Neurodevelopmental and visual outcomes of infants at risk of neurodevelopmental disability following dietary supplementation in infancy

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1 ABSTRACT

Background: Docosahexaenoic acid (DHA), choline and uridine-5-monophosphate (UMP) are important brain nutrients which form phosphatidylcholine, the most abundant brain membrane phospholipid. DHA, choline and UMP supplementation increases rodent brain phospholipids, synaptic components, functional brain connectivity and cognitive performance. This novel pilot study supplemented infants at risk of neurological impairment (ARNI) with a nutrient combination containing these neurotrophic compounds.

Aims: 1) In a double blind randomised control trial (RCT), investigate if intake of a specific nutrient combination improves neurodevelopmental and visual outcome in infants ARNI. 2) Using novel measures of cortical visual function, investigate the effect of perinatal brain injury severity, gestational age at birth and sex upon visuocognitive development in infants at risk of neurodevelopmental impairment.

Method: Recruitment was from UK neonatal units. Eligibility: ≤31 weeks, weight <9th percentile; <31 weeks with ≥ Grade II intraventricular haemorrhage (IVH) or preterm white matter injury (PWMI); 31-40 weeks with ≥ Grade II IVH or PWMI, ≥ Sarnat Grade II HIE or defined brain MRI abnormalities. Stratification was by sex, gestation and brain injury severity. Randomised infants received neurotrophic supplementation or placebo, for 2 years. Primary outcome was Bayley Scales of Infant Development III (BSID III) composite cognitive score (CCS) after 2 years. Secondary outcomes included BSID III composite language score (CLS) and BSID III composite motor score (CMS). Cortical visual measures were pattern reversal visual event related potential (PR-VERP) latency (transient and calculated), orientation

reversal visual event related potentials (OR-VERP), and the Fixation Shift test (FS). Functional behavioural vision was assessed using the Atkinson Battery of Child Development for Examining Functional Vision (ABCDEFV). Local Ethics Committee approval was granted.

Results: 62 neonates were recruited. After 2 years, mean CCS in the intervention group was 87.7 (SD 20.4) and 81.6 (SD 18.5) in the placebo group (mean difference = 2.28, p=0.13; -0.2, 18.2). Mean CLS in the intervention group was 91.5 (SD 20.1) and 83.2 (SD 19.6) in the placebo group (mean difference = 2.74, p=0.1; -2.4, 18.3). CMS was similar in both groups. In relation to trial visual outcome measures, more infants in the placebo group gave a statistically significant OR-VERP response than in the intervention group (p=0.03). There were no statistically significant differences between the placebo and intervention on any other trial visual outcome measure. Cohort analyses indicate that transient PR-VERP latency is prolonged in children at risk of neurodevelopmental disability compared to typically developing infants (mean difference = -23.3, p=0.015, 95% CI -42.10 - -4.54). Calculated PR-VERP latency is prolonged to an even greater extent in children at risk of neurodevelopmental disability compared to typically developing infants (mean difference -148.6, p=0.000, 95% CI -179.7- -117.43), and remains prolonged across the age range tested.

Conclusions: 1) The difference in CCS and CLS between intervention and placebo groups represents a clinically significant effect size. Use of neurotrophic micronutrient supplementation in infants ARNI warrants exploration in a large multicentre RCT. 2) Calculated PR-VERP latency may be a more appropriate outcome

measure of cortical visual function than transient PR-VERP latency in infants at risk of neurodevelopmental disability.

