening and ROP treatments.

In this introductory Chapter, further background information will be provided regarding ROP, including the pathogenesis and epidemiology as well as details of screening and treatment. An overview of brain development will be given, with particular emphasis on the development of nociceptive pathways. Next, a discussion of early life pain will include the history of pain research in infants, the phenomenon of pain perception, and the consequences of early life pain. Further detail will be provided regarding previous studies into multimodal pain assessment including behavioural, physiological and brain-derived measures, upon which the work in this thesis rests. The Chapter will end with an overview of the thesis aims.

1.2 Retinopathy of Prematurity

1.2.1 Overview

ROP is a retinal vascular condition which is a cause of childhood blindness (Blencowe *et al*, 2013; Tan *et al*, 2015; Norman *et al*, 2019). The disease affects infants of low gestational age and birthweight (Appelbaum, 1952; Hellström *et al*, 2013; Razak and Faden, 2020). In the UK and many other developed countries, a programme of screening exists to detect ROP at an early stage (Royal College of Ophthalmologists, 2008; Fierson, 2013; Roohipoor *et al*, 2016). In most infants, ROP resolves spontaneously and does not progress to treatment-indicated disease (Foos, 1987; International Committee for the Classification of Retinopathy of Prematurity, 2005; Hellström *et al*, 2013). Severe ROP is treated with diode laser photocoagulation and intravitreal injection of anti-vascular endothelial growth factor (anti-VEGF).

1.2.2 Pathogenesis

The retina begins to develop from the fifth week of life (O'Rahilly, 1975). Around the sixteenth week of life, blood vessels form at the centre of the retina near the optic nerve, and grow outward in an organised branching pattern over the retina to reach the periphery (*ora serrata*) (Ashton, 1970). The relatively low oxygen concentration in avascular retina leads to the release of VEGF (Alon *et al*, 1995), a cytokine which mediates blood vessel growth in the presence of insulin-like growth factor 1 (Hellström *et al*, 2001). Retinal angiogenesis is usually complete by the eighth month of life (Ashton, 1970).

The pathogenesis of ROP can be described in two distinct phases: firstly, slowing of blood vessel growth due to high oxygen concentration; secondly, uncontrolled blood vessel growth due to low oxygen concentration (Smith, 2003; Hellström *et al*, 2013). When an infant is born prematurely, retinal angiogenesis is incomplete. During resuscitation of the premature infant, high-concentration oxygen is administered in order

to improve chances of survival for the infant (SUPPORT, 2010; BOOST II, 2013). High tissue oxygen concentration in the avascular retina results in reduction of VEGF and premature cessation of angiogenesis (Alon *et al*, 1995; Owen and Hartnett, 2014; Darlow and Husain, 2019).

During the preterm period, infant growth results in increased metabolic demand from the retina. The avascular retina becomes relatively hypoxic, signalling release of VEGF (Hartnett and Penn, 2013). Angiogenesis restarts at the junction between perfused and non-perfused retina (Smith *et al*, 1994) but proceeds in an aberrant manner; new vessels grow into the vitreous gel rather than along the retinal surface (Foos, 1987). The new vessels are permeable, due to loss of the blood-retinal barrier (Smith *et al*, 1994), resulting in retinal haemorrhage and disruption of the vitreous structure (Coats, 2005). Retinal detachment is caused by fibrovascular proliferation and contraction of vitreous collagen fibres (Faris *et al*, 1971; Chan-Ling *et al*, 1992), and results in vision loss.

1.2.3 Epidemiology

In 2010, an estimated 32,000 infants worldwide were visually impaired or blind from ROP (Blencowe *et al*, 2013). In developed countries, approximately 66% of preterm or low birthweight infants develop ROP (Tasman, 1988). The epidemiology of ROP is influenced by trends in neonatal care. High-income countries experienced the "first epidemic" of ROP, due to the widespread use of 100% oxygen therapy for premature infants (Campbell, 1951; Patz *et al*, 1952; Ashton *et al*, 1954; Flower and Patz, 1971; Patz, 1975; Owen and Hartnett, 2014; Gilbert *et al*, 2019). The epidemic ended after oxygen therapy was optimised, both in terms of reduced target oxygen saturations following landmark clinical trials (SUPPORT, 2010; BOOST II, 2013), and improved oxygen saturation monitoring through pulse oximetry (Wasunna and Whitelaw, 1987). It is now well understood that 100% oxygen administration in the resuscitation of premature infants is a significant risk factor for ROP, while maintain-

ing oxygen saturations below 90% is significantly associated with increased risk of neonatal mortality (SUPPORT, 2010). Therefore, for infants requiring supplemental oxygen where pulse oximetry is available, it is recommended to maintain a target range of 89-95% oxygen saturation (World Health Organisation, 2016; Sweet *et al*, 2017; Darlow and Husain, 2019).

However, ROP continues to occur despite optimal clinical practice for oxygen supplementation (Hellström *et al*, 2013). This "second epidemic" of ROP is due to the survival of previously non-viable premature infants (Wood *et al*, 2000; Gilbert *et al*, 2019). Further risk factors for progression to severe ROP have been characterised including low levels of insulin-like growth factor 1 (Hellström *et al*, 2001; Perez-Munuzuri *et al*, 2010), systemic illness (Klinger *et al*, 2010; Lundgren *et al*, 2016; Kim *et al*, 2018), and poor postnatal weight gain (Hellström *et al*, 2009; Van der Veen *et al*, 2013; Lin and Binebaum, 2019).

1.2.4 Screening and Treatment

In developed countries, approximately 1-6% of preterm or low birthweight infants develop ROP requiring treatment (Tasman, 1988; Slidsborg *et al*, 2008; Adams *et al*, 2017; Gerull *et al*, 2018). If timely treatment is not delivered, the risk of visual impairment is 64% (Palmer *et al*, 2005) and the risk of blindness is 43% (Tasman, 1988). Therefore, screening for ROP is recommended to identify disease at a stage at which treatment is likely to be effective (Fierson, 2013).

In the UK, ROP screening is performed for all infants born at less than 32 weeks gestation, or birthweight less than 1501g (Royal College of Ophthalmologists, 2008). Screening is performed between 3-4 weeks postnatal age in infants born extremely premature (less than 27 weeks gestation). In infants born very premature (between 27 to 32 weeks gestation), and in infants born at more than 32 weeks gestation but with birthweight less than 1501g, the first ROP screening takes place between 4-5 weeks postnatal age. Screening is repeated at weekly intervals when the retinal

vasculature is very immature, or when there are signs of ROP development. Otherwise screening is repeated fortnightly until the retinal vessels have reached the *ora serrata*, which usually occurs after 37 weeks gestational age, or until there are signs of ROP regression.

Epidemiological evidence suggests screening is effective in reducing visual impairment from ROP. Between 1969 to 1985, ROP was the cause of 5% of severe childhood visual impairment in the UK (Rahi and Dezateux, 1998). In 1988, effective treatment for ROP was identified (Tasman, 1988c), and screening was introduced in the UK in 1990. Subsequently, the incidence of severe visual impairment from ROP decreased to 3% in 2000 (Rahi and Cable, 2003). It is inferred from the epidemiological trends that UK ROP screening programme is effective since, despite increased survival of extremely preterm infants at risk for ROP (Wood *et al*, 2000), there is a decreased incidence of ROP-related visual impairment (Haines *et al*, 2005)Haines et al. (2005).

Unfortunately, BIO ROP screening is considered to be painful and stressful for infants (see Table 1.1). BIO ROP screening involves viewing the posterior segment of the eye using a headset and bright illumination. An eyelid speculum is used to open the eyelids and a scleral indenter is used to stabilise and rotate the eye. Previous studies suggest that each of these elements may be painful or aversive.

Table 1.1: Infant responses to binocular indirect ophthalmoscopy screening for retinopathy of prematurity

Increase in heart rate and blood pressure[1-3]
Decrease in oxygen saturations[1-3]
Increase in apnoeas 24-72 hours after screening[3-5]
Clinical pain score indicating moderate to severe pain[5-14]

1. Laws *et al*, 1996; 2. Rush *et al*, 2004; 3. Jiang *et al*, 2016; 4. Mitchell *et al*, 2011; 5. Hartley *et al*, 2018; 6. Sun *et al*, 2010; 7. Cogen *et al*, 2011; 8. Moral-Pumarega *et al*, 2012; 9. Mandel *et al*, 2012; 10. Dilli *et al*, 2014; 11. Rosali *et al*, 2015; 12. Kabatas *et al*, 2016; 13. Nayak *et al*, 2020; 14. O'Sullivan *et al*, 2010.

A study of non-indented BIO ROP screening in 15 infants identified greater increase in heart rate (HR) and blood pressure following screening in which a speculum

was used than without, as well as increased brow bulge and fellow eye squeeze(Mehta *et al*, 2005). In a group of 17 infants undergoing BIO ROP screening with an eyelid speculum, a higher PIPP score was measured when scleral depression was used than without (10.4 with vs 7.3 without scleral depression) (Cogen *et al*, 2011). In a larger study of 92 infants undergoing BIO ROP screening, use of an eyelid speculum and indenter was associated with a significantly higher HR and Neonatal Infant Pain Score during the procedure than without speculum and indenter (187 vs 167 bpm, $p < 0.001$; 5 vs 3.8, $p < 0.001$ respectively)(Kirchner *et al*, 2009). A review of 29 studies of pain relief for ROP screening (Disher *et al*, 2018) highlighted that the lowest pain score for BIO ROP screening was obtained in a group of 15 infants examined without the use of an eyelid speculum (median PIPP score = 6) (Olsson and Eriksson, 2011).

In view of this evidence, researchers have questioned whether an eyelid speculum and scleral depression are necessary components of BIO ROP screening (Mehta *et al*, 2005; Kirchner *et al*, 2009; Disher *et al*, 2018). The presence of 'plus disease', an indicator of treatment-warranted ROP, is determined by the appearance of retinal vessels near the optic nerve, which can be viewed without a speculum and scleral depression. However, accurate grading of ROP requires inspection of the retinal periphery to detect signs which may occur even in the absence of well-defined plus disease. Vascular ridge formation, retinal traction and detachment occur in the retinal periphery and lead to sight impairment or blindness unless treated in a timely manner (Tasman, 1988; International Committee for the Classification of Retinopathy of Prematurity, 2005; Royal College of Ophthalmologists, 2008). The retinal periphery may not be visualised adequately during BIO ROP screening without use of a speculum and indenter (Dhillon *et al*, 1993; Rani and Jalali, 2010).

Bright light is an anxiogenic and aversive stimulus in neonatal and adult animal models (Warthen *et al*, 2011; Delwig *et al*, 2012). It has been suggested that bright lighting conditions may also be stressful for preterm infants (Blackburn, 1998; Weber and Harrison, 2019). A study of 27 sleeping preterm infants identified a significant

reduction in oxygen saturation after a sudden increase in light (switching on an overhead fluorescent lamp) (Shogan and Schumann, 1993). A later study of 8 preterm infants also identified oxygen desaturation occurred after a rapid increase in light (switching on a fluorescent or incandescent light) but not after a gradual increase in light (incandescent light with dimmer switch) (Ozawa *et al*, 2010). This evidence suggests that the bright retinal illumination used in BIO ROP screening may be stressful for infants, and accordingly the use of a dimmer switch is advocated by the American Academy of Pediatrics to avoid sudden changes in procedural lighting.

1.2.5 Analgesic Strategies for ROP Screening and Treatment

A wide variety of pain relief strategies have been investigated for BIO ROP screening (see Table 1.2). Pharmacological approaches include local anaesthetic eye drops, nitrous oxide, oral paracetamol, and oral morphine. Non-pharmacological approaches include skin-to-skin care, swaddling, non-nutritive sucking, and sweet taste. Unfortunately, no ideal strategy for pain relief during BIO ROP screening has been identified. Topical anaesthetic is administered routinely prior to eye examination and produces temporary corneal anaesthesia, however it does not fully relieve pain associated with the procedure (Marsh *et al*, 2005; Dempsey and McCreery, 2011; Sun *et al*, 2010). Oral sucrose, though frequently used for procedural pain in infants (Stevens *et al*, 2016), does not significantly reduce pain scores associated with BIO ROP screening (Boyle *et al*, 2006; Kandasamy *et al*, 2011). Analysis of pooled results of four studies of sucrose for pain relief in BIO ROP screening (Gal *et al*, , 2005; Grabska *et al*, 2005; Boyle *et al*, 2006; Mitchell *et al*, 2011) identified only a modest reduction in PIPP scores (1.38 points) (Sun *et al*, 2010).

Table 1.2: Strategies for pain relief for binocular indirect ophthalmoscopy screening for retinopathy of prematurity

Study	Strategy	Outcome measure	Conclusion
Rush *et al* (2005)	LA + swaddle + NNS + sucrose + holding	HR, SpO_2	No additional effect compared to LA alone
Grabska *et al* (2005)	LA + swaddle + NNS + sucrose	HR, SpO_2, BP, PIPP score	No additional effect compared to LA + swaddle + NNS + placebo
O'Sullivan *et al* (2010)	LA + swaddle + NNS + sucrose	NPASS score	Effective compared to LA + swaddle + NNS + placebo ($p = 0.02$)
Mandel *et al* (2012)	LA + swaddle + sucrose + nitrous oxide	PIPP score	No additional effect compared to LA + swaddle + sucrose
Gal *et al* (2005)	LA + swaddle + sucrose	PIPP score	Effective compared to LA + swaddle + placebo ($p = 0.01$)
Rosali *et al* (2015)	LA + swaddle + breast milk	PIPP score	Effective compared to LA + swaddle alone ($p < 0.05$)
Marsh *et al* (2005)	LA + swaddle	PIPP score	Effective compared to swaddle + placebo ($p = 0.001$)
Kristoffersen *et al* (2019)	LA + NNS + sucrose + skin to skin	PIPP score	No additional effect compared to LA + NNS + sucrose alone
Dilli *et al* (2014)	LA + NNS + sucrose	PIPP score	Effective compared to LA + NNS + placebo ($p = 0.001$)
Boyle *et al* (2006)	LA + NNS	PIPP score	Effective compared to LA alone ($p = 0.003$)
Kleberg *et al* (2008)	LA + NIDCAP care	HR, SpO_2, PIPP score	No additional effect compared to LA alone
Nayak *et al* (2020)	LA + breast milk	PIPP score	No additional effect compared to LA + placebo
Kabatas *et al* (2016)	LA + oral paracetamol	PIPP score	Effective compared to LA + placebo ($p = 0.001$)
Hartley *et al* (2018)	LA + oral morphine	PIPP score	No additional effect compared to LA + placebo
Cogen *et al* (2011)	LA	PIPP score	Effective compared to placebo ($p = 0.04$)

LA = local anaesthetic eye drops; NNS = non-nutritive sucking; HR = heart rate; SpO2 = oxygen saturation; BP = blood pressure; NPASS = neonatal pain and agitation score; PIPP = premature infant pain profile; NIDCAP = newborn individualized developmental care and assessment program

1.3 Early Brain Development

1.3.1 Overview

The development of the brain begins at approximately two weeks post-conception, with differentiation of neural stem cells. The neural stem cells, or neural progenitor cells, develop from embryonic epiblast cells during gastrulation, a complex process in which the embryo transforms into a multilayered structure (Stiles and Jernigan, 2010). During the third week post-conception the neural plate develops (Tierney and Nelson, 2009). The neural plate folds to form the neural tube (Wilde, 2014), which closes by four weeks post-conception. The anterior end of the neural tube develops into three brain vesicles - hindbrain, midbrain, and forebrain - which eventually develop into distinct regions of the central nervous system (CNS) - brainstem, midbrain, and cerebral hemispheres and deeper structures including the thalamus, respectively (Gilbert, 2003).

At a cellular level, the inner layer of the neural tube (ventricular zone) gives rise to neuroblasts and glioblasts, which migrate into the middle layer of the neural tube (intermediate zone). These cells form two collections; the alar plate, which will develop into the spinal cord dorsal horn sensory neurons, and the basal plate, which will form the ventral horn motor neurons (Gilbert, 2003). Marked neuronal proliferation occurs during this process, which is completed by approximately 22 weeks (Stiles and Jernigan, 2010; Kostovic *et al*, 2019; Wallois *et al*, 2021)

During the second trimester, neurons migrate from the neural tube to form cortical layers; cells in earlier migrations are destined for deep cortical layers, while later migrations pass through to superficial cortical layers (Tierney and Nelson, 2009). Neuronal migration is completed by approximately 25 weeks post-conception (Wallois *et al*, 2021), and the six cortical layers are fully established by approximately 32 weeks (Kostovic and Judas, 2010). Differentiation of neurons occurs during the third trimester; cells then undergo axon development and dendrite outgrowth (Kostovic *et*

al, 2019) allowing communication with other neurons via synapses. A period of rapid synapse formation begins, which continues into postnatal life (Wallois *et al*, 2021). After birth nerve fibres are myelinated, which facilitates faster conduction (Kostovic *et al*, 2019).

1.3.2 Subplate Development

In the second trimester (12-18 weeks), a structure termed the subplate develops in the neonatal brain. The subplate is a transient layer of neuronal cells located in the cortical white matter (Kanold and Luhmann, 2010; Kostovic and Judas, 2010), and is essential for cortical development.

Afferent neurons from the thalamus enter the subplate around 23-25 weeks and synapse with subplate neurons. The subplate sends output to layer IV of the cortex, thereby forming the first thalamo-cortical connections (Kostovic and Judas, 2010; Kostovic *et al*, 2019). The subplate also receives input from the cortical plate, and acts as a temporary relay back to the thalamus, as well as to the contralateral hemisphere via the corpus callosum after 30 weeks post conception (Kostovic and Judas, 2010; Wallois *et al*, 2021). These connections allow early processing of external input, hence subplate neurons are the first cortical neurons to respond to sensory stimuli (Wess *et al*, 2017).

In the third trimester, afferent neurons from the thalamus synapse directly with layer IV cortical neurons (Burkhalter *et al*, 1992; Kostovic and Judas, 2010), and afferent neurons from cortical layers V and VI synapse directly with thalamic neurons (Stile and Jernigan, 2010). These connections allow cortical processing of sensory input; evoked potentials can be recorded from the primary somatosensory, visual and auditory cortex at this stage (Vanhatalo and Lauronen, 2006; Vanhatalo and Kaila, 2006). From 34 weeks post conception, the subplate begins to break down by apoptosis and has disappeared by the first postnatal month (Kostovic and Judas, 2010; Kostovic *et al*, 2019).

1.3.3 Development of Nociceptive Pathways

The nociceptive pathway commences with the detection by peripheral nociceptors of a noxious stimulus, such as heat, cold, pressure or a chemical signal of actual or potential tissue damage (Dubin and Patapoutian, 2010). Cutaneous sensors develop between 7 to 15 weeks gestation (Lowery *et al*, 2007).

Nociceptive signals are conducted along afferent nociceptive neurons termed C-fibres and A-fibres, which are present by 20 weeks gestation (Lowery *et al*, 2007). C-fibres are narrow-diameter, unmyelinated neurons with a conduction velocity of 0.4-1.4 m/s (Djouhri and Lawson, 2004; Woolf and Ma, 2007) which project to the spinal cord dorsal horn layers I and II. A-fibres are myelinated with a faster conduction velocity (5-30 m/s), and project to dorsal horn layers I and V (Djouhri and Lawson, 2004).

Reflex withdrawal from a noxious stimulus is mediated by synapses with second-order neurons in the spinal cord (Dubin and Patapoutian, 2010). The spinal reflex response to noxious stimulation develops by 19 weeks gestation (Lowery *et al*, 2007).

Central responses to a noxious stimulus are mediated by ascending pathways from the dorsal horn to the thalamus, which develop by 20 weeks gestation (Lowery *et al*, 2007). Nociceptive signals are transmitted from the thalamus to the cortex initially via the subplate described above. These connections are considered immature prior to 25 weeks gestation, and therefore unlikely to process external noxious stimuli (Kostovic and Judas, 2010); after 25 weeks gestation, neuronal responses can be measured in response to an external noxious stimulus in premature infants (Slater *et al*, 2006; Bartocci *et al*, 2006; Fabrizi *et al*, 2011). Pain processing and perception are facilitated by maturing thalamo-cortical connections from approximately 30 weeks gestation (Verriotis *et al*, 2016).

1.4 Early Life Pain

1.4.1 History

Until the 1980's, there was a misconception that infants did not feel pain (Lippmann *et al*, 1976). The medical community believed that newborn babies were not capable of perceiving or localising pain, and that they were not capable of discriminating pain from other stimuli (Richards, 1985; Anand *et al*, 1987). A change in opinion was brought about partly due to clinical research and partly due to parental advocacy. A landmark randomised controlled trial was led by Anand at the John Radcliffe Hospital, Oxford, in 1987, which investigated the effect of opioid anaesthesia on infants undergoing cardiac surgery (Anand *et al*, 1987). There was a greater 'stress response' in the infants who did not receive fentanyl anaesthesia – for example, there was a significant increase in adrenaline levels persisting until 24 hours post operatively, as well as significant differences in glucose, lactate and mineralocorticoid hormones. Importantly, the group that did not receive anaesthesia also had a poorer clinical course post-operatively, including increased ventilation requirements, increased number of bradycardias and increased incidence of metabolic acidosis.

At a similar time, a premature infant named Jeffrey Lawson underwent cardiac surgery while paralysed but not anaesthetised, as per usual practice. His mother, on learning this was the current standard of care, became a strong advocate for anaesthesia for premature infants undergoing surgery and other clinical procedures (Lawson, 1986). Opinion changed quickly such that by the end of 1987 the New England Journal of Medicine published a review of pain in infants citing over 200 references in support of their argument (Anand and Hickey, 1987), and the American Academy of Pediatrics published a statement that the available anaesthetic agents were safe for use in neonates (Poland *et al*, 1987).

Pain in infants is now understood to be a serious clinical issue. Infants born prematurely spend on average 81 days in the NICU (Lee *et al*, 2013), and during

their admission may undergo between 2 to 17 painful procedures daily (Carbajal *et al*, 2008; Simons *et al*, 2003; Cruz *et al*, 2016). It is therefore of critical importance to better detect and treat pain in infants.

1.4.2 Pain Perception

As stated in Section 1.1, pain is defined by the International Association for the Study of Pain as 'an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage'. Pain, therefore, is a personal, subjective experience which can be communicated both verbally and through pain-related behaviours. Pain is different to nociception, which is the conduction of a noxious stimulus along the ascending nociceptive neuronal pathways described in Section 1.3.3.

Pain is currently considered to arise from a functional network of brain regions termed the 'dynamic pain connectome' (Kucyi and Davis, 2015). These are defined as the ascending nociceptive pathway discussed in Section 1.3.3, the descending anti-nociceptive pathway, the salience network, and the default mode network. The descending anti-nociceptive pathway is considered to be active during 'mind-wandering' from pain stimulation, and may comprise the amygdala, mid-prefrontal cortex, peri-aqueductal grey, and the rostoventral medulla. The salience network is considered to be active during attention to pain, and may include the temporoparietal junction, the anterior insula, and the dorsolateral prefrontal cortex. The default mode network is considered to be active in the absence of pain stimulation. It may comprise the posterior cingulate, the medial temporal lobe, the anteromedial prefrontal cortex, and the dorsomedial prefrontal cortex. These regions are not specific to pain, but together are considered capable of generating the conscious experience of pain through a coordinated change in neuronal activity (Verriotis *et al*, 2016; Kucyi and Davis, 2017).

It is not known at which stage of development the brain becomes able to generate

the experience of pain (Verriotis *et al*, 2016). In order to investigate physical pain in infants, in the absence of verbal confirmation, surrogate markers of pain are required such as measurement of behavioural responses, physiological responses, and neuronal responses to painful stimuli.

1.4.3 Consequences of Early Life Pain

Visual, auditory and tactile pathways receive afferent input during gestation, whereas nociceptive pathways are not routinely activated prenatally (Fitzgerald, 2005; Fabrizi *et al*, 2016). Painful stimuli during the preterm period may negatively influence neurodevelopment, including the structure of the developing brain, activity of nociceptive pathways, and individual pain sensitivity (Williams and Lascelles, 2020).

Higher number of painful procedures in the early preterm period (before 32 weeks gestation) is associated with reduced white matter maturity in infants reaching term-equivalent age (Brummelte *et al*, 2012), and at 7 years of age (Vinall *et al*, 2014). These factors are associated with lower intelligence quotient at the same age (Vinall *et al*, 2014). Greater number of painful procedures during the early preterm period is also associated with reduced thalamus growth and altered thalamo-cortical pathways (Duerden *et al*, 2018).

Alteration in nociceptive activity has been identified in children aged 7 to 11 years old who had been born before 32 weeks gestation. Higher numbers of painful procedures during NICU admission is associated with reduced descending inhibitory modulation of noxious input, and reduced autonomic response to pain (Goffaux *et al*, 2008). During experimental noxious stimulation in children aged 11 to 16 year old who had been born prematurely, increased activity has been identified in pain-related brain regions including the thalamus, anterior cingulate cortex and insula (Hohmeister *et al*, 2010).

Pain sensitivity may be altered in infants who experience early life pain (Slater *et al*, 2010c). Infants who had previously undergone circumcision without analgesia have

been observed to have increased pain sensitivity following vaccination (Taddio *et al*, 1997). Similarly infants who had undergone surgery within the neonatal period were noted to have increased pain sensitivity during repeat surgery in the same dermatome aged 0 to 3 years old (Peters *et al*, 2005).

1.5 Infant Pain Assessment

1.5.1 Behavioural Measures

Infants respond to an acute noxious stimulus with pain-related behaviours such as crying, body movement, and facial expression (eye squeeze, brow bulge, nasolabial furrow, grimacing) (Grunau and Craig, 1987; Rushforth and Levene, 1994).

Several clinical pain scores exist for preterm infants which quantify infant behavioural and physiological responses to a noxious stimulus. Pain scoring provides a surrogate indicator of the infant pain experience, and can be used to evaluate the severity of pain (Buyuktiryaki *et al*, 2018; Sethi *et al*, 2020) and the effectiveness of pain-relieving interventions (Boyle *et al*, 2006; Hartley *et al*, 2018). Examples of clinical pain scores for premature infants include the Behavioural Indicators of Infant Pain score (Holsti *et al*, 2008); the COMFORT scale (Ambuel *et al*, 1992); the Echelle Douleur Inconfort Nouveau-Ne score (Debillon *et al*, 2001), the Neonatal Infant Pain Score (Lawrence *et al*, 1993), and the Premature Infant Pain Profile - Revised (PIPP-R score) (Stevens *et al*, 1996; Stevens *et al*, 2010; Stevens *et al*, 2014).

In this thesis the PIPP-R score is used. This scoring system incorporates the age and baseline behavioural state of the infant in addition to behavioural and physiological variables. Gestational age is considered because older infants display more consistent behavioural responses than younger infants (Green *et al*, 2019). Baseline behavioural state is considered because alert awake infants are more likely to respond to a stimulus than infants in quiet sleep (Grunau and Craig, 1987). The PIPP-R score was validated predominantly in acute skin-breaking procedures (see Table 1.3), and

has been used widely to characterise the infant response to acute clinical procedures such as heel lancing and venepuncture (Stevens *et al*, 1996; Ballantyne *et al*, 1999; Stevens *et al*, 2010; Stevens *et al*, 2014; Gibbins *et al*, 2014). BIO ROP screening is a complex non-skin-breaking procedure of longer duration than heel lancing, therefore cautious interpretation of the PIPP score may be required (Sun *et al*, 2010).

Table 1.3: Validation studies for the Premature Infant Pain Profile (PIPP) and the Premature Infant Pain Profile - Revised (PIPP-R) clinical pain scores

Study	Procedure	Score
Stevens *et al* (1996)	Heel lance, circumcision	PIPP
Ballantyne *et al* (1999)	Heel lance, venepuncture, intravenous line insertion	PIPP
Jonsdottir & Kristjansdottir (2005)	Heel lance	PIPP
Vederhus *et al* (2006)	Heel lance, cannulation	PIPP
Ahn & Jun (2007)	Heel lance, venepuncture, endotracheal suction	PIPP
Eriksson *et al* (2008)	Heel lance	PIPP
Stevens *et al* (2014)	Heel lance	PIPP-R
Gibbins *et al* (2014)	Heel lance, venepuncture	PIPP-R

There are limitations to the use of behavioural measures as surrogate markers for pain in infants. Firstly, these measures must be used with caution in infants younger than 34 weeks, because the ability to respond discriminatively to stimuli emerges around 33 weeks (Fabrizi *et al*, 2011a; Green *et al*, 2019). In a study of 105 infants aged 28 to 42 weeks, younger infants were as likely to display pain-associated facial behaviours (brow bulge, eye squeeze, nasolabial furrow) to noxious or innocuous stimuli, whereas older infants were more likely to display facial behaviours to noxious stimuli only (Green *et al*, 2019).

Secondly, changes in infant physiology and behaviour are not a specific response to painful stimuli; similar changes may be evoked as a stress or distress response (Stevens *et al*, 1993; Holsti *et al*, 2005a; Ahola Kohut and Pillai Riddell, 2009). For example, nappy change is a routine procedure that is not considered to be painful

(Blauer and Gerstmann, 1998; Mörelius *et al*, 2006; Lyngstad *et al*, 2014). However, in a study of 12 term infants, the mean PIPP score for nappy change was 9 (range 7.8 to 10.9), indicating moderate pain (Ballantyne *et al*, 1999). In a larger study of 30 term infants, there was a significant increase in pain scores during nappy change ($p < 0.01$) (Mörelius *et al*, 2006). Studies of pre-term infants have also identified significant increases in HR and decreases in oxygen saturation during nappy change (Wang and Chang, 2004; Lyngstad *et al*, 2014).

1.5.2 Physiological Measures

Autonomic responses to noxious stimulation in infants include increase in HR and decrease in oxygen saturation (Stevens and Johnston, 1994; Stevens *et al*, 2016).

For all preterm infants receiving oxygen therapy, the recommended lower limit for oxygen saturation is 89% and the upper limit is 95%. While for infants who have been weaned off oxygen therapy onto room air, the upper limit is 100%. The target range for neonatal oxygen saturation has been established on the basis of evidence from meta-analysis of several randomised controlled trials. A Cochrane systematic review (Askie *et al*, 2017) included data from 4965 infants born before 28 weeks gestation enrolled in five randomised controlled trials. It investigated the effect of a lower oxygen saturation target range (85-89%) or a higher target range (91-95%) on major infant disability (bilateral blindness, deafness, or cerebral palsy) or death at 18-24 months. Lower oxygen saturation target range was associated with decreased incidence of ROP treatment ($p < 0.003$). However, it was also associated with significantly increased incidence of death at 36 weeks postmenstrual age, at hospital discharge, and at 18-24 months ($p = 0.01$). Oxygen therapy is therefore implicated in the development of ROP requiring treatment, nonetheless a lower oxygen saturation target range is not recommended due to the risk of increased mortality.

A period during which oxygen saturation falls below 80% for $\geq$ 10s is termed a desaturation. Oxygen desaturation in preterm infants may be indicative of lung

pathology, circulatory shunting, or inadequate gas exchange which may occur during periods of stress and pain (Pokela, 1994; Stevens *et al*, 1996; Fairchild and O'Shea, 2010; Martin *et al*, 2012). Between 3-26% of infants undergoing BIO ROP screening experience oxygen desaturations during or after the procedure (Laws *et al*, 1996; Belda *et al*, 2004; Mehta *et al*, 2005; Moral-Pumarega *et al*, 2012; Dilli *et al*, 2014), indicating that BIO ROP screening may be a source of pain or stress for infants.

Continuous electrocardiography (ECG) monitoring in the NICU allows identification of significant excursions from normal heart rate and rhythm. A tachycardia occurs when the HR exceeds 200 beats per minute (bpm) for $\geq$ 15s, and a bradycardia is a period during which HR falls below 100 bpm for $\geq$ 15s. Increased heart rate may be indicative of hypovolaemia, sepsis, stress, or pain (Anand *et al*, 1987; Anand *et al*, 1999; Mitchell *et al*, 2011; Moral-Pumarega *et al*, 2012). Bradycardias may arise concurrently with a desaturation event due to an apnoea, with valsalva manoeuvre, or due to vagal stimulation, including the oculocardiac reflex due to pressure on the eyeball (Phillips *et al*, 1964; Gamble *et al*, 2007; Eichenwald, 2016). Increase in apnoeic episodes in premature infants occurs due to acute illness or stress (Fairchild *et al*, 2016).

BIO ROP screening has been associated with a 7-26 bpm increase in HR during the procedure (Laws *et al*, 1996; Rush *et al*, 2004; Jiang *et al*, 2016), a further indication that the examination may be painful for infants. Bradycardia related to the oculocardiac reflex has been observed in up to 31% of infants undergoing BIO ROP screening (Clarke *et al*, 1985). The reflex may be elicited by components of the screening examination including instillation of eyedrops, eyelid speculum insertion, scleral indentation, or stretching of the rectus muscles (Clarke *et al*, 1985). BIO ROP screening has also been associated with increase in new-onset apnoeic episodes in the 24 hours following the examination (Mitchell *et al*, 2011; Jiang *et al*, 2016; Hartley *et al*, 2018); the incidence of new onset apnoea is related to gestational age at examination, occurring in 24% of infants aged less than 31 weeks, and 1% of infants

older than 37 weeks (Jiang *et al*, 2016). Acute increase in apnoea events is a further indication that ROP screening may be stressful for infants.

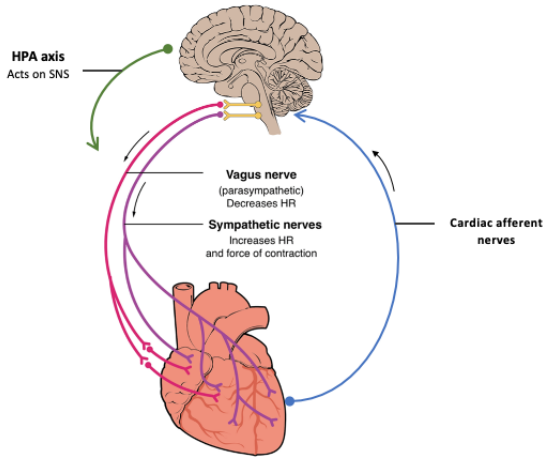
Increased frequency of physiological instability events has been associated with negative sequelae such as severe ROP, disability and death (Butcher-Puech *et al*, 1985; DiFiore *et al*, 2012; Poets *et al*, 2015). The mechanism by which these events cause adverse outcomes is not fully understood (Fairchild *et al*, 2019). However, in view of the negative long-term consequences of instability events, minimising infant pain or stress during clinical procedures is vital.

Cardiac monitoring in the NICU has further value in allowing assessment of neonatal cardiac autonomic reactivity. Heart rate variability (HRV) describes the time-varying intervals between consecutive heart beats (Quintana and Heathers, 2014), and reflects the influence of the sympathetic and parasympathetic divisions of the autonomic nervous system on the cardiac rhythm (Task Force of the European Society of Cardiology, 1996).

The autonomic nervous system divisions have distinct functions in the maintenance of cardiovascular homeostasis, as illustrated in Figure 1.1. The sympathetic nervous system generates a 'fight or flight' stress response to perturbations within the environment, acting within 5 seconds to increase HR. Conversely the parasympathetic nervous system generates a 'rest and digest' response, acting within 0.5 seconds to decrease HR and promote systemic energy conservation. Parasympathetic HR modulation is mediated by the vagus nerve. HRV results from the complex interaction between the divisions of the autonomic nervous system, together with modulation from the intrinsic cardiac nervous system, and feedback to higher centres from cardiac afferent pathways (Nunan *et al*, 2010; Dilsizian and Narula, 2017; Shaffer and Ginsberg, 2017).

HRV also reflects the influence of the hypothalamic-pituitary-adrenal axis on the autonomic nervous system (Kim *et al*, 2018). The endocrine stress response is initiated by corticotrophin-releasing hormone released from the hypothalamus, which

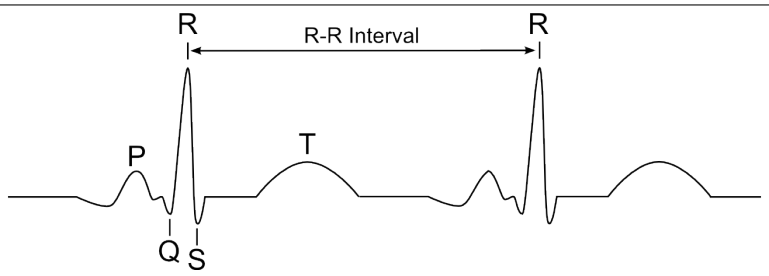
stimulates cortisol release from the adrenal glands (Egliston *et al*, 2007). Cortisol enhances the sympathetic cardiovascular stress response, for example increasing HR (Sapolsky *et al*, 2000), which in turn influences HRV (Rotenberg and McGrath, 2016).



Heart rate variability results from complex interactions between the parasympathetic (shown in red) and sympathetic (shown in purple) divisions of the autonomic nervous system, together with modulation from the intrinsic cardiac nervous system (not shown), feedback to higher centres from the cardiac afferent nervous system (shown in blue), and the action of the hypothalamic-pituitary-adrenal (HPA) axis (shown in green), mediated by cortisol, on the sympathetic nervous system. OpenStax College, CC BY 3.0 via Wikimedia Commons.

Figure 1.1: Simplified schematic of cardiac autonomic innervation

HRV analysis requires identification of consecutive heart beats from an ECG recording. As illustrated in Figure 1.2, each heartbeat is composed of a P wave, a QRS complex, and a T wave. The heartbeat originates in the sinoatrial node of the heart, depolarising first the atria (P wave) then the ventricles (QRS complex). The T wave is generated by ventricular repolarisation. Although sinoatrial node activity coincides most closely with the timing of the P-wave, the QRS complex has greater amplitude and is better detected than the P-wave. Beat-to-beat time difference is therefore calculated using consecutive QRS complexes. The interbeat interval is termed the RR interval, or the normal-to-normal (NN) interval after artefact has been removed from the ECG recording. The derivation of HRV indices included in this thesis is described in Section 2.5.4.



Each heartbeat is composed of a P wave, generated by atrial depolarisation; a QRS complex, generated by ventricular depolarisation; and a T wave, generated by ventricular repolarisation. The R-R interval is the interbeat interval. Tedburke, CC BY-SA 4.0, via Wikimedia Commons.

Figure 1.2: Schematic of two heartbeats on electrocardiogram recording

In adults, HRV analysis is used as a tool for evaluating cardiac physiology and assessing cardiac health. The Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology endorsed the use of HRV analysis in assessing risk of cardiac arrhythmias following acute myocardial infarction and cardiac transplant, and in assessing the quality of cardiac innervation in patients with diabetic neuropathy. Cardiac status in conditions such as congestive heart failure, left ventricular hypertrophy, and valvular disease can be evaluated using HRV analysis (Coumel *et al*, 1991; Stys and Stys, 1998). HRV analysis has been used as a research tool to evaluate autonomic functioning in a range of physiological and psychological states (see Table 1.4).

In relation to pain in adults, the high frequency component of HRV has been identified as a measure of nociception; noxious stimulation during surgery evokes a decrease in high frequency HRV, which may be modulated by analgesia (Jeanne *et al*, 2009). The high frequency component of HRV reflects parasympathetic tone (Szental *et al*, 2015); parasympathetic tone prior to a surgical procedure has been identified as a predictor of intraoperative pain experience (Adjei, 2019). Stress in adults has also been observed to evoke a decrease in high frequency HRV, indicating a relative reduction in parasympathetic activity compared to sympathetic activity (Kim *et al*, 2018).

Table 1.4: Applications of heart rate variability analysis in adults

Evaluation of physiological change due to:
Exercise[1,2]
Obstructive sleep apnoea[3,4]
Renal failure[5,6]
Tobacco smoking[7,8]
Alcohol consumption[9,10]
Evaluation of psychophysiological change due to:
Surgical pain[11,12,13]
Stress[14,15]
Attention[16,17]

1. Tulppo *et al*, 1996; 2. Verlinde *et al*, 2001; 3. Drinnan *et al*, 2000; 4. Roche *et al*, 2003; 5. Forsström *et al*, 1986; 6. Axelrod *et al*, 1987; 7. Hayano *et al*, 1990; 8. Barutcu *et al*, 2005; 9. Malpas *et al*, 1991; 10. Rossinen *et al*, 1997; 11. Jeanne *et al*, 2009; 12. Szental *et al*, 2015; 13. Adjei, 2019; 14. Houtveen *et al*, 2002; 15. Kim *et al*, 2018; 16. Richards, 1985; 17. Weber *et al*, 1994.

HRV has been used to characterise the development of autonomic nervous system control in infants. HRV is low at birth then increases during the first few days of postnatal life as autonomic nervous system regulation matures (Javorka *et al*, 2017; Cardoso *et al*, 2017). Increasing gestational age at birth, and increasing post-conceptional age (that is, gestational age combined with postnatal age), are associated with higher HRV (van Ravenswaaij-Arts *et al*, 1991; Longin *et al*, 2005; Cardoso *et al*, 2017; Joshi *et al*, 2019). At birth, healthy infants have higher HRV than infants with birth complications such as low birth weight or hypoxia (Rother *et al*, 1987; Aziz *et al*, 2012). Preterm infants at term-equivalent age have lower HRV than term infants (Hunt, 2006; Fyfe *et al*, 2015; Burtchen *et al*, 2019). This may reflect autonomic dysregulation increasing the risk of sudden infant death syndrome in this population (Schechtman *et al*, 1989). Reduced HRV has been associated with a range of neonatal pathologies, listed in Table 1.5.

Table 1.5: Pathological associations of reduced heart rate variability in neonates

Neurological
Hypoxic ischaemic encephalopathy[1,2,3]
Ventricular haemorrhage[4]
Brain death[5,6]
Cardiac
Congenital heart defect[7]
Patent ductus arteriosus[8,9]
Coarctation of the aorta[10]
Infective / Inflammatory
Sepsis / systemic inflammatory response syndrome[11,12,13]
Necrotising enterocolitis[14]

1. Massaro *et al*, 2014; 2. Goulding *et al*, 2015; 3. Goulding *et al*, 2017; 4. Tuzcu *et al*, 2009; 5. Kero *et al*, 1978; 6. van der Moer *et al*, 1985; 7. Butera *et al*, 2004; 8. Prietsch *et al*, 1992; 9. Goudjil *et al*, 2013; 10. Polson *et al*, 2006; 11. Kovatchev *et al*, 2003; 12. Fairchild and O'Shea, 2010; 13. Moorman *et al*, 2011; 14. Stone *et al*, 2013.

The infant stress response to a painful stimulus has been investigated using HRV analysis. Studies of healthy term infants undergoing heel lance blood sampling identified a significant decrease in high frequency HRV (Jones *et al*, 2017) and nonlinear HRV indices (Weissman *et al*, 2012; Kramaric *et al*, 2019) between the baseline period and the clinical procedure. Preterm infants also demonstrated a significant reduction in high frequency, linear, and nonlinear HRV indices following a painful procedure (heel lance blood sampling, intramuscular injection, cannulation, tracheal suctioning, surfactant administration, or chest drain insertion) (Buyuktiryaki *et al*, 2018; Okur *et al*, 2019; Cremillieux *et al*, 2018). One study investigated the effect of prolonged pain on HRV in infants over 34 weeks gestation: high frequency HRV and the Échelle Douleur Inconfort Nouveau-Né (EDIN) clinical pain score were calculated at median 5 hours after surgery; reduction in high frequency HRV was predictive of a score $\geq$5, indicating moderate pain (Faye *et al*, 2010).