2 CONTENTS

3 LIST OF ABBREVIATIONS

AA: Arachidonic acid

ABCDEFV: Atkinson Battery of Child Development for Examining Functional Vision

AGA: Appropriate for gestational age

ARNI: At risk of neurodevelopmental impairment

αLA: Alpha linolenic acid

BG: Basal ganglia

BGT: Basal ganglia-Thalamus

BSID: Bayley Scales of Infant Development

CCS: Composite cognitive score

CGA: Corrected gestational age

CIMT: Constraint induced movement therapy

CLS: Composite language score

CMS: Composite motor score

CoP: Cortical plate

CNS: Central nervous system

CP: Cerebral palsies

CRF: Case report form

CT: Computed tomography

cUSS: Cranial ultrasound scan

CVI: Cerebral visual impairment

DHA: Docosahexaenoic acid

DGH: District general hospital

DTI: Diffusion tensor imaging

EBM: Expressed breast milk

EE: Ethyl esters

EEG: Electroencephalogram

ELBW: Extremely low birth weight

ELOVL: add full name in thesis

EP: Extreme prematurity

EPA: Eicosapentaenoic acid

FA: Fatty acid

FADS: Fatty acid desaturase

FEF: Frontal eye fields

FFA: Free fatty acids

fMRI: Functional magnetic resonance imaging

FS: Fixation shift

FSC: Fixation shift competition

FSNC: Fixation shift non- competition

FrA: Fractional anisotropy

GMFCS: Gross motor function classification scale

GMH: Germinal matrix haemorrhage

GMI: Grey matter injury

HIE: Hypoxic ischaemic encephalopathy

HOME: Home Observation for Measurement of the Environment

ICF: International classification of function, Disability and Health

IDA: Iron deficiency anaemia

IQ: Intelligence quotient

IUGR: Intra-uterine growth retardation

IVH: Intraventricular haemorrhage

IZ: Intermediate zone

JRH: John Radcliffe Hospital

LA: Linoleic acid

LCPUFA: Long chain polyunsaturated fatty acid

LGN: Lateral geniculate nucleus

MDI: Mental development index

MRI: Magnetic resonance imaging

NDV: Near detection vision

NMDA: N-methyl-D-aspartate

NNU: Neonatal units

NOT: Nucleus of the optic tract

NTD: Neural tube defect

OFC: Occipitofrontal circumference

OKN: Opto-kinetic nystagmus

OR-VERP: Orientation reversal -Visual event related potential

PAM: Perception-action model

PBI: Perinatal brain injury

PC: Phosphatidylcholine

PCW: Post conceptual weeks

PDI: Psychomotor development index

PL: Phospholipid

PLIC: Posterior limb of the internal capsule

PP: Per protocol

PPARG: Peroxisome Proliferator Activated Receptor Gamma

PPC: Posterior parietal cortex

PPV: Positive predictive value

PR-VERP: Pattern reversal -Visual event related potential

PUFA: Polyunsaturated fatty acid

PVL: Periventricular leucomalacia

PVHI: Periventricular haemorrhagic infarction

PVI: Profound visual impairment

PWMI: Preterm white matter injury

QoL: Quality of life

RAI: Recommended average intake

RBH: Royal Berkshire Hospital

RCT: Randomised control trial

RDI: Recommended daily intake

RMR: Resting metabolic rate

r/s: reversals per second

SAP: Statistical analysis plan

SC: Superior colliculus

SF: Spatial frequency

SGA: Small for gestational age

SNR: Signal to noise ratio

SP: Sub-plate

SVI: Severe visual impairment

SVZ: Sub-ventricular zone

TAG: Triacylglycerol

TDI: Typically developing infants

TEA: Term equivalent age

TF: Temporal frequency

TMF: Trial master file

TPN: Total parenteral nutrition

UMP: Uridine-5-Monophosphate

VABS: Vineland Adaptive Behaviour Scale

VI: Visual impairment

VIn: Ventricular Index

VLBW: Very low birth weight

VR: Virtual reality

VZ: Ventricular zone

WMI: White matter injury

WPH: Wexham Park Hospital

WPPSI-III: Wechsler Preschool and Primary Scale of Intelligence-III

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6 ACKNOWLEDGEMENTS

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From the Dolphin team I would like to give thanks to Christine Montague-Johnson, Bonnie Baker and Karen Laler. Without their hard work, dedication and enthusiasm this project would not have been possible. I would also like to thank Dr Jin Lee who first showed me how to perform and analyse Visual Event Related Potentials, and who supervised the initial VERP data collection.

I would like to thank my family, Mum and Dad, Andrew, Ailsa and Euan for all their love and support through this process, without which this thesis would not have been feasible.

Finally, this thesis is dedicated to the families who participated in the study, and to whom the greatest thanks is extended.

7 STATEMENT OF ORIGINALITY

The Dolphin Study protocols were written by Professor Peter Sullivan and Dr Jeremy Parr between 2007 and 2009. I contributed to:

Trial design and protocol development:

- instigating amendments including the measurement of participant blood fatty acid levels
- inclusion of children with more severe patterns of perinatal brain injury to enable all children at risk of developing neurodevelopmental impairments, specifically cerebral palsy, to participate in the trial

- devising the neurological severity grading system drawing on current literature, with the support of Dr Gerardine Quagehbeur, consultant paediatric neuroradiologist.
- vision testing protocol development in collaboration with Professors Oliver Braddick and Janette Atkinson.

Trial management:

For the first 2 years of the trial I took lead responsibility for recruitment to the study, including the opening of additional neonatal centres at the Royal Berkshire Hospital in Reading and Wexham Park Hospital in Slough to boost recruitment.

Testing:

I undertook:

- All Bayley Scales of Infant Development testing between the dates of September 2009 and November 2011 (34 assessments).
- All vision assessments with the support of Professors Janette Atkinson and Oliver Braddick of the Department of Experimental Psychology, University of Oxford during the period September 2009 to November 2011 and October 2012 to June 2013 (90 assessments).