1.5.3 Brain-derived Measures

Near-Infrared Spectroscopy

Near-infrared spectroscopy is a non-invasive indirect neuroimaging technique which measures cortical haemodynamic changes occurring as a result of brain activity. Neural activation increases cellular metabolic demand, which results in increased local blood flow and oxygenation (Lloyd-Fox *et al*, 2010). Near-infrared spectroscopy is portable and can be used safely in the clinical environment to monitor awake alert infants (Verriotis *et al*, 2016). It has good motion tolerance and is capable of more precise spatial localisation than electroencephalography (Verriotis *et al*, 2016). Near-infrared spectroscopy may provide more precise temporal resolution than magnetic resonance imaging (Lloyd-Fox *et al*, 2010), although the haemodynamic activity detected is a delayed representation of cortical activity. Near-infrared spectroscopy has been used to characterise infant responses to noxious stimuli including heel lancing, venepuncture and chest drain removal (Slater *et al*, 2006; Bartocci *et al*, 2006; Bembich *et al*, 2015; Verriotis *et al*, 2016).

Magnetic Resonance Imaging

Magnetic resonance imaging is a non-invasive neuroimaging technique that measures haemodynamic changes in the brain. Blood oxygen level dependent activity is used as an indirect marker of neural activation. It has greater spatial localisation than near-infrared spectroscopy and electroencephalography, being able to image the cortex and subcortical regions (Verriotis *et al*, 2016). Magnetic resonance imaging cannot be performed at the bedside; infants are removed from the clinical environment and closely supervised while undergoing scanning (Goksan *et al*, 2015; Williams *et al*, 2015). Infants can undergo scanning while awake but frequently require sedation (Williams *et al*, 2015; Verriotis *et al*, 2016). Magnetic resonance imaging has been used to characterise the infant response to brushing and to experimental non tissue-

damaging stimuli (Goksan *et al*, 2015; Williams *et al*, 2015; Goksan *et al*, 2018; Duff *et al*, 2020; Baxter *et al*, 2021). Magnetic resonance imaging has identified activity in regions such as the primary somatosensory cortex, insula, and thalamus during experimental noxious stimulation in infants, which are also active during pain experience in adults (Goksan *et al*, 2015; Duff *et al*, 2020; Baxter *et al*, 2021).

Electroencephalography

EEG is a non-invasive direct neuroimaging technique which measures electrical activity generated by synchronised populations of cortical neurons (Wallois *et al*, 2021). It provides more precise temporal resolution than near-infrared spectroscopy and magnetic resonance imaging (Wallois *et al*, 2021). EEG is portable and can be safely applied at the bedside to awake alert infants, including fragile infants in the NICU setting (Lloyd *et al*, 2015; El Ters *et al*, 2018; Whitehead *et al*, 2018).

Clinical conditions such as intraventricular haemorrhage, periventricular leukomalacia, seizure activity, and hypoxic ischaemic encephalopathy can be diagnosed and monitored using EEG (Pressler *et al*, 2001; Pavel *et al*, 2020; Wallois *et al*, 2020; Wallois *et al*, 2021). EEG is also a well–established research tool used in premature infants, for example in assessment of sleep state (Dereymaeker *et al*, 2017a) and brain maturation (Dereymaeker *et al*, 2017; Stevenson *et al*, 2017; Pillay *et al*, 2020).

With regard to pain research, EEG has been used to characterise infant neuronal activity associated with acute noxious stimulation. The infant nociceptive response has been characterised in experimental non tissue-damaging pinprick (Hartley *et al*, 2015) and clinical procedures such as heel lancing and vaccination (Slater *et al*, 2010b; Slater *et al*, 2010c; Fabrizi *et al*, 2011a; Verriotis *et al*, 2015).

A template of noxious-related brain activity has been identified from raw EEG at the Cz electrode 400-700 ms after noxious stimulus application (Hartley *et al*, 2017). The template was derived from principal component analysis of event-related potentials from 18 term infants in response to heel lancing or experimental weighted

noxious probe, and is not evoked by visual, auditory or tactile stimuli. The template can be scaled to fit independent data, in order to quantify brain activity evoked by an acute noxious stimulus: a magnitude < 1 implies the observed response is less than the template response and *vice versa* (Gursul *et al*, 2018; Kasser *et al*, 2019). The template is validated for use in preterm infants aged 34 weeks or older, and has been used as an outcome measure in studies of topical and oral analgesic efficacy for acute clinical procedures (Hartley *et al*, 2017; Hartley *et al*, 2018; Cobo *et al*, 2021).

Unlike heel lancing, which is a phasic skin-breaking stimulus of milliseconds duration that can be accurately time-locked in the EEG signal, and which evokes a specific event-related potential, BIO ROP screening is a tonic complex stimulus lasting several minutes. Brain activity responses to BIO ROP screening therefore may be better characterised by quantitative analysis of EEG than measurement of evoked potentials (Nir *et al*, 2012a). A common method of EEG analysis is data transformation to the frequency domain (Nir *et al*, 2010), then characterisation of the component frequency bands: delta (0.5-3 Hz), theta (3-8 Hz), alpha (8-12 Hz), beta (12-30 Hz), and gamma (30-100 Hz).

Frequency-domain EEG analysis has been used in studies of experimental and clinical tonic pain in adults (Chen *et al*, 1983; Peng *et al*, 2014; Li *et al*, 2016; Gram *et al*, 2017). However, these studies show conflicting results which may be due to small sample size, as well as differences in choice of noxious stimulus, study population, experimental design, and analysis approach. Table 1.6 shows an example of conflicting observations (theta activity increase or decrease) in two studies with differences in experimental design. These conflicting results highlight the need for caution in EEG analysis and interpretation (O'Toole and Boylan, 2019). Recommendations for a consistent approach to quantitative analysis of preterm EEG include ensuring the recording is free from artefact, of appropriate duration, and that the analysis includes a wide range of features, that is, computed values which represent the signal quantitatively (Palaniappan, 2010).

Table 1.6: Differences in experimental design of studies of tonic pain in adults

Study	Misra *et al*, 2017	Gram *et al*, 2015
Stimulus	Thermal stimulus	Cold pressor test
Sample size	30	39
Sample age	20 yrs ±2	22-65 yrs
Experimental design	5.5s epoch; 5.5s window; 0s overlap	2 min epoch; 15s window; 7.5s overlap
EEG analysis	Sinusoidal wavelet transform	Complex Morlet wavelet transform
Result	Theta increase	Theta decrease

In this study, these considerations have been taken into account and a wide range of quantitative features of EEG are analysed including those from the time-domain and frequency-domain, aiming to better represent the complexities of the preterm EEG (O'Toole and Boylan, 2019). This analysis approach has been used in studies of infant brain maturation (Janjarasjitt *et al*, 2008; Zhang *et al*, 2009; De Wel *et al*, 2017; Stevenson *et al*, 2017; Dereymaeker *et al*, 2017; Koolen *et al*, 2017; Pillay *et al*, 2018), seizure detection (Greene *et al*, 2008; Temko *et al*, 2011), and hypoxic ischaemic encephalopathy (Hathi *et al*, 2010; Korotchikova *et al*, 2011), and may be applicable to investigating infant nociceptive responses (Roué *et al*, 2021).

Characteristics of the normal EEG in preterm and full-term infants

Prior to gestational age 29 weeks, the EEG pattern is termed tracé discontinu (St. Louis *et al*, 2016). This pattern is discontinuous, containing periods of high amplitude, mixed frequency activity called bursts, separated by periods of lower amplitude called interburst intervals (St. Louis *et al*, 2016). An interburst interval may last up to 30 seconds in infants of 29 weeks gestational age (André *et al*, 2010). Burst activity includes monorhythmic slow delta waves in the occipital region, and theta activity initially in the occipital region and later in the temporal regions (Bourel-Ponchel *et al*, 2021). An EEG pattern termed a delta brush occurs, in which slow delta waves are superimposed with faster frequency activity of between 8-20 Hz (André *et al*, 2010;

Bourel-Ponchel *et al*, 2021). The incidence of delta brushes peaks between 32 and 35 weeks gestational age, after which they occur less frequently, disappearing by term (Boylan *et al*, 2008; André *et al*, 2010). EEG response to external stimuli, termed reactivity, begins to emerge between 28 to 30 weeks gestational age (André *et al*, 2010; Bourel-Ponchel *et al*, 2021).

Between 30 to 32 weeks gestational age, during quiet sleep, the EEG displays tracé discontinu with bursts of slow delta activity in temporal and occipital regions, as well as temporo-occipital delta brushes (André *et al*, 2010; Bourel-Ponchel *et al*, 2021). Rhythmic temporal theta activity is demonstrated during sleep during this period; it disappears from active sleep at 32 weeks (André *et al*, 2010; St. Louis *et al*, 2016). Interburst intervals occurring during quiet sleep last $\leq$15 seconds at gestational age 32 weeks (André *et al*, 2010). Active wakefulness can be identified in the EEG at this age by the presence of near-continuous activity (Bourel-Ponchel *et al*, 2021), and by the occurrence of muscle artefacts indicating limb movements (André *et al*, 2010).

At gestational age 33-34 weeks, during wakefulness and active sleep, burst activity comprises frequent delta brushes in the temporal and occipital regions (André *et al*, 2010; Bourel-Ponchel *et al*, 2021). Temporal theta disappears during quiet sleep at this age (André *et al*, 2010). Interburst intervals occurring during quiet sleep last $\leq$10 seconds at 34 weeks gestational age (André *et al*, 2010). Behavioural states of quiet sleep, active sleep, and wakefulness can be determined from the EEG at this period: during wakefulness, EEG activity is continuous and muscle artefacts may be present; during sleep, EEG activity is discontinuous with the longest interburst intervals occurring during quiet sleep (Bourel-Ponchel *et al*, 2021).

At gestational age 35-36 weeks, during active sleep and wakefulness, the interburst interval disappears and burst activity becomes continuous (Bourel-Ponchel *et al*, 2021). Two further EEG patterns appear by this age, termed frontal transients and anterior slow dysrhythmia. Frontal transients are frontal sharp waves which are typically synchronous and symmetrical, meaning the activity is of similar amplitude

and frequency in each hemisphere (André *et al*, 2010; Bourel-Ponchel *et al*, 2021). Anterior slow dysrhythmia is a normal pattern of delta activity in anterior regions, which is also synchronous and symmetrical (Boylan *et al*, 2008; St. Louis *et al*, 2016).

Between 37 to 40 weeks gestational age, tracé alternant occurs during quiet sleep (Bourel-Ponchel *et al*, 2021). This pattern comprises bilateral, synchronous and symmetrical delta bursts on theta activity (André *et al*, 2010; Bourel-Ponchel *et al*, 2021). Bursts are separated by interburst intervals lasting $\leq$5 seconds (André *et al*, 2010; St. Louis *et al*, 2016). During active sleep and wakefulness, polyfrequency activity at 25–50μV, termed activité moyenne occurs, as well as frontal transients and anterior slow dysrhythmia (Boylan *et al*, 2008; André *et al*, 2010; Bourel-Ponchel *et al*, 2021).

1.6 Thesis Aims

- Characterise the effect of BIO ROP screening on infant brain activity using quantitative EEG analysis.
- Evaluate noxious-related change in infant brain activity following BIO ROP screening.
- Characterise the effect of BIO ROP screening on infant physiology using continuous recordings of HR and oxygen saturations.
- Evaluate stress-related change in infant heart rate variability following BIO ROP screening.
- Characterise the effect of Optos ROP screening on preterm infants using behavioural, physiological and EEG data.
- Characterise the effect of ROP treatment on preterm infants using behavioural, physiological and EEG data.

Chapter 2

General Methods

This thesis comprises three studies including a total of 62 infants. Here the general methods used across all studies are presented. Each study is described in detail in individual chapters.

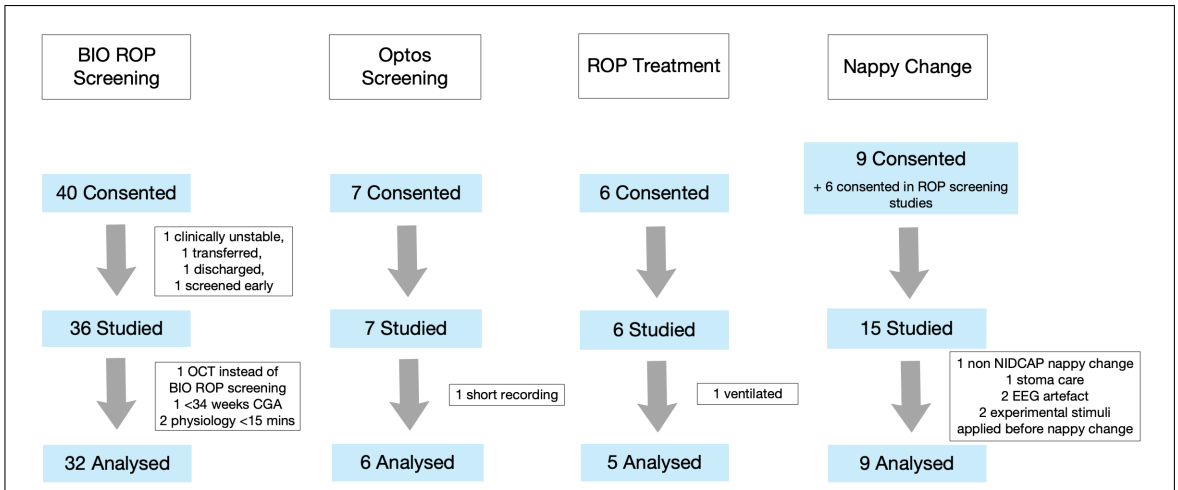
2.1 Ethics

Studies were performed under ethical approval obtained from the National Research Ethics Service for the applications "Imaging pain in the developing human brain" (reference 12/SC/0447) and "Is morphine an effective analgesic for procedural pain in infants?" (reference 15/EM/0310), and from the Medicines and Healthcare products Regulatory Agency for the randomised controlled trial 'A blinded randomised placebo-controlled trial investigating the efficacy of morphine analgesia for procedural pain in infants' (EudraCT number 2014-003237-25). All studies conformed to the principles of the Declaration of Helsinki and to Good Clinical Practice requirements.

2.2 Recruitment

Infants were recruited to the studies carried out in this thesis between October 2017 and March 2020 in the John Radcliffe Hospital NICU, Oxford University NHS Found-

ation Trust. As shown in Figure 2.1, 62 infants were recruited specifically for this thesis: 36 infants were studied during BIO ROP screening with or without nappy change, 7 infants were studied during Optos photography screening with or without nappy change, 6 infants were studied during ROP treatment, and 9 infants were studied during nappy change alone. Four infants were withdrawn after recruitment.



The figure illustrates the total number of infants consented, studied and included in analysis for each clinical procedure. Reasons for participant withdrawal are noted in boxes. BIO = binocular indirect ophthalmoscopy; ROP = retinopathy of prematurity; OCT = ocular coherence tomography; CGA = corrected gestational age; NIDCAP = newborn individualized developmental care and assessment program; EEG = electroencephalography.

Figure 2.1: Recruitment diagram

This thesis also includes data for 31 infants which were collected previously: 8 infants underwent ROP screening as part of the placebo arm of the Poppi trial (Hartley *et al*, 2018), and 23 infants were studied during heel lance blood sampling. Inclusion and exclusion criteria for study participation are shown in Table 2.1.

Table 2.1: Eligibility criteria for participation in studies

Inclusion criteria
Patient eligible for retinopathy of prematurity screening
Patient treated under NHS
Clinically stable at time of study
English language ability sufficient for valid informed consent
Maternal age 16 years or older
Maternal custody of infant
Maternal consent or paternal consent if parents married or father named on birth certificate

Exclusion criteria
Neurological, developmental or genetic condition affecting pain processing
Grade 3 or 4 intraventricular haemorrhage
Patient treated with analgesic or sedative medication (except for ROP treatment studies)
History of maternal substance misuse during pregnancy
Infant 'barrier nursed' for infection control
Safeguarding concerns for the infant

Screening Methods

When an infant was identified as eligible, a member of the research team discussed approaching the parents with the NICU nursing staff. The nurse caring for the infant was well-placed to know whether there were any reasons not to approach the parents at that time - for example, if the infant was likely to be transferred to another hospital or discharged within the timeframe of the study, if the infant was scheduled to have surgery (and therefore have analgesic or sedative treatment within the timeframe of the study), or if the parents had previously stated they did not wish to participate in research.

Approaching parents involved introducing oneself as a member of the research

team, then asking if the parents would like to hear about research studies for which their baby was eligible. If so, it was explained that the study aimed to improve understanding of the infant experience during clinical procedures, details of the study were explained, and a Patient Information Leaflet was given (Appendix A). If parents accepted the leaflet and expressed interest in taking part, they were given some time for reflection (approximately 1-2 days, unless the procedure to be studied was sooner) and re-approached afterward. At that time, any further questions were answered. It was explained that study participation was voluntary, parental consent could be withdrawn at any time, and study participation (or not) would not affect the infant's clinical care. If parents had understood the study procedure, considered their options, and communicated their decision, valid written informed consent was obtained using the study Consent Form (Appendix B). The infant was assigned a study participant number, and three copies of the consent form were distributed: one given to parents, one filed in the medical notes, and one retained by the research group.

Impact of COVID-19

The recruitment of subjects into both studies was curtailed by the outbreak of the coronavirus pandemic. Clinical activity continued, including ROP screening and treatment, however non-essential staff including research staff were not permitted access to the NICU. This resulted in limited recruitment to later studies involving Optos ROP screening and ROP treatment. The analyses in Chapter 5 therefore are underpowered to achieve statistical significance, but demonstrate approaches that could be applied to a larger group.

2.3 Clinical Procedures

All procedures were clinically indicated and scheduled by the clinical team caring for the infant.

2.3.1 Binocular Indirect Ophthalmoscopy

Binocular indirect ophthalmoscopy is a method of viewing the posterior segment of the eye, namely the retina and optic nerve. A headset is used to direct a bright light source through a handheld condensing lens to obtain a stereoscopic, real, inverted image of the ocular structures. In order to optimally view the retinal periphery (Dhillon *et al*, 1993), an eyelid speculum is inserted and a scleral indenter is used to stabilise and rotate the eye. Screening for ROP using BIO is considered the 'gold standard' approach to ROP screening because it affords a dynamic stereoscopic view of the entire fundus including the *ora serrata* (RCOphth, 2008; Fierson, 2013).

2.3.2 Non-Contact Ultra-Widefield Scanning Laser Ophthalmoscopy

Optomap (Optos Plc. Dunfermline, UK) is a non-invasive retinal imaging system that is used as an adjunct to BIO ROP screening in the NICU of the John Radcliffe Hospital, Oxford University NHS Foundation Trust. The 'flying baby' technique is used for Optos imaging of premature infants (Patel *et al*, 2013). The infant is placed securely along the ophthalmologists upturned forearm, such that the body is supported along its length, and the infant's head is supported by the ophthalmologists's hand. The free hand of the ophthalmologist is used to fine-tune the head position of the infant in front of the Optos (see Figure 2.2).

General advantages of fundus photography as an adjunct to BIO ROP screening include obtaining an objective record of the retinal findings for monitoring and treatment planning, and the ability to share images between remote locations for the purposes of telemedicine (RCOphth, 2008; Fierson, 2013). A specific advantage of Optos imaging is the 'ultra-widefield' capability - images are approximately 200 degrees horizontally (Kato *et al*, 2019) which visualises the retina and choroid beyond the equator of the eye (see Figure 2.3).

Optos imaging is used by the local clinical team for two indications. Firstly, Optos is used to perform ROP screening in infants in the Low Dependency Unit. This group of infants are clinically stable, and therefore the handling that is required to position the infant for Optos screening is considered to be safe. Optos ROP screening may be a less aversive experience for infants than other methods of screening since bright light and scleral depression are not used and the eye is not touched (Patel *et al*, 2013). The procedure provides a valuable digital record of the infant's retinal vessel development. This is useful both on an individual level, for example, if the infant is transferred to another hospital for ongoing care; and on a population level, because the images aid clinical understanding of normal retinal vessel development during the preterm period. Optos ROP screening is not used in infants who are due their final screening examination; in this case BIO ROP screening is performed. This is because it is imperative to fully assess the retina at the final screening, and BIO ROP screening is considered the 'gold standard' for visualising the *ora serrata*.

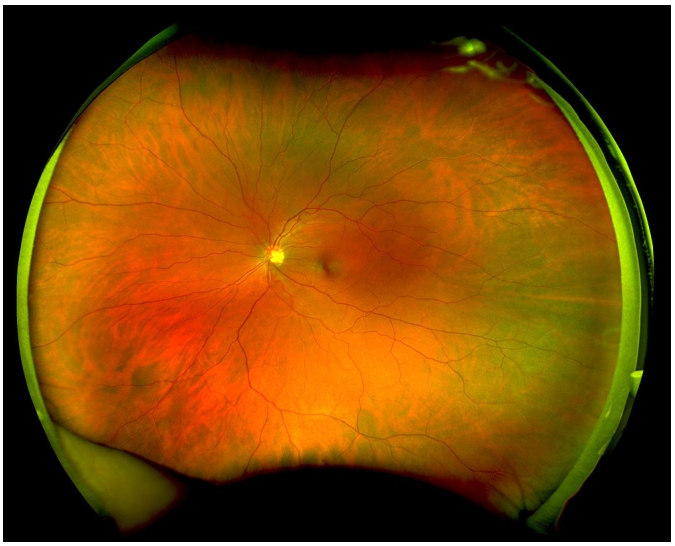
Secondly, Optos imaging is used to record ROP disease in affected infants and to monitor disease progression or regression. The images provide an objective record of the infant's retinal disease and are used for teleconsultation, treatment planning, and assessing the effect of ROP treatment. While there have been no reports of adverse events associated with Optos imaging for ROP (Mao *et al*, 2020; Fung *et al*, 2021), it has been suggested that the handling required for Optos imaging may risk physiological instability in premature infants (Patel *et al*, 2013; Fung *et al*, 2018). Imaging is performed therefore in the presence of clinical NICU staff, and is not attempted in the most fragile infants.

2.3.3 Laser Treatment

Laser treatment for ROP aims to reduce retinal demand for oxygen by thermal ablation (photocoagulation) of the peripheral retina. Laser treatment is performed using a binocular indirect diode laser headset together with a condensing lens, speculum and

The infant is held with the body resting along the ophthalmologist's right forearm. The clinician's right hand supports the infant's neck and chin, and the left hand steadies the infant's head such that the forehead is resting in the required location on the Optos camera. An eyelid speculum may be used to aid eyelid opening. Image from Patel *et al* (2013) used with permission.

Figure 2.2: 'Flying baby' technique for Optos imaging in the Neonatal Intensive Care Unit



Optomap, CC BY-SA 4.0, via Wikimedia Commons.

Figure 2.3: Example of an Optos ultra-widefield retinal image

indenter as for BIO ROP screening. Diode laser is the standard treatment for ROP because it results in fewer unfavourable structural outcomes (e.g. macular dragging, retinal detachment) and better visual acuity outcomes than cryotherapy, the historic alternative treatment (Fallaha *et al*, 2002; Ng *et al*, 2002; Connolly *et al*, 2002).

Diode laser treatment is known to be painful in adults (Balles *et al*, 1990; Friberg and Venkatesh, 1995; Lira *et al*, 2010), hence various analgesic strategies have been used in infants undergoing laser treatment for ROP. The optimal analgesic strategy would provide effective pain relief while minimising adverse systemic side effects.

Local anaesthesia for laser treatment provides insufficient pain relief when used alone (Batton *et al*, 2006). A study of 15 infants who underwent laser treatment for ROP with local anaesthesia alone identified high clinical pain scores during the procedure (Sato *et al*, 2015). Two studies observed life-threatening cardiorespiratory instability post-procedure in infants treated with local anaesthesia alone for ROP laser (Haigh *et al*, 1997; Jiang *et al*, 2017). A survey of practice in the UK identified that treating clinicians do not use local anaesthetic alone for laser ROP treatment (Chen *et al*, 2007).

Intravenous sedation is used in several UK centres (Chen *et al*, 2007). However, intravenous sedation may not provide adequate pain relief when used in combination with local anaesthesia. Two studies of infants undergoing laser treatment for ROP with intravenous fentanyl sedation identified moderate clinical pain scores during the procedure (Sato *et al*, 2015; Sethi *et al*, 2020) and physiological instability during and after the procedure (Sethi *et al*, 2020).

Intubation and mechanical ventilation with sedation or inhaled general anaesthesia are used in many UK centres (Chen *et al*, 2007). This approach is performed in the operating theatre, and a paediatric anaesthetist is required to deliver the anaesthesia. Intubation with sedation or general anaesthesia provides effective pain relief for ROP treatment (Sato *et al*, 2015). However, several studies have reported post-operative physiological instability and difficulty in weaning infants from ventilatory support

following the procedure (Haigh *et al*, 1997; Sato *et al*, 2015; Jiang *et al*, 2017).

In our centre, oral sedation and analgesia (chloral hydrate 50mg/kg, oral morphine 100μg/kg, oral paracetamol 15mg/kg) are given 45 minutes to 1 hour prior to the start of the laser procedure by the NICU clinical staff, and anaesthetic eye drops (oxybuprocaine) and sub-Tenon's anaesthesia (bupivacaine) are given by the ophthalmologist just prior to the procedure (Woodhead *et al*, 2007; Ah-Chan *et al*, 2008; Parulekar *et al*, 2008).

Chloral hydrate and morphine are known to evoke changes in the EEG of term infants. Chloral hydrate is a mild sedative which induces sleep in premature infants (Litman *et al*, 2010); it does not have analgesic properties (Boswinkel and Litman, 2005). Chloral hydrate is converted by the liver and by red blood cells to its active metabolite trichloroethanol, and cleared by excretion in urine (Jacqz-Aigrain and Burtin, 1996). When used as a single oral dose of 25 - 50 mg/kg, chloral hydrate is considered safe in premature infants. Chloral hydrate sedation evokes morphological changes in event-related potentials in the EEG of term neonates (Zhang *et al*, 2019).

Morphine is an opioid which provides sedation in preterm infants when administered via intravenous infusion (Scott *et al*, 1999; Pacifici, 2016). Morphine is also commonly prescribed for the treatment of infant pain, however morphine infusion is not effective in reducing infant pain during invasive ventilation (Bellu *et al*, 2010), and the efficacy of oral morphine in the treatment of pain from ROP screening has not been established (Hartley *et al*, 2018). Morphine is converted by the liver and kidneys to its active metabolite morphine-6-glucuronide, and cleared by excretion in urine (Jacqz-Aigrain and Burtin, 1996). Neonates may be less sensitive to morphine analgesic effects than adults, because infants have a lower rate of morphine metabolism, and immature target receptors (Jacqz-Aigrain and Burtin, 1996). Morphine sedation results in EEG depression with increased inter-burst intervals and longer periods of discontinuity (Bell *et al*, 1993; Norman *et al*, 2013; Young and Da Silva, 2000).

The procedure is performed on the NICU in a treatment room. There is no pre-emptive increase to the infant's level of ventilatory support and a paediatric anaesthetist is not required. This peri-procedural management was developed in response to difficulties in delivering timely treatment when co-ordination with anaesthetic support was required.

2.3.4 Intravitreal Injection Treatment

Intravitreal injection of inhibitory monoclonal antibodies targeting VEGF is a more recent treatment option for ROP, which inhibits the neovascularisation process central to ROP pathogenesis (Mintz-Hittner and Best, 2009; Mintz-Hittner *et al*, 2011). Intravitreal injection is performed via the sclera using a narrow gauge sterile needle to introduce anti-VEGF into the vitreous. An eyelid speculum and toothed forceps are used to access and stabilise the eye. Anti-VEGF is used alone (Mintz-Hittner and Kuppel, 2008) or as an adjunct to ROP treatment (Law *et al*, 2010; Axer-Siegel *et al*, 2011), and has been shown to reduce unfavourable structural outcomes and maintain longterm good visual acuity (Martinez-Castellanos *et al*, 2013; Stahl *et al*, 2019).

Intravitreal injection is the treatment choice for ROP developing in a very preterm infant (World Health Organisation, 2012). In this case, much of the retina is still avascular, and laser ablation would result in a greatly reduced visual field, whereas intravitreal injection spares the peripheral retina. Caution is required in intravitreal injection in extremely preterm infants because systemic absorption of anti-VEGF may adversely influence vascular development in the brain, resulting in impaired neurodevelopment (Morin *et al*, 2016; Lien *et al*, 2016). Intravitreal injection is also used for treating ROP in unwell or unstable infants.

In our centre, laser is the first-line treatment for most infants while intravitreal injection is used for select indications; this is in line with UK practice (Adams *et al*, 2017). Laser treatment is a lengthy procedure lasting approximately an hour, whereas intravitreal injection can be performed within minutes. Elements of laser

treatment that are considered to be painful or stressful, such as administration of sub-Tenon's anaesthetic, scleral indentation, and bright retinal illumination, are not used in the intravitreal injection procedure and therefore the treatment is considered suitable for infants who may not tolerate laser treatment safely. A third indication for intravitreal injection is in combination with laser treatment for managing aggressive posterior ROP (Law *et al*, 2010; Axer-Siegel *et al*, 2011), a situation in which both treatments are required to halt progression of unusually pernicious disease.

Intravitreal injection treatment causes mild pain in adults and local anaesthetic eye drops are widely used to treat pain associated with the procedure (Rifkin and Schaal, 2012; Moisseiev *et al*, 2014; Han *et al*, 2020). One study of 9 infants undergoing intravitreal injection for ROP treatment with local anaesthetic eye drops identified moderate clinical pain scores during the procedure. It was noted that pain behaviour was temporally related to eyelid speculum insertion, while the injection itself appeared well-tolerated (Castellanos *et al*, 2013). For intravitreal injection treatment in our centre, oral paracetamol 15mg/kg is given 45 minutes to 1 hour prior to the procedure. Oral chloral hydrate is used on a case-by-case premise. Anaesthetic eye drops are given by the ophthalmologist just prior to the procedure, which is performed on the NICU in a treatment room.

2.3.5 Heel Lance

Heel lancing is a standard method of capillary blood sampling in term and preterm infants. In our centre, heel lancing is performed using sterile, single-use BD Quikheel lancets (Becton, Dickinson and Company), either in the 'Infant' size (width 2.50mm, depth 1.0mm) for term infants, or 'Preemie' size (width 1.75mm, depth 0.85 mm) for premature infants. A heel lance blood sample is performed by cleaning the infant's heel, pressing the lancet against the side of the heel, and depressing the lancet trigger to release a retractable blade.

During studies, 30 seconds elapsed prior to gentle pressure on the foot to stimulate

blood flow from the lancet incision. This interval allowed pain score assessment in response to the heel lance stimulus alone.

2.3.6 Nappy Change

Nappy change is part of routine care in the NICU that takes place approximately every 3 hours for clinically stable infants, every 6 hours for infants that are clinically unstable, and can be deferred up to 12 hours if necessary. The nappy changes analysed in this thesis were performed by a member of the research team trained to deliver care to premature infants in a neurodevelopmentally supportive manner, in line with the principles of the Newborn Individualized Developmental Care and Assessment Program (NIDCAP) (Als and McAnulty, 2011). Nappy change included the following steps: alert infant to nappy change with gentle tone and light touch; place infant in lateral position and use containment holding to settle infant (see Figure 2.4); loosen and remove soiled nappy; clean and dry perineal area; replace fresh nappy, fasten clothing and return infant to original position.

In Chapter 3, the change in EEG features following BIO ROP screening is characterised. BIO ROP screening is a complex stimulus including elements which are considered to be painful (eyelid speculum insertion, scleral indentation), as well as elements which are considered to be stressful but not painful (bright light, manual restraint). In order to better interpret the findings of Chapter 3, nappy change was included as a negative control procedure for noxious-related changes in brain activity. Nappy change is a frequently performed clinical procedure of similar duration to BIO ROP screening. It is not considered to be painful, but has been observed in previous studies to evoke similar trends in physiological stress responses as BIO ROP screening (Wang and Change, 2004; Lyngstad *et al*, 2014). I hypothesised that EEG changes following BIO ROP screening which were related to the infant nociceptive response would not be observed following nappy change.

During containment holding, the infant's head is gently cupped, and the infant's feet are held such that the legs are bent and tucked in. The contained position recreates the foetal position *in utero*. Image from https://www.nationwidechildrens.org/family-resources-education/health-wellness-and-safety-resources/helping-hands/hand-containment.

Figure 2.4: Containment holding for preterm infants

2.4 Recording Techniques

2.4.1 Electroencephalography

To apply EEG electrodes, the infant's head was measured using a paper measuring tape, and a chinagraph pencil was used to mark the electrode locations. The infant's scalp was gently cleaned using a cotton bud and preparation gel (Nuprep gel, D.O. Weaver and Co.). Single use sterile electrodes (Ambu Neuroline disposable Ag/AgCl cup electrodes) were placed onto the scalp using conductive paste (Elefix EEG paste, Nihon Kohden) and secured using paper hypoallergenic surgical tape.

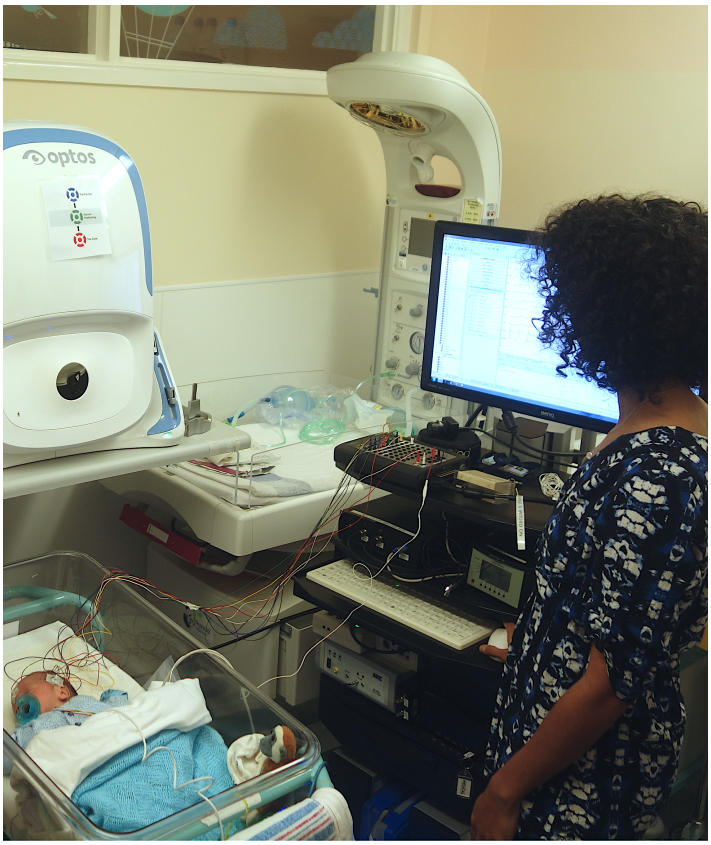
The practical application of EEG in the neonatal setting is challenging due to various factors. The infant may be nursed in an incubator which restricts the space for applying EEG electrodes. The electrode array must be placed efficiently to minimise the time spent handling the infant. An electrode cap was not used, allowing individualised electrode placement and avoiding cap-related scalp indentation. Premature infants are vulnerable to infection, therefore strict hand hygiene is necessary as well as the use of single-use disposable equipment where possible (Lloyd *et al*, 2015). Due to the fragile nature of preterm infant skin, gentle cleaning is necessary to avoid skin abrasions, and any adhesive must be removed with care (St. Louis *et al*, 2016). The

premature infant's head size is small therefore care must be taken during electrode placement to avoid salt bridge formation between closely adjacent electrodes. The EEG studies in this thesis were performed by members of the research team trained in neonatal EEG placement, in the presence of at least one clinical member of the research team trained in Neonatal Life Support. An example of study equipment set-up is shown in Figure 2.5.

An array of 8 electrodes was placed to form a longitudinal and transverse montage: Fz (reference), FPz (ground), FCz, Cz, CPz, C3, C4, T3, T4 and Oz (see Figure 2.6). The reduced array was chosen due to the small head size of the premature infants, time considerations in the NICU, and precedent from previous studies of infant pain carried out by the research group (Hartley *et al*, 2018; Green *et al*, 2019). A decision was taken after data collection to analyse recordings from a single central recording electrode (Cz). Data from one subject included in this thesis but studied previously was originally referenced to FPz and re-referenced to Fz during analysis. The electrode impedance was below 10 kOhms for all studies.

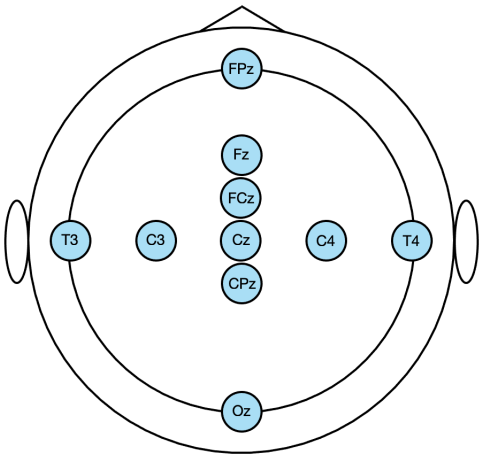
EEG was recorded and amplified using the SynAmps RT system (Compumedics Neuroscan). The signal was sampled at 2000 Hz using the CURRYscan7 neuroimaging suite (Compumedics Neuroscan). Neonatal EEG frequencies may be as slow as 0.01 Hz (St. Louis *et al*, 2016), however this lower range overlaps with sweat artefact low frequency oscillations (White and Van Cott, 2010; Reis *et al*, 2014), therefore the signal was high-pass filtered at 0.5Hz. A low-pass limit of 70 Hz was used, with a notch filter at 49-51 Hz added to removed 50 Hz mains interference. All equipment was compliant with international safety standards for medical devices (IEC 60601-1).

For studies involving ROP screening, ROP treatment or nappy change, a marker was added to the EEG signal at the time of the study to indicate the timing of the pain score baseline period, the start of the procedure, and the end. For ROP screening and treatment studies, the start was defined as instillation of anaesthetic eye drops prior to the procedure, and the end was defined as removal of the eyelid speculum



Clockwise from top left: Optos imaging system; electroencephalography recording system; wires for pulse oximeter and electrocardiogram leads (white); electroencephalography electrodes. Shown with parental permission.

Figure 2.5: Example of equipment for a study involving Optos screening



Schematic of infant's head seen from above. An array of 8 electrodes was placed to form a longitudinal and transverse montage: Fz (reference), FPz (ground), FCz, Cz, CPz, C3, C4, T3, T4 and Oz.

Figure 2.6: Electrode placement

from the second eye. For nappy change studies, the start was defined as opening the nappy fastenings, and the end was defined as closing the nappy fastenings.

The pain score baseline for BIO ROP screening studies was recorded median 405s following the start of the EEG recording, and median 198s prior to the start of screening. The nappy change pain score baseline was recorded median 452s following the start of the EEG recording, and median 164s before screening.

Additional markers indicating drug administration or handling were also marked where relevant. For studies involving heel lancing, the deployment of the lance was time-locked to the EEG signal using methods described previously (Worley *et al*, 2012). A K-shear accelerometer (Kistler Instruments Ltd.) held against the lance allowed detection of the lance deployment, which in turn triggered the contemporaneous addition of a marker in the EEG recording.

2.4.2 Physiological Monitoring

Infants in the Intensive Care and High Dependency areas of the NICU have routine continuous monitoring of HR and oxygen saturations via ECG and pulse oximetry respectively. For such infants, the monitoring leads were disconnected from the Unit monitors and connected to a research monitor (IntelliVue MX800 patient monitor, Philips). The research monitor was connected to a laptop which recorded the physiological data using ixTrend software (ixellence GmbH, now ixitos GmbH, Germany). Infants in the Low Dependency area of the NICU do not have routine physiological monitoring. For these infants, the research team attached ECG leads to the chest and a pulse oximeter to the foot. All non-routine monitoring was removed at the end of the study. As for the EEG signal, markers were added manually in ixTrend to indicate the timing of the PIPP-R baseline period, the start and end of the procedure, and any other important events such as drug administration or handling.

2.4.3 Facial Video

After the EEG and physiological monitoring equipment had been set up and the infant was settled, a hand-held digital camera was used on video mode to record the infant's face for 15 seconds. This provided a record of the infant's baseline behavioural state. Immediately following the clinical procedure, a second facial video was recorded for 30 seconds to record any pain-related facial behaviours (eye squeeze, brow bulge, and nasolabial furrow). The two videos were used to calculate a PIPP-R score, together with corrected gestational age, HR and oxygen saturations as described in Section 2.5.3.

2.5 Analysis

2.5.1 Subjects

BIO ROP screening Twenty-seven infants had EEG recorded during BIO ROP screening, of whom 17 were included in analysis. Reasons for exclusion were: 2 infants' EEG recording had artefact precluding analysis; 1 infant did not have BIO ROP screening but underwent optical coherence tomography scanning instead; 2 infants were diagnosed with excluded conditions on the day of study (interventricular haemorrhage, meningitis); and 5 infants were aged $<$ 34 weeks corrected gestational age at study. A *post hoc* decision was taken to include only infants of corrected gestational age 34 weeks or older in analysis of brain activity. The intention was to align inclusion criteria with previous studies of event-related potentials (ERP). The template of noxious-related ERP described in Section 1.5.3 was characterised in infants of corrected gestational age 34 weeks or older, because the ability to respond discriminatively to stimuli is reported to emerge around 33 weeks (Fabrizi *et al*, 2011a; Green *et al*, 2019). Younger infants are more likely to display non-specific neuronal activity to a noxious stimulus, whereas after approximately 34 weeks corrected gestational

age noxious-specific activity is likely to be generated (Fabrizi *et al*, 2011; Hartley *et al*, 2017).

Additionally, data were included from an independent group of infants who had EEG recorded during BIO ROP screening as part of the placebo arm of a randomised controlled trial. The placebo group comprised 15 infants, of whom 8 had sufficient duration artefact-free EEG before and after screening recorded for inclusion in this study.

Optos ROP screening Seven infants had EEG recorded during Optos ROP screening, of whom 6 were included in analysis. EEG data from one infant was excluded due to insufficient duration of post-procedure recording for analysis.

ROP treatment Six infants had EEG recorded during ROP treatment, of whom 5 were included in analysis. One infant was excluded because the procedure was performed while intubated and ventilated; a decision was taken to include only non-ventilated infants because this represented usual clinical practice in our centre.

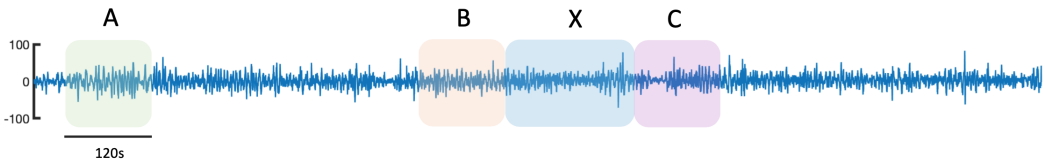
Nappy change Fifteen infants had EEG recorded during nappy change, of whom 9 were included in analysis. Reasons for exclusion were: 2 infants' EEG recording had artefact precluding analysis, 2 had other experimental stimuli applied prior to nappy change (light, sound, touch) and were excluded to avoid confounding the response to experimental stimuli with the response to nappy change; 1 infant was excluded due to a different nappy change technique (the infant's legs were lifted rather than turning the infant on its side); and 1 infant had stoma care performed in addition to nappy change. The number of infants recruited for study during nappy change was low, because recruitment was stopped in March 2020 following the outbreak of COVID-19. This meant that access to the NICU was restricted for clinical researchers, and clinical studies were prohibited as part of wider restrictions introduced to limit the spread of coronavirus.

Heel lance Data were included from a database of infants who had EEG recorded during heel lancing as part of previous research studies. Twenty-three healthy infants had sufficient duration artefact-free EEG before and after heel lancing for inclusion in this study. Infants with normal blood test results were included because previous research suggests that intercurrent illness may affect nociceptive responses (Sellam *et al*, 2011).

2.5.2 Electroencephalography

EEG epochs at the Cz electrode of 120 seconds duration were identified using CURRY-scan7 and exported as .dat files. Recording length for analysis of the effect of complex stimuli on EEG is not standardised, with 1-5 minute recordings having been reported (Le Pera *et al*, 2000; Ishitani *et al*, 2005; Nir *et al*, 2012); in this thesis, a period of 120 seconds was analysed.

For BIO ROP screening studies, three 120s epochs were selected (see Figure 2.7): one epoch (A) was selected at the start of EEG recording as early as possible following electrode placement with the intention that subjects be in a similar state of arousal during the baseline recording. This baseline epoch was selected median 17s (lower quartile (LQ) 5s, upper quartile (UQ) 142s) following the start of the EEG recording. A second epoch (B) was chosen immediately prior to screening in order to identify changes which occurred due to non-noxious stimulation - tactile stimulation from handling, auditory stimulation from voices of the ophthalmology team, and visual stimulation from the ophthalmoscope near the infant. The third 120s epoch (C) was selected immediately following the procedure with the intention of capturing post-procedure changes in brain activity before recovery to baseline.



The figure illustrates the timing of epoch selection in the studies presented in this thesis. Epoch A = baseline epoch of 120 seconds duration selected as early as possible in the study recording, with the intention that study subjects be in a similar state of arousal following electrode application. Epoch B = 120 second epoch immediately before the start of the study procedure. This epoch was selected only for studies of BIO ROP screening, with the intention of identifying EEG changes related to non-noxious stimulation associated with the arrival of the clinical team. Epoch X = epoch representing the approximate duration of the study procedure, during which EEG was recorded but not analysed due to excessive motion artefact. Epoch C = post-procedure epoch of 120 seconds duration selected immediately following the procedure, with the intention of capturing post-procedure changes in brain activity before recovery to baseline.

Figure 2.7: Schematic of electroencephalogram epoch selection

For Optos ROP screening, ROP treatment, heel lance, and nappy change studies an epoch of 120s duration was selected before (A) and after (C) the procedure. The baseline epoch was selected median 334s (LQ 278s, UQ 413s) following the start of the EEG recording for Optos ROP screening; median 16s (LQ 5s, UQ 94s) for ROP treatment; median 5s (LQ 5s, UQ 30s) for heel lancing, and median 61s (LQ 8s, UQ 243s) for nappy change subjects. EEG recorded during the study procedures was frequently affected by movement and muscle artefact, therefore EEG from this period was not analysed.

Forty-five quantitative features of EEG were calculated for each 120s epoch using MATLAB code developed by Pillay (Pillay *et al*, 2018). The features are listed in Table 2.2.

Table 2.2: Quantitative features of the electroencephalogram investigated in this thesis

Frequency-domain	*Fourier transformation*
	Average power of full electroencephalogram signal
	Relative power of delta, theta, alpha & beta bands
	Average frequency of full electroencephalogram signal power spectrum
	Average frequency of delta, theta, alpha and beta bands
	Spectral entropy
	Wavelet transformation
	Mean & standard deviation of detail levels 3 to 5 and approximation level 5 coefficients
	Sum of squared detail levels 3 to 5 and approximation level 5 coefficients
Time-domain	*Amplitude*
	Mean & standard deviation of the signal amplitude
	Mean & standard deviation of the amplitude in delta, theta, alpha and beta bands
	Amplitude range (max-min)
	Sum of amplitudes
	Sum of squared amplitudes
	Maximum value of first derivative of the signal
	Coastline distance
	Zero crossing rate
	Range electroencephalogram
	Mean & standard deviation
	Median & interquartile range
	Lower & higher margins

Frequency domain features

Fourier transformation Frequency domain analysis is commonly performed using Fourier transformation of the EEG signal, which decomposes the signal into its component frequencies. The frequencies are then divided into classical frequency bands: delta (0.5-3 Hz), theta (3-8 Hz), alpha (8-12 Hz), beta (12-30 Hz). However, there are limitations to applying the Fourier transform to an EEG signal. Fourier analysis requires the data to be strictly periodic or stationary, that is, unchanging with regard to time. EEG does not fulfil this requirement because the signal changes over time, hence cautious interpretation of EEG analysis using Fourier transformation is required. Nonetheless, the Fourier transform is widely used in EEG research and therefore the approach was included in this thesis.

Average power of full EEG signal & relative delta, theta, alpha, and beta power The Welch method (Welch, 1967) (MATLAB command 'pwelch') was used to estimate the power spectral density, that is, the power of the signal at each component frequency. Welch's method was chosen because it minimises noise in the signal by windowing the total signal (Proakis and Manolakis, 1996). A Hamming window was used with 50% overlap to better reduce ringing artefact than a simple rectangular window. In Welch's method, after windowing, the Discrete Fourier Transform is used in calculating a periodogram (that is, a power spectral density estimate) per window. The resultant modified periodograms are averaged to give a periodogram for the whole signal.

The MATLAB command 'bandpower' was used to calculate the average power of the signal from the periodogram within the full filtered range (0.5 to 30 Hz). Relative bandpowers were calculated using the MATLAB command 'bandpower' for each frequency band, then dividing by the full power of the signal within the filtered range.

Average frequency of full EEG signal & delta, theta, alpha and beta bands The MATLAB command 'meanfreq' was used to calculate the average frequency of the signal from the periodogram within the full filtered range. The average

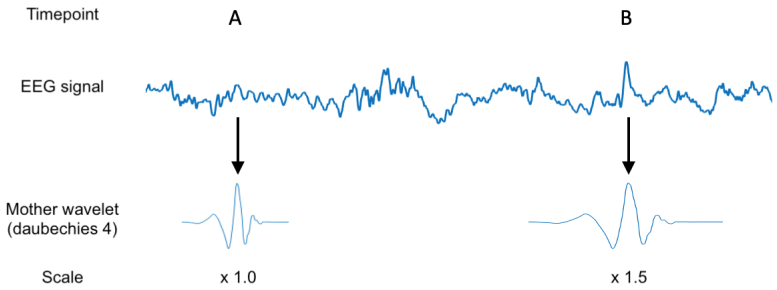
delta, theta, alpha and beta frequencies were similarly calculated.

Spectral entropy Power spectral entropy is a measure of complexity of the EEG signal. High entropy implies a complex signal and low entropy an ordered or predictable signal (Gomez and Hornero, 2010). EEG complexity may reflect neural network connectivity (Bosl *et al*, 2011; Sporns *et al*, 2000) and brain maturation in infants (Zhang *et al*, 2009; Scher *et al*, 2010). Previous studies have observed that EEG complexity increases with age through the neonatal period (Janjarasjitt *et al*, 2008; Lippe *et al*, 2009; Zhang *et al*, 2009), and decreases in pathological states (Costa *et al*, 2005; Bosl *et al*, 2011; Al-Nuaimi *et al*, 2018).

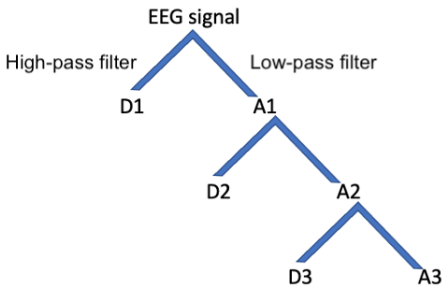
Spectral entropy was estimated using Powell and Percival's approach (Powell and Percival, 1979) to calculate Shannon's entropy from the periodogram (Shannon, 1948).

Wavelet transformation Wavelet transformation is a method of spectral analysis that takes into account the non-stationary or time-varying nature of the EEG signal. The frequency content of the signal is represented temporally as a series of wavelet functions. A "mother wavelet" is selected which can be scaled to approximate the signal as it changes over time (see Figure 2.8a). The Daubechies order 4 wavelet was chosen because of precedent in previous studies of preterm infant EEG (Greene *et al*, 2008; Sen *et al*, 2014; Pillay *et al*, 2018). The EEG signal is low-pass filtered using the mother wavelet function to produce approximation coefficients, and high-pass filtered to produce detail coefficients. The approximation coefficients of the signal at each scale of the wavelet are termed A_n for the n^{th} level of signal decomposition. The detail coefficients of the signal between each scale are termed D_n (see Figure 2.8b). The EEG signal was resampled to 250 Hz for wavelet transformation. The frequencies contained in each decomposition level are shown in Table 2.8c.

The MATLAB command 'wavedec' with the option 'db4' was used to perform the Discrete Wavelet Transformation. Measures calculated from detail levels 3 to 5 and approximation level 5 coefficients included the mean, standard deviation, and sum of



(a) A 'mother wavelet' is selected, which can be scaled to approximate the EEG signal as it changes over time. In this schematic, the Daubechies order 4 wavelet is scaled to approximate the EEG signal at timepoints A and B.



(b) The EEG signal is low-pass filtered using the mother wavelet function to produce approximation coefficients (termed A_n for the n^{th} level of signal decomposition). The signal is high-pass filtered to produce detail coefficients (termed D_n for the n^{th} level of signal decomposition).

Discrete Fourier Transform		Discrete Wavelet Transform	
Band	Range	Level	Range
Beta	12 - 30 Hz	Detail 3	15.6 - 31.3 Hz
Alpha	8 - 12 Hz	Detail 4	7.8 - 15.6 Hz
Theta	3 - 8 Hz	Detail 5	3.9 - 7.8 Hz
Delta	0.5 - 3 Hz	Approximation 5	0 - 3.9 Hz

(c) This table highlights that, for a sampling frequency of 250 Hz, the frequencies contained in wavelet decomposition levels 3 to 5 overlap with the Fourier frequency bands. For example, level 3 detail coefficients contains beta frequencies, and level 4 detail coefficients contains alpha and beta frequencies.

Figure 2.8: Description of the Discrete Wavelet Transformation

squared coefficients. The sum of squared coefficients is a measure of signal power in the coefficients frequency range (Bradshaw and Spies, 1992).

Time domain features

Amplitude MATLAB was used to calculate amplitude features of the full signal and the delta, theta, alpha and beta bands, including mean, standard deviation, range, sum, sum of squared amplitudes and the maximum value of the first derivative of the signal. The coastline distance was calculated, which is the sum of the absolute differences between consecutive data points (Yetton *et al*, 2016). The zero-crossing rate was calculated by counting the number of times the signal crossed zero per 1 second window, then calculating the standard deviation across all windows.

Range EEG The range EEG is a compressed representation of the EEG, similar to amplitude-integrated EEG that is used in clinical practice (O'Reilly *et al*, 2012; Navakatikyan *et al*, 2016). It estimates inter-burst intervals by measuring peak-to-peak amplitude in the EEG signal, and reflects EEG discontinuity. The range EEG was summarised using the mean, standard deviation, median, IQR, 5th percentile, and 95th percentile.

Event-related potentials

EEG epochs at the Cz electrode of 5 second duration were selected before and after heel lancing using CURRYscan7 and exported as .dat files. For each subject, the ERP evoked in response to heel lancing was compared to a template of noxious-related infant brain activity that has been developed previously, and is described in Section 1.5.3. MATLAB was used to scale the template to fit the infant brain activity evoked by heel lancing, thereby quantifying the ERP magnitude for each subject. When projecting the template onto a single subject's ERP, latency-corrected adaptive filtering (Woody, 1967) was used to account for temporal variability, and thereby

maximise correlation between the template and subject. A template magnitude $<$ 1 implied the observed ERP response was less than the average response evoked by heel lancing in healthy term infants, and *vice versa*.

Artefact identification

EEG recordings were visually inspected for artefact including transient electrode artefact, single electrode artefact in Cz, movement artefact, muscle artefact, and electrical interference. If artefact were present, an alternative 120s epoch was selected as soon as possible from that point in the recording. For ERP analysis, the subject was rejected if artefact occurred in either 5s epoch. Examples of EEG artefact are shown in Figure 2.9.

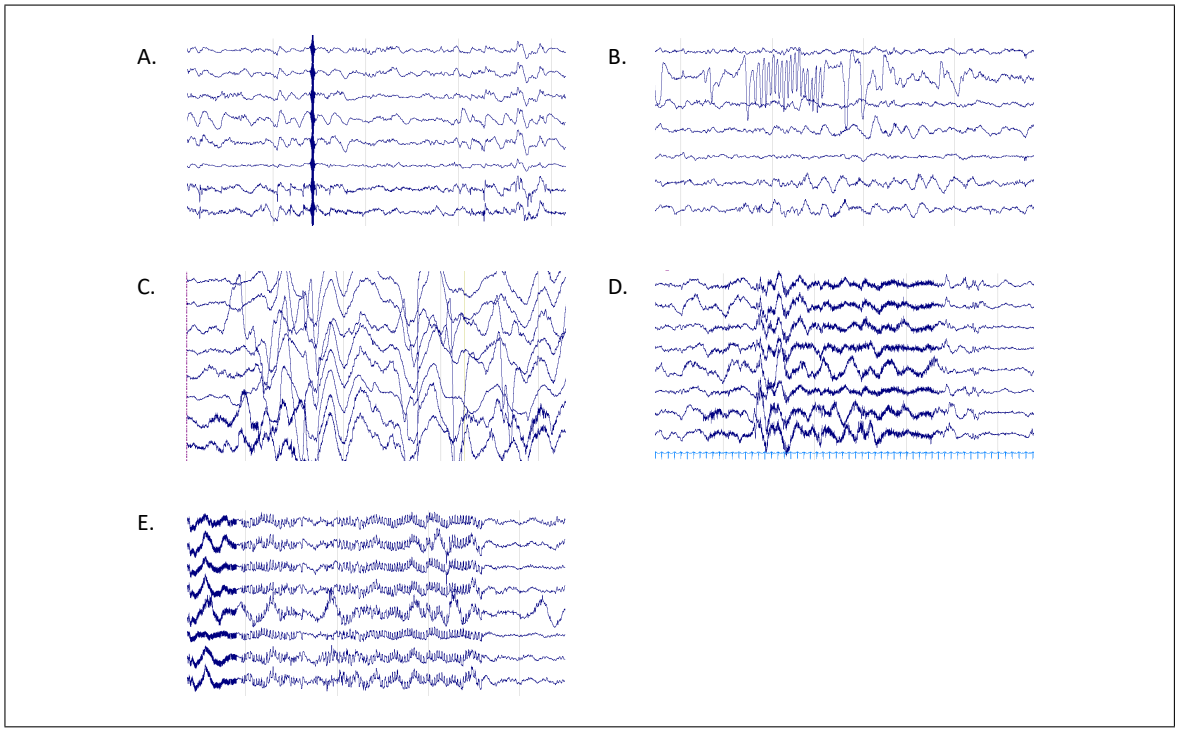


Figure 2.9: Examples of EEG artefact: A. Transient electrode B. Single electrode C. Movement D. Muscle E. Electrical.

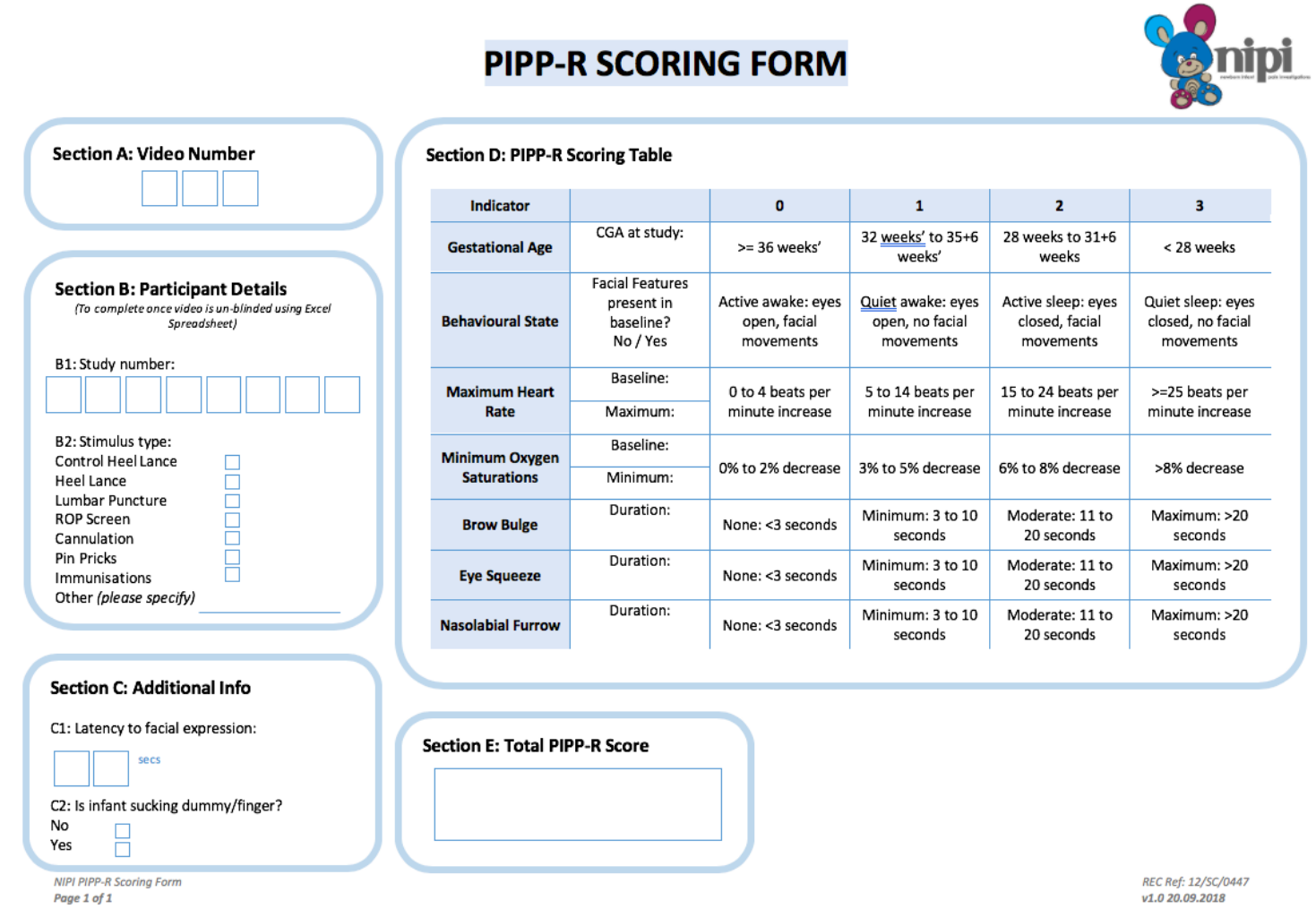
2.5.3 Behaviour

Pain score

A Premature Infant Pain Profile-Revised (PIPP-R) score (Stevens *et al*, 2014) was calculated for all subjects using corrected gestational age at study, the facial video of the baseline behavioural state, the physiological recording of change in heart rate and oxygen saturation, and the facial video following the procedure. The subject's corrected gestational age at study was assigned a score of 0 to 3. Younger infants received a higher score due to the influence of corrected gestational age on behavioural responses - at earlier corrected gestational age, infants are less likely to display a behavioural response to noxious stimulation (Johnston *et al*, 1993; Johnston *et al*, 1996; Stevens *et al*, 1996).

Using the pre-procedure 15 second facial video recording, the subject's baseline behavioural state was identified (active awake, quiet awake, active sleep, or quiet sleep) and assigned a score from 0 to 3 according to the PIPP-R scoring chart (see Figure 2.10). Using the post-procedure 30 second facial video recording, the cumulative duration of each component of facial expression (brow bulge, eye squeeze, nasolabial furrow) was timed with a stopwatch, and each component was assigned a score from 0 to 3 according to the scoring chart. For ROP screening and treatment studies, the post-procedure video was recorded immediately after eyelid speculum removal to allow assessment of eye squeeze.

The continuous physiological recordings were exported as .csv files and analysed in MATLAB to calculate the difference between the mean value during the 15 second pre-procedure period and the maximum value during the 30 second post-procedure period. A score of 0 to 3 was assigned according to the number of beats per minute increase in HR and the percentage decrease in oxygen saturation. Finally, a composite score was calculated for each subject (maximum possible score = 21). An example of the PIPP-R scoring form used is shown in Figure 2.10.



PIPP-R SCORING FORM

Section A: Video Number

Section B: Participant Details

(To complete once video is un-blinded using Excel Spreadsheet)

B1: Study number:

B2: Stimulus type:

- Control Heel Lance
- Heel Lance
- Lumbar Puncture
- ROP Screen
- Cannulation
- Pin Pricks
- Immunisations
- Other *(please specify)*

Section C: Additional Info

C1: Latency to facial expression: secs

C2: Is infant sucking dummy/finger?

- No
- Yes

Section D: PIPP-R Scoring Table

Indicator		0	1	2	3
Gestational Age	CGA at study:	>= 36 weeks'	32 weeks' to 35+6 weeks'	28 weeks to 31+6 weeks	< 28 weeks
Behavioural State	Facial Features present in baseline? No / Yes	Active awake: eyes open, facial movements	Quiet awake: eyes open, no facial movements	Active sleep: eyes closed, facial movements	Quiet sleep: eyes closed, no facial movements
Maximum Heart Rate	Baseline: / Maximum:	0 to 4 beats per minute increase	5 to 14 beats per minute increase	15 to 24 beats per minute increase	>=25 beats per minute increase
Minimum Oxygen Saturations	Baseline: / Minimum:	0% to 2% decrease	3% to 5% decrease	6% to 8% decrease	>8% decrease
Brow Bulge	Duration:	None: <3 seconds	Minimum: 3 to 10 seconds	Moderate: 11 to 20 seconds	Maximum: >20 seconds
Eye Squeeze	Duration:	None: <3 seconds	Minimum: 3 to 10 seconds	Moderate: 11 to 20 seconds	Maximum: >20 seconds
Nasolabial Furrow	Duration:	None: <3 seconds	Minimum: 3 to 10 seconds	Moderate: 11 to 20 seconds	Maximum: >20 seconds

Section E: Total PIPP-R Score

NIPI PIPP-R Scoring Form
Page 1 of 1

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v1.0 20.09.2018

Gestational age at study, baseline behavioural state, change in heart rate and oxygen saturations, and duration of facial expression are graded to provide a total score.

Figure 2.10: Premature Infant Pain Profile - Revised scoring form

2.5.4 Physiology

Subjects

Thirty-six subjects had physiological data recorded during BIO ROP screening, of whom 22 were included in analysis. Reasons for exclusion were: 1 infants' physiology recording had artefact precluding analysis; 1 infant did not have BIO ROP screening but underwent optical coherence tomography scanning instead; 12 infants had less than 15 minutes physiology recorded. A *post hoc* decision was taken to exclude infants with less than 15 minutes duration of physiological recording. The intention was to select a recording duration that allowed calculation of heart rate variability measures across consecutive 5 minute windows, while excluding as few infants as possible. Seven infants had physiological data recorded during Optos ROP screening, of whom 6 were included in analysis; 6 infants had physiological data recorded during ROP treatment, of whom 5 were included in analysis. Reasons for exclusions are stated in Section 2.5.1.

Physiological instability

Physiological instability events (bradycardia, tachycardia, desaturation, and apnoea) do not have standardised parameters for preterm infants. Bradycardia has been defined as a reduction in HR below 80-100 bpm for more than 10-20 seconds, and tachycardia as an increase in HR above 180-200 bpm for more than 10-15 seconds (Litman *et al*, 2010; O'Sullivan *et al*, 2010; Mitchell *et al*, 2011; Hartley *et al*, 2018). Oxygen desaturation has been defined as a reduction in oxygen saturation below 80-90% for more than 10 seconds, and apnoea as cessation in breathing for more than 20 seconds or a shorter pause associated with bradycardia (Litman *et al*, 2010; O'Sullivan *et al*, 2010; Mitchell *et al*, 2011; Eichenwald, 2016; Chandrasekharan *et al*, 2018; Hartley *et al*, 2018). The definitions used in this thesis were used in a randomised controlled trial (Hartley *et al*, 2018), and are listed in Table 2.3.

Table 2.3: Instability event definitions used in this thesis

Bradycardia	Heart rate less than 100 beats per minute for $\geq$ 15 seconds
Tachycardia	Heart rate greater than 200 beats per minute for $\geq$ 15 seconds
Desaturation	Oxygen saturations less than 80% for $\geq$ 10s seconds
Apnoea	Respiratory pause for $\geq$ 20 seconds associated with bradycardia

For ROP screening and treatment studies, a 15 minute epoch of continuous physiological recording was selected before and after the procedure, exported as a .csv file, and analysed in MATLAB to identify instability events. For BIO ROP studies, instability events were also calculated in 1, 6 and 10 hour epochs before and after screening.

Heart rate variability

HRV measures were calculated using 15 minute epochs of continuous ECG recordings before and after ROP screening studies. ECG recordings were pre-processed in R (Version 3.6.3, The R Foundation for Statistical Computing) then converted into a time series of R peaks using MATLAB. The time series was filtered to remove outlying RR intervals occurring due to artifactual beats (RR interval $<$ 0.2s) or missed beats (RR interval $>$1.5s). Interpolation was performed using the RHRV package (Garcia Martinez *et al*, 2017; Version 4.2.5) to transform the RR time series into a regularly sampled signal (NN time series).

Frequency domain measures The NN time series was decomposed into its component frequencies using the Fourier transform (window size 300 seconds; slide 30 seconds; Hamming window). The standardised frequency bands used in HRV analysis are: ultra-low ($<$0.003 Hz), very low (0.003-0.05 Hz), low (0.05-0.15Hz), and high frequency (0.15-0.4Hz). Application of the Fourier transform to ECG data assumes the data are stationary or cyclical; however this assumption is not met by ECG data because the signal changes over time. Theoretically therefore caution is required in interpreting frequency domain analysis using the Fourier transform, although this

approach is used commonly in practice. Wavelet transformation is an alternative method of analysing the RR time series in the frequency domain; this approach was not included in the preliminary investigation of BIO ROP screening in this thesis, but may be considered in future.

The physiological basis of frequency domain HRV analysis is the different rate of response of the parasympathetic and sympathetic arms of the autonomic nervous system. The parasympathetic nervous system responds within less than 1 second and its activity is reflected by the high frequency band (Pagani *et al*, 1993; Task Force of the European Society of Cardiology, 1996; Shaffer and Ginsberg, 2017). In adults, the high frequency band is also influenced by the synchronisation of the heart rate and respiration, known as respiratory sinus arrhythmia (Shaffer and Ginsberg, 2017). For this reason, the upper bound of the high frequency band is adjusted in infants, who have a higher respiration rate, to 0.15-1.5Hz (Shaffer and Ginsberg, 2017; Kozar *et al*, 2018). However, several studies have found little or negligible influence of respiratory sinus arrhythmia on HRV activity in infants (Giddens and Kitney, 1985; Longin *et al*, 2005; Joshi *et al*, 2019), and therefore the importance of accounting for this characteristic in infant HRV analysis is uncertain.

The sympathetic nervous system responds within 5 or more seconds and its activity is reflected by the low frequency band. The low frequency band has been considered to arise predominantly from the sympathetic nervous system (Malliani *et al*, 1991; Montano *et al*, 1994), or to represent the balance between parasympathetic and sympathetic contributions (Akselrod *et al*, 1981; Appel *et al*, 1989). The ratio of low frequency to high frequency activity (LF/HF ratio) is influenced by the effect of respiratory sinus arrhythmia on the high frequency band, the possible contribution of the parasympathetic nervous system to the low frequency band, and the non-reciprocal interaction of the sympathetic and parasympathetic systems in cardiac homeostasis (Billman, 2013).

The ultra-low frequency and very-low frequency bands have uncertain physiolo-

gical basis, and may reflect circadian rhythm and the activity of the intrinsic cardiac nervous system respectively (Kleiger *et al*, 2005; Shaffer and Ginsbery, 2017). These bands are not characterised in this thesis due to their uncertain physiological correlates. Table 2.4 lists the frequency-domain HRV measures calculated for each ECG epoch.

Table 2.4: Frequency-domain heart rate variability measures investigated in this thesis

Absolute power of the low frequency band (0.05–0.15 Hz)
Absolute power of the high frequency band (0.15–1.5 Hz)
Ratio of low to high frequency power

Time domain measures

Statistical measures of the NN interval The standard deviation of the NN interval (SDNN) is a measure of all components contributing to variability in the heart rate recording (Task Force of the European Society of Cardiology, 1996). Since variance increases with recording duration, a standardised recording duration was used in this study (15 minutes). SDNN is strongly related to the total power of the signal, since variance is equivalent to total power in spectral analysis (Task Force of the European Society of Cardiology, 1996).

Other windowed indices include the standard deviation of the average NN interval over 5 minutes (SDANN) and the average of the standard deviation of the NN interval over 5 minutes (SDNN index). Two measures of the differences between NN intervals were included: the standard deviation of successive differences between NN intervals (SDSD), and the number of interval differences greater than 50ms as a proportion of the total number of NN intervals (pNN50). The SDANN is produced by ultra-low frequency activity, the SDNN index reflects very-low frequency activity, and SDSD and pNN50 are closely correlated with high frequency activity (Shaffer and Ginsberg, 2017).

Geometric measures The HRV triangular index is calculated from a histogram of NN interval, where the total number of NN intervals is divided by the number of NN intervals in the modal bin. This measure is dependent on the width of the histogram bins; 128 Hz is the most frequent sampling rate and was used in this study, hence the bin width was 7.8 ms. The triangular interpolation of the NN interval histogram (TINN) is the baseline width of the NN histogram measured as the base of a triangle. These measures reflect all variability in the recording and therefore are measures of the total power of the signal (Task Force of the European Society of Cardiology, 1996).Table 2.5 lists the time-domain HRV measures calculated for each ECG epoch.

Table 2.5: Time-domain heart rate variability measures investigated in this thesis

Statistical measures of the NN interval
SDNN: Standard deviation of NN intervals
SDANN: Standard deviation of the average NN intervals for each 5 min segment
SDNN index: Mean of the standard deviation of the NN intervals for each 5 min segment
SDSD: Standard deviation of successive differences between NN intervals
pNN50: Number of successive NN intervals that differ by more than 50 ms / Total number of NN intervals
Geometric measures
HRV triangular index: Integral of the NN interval histogram divided by its height
TINN: Triangular interpolation of the NN interval histogram

NN interval = 'normal to normal'; interbeat interval from which artifact has been removed

Chapter 3

Does binocular indirect ophthalmoscopy evoke noxious-related change in the infant brain?

3.1 Abstract

Introduction

ROP is a disorder of the retinal vasculature which develops in premature infants and is a cause of childhood blindness. In order to detect sight-threatening disease, BIO screening for ROP is performed repeatedly during the neonatal period. Unfortunately, BIO ROP screening is considered to be painful and stressful for infants, as inferred from changes in behaviour and physiology evoked by the procedure. Pain and stress during the preterm period can lead to negative consequences for infant development. Reduction of pain and stress during BIO ROP screening have therefore been attempted using pharmacological and non-pharmacological strategies. However,

it is challenging to evaluate the effectiveness of such interventions, due to limitations in the accurate measurement of infant pain and stress. Physiological and behavioural measures yield inconsistent results and do not distinguish between infant pain and stress. Measurement of infant brain activity may be a useful additional approach to quantifying infant pain and stress evoked by BIO ROP screening. To address this question, the hypothesis that BIO ROP screening evokes noxious-related changes in infant brain activity was tested.

Methods

A wide range of EEG features were investigated for change following BIO ROP screening in an exploratory analysis. The findings were tested in confirmatory analyses comprising 1) an independent group of preterm infants undergoing BIO ROP screening, 2) a negative control group of preterm infants undergoing nappy change, and 3) a positive control group of infants undergoing heel lance blood sampling. The findings were further investigated by relating the change in EEG features following heel lancing to the magnitude of the noxious event-related potential (ERP), a previously-defined measure of infant nociception. Finally, the relationships between changes in infant brain activity, behaviour and physiology evoked by BIO ROP screening were characterised using data from the infants included in the first two analyses.

Results

Forty-five quantitative features of EEG were calculated in 17 infants undergoing BIO ROP screening. A subset of 10 features relating to alpha and beta frequency bands (12 - 30 Hz) changed significantly from baseline to post-procedure ($p < 0.05$). The features were grouped into three clusters using cluster analysis, and the feature with the greatest effect size per cluster was identified: standard deviation of level 3 detail coefficients, sum of squared level 4 detail coefficients, and relative beta power. These

findings were confirmed in an independent group of 8 infants undergoing BIO ROP screening.

Non-noxious stimulation (nappy change in 9 infants) did not evoke a significant change in EEG activity, whereas a different noxious stimulus (heel lance blood sampling in 23 infants) evoked a significant increase in one of the three features, relative beta power ($p = 0.0061$). There was a significant relationship between the change in relative beta power and the magnitude of the noxious ERP following heel lancing (Spearman's rho = 0.52, $p = 0.033$). Finally, there was no significant relationship between changes in infant brain activity, behaviour and physiology evoked by BIO ROP screening.

Conclusions

BIO ROP screening evokes a significant increase in higher frequency brain activity (12 - 30 Hz) in preterm infants. The increase in relative beta power evoked by BIO ROP screening may be noxious-related. The research described in this Chapter aims to contribute to understanding of the noxious-related changes in brain activity evoked by BIO ROP screening. Improved understanding of the infant experience of ROP screening may allow clinicians to better identify and treat infant pain and stress during essential clinical procedures.

3.2 Methods

3.2.1 Participating Infants

Seventeen subjects undergoing BIO ROP screening were included in Analysis 1, and an independent group of 8 subjects undergoing BIO ROP screening were included in Analysis 2. Nine subjects studied during nappy change were included in Analysis 3, and 23 subjects studied during heel lancing were included in Analysis 4. Demographic

information for all subjects included in Chapter 3 is shown in Table 3.1.

3.2.2 Experimental Design

This section contains an overview of the analyses comprising this Chapter. In Analysis 1, 45 quantitative features of EEG were calculated in 17 infants undergoing BIO ROP screening. The change in each EEG feature after BIO ROP screening was quantified, clusters of related features identified, and the features with greatest effect size for change post-procedure were selected from each cluster for further testing. The change in each of the subset of features of EEG identified in Analysis 1 was calculated in 8 infants undergoing BIO ROP screening (Analysis 2), 9 infants during nappy change (Analysis 3), and 23 infants undergoing heel lance blood sampling (Analysis 4). Additionally in Analysis 4, the magnitude of the noxious-related ERP was calculated, and related to the change in each of the subset of EEG features. Finally, for the subjects undergoing BIO ROP screening and nappy change, the PIPP-R score was calculated. For infants undergoing BIO ROP screening, the relationships between the change in each of the subset of EEG features and behavioural and physiological measures were characterised.

3.2.3 Recording Techniques

Brain activity was recorded using EEG in all studies. Behavioural and physiological measures were recorded for infants undergoing BIO ROP screening and nappy change. Chapter 2 (General Methods) describes the recording techniques in detail.

3.2.4 Analysis

Exploratory Analysis

Forty-five quantitative EEG features (listed in Table 2.2) were analysed in 17 subjects undergoing BIO ROP screening. Three 120s EEG epochs were analysed for each

Table 3.1: Demographic information for subjects included in Chapter 3

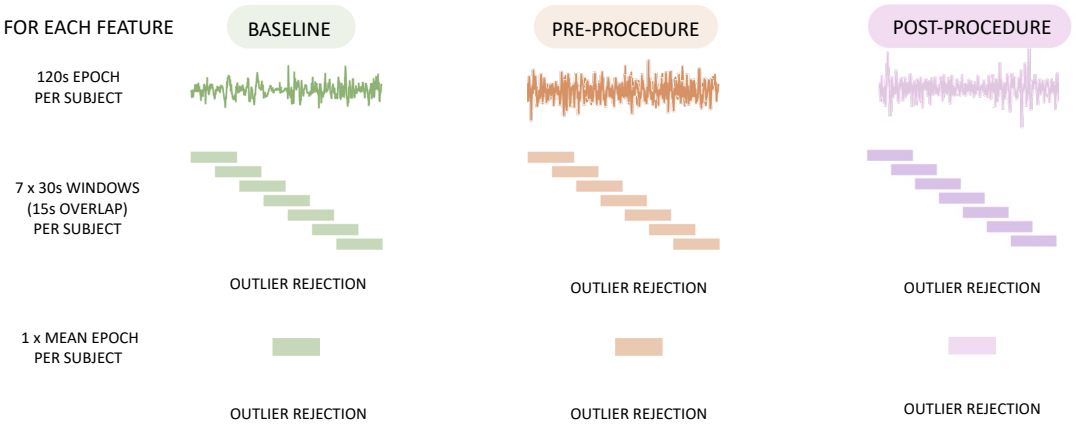
	Analysis 1	Analysis 2	Analysis 3	Analysis 4
Number of infants analysed	17	8	9	23
Corrected gestational age at study (weeks)	34.86 (34.71, 40.29)	35.43 (34.93, 37.71)	34.71 (34.71, 38.71)	39.43 (36.79, 41.14)
Birthweight (grammes)	1160 (880, 1912)	1100 (1009, 2110)	1140 (880, 3535)	3260 (1100, 4470)
Number of males	6 (35%)	5 (63%)	5 (56%)	13 (57%)
Apgar score	8.5 (7, 10)	9 (6.5, 10)	10 (8, 10)	10 (8, 10)
Normal vaginal delivery	5 (29%)	2 (25%)	5 (56%)	13 (57%)
Procedure duration (minutes)	2.9 (2.0, 4.0)		2.5 (2.1, 3.8)	-

Figures are median (lower quartile, upper quartile) or number (percentage). Apgar score at 5 minutes for studies 1, 3 and 4; at 10 minutes for Analysis 2.

subject (baseline, pre-procedure, post-procedure) as discussed in Section 2.5.2. Each epoch was analysed in consecutive 30s windows with 15s overlap (see Figure 3.1). Epoch length and window duration are not standardised in investigation of tonic noxious stimuli; epoch length of 2-3 minutes has been reported (Gram *et al*, 2015; Ki *et al*, 2016; Furman *et al*, 2018) and window duration of 1-25s has been reported (Chen *et al*, 1983; Peng *et al*, 2014; Gram *et al*, 2015; Zhang *et al*, 2016; Li *et al*, 2016; Furman *et al*, 2018). For this analysis, 30s was used as the window duration because range EEG features could not be calculated across a shorter window. Overlapping windows were used in order to increase the number of samples (Coggeshall and Wu, 2011; Dehghani *et al*, 2019).

The consecutive 30s windows comprising each 120s epoch were tested for outlying values for each feature. On average, 5% of the windows were rejected for each feature. In this thesis, outlying values were defined as more than three scaled median absolute deviations away from the median; a robust method of outlier detection (Hampel, 1974; Leys *et al*, 2013) (MATLAB command 'isoutlier').

Following outlier rejection, the mean value was calculated across remaining windows for each feature. This resulted in 17 mean values at baseline, pre-procedure and post-procedure, for each feature. Outlier detection was performed again for the mean baseline group, and subjects identified were rejected in the mean baseline, pre-procedure and post-procedure groups. On average, 2% of subjects were rejected at this stage for each feature.



This schematic illustrates the process of EEG epoch analysis. For each subject, an artefact-free 120 second epoch was chosen at three timepoints (baseline, pre-procedure, and post-procedure). Each epoch was analysed in consecutive 30 second windows, with 15 second overlap, resulting in seven windows per epoch. The mean value for each epoch was calculated after rejecting outlying windows. Finally for each time-point, outlying mean epoch values were rejected for the group of 17 subjects. The analysis was performed for each feature.

Figure 3.1: Schematic of electroencephalogram analysis procedure

Next, it was investigated whether there was a significant change from baseline to pre-procedure for each feature. As discussed in Section 2.5.2, the pre-procedure epoch was chosen in order to identify changes in brain activity which occurred due to non-noxious stimulation before the start of the procedure, for example, tactile stimulation from handling, auditory stimulation from voices of the ophthalmology team, and visual stimulation from the ophthalmoscope near the infant. Any features that demonstrated significant change baseline to pre-procedure were excluded from further analysis, because the change would be evoked by non-noxious stimuli occurring before the procedure of interest. The remaining features were investigated for significant change from baseline to post-procedure using the same method.

Features which demonstrated significant change from baseline to post-procedure were analysed to identify related features using cluster analysis. Frequency domain features were expected to be related since both the Fourier and the Wavelet transforms decompose the EEG signal into its component frequencies. The relationship between Fourier bands and wavelet levels is shown in Figure 2.8c. For each cluster identified, the feature with greatest effect size was selected for further investigation in confirmatory analysis. The statistical approaches used in this analysis are detailed

in Section 3.2.5.

Confirmatory Analyses

For an independent group of 8 subjects undergoing BIO ROP screening, a group of 9 subjects undergoing nappy change, and a group of 23 subjects undergoing heel lance blood sampling, the subset of features identified in the first analysis was calculated in two 120s epochs (baseline, post-procedure) for each subject. Outlier rejection was performed as for Analysis 1. For each feature, it was investigated whether there was a significant increase from baseline to post-procedure.

Additionally, for the heel lancing group, the magnitude of the noxious-related ERP was calculated for each subject. As discussed in Section 1.5.3, a template of noxious-related brain activity has been characterised in infants undergoing experimental and clinical noxious stimulation (Hartley *et al*, 2017). The template was projected onto individual EEG responses evoked by heel lancing, allowing calculation of the response magnitude for each subject. In order to investigate the relationship between the magnitude of the noxious-related ERP and the change from baseline to post-procedure for each EEG feature, correlation analysis was performed. Subjects were rejected prior to correlation analysis if outlying values were identified within the ERP responses or the EEG responses.

Finally, for all infants studied during BIO ROP screening (25 infants), nappy change (9 infants), and heel lancing (23 infants), a PIPP-R score was calculated as described in Chapter 2 (General Methods). Additionally, for the BIO ROP screening group, the relationships between EEG feature change post-procedure and PIPP-R score, mean baseline HR (15s before the baseline marker), and mean change in HR (30s after the procedure end marker) were characterised.

3.2.5 Statistical Analysis

Exploratory Analysis

The differences in baseline to pre-procedure epoch were tested for whether they were drawn from a normal distribution using the Anderson-Darling test (MATLAB command 'adtest'). For normally distributed differences, a paired-sample two-tailed t-test was performed; for non-normally distributed values, a Wilcoxon signed rank test was performed.

The False Discovery Rate (Benjamini and Hochberg, 1995) method was used to correct for multiple hypothesis testing. This method was selected because False Discovery Rate has greater power and a lower type II error rate than the more conservative Bonferroni method. A less conservative approach was acceptable because the results of Analysis 1 were tested in subsequent confirmatory analyses. The False Discovery Rate method is described here in brief: p-values (p) were ranked from least to greatest, then corrected p-values (p_c) were calculated using:

$$p_c = Q\left(\frac{i}{m}\right)$$

where $Q = 0.2$ (accepted type I error rate), i = rank, and m = number of tests. The greatest rank where $p \leq p_c$ was identified and all hypotheses above this rank were accepted.

After excluding any features that demonstrated significant change baseline to pre-procedure, the remaining features were investigated for significant change from baseline to post-procedure using the same method of parametric or non-parametric testing with False Discovery Rate control for multiple testing.