Data entry and analysis:

- Analysed all VERP data and completed VERP data entry to the database
- Generated neurodevelopmental and vision related trial and cohort vision hypotheses

- Planned appropriate statistical analyses in collaboration with Professors Sullivan, Braddick and Atkinson and Dr Parr, and Drs Jane Holmes and Stephen Gerry (trial statisticians, University of Oxford Centre for Statistics in Medicine).
- Wrote the vision sections of the statistical analysis plan (SAP), and had substantial input to the neurodevelopmental aspects of the SAP in discussion with Dr Jane Holmes and Dr Stephen Gerry.
- Conducted blood fatty acid statistical analyses (excluding Dolphin trial breastfeeding analyses).

Good clinical practice:

- As expected in any clinical trial, trial analyses were conducted by the trial statistician, Dr Jane Holmes, Centre for Statistics in Medicine, University of Oxford.
- Due to the complexities of the data all cohort vision analyses were conducted by Dr Jane Holmes and Dr Stephen Gerry, Centre for Statistics in Medicine, University of Oxford.

This thesis reflects my intellectual contribution to the Dolphin study, and to the development of cohort vision studies. I confirm that this thesis is my original work.

8 PREFACE

This thesis describes the Dolphin trial of combination neurotrophic supplementation of neonates with risk factors for neurodevelopmental disability. The overall aim of the trial was to establish whether neurotrophic supplementation could ameliorate level of disability by supporting the developing brain's inherent mechanisms of plasticity. The introduction describes normal brain development and outlines the role of the cortical subplate in providing the substrate for normal brain development, and repair following early brain injury. The neurodevelopmental impact of perinatal brain injury, with a particular focus on cerebral palsy,

neurocognitive and visual outcomes are described, followed by a discussion of the potential for brain plasticity following injury. The literature relevant to trial design is then described, followed by a description of the current evidence on the role of nutrition in neurodevelopment. Thesis hypotheses are then presented, followed by trial methodology and results. Cohort fatty acid and vision analyses are also presented, followed by a discussion of findings and general conclusions.

9 GENERAL INTRODUCTION

9.1 Brain development between 24 weeks post conception and age 2 years

This section outlines normal brain development between 24 weeks post conception until around 2 years of age. Understanding the typical course of infant brain development is important in the context of the Dolphin study, as the aim of the study was to support processes of normal brain development through provision of nutritional factors required by the brain through the course of its rapid development in the first two years of life, thus ameliorating the level of disability experienced by

infants at risk of neurodevelopmental impairment. The importance of the cortical subplate as a rich substrate to synaptic development is highlighted, as animal research shows that provision of Dolphin study nutrients, docosahexaenoic acid, choline and uridine-5-monophosphate, in combination, increases synaptic elements in the rodent brain(1). The support of synaptic development may be one mechanism by which combination neurotrophic supplementation improves cognitive performance in supplemented rodents(2).

Before 24 post conception weeks (PCW) cerebral connections have a laminar arrangement in transient fetal zones containing growing axons, synapses and dendrites: the ventricular, sub-ventricular, intermediate zones, sub-plate and cortical plate(3). Transient zones support the growth of the major afferent fibre tracts from the thalamus(4-6).

The subplate is the largest of the transient zones and is rich in pre- and post-synaptic elements, and extracellular matrix and guidance molecules providing support and scaffolding to immature neurons(7). Afferent fibres wait in the subplate through mid-fetal life(7, 8), having journeyed through the internal capsule, periventricular crossroads and intermediate zones before reaching their subplate target in different cortical regions(5, 9-11). Synapses are present in the subplate throughout fetal development(7).

Between 24-32 PCW, thalamocortical fibres grow into the cortical plate(12). This occurs alongside synaptogenesis in the subplate, establishing co-existent transient subplate circuitry, and permanent sensory-driven thalamocortical circuitry(13, 14).

Thalamocortical afferents and cortical plate synapses communicate sensory signals from the periphery to the cortex (Figure 1).

After the 34th PCW the SP begins to resolve, but a remnant remains during the first few months of life in central and occipital cortex and until at least 13 months of age in the prefrontal cortex(15). As the subplate resolves, callosal and long cortico-cortical pathways grow into the cortex(3).

By term (37 weeks), long afferents reach their cortical destination. Axonal arborisation occurs within the cortical plate alongside growth of short cortico-cortical connections(3). Explosive synaptogenesis occurs in all layers, with active production of dendrites(16), dendritic spines(17) and synapses(18). During this stage, sensory driven cortical activity refines connectivity within the cortex(13).

In the perinatal period the transient subplate circuitry coexists with immature permanent cortical circuitry and neurons from the subplate continue to contribute to the development of short cortico-cortical connections in the first year of life(15). The persistence of transient neuronal circuitry beyond the neonatal period carries important implications for brain plasticity potential. Although the subplate begins to disappear from the late preterm phase, many of its neurons survive postnatally(19).