Features which demonstrated significant change from baseline to post-procedure were analysed to identify related features. Linkage analysis was used to group features into a hierarchical cluster tree based on the correlation coefficients (MATLAB command 'linkage'; using parameters 'single' (shortest distance) and 'correlation' (1 - sample correlation between points)). Cluster accuracy was assessed using the cophen-

etic correlation coefficient (Sokal and Rohlf, 1962), where the closer the coefficient is to 1, the more accurate the clustering solution (Saracli *et al*, 2013). Finally, Cohen's d effect size (Cohen, 1988; Lakens, 2013) was calculated for the change from baseline to post-procedure for each feature, using the MATLAB function 'computeCohen_d (paired)'. Hedge's correction (Hedges and Olkin, 1985) for a small sample size was applied:

$$\text{Hedge's } g = d\left(1 - \frac{3}{4(n_1+n_2)-9}\right)$$

where d= Cohen's d, n_1= sample size baseline, and n_2 = sample size post-procedure.

Confirmatory Analyses

The differences in baseline to post-procedure were tested for whether they were drawn from a normal distribution using the Anderson-Darling test, then a paired-sample one-tailed t-test was performed for normally-distributed values, and a Wilcoxon signed rank test for non-normally distributed values. The MATLAB Multiple Testing Toolbox (Martinez-Cagigal, 2021) was used to apply the Hochberg method to correct for multiple hypothesis testing. This method controls the Family Wise Error Rate, and was selected because it strongly controls Type 1 errors, which is important in confirmatory analysis of a small number of hypotheses. The Hochberg method also has greater power than the Holm and Bonferroni methods of controlling Family Wise Error Rate (Benjamini and Hochberg, 1995; Chen *et al*, 2017).

For infants undergoing heel lancing, the magnitude of the noxious-related ERP was calculated using methods developed by Hartley (Hartley *et al*, 2017). The relationship between the magnitude of the noxious-related ERP and the change from baseline to post-procedure for each EEG feature was characterised using Spearman's correlation, since the magnitude of the noxious-related ERP was non-normally distributed.

The same method was used to characterise the relationships between EEG feature change post-procedure and PIPP-R score, baseline HR, and post-procedure change

in HR for all infants studied during BIO ROP screening. Pearson's linear correlation coefficient for normally distributed values, and Spearman's rho for non-normally distributed values.

3.3 Results

3.3.1 BIO ROP screening evokes significant change in infant brain activity

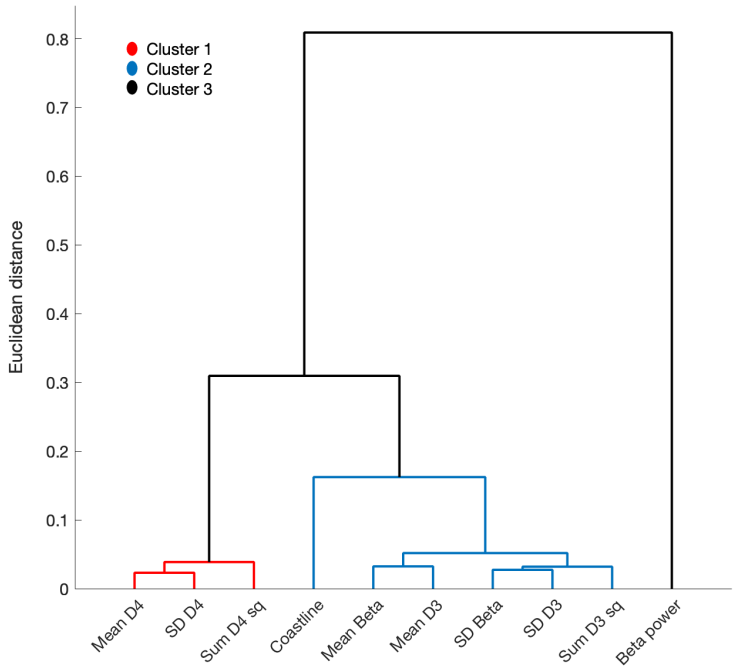
Forty-five quantitative features of EEG were calculated in 17 infants undergoing BIO ROP screening. Non-noxious stimuli arising prior to the procedure of interest did not evoke significant change in EEG features: no features demonstrated a significant change from baseline to pre-procedure epochs. A subset of 10 features relating to alpha and beta frequency bands changed significantly from baseline to post-procedure (see Table 3.2).

Table 3.2: Electroencephalographic features which demonstrated significant change from baseline following BIO ROP screening in a group of 17 preterm infants

Feature	Uncorrected p-value	Corrected p-value
Sum of squared level 3 detail coefficients	0.00085	0.0044
Standard deviation of level 3 detail coefficients	0.0010	0.0089
Standard deviation of beta amplitude	0.0017	0.013
Mean of level 3 detail coefficients	0.0019	0.018
Mean of beta amplitude	0.0031	0.022
Standard deviation of level 4 detail coefficients	0.010	0.027
Sum of squared level 4 detail coefficients	0.011	0.031
Relative beta power	0.022	0.036
Coastline distance	0.033	0.040
Mean of level 4 detail coefficients	0.043	0.044

For each EEG feature, the uncorrected p-value (paired-sample two-tailed t-test or Wilcoxon signed rank for non-parametric features) and corrected p-value following False Discovery Rate correction are shown.

Linkage analysis identified three clusters (see Figure 3.2): cluster 1 contained features characterising the signal in the alpha frequency range (7.8 - 15.6 Hz) derived from wavelet analysis. Cluster 2 contained features characterising the signal in the beta frequency range (12 - 31.3 Hz), derived from the Fourier transform and wavelet analysis. Coastline distance, a feature relating to signal amplitude, was also included in cluster 2. Cluster 3 contained relative beta power, a feature characterising the signal power in the frequency range 12 - 30 Hz derived from the Fourier transform. The cophenetic correlation coefficient for the hierarchical structure determined by linkage analysis was 0.94, indicating a good representation of the data.



This figure shows a dendrogram plot of the hierarchical cluster tree created by linkage analysis. Three clusters are identified: cluster 1 (red) comprises 3 features (mean D4 = mean of level 4 detail coefficients; SD D4 = standard deviation of level 4 detail coefficients; sum D4 sq = sum of squared level 4 detail coefficients). Cluster 2 (blue) comprises 6 features (coastline = coastline distance; mean beta = mean of beta amplitude; mean D3 = mean of level 3 detail coefficients; SD beta = standard deviation of beta amplitude; SD D3 = standard deviation of level 3 detail coefficients; sum D3 sq = sum of squared level 3 detail coefficients). Cluster 3 (black) comprises 1 feature (beta power = relative beta power).

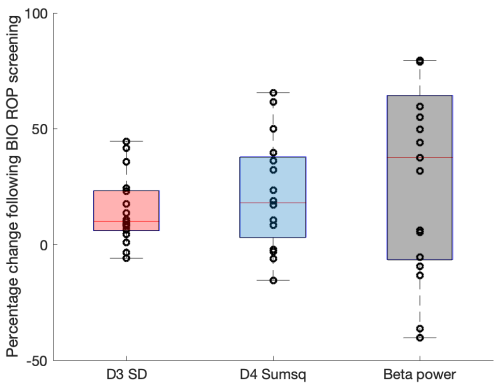
Figure 3.2: Linkage analysis of the change in 10 electroencephalographic features following BIO ROP screening in a group of 17 infants

The corrected Cohen's d effect size for change from baseline to post-procedure is listed in Table 3.3 for the subset of 10 features. The feature with greatest effect size

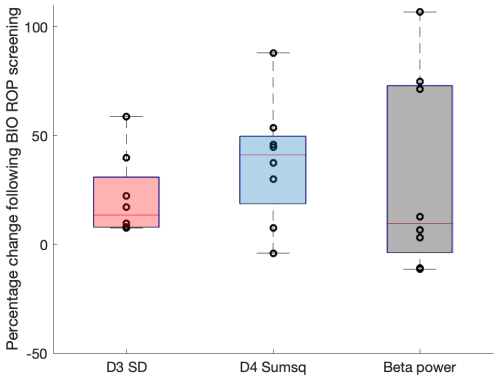
in cluster 1 was the sum of squared detail coefficients in wavelet transform level 4, a measure of signal power in the alpha and low beta frequency range (7.8 - 15.6 Hz). For cluster 2, the standard deviation of the detail coefficients in wavelet transform level 3, a measure of signal amplitude in the beta frequency range (12 - 31.3 Hz); and for cluster 3, the relative beta power. The median increase from baseline to post-procedure was 11% for the standard deviation of level 3 detail coefficients, 18% for the sum of squared level 4 detail coefficients, and 39% for relative beta power (see Figure 3.3a).

Table 3.3: Cohen's d effect size for the change in 10 electroencephalographic features following BIO ROP screening in a group of 17 infants. The feature with greatest effect size for each cluster is highlighted.

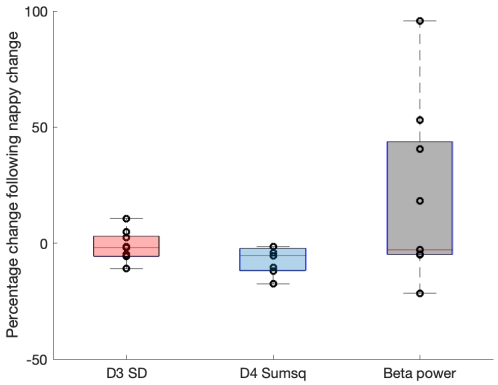
Cluster	Feature	Effect size
1	**Sum of squared level 4 detail coefficients**	0.70
	Standard deviation of level 4 detail coefficients	0.69
	Mean of level 4 detail coefficients	0.52
2	**Standard deviation of level 3 detail coefficients**	0.95
	Sum of squared level 3 detail coefficients	0.91
	Standard deviation of beta amplitude	0.89
	Mean of level 3 detail coefficients	0.83
	Mean of beta amplitude	0.82
	Coastline distance	0.57
3	**Relative beta power**	0.57



(a) Percentage change in three electroencephalographic features following BIO ROP screening in a group of 17 infants



(b) Percentage change in three electroencephalographic features following BIO ROP screening in a group of 8 infants



(c) Percentage change in three electroencephalographic features following nappy change in a group of 9 infants

Figure 3.3: Percentage change in three electroencephalographic features following clinical procedures: standard deviation of level 3 detail coefficients (D3 SD), sum of squared level 4 detail coefficients (D4 Sumsq), and relative beta power.

3.3.2 Changes in infant brain activity evoked by BIO ROP screening are confirmed in an independent dataset

The first analysis presented in this Chapter was exploratory in nature; the aim was to investigate whether there is a change in infant brain activity following BIO ROP screening.A wide range of candidate features of EEG was reduced to a subset of features for further investigation. This confirmatory analysis tested the hypothesis that BIO ROP screening evokes a change in the subset of features highlighted in Table 3.3. The change in these features was calculated in an independent group of 8 infants undergoing BIO ROP screening. Each feature again demonstrated a significant change from baseline to post-procedure (see Table 3.4), confirming the robustness of the observations. One feature which did not change significantly following BIO ROP screening, relative delta power (p-value = 0.48), was re-tested in the independent dataset group as a control: again there was no significant change in relative delta power following BIO ROP screening (p-value = 0.96). The median increase following screening was 13% for the standard deviation of level 3 detail coefficients, 41% for the sum of squared level 4 detail coefficients, and 9% for relative beta power (see Figure 3.3b).

Table 3.4: Electroencephalographic features which demonstrated significant change following BIO ROP screening were re-tested in an independent group of 8 infants undergoing BIO ROP screening

Feature	Uncorrected p-value	Corrected p-value
Standard deviation of level 3 detail coefficients	0.0039	0.012
Sum of squared level 4 detail coefficients	0.0085	0.017
Relative beta power	0.048	0.048

For each EEG feature, the uncorrected p-value (paired-sample one-tailed t-test or Wilcoxon signed rank for non-parametric features) and corrected p-value following Hochberg correction are shown.

3.3.3 Changes in infant brain activity following BIO ROP screening are not evoked by a non-noxious control procedure

A negative control procedure (nappy change) was used to test the hypothesis that EEG feature change observed following BIO ROP screening was non-noxious-related. The change in the subset of features highlighted in Table 3.3 was calculated in 9 infants during nappy change. The number of infants included in this analysis is small; although more data was intended to be collected, further recruitment was limited by restrictions on carrying out clinical studies during the COVID-19 pandemic.

Nappy change did not evoke a significant change in EEG activity from baseline to post-procedure (see Table 3.5), which may suggest the results of the previous analyses were not due to non-noxious stimulation arising during screening. There was a median reduction following nappy change of 2% for the standard deviation of level 3 detail coefficients, 5% for the sum of squared level 4 detail coefficients, and 4% for relative beta power (see Figure 3.3c). For all infants in BIO ROP screening and nappy change groups, the PIPP-R score, baseline HR and change in HR post-procedure are listed in Table 3.6. The median PIPP-R score for infants undergoing BIO ROP screening was 9, indicating moderate pain (Fatollahzade *et al*, 2020), whereas the median PIPP-R score for nappy change was 5, indicating minimal or no pain (Stevens *et al*, 1996).

Table 3.5: Electroencephalographic features which demonstrated significant change from baseline following BIO ROP screening were re-tested in a group of 9 infants undergoing nappy change

Feature	Uncorrected p-value	Corrected p-value
Standard deviation of level 3 detail coefficients	0.84	0.99
Sum of squared level 4 detail coefficients	0.99	0.99
Relative beta power	0.25	0.74

For each EEG feature, the uncorrected p-value (paired-sample one-tailed t-test or Wilcoxon signed rank for non-parametric features) and corrected p-value following Hochberg correction are shown.

Table 3.6: Behavioural and physiological outcomes for infants undergoing BIO ROP screening and nappy change

	BIO ROP screening (n=25)	Nappy change (n=9)
Premature Infant Pain Profile - Revised score	9 (7.5, 11.5)	5 (4.5, 5.25)
Mean baseline heart rate (bpm)	154 (142, 163)	154 (152, 157)
Mean post-procedure heart rate change (bpm)	40 (27, 60)	20 (11.5, 27)

Figures are median (lower quartile, upper quartile). bpm = beats per minute.

3.3.4 Changes in infant brain activity following BIO ROP screening are evoked by a noxious control procedure

In the fourth analysis, a positive control procedure (heel lancing) was used to test the hypothesis that EEG feature change identified following BIO ROP screening is noxious-related. Firstly, the change in the subset of features highlighted in Table 3.3 was calculated in 23 infants during heel lancing; a significant change following heel lancing was identified in relative beta power only (see Table 3.7). There was a median increase following heel lancing of 5% for the standard deviation of level 3 detail coefficients, 7% for the sum of squared level 4 detail coefficients, and 12% for relative beta power (see Figure 3.4). The median PIPP-R score for heel lancing was 5, and the median post-procedure change in HR was 13 bpm (see Table 3.8).

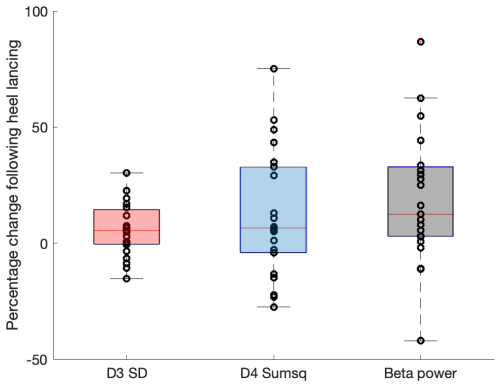
Next, a template of noxious-related brain activity was used, which was characterised in response to heel lancing as discussed in Section 1.5.3. The template is specific to noxious stimuli, and is validated for use in independent data to quantify the noxious-related response to a stimulus. In this analysis, the template was fitted to the brain activity evoked by heel lancing in a group of 23 infants in order to calculate the magnitude of the noxious-related ERP. The relationship was characterised between the change in EEG features and the magnitude of the noxious-related ERP. Following heel lancing, there was a significant positive correlation between the change in relative beta power and the magnitude of the noxious-related ERP (Spearman's

rho = 0.52, p-value = 0.033, see Figure 3.5). There was no significant relationship between the magnitude of the noxious-related ERP and either the standard deviation of level 3 detail coefficients (Spearman's rho = -0.036, p-value = 0.87) or the sum of squared level 4 detail coefficients (Spearman's rho = 0.045, p-value = 0.85). These findings suggest the change in relative beta power may be noxious-related.

Table 3.7: Electroencephalographic features which demonstrated significant change from baseline following BIO ROP screening were re-tested in a group of 23 infants undergoing heel lancing

Feature	Uncorrected p-value	Corrected p-value
Standard deviation of level 3 detail coefficients	0.046	0.92
Sum of squared level 4 detail coefficients	0.31	0.31
Relative beta power	0.0020	0.0061

For each EEG feature, the uncorrected p-value (paired-sample one-tailed t-test or Wilcoxon signed rank for non-parametric features) and corrected p-value following Hochberg correction are shown.



D3 SD = standard deviation of level 3 detail coefficients; D4 Sumsq = sum of squared level 4 detail coefficients; Beta power = relative beta power.

Figure 3.4: Percentage change in three electroencephalographic features following heel lancing in a group of 23 infants

Table 3.8: Behavioural and physiological outcomes for infants undergoing BIO ROP screening and heel lancing

	BIO ROP screening (n=25)	Heel lancing (n=23)
Premature Infant Pain Profile - Revised score	9 (7.5, 11.5)	5 (4, 9)
Mean post-procedure heart rate change (bpm)	40 (27, 60)	13 (5, 28)

Figures are median (lower quartile, upper quartile). bpm = beats per minute.

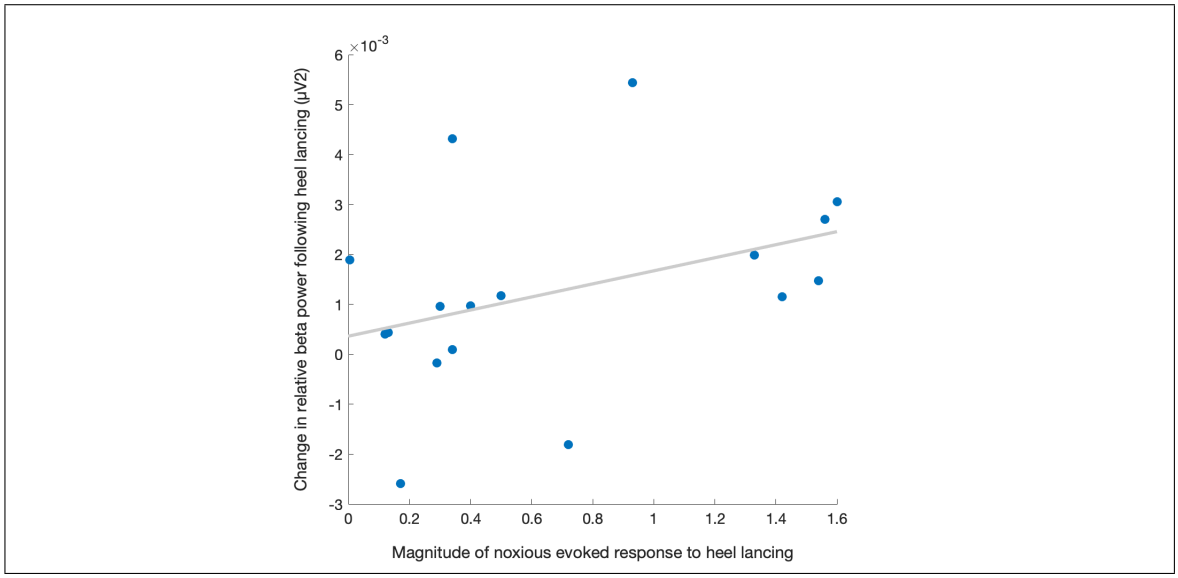


Figure 3.5: Change in relative beta power and the magnitude of the noxious event related potential evoked by heel lancing were positively correlated in 23 infants (Spearman's rho = 0.52, p-value = 0.033)

3.3.5 Changes in brain activity evoked by BIO ROP screening are not related to changes in behaviour and physiology

The change in features of EEG evoked by BIO ROP screening was also compared to behavioural and physiological surrogate measures of pain. For the 25 subjects undergoing BIO ROP screening there was no significant relationship between the change in the subset of EEG features identified following BIO ROP screening and PIPP-R score, baseline HR or change in HR following BIO ROP screening (see Table 3.9 and Figure 3.6). There was a strong correlation between the baseline HR and the

change in HR following BIO ROP screening (rho = -0.77, *p*-value = 0.0000062; see Figure 3.7). Notably, one subject had a baseline HR of 170 bpm, with no increase following screening. This is an example of the ceiling effect of the maximum HR achievable for each subject, wherein the HR cannot further increase from baseline in response to stimulation.

Table 3.9: Correlations between electroencephalographic feature change and behavioural & physiological measures following BIO ROP screening in 25 infants

	PIPP-R score		Baseline HR		HR change	
	rho	*p*-value	*rho*	*p*-value	*rho*	*p*-value
Standard deviation of level 3 detail coefficients	-0.059	0.81	0.26	0.25	-0.17	0.46
Sum of squared level 4 detail coefficients	-0.057	0.80	-0.13	0.54	0.10	0.64
Relative beta power	0.011	0.96	0.16	0.47	-0.0071	0.97

PIPP-R = Premature Infant Pain Profile-Revised; HR = heart rate; Pearson's linear correlation or Spearman's rho for non-parametric measures is shown.

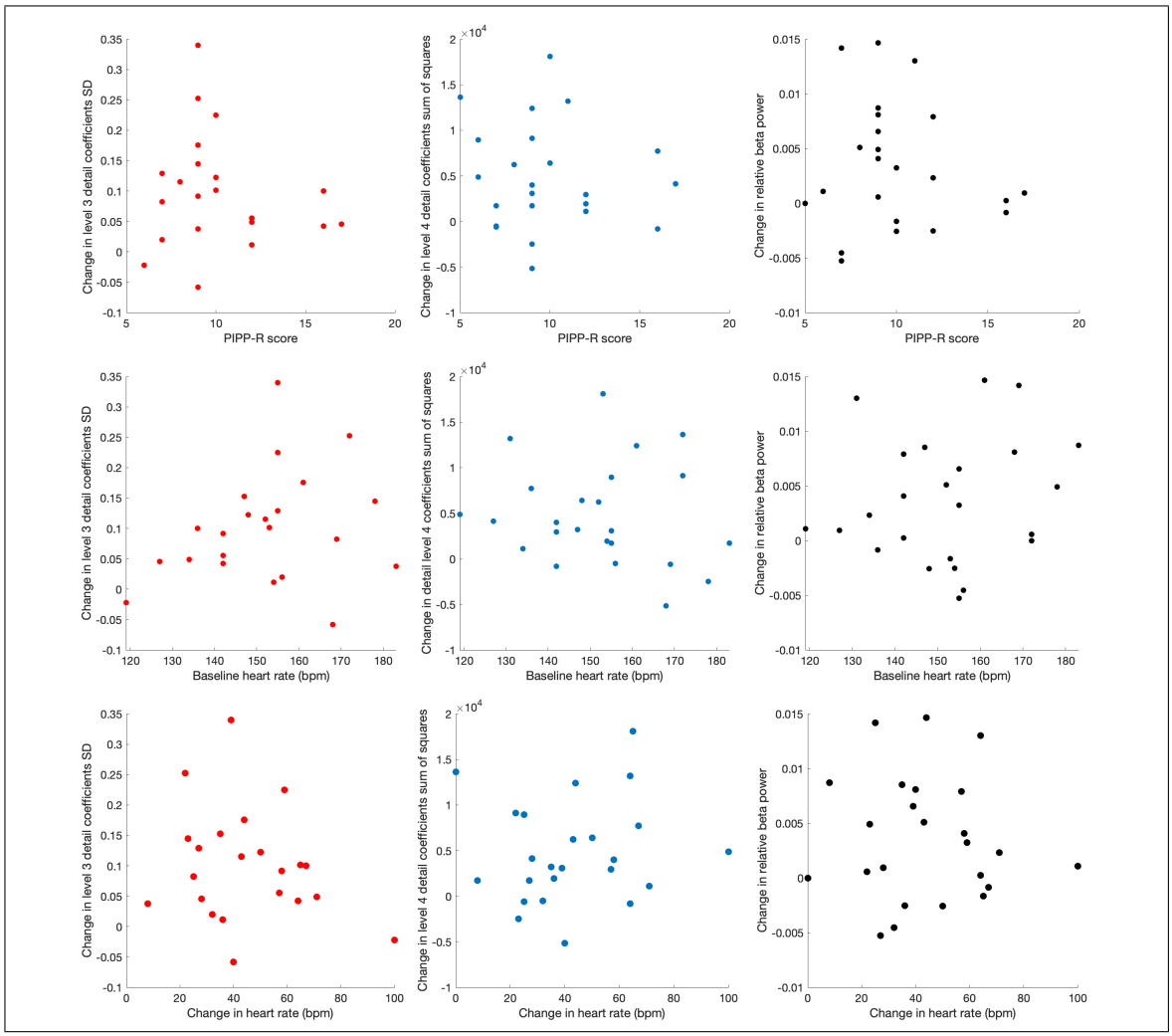


Figure 3.6: Change in three electroencephalographic features was not significantly correlated to Premature Infant Pain Profile-Revised (PIPP-R) score, baseline heart rate, or change in heart rate following BIO ROP screening in 25 infants. (SD = standard deviation; bpm = beats per minute).

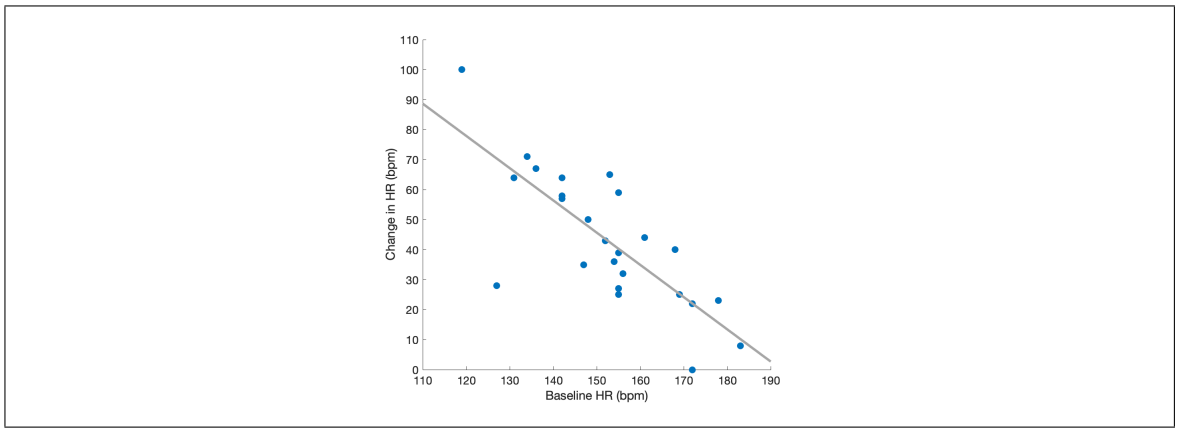


Figure 3.7: Baseline heart rate and change in heart rate following BIO ROP screening were strongly negatively correlated in 25 infants (Pearson's rho = -0.77, p-value = 0.0000062)

3.4 Discussion

3.4.1 Overview

The effect of BIO ROP screening on infant brain activity was characterised in 17 infants: screening evoked a significant change in 10 features of EEG which related to the alpha and beta bands (7.8 - 15.6 Hz, and 12 - 31.3 Hz). This observation was confirmed in an independent set of 9 infants undergoing BIO ROP screening.

BIO ROP screening evokes changes in infant brain activity that may be noxious-related: the increase in relative beta power observed following BIO ROP screening was also observed following a different noxious procedure (heel lancing), and was not observed following a non-noxious stressful procedure (nappy change). Moreover, following heel lancing, the increase in relative beta power was significantly correlated to a previously-characterised noxious-related ERP.

3.4.2 A shift to higher frequency brain activity is observed following BIO ROP screening

Following BIO ROP screening, an increase in relative beta power and beta amplitude was observed, as well as an increase in signal power at 7.8 - 15.6 Hz, spanning alpha

and beta frequencies. Increase in relative beta power may be a measure of nociception in infants, since this change was identified again following a different noxious procedure (heel lancing) and was not identified following a non-noxious control procedure (nappy change). Moreover, the change in relative beta power was significantly correlated to the magnitude of the noxious-related ERP evoked by heel lancing, a validated measure of nociception in infants.

An association between increased high frequency activity and nociception in infants has been identified previously: in a study of 18 infants, an increase in beta and gamma oscillations was observed at the Cz electrode following heel lancing (Fabrizi *et al*, 2016). Studies of tonic pain in adults have also observed an increase in beta and gamma frequency activity in response to experimental tonic noxious stimuli such as intramuscular capsaicin injection, cold pressor test and thermal pain. (Chang *et al*, 2001; Gram *et al*, 2015; Peng *et al*, 2014; Li *et al*, 2016; Misra *et al*, 2017). Clinical tonic pain in adults has been investigated in the context of noxious stimulation during anaesthesia. In a study of 20 anaesthetised patients, a noxious clinical procedure (laryngoscopy and intubation) evoked a reduction in relative delta and increase in relative beta activity (Wilder-Smith *et al*, 1995). A similar approach has been used in animal models. A shift toward higher frequency EEG activity was observed in an anaesthetised dog during noxious pressure stimulation. The change in brain activity was associated with physiological markers of nociception such as increased HR and BP (Otto, 2008).

Higher frequency brain activity may have a role in nociceptive perception and processing. Peng *et al* observed that tonic pain evokes widespread cortical increase in high frequency brain activity, and suggested this may reflect synchronisation between cortical regions involved in pain processing (Peng *et al*, 2014). More specifically, Ploner *et al* observed that tonic pain evokes high frequency brain activity first over sensorimotor cortical regions, and later over cortical regions encoding emotional processing (Ploner *et al*, 2017; Gross *et al*, 2007; Schulz *et al*, 2015). In this study, the

increase in high frequency brain activity at the Cz electrode may arise from the underlying sensorimotor cortex (Slater *et al*, 2010c; Marshall and Meltzhoff, 2015; De Klerk *et al*, 2015; Dall'Orso *et al*, 2018). High frequency brain activity over sensorimotor regions in response to a tonic noxious stimulus may relate to the transformation of nociception into a motor response (Schulz *et al*, 2012).

The changes observed in the sum of squared level 4 detail coefficients may be due to differences in alpha and beta oscillatory responses contained within the feature. As discussed above, beta oscillations increase in response to noxious stimulation. Hence, following BIO ROP screening, a beta-mediated noxious-related increase in the sum of squared level 4 detail coefficients may be observed.

However, following nappy change, a decrease in the sum of squared level 4 detail coefficients was observed. This decrease may have been mediated by a decrease in alpha oscillations. A large body of evidence underpins the role of alpha power in adults: increase in alpha oscillations represents active cortical suppression of distracting information, while decreased alpha power reflects removal of this suppression during attention (Fries *et al*, 2001; Foxe and Snyder, 2011; Klimesch, 2012). An association between alpha suppression and arousal in infants has been identified previously: in a study of 59 infants, a decrease in alpha oscillations was observed in central electrodes during sustained attention (Xie *et al*, 2018). As discussed in Section 2.3.6, nappy change is a salient stimulus, evoking behavioural and physiological arousal-related changes. Therefore, following nappy change, an alpha-mediated arousal-related decrease in the sum of squared level 4 detail coefficients may have been observed.

No significant change in the sum of squared level 4 detail coefficients was identified following heel lancing, which may be indicative of the balance between beta-mediated increase in response to nociception, and alpha-mediated decrease in response to stimulus saliency.

The high frequency brain activity observed following BIO ROP screening is unlikely to have been generated by muscle artefact. All EEG recordings were visually

inspected for artefact and contaminated segments were manually rejected. Recordings were obtained from a centrally located electrode, which is the location least vulnerable to muscle contamination (Ma *et al*, 2012; Li *et al*, 2016).

3.4.3 Relating brain activity to behaviour and physiology

If the changes in brain activity following BIO ROP screening are related to nociceptive cortical processing, then a relationship may be expected between brain activity and other measures of nociception (Hartley *et al*, 2017). However, in this study there were no significant correlations between the change in brain activity after BIO ROP screening and the baseline HR, change in HR, or PIPP-R score.

Limitations to the PIPP-R score have been discussed; the score is not specific to BIO ROP screening and may not fully capture noxious-related changes in behaviour and physiology evoked by the procedure. The screening procedure is comprised of varied stimuli, including tactile (head restraint, eyelid speculum, scleral indentation) and visual (bright retinal illumination) stimulation. The behavioural, physiological and neurological responses observed may be evoked differently by the component stimuli. The experimental approach did not characterise the effect of individual components of BIO ROP screening on the outcome measures and so conclusions cannot be drawn regarding this hypothesis.

PIPP-R scores may range from 0 to 21, however in this study there was a narrower range of PIPP-R scores (5 to 17). This study may have been underpowered due to small sample size; a larger sample with greater variability may be required to better identify significant relationships between brain activity and PIPP-R score (Haegerstrom-Portnoy *et al*, 2000; Armstrong, 2019).

Moreover, in this study the baseline EEG epoch and the baseline physiological and behavioural observations were not precisely temporally aligned. As described in Chapter 2 (General Methods), the baseline EEG epoch was selected retrospectively by identifying a period of 120s as early as possible in the EEG recording that was

unaffected by artefact. The PIPP-R baseline was marked contemporaneously during each study when the infant was settled. It is possible that a difference in infants' activity state may have influenced the evaluation of the relationship between brain activity and physiological or behavioural measures.

Additionally, the baseline HR was strongly related to the change in HR after screening. The post-procedure change in HR may have been limited by the physiological maximum HR achievable. This 'ceiling effect' may have influenced the relationship between change in brain activity and change in HR after screening. In Chapter 4, heart rate variability (HRV) is investigated, which may avoid a physiological 'ceiling effect' and therefore may better characterise physiological changes during BIO ROP screening.

3.4.4 Strengths and Limitations

To date, this is the first study of clinical tonic pain in preterm infants using exploratory analysis of quantitative features of EEG. A wide range of EEG features was included with the intention of representing the complexities of the preterm EEG. The feasibility of recording EEG in infants during a complex clinical procedure is demonstrated, and an approach toward defining a novel measure of nociception in infants is described.

Limitations include a small sample size, particularly in Analysis 3 (nappy change), meaning that the study was underpowered for statistical significance. Recruitment and study of preterm infants is challenging, given the fragile nature of newborn infants, and the care required to sensitively approach parents or caregivers regarding consent for study. Additionally, following the outbreak of the coronavirus pandemic, recruitment and study of infants in the NICU was suspended to reduce risk of infection spread.

A limitation of the study design was the selection of EEG epochs for analysis without determination of the infants' behavioural state. This is particularly relevant

for the baseline EEG epoch, which was selected at the start of the recording period, but not precisely aligned to the time of the PIPP-R scoring facial video identifying the infants' behavioural state (active awake, quite awake, active sleep, or quiet sleep). It is possible that the study observations are associated with a change in behavioural state evoked by the clinical procedures.

The observations from this study are based on recordings from a single central electrode. This approach was based on precedent from previous studies (Hartley *et al*, 2017; Gursul *et al*, 2018; Green *et al*, 2019). However, a greater number of electrodes may better identify cortical regions involved in nociceptive processing (Tokariev *et al*, 2016; Wallois *et al*, 2021).

Nappy change was selected as a negative control for BIO ROP screening. Nappy change is a frequently performed clinical procedure that is not considered to be painful, but has been observed in previous studies to evoke similar trends in physiological stress responses as BIO ROP screening (Wang and Change, 2004; Lyngstad *et al*, 2014). Both procedures are of similar duration; in this study nappy change duration was median 2.5 minutes, and BIO ROP screening was 2.9 mins duration. However, there are notable differences between the procedures, for example, the location of tactile stimulation differs: in nappy change, bottom care is performed, whereas in BIO ROP screening the head and eyes are touched. The type of tactile stimulation also differs: in nappy change, intermittent gentle touch is used to clean the skin, whereas in BIO ROP screening, manual head restraint is firm and continuous. In this study, the median PIPP-R score was 5 for nappy change, and 9 for BIO ROP screening. It is possible that the developmental-care based nappy change was less salient than BIO ROP screening. While it has been argued that increase in higher frequency EEG activity is unlikely to reflect stimulus saliency (Chang *et al*, 2001; Zhang *et al*, 2012; Peng *et al*, 2018), comparison with other clinical procedures may be appropriate. In Chapter 5, an alternative screening procedure is investigated which may better compare to the stimulus saliency of BIO ROP screening than nappy change.

3.4.5 Future Directions

An increase in power in the frequency range 12-30 Hz following clinical tonic noxious stimulation may represent an additional surrogate marker for procedural pain in infants. Characterisation of other complex painful procedures such as immunisation, intravenous line placement, or alternative ROP screening methods, using quantitative analysis of EEG may improve understanding of the effect of tonic noxious stimulation on infant brain activity. Efficacy of pain-relief strategies could also be investigated using the multimodal approach demonstrated in this Chapter, to better characterise the effect of non-pharmacological and pharmacological treatments for procedural pain in infants. Since hospitalised infants undergo multiple painful procedures daily (Cruz *et al*, 2016), further research is vital to better detect and treat infant pain. The ongoing observational prospective study by Roué (Roué *et al*, 2021) will evaluate infant pain using multimodal assessment including quantitative analysis of EEG, physiological measures (HR, HRV, oxygen saturations), facial myography, and skin conductance, and may provide further insight into infant pain responses.

3.5 Conclusions

This Chapter demonstrates a shift to higher frequency brain activity following BIO ROP screening. This finding may be noxious-related, and may represent a novel surrogate marker for procedural pain in infants. Improved measurement of pain in infants may lead to better evaluation of pain-relief strategies and thereby improved treatment of infant pain.

Chapter 4

Characterising the infant response to binocular indirect ophthalmoscopy using physiological measures

4.1 Abstract

Introduction

Infants admitted to the Intensive Care and High Dependency areas of the NICU undergo continuous physiological monitoring. Pulse oximetry is used to monitor oxygen saturations, an indicator of airway and respiratory status, and ECG is used to monitor heart rate and rhythm, indicators of circulatory status. In this Chapter the effect of BIO ROP screening on infant physiology was characterised using detailed continuous recordings of HR and oxygen saturations. Heart rate variability (HRV), a measure of neonatal cardiac autonomic reactivity, was also quantified. Since BIO ROP screening is considered to be a painful and stressful experience, a reduction in the high frequency component of HRV following BIO ROP screening was hypothesised, as has been noted after other noxious clinically-indicated procedures in infants.

Methods

The trend in HR and oxygen saturation before, during and after BIO ROP screening was evaluated, and the number of instability events (bradycardia, tachycardia, desaturation, apnoea) occurring before and after the procedure was calculated. Time and frequency-domain HRV measures were quantified before, during and after BIO ROP screening. The COVID-19 pandemic occurred during recruitment for this Chapter, and therefore the number of infants included is small. The investigations are an example of an approach which could be performed in a larger sample size.

Results

Physiological data from 22 infants was characterised during BIO ROP screening. The average HR increased throughout screening then rapidly decreased once the procedure was completed. The average oxygen saturation showed a trend toward decreased saturations during screening. Screening was not associated with an increase in instability events. Following BIO ROP screening there was a significant reduction in two indicators of parasympathetic activity: pNN50 ($p = 0.013$, high baseline HRV group; $p = 0.008$, low baseline HRV group), and the absolute power of the high frequency band ($p = 0.032$, low baseline HRV group).

Conclusion

These findings suggest that BIO ROP screening evokes a physiological stress response in preterm infants. Measurement of HRV during BIO ROP screening and other stressful clinically-necessary procedures may help clinicians better recognise and manage infant stress. It is vital to monitor and ameliorate stress in infants, since stress during the neonatal period may adversely affect neurodevelopment and exert negative

influence into adulthood.

4.2 Methods

4.2.1 Participating Infants

Physiological recordings from 22 infants during BIO ROP screening were included in analysis. Demographic information for all subjects is shown in Table 4.1.

Table 4.1: Demographic information for subjects included in Chapter 4

Number of infants analysed	22
Corrected gestational age at study (weeks)	34.43 (32.86, 44.57)
Postnatal age at study (weeks)	7.71 (6.14, 9.71)
Birthweight (g)	865 (756, 1510)
Number of males	11 (48%)
Apgar score at 5 minutes	8 (7, 10)
Normal vaginal delivery	10 (43%)

Figures are median (lower quartile, upper quartile) or number (percentage).

4.2.2 Experimental Design

In the first section of this study, HR and oxygen saturation trends were characterised in 22 infants undergoing BIO ROP screening. In the second section, the change in physiological instability following BIO ROP screening was investigated by calculating the number of instability events before and after BIO ROP screening. Fifteen minute, 1 hour, 6 hour, and 10 hour recording periods before and after screening were analysed. Next, the change in HRV following BIO ROP screening was calculated by analysing time and frequency domain HRV measures for 15 minutes before and after the procedure. Finally, the relationship between change in each HRV measure and baseline HRV was characterised to investigate whether HRV measures are limited by physiological maximal values, that is, a 'ceiling effect'.

4.2.3 Recording Techniques

Continuous ECG and oxygen saturation recordings were obtained during BIO ROP screening. Chapter 2 (General Methods) describes the recording techniques in detail.

4.2.4 Analysis

MATLAB was used to analyse physiological data contained in the output file of the recording software.

Heart rate and oxygen saturation

HR data were selected from the start of BIO ROP screening (placement of eye drops) to the end of the procedure (removal of eyelid speculum) for each subject. The group mean HR and 95% confidence intervals were calculated at each time point in order to visualise the trend in HR. The time taken for HR recovery following BIO ROP screening was analysed: the mean baseline HR was calculated for each subject during a 15 minute period immediately prior to placement of eye drops, then following BIO ROP screening, the time taken for the HR to return to baseline was calculated using a 15 minute period immediately following speculum removal. Non-parametric cluster analysis of the same 15 minute epochs was used to identify temporal differences between the baseline and post-procedure HR. The statistical approach used for non-parametric cluster analysis is detailed in Section 4.2.5. The above analyses were also performed for the continuous oxygen saturation recordings.

In order to investigate the effect of infant age on HR change and recovery following BIO ROP screening, the relationship was characterised between corrected gestational age at study and the following parameters: baseline HR, time to recovery of baseline HR, and percentage increase in HR following BIO ROP screening. Pearson's linear correlation coefficient was calculated for normally distributed values, and Spearman's

rho was calculated for non-normally distributed values.

Instability events

The number of instability events which occurred before and after BIO ROP screening was calculated, with the intention of characterising whether the procedure was associated with an increase in physiological instability. The definition of each instability event - tachycardia, bradycardia, desaturation, and apnoea - is listed in Table 2.4. Events occurring within symmetrical 15 minute, 1 hour, 6 hour and 10 hour intervals before and after BIO ROP screening were analysed. The standardised difference in number of instability events after BIO ROP screening was also calculated for each time interval. The standardised difference in instability events was calculated by dividing the difference between the number of events in the baseline and post-procedure intervals by the total number of events. A negligible amount (0.01) was added to each event total to avoid division by zero.

Cluster analysis

The HR trace was visualised for each subject, which led to the observation that there were differences in the visual characteristics of the baseline HR between subjects. Baseline HRV may influence the change in HRV after BIO ROP screening, therefore the preliminary observation of between-subject differences in baseline HRV was further investigated using cluster analysis of time-domain HRV measures. The intention was to cluster all subjects by baseline HRV before analysing the effect of BIO ROP screening.

Time-domain HRV measures were calculated for baseline and post-procedure for each subject. A 5 minute window was chosen with a 30s slide, as is typical for HRV analysis. For each subject, outlying window values were identified and rejected (see Section 3.2.4 for approach). On average, 7% of data were rejected at this stage. Following outlier rejection, the subject mean baseline value was calculated for each

HRV measure.

The k-means method was used to separate the baseline mean values into two clusters (MATLAB command 'kmeans'). This clustering approach identifies the centre of each cluster - as distant as possible from each other - and assigns each data point to the nearest cluster centre (Orhan *et al,* 2011). The cluster iterations were initialised using the k-means++ algorithm, and the optimal partition was chosen out of 15 initialisations. Proximity of data points was based on the squared Euclidean distance. The quality of clusters was evaluated using silhouette values, in which values range from -1 to 1, and higher values indicate a better clustering result.

HRV change during BIO ROP screening

In order to visualise the time course of each HRV measure before, during, and after BIO ROP screening, an epoch from 40 minutes before BIO ROP screening to 15 minutes after screening was selected for each subject. HRV measures were calculated using a 5 minute window with a 30s slide. Subjects were grouped by high or low baseline HRV based on the k-means cluster analysis detailed above. For each window, outlying subject values were identified and rejected. On average, 8% of data were rejected in the high baseline HRV group, and 6% of data in the low baseline HRV group. The mean and 95% confidence intervals were calculated for each HRV measure for the high and low baseline HRV groups.

Finally, non-parametric cluster analysis was performed, in order to characterise the temporal change in HRV following BIO ROP screening. The analysis approach described in Section 4.2.5 was used to analyse the difference between the baseline and post-procedure periods for each HRV measure.

HRV 'ceiling effect'

In Chapter 3, it was observed that the change in HR following BIO ROP screening was strongly related to the baseline HR, and the magnitude of change in HR was limited

by the physiological maximum limit. In this Chapter, the relationship between change in HRV following BIO ROP screening and baseline HRV was characterised in order to investigate whether HRV measures demonstrate a similar 'ceiling effect'. Non-parametric cluster analysis identified the specific time window during which HRV differences occurred between the baseline and post-procedure periods. The same time window was chosen to calculate the the baseline HRV and the change in HRV following BIO ROP screening.

Outlying data were identified and rejected before correlations were performed. On average, 12% of data was rejected for each correlation. Pearson's linear correlation coefficient was calculated for normally distributed values, and Spearman's rho was calculated for non-normally distributed values.

4.2.5 Statistical Analysis

Non-parametric cluster analysis of the HR and oxygen saturation recordings was performed using Maris and Oostenveld's method (Maris and Oostenveld, 2007). Firstly, for each second, a t-statistic was calculated using the formula:

$$t = \frac{(m_p - m_b)}{\sqrt{\frac{\left(\sigma_p^2(n_p-1)+\sigma_b^2(n_b-1)\right)}{(n_p+n_b-2)}\sqrt{\left(\frac{1}{n_p}+\frac{1}{n_b}\right)}}}$$

where m_b = mean of baseline epochs; m_p = mean of post-procedure epochs; σ_b = standard deviation of baseline epochs; σ_p = standard deviation of post-procedure epochs; n_b = number of subjects in baseline group; n_p = number of subjects in post-procedure group.

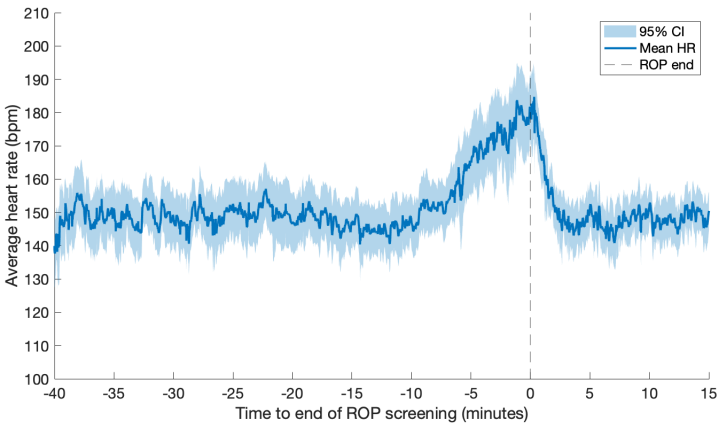
Next, the baseline and post-procedure epochs were combined into a single set from which 1000 random partitions were created. A permutation t-statistic was calculated for each random partition using the same formula as above. Finally, a Monte Carlo estimate of the p-value was calculated, which was the proportion of random partitions in which the observed t-statistic was larger than the permutation t-statistic. The baseline and post-procedure were deemed significantly different if the Monte Carlo p-value estimate was less than 0.05. This was repeated for each second to identify

clusters of significant t-statistics. The same non-parametric cluster approach was used to compare the baseline and post-procedure HRV time courses for time and frequency domain measures.

4.3 Results

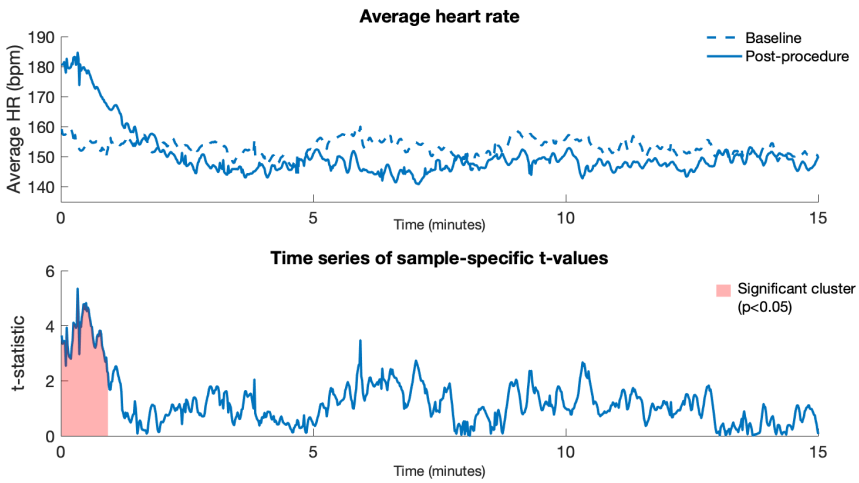
4.3.1 Heart rate increases during BIO ROP screening with rapid recovery post-procedure

The average HR for 22 infants undergoing BIO ROP screening is shown in Figure 4.1 (mean and 95% confidence intervals). The group mean baseline HR was 153 bpm (standard deviation 13 bpm). HR increased throughout BIO ROP screening then rapidly decreased once the procedure was completed. Non-parametric cluster analysis of the baseline and post-procedure HR time courses identified the post-procedure HR was significantly higher than baseline from 0 to 56 seconds after ROP screening (p = 0.007; see Figure 4.2).



95% CI = 95% confidence intervals; mean HR = mean heart rate; ROP end = removal of speculum from second eye examined. Time-point zero indicates the end of screening as identified by the removal of the eyelid speculum.

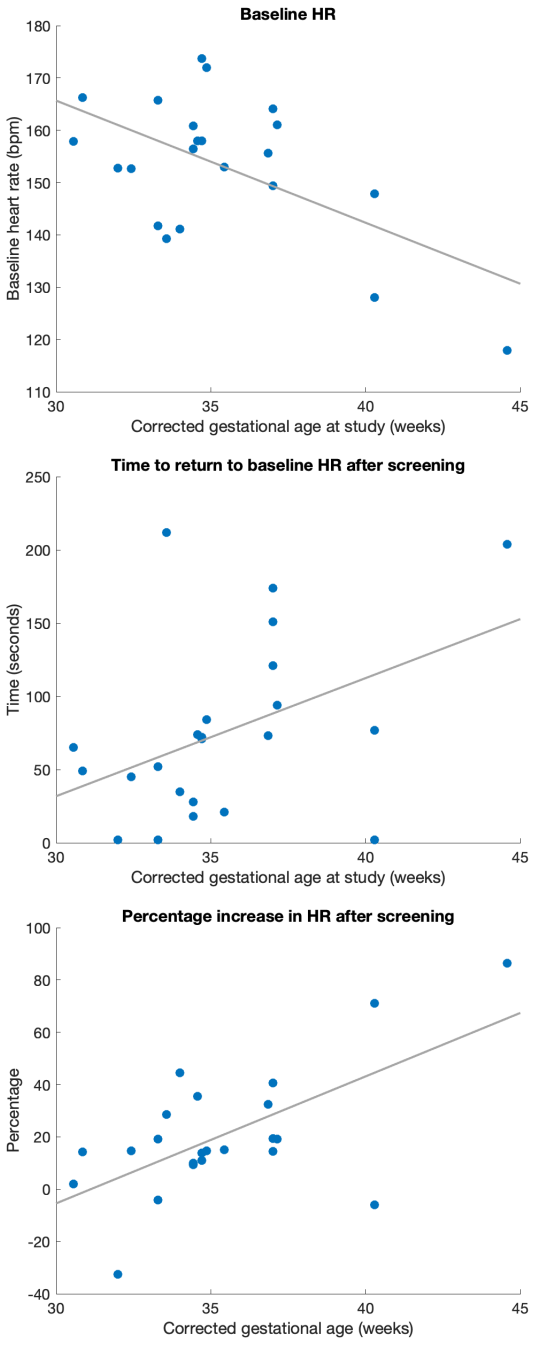
Figure 4.1: Trend in heart rate during BIO ROP screening in 22 infants (mean and 95% confidence intervals)



The top figure shows the average heart rate for a baseline epoch immediately preceding screening (dashed line), and a post-procedure epoch immediately following screening (bold line). The bottom figure shows non-parametric cluster analysis of the two epochs. A significant cluster (highlighted in red) indicates that the heart rate was significantly higher than baseline for 56 seconds post-procedure ($p = 0.007$)

Figure 4.2: Non-parametric cluster analysis of heart rate before and after BIO ROP screening in 22 infants

There was a significant negative relationship between corrected gestational age at study and baseline HR (Pearson's correlation coefficient = 0.57, p = 0.0046). Corrected gestational age was also significantly related to the time to return to baseline HR (Spearman's rho = 0.48, p = 0.022) and the percentage increase in HR following BIO ROP screening (Spearman's rho = 0.43, p = 0.039). Correlations are shown in Figure 4.3.



HR = heart rate; bpm = beats per minute.

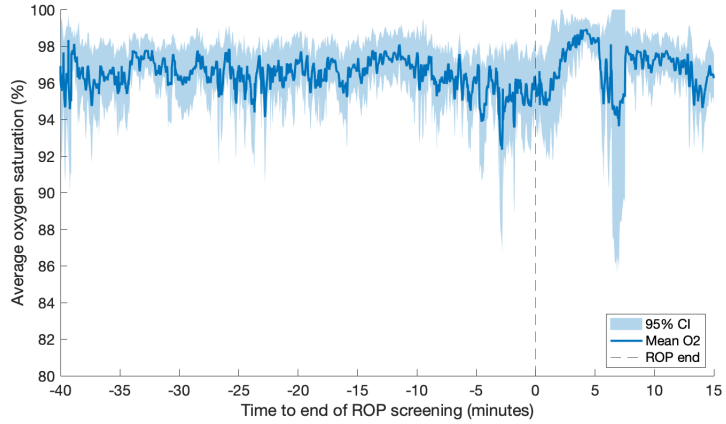
Figure 4.3: Corrected gestational age at study (weeks) was significantly correlated to baseline heart rate (p = 0.0046), time to return to baseline heart rate (p = 0.022), and percentage increase in heart rate (p = 0.039) following BIO ROP screening in 22 infants.

4.3.2 Oxygen saturation does not change significantly during or after BIO ROP screening

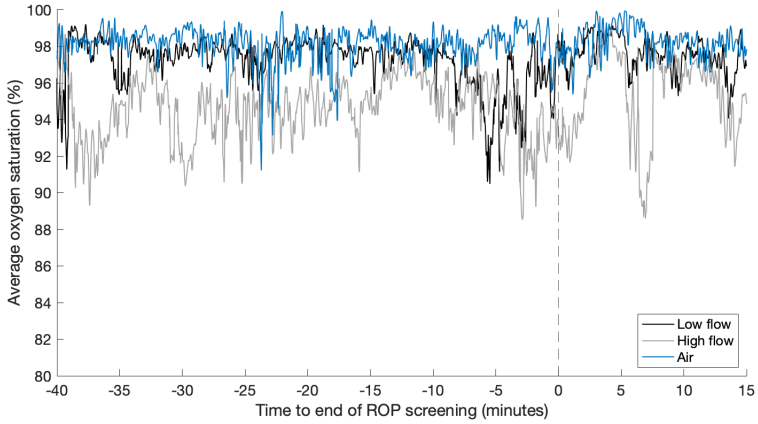
The average oxygen saturation for 22 infants undergoing BIO ROP screening is shown in Figure 4.4a (mean and 95% confidence intervals). The baseline oxygen saturation was mean 96.8% (standard deviation 2.4%). The trend in oxygen saturation is also shown grouped by method of ventilation (low flow oxygen, high flow oxygen, or spontaneous ventilation in air; Figure 4.4b). The mean baseline oxygen saturation was 94.6% for infants receiving high flow oxygen (n = 10), 97.4% for infants receiving low flow oxygen (n = 7), and 98.4% for infants not receiving supplementary oxygen (n = 5).

The averaged time course shows a trend toward decreased oxygen saturation during eye screening. This corresponds with previously published observations that oxygen saturations decrease during BIO ROP screening (Laws *et al*, 1996; Rush *et al*, 2004; Jiang *et al*, 2016). Following screening, the average oxygen saturations rose to above baseline levels, again corresponding with previously published data (Rush *et al*, 2004). At 7 minutes post-screening, the average oxygen saturation decreases to 86%, driven by an oxygen desaturation event in the group of infants receiving high flow oxygen. This group may be expected to demonstrate greater physiological instability, since infants requiring high flow oxygen have poorer clinical status.

Non-parametric cluster analysis of the baseline and post-procedure oxygen saturation time courses did not identify significant difference between the baseline and post-procedure epoch (see Figure 4.5). Similarly, no significant differences were identified between baseline and post-procedure oxygen saturations when analysed by ventilation method.

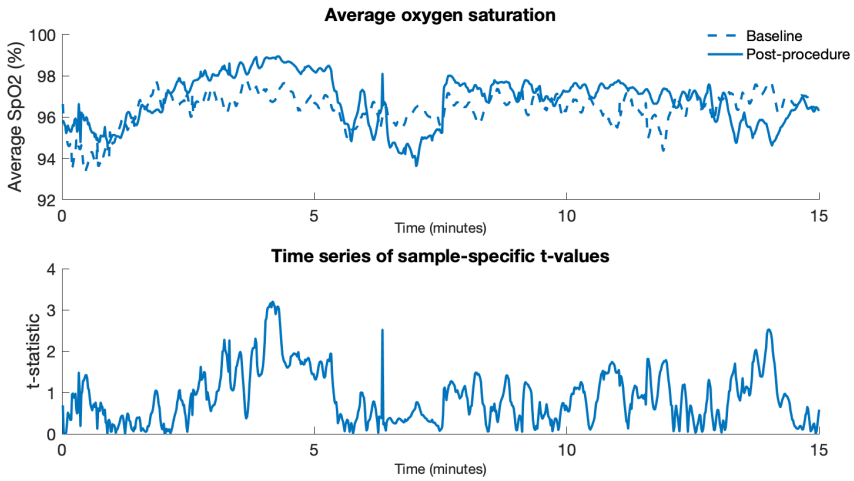


(a) Mean oxygen saturation and 95% confidence intervals (95% CI = 95% confidence intervals; mean O2 = mean oxygen saturations; ROP end = removal of speculum from second eye examined).



(b) Mean oxygen saturation grouped by method of ventilation (low flow, high flow, or spontaneous ventilation in air). Time-point zero indicates the end of screening as identified by the removal of the eyelid speculum.

Figure 4.4: Trend in oxygen saturation during BIO ROP screening in 22 infants



The top figure shows the average oxygen saturations for a baseline epoch immediately preceding screening (dashed line), and a post-procedure epoch immediately following screening (bold line). The bottom figure shows non-parametric cluster analysis of the two epochs. No significant clusters were identified, indicating that post-procedure oxygen saturations were not significantly different from baseline.

Figure 4.5: Non-parametric cluster analysis of oxygen saturation before and after BIO ROP screening in 22 infants

4.3.3 BIO ROP screening was not associated with a change in instability events

Instability events (bradycardia, tachycardia, desaturation, apnoea) are presented for 15 minute, 1 hour, 6 hour, and 10 hour intervals before and after BIO ROP screening in Table 4.2. The standardised difference in number of instability events after BIO ROP screening for each time interval is also listed. The duration of physiological recording available varied per subject; fewer subjects had recordings of 1 hour, 6 hours and 10 hours.

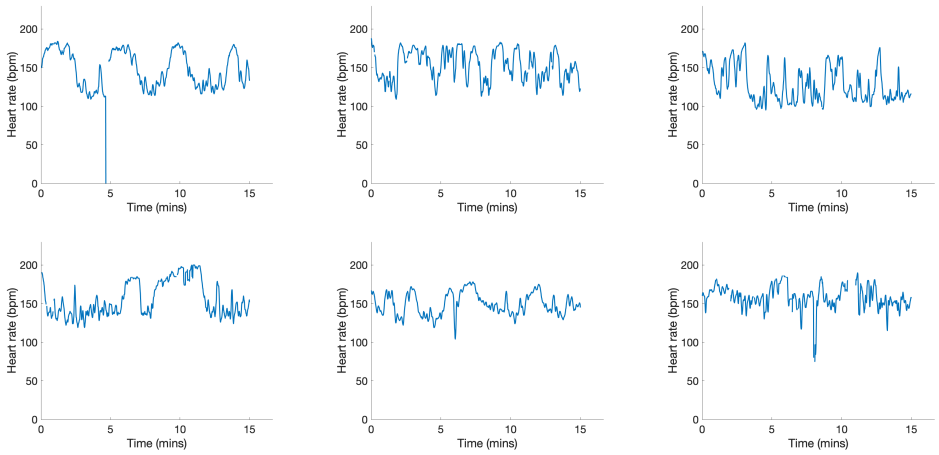
Table 4.2: Number of instability events which occurred before and after BIO ROP screening, and standardised difference in number of events following screening

Interval	Bradycardia			Tachycardia			Desaturation			Apnoea		
	Pre	Post	SDE	Pre	Post	SDE	Pre	Post	SDE	Pre	Post	SDE
15 minutes (n=22)	0	2	0 (0 to 0)	0	0	0 (0 to 0)	3	4	0 (0 to 0)	0	1	0 (0 to 0)
1 hour (n=7)	0	1	0 (0 to 0)	0	1	0 (0 to 0)	5	5	0 (-0.74 to 0.25)	0	0	0 (0 to 0)
6 hours (n=6)	5	2	0 (-0.33 to 0)	0	2	0 (0 to 0.98)	19	24	0 (-0.20 to 0.45)	5	1	0 (-0.50 to 0)
10 hours (n=5)	5	2	0 (-0.11 to 0)	0	4	0 (0 to 0.98)	40	41	0 (-0.16 to 0.16)	5	2	0 (-0.11 to 0)

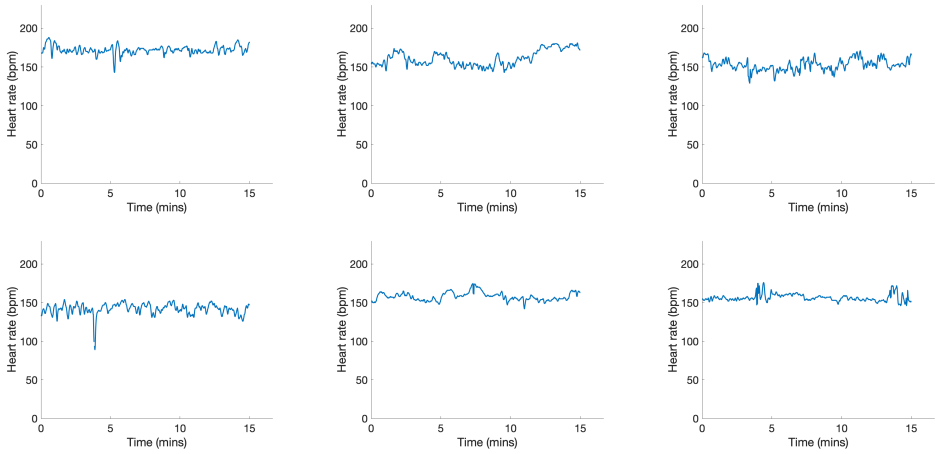
Twenty-two infants had 15 minutes, seven subjects had 1 hour, six subjects had 6 hours, and five subjects had 10 hours of physiological recording before and after BIO ROP screening. Median (interquartile range) shown. SDE = standardised difference in number of events after BIO ROP screening

4.3.4 Older infants have higher resting heart rate variability

Subjects were clustered by baseline HRV before investigating the effect of BIO ROP screening on HRV. Differences in the visual characteristics of the baseline HR were observed between subjects, which was reflected in HR standard deviation. 12 of 22 subjects could be grouped into high or low baseline HR standard deviation (see Figure 4.6).



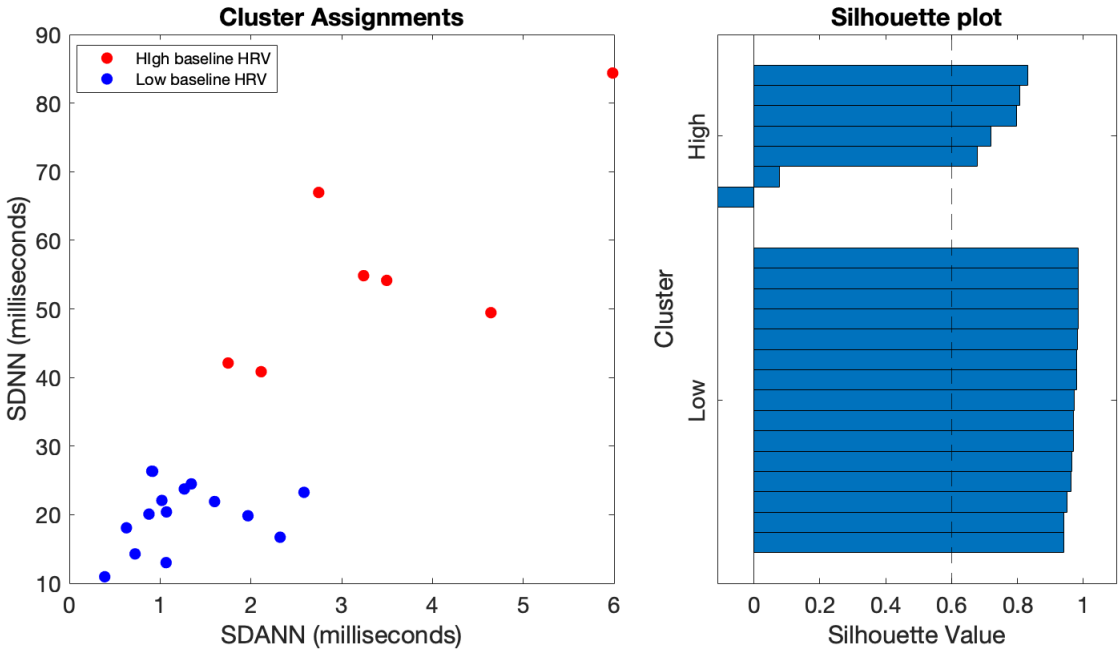
(a) Examples of subjects with high baseline heart rate standard deviation (≥14 beats)



(b) Examples of subjects with low baseline heart rate standard deviation (≤8 beats)

Figure 4.6: Single subject heart rate traces showed differences in standard deviation during the 15 minute baseline period prior to BIO ROP screening

The preliminary finding of between-subject differences in baseline HR standard deviation was further investigated using cluster analysis of the seven time-domain HRV measures listed in Table 2.5. Multidimensional k-means clustering of all infants resulted in one large and one small cluster. The sample size in the small cluster was too small for further analysis (2 subjects), hence alternative partitioning of the data was investigated. Two-dimensional k-means clustering was performed, in which all pairs of HRV measures were analysed consecutively. For the pair of measures standard deviation of the NN interval and standard deviation of the average NN interval, seven infants were allocated to the high baseline HRV cluster, and 15 infants to the low baseline HRV cluster (see Figure 4.7). Twenty of twenty-two subjects' silhouette values were greater than 0.6, which indicated the clusters were well-separated.



The left-hand plot shows that clustering based on the standard deviation of the NN interval (SDNN) and the standard deviation of the average NN interval (SDANN) yielded two clusters of sufficient size for further analysis. The right-hand plot shows that twenty of twenty-two subjects' silhouette values were greater than 0.6, which indicates the clusters were well-separated.

Figure 4.7: 2-dimensional k-means clustering was used to group 22 subjects into high or low heart rate variability based on the 15 minute baseline period prior to BIO ROP screening.

There was a significant difference in corrected gestational age between the high baseline HRV group (mean 38.4 weeks, standard deviation 3.5 weeks) and the low baseline HRV group (mean 33.8 weeks, standard deviation 1.9 weeks; two sample two-tailed t-test, $p = 0.00067$). Two HRV measures, the triangular interpolation of the NN interval histogram and the HRV triangular index, demonstrated a significant relationship between baseline values and corrected gestational age at study ($p = 0.027$ and $p = 0.027$; Figure 4.8).

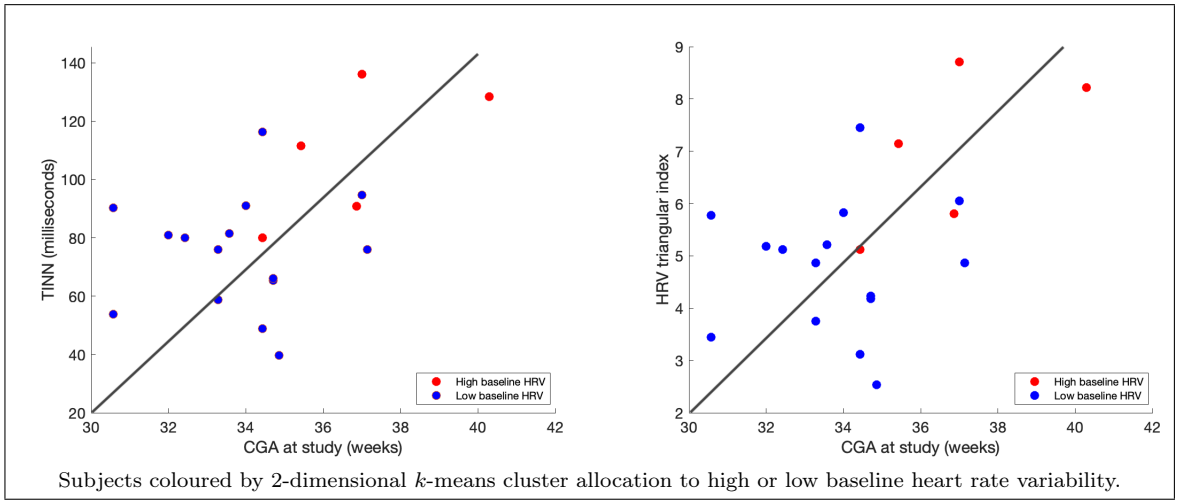
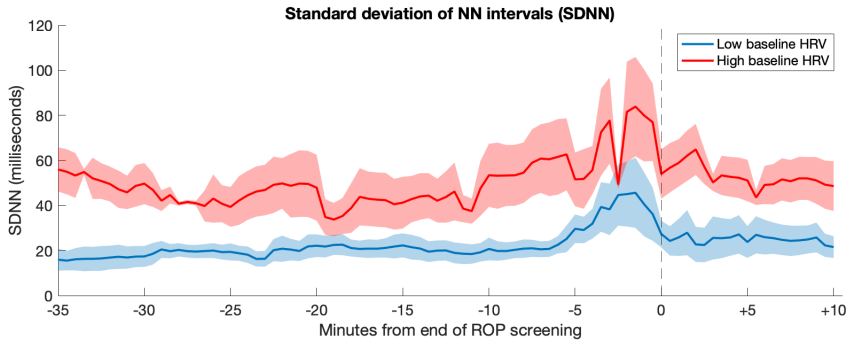


Figure 4.8: Corrected gestational age at study (weeks) was significantly related to the triangular interpolation of the NN interval histogram (TINN; p = 0.027) & the HRV triangular index (p = 0.027) in 22 infants during the 15 minute baseline period prior to BIO ROP screening.

4.3.5 BIO ROP screening evokes a reduction in time domain heart rate variability measures

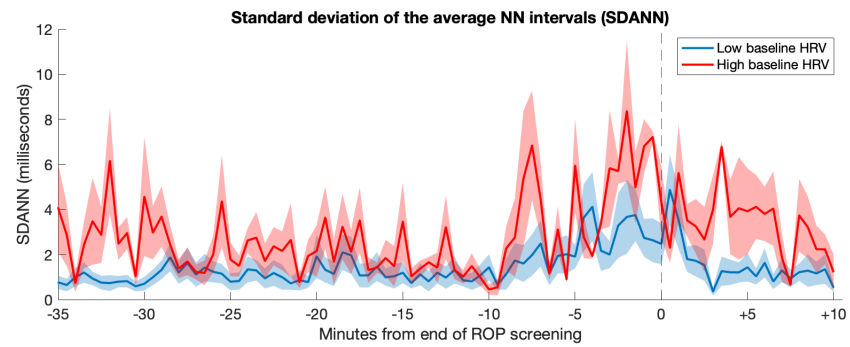
The time course for each HRV measure during BIO ROP screening is shown in Figures 4.9 to 4.15; subjects were grouped by baseline HRV. The low baseline HRV group appeared more stable during the baseline period than the high baseline HRV group, however this may be due to the larger group size. There appeared to be an increase in several measures of HRV temporally related to BIO ROP screening in the both baseline HRV groups, and a decrease in the triangular interpolation of the NN interval

histogram and the HRV triangular index in the high baseline HRV group during the same period. However the signal was at risk of contamination by motion artefact during screening (Perez-Riera *et al,* 2018), hence the impact of the screening procedure on HRV was further investigated by comparing the HR recording prior to screening and after screening.



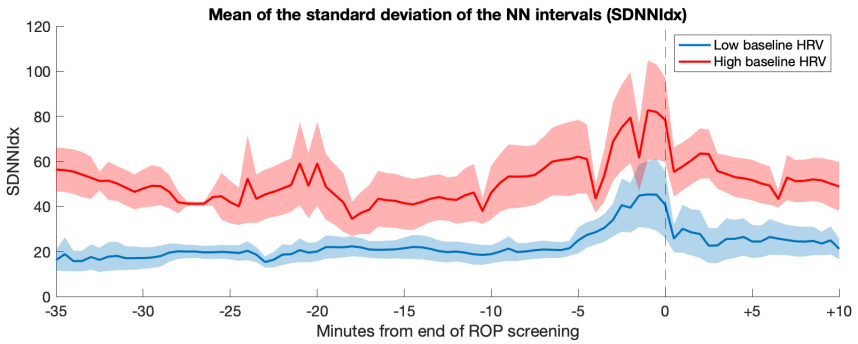
Time-point zero indicates the end of screening as identified by the removal of the eyelid speculum. Mean and 95% confidence interval shown for each HRV measure. Subjects grouped by high or low baseline heart rate variability.

Figure 4.9: Time course for the standard deviation of NN intervals from 35 minutes before BIO ROP screening to 10 minutes after screening



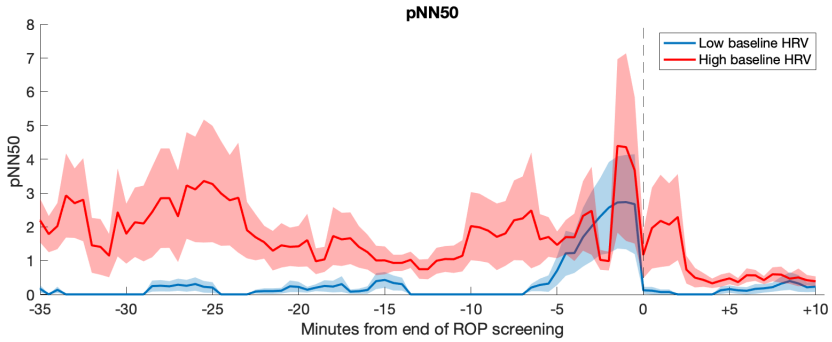
Time-point zero indicates the end of screening as identified by the removal of the eyelid speculum. Mean and 95% confidence interval shown for each HRV measure. Subjects grouped by high or low baseline heart rate variability.

Figure 4.10: Time course for the standard deviation of the average NN intervals from 35 minutes before BIO ROP screening to 10 minutes after screening



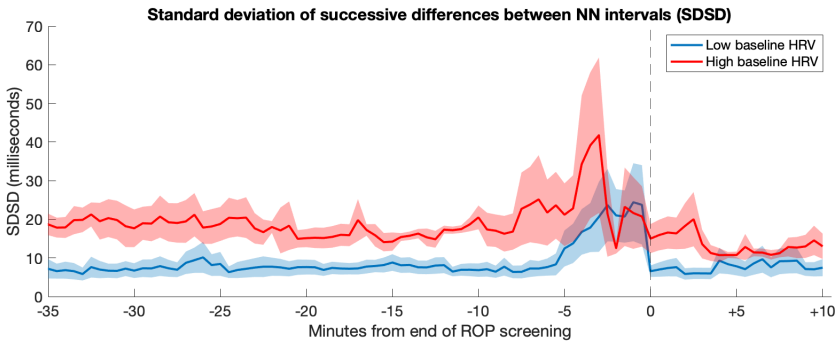
Time-point zero indicates the end of screening as identified by the removal of the eyelid speculum. Mean and 95% confidence interval shown for each HRV measure. Subjects grouped by high or low baseline heart rate variability.

Figure 4.11: Time course for the mean of the standard deviation of the NN intervals from 35 minutes before BIO ROP screening to 10 minutes after screening



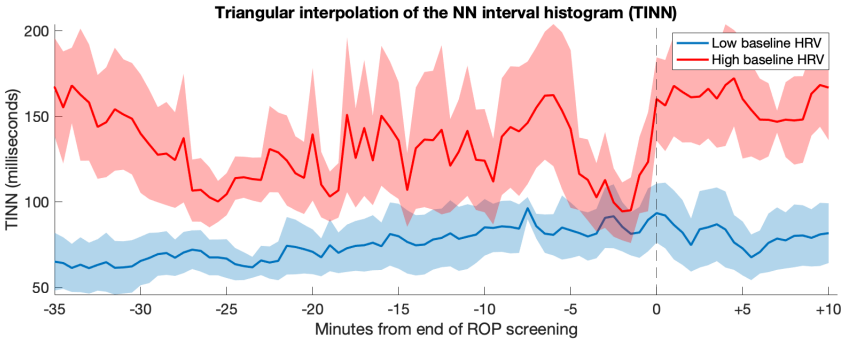
Time-point zero indicates the end of screening as identified by the removal of the eyelid speculum. Mean and 95% confidence interval shown for each HRV measure. Subjects grouped by high or low baseline heart rate variability. pNN50 = Number of successive NN intervals that differ by more than 50 ms / Total number of NN intervals.

Figure 4.12: Time course for pNN50 from 35 minutes before BIO ROP screening to 10 minutes after screening



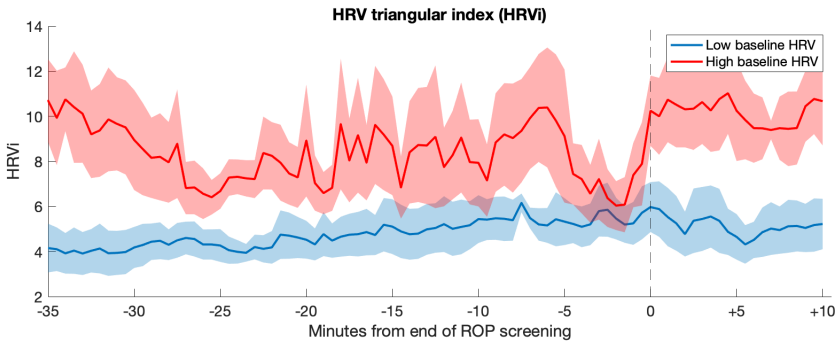
Time-point zero indicates the end of screening as identified by the removal of the eyelid speculum. Mean and 95% confidence interval shown for each HRV measure. Subjects grouped by high or low baseline heart rate variability.

Figure 4.13: Time course for standard deviation of successive differences between NN intervals from 35 minutes before BIO ROP screening to 10 minutes after screening



Time-point zero indicates the end of screening as identified by the removal of the eyelid speculum. Mean and 95% confidence interval shown for each HRV measure. Subjects grouped by high or low baseline heart rate variability.

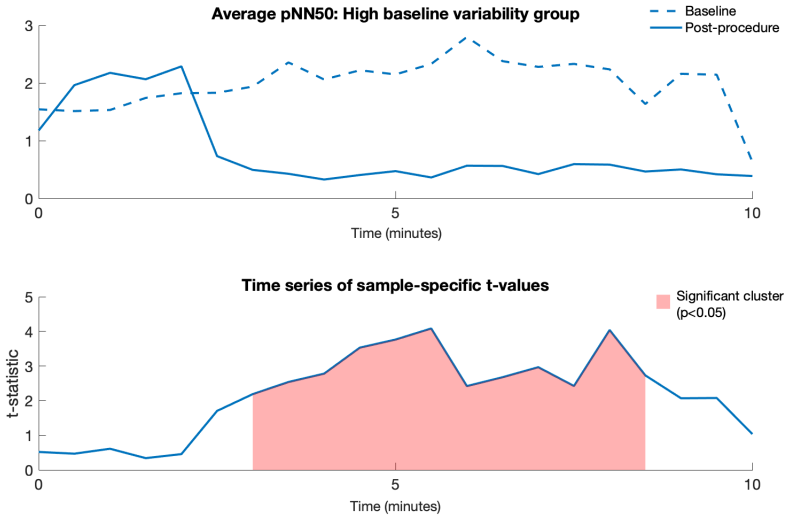
Figure 4.14: Time course for the triangular interpolation of the NN interval histogram from 35 minutes before BIO ROP screening to 10 minutes after screening



Time-point zero indicates the end of screening as identified by the removal of the eyelid speculum. Mean and 95% confidence interval shown for each HRV measure. Subjects grouped by high or low baseline heart rate variability.

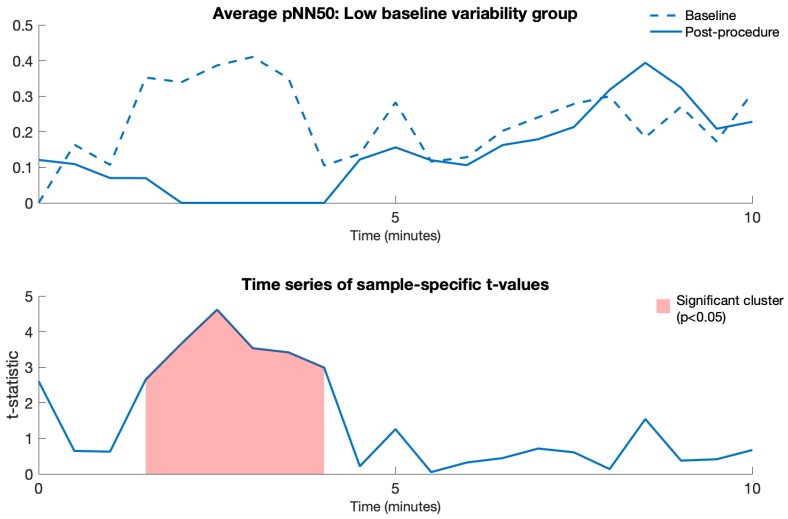
Figure 4.15: Time course for the HRV triangular index from 35 minutes before BIO ROP screening to 10 minutes after screening

Following ROP screening, non-parametric cluster analysis identified a significant decrease in pNN50 (that is, the number of successive NN intervals that differ by more than 50 ms divided by the total number of NN intervals). The difference in pNN50 was observed between 3.0 to 8.5 minutes in the group with high baseline HRV ($p = 0.013$; Figure 4.16), and from 1.5 to 4.0 minutes in the low baseline HRV group ($p = 0.008$; Figure 4.17). A significant increase in standard deviation of the average NN interval following BIO ROP screening was also identified. The difference in standard deviation of the average NN interval was observed between 0 to 1.0 minute in the low baseline HRV group ($p < 0.0001$; Figure 4.18).



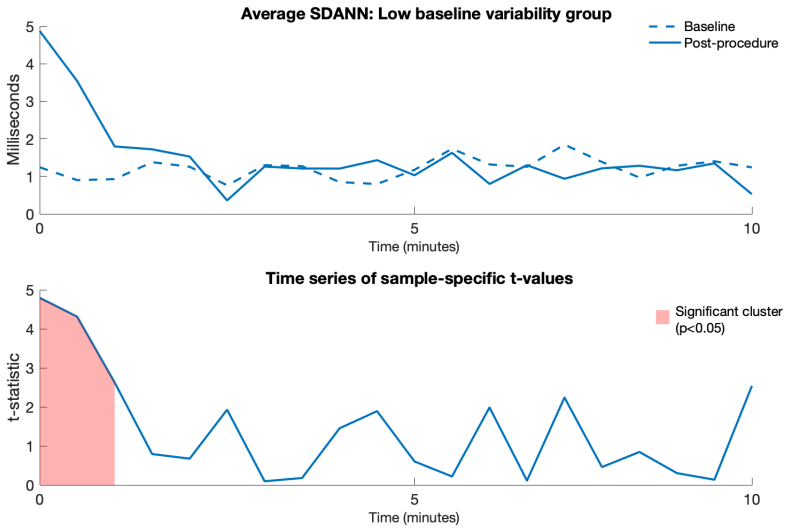
The top figure shows the average pNN50 for a baseline epoch immediately preceding screening (dashed line), and a post-procedure epoch immediately following screening (bold line). The bottom figure shows non-parametric cluster analysis of the two epochs. A significant cluster (highlighted in red) indicates that the pNN50 was significantly higher than baseline for 3.0 to 8.5 minutes post-procedure (p = 0.013). pNN50 = Number of successive NN intervals that differ by more than 50 ms / Total number of NN intervals.

Figure 4.16: Non-parametric cluster analysis of pNN50 before and after BIO ROP screening in 22 infants - high baseline heart rate variability group



The top figure shows the average pNN50 for a baseline epoch immediately preceding screening (dashed line), and a post-procedure epoch immediately following screening (bold line). The bottom figure shows non-parametric cluster analysis of the two epochs. A significant cluster (highlighted in red) indicates that the pNN50 was significantly higher than baseline for 1.5 to 4.0 minutes post-procedure (p = 0.008). pNN50 = Number of successive NN intervals that differ by more than 50 ms / Total number of NN intervals.