Cortical synaptogenesis is predominantly a postnatal process(20). In the first postnatal months, there is a rapid increase in the number of synapses, but with persistence of transient neuronal circuitry well into the first year of life(21). The timing of maximal synaptic formation varies between brain regions. For example, in the visual cortex synaptogenesis is most rapid between the 2nd and 4th month of life. Maximal synaptic density in visual cortex is achieved around 8 months of age,

beyond which selective pruning of synapses further refines cortical circuitry(22). In the prefrontal cortex maximal synaptic density is achieved by 3 years of age, followed by more gradual selective synapse elimination(20).This ongoing process of synaptogenesis and synapse elimination may be crucial to the brain's potential for plasticity, and may provide the substrate for interventions which change the developing brain's injury response.

Although the production and migration of neurons are largely prenatal events, the proliferation and migration of glial precursors, and differentiation of astrocytes and oligodendrocytes are dominant features of brain development postnatally, and through the preschool years(23). Progressive myelination occurs throughout the brain during the first 2 years of life. MRI studies have described sequential postnatal myelination, occurring first in sensorimotor pathways and commissural tracts, followed by other ascending pathways of the corona radiata and lastly in cortico-cortical association tracts(23). As well as their role in myelination, oligodendrocytes also produce a number of trophic factors which support axonal integrity and neuronal survival(23).

Figure 1. Transient fetal organisation in of the developing cerebral wall during the 20th post-conception week, fetal phase, early preterm phase and late preterm phase.

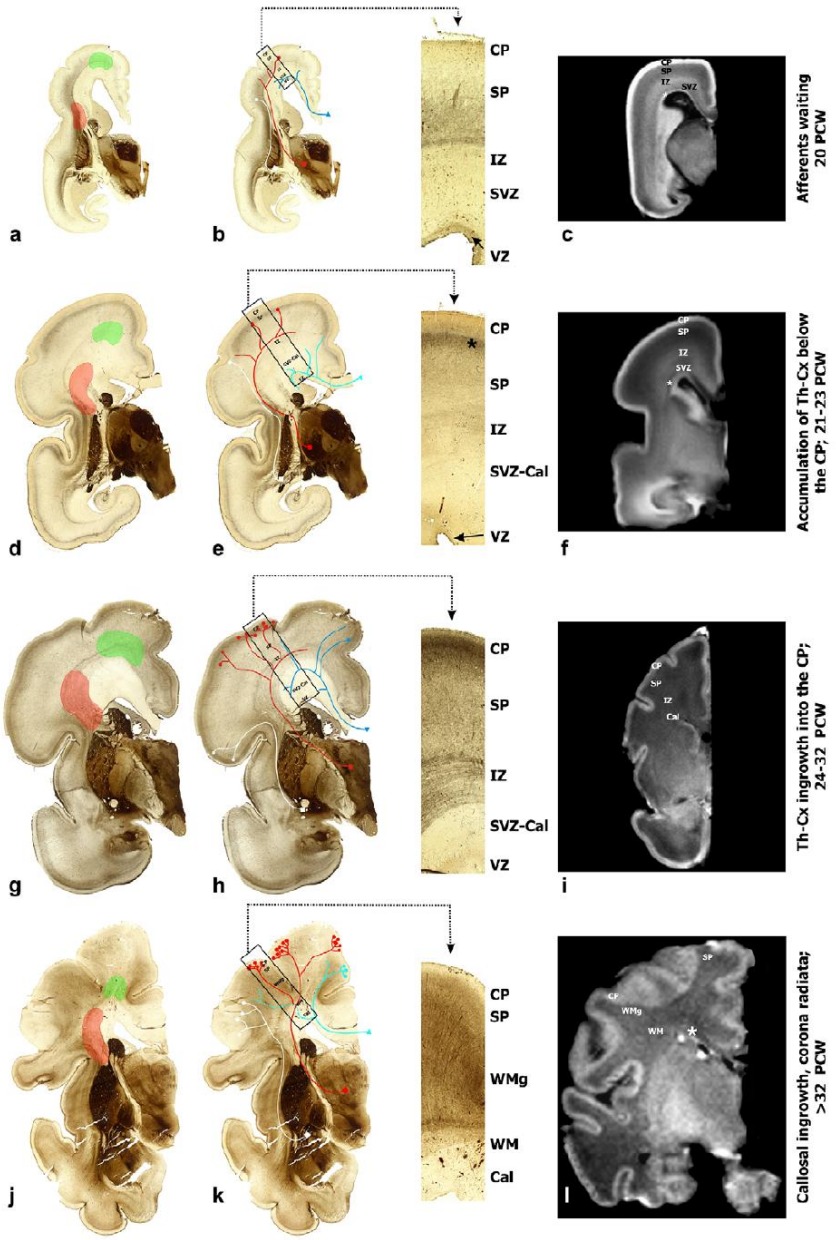


Figure 1. Transient fetal organisation in of the developing cerebral wall during the 20th post-conception week (PCW) (a, b, c), fetal phase (d, e, f), early preterm phase (g, h, i) and late preterm phase (j, k, l). The first main periventricular crossroads of pathways are shown in red, the second, frontal crossroads are shown in green (a, d, g, j). Growth of the cortical afferents (b, e, h, k): thalamocortical afferents are shown

in red, callosal fibres in blue, and basal forebrain fibres in white lines. Insets illustrate the laminar organisation of transient zones of the cerebral wall (from pia to ventricle). CP, cortical plate; SP, subplate zone; IZ, intermediate zone; SVZ, subventricular zone; SVZ-Cal, subventricular zone with callosal fibres; WM, white matter; WMg, gyral white matter. Accumulation of the thalamocortical afferents in the subplate is marked with a black asterisk (e). "Retracting" callosal axons are shown as blue dotted lines (k). The transient zones are visible on in vitro MRI (c, f, i, l), as are fibre-rich periventricular zones (white asterisks in c, f, l). Reproduced with kind permission from Elsevier(3).

9.2 Perinatal brain injury

9.2.1 Mechanisms of perinatal brain injury

The developing brain is extremely vulnerable to injury, via processes of hypoxia-ischaemia and/or inflammation. An appreciation of the mechanisms by which brain injury results will help to identify potential therapeutic interventions. Irrespective of the aetiology of the insult, it appears that a final common pathway of injury develops, involving primary, secondary and tertiary mechanisms of injury(24).

The mechanisms of early brain injury are multifactorial, and are summarised in the review by Johnston et al(25). This section considers the mechanisms of hypoxia and inflammation, which are associated with preterm birth and hypoxic ischaemic encephalopathy (HIE). Both conditions are risk factors for neurodevelopmental impairment and were eligibility criteria for the Dolphin study (see General Methods section 10).

Inadequate brain oxygen delivery appears to play a key role in the development of neuronal damage, triggering an “excito-oxidative cascade” of events(25). Hypoxia causes reduced cellular glucose delivery, limiting anaerobic respiration. Anaerobic respiration provides the energy to drive peri-synaptic neurotransmitter re-uptake

pumps. Pump failure ensues with accumulation of glutamate in the synaptic cleft, and neuronal depolarisation. Neuronal membrane depolarisation and glutamate accumulation open N-methyl-D-aspartate (NMDA) and other calcium channels causing neuronal calcium influx. Calcium influx through NMDA receptors increases production of nitric oxide, an oxygen free radical, by activating nitric oxygen synthetase. Oxygen free radicals interfere with mitochondrial respiration, causing further production of toxins, resulting in damage to DNA and mitochondrial failure. This initial energy failure caused by acute hypoxic-ischaemia is termed "primary energy failure"(26). As mitochondria progressively fail, "secondary energy failure" ensues(26). During this process lactic acid accumulates and pro-apoptotic proteins move into the cytoplasm, triggering delayed cell death(26).

As in hypoxia, the aetiology of brain injury caused by inflammation is complex, but is likely to involve increased levels of circulating cytokines e.g. TNFα and IL-6(25). These cytokines may up-regulate prostaglandin synthesis in cerebral endothelial or periventricular cells, increasing permeability of the blood brain barrier, with influx of proteins and macrophages into the brain. Macrophages have powerful oxidative capabilities, increasing levels of harmful reactive oxygen species in addition to pro-inflammatory cytokines. The interplay between hypoxic and inflammatory processes in fetal and neonatal brain injury is not yet fully elucidated, however a combination of hypoxia and inflammation appears to worsen the degree of brain injury, compared to hypoxia alone(26).

In addition to the primary and secondary mechanisms of brain injury outlined above, Fleiss et al propose that injurious mechanisms continue to affect and sensitise the

developing brain for years after initial injury(24). Fleiss et al propose that tertiary damage is mediated by a number of processes which include aberrant gliosis, ongoing inflammation and epigenetic changes(24). Furthermore, the authors suggest that identification of these processes may allow the development of targeted therapies aimed at reducing inflammation, creation of a more favourable epigenetic environment or promotion of brain plasticity(24).