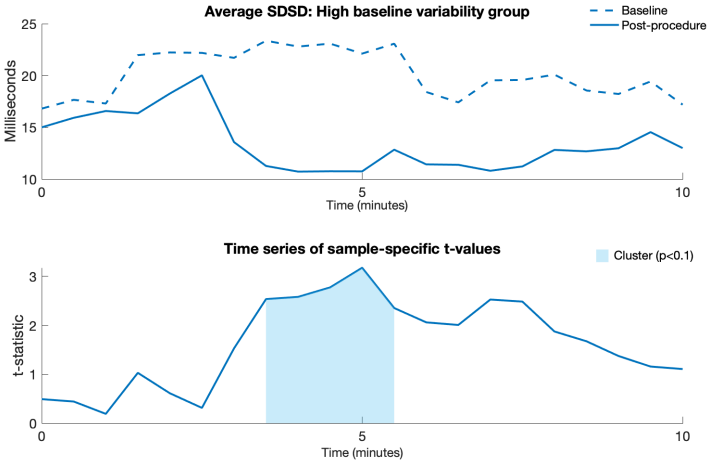
Figure 4.17: Non-parametric cluster analysis of pNN50 before and after BIO ROP screening in 22 infants - low baseline heart rate variability group



The top figure shows the average SDANN for a baseline epoch immediately preceding screening (dashed line), and a post-procedure epoch immediately following screening (bold line). The bottom figure shows non-parametric cluster analysis of the two epochs. A significant cluster (highlighted in red) indicates that the SDANN was significantly higher than baseline for 1.0 minutes post-procedure ($p < 0.0001$)

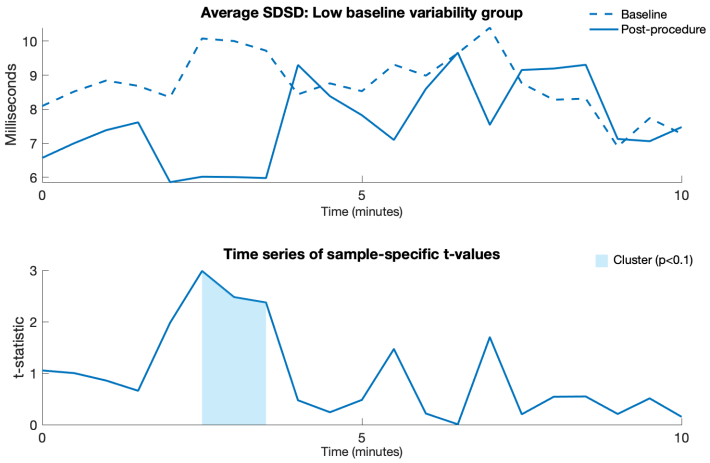
Figure 4.18: Non-parametric cluster analysis of the standard deviation of the average NN interval (SDANN) before and after BIO ROP screening in 22 infants - low baseline heart rate variability group

There were no significant clusters in other time domain HRV measures, however the standard deviation of successive differences between NN intervals showed a trend toward decrease post-procedure. The difference in the standard deviation of successive differences between NN intervals was observed between 3.5 to 5.5 minutes in the group with high baseline HRV ($p = 0.078$; Figure 4.19), and from 2.5 to 3.5 minutes in the low baseline HRV group ($p = 0.071$; Figure 4.20).



The top figure shows the average SDSD for a baseline epoch immediately preceding screening (dashed line), and a post-procedure epoch immediately following screening (bold line). The bottom figure shows non-parametric cluster analysis of the two epochs. A cluster (highlighted in blue) indicates that there was a trend toward higher SDSD between 3.5 to 5.5 minutes post-procedure (p = 0.078).

Figure 4.19: Non-parametric cluster analysis of the standard deviation of successive differences between NN intervals (SDSD) before and after BIO ROP screening in 22 infants - high baseline heart rate variability group

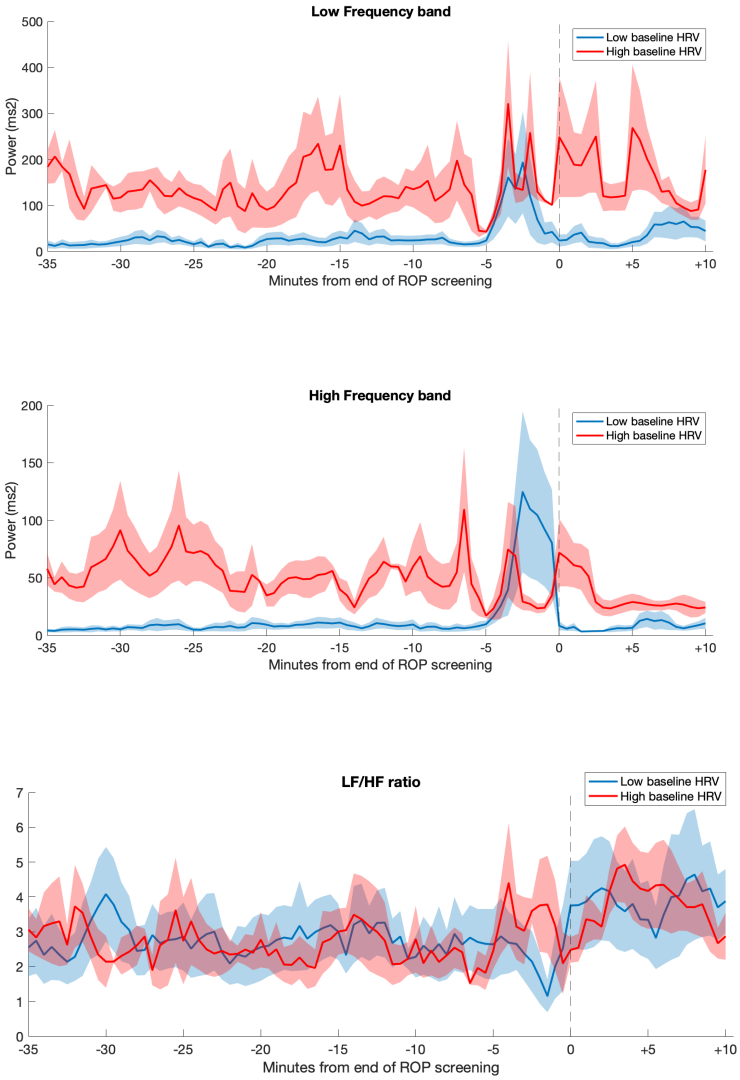


The top figure shows the average SDSD for a baseline epoch immediately preceding screening (dashed line), and a post-procedure epoch immediately following screening (bold line). The bottom figure shows non-parametric cluster analysis of the two epochs. A cluster (highlighted in blue) indicates that there was a trend toward higher SDSD between 2.5 to 3.5 minutes post-procedure (p = 0.071).

Figure 4.20: Non-parametric cluster analysis of the standard deviation of successive differences between NN intervals (SDSD) before and after BIO ROP screening in 22 infants - low baseline heart rate variability group

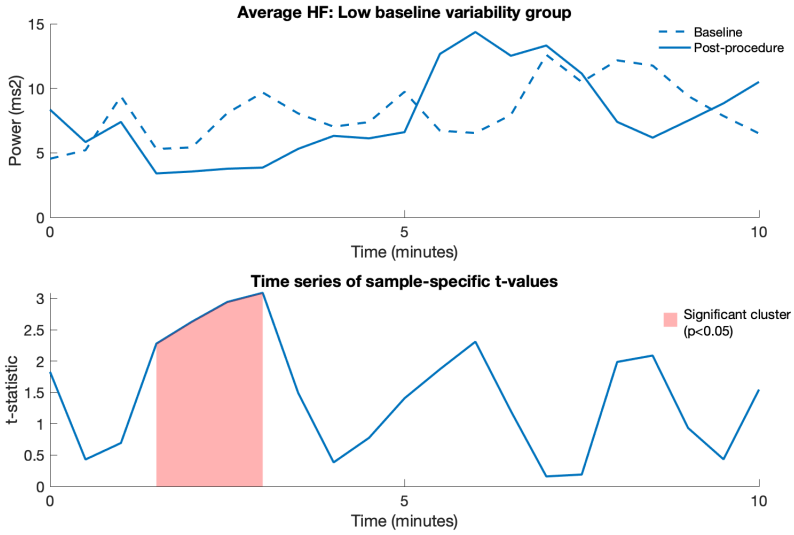
4.3.6 BIO ROP screening evokes a reduction in frequency domain heart rate variability measures

The time course for each frequency-domain HRV measure during BIO ROP screening is shown in Figure 4.21 (mean & 95% CI). Non-parametric cluster analysis identified a significant decrease in the power of the high frequency band following BIO ROP screening, shown in Figure 4.22. The difference in the power of the high frequency band was observed between 1.5 to 3 minutes in the low baseline HRV group (p = 0.032). Baseline high frequency activity was significantly related to baseline pNN50 (Pearson's correlation coefficient = 0.94, p-value <0.0001; Figure 4.23).



Mean and 95% confidence interval shown for each HRV measure. Subjects grouped by high or low baseline heart rate variability.

Figure 4.21: Time course for each heart rate variability measure from 35 minutes before BIO ROP screening to 10 minutes after screening. Time-point zero indicates the end of screening as identified by the removal of the eyelid speculum.



The top figure shows the average high frequency power for a baseline epoch immediately preceding screening (dashed line), and a post-procedure epoch immediately following screening (bold line). The bottom figure shows non-parametric cluster analysis of the two epochs. A significant cluster (highlighted in red) indicates that the high frequency power was significantly lower than baseline for 1.5 to 3 minutes post-procedure (p = 0.032).

Figure 4.22: Non-parametric cluster analysis of high frequency power before and after BIO ROP screening in 22 infants - low baseline heart rate variability group

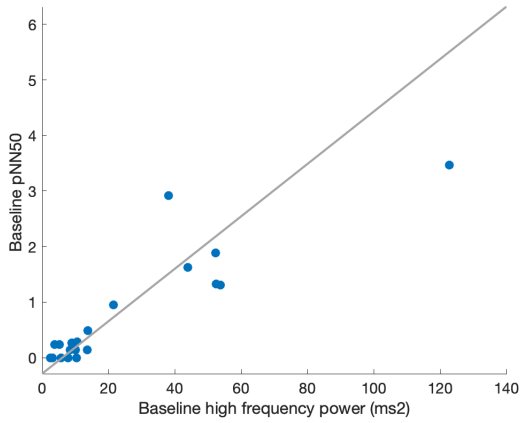
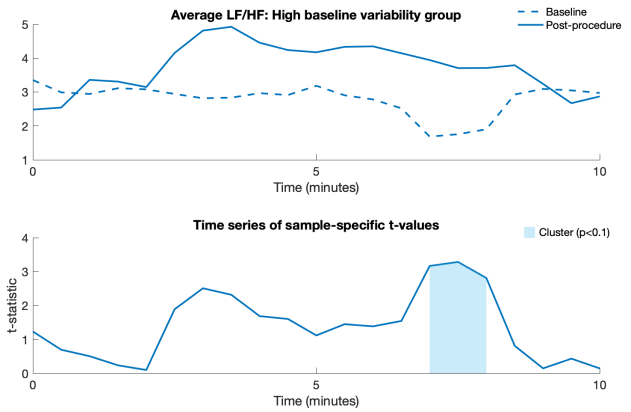


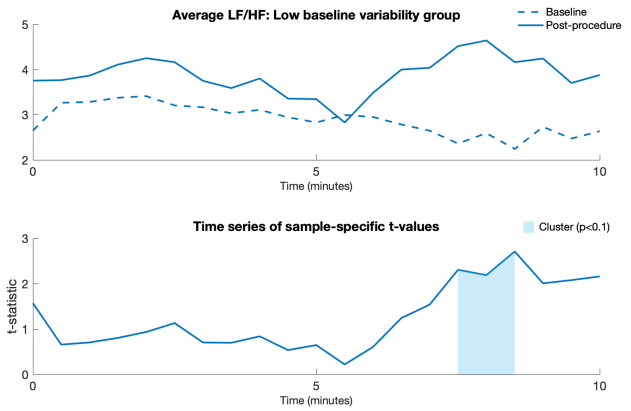
Figure 4.23: Baseline high frequency power was significantly related to baseline pNN50 (Pearson's correlation coefficient = 0.94, p < 0.0001)

There were no significant clusters in other frequency domain HRV measures, however the ratio of low to high frequency power showed a trend toward an increase post-procedure. The difference in the ratio of low to high frequency power was observed from 7 to 8 minutes in the high baseline HRV group ($p = 0.096$, Figure 4.24). and from 7.5 to 8.5 minutes in the low baseline HRV group ($p = 0.10$, Figure 4.25).



The top figure shows the average LF/HF for a baseline epoch immediately preceding screening (dashed line), and a post-procedure epoch immediately following screening (bold line). The bottom figure shows non-parametric cluster analysis of the two epochs. A cluster (highlighted in blue) indicates that there was a trend toward higher LF/HF between 7 to 8 minutes post-procedure (p = 0.096).

Figure 4.24: Non-parametric cluster analysis of the ratio of low to high frequency power (LF/HF) before and after BIO ROP screening in 22 infants - high baseline heart rate variability group



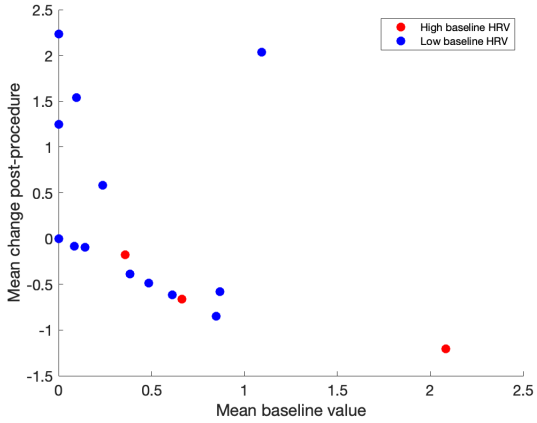
The top figure shows the average LF/HF for a baseline epoch immediately preceding screening (dashed line), and a post-procedure epoch immediately following screening (bold line). The bottom figure shows non-parametric cluster analysis of the two epochs. A cluster (highlighted in blue) indicates that there was a trend toward higher LF/HF between 7.5 to 8.5minutes post-procedure (p = 0.10).

Figure 4.25: Non-parametric cluster analysis of the ratio of low to high frequency power (LF/HF) before and after BIO ROP screening in 22 infants - low baseline heart rate variability group

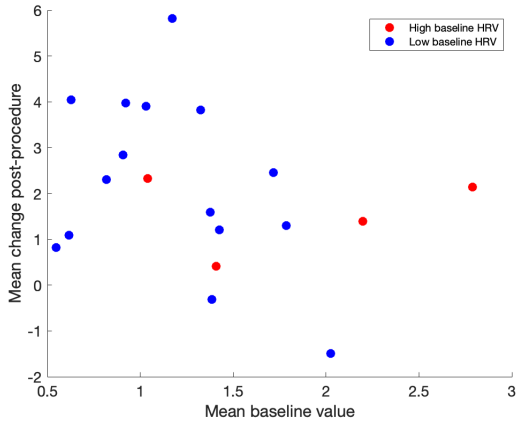
4.3.7 Heart rate variability demonstrates physiological limits

The time domain HRV measures which demonstrated a significant difference following BIO ROP screening were further investigated for the presence of a physiological limit or 'ceiling effect' to the change in HRV. For pNN50, the difference in baseline and post-procedure time courses occurred 3.0 to 8.5 minutes following screening in the high baseline HRV group and 1.5 to 4.0 minutes after screening in the low baseline HRV group. For this exploratory analysis, the groups were combined to increase the sample size, and a time window of 3.0 to 4.0 minutes was chosen. There was a negative relationship between mean baseline pNN50 and mean change in pNN50 post-procedure (Spearman's rho = -0.65, $p = 0.0046$; Figure 4.26a).

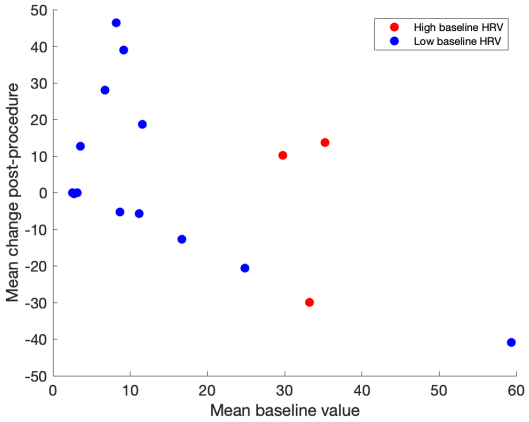
For the standard deviation of the average NN interval, at 0 to 1.0 minutes after BIO ROP screening, there was a trend toward a negative relationship between mean baseline standard deviation of the average NN interval and mean change in standard deviation of the average NN interval (rho = -0.32, $p = 0.18$; Figure 4.26b). Similarly for the power of the high frequency band, at 1.5 to 3.0 minutes after BIO ROP screening, there was a trend toward a negative relationship between mean baseline high frequency power and mean change in high frequency power (rho = -0.37, $p = 0.16$; Figure 4.26c).



(a) Baseline pNN50 was significantly related to the change following BIO ROP screening ($p = 0.0046$)



(b) Baseline standard deviation of the average NN interval was not significantly related to change following BIO ROP screening



(c) Baseline power in the high frequency band was not significantly related to change following BIO ROP screening

Figure 4.26: Correlation between baseline and change in HRV measure following BIO ROP screening

4.4 Discussion

4.4.1 Overview

This Chapter provides a detailed characterisation of the infant physiological response to BIO ROP screening.

Heart rate As hypothesised, there was an increase in HR during BIO ROP screening, followed by a return to baseline HR within 1 minute of the end of the procedure. There was a significant negative relationship between corrected gestational age at study and baseline HR ($p = 0.0046$), which corresponds with previous observations that resting HR is lower in older infants than younger infants (Alonzo *et al,* 2018). Older gestational age was significantly related to greater increase in HR following BIO ROP screening ($p = 0.039$). Since HR rise is limited by the physiological maximum, older infants, who have lower resting HR than younger infants, have potential for greater HR increase.

Oxygen saturation The baseline oxygen saturation for infants receiving high flow oxygen therapy was within the recommended target limits. For infants receiving low flow oxygen, the baseline oxygen saturation was higher than the recommended upper limit. This may be due to pre-emptive increase in oxygen flow prior to the procedure by bedside clinical staff, which was noted on occasion by the author. This deviation from standard NICU practice was intended was to prevent oxygen desaturations during the procedure, and may have been the underlying cause of higher-than-target oxygen saturation in this group.

No change in oxygen saturation was observed during BIO ROP screening; when analysed by ventilation method there was a visual trend suggesting a decrease in saturations at the start of screening in infants receiving supplementary oxygen. Healthier infants do not require supplementary oxygen, and therefore ventilation method may be considered a surrogate measure for infant health status. Oxygen saturation may

reduce during BIO ROP screening in vulnerable infants but not in more robust infants. The sample size in each ventilation group was small, and therefore a larger cohort is needed to test this hypothesis.

Physiological instability events There was no change in the standardised number of instability events following BIO ROP screening; however there were few instability events captured in each symmetrical interval, particularly the shorter duration intervals. A larger sample with longer duration recording is needed to accurately characterise the occurrence of instability events before and after BIO ROP screening. One infant had an apnoea episode following screening; this infant was in the lowest quartile for corrected gestational age, which corresponds with previous findings that younger infants display greater physiological instability following BIO ROP screening (Royal Collage of Ophthalmologists, 2008; Jiang *et al,* 2016).

4.4.2 Effect of age on heart rate variability

Baseline HRV was higher in older infants, which corresponds with previous observations that HRV increases with gestational age (van Ravenswaaij-Arts *et al,* 1993; Chiera *et al,* 2020). The median resting HRV for infants in this study, who were on average 7.71 weeks postnatal age, was between the 5-50th centile for pNN50, and between the 50-95th centile for power in low and high frequency bands, of normative data collected from healthy term infants during the first week of life (Longin *et al,* 2005), and below the 3rd centile for normative data collected from healthy term infants during the first month of life (Patural *et al,* 2019)(see Table 4.3). These findings correspond with previous observations that preterm infants at term-equivalent age have lower HRV than term infants (Hunt, 2006; Fyfe *et al,* 2015; Burtchen *et al,* 2019).

There was a negative relationship between HRV at baseline and change in HRV following BIO ROP screening. This suggests that there may be a physiological limit

to HRV increase. Caution may be needed in interpreting the magnitude of change in HRV, since infants with higher baseline HRV (such as older or healthier infants) have limited potential for HRV rise.

Table 4.3: Median HRV values from study infants aged average 7.7 weeks postnatal age, presented alongside normative values for healthy term infants aged 1 week (Patural *et al*) and 4 weeks postnatal age (Longin *et al*).

	Study data	Normative data					
		Patural *et al*, 2019			Longin *et al*, 2005		
Centile	**50th**	3rd	50th	97th	5th	50th	95th
HRV measure							
Standard deviation of NN intervals	**23.82**	25.5	47.5	75.9			
pNN50	**0.25**	0.56	3.62	27.49	0	1.00	23.5
HRV triangular index	**4.99**	11.7	20.5	32.9			
Triangular interpolation of the NN histogram	**78.02**	183	320	513			
Low Frequency power (0.05-0.15 Hz)	**33.87**	137	435	1513	8.60	25.65	84.25
High Frequency power (0.15-1.5 Hz)	**10.48**	66.7	247.5	1555.4	2.09	6.40	33.50
LF/HF ratio	**3.04**	0.99	2.69	5.26	1.83	3.85	6.70

4.4.3 BIO ROP screening causes physiological stress

As discussed in the Chapter Introduction, high frequency HRV activity predominantly reflects the parasympathetic modulation of the heart rate through vagal tone. In infants, parasympathetic activity emerges around 32 weeks gestation, increases greatly around 37 weeks gestation, and continues developing in the postnatal period (Fyfe *et al,* 2015; Mulkey and du Plessis, 2018; Chiera *et al,* 2020). The infants included in this study were median 34.43 weeks gestation and therefore at a stage of ongoing parasympathetic neurodevelopment.

Several studies have observed reduction in parasympathetic activity in preterm infants following a painful clinical procedure such as heel lancing, intramuscular in-

jection and chest drain insertion (Buyuktiryaki *et al,* 2018; Cremillieux *et al,* 2018; Okur *et al,* 2019). The reduction in parasympathetic activity in infants undergoing physiological stress is considered to result from failure of the immature infant parasympathetic nervous system to maintain equilibrium with the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis (Chiera *et al,* 2020; Hashiguchi *et al,* 2020).

In this study, a reduction in pNN50 and high frequency activity was observed following BIO ROP screening. These measures were highly correlated, which corresponds with previous findings that the pNN50 is related to high frequency HRV activity (Taskforce of the European Society of Cardiology, 1996; Kleiger *et al,* 2005; Shaffer and Ginsberg, 2017). The ratio of low to high frequency power increased following BIO ROP screening, reflecting the decrease in high frequency activity observed. BIO ROP screening was also associated with an increase in standard deviation of the average NN interval, and a trend toward decrease in the standard deviation of successive differences between NN intervals. These measures are most closely related to ultra-low frequency and high frequency activity respectively. The physiological source of the ultra-low frequency band ($<$0.003 Hz) is uncertain, although slow-acting biological processes such as circadian rhythm have been suggested (Shaffer *et al,* 2014). In sum, a decrease in HRV indices of parasympathetic activity was identified following BIO ROP screening, which may suggest that the procedure is a painful physiological stressor for preterm infants.

4.4.4 Strengths and Limitations

This study provides a detailed analysis of the physiological changes that occur during and after BIO ROP screening. HRV measures are used to evaluate the impact of the procedure in preterm infants, and identified an autonomic response indicative of pain and stress. The study was limited by a small sample size; as discussed previously, recruitment to the study was curtailed by the outbreak of the COVID-19 pandemic.

A second limitation of the study was the duration of the physiological recordings was between 15 minutes and 10 hours; a longer period such as 24 hours may better identify new-onset apnoea events, changes related to circadian rhythm, or cycles of care within the NICU. Analysis of the baseline or resting state may lead to greater understanding of the development of physiological instability after BIO ROP screening. Infants who develop new-onset apnoea after BIO ROP screening may display behaviours that may predict physiological instability, such as pauses in breathing, or increased desaturations (Mitchell *et al,* 2011). Further research is needed with larger sample size and longer recording duration to investigate this question.

A limitation of the study design was that the infants' behavioural state was not determined prior to the clinical procedures. It is possible that the study observations are associated with a change in behavioural state evoked by the clinical procedures. In future, the baseline behavioural state of the infant should be documented, to ensure the effect of behavioural state change can be understood.

The influence of respiration on heart rate, that is, respiratory sinus arrhythmia, has not been specifically considered in this study. Respiratory sinus arrhythmia is considered to influence high frequency activity in adults. However, previous data suggest that respiration has little or negligible effect on HRV in infants (Giddens and Kitney, 1985; Longin *et al,* 2005; Joshi *et al,* 2019), therefore the importance of accounting for this characteristic in infant HRV analysis is uncertain.

Frequency analysis using the Fourier transform assumes the HRV signal is stationary or cyclical. Since this assumption is not met, wavelet transform and non-linear analysis methods may contribute additional insights into infant HRV (Quintana and Heathers, 2014). Non-linear approaches have been used previously in the investigation of infant pain (Weissman *et al,* 2012; Cremillieux *et al,* 2018). This study included only HRV indices recommended by the Task Force with a known physiological basis, however, non-linear indices may be considered in future work as understanding of these measures advances.

4.4.5 Future Directions

The findings from this study suggest that BIO ROP screening is a source of physiological stress for preterm infants. In future, it would be interesting to further characterise the relationship between stress and pain in neonates. In adults, stress increases pain sensitivity to noxious stimuli (Crettaz *et al,* 2013; Jennings *et al,* 2014). In newborn infants aged 36-42 weeks, greater stress (as indicated by salivary cortisol) results in increased noxious-related brain activity following an acute noxious stimulus (Jones *et al,* 2017). Preterm infants at term-equivalent age display greater magnitude of noxious-related brain activity following an acute noxious stimulus than term born infants (Slater *et al,* 2010). This difference in sensitivity may be due to immature pain processing, prior pain experience, or greater physiological stress. It would be interesting to investigate whether stress evoked by a complex procedure such as BIO ROP screening results in heightened responses to subsequent acute noxious stimulation in preterm infants. Multimodal data could be recorded in infants undergoing clinically-required heel lancing before and after BIO ROP screening. This could provide insight into the cumulative effect of procedures on infants in the NICU, and allow clinicians to optimise timing of care and interventions.

Following discharge from the NICU, ex-preterm infants display raised cortisol levels up to 18 months of age; this may be due to their early life experience including physiological stress and mechanical ventilation (Grunau *et al,* 2007). Previous studies have suggested that postnatal stress may increase the risk of metabolic and cardiovascular conditions later in life, due to altered functioning of the hypothalamic-pituitary-adrenal axis (Reynolds, 2013; Spencer, 2013). Raised cortisol levels may contribute to the neurodevelopmental abnormalities that occur in this population-Grunau et al. (2007). It is therefore vital to identify and ameliorate stress in infants admitted to the NICU.

A promising approach in identifying stress in infants is the use of automated HRV algorithms which measure parasympathetic activity in infants in real-time. For ex-

ample, Butruille *et al* developed the Newborn Infant Parasympathetic Evaluation index (Butruille *et al, 2015)*, which is based on the high frequency component of HRV. Decrease in the Newborn Infant Parasympathetic Evaluation index has been used as a surrogate measure of infant pain after assisted birth (Rakza *et al,* 2018), and after surgical interventions (Faye *et al,* 2010). Increase in the Newborn Infant Parasympathetic Evaluation index has been used as a surrogate measure of infant comfort during kangaroo care and facilitated tucking (Butruille *et al,* 2017). Measurement of the the Newborn Infant Parasympathetic Evaluation index or similar real-time algorithm in infants undergoing BIO ROP screening may improve NICU staff ability to monitor infants physiological stress during the procedure (Chiera *et al,* 2020), and may create opportunity for caregivers to respond with comfort measures or analgesia in order to improve the infants experience of screening.

4.5 Conclusions

In sum, this study suggests that BIO ROP screening causes physiological stress in infants, and may be a painful physiological stressor. HRV analysis may provide insight into the infant experience of stressful clinical procedures. Using HRV to monitor infant stress in the clinical environment may allow caregivers to better detect and manage infant stress during the critical neonatal period of neurodevelopment. Improved monitoring and treatment of infant stress may confer benefit into adulthood.

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Chapter 5

Is Optos ROP screening less painful and stressful than BIO ROP screening?
Does oral sedation and analgesia reduce pain and stress associated with ROP treatment?

5.1 Abstract

Introduction

Retinopathy of prematurity is detected through a programme of repeated retinal screening of preterm infants. In the John Radcliffe Hospital NICU, BIO ROP screening is supported by the use of Optos ROP screening, an ultra-widefield retinal imaging system. Optos imaging differs from BIO ROP screening in that it does not involve

stimuli such as bright retinal illumination, scleral indentation, or contact with the eye. In this Chapter, the hypothesis that Optos ROP screening is less painful and stressful than BIO ROP screening was tested by characterising the infant response to Optos screening using a multimodal approach and comparing the results to those from BIO ROP screening presented in Chapter 3 and Chapter 4.

Treatment options for ROP comprise diode laser photocoagulation and intravitreal injection of anti-VEGF, as discussed in Section 2.3. In the John Radcliffe NICU, ROP treatments are performed under oral sedation and analgesia (chloral hydrate and morphine). In this Chapter, the hypothesis that pharmacological sedation reduces EEG complexity was tested by quantifying baseline EEG complexity prior to ROP treatment and comparing the results to non-medicated infants. Additionally, the hypothesis that ROP treatment under sedation and oral analgesia is less painful and stressful than BIO ROP screening was tested by characterising the infant response to ROP treatment using a multimodal approach and comparing the results to those from BIO ROP screening presented in Chapter 3 and Chapter 4.

Method

Quantitative EEG analysis was performed to characterise the change in standard deviation of level 3 detail coefficients, sum of squared level 4 detail coefficients, and relative beta power following Optos screening and following ROP treatment. EEG complexity was quantified by measuring the entropy of the signal in medicated infants in the baseline period prior to ROP treatment, and compared to that of non-medicated age-matched infants. The changes in HR, oxygen saturations, and physiological instability events occurring after Optos ROP screening and ROP treatment were calculated. HRV analysis was performed to characterise the change in measures of parasympathetic activity (pNN50 and absolute high frequency power) following Optos screening and following ROP treatment. The recruitment of subjects into both studies was curtailed by the outbreak of COVID-19, therefore the number of subjects studied during

Optos ROP screening and ROP treatment was small. The analyses in this Chapter demonstrate approaches that could be applied to a larger sample size.

Results

Six infants were studied during Optos ROP screening. No significant difference was identified between infants undergoing Optos screening and an age-matched group of infants undergoing BIO ROP screening regarding the change in standard deviation of level 3 detail coefficients, sum of squared level 4 detail coefficients, or relative beta power, nor with regard to the percentage change in HR and oxygen saturation following screening. There was a trend toward a lower clinical pain score following Optos ROP screening compared to BIO ROP screening ($p = 0.12$). Screening was not associated with an increase in instability events. No significant reduction was observed in pNN50 or absolute high frequency power following Optos ROP screening.

Five infants were studied during ROP treatment. Each feature of complexity was significantly lower in infants who had received medication compared to an age-matched group of non-medicated infants ($p < 0.05$). No significant increase in standard deviation of level 3 detail coefficients, sum of squared level 4 detail coefficients, or relative beta power was identified following ROP treatment. There was significantly lower HR ($p = 0.031$) and a trend toward lower oxygen saturation ($p = 0.084$) and clinical pain score ($p = 0.074$) in medicated infants undergoing ROP treatment compared to a non-medicated age-matched group undergoing BIO ROP screening. Treatment was not associated with an increase in instability events. There was a trend toward a reduction in pNN50 following ROP treatment ($p = 0.15$); no significant reduction in absolute power of the high frequency band was identified.

Conclusions

This Chapter demonstrates an approach to evaluating the infant response to Optos imaging, an alternative method of ROP screening to BIO ROP screening. The findings

were consistent with the hypothesis that Optos screening may be less stressful and painful than BIO ROP screening. An approach to characterising the infant response to treatment for ROP under oral sedation and analgesia is presented. The findings were consistent with the hypothesis that oral sedation and analgesia may reduce brain activity complexity, and may be effective in reducing pain and stress related to the procedure. However, more data are required to establish the significance of the results presented in this Chapter.

5.2 Methods

5.2.1 Participating Infants

EEG and physiological recordings from 6 subjects undergoing Optos ROP screening were included in Study 1, and from 5 subjects undergoing treatment for ROP in Study 2. Demographic information for all subjects included in Chapter 5 is shown in Table 5.1.

Table 5.1: Demographic information for subjects included in Chapter 5

	Study 1	Study 2
Number of infants analysed	6	5
Corrected gestational age at study (weeks)	36.21 (35.89, 37.5)	37.86 (36.0, 39.14)
Birthweight (g)	1050 (1012.5, 1107.5)	660 (600, 800)
Number of males	6 (100%)	3 (60%)
Apgar score at 5 minutes	9.5 (9.13, 10)	5 (5, 7)
Normal vaginal delivery	4 (67%)	4 (80%)
Procedure duration (minutes)	5.39 (4.62, 6.16)	61.13 (45.69, 68.75)

Figures are median (lower quartile, upper quartile) or number (percentage).

5.2.2 Experimental Design

This section contains an overview of the studies comprising this Chapter. In Study 1, data collected from 6 infants undergoing Optos ROP screening were considered. Firstly, EEG recordings were analysed for three frequency-domain features - relative beta power, standard deviation of level 3 detail coefficients, and sum of squared level 4 detail coefficients - and the change in each EEG feature after Optos ROP screening was quantified. The percentage change in each feature was compared to an age-matched subgroup of infants undergoing BIO ROP screening. Secondly, physiological and behavioural data were characterised and compared to age-matched infants undergoing BIO ROP screening. Finally, heart rate variability analysis was performed for two measures - pNN50 and absolute high frequency power, and the change in each measure after Optos ROP screening was quantified.

In Study 2, data collected from 5 infants undergoing treatment for ROP were considered (4 infants treated with laser, 1 infant treated with intravitreal injection). Firstly, EEG recordings were analysed for 13 features related to signal complexity during the baseline period prior to ROP treatment, and compared to the same period in age-matched non-medicated infants. Next, the change in three frequency-domain features - relative beta power, standard deviation of level 3 detail coefficients, and sum of squared level 4 detail coefficients - was quantified after ROP treatment. Physiological and behavioural data were characterised and compared to age-matched infants undergoing BIO ROP screening. Finally, heart rate variability analysis was performed for two measures - pNN50 and absolute high frequency power, and the change in each measure after ROP treatment was quantified.

5.2.3 Recording Techniques

EEG, ECG, oxygen saturation recordings, and behavioural measures, were obtained during Optos ROP screening and during treatment for ROP. Chapter 2 (General

Methods) describes the recording techniques in detail.

5.2.4 Analysis

Study 1 - Optos ROP screening

Electroencephalography Three frequency-domain features of EEG were calculated in 120s epochs using MATLAB code developed by Pillay (Pillay *et al*, 2018). Two features - standard deviation of level 3 detail coefficients and sum of squared level 4 detail coefficients - were derived using the Discrete Wavelet Transform, and one feature - relative power of the beta band - was derived using the Discrete Fourier Transform. Both analysis approaches are described in detail in Section 2.5.2.

A baseline and post-procedure 120s epoch was analysed for each subject (see schematic in Figure 2.7). Epochs were analysed in consecutive 30s windows with 15s overlap, then the 30s windows were tested for outlying values for each feature. Outlying values were defined as more than three scaled median absolute deviations away from the median (Hampel, 1974; Leys *et al*, 2013) (MATLAB command 'isoutlier'). On average, 5% of the windows were rejected for each feature. Following outlier rejection, the mean value was calculated across remaining windows for each feature.

For each feature, it was investigated whether there was a significant increase from baseline to post-procedure. The differences from baseline to post-procedure were firstly tested for whether they were drawn from a normal distribution using the Anderson-Darling test, then a paired-sample one-tailed t-test was performed for normally-distributed values, or a Wilcoxon signed rank test for non-normally distributed values.

The MATLAB Multiple Testing Toolbox was used to apply the Hochberg method to correct for multiple hypothesis testing. This method controls the Family Wise Error Rate, and was selected because it strongly controls Type 1 errors, which is important in confirmatory analysis of a small number of hypotheses. The Hochberg

method also has greater power than the Holm and Bonferroni methods of controlling Family Wise Error Rate (Benjamini and Hochberg, 1995; Chen *et al*, 2017).

Finally, the percentage change in each feature following Optos ROP screening was compared to an age-matched subgroup of infants following BIO ROP screening. A paired-sample two-tailed t-test was performed for normally-distributed values, or a Wilcoxon signed rank test for non-normally distributed values.

Physiology MATLAB was used to analyse physiological data contained in the output file of the recording software. The mean HR and oxygen saturation and 95% confidence intervals were calculated at each time point during Optos ROP screening. Non-parametric cluster analysis of the baseline and post-procedure HR and oxygen saturation recordings was performed using the approach described in Section 4.2.5.

A PIPP-R score following Optos ROP screening was calculated as described in Chapter 2 (General Methods). Components of the PIPP-R score were used to calculate the percentage change in HR and oxygen saturation following screening. Subjects studied during Optos ROP screening were age-matched to subjects studied during BIO ROP screening; the intention was to compare the two procedures effect on infant physiology and behaviour.

The number of instability events (bradycardia, tachycardia, desaturation, apnoea) which occurred within 15 minutes before and after Optos ROP screening was calculated. The definition of each instability event is listed in Table 2.3. The standardised difference in number of instability events after Optos ROP screening was also calculated for the same interval. The standardised difference in instability events was calculated by dividing the difference between the number of events in the baseline and post-procedure intervals by the total number of events. A negligible amount (0.01) was added to each event total to avoid division by zero.

Two HRV measures - pNN50 and absolute high frequency power - were calculated in the baseline and post-procedure recordings. The HRV measures' definition and

derivation are described in Section 2.5.4. An epoch of 3.5 minutes was used from 1.5 to 4 minutes in each recording, based on the timing of the post-procedure reduction in parasympathetic activity identified in Chapter 4. Epochs were analysed in consecutive 5 minute windows with 30s overlap, then the 5 minute windows were tested for outlying values for each measure; on average, 4% of the windows were rejected for each measure. Following outlier rejection, the mean value was calculated across remaining windows for each measure.

For each measure, it was investigated whether there was a significant decrease from baseline to post-procedure. The differences from baseline to post-procedure were firstly tested for whether they were drawn from a normal distribution using the Anderson-Darling test, then a paired-sample one-tailed t-test was performed for normally-distributed values, or a Wilcoxon signed rank test for non-normally distributed values.

Study 2 - ROP treatment

Electroencephalography Twelve features of EEG which reflect signal complexity were calculated: multi-scale entropy scales 1 to 10, maximum multi-scale entropy, and the area under the multi-scale curve (multi-scale entropy AUC). EEG complexity analysis has been used previously to characterise non-pharmacological sleep in preterm infants (Janjarasjitt *et al*, 2008; Zhang *et al*, 2009; De Wel *et al*, 2017). Measures of entropy quantify the regularity of the EEG signal; low entropy indicates a more regular and less complex signal, higher values indicate a more disordered, complex signal (Richman and Moorman, 2000). Multi-scale entropy is a measure of EEG complexity in which sample entropy is calculated for a sample of the EEG signal. The EEG signal is sampled at increasingly coarse scales (Costa *et al*, 2005). Sample entropy is measured by dividing the signal into consecutive sections, termed templates. Each template is compared to the others and the total number of similar templates is quantified. Templates are identified as similar if the maximum of the

differences between each pair of template values is below a specified threshold value (Pincus *et al*, 1991). In this thesis, the template length was 2s and the threshold value was 0.2*SD (where SD = standard deviation of the signal amplitude). These values were selected due to precedent from other EEG studies in premature infants (Lu *et al*, 2015; De Wel *et al*, 2017).

Complexity features were calculated during a 120s baseline epoch for two groups of infants: 5 infants who received ROP treatment (4 treated with laser, 1 treated with intravitreal injection) and 5 age-matched infants who had not received medication prior to BIO ROP screening. The intention was to investigate the effect of medications given for ROP treatment on baseline brain activity by comparing infants who had received medication with non-medicated infants. Epochs were analysed in consecutive 30s windows with 15s overlap, then the 30s windows were tested for outlying values for each feature. On average, 5% of the windows were rejected for each feature in the treatment group, and 2% in the age-matched control group. For each feature, it was investigated whether complexity in the ROP treatment group was significantly lower than the non-medicated group, using the same statistical approach as for Study 1 EEG analysis.

A further three features of EEG - standard deviation of level 3 detail coefficients, sum of squared level 4 detail coefficients, and relative power of the beta band - were calculated during a baseline and post-procedure 120s epoch for the 5 infants undergoing treatment for ROP. Epochs were analysed in consecutive 30s windows with 15s overlap, then the 30s windows were tested for outlying values for each feature. On average, 6% of the windows were rejected for each feature. For each feature, it was investigated whether there was a significant increase from baseline to post-procedure using the same statistical approach as for Study 1 EEG analysis.

Physiology MATLAB was used to analyse physiological data contained in the output file of the recording software. HR and oxygen saturation data were visualised

during ROP treatment and instability events were identified for each subject. The timing of medication administration and the procedure duration varied between subjects so group parameters were not calculated for the procedure time course.

A PIPP-R score following ROP treatment was calculated as described in Chapter 2 (General Methods). Subjects studied during treatment for ROP were age-matched to subjects studied during BIO ROP screening, in order to compare the two procedures effect on infant physiology and behaviour.

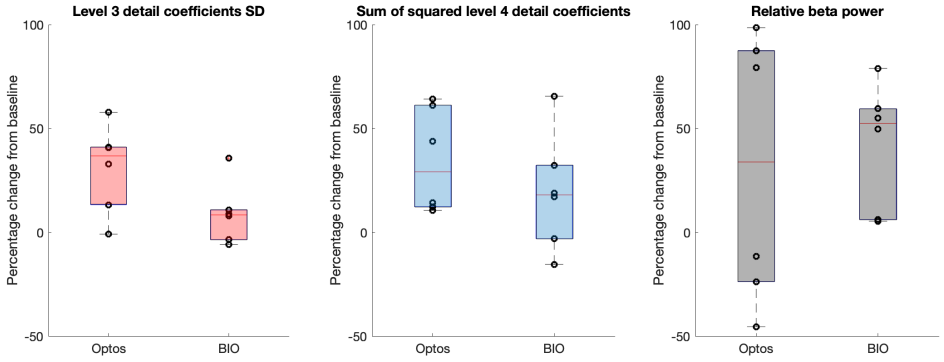
.Measures of parasympathetic activity were be used to characterise the effect of BIO ROP treatment under pharmacological sedation on infant HRV. In an adult study, morphine evoked a decrease in the ratio of low to high frequency power which reflected increased parasympathetic activity (Michaloudis *et al*, 1998). Similarly in an animal model, chloral hydrate evoked an increase in high frequency activity (Moldovan *et al*, 2004). Two HRV measures - pNN50 and absolute high frequency power - were calculated in the baseline and post-procedure recordings using the same approach as for Study 1 physiology analysis. On average, 8% of the windows were rejected for each measure. Following outlier rejection, the mean value was calculated across remaining windows for each measure. For each measure, it was investigated whether there was a significant decrease from baseline to post-procedure using the same statistical approach as for Study 1 physiology analysis.

5.3 Results

Optos Screening

5.3.1 Optos ROP screening may not evoke noxious-related brain activity

The increase in three frequency-domain features of EEG was calculated following Optos ROP screening. There was a significant increase in the standard deviation of level 3 detail coefficients (p = 0.020) and sum of squared level 4 detail coefficients (p = 0.027). No significant increase in relative beta power was identified following Optos ROP screening (p = 0.23). There was no significant difference in percentage feature-change following ROP screening between the Optos and age-matched BIO groups for the standard deviation of level 3 detail coefficients (p = 0.14), sum of squared level 4 detail coefficients (p = 0.43), or relative beta power (p = 0.76; Figure 5.1).