9.2.2 Genetic modifiers of neurologic vulnerability following preterm birth

Preterm brain injury is only partly explained by environmental stressors such as hypoxia-ischaemia and inflammation. Recent research has revealed an important contribution of heritable factors to the development of preterm brain injury. Genetic measures have been combined with neuroimaging modalities including magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI) to identify abnormal brain morphology and microstructure associated with neurodevelopmental outcome. DTI provides information about connectivity in the developing brain by measuring the anisotropic diffusion of water in white matter tracts. Fractional anisotropy (FrA) quantifies how strongly directional the local tract structure is. Tract based spatial statistics (TBSS) can be employed to analyse FrA images from multiple subjects in order to identify brain areas associated with development and disease. A recent study used TBSS and deformation- based morphometry to assess how the presence of specific alleles at single nucleotide polymorphisms (SNPs) in genes associated with cognitive dysfunction, neuronal/glial development, and endogenous long chain polyunsaturated fatty acids (LCPUFA) production may modify risk for brain injury in 83 preterm infants(27). Genes

encoding endogenous LCPUFA production were selected in recognition of their important role in brain development. The study associated carriage of two minor alleles with preterm white matter injury measured using DTI. These alleles were rs2518824 in the armadillo repeat gene deleted in velocardiofacial syndrome (ARVCF) gene, previously linked to neuronal migration and schizophrenia, and rs174576 in the fatty acid desaturase 2 (FADS2) gene(27). The FADS 2 gene encodes rate-limited endogenous LCPUFA synthesis, and polymorphisms in FADS2 have been shown to modify the effect of breastfeeding on child intelligence quotient (IQ)(28, 29).

This work adds to the growing body of work linking fatty acid status, FADS genotype and cognitive outcome in children(30, 31). In one study child development was assessed using the Ages and Stages Questionnaire 3 at 3 years of age. Developmental profiles were then correlated to red blood cell docosahexaenoic acid (DHA) status at 9 months and 3 years and to FADS-tag SNPs previously found to have opposing effects on infant erythrocyte DHA levels. Minor allele carriers of FADS SNP rs1535 had higher RBC DHA levels whereas minor allele carriers of rs174448 and rs174575 had lower RBC DHA levels, at 9 months but not 36 months(32), possibly suggesting a programming effect from early DHA exposure(31). Investigators found that FADS SNP alleles were correlated in a sex specific way. In girls, the rs1535 FADS SNP was associated with lower communication and problem solving skills despite being associated with higher levels of DHA biosynthesis at 9 months. SNPs rs174448 and rs174575 which are associated with reduced DHA biosynthesis at 9 months were associated with improved communication and problem solving skills in girls.

Opposite associations were identified in boys(31). The developmental effects of DHA increasing FADS SNPs may be further mediated by Peroxisome Proliferator Activated Receptor Gamma (PPARG) genotype in a sex specific manner(31). PPARG is involved in the regulation of lipid metabolism.

Taken together, these examples provide early evidence of interaction between fatty acid status, genetic profile and cognitive outcomes. Further studies are required to elucidate the precise mechanisms of these interactions and establish the effects of dietary interventions on cognitive outcomes.

9.2.3 The timing of brain insult dictates the pattern of brain injury

Human cerebral development is characterised by a changing pattern and intensity of axonal growth throughout fetal development (described in section 9.1). The timing of specific events in neural development underlies the different patterns of vulnerability in the preterm and term infant.

First and second trimester brain insults disrupt migration of cortical neurons(33), causing cortical malformations such as polymicrogyria, schizencephaly and holoprosencephaly. During the early third trimester (23-32 weeks) selective vulnerability of the periventricular white matter (PVWM) results from active growth of long cerebral pathways(3). Periventricular leucomalacia (PVL) and periventricular haemorrhagic infarction (PVHI) complicating intraventricular haemorrhage (IVH) are the predominant brain lesions in preterm children.

Preterm brain injury may be focal or diffuse. Focal injuries cause well defined necrotic lesions associated with cyst formation in the periventricular white matter, referred to as cystic PVL. Cystic PVL occurs in less than 5% of preterm children(34).

Diffuse necrosis causes non-cystic PVL. PVL may be accompanied by germinal matrix or intraventricular haemorrhage. Cortical and subcortical gray matter injury may co-occur with white matter injury in preterm infants. At least one third of infants with PVL (cystic and non-cystic) have evidence of neuronal loss and gliosis in basal ganglia and dentate cerebellar nuclei(35), and thalamus(36), as reviewed in Rees et al(26). The authors also review a number of MRI studies corroborating these findings, identifying reduced volume of the thalamus and basal ganglia(37, 38), hippocampus(39) and cerebellum(40) in ex-preterm infants(26). Brain imaging studies also identify a reduction in the rate of growth of cortical surface area in preterm infants which is directly related to gestational age, and is more pronounced in males compared to females. Cortical surface area has been directly related to neurodevelopmental performance assessed aged 2 years, suggesting a possible mechanism for the neurodevelopmental consequences of preterm birth(41)

In term infants, grey matter neurons and early myelinating tissue have high metabolic demand, and are most vulnerable to injury. Term hypoxic ischaemic encephalopathy (HIE) affects 1 to 3 per 1000 births(42). Basal ganglia (BG) lesions are characteristic of acute perinatal hypoxia ischaemia(43), and may be associated with damage to the posterior limb of the internal capsule (PLIC), hippocampus, brainstem and central cortical grey matter(43). Half of affected infants will have associated white matter abnormalities(44). Less commonly isolated white matter injury may occur(45). A water-shed distribution of injury affecting the white matter of the intervascular boundary zone can also occur(45). Cerebral arterial infarction is more common in term babies, but may also occur in very preterm infants(46).