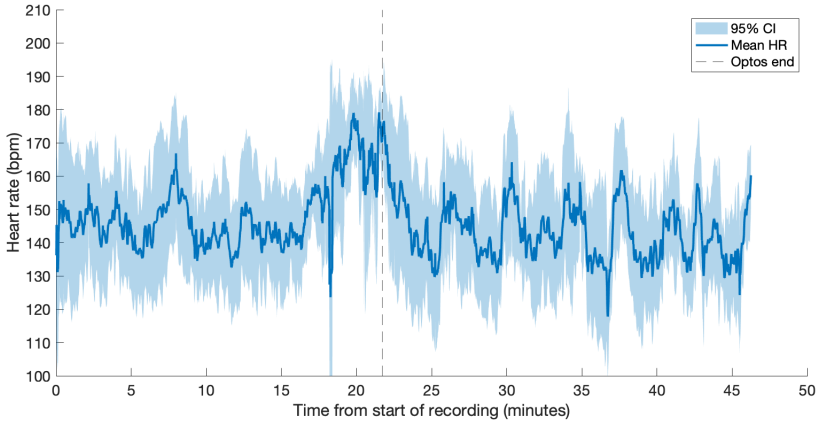


This figure shows the percentage increase in each feature following Optos screening in a group of 6 infants, and following BIO ROP screening in an independent age-matched subgroup of 6 infants. No significant difference in percentage feature-change following ROP screening was identified between the two groups. SD = standard deviation.

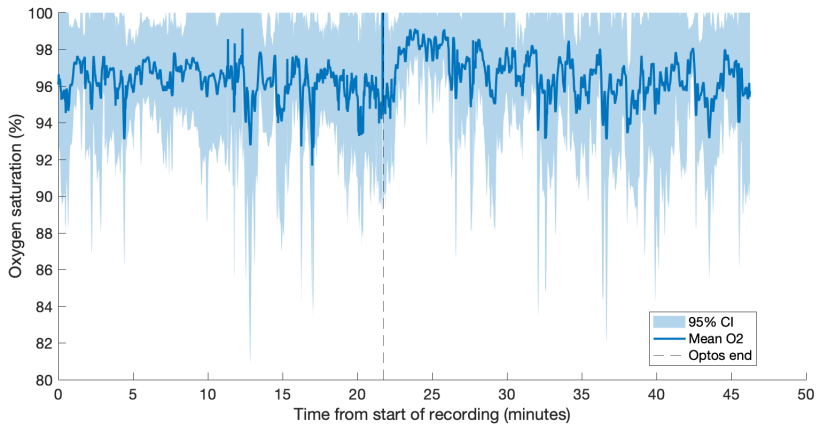
Figure 5.1: Percentage change in electroencephalographic features in a group of 6 infants following Optos ROP screening, and an age-matched group of 6 infants following BIO ROP screening

5.3.2 Physiological and behavioural changes following Optos ROP screening

The trend in HR and oxygen saturation during Optos ROP screening are shown in Figure 5.2. Non-parametric cluster analysis of the baseline and post-procedure time courses did not identify significant changes in HR and oxygen saturation following Optos screening (see Figure 5.3). No significant difference was identified between infants undergoing Optos screening and an age-matched group of infants undergoing BIO ROP screening with regard to the percentage change in HR and oxygen saturation following screening ($p = 0.70$ and $p = 0.67$ respectively; see Figure 5.4). There was a trend toward a lower PIPP-R score following Optos ROP screening compared to BIO ROP screening (age-matched subgroup) (paired sample one-tailed t-test; $p = 0.12$; Figure 5.5).



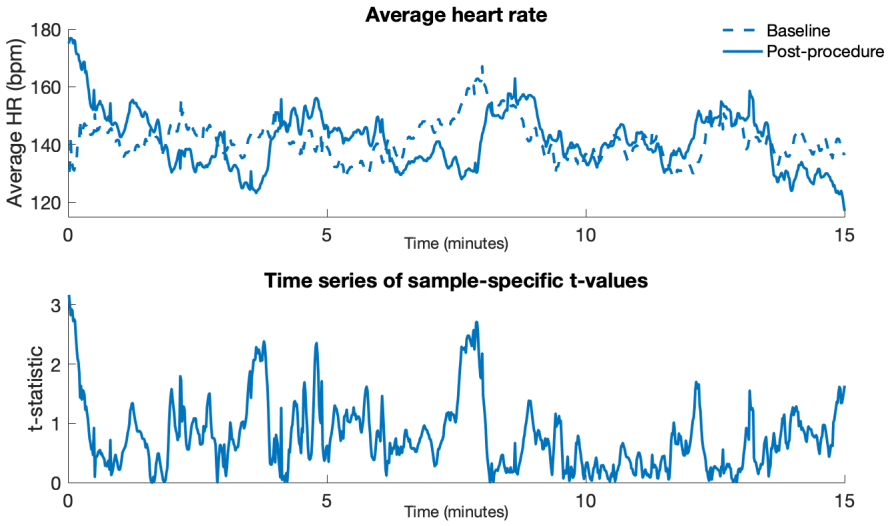
(a) Heart rate



(b) Oxygen saturation

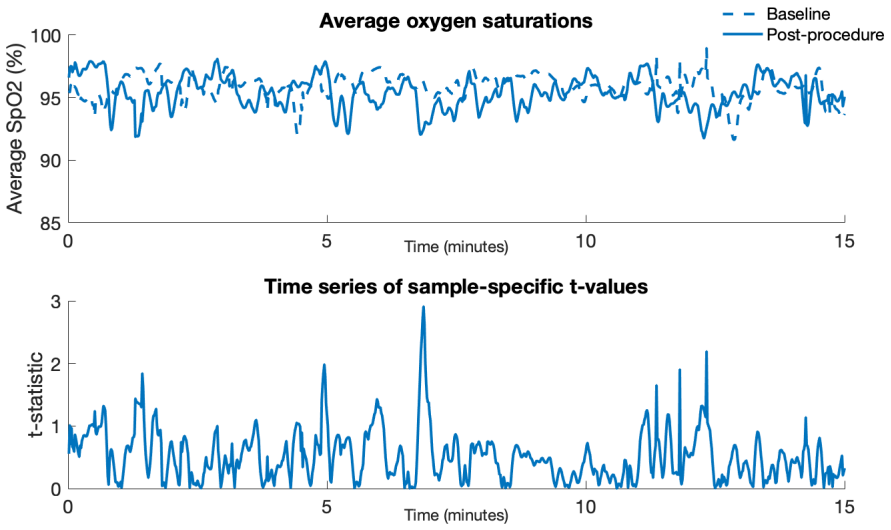
95% CI = 95% confidence intervals; mean HR = mean heart rate; mean O2 = mean oxygen saturations; Optos end = end of screening as indicated by removal of speculum from second eye examined.

Figure 5.2: Trend in physiology during Optos ROP screening in 6 infants (mean and 95% confidence intervals).



(a) Heart rate

The top figure shows the average heart rate for a baseline epoch immediately preceding screening (dashed line), and a post-procedure epoch immediately following screening (bold line). The bottom figure shows non-parametric cluster analysis of the two epochs.



(b) Oxygen saturation

The top figure shows the average oxygen saturation for a baseline epoch immediately preceding screening (dashed line), and a post-procedure epoch immediately following screening (bold line). The bottom figure shows non-parametric cluster analysis of the two epochs.

Figure 5.3: Non-parametric cluster analysis of physiology before and after Optos ROP screening in 6 infants

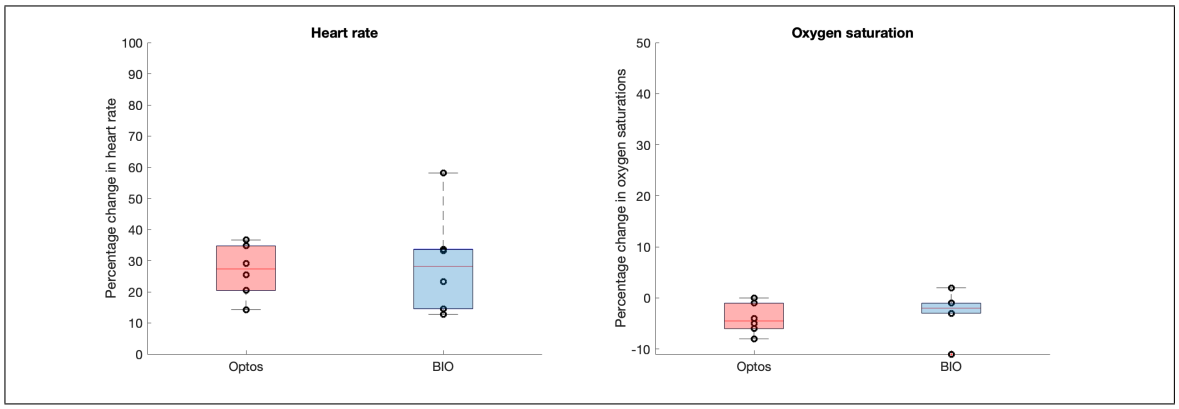


Figure 5.4: Percentage change in physiological parameter in 6 infants following Optos ROP screening, and in an age-matched group of 6 infants following BIO ROP screening

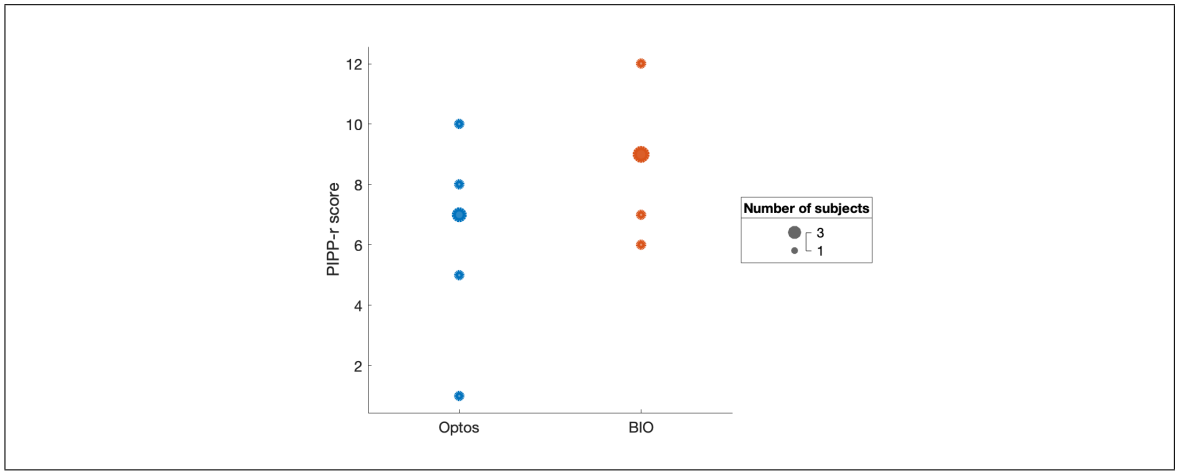


Figure 5.5: PIPP-R scores in 6 infants following Optos ROP screening, and in an age-matched group of 6 infants following BIO ROP screening

5.3.3 Optos ROP screening may not be associated with a change in instability events

The total number of instability events (bradycardia, tachycardia, desaturation, apnoea) in the 15 minutes before and after Optos ROP screening, and the standardised difference in number of instability events following screening for the same time interval are listed in Table 5.2.

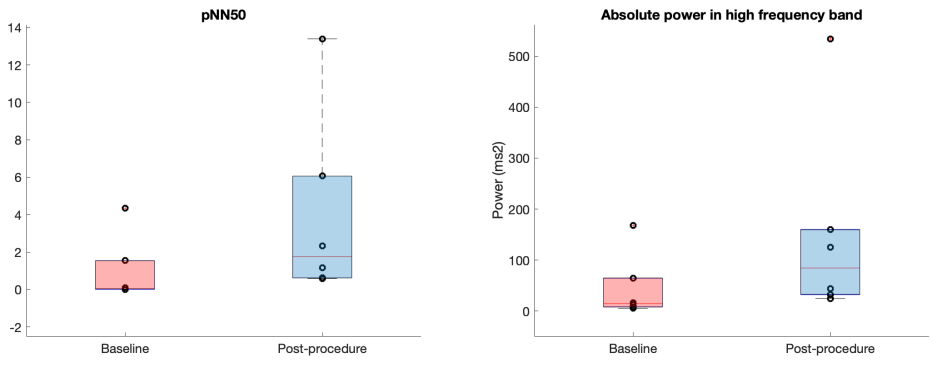
Table 5.2: Number of instability events which occurred 15 minutes before and after Optos ROP screening, and standardised difference in number of events following screening

	Events before screening	Events after screening	Standardised difference in events
Bradycardia	0	0	0 (0 to 0)
Tachycardia	0	0	0 (0 to 0)
Desaturation	1	0	0 (0 to 0)
Apnoea	0	0	0 (0 to 0)

Median (interquartile range) shown.

5.3.4 Optos ROP screening may not evoke stress-related parasympathetic activity

Two measures of HRV - pNN50 and absolute high frequency power - were calculated in the baseline and post-procedure recordings using an epoch of 3.5 minutes, based on the timing of the post-procedure reduction in parasympathetic activity identified in Chapter 4. A reduction was not observed in either measure following Optos ROP screening (paired sample one-tailed t-test; $p > 0.05$; Figure 5.6).

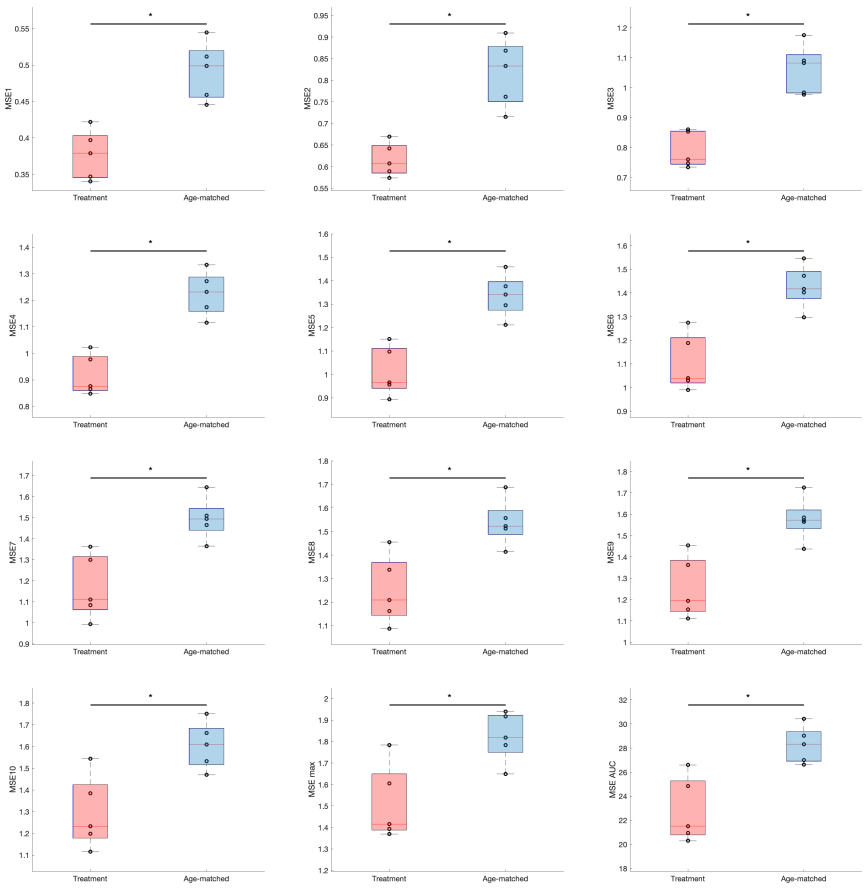


pNN50 = Number of successive NN intervals that differ by more than 50 ms / Total number of NN intervals

Figure 5.6: Heart rate variability measures reflecting parasympathetic activity before and after Optos ROP screening

5.3.5 Medication prior to ROP treatment may reduce brain activity complexity

The difference in 12 complexity features of EEG was calculated from baseline recordings in 5 infants prior to treatment for ROP and 5 age-matched infants prior to BIO ROP screening. Each feature of complexity was significantly lower in infants who had received medication prior to ROP treatment compared to non-medicated infants ($p < 0.05$; Figure 5.7).

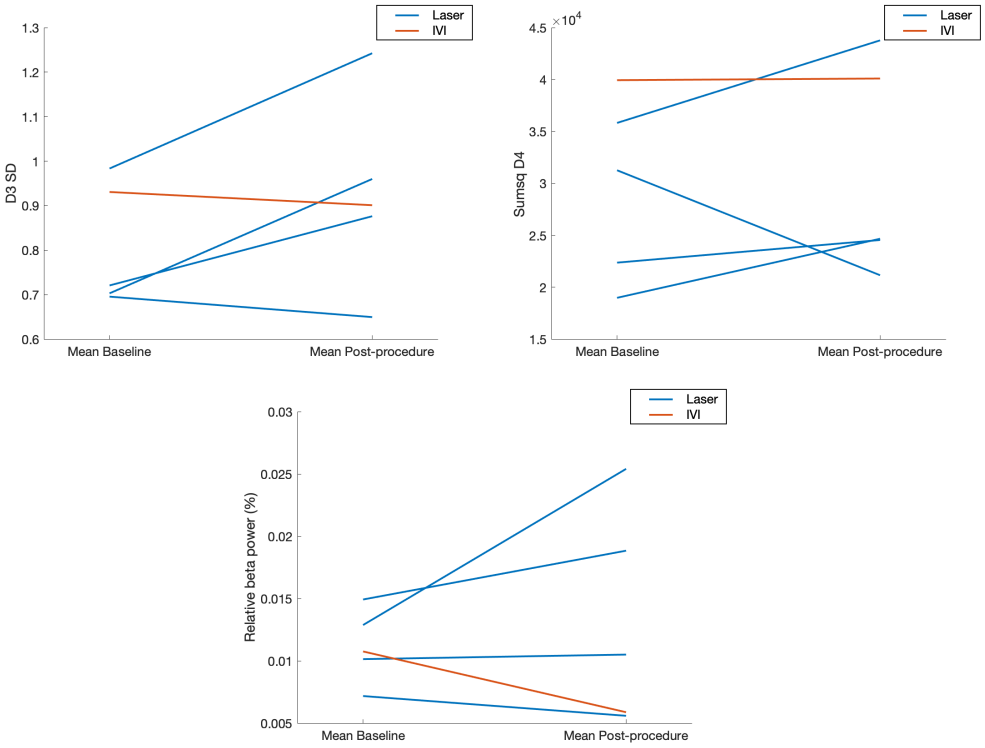


MSE 1-10 = multiscale entropy scales 1 to 10; MSE max = maximum multiscale entropy; MSE AUC = area under multiscale curve

Figure 5.7: Measures of baseline complexity in infants who had received medication prior to ROP treatment compared to age-matched non-medicated infants prior to BIO ROP screening. Bar and asterisk indicate significant difference ($p < 0.05$).

5.3.6 Treatment for ROP screening may not evoke noxious-related brain activity

The increase in three frequency-domain features of EEG was calculated in 5 infants following treatment for ROP. There was no significant increase in standard deviation of level 3 detail coefficients (p = 0.11), sum of squared level 4 detail coefficients (p = 0.11), or relative beta power (p = 0.24). The change from baseline is shown in Figure 5.8 for each feature.



D3 SD = standard deviation of level 3 detail coefficients; Sumsq D4 = sum of squared level 4 detail coefficients.

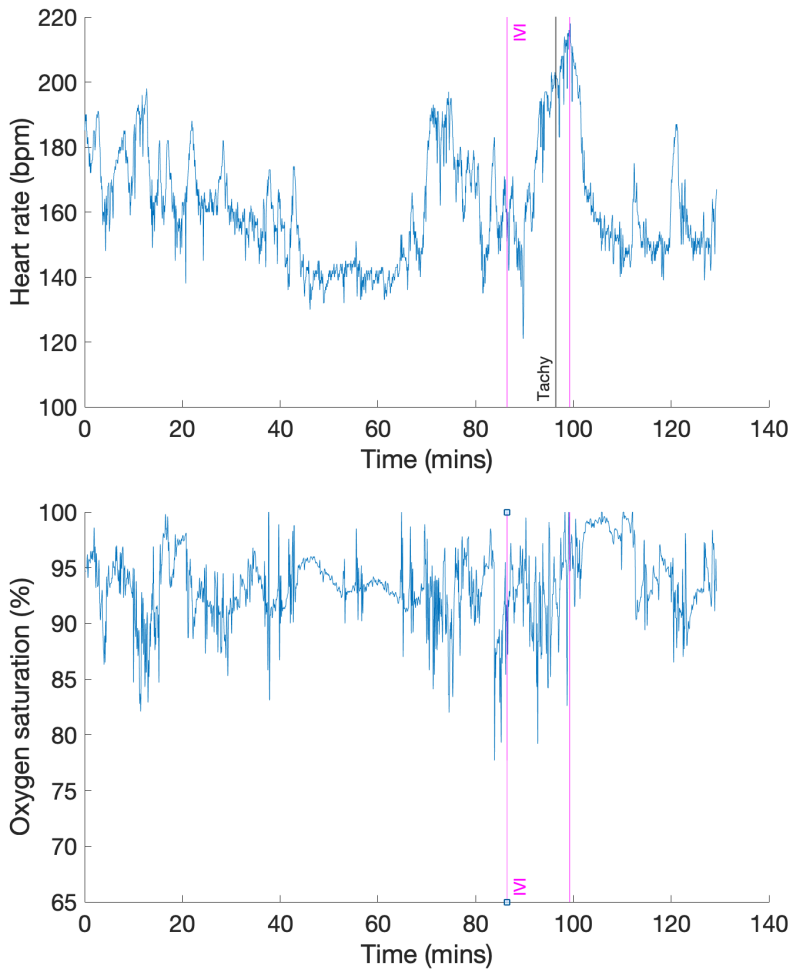
Figure 5.8: Change from baseline for three EEG features following ROP treatment. Infants were treated with either laser (n = 4) or intravitreal injection (n = 1).

5.3.7 Characterisation of physiological changes and instability events during ROP treatment

The HR and oxygen saturations for each subject undergoing ROP treatment are shown in Figures 5.9 to 5.13.

Intravitreal injection (Figure 5.9)

The subject was 45+0 weeks corrected gestational age at study and was spontaneously ventilating in room air. The HR demonstrated a gradual decrease during the initial 60 minutes which may correspond to the onset of medication effect (given before the start of the recording). Intravitreal injection was associated with an increase in HR and one tachycardia; the HR rapidly decreased after the procedure. There was one transient decrease in oxygen desaturation prior to the start of the procedure. Oxygen saturations remained within baseline levels during the intravitreal injection, then increased following the procedure.



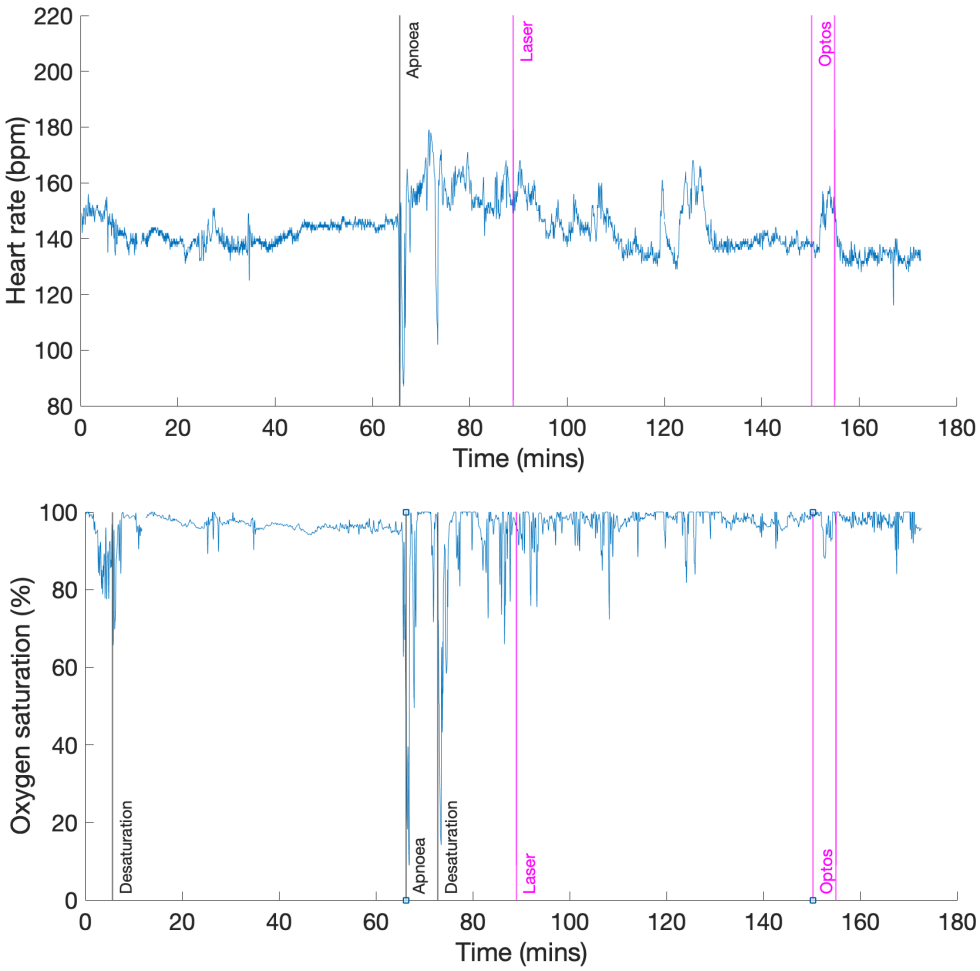
Procedure start indicated by line labelled IVI (intravitreal injection); procedure end indicated by second pink line. Tachy = tachycardia.

Figure 5.9: Physiological changes during intravitreal injection in a single infant

Laser treatment

Participant 1 (Figure 5.10)

The subject was 36+0 weeks corrected gestational age at study and was receiving high flow oxygen therapy. There was one apnoea event within 30 minutes of the start of the procedure, which may correspond to the onset of action of chloral hydrate. Laser treatment was not associated with an increase in HR; Optos examination following treatment was associated with a small HR rise. There was one oxygen desaturation at the start of the recording not associated with bradycardia. Oxygen saturations remained within baseline levels during the procedure.

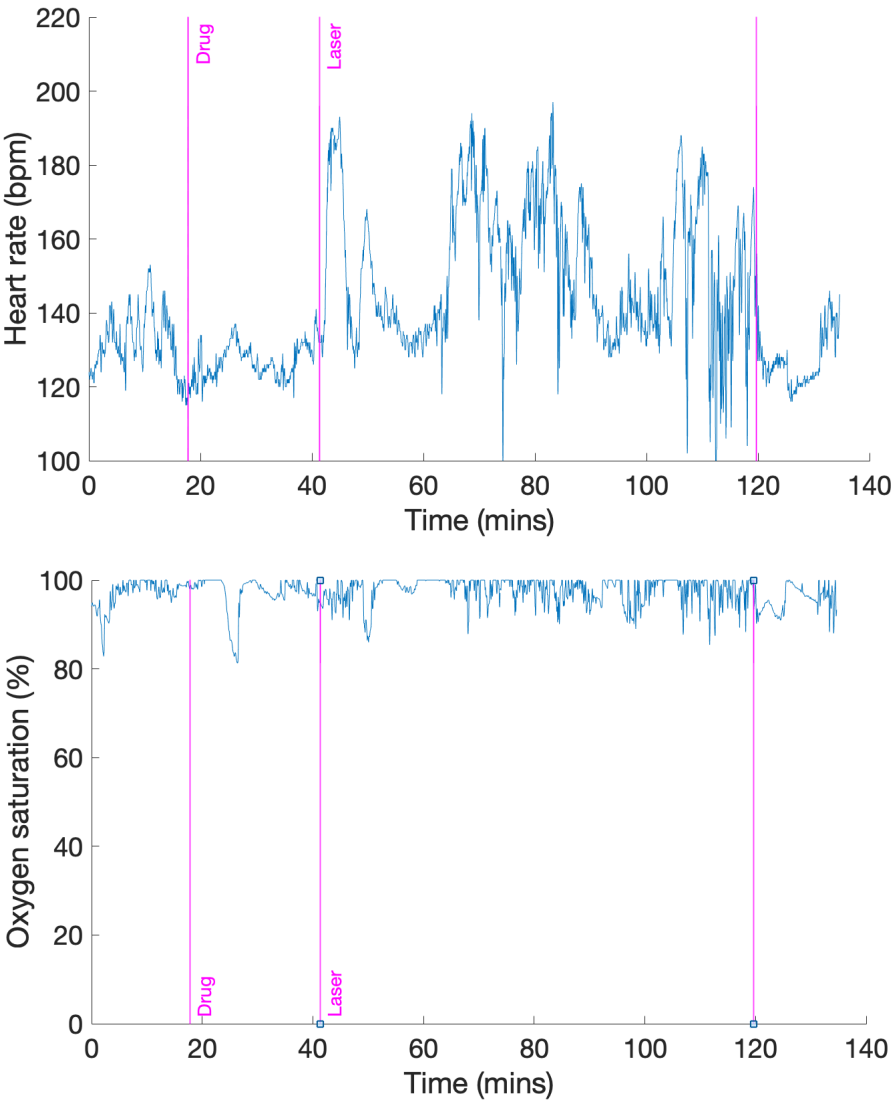


Procedure start indicated by line labelled Laser; Optos imaging indicated by line labelled Optos; procedure end indicated by third pink line. Instability events indicated by labelled black lines.

Figure 5.10: Physiological changes during laser treatment for Participant 1

Participant 2 (Figure 5.11)

The subject was 37+6 weeks corrected gestational age at study and was receiving high flow oxygen therapy. There was no change in HR following drug administration. Laser treatment was associated with an increase in HR; the HR rapidly decreased after the procedure. There was one small decrease in oxygen saturation following drug administration; oxygen saturations remained within baseline levels during the laser procedure.

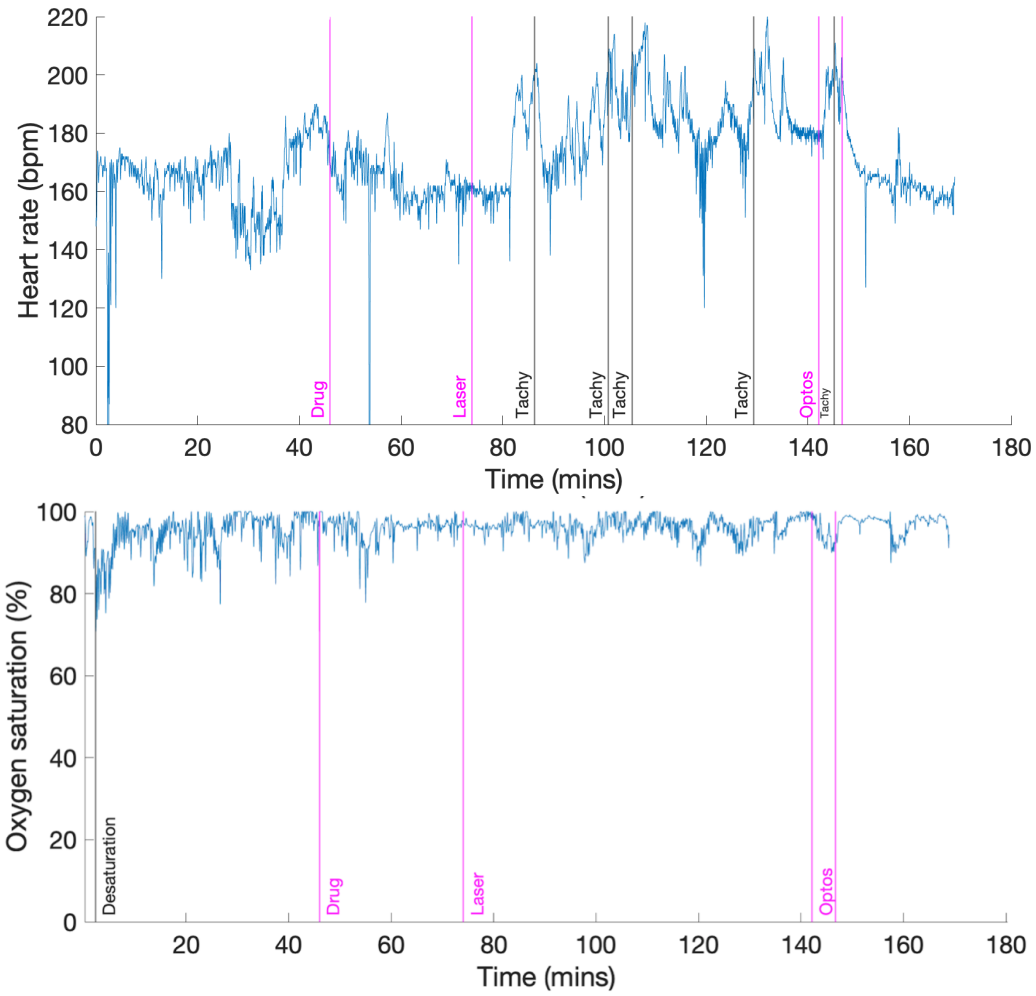


Medication administration indicated by line labelled Drug; procedure start indicated by line labelled Laser; procedure end indicated by third pink line.

Figure 5.11: Physiological changes during laser treatment for Participant 2

Participant 3 (Figure 5.12)

The subject was 39+1 weeks corrected gestational age at study and was receiving low flow oxygen therapy. Following drug administration, there was a gradual decrease in HR. Laser treatment was associated with an increase in HR and four tachycardias; Optos examination following treatment also was associated with a tachycardia. The HR steadily decreased after the procedure. There was one oxygen desaturation before drug administration. Oxygen saturations remained within baseline levels during the laser procedure and Optos examination.

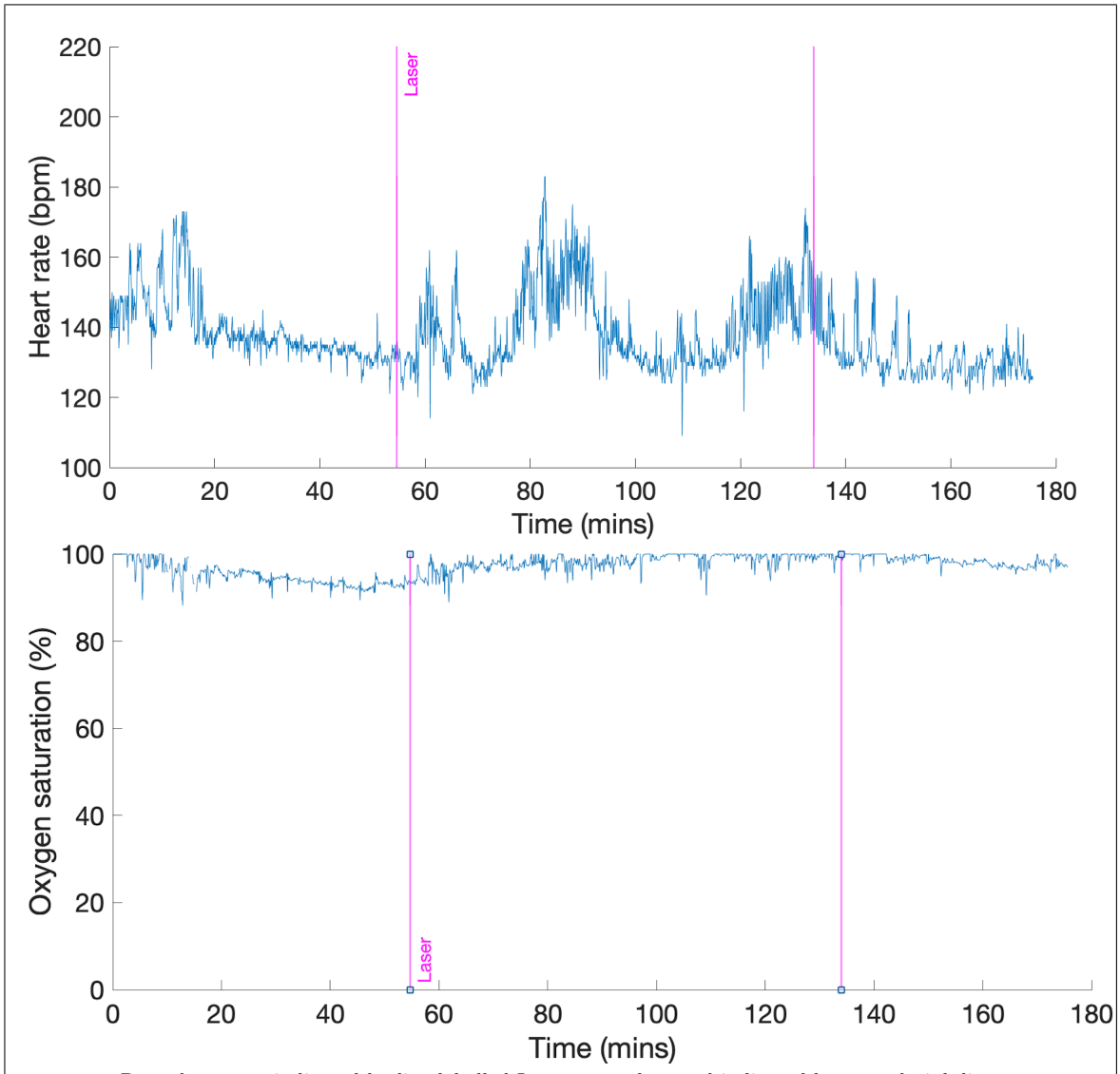


Medication administration indicated by line labelled Drug; procedure start indicated by line labelled Laser; Optos imaging indicated by line labelled Optos; procedure end indicated by fourth pink line. Instability events indicated by labelled black lines. Tachy = tachycardia.

Figure 5.12: Physiological changes during laser treatment for Participant 3

Participant 4 (Figure 5.13)

The subject was 40+1 weeks corrected gestational age at study and was receiving low flow oxygen therapy. The HR demonstrated a gradual decrease from 20 minutes which may correspond to the onset of medication action (given before the start of the recording). Laser treatment was associated with moderate increases in HR; the HR rapidly decreased after the procedure. There was a gradual modest decrease in oxygen saturation prior to the procedure; during laser treatment saturations remained within baseline levels.



Procedure start indicated by line labelled Laser; procedure end indicated by second pink line.

Figure 5.13: Physiological changes during laser treatment for Participant 4

There was a significant difference between percentage change in HR following ROP treatment and following BIO ROP screening (age-matched subgroup) (paired sample two-tailed t-test, p = 0.031; Figure 5.14). There was a trend toward a difference between percentage change in oxygen saturation following ROP treatment and following BIO ROP screening (age-matched subgroup) (paired sample two-tailed t-test, p = 0.084; Figure 5.14).There was a trend toward a higher PIPP-R score in the BIO ROP screening subgroup than in the treatment group (paired sample one-tailed t-test; p = 0.074), as shown in Figure 5.15.

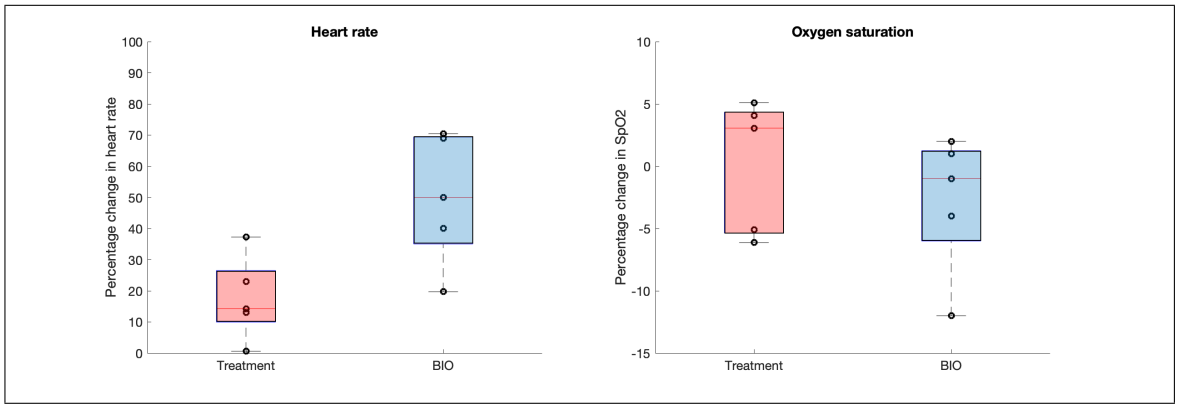


Figure 5.14: Percentage change in physiological parameters in 5 infants following ROP treatment, and in an age-matched group of 5 infants undergoing BIO ROP screening

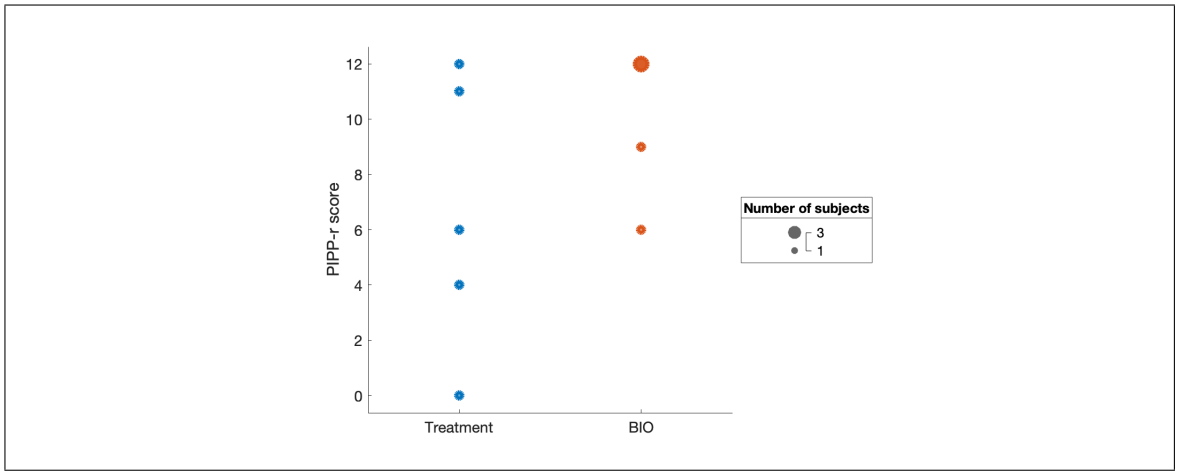


Figure 5.15: PIPP-R scores in 5 infants following ROP treatment, and in an age-matched group of 5 infants following BIO ROP screening

5.3.8 ROP treatment may not evoke stress-related parasympathetic activity

Two measures of HRV - pNN50 and absolute high frequency power - were calculated in the baseline and post-procedure recordings using an epoch of 3.5 minutes, based on the timing of the post-procedure reduction in parasympathetic activity identified in Chapter 4.. There was a trend toward a reduction in pNN50 following ROP treatment (paired sample one-tailed t-test; p = 0.15; Figure 5.16); there was no reduction in high frequency activity (paired sample one-tailed t-test; p = 0.53; Figure 5.16).