9.3 Plasticity in the developing brain

Plasticity refers to the brain's inherent capacity to alter its structure and function in response to external experience and environment. Plasticity underpins new learning and memory and is a beneficial property in normal brain development(47). Plasticity is an important concept for any PBI early intervention trial and underpins the ethos of the Dolphin study which aims to reduce the level of disability of children with risk factors for neurodevelopmental impairment through optimal dietary provision of nutrients required for normal brain development.

"Critical periods" are temporal windows during which there is increased plasticity associated with maximal neural sensitivity to specific stimuli. These are periods of rapid neural maturation and extensive neural connectivity. Brain development is not uniform across cortical regions; regions serving unique functions mature at different rates, and critical periods are specific to particular cortical modules.

Cortical development through early childhood and into adolescence are characterised by complex changes in brain structure. Early MRI studies showed that region specific gray matter volume was higher in school aged children compared to adults, which is then followed by overall cortical thinning as brain development proceeds through adolescence. There is now extensive evidence of posterior-to-anterior gradients in brain development, as reviewed by Brown et al 2012(23). Detailed high resolution MRI mapping studies, show that cortical modules develop in a specific order; lower order somatosensory and visual cortices develop ahead of higher-order association cortices capable of integrating functions across lower order cortices. Phylogenetically older cortical systems also develop ahead of newer

regions. These studies confirm progressive cortical thinning as brain development proceeds, but also identify more limited areas of cortical thickening (23, 48, 49).

It appears that childhood intelligence is directly related to the rate of change of cortical thickness and subsequent cortical thinning, rather than cortical thickness itself(50). Children who have the highest intelligence quotient (IQ) experience the most rapid increase in cortical thickness through early childhood, particularly in frontal regions associated with the maturation of intelligent functions. This is followed by rapid cortical thinning towards early adolescence. Rapid neuroanatomical change implies adequate neural substrate, and the importance of the subplate (abundant in neurons, developing cortical afferents, and their synapses) and proliferation in myelin in the developing brain, followed by selective pruning processes are implicated as factors which may contribute to the observed rapid changes in cortical thickness(50).

The role of plasticity following childhood brain injury is less clear; plasticity may contribute to aberrant neuronal development associated with poorer functional outcomes(51, 52). Two predominant theories regarding brain plasticity have evolved. “Early plasticity” models argue that the developing brain has greater potential for recovery following early brain injury, facilitated by abundant neural substrate during early brain development. “Early vulnerability” models infer “derailment” of early neural processes, especially during critical periods, with adverse impact on subsequent neurodevelopment. Anderson et al 2014 propose that these two apparently opposite models actually represent two extremes of a “recovery continuum”(53).

The immature brain appears to be particularly vulnerable to diffuse brain injury(52, 54). Better outcomes have been reported following focal brain injury, implying that undamaged areas assume the roles of damaged areas(47, 55-57) Emerging evidence suggests that injury sustained before the age of 3 years is associated with poorer neurodevelopmental outcome than injury sustained in later childhood(53, 58-60). However, nature, extent and timing of injury do not fully predict developmental outcome following childhood brain injury. Additional factors such as pre-injury developmental stage and cognitive ability, genotype, sex, family and social status, comorbidity, and access to intervention and rehabilitation may all exert individual or synergistic effect on outcome(47). One study has identified comorbid epilepsy as a poor prognostic factor for IQ outcomes, and family function and social risk factors as poor prognostic factors for executive function and behavioural outcomes.

The Dolphin study aims to take advantage of the brain's inherent plasticity potential, in order to improve the neurodevelopmental outcomes of neonates with risk factors for neurodevelopmental disability.

9.3.1 Mechanisms of recovery from brain injury

Mechanisms of brain recovery fall into two categories, "restitution" and "substitution"(61). Restitution refers to the recovery or reactivation of damaged areas, with subsequent restoration of function, whereas substitution mechanisms result in recovery following transfer of specific functions previously executed by now damaged areas, to alternative healthy sites.

9.3.1.1 Restitution

The evidence for restitution mechanisms comes from animal models, and describes processes of neuronal regeneration, neuronal sprouting, denervation sensitivity and modulation of molecular genetic activity. Evidence regarding the role of these recovery mechanisms following human brain injury is lacking.

Diaschisis refers to the period of rapid recovery of functions following brain injury, resulting from restitution of neural circuits both close to and distant from the site of injury which were suppressed but not destroyed at the time of injury(62). Following this phase several mechanisms may contribute to further recovery.