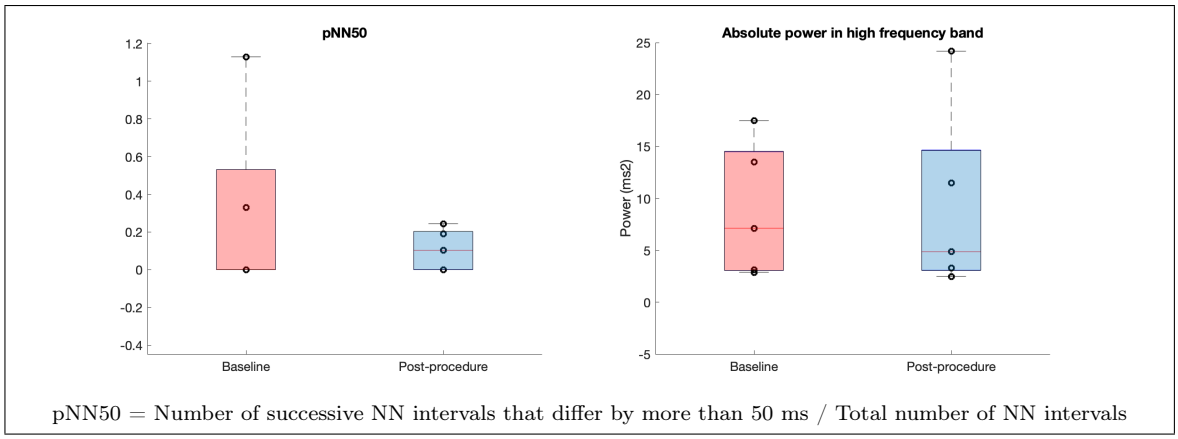


Figure 5.16: Heart rate variability measures reflecting parasympathetic activity before and after ROP treatment

5.4 Discussion

5.4.1 Overview

The first study of this Chapter demonstrates an approach to evaluating the infant response to Optos imaging, an alternative method of ROP screening to BIO screening. The findings from Study 1 were consistent with the hypothesis that Optos ROP screening is less stressful and painful than BIO ROP screening.

An approach to characterising the infant response to treatment for ROP is presented in the second study of this Chapter. The findings from Study 2 were consistent

with the hypothesis that oral sedation and analgesia reduce brain activity complexity, and reduce pain and stress from ROP treatment. However, more data are required to draw firm conclusions from the results presented in this Chapter due to the small sample size.

5.4.2 Optos ROP screening may be less painful and stressful than BIO ROP screening

Unlike BIO ROP screening, Optos screening does not involve stimuli such as bright retinal illumination, scleral indentation, or contact with the eye, which may be stressful or painful for infants. In view of this, I hypothesised that Optos ROP screening is less stressful and painful than BIO ROP screening.

In Chapter 3, it was observed that BIO ROP screening evokes an increase in higher frequency brain activity (12 - 30 Hz), which may be a surrogate measure of the infant pain response. Specifically, three frequency-domain features of EEG - relative beta power, standard deviation of level 3 detail coefficients, and sum of squared level 4 detail coefficients - increased following BIO ROP screening, of which increase in relative beta power was observed to be noxious-related.

In this Chapter, EEG of infants undergoing Optos ROP screening was analysed: firstly, no significant increase in relative beta power was identified after the procedure. This finding may suggest that Optos ROP screening does not evoke noxious-related brain activity in infants. Secondly, a significant increase in standard deviation of level 3 detail coefficients was observed, which reflects an increase in beta amplitude, and a significant increase in sum of squared level 4 detail coefficients was observed, which reflects an increase in frequency range 7.8 - 31.3 Hz (comprising alpha and beta bands).

A hypothesis for interpreting these findings is that the increase in sum of squared level 4 detail coefficients in this study may be alpha-mediated. As discussed previously, during attention to a stimulus, alpha activity increases in cortical regions

under active suppression. Differences in the Optos screening method compared to BIO ROP screening, including physical handling (lifting from the cot), may result in different patterns of arousal-related brain activity. An alternative hypothesis is that increase in these two features may be evoked by a non-noxious stimulus common to both procedures, for example, eye drop administration, eyelid speculum insertion, or auditory stimulation from clinician's voices during the procedure. Further investigation is required to better understand the change in standard deviation of level 3 detail coefficients and sum of squared level 4 detail coefficients following Optos ROP screening.

In Chapter 4, it was observed that BIO ROP screening evokes an increase in HR, and an increase in physiological stress. Specifically, there was a reduction in measures of parasympathetic activity (pNN50 and absolute high frequency power) reflecting the physiological stress state. In this Chapter, a significant increase in HR was not identified following Optos ROP screening, and no significant decrease in pNN50 or high frequency activity was identified. This finding may suggest that Optos ROP screening does not evoke stress-related parasympathetic changes in infants, however the study was underpowered to detect this given the small sample size. The trend toward lower clinical pain scores in infants undergoing Optos ROP screening compared to BIO ROP screening supports the hypothesis that Optos screening is less painful and stressful than BIO ROP screening, since pain scores are a surrogate marker of the infant pain and stress response.

5.4.3 Oral sedation and analgesia may reduce brain activity complexity, and may reduce pain and stress from ROP treatment

Infants receive oral analgesia and sedation prior to ROP treatment; I hypothesised that these medications would reduce EEG complexity. Analysis of background EEG

of infants who had received medication prior to ROP treatment identified significantly lower complexity, as quantified by multi-scale entropy, than the background EEG of non-medicated infants. This finding provides insight into the effect of oral analgesic and sedative medications on the infant brain.

I hypothesised that the medications given prior to ROP treatment reduce pain and stress associated with the procedure. Again, the findings from Chapter 3 and 4 were used to investigate this hypothesis. In this Chapter, EEG of infants undergoing ROP treatment was analysed, and no significant increase in relative beta power, standard deviation of level 3 detail coefficients, or sum of squared level 4 detail coefficients was identified after the procedure. This finding may suggest that ROP treatment with pre-medication of oral analgesia and sedation does not evoke noxious-related brain activity in infants, however the small sample size means the study was underpowered to detect this.

Heart rate variability analysis identified no significant decrease in pNN50, and no reduction in high frequency activity, following ROP treatment. This finding may suggest that ROP treatment with pre-medication of oral analgesia and sedation does not evoke stress-related parasympathetic changes in infant, however as stated previously the study was underpowered to detect this. There was greater increase in HR, and a trend toward higher PIPP-R score, following BIO ROP screening than following ROP treatment. This finding supports the hypothesis that medications reduce pain and stress associated with ROP treatment.

Prior to laser treatment, the youngest subject (corrected gestational age at study 36+0) had an apnoeic episode after receiving medication. Apnoea may occur as a sign of chloral hydrate toxicity (Jacqz-Aigrain and Burtin, 1996). While chloral hydrate sedation is considered to be safe for a single dose in the range 25 - 50 mg/kg (Jacqz-Aigrain and Burtin, 1996; Litman *et al*, 2010), the active metabolite trichloroethanol has an extended half-life in neonates, and may accumulate in premature infants with hyperbilirubinaemia, or compromised liver or kidney function (Mayers *et al*, 1992;

Jacqz-Aigrain and Burtin, 1996). The affected infant had a history of jaundice and anaemia requiring transfusion, and therefore may have been at risk of trichloroethanol accumulation. There were no episodes of bradycardia in infants receiving treatment for ROP, which may occur as a sign of morphine toxicity (Jacqz-Aigrain and Burtin, 1996; Hartley *et al*, 2018).

5.4.4 Strengths and Limitations

This Chapter presents a preliminary investigation of important clinical questions, namely characterising the infant experience of Optos ROP screening and ROP treatments. Detailed analysis of infant brain activity, physiology and behaviour allow a multimodal approach toward better understanding the effect of these essential procedures on preterm and ex-preterm infants. The findings from Study 1 suggest that Optos ROP screening is well-tolerated in eligible infants and may be a less painful and stressful screening modality compared to BIO ROP screening. Further investigation with a larger sample is needed to confirm the findings from this preliminary study. The findings of the study may not be generalisable to all infants requiring ROP screening. As discussed in the introduction, Optos ROP screening is avoided in infants for whom handling is not considered safe by the clinical team. Where possible, infants with a range of ventilatory support, including CPAP and intubation, should be included in future studies of Optos ROP screening, because clinicians would value evidence as to the feasibility and safety of Optos ROP screening in such infants.

The results from Study 2 suggest that oral analgesia and sedation reduce pain and stress associated with ROP treatment. The individual effect of each systemic medication - chloral hydrate, morphine, paracetamol - have not been elucidated, and the findings relate to the combined effect of the medications. Local anaesthetic (sub-Tenon's injection and eye drops) may also have a contributory role in modifying the infant response to ROP treatment, although evidence suggests that when used individually, local strategies provide inadequate pain relief during ROP treatment. Based

on the study sample, the safety of chloral hydrate as a single oral dose appears to depend on the degree of prematurity and presence of comorbidities, which is consistent with previous studies. There were no adverse effects from oral morphine in this sample, however, oral morphine has previously been identified to cause physiological instability in non-ventilated preterm infants (Hartley *et al*, 2018). Further investigation with a larger sample is required to confirm the observations from this study.

Heart rate variability analysis in this Chapter was limited by the small sample size - a larger sample of subjects studied during Optos screening and ROP treatment would have enabled subjects to be grouped into low- and high baseline HRV clusters, as for Chapter 4, before age-matching to subjects undergoing BIO ROP screening within the same baseline HRV cluster.

The recruitment of infants undergoing ROP treatment was challenging prior to the outbreak of the coronavirus pandemic. Recruitment was necessarily opportunistic, because development of treatment-indicated ROP is unpredictable. Infants requiring treatment for ROP are often transferred from other centres, and may not travel with the parents or caregiver. In this scenario, consent could not be obtained for participation in research because telephone consenting was not covered by the study ethical approval. Treatment for ROP must be delivered within 72 hours of a decision to treat, and therefore the process of approach and consent was subject to time constraints.

In this study, laser and intravitreal injection treatments were analysed together due to the small sample size, despite procedural differences between the two treatments including differences in medication. A larger sample of subjects undergoing ROP treatment would enable analysis of laser and intravitreal injection treatments separately. This would be of interest to clinicians who would value characterisation of the infant response to laser and intravitreal injection treatments separately.

Age-matched EEG analysis of subjects undergoing ROP treatment to subjects undergoing BIO ROP screening was not possible, as for Optos ROP screening, because

infants receiving treatment were older than the BIO ROP screening cohort.

EEG analysis was performed using a single electrode - Cz - in order to maintain a consistent analysis approach with Chapter 3 and previous studies examining the effect of nociception and analgesia. However, the effect of sedative medication may be better characterised using multiple electrodes and further investigation is required to test this approach.

5.4.5 Future Directions

The findings from this Chapter suggest that Optos ROP screening may be a less painful and stressful method compared to BIO ROP screening. Optos imaging is not as widely used for ROP screening as other methods such as RetCam (Clarity Medical Systems, Pleasanton, USA). RetCam is digital widefield imaging system in which a probe is placed in contact with the eye using a coupling gel. It is a popular method of ROP screening because it does not require specialist skill to acquire images and can be used by non-ophthalmologists. However, the field of view requires several images to be obtained per examination to create a composite of the entire fundus. For this reason, RetCam image acquisition takes longer than BIO ROP screening and Optos ROP screening. Table 5.3 compares RetCam screening with BIO and Optos ROP screening.

Table 5.3: Comparison of screening methods for retinopathy of prematurity

	BIO	RetCam	Optos
Field of view	30º	120º	200º
Handling / restraint	✓	✓	✓
Eyelid speculum	✓	✓*	✓*
Eye contact	✓	✓	
Bright retinal illumination	✓		

*Use of an eyelid speculum is optional with RetCam and Optos methods.
BIO = binocular indirect ophthalmoscopy; RetCam = contact widefield photography; Optos = non-contact ultra-widefield photography.

Previous studies have compared the infant response to RetCam imaging with BIO ROP screening using physiological and behavioural measures. RetCam imaging was associated with lower clinical pain scores than BIO ROP screening in a study of 24 infants (Moral-Pumarega *et al*, 2012), however no difference in clinical pain score was identified between the two methods in a separate study of 52 infants (Dhaliwal *et al*, 2010). One study observed more oxygen desaturations with RetCam than BIO screening, and the authors' opinion was that RetCam was more distressing for infants than BIO ROP screening (Mehta *et al*, 2005). The method and analysis approaches demonstrated in this Chapter could be used to characterise the infant response to RetCam ROP screening, and thereby aid clinicians better understand how the established screening approach of RetCam compares to the 'gold standard' of BIO ROP screening, and the promising alternative approach of Optos imaging.

The findings from this Chapter suggest that oral analgesia and sedation may reduce pain and stress from ROP treatment. Further investigation in a larger cohort is required to confirm the robustness of this observation. Effective oral analgesia and sedation for ROP treatment has the potential to allow treatment delivery without the need for invasive ventilation. Advantages of this include reduced risk of prolonged

intubation post-procedure in infants with chronic lung disease, and improved availability of timely ROP treatment delivery in settings where paediatric anaesthetic support is not available. Timely treatment for ROP is essential in achieving good structural and visual outcomes for infants, and in preventing childhood blindness from ROP.

5.5 Conclusions

This Chapter suggests that Optos ROP screening may be a less painful and stressful method of ROP screening compared to BIO ROP screening, and that oral sedation and analgesia may reduce pain and stress associated with ROP treatment. These exciting preliminary findings warrant further investigation in a larger cohort, and if robust, have the potential to improve the infant experience of ROP screening and treatment.

Chapter 6

General Discussion

6.1 Thesis Summary

Quantitative EEG analysis was used to characterise the effect of BIO ROP screening on infant brain activity, and to evaluate noxious-related change in brain activity

- BIO ROP screening evokes a significant increase in higher frequency brain activity (12 - 30 Hz) in preterm infants.
- Increase in relative beta power may be a measure of nociception in preterm infants, since this change was identified following different noxious procedures (BIO ROP screening, heel lancing) and was not identified following a non-noxious control procedure (nappy change). Moreover, the change in relative beta power was significantly correlated to the magnitude of the noxious-related ERP evoked by heel lancing, a previously-defined measure of nociception in preterm infants.

Physiological recordings were used to characterise the effect of BIO ROP screening on infant physiology, and to evaluate stress-related change in heart rate variability

- Infant heart rate increased significantly during BIO ROP screening with rapid recovery post-procedure. No significant change in oxygen saturations was identified during or after BIO ROP screening. No significant change in instability events was identified following BIO ROP screening.
- A reduction in HRV measures of parasympathetic nervous system activity was observed following BIO ROP screening. This indicates that BIO ROP screening evokes a physiological stress response in preterm infants.

Quantitative EEG analysis and physiological recordings were used to characterise the effect of Optos ROP screening and ROP treatment on infant brain activity and physiology

- The effects of Optos ROP screening and ROP treatment were investigated in a small cohort of infants; the sample size was too small to identify statistical significance.
- Optos ROP screening may not evoke noxious-related brain activity, nor stress-related parasympathetic nervous system activity. Clinical pain score may be lower following Optos ROP screening than BIO ROP screening. These findings were consistent with the hypothesis that Optos screening may be less painful and stressful than BIO ROP screening.
- Medication prior to ROP treatment (oral analgesia, oral sedation, and local anaesthesia) may reduce brain activity complexity. Noxious-related brain activity and stress-related parasympathetic nervous system activity were not identified following ROP treatment in infants who received pre-medication. Clinical pain score may be lower following ROP treatment than BIO ROP screening. These

findings were consistent with the hypothesis that pre-medication for ROP treatment may be effective in reducing pain and stress related to the procedure.

The following sections of this Chapter will discuss various aspects of the research presented in this thesis. Firstly, the topic of recruitment in an infant population will be addressed. Secondly, aspects of the methodology used in this thesis will be examined, including the single-centre design, and practical considerations in recording physiology and brain activity. Limitations of the research, including regarding analysis approaches used in this thesis, will be considered. Finally there will be a general discussion of the results interpretation and future directions for further research.

6.2 Recruitment

Recruitment in the NICU setting is a challenge. From the perspective of the clinical team caring for the infant in the NICU, the key priority is to protect the infant from adverse experiences and maintain access to the continuous care and support required. In terms of BIO ROP screening, this procedure is unpopular with NICU staff who are by the bedside during the examination. They have first-hand experience of the infant's behavioural changes during screening, such as crying and body tension, which intuitively indicate distress or stress to the observer. Additionally, the bedside staff were familiar with tachycardia events during the procedure, and risk of oxygen desaturation and apnoea afterward. For these reasons, clinical staff were sometimes reluctant to allow researchers to approach parents for consent for research studies during ROP screening, appearing to consider the research study an extra burden on their fragile patients during an already challenging procedure.

Parents of infants admitted to the NICU endure an extremely stressful life event, in which their infant is born or delivered at an unexpected time. Moreover, their infant requires emergency resuscitation to preserve life, and is immediately cared for in a highly medicalised environment. The infant's physical vulnerability often means

that parental contact with their newborn is limited by environmental factors such as the incubator, intravenous lines, and monitoring wires. Additionally, the need for infection control and temperature control contribute to minimised touching of their infant. As a consequence of the above, parents may be hesitant to consent to a research team handling their infant.

ROP screening takes place at 4 to 5 weeks post birth, and is repeated at fortnightly intervals for the majority of infants. Parents were often reluctant to consent for their infant to participate in research during the infant's first ROP screen, intimating that they would like to wait and see how their infant responded to the examination without the addition of the research study. Parents appeared reassured to know that a study would not take place (despite consent) if at the time of the study, the infant appeared clinically unstable. In general, parents did not wish to expose their infants to additional challenges at an already difficult time.

However, in the NICU there were several research trials underway. For this reason, the idea of participating in research was familiar to parents of infants admitted to the NICU. Parents were often aware that the care their infants received was built on a foundation of research, and that by participating in research they felt able to give something back to the NICU unit which was supporting their infant. Parents were emotionally invested in contributing to better care and better understanding of the infant experience of preterm birth by consenting to their infant's participation in research.

From the researcher's perspective, succeeding in gaining consent for an infant to participate in research required several conversations. As a first step, the list of all patients admitted to the NICU was required, in order to check which infants were due eye screening. This list contained confidential patient information and therefore access to this list required smart-card access to the NICU and discussion with the NICU team to obtain the list. Subsequently the medical notes of potential study participants were reviewed; again this was confidential information and therefore

NICU staff would require the researcher to identify themselves and explain why the notes were required before allowing access. Next, if an infant was identified as eligible, the member of the clinical team caring for the infant at the bedside was approached by the researcher to check if it would be appropriate to consider recruitment for a study taking place at the next ROP screening, which occurred weekly. This conversation was very useful in gaining the trust of the bedside member of staff, who had a key gate-keeper role in terms of access to the patient and parents. It was advantageous once the nursing team were familiar with the members of the research team, and the broad details of the research studies undertaken. Next, the researcher needed to attend the NICU at the time at which the parents usually visited their infant. Since time between parent and infant should be protected, liaison with the bedside staff was needed to identify the appropriate time to approach parents during their visit. If parents had received difficult news, or the infant was navigating a challenging period, it would not be a suitable time to suggest participation to parents who might be feeling distressed or stressed. In discussing the research study with parents, it was important to introduce oneself clearly and be candid that participation (or not) in research did not affect the clinical care of their infant. Parents were given an information leaflet and time to reflect before signing their consent. After consent was signed, it was important to be mindful that the infant's clinical condition could deteriorate during the time between obtaining consent and the next weekly ROP examination. Thus each study participant in the research presented in this thesis was the result of multiple interactions with the care team and parents.

In the author's case, being both a member of the ophthalmology team and the lead researcher in the studies presented in this thesis required particular care when communicating with parents of infants in the NICU. It was important to separate the roles where possible, to avoid parents feeling pressure to consent to participation and to safe-guard the patient-doctor relationship. To this end, information regarding the procedure of ROP screening and treatment was given first, and at a separate occasion

to the approach regarding research participation. The duality of the author's role was most challenging during ROP treatment. ROP treatment must be performed within 72 hours of diagnosis of treatment-indicated ROP, hence the time-frame was shorter and therefore there was less time between discussions with parents.

Since Oxford is a centre of ROP expertise, in many cases infants were transferred for treatment from other hospitals in the country. Transfer involves the infant arriving by ambulance transport, with parents arriving separately by private transport. Thus, parents may be unable to attend if they do not have access to transport, or if they have responsibility for care of other children, or they may plan to arrive after the treatment has taken place. Consent for ROP treatment could be obtained by telephone consultation, whereas consent for research participation had to be face-to-face due to the stipulations of the research study ethical approvals. Thus it was not possible to recruit all patients who were received ROP treatment during the research period.

The NICU population are vulnerable to infection, which can have devastating consequences such as permanent disability or death. During the period of recruitment there were two infectious outbreaks in the NICU with unknown source. During these outbreaks, research studies were not permitted on the Unit, in order to minimise access to infants and thereby reduce risk of infection. After discussion with Infection Control, it was determined that the procedures used by the research team for cleaning equipment after studies were appropriate, and no additional measures needed to be adopted.

6.3 Methodology

6.3.1 Single-centre design

The ROP Service at the John Radcliffe Hospital is a national referral centre, and as such, local patient investigation and management may differ from other centres

in the UK due to patient complexity and caseload. Firstly, regarding screening: as mentioned in Chapter 3, insertion of an eyelid speculum and indentation with a scleral depressor are standard practice in our centre, but may not always be included in the BIO ROP examination in other centres. Although BIO examination is considered the gold-standard examination method and is standard practice in our centre, it is not a universally performed screening method; examination of the fundus with a contact fundal imaging device termed the RetCam is another commonly-used method of ROP screening. Optos imaging is a seldom-performed method of ROP screening. Reasons for this include the cost of the imaging system, its portability to the NICU from the Ophthalmology department, and practitioner reluctance to handle and lift preterm infants toward the camera - including both ophthalmologists and NICU clinical staff.

Secondly, regarding ROP treatment: locally, ROP treatment is performed under oral sedation, oral analgesia and local anaesthesia; in other centres, ROP treatment is performed using intubation and sedation, or general anaesthesia. The infants included in this study received standard-of-care management, and no clinical practice was altered in order to perform the study. Oral morphine was included in the ROP laser treatment protocol until the results of the Poppi trial were published, which advised that it should not be used for procedural pain due to potential for harm without evidence of analgesic efficacy. Following the Poppi trial results, oral morphine was given on as case-by-case basis to infants who were considered appropriate by the NICU clinical team.

In view of these considerations, clinicians may therefore question whether BIO ROP examination without scleral indentation or eyelid speculum insertion, or a different method of ROP screening such as RetCam, may result in the a different pattern of brain activity and autonomic nervous system activity than that observed in this thesis. Similarly, clinicians may be interested to understand whether infant physiological responses and brain activity, including measures of complexity, following ROP treatment are influenced by different peri-operative pain-relief strategies, such as in-

tubation and sedation, or general anaesthesia. Further research including multiple centres is required to address these important clinical questions.

6.3.2 Practical considerations in data recording

As described in Chapter 2, the study design compared periods of EEG recording before and after the clinical procedure. During the procedure there was significant EEG artefact, especially motion artefact.

The method used for recording EEG in this thesis involved eight recording electrodes which were applied manually to each infant by the author, a trained member of the research team, or a research electrophysiologist. A similar EEG montage has been used in previous studies of infant brain activity (Hartley *et al*, 2015; Green *et al*, 2019; Cobo *et al*, 2021). An advantage of the manual approach is that the electrodes were measured and sited precisely in accordance with the 10-20 system nomenclature. However, manually-affixed electrodes were vulnerable to becoming dislodged by infant movement or clinician touch during the procedure. More securely fixed electrodes may have allowed EEG recording to continue for longer before and after the procedure, without concern that the need to avoid disturbing the electrodes was interfering with routine care and parental access. Other groups have used an electrode cap to record EEG in infants aged 6 to 24 months (Bosl *et al*, 2011; De Klerk *et al*, 2015) which may limit electrode motion (Wallois *et al*, 2021) and moreover obviates the need for scalp preparation and electrode fixation with paste, tape or bandages.

Physiological recordings were obtained by exporting data from the patient monitor to a laptop at the bedside, as described in the General Methods. The majority of infants in the NICU have continuous physiological monitoring, generating large amounts of data per patient stay. However, with the method used in this thesis, recordings were often interrupted for practical reasons, such as relocating an infant to a different bed space. Automatic recording of physiological data during an infant's admission is performed in other centres (Poppe *et al*, 2020; den Boer *et al*, 2021),

which avoids the practical problems associated with a separate bedside recording device. Achieving a similar approach in the local unit would greatly increase capacity for physiological data collection.

6.4 Limitations

6.4.1 Analysis approaches

The characterisation of brain activity presented in this thesis was based on data from a single recording electrode, which was positioned at Cz and therefore considered to record activity from the primary somatosensory cortex. However, data from seven other electrodes were recorded during the studies, and could be used in future to characterise brain activity in other cortical regions associated with painful procedures. Given that pain is considered to arise from activity across the dynamic pain connectome, it may be advantageous to analyse data from a greater number of recording electrodes (Wallois *et al*, 2021). Detecting neural activity more widely across brain regions rather than in one locus may better characterise brain activity evoked by noxious stimulation (Gursul *et al*, 2019).

6.4.2 Baseline state identification

Infant sleep state was not determined from the EEG recordings, which were generally too short to capture an entire sleep-wake cycle (St Louis *et al*, 2016). The PIPP-R score baseline behavioural state provided information as to the infant's likely sleep state. However, as detailed in the General Methods, the EEG baseline period did not align precisely with the PIPP-R baseline, because the EEG baseline period was identified retrospectively as an artefact-free period during the EEG recorded prior to the procedure. Since sleep state markedly influences both EEG and heart rate (Grigg-Damberger, 2016; Dereymaeker *et al*, 2017), the study design could be improved by

identifying the infant behavioural state more precisely.

6.4.3 Sample size

Recruitment to the studies presented in this thesis was curtailed by the outbreak of the COVID-19 pandemic in February 2020. The recruitment of infants undergoing Optos screening was particularly affected, as all recruitment to the NICU was stopped during this phase of research. Clinicians' access to the Unit was maintained, but researchers were no longer allowed access in order to minimise the risk of introducing COVID-19 into the Unit. Parental access was also severely limited which would have affected the consenting process; parents of neighbouring infants in the Unit were given staggered visiting slots in order to maintain 2 metre distance between parents, and to reduce the total number of people in the Unit.

6.5 General Discussion

During NICU admission, infants undergo multiple painful clinically-necessary procedures daily. Pain during early life has deleterious consequences for the developing brain and neural pathways, therefore detecting pain in infants and delivering effective pain relief is essential. Identifying pain in a non-verbal population is challenging, hence surrogate measures of pain are required to estimate the pain experience in infants.

In this thesis, the clinical procedures of ROP screening and treatment are investigated using a multimodal approach to infant pain assessment. Particular attention is given to defining brain-derived responses and autonomic responses to pain and stress in infants. The aim of the research was to use quantitative analysis of EEG features to better understand the infant response to complex noxious stimulation, and to analyse heart rate variability to better understand the infant physiological stress response.

The research presented in this thesis contributes to better understanding of nociceptive processing in preterm infants: higher frequency brain activity (12 - 30 Hz) is ob-

served following noxious clinical procedures, and an approach toward defining a novel measure of nociception in infants is described. The work also adds to knowledge of infant physiological stress during eye screening: a reduction in HRV measures associated with parasympathetic nervous system activity is observed, indicative of an autonomic stress response. These findings have not been reported previously and therefore represent a novel contribution to the scientific field.

The thesis may inform future research and clinical practice: the shift to higher frequency brain activity associated with BIO ROP screening may be a marker of nociception in infants. It may represent a useful outcome measure to include in future investigations into infant pain, particularly pain associated with clinical procedures. The reduction in EEG complexity observed during pre-medication with oral sedation, oral analgesia and local anaesthesia may also represent a useful outcome measure in clinical research. Peri-procedural approaches to pain-relief could be evaluated in terms of the effect on EEG complexity, providing an indication of effectiveness and ultimately improving infants' experience of clinical procedures. The multimodal approach to pain assessment could be applied to a larger cohort to investigate which method of ROP screening is optimal and thereby inform clinical management decisions.

Regarding clinical practice, HRV measures of parasympathetic nervous system activity may be useful indicators of infant stress during eye screening. Automated heart rate variability monitoring is available in the NICU, and this research supports its implementation during ROP screening. Measurement of real-time parasympathetic activity in infants undergoing BIO ROP screening may improve NICU staff ability to monitor infants physiological stress during the procedure, and may create opportunity for caregivers to respond with comfort measures or analgesia in order to improve the infants experience of screening.

There are several directions in which the research could be developed in future. Firstly, it may be beneficial to standardise certain characteristics which are con-

sidered to influence pain sensitivity in infants. For example, prior exposure to painful experiences may influence infant responses to pain - previous studies suggest that prior pain may evoke increased brain activity (Slater *et al*, 2010) and reduced behavioural responses (Johnston and Stevens, 1996; Grunau *et al*, 2005; Morison *et al*, 2003) following a noxious stimulus. Since infants admitted to the NICU accumulate considerable pain experience, future studies of noxious-related responses from this population could be improved by accounting for previous pain.

An approach to accounting for individual pain sensitivity has been recently described (Cobo *et al*, 2021). The magnitude of brain activity evoked by an experimental pinprick stimulus has been used to predict sensitivity to heel lancing in infants, based on the observation that the magnitude of brain activity evoked by pinprick and heel lance is highly correlated. The analgesic effect of paracetamol was characterised by demonstrating that the magnitude of brain activity evoked by heel lancing with paracetamol treatment was lower than the predicted magnitude of brain activity to heel lancing without paracetamol. This paradigm was also used to demonstrate the analgesic effect of gentle stroking.

This approach could be applied to ROP screening procedures, if baseline sensitivity to an experimental pinprick were strongly correlated to the measures of brain activity identified in this thesis. The infant response to alternative methods of screening, such as Optos screening, could be compared to the predicted response to BIO ROP screening, using the baseline sensitivity to experimental pinprick. This experimental design would be advantageous in avoiding subjecting infants to both types of screening, as has been the case in previous comparisons of screening methods (Fung *et al*, 2018).

Another exciting direction would be to investigate the effect of parental voice as a potential non-pharmacological analgesic or comfort measure during ROP screening or treatment. Recently, maternal voice was demonstrated to significantly reduce clinical pain score and increase endogenous oxytocin during heel lancing in preterm

infants (Filippa *et al*, 2021). This intervention may be welcomed by parents of infants undergoing ROP screening in the NICU, who are encouraged to be involved with the care of their infant where possible.

Accounting for inter-individual variation in pain responses has been demonstrated to reduce the sample size required (Cobo *et al*, 2021). Using this paradigm, the robustness of the findings presented in this thesis could be confirmed by demonstrating repeatability, without the challenges of recruiting a larger cohort from the NICU population as discussed above.

An interesting future direction would be to develop an automated process by which EEG and ECG changes related to pain and stress could be identified. Feature extraction from a biological signal is often performed in order to facilitate machine learning. EEG features have been used to classify sleep state and detect seizure activity in infants (Dereymaeker *et al*, 2017; Koolen *et al*, 2017; Pillay *et al*, 2018; Pavel *et al*, 2020). Automated pain assessment has been attempted using EEG features in adults following experimental thermal noxious stimulation (Misra *et al*, 2017), and using HRV features in infants after surgery and after delivery (Butruille *et al*, 2015). The research presented in this thesis contributes to this field by characterising both EEG and HRV features related to nociception in infants, and by focussing on pain resulting from clinical procedures.

Combining EEG with near-infrared spectroscopy neuroimaging would be advantageous in future, firstly in order to improve data quality during the clinical procedure, since near-infrared spectroscopy is less vulnerable to motion artefact. In addition, using haemodynamic and electrophysiological neuroimaging methods may allow more comprehensive understanding of the changes that occur in the infant brain in response to nociception (Verriotis *et al*, 2016).

The results discussed in this thesis could be used as outcome measures to characterise the infant response to other noxious clinical procedures such as lumbar puncture or chest drain insertion. It would be interesting to investigate whether the infant re-

sponse to procedural pain demonstrates intensity encoding. Many clinical procedures performed in infants have a counterpart in adult medical care, and adult pain ratings could form the basis of an expected severity for a given procedure. Characterising the intensity of clinical procedures would allow improved understanding of the impact of frequently-performed procedures on the NICU population.

In future, the results presented in this thesis could be included as outcome measures in multimodal characterisations of different methods of ROP screening. It is of particular interest to ophthalmologists and NICU staff to better understand which screening method is optimal in terms of pain and stress experienced by the infant. A randomised controlled trial of BIO ROP screening, RetCam screening and Optos screening, for example, would answer important clinical questions regarding the impact of screening techniques on infants, and assist clinical decision-making. With increased survival of preterm infants, demand for ROP screening is likely to increase and outstrip the capacity of trained ophthalmologists (Brady *et al*, 2020). Therefore, 'telemedicine' screening methods that can be performed by non-specialists and which facilitate image-sharing will be increasingly adopted, rather than the BIO technique which requires specialist skill. It is essential to understand the impact of such methods on the infant population.

6.6 Concluding Remarks

To conclude, the research presented in this thesis contributes to understanding of the infant response to clinically-required noxious stimulation. Features of brain activity and heart rate variability related to procedural pain and stress have been characterised, which may be useful outcome measures in future investigations of infant nociception evoked by clinical procedures. The features identified may also be used in the development of automated pain detection using machine learning approaches. Improved diagnosis of pain in infants may lead to better evaluation of pain-relief

strategies and thereby improved treatment of infant pain. Improved treatment of infant pain is critical in order to avoid the negative effects of early life pain arising from clinically-necessary procedures performed in the NICU.

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Appendix A

Patient Information Leaflet

Investigating pain in the developing human brain

Parent Information Leaflet

DEPARTMENT OF PAEDIATRICS
Medical Sciences Division

Oxford University Hospitals NHS
NHS Foundation Trust

Your child is, or may be, eligible to take part in a research study. Before you decide, it is important that you understand why the research is being done and what it involves. Please read the following information carefully and ask us if anything is unclear or if you would like more information.

1. **Study title:** Investigating pain in the developing human brain

2. What is the purpose of the study?
Infants in hospital often need to have many procedures as part of their routine medical treatment, which may cause discomfort. As they cannot tell us how much these procedures hurt, it is difficult to know how much pain they are feeling and to make sure that they receive the right medicines. We know that infants can process discomfort and pain in their brains and we have developed a method of assessing this pain-related brain activity. We also know that infants show that they are in pain using different behaviours. These may be indicated by changes in heart rate and breathing rate in response to pain.

The aim of this research is to understand more about how infants experience pain, so that better ways of treating pain can be developed. We are also interested in how infants respond to different stimuli from their environment, such as light and sound, and how this might change across development.

3. Does my child have to take part?
No, it is your decision whether or not your child takes part. If you decide to allow your child to take part, you will be asked to sign a consent form. If you decide you do not want your child to take part, this will not affect your child's care.

If you decide you would like your child to take part, you can change your mind at any time and withdraw your child from the study by telling the research team. You do not have to give a reason. You will be asked if we can use the data/images that have already been collected for analysis (all data) and for publication of results (anonymised data only).

4. What is involved in the study?
In this study we would like to understand how infants respond to clinically-required procedures, such as blood tests. **No clinical procedures will be carried out solely for research purposes.** We will only study your child during a procedure that is needed for clinical purposes.

As we are interested in how infants respond to different stimuli in their environment, we may also ask to study your baby during a control procedure and in response to sharp touch. These do not pierce the skin, but they stimulate the receptors that we are interested in generally without waking or upsetting the infant.

We will assess your child's response by measuring their activity. We may also video your child's face, and measure other responses such as muscle activity, heart rate and oxygen saturations. We will monitor your child before, during and after the clinical procedure. On rare occasions we may ask to monitor your child for up to 24 hours before and/or after the clinical procedure.

Clinical procedures will be completed in the routine way. The study will not interfere with your child's clinical care, nor will there be any delay if an emergency procedure is required. We may also explore the impact of pain relief and comfort measures on your child and may ask you to complete a questionnaire following the study.

As we are interested in how your child's response to pain changes as they grow, we may ask if we can study your child more than once during their stay in hospital. We will also ask you if we can contact you in the future, to ask if you would be happy for your child to take part in other research studies. If you agree that we can contact you in the future about other research studies, we will also record your contact details. Your contact details will not be passed onto anyone outside of the research team. You can opt-out of this at any point by contacting Dr Rebeccah Slater (details below). Your agreement for us to contact you does not form any obligation to participate in future research.

We may use the following recording measures for your child:

Measuring brain activity

Electroencephalography (EEG)**:** EEG is a portable imaging system to measure brain activity. It involves gently placing electrodes (small metal discs) on the head using a paste that can be washed off with soap and water. EEG is routinely used on the neonatal unit, children's wards and clinics.

Near Infrared Spectroscopy (NIRS): NIRS is a non-invasive technique to measure brain activity. It involves placing lights and detectors on the head to record changes in blood and tissue oxygen levels.

Ultrasound: an ultrasound machine uses sound waves to create images of the brain. Ultrasound is routinely used to monitor babies' development during pregnancy and to assess brain development on the neonatal unit. In our research we also use a special type of ultrasound called functional ultrasound. This can measure which areas of the brain are active. An ultrasound scan involves placing an ultrasound probe on your child's head. To make contact, some gel will be applied between the head and the probe.

Measuring other responses

Electromyography (EMG): EMG is a safe non-invasive technique to record muscle activity. Small electrodes will be placed on the skin over the muscle to see if your child pulls away during the stimulation (and clinical procedure if relevant).

Vital sign monitoring: Small adhesive electrodes may be placed on your child's chest to measure changes in heart rate (this is called an ECG) and breathing rate. A small probe may also be wrapped around your child's foot to measure changes in blood oxygen levels.

Videoing your child: We may also video your child during the study. This is so that we can assess changes in facial expression and body movements, and to record the exact timing of the stimulation or clinical procedure.

We may also approach you to ask if you are happy for us to use these images for teaching, publicity and/or scientific journals. If you agree, we will take separate consent for this as your child's face would be visible in the video footage. This is not a mandatory part of the study. If you choose not to allow us to use the images in this way, this will not affect your child's care or prevent your child from participating in this research.

5. Are there any additional risks or benefits for my child?

Obtaining video footage of your child is non-invasive and does not present any risk to your child. EEG, EMG and ECG have been used clinically for over 20 years without any adverse effects. Ultrasound is a tool that is routinely used in clinical practice. All studies have a dedicated team of healthcare professionals and researchers that will ensure the safety of your child at all times. We are not aware of any risks for your child taking part in this study.

The data collected are for research, so will not be reviewed by a doctor routinely. If any clinically significant findings are identified at the time of the study then the research team will report these to the clinical care team to handle as appropriate.

There are no direct benefits of participating in this research. This study is designed to gather information, to help guide improvements in care for infants in the future. If your child becomes distressed, the research study will be paused or stopped. Any clinically required procedures will still go ahead if the treating clinician feels that this is appropriate.

6. What information will be collected about my child?
We will collect clinical information about your child, for example their gestational age at birth and any medications they have received. This information helps us to determine which factors may influence the way an infant copes with pain. We will also collect information about your child's brain and it's activity, and may collect information about your child's muscle activity, vital signs (such as heart rate and breathing rate), and recordings of their facial expressions and body movements.

All information and videos that are collected during this research study will be kept strictly confidential. Each infant will be allocated a study number which will be used to label all data. This study has been registered with the data protection registration office and forms part of an educational programme.

7. What will happen to the results?
Results will be analysed and published in a journal. All publications will be made available on our website **https://neuroimaging.paediatrics.ox.ac.uk**. The findings may also be used for teaching or academic research presentations. No identifying information will be presented about you or your child, unless you have provided specific consent for us to use videos/images of your child in this way.

8. What will happen to my child's data?
We will be using information collected from your child and their medical records in order to conduct this study. Research is a task that we perform in the public interest. The University of Oxford, as Sponsor, is the data controller. This means that we, as University of Oxford researchers, are responsible for looking after the information collected and using it properly. We will use the minimum personally-identifiable information possible. We will keep identifiable information about your child for up to 5 years after the study has finished. This excludes any research documents with personal information, such as consent forms, which will be held securely at the University of Oxford for 25 years after the end of the study.

Data protection regulation provides you with control over your personal data and how it is used. When you agree to your information being used in research, however, some of those rights may be limited in order for the research to be reliable and accurate. Further information about your rights with respect to your personal data is available at
http://www.admin.ox.ac.uk/councilsec/compliance/gdpr/individualrights/

You can find out more about how we use your information from the contacts in section 12.

Research data may be shared with other researchers doing similar work, both here and abroad. Responsible members of the University of Oxford or the Oxford University Hospitals NHS Trust may be given access to data for monitoring and/or audit of the study to ensure we are complying with regulations.

9. Who is organising and funding this research?
This study is sponsored by University of Oxford and has been funded by The Wellcome Trust. Your doctor will not be paid for including you in this study.

10. Who has reviewed the study?

All research that involves NHS patients has to be approved by a Research Ethics Committee. Approval means that the Committee is satisfied that yours and your child's rights will be respected, that any risks have been reduced to a minimum and balanced against possible benefits, and that you have been given sufficient information on which to make an informed decision about whether to take part. The South Central Oxford C Research Ethics Committee has reviewed and approved this study.

11. Comments or concerns during the study

The University has arrangements in place to provide for harm arising from participation in the study for which the University is the Research Sponsor. NHS indemnity operates in respect of the clinical treatment with which your child is provided. If you wish to complain about any aspect of the way in which you have been approached or treated during the course of this study, you should contact Prof Rebeccah Slater (details below) or the University of Oxford Clinical Trials and Research Governance (CTRG) office (tel: 01865 (6)16480, email: ctrg@admin.ox.ac.uk).

12. Contact for further information

Prof Rebeccah Slater (Study Lead) *Professor of Paediatric Neuroscience* *University of Oxford*	01865 234537 rebeccah.slater@paediatrics.ox.ac.uk
Dr Eleri Adams (Clinical Lead) *Consultant Neonatologist* *Oxford University Hospitals NHS Trust*	01865 221356 eleri.adams@ouh.nhs.uk

Picture shows example of an EEG study.

Thank you for reading this information leaflet.

Appendix B

Consent Form

Consent Form

Study ID: | | | | | | | | |

Infant's name:

Study Title: Investigating pain in the developing human brain

Chief Investigator: Prof Rebeccah Slater (01865 234537, rebeccah.slater@paediatrics.ox.ac.uk)

Please initial each box

Please complete in black ballpoint pen.

1	I confirm that I have read and understood the information sheet (clinical procedures) **(v9.0, dated 31/05/2019)**, for the above study. I have had the opportunity to ask questions and have had these answered satisfactorily.	☐
2	I understand that my child's participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my child's medical care or legal rights being affected.	☐
3	I understand that relevant sections of my child's medical notes and data collected during the study may be looked at by individuals from the University of Oxford or Oxford University Hospitals NHS Trust, where it is relevant to my child's taking part in this research. I give permission for these individuals to access to my child's records.	☐
4	I agree to my child being videoed during the study. I understand that recorded images will not be used for public use, only analysis. No identifiable information, including video recordings or imaging, will be used in any publications/presentations. Only anonymised data will be published or presented at meetings.	☐
5	I agree for the collected data to be used for teaching or academic research presentations.	☐
6	I agree to my child taking part in the above study.	☐
	OPTIONAL	
7	I agree to my child being studied on more than one occasion, up to a maximum of 5 occasions.	☐
8	I consent to being approached in the future about other research studies that my child may be eligible for.	☐
9	I agree to the images/videos of my child recorded during this study being used for publications and presentations.	☐

Name of parent: _____

Relationship to baby: _____

Signature: _____

Date: _____

Name of investigator taking consent: _____

Signature: _____

Date: _____

1 to be kept as part of the study documentation (original)
1 form for parent
1 to be kept with hospital notes