9.3.1.2 Neuronal regeneration

Injured neurons, axons and dendrites can regrow and re-connect to appropriate cortical targets. This has been documented both in the peripheral nervous system and the central nervous system in animal studies(63-65). There is little evidence that these processes occur in the human cortex.

9.3.1.3 Sprouting

Surviving neural components e.g. axons can sprout, developing new synaptic connections in target areas(66). However, it is unclear how efficient this process is and sprouting may result in partial recovery or worsened function if newly established connections are aberrant(47).

9.3.1.4 Denervation super sensitivity

Post-synaptic cells which lose their normal synaptic inputs following injury may develop a hypersensitive response to circulating neurotransmitters by developing

additional receptors(67). This activates pathways distal to the lesion and may lead to functional recovery in the case of small localised brain lesions(47).

9.3.1.5 Substitution

Evidence for substitution brain injury recovery mechanisms come from animal and functional imaging studies. Functional re-organisation may involve: i) interhemispheric reorganisation whereby functions of damaged areas are taken up by analogous regions in the contralateral hemisphere ii) intrahemispheric reorganisation whereby functions of damaged areas are adopted by adjacent areas within the same hemisphere or iii) intrahemispheric maintenance where functions are maintained within damaged areas, resulting in greatest dysfunction. Factors governing which of these recovery mechanisms is adopted by the injured brain remains unclear, but may include timing of injury in relation to developmental stage, the extent of the lesion (diffuse vs focal); size of lesion, laterality of lesion and the distribution of neural networks underpinning discrete functions(47). The functional consequences of these mechanisms remains unclear.

9.3.2 Plasticity in the motor cortex

Developing corticospinal projections synapse with spinal cord alpha motor neurons by 20 weeks gestation(68). Each hemisphere develops ipsilateral and contralateral projections(69). As normal development proceeds ipsilateral projections recede and contralateral projections take over motor functions. Cat model data suggest that competitive neuronal activity determines which projections persist or are withdrawn(70). When unilateral brain injury occurs before or during synaptogenesis

of corticospinal axons with spinal alpha motor neurons, ipsilateral neural activity dominates and contralateral neural activity decreases. Such re-organisation appears possible throughout the pre- and peri-natal period(71) and first few months of life(72) and is reported in one case up to age 2 years(73). Paretic hand function appears to be best when injury occurs early; many children with injuries around term have little useful hand function despite persistence of ipsilateral projections(71). Growth of ipsilateral corticospinal projections may represent useful “plasticity”, or aberrant neuronal development compounding disability by promoting loss of viable contralateral projections(72). Whether motor reorganisation utilises ipsilesional or contralesional pathways can be influenced by the site and extent of damage, but also by the interplay of motor output from the damaged hemisphere and somatosensory feedback from the hemiparetic limb(74).

Further evidence of motor cortex plasticity is provided by the results of interventions in children with congenital hemiplegia. Motor outcomes following non-invasive therapies for children with unilateral cerebral palsy (CP) have been evaluated in a recent systematic review(75). Seven case series studies met the criteria for inclusion in the review; six reported outcomes for constraint induced movement therapy (CIMT)(76-81), with or without adjunct therapies, and a seventh reported outcomes following a novel virtual reality (VR) therapy(82). Standard CIMT involves the wearing of a restraint on the unaffected hand for 90% of waking hours, plus 6 hours/day of intensive rehabilitative therapy for a period of 2 weeks(83), however less intensive programmes have been developed for young children(84). The VR therapy used new technologies to simulate patient perception of real-life activities. The included

studies variously used a combination of upper limb functional measures, neuroimaging (MRI, functional MRI (fMRI), magnetoencephalography and voxel based morphometry) to assess grey matter change, and neurophysiology techniques (transcranial magnetic stimulation) to assess response to therapy. Enlargement of the primary hand motor cortex contralateral to the paretic hand following intervention was reported across the reviewed studies(76-82), despite variation in study design and outcome measures used. In addition to enhanced activation of contralateral primary motor and sensory cortex, increased activation was also identified in the supplementary motor cortex(81), premotor cortex(80) and cerebellum(80, 82). The four studies which assessed correlation between neuroimaging change and functional outcome demonstrated improved motor skills in the paretic hand(76-78, 82). On the evidence available, both CIMT and VR induce neuroplasticity in sensorimotor cortex, with associated functional benefit.

9.3.3 Plasticity in visual cortex

First trimester injury causing occipital (primary visual) cortical dysplasia does not always result in visual field deficit(85), suggesting that either dysplastic tissue can effectively process visual information, or that other cortical areas take on visual functions. Removal of dysgenic cortex can be associated with increased risk of visual field defects(86). In preterm infants (23-32 weeks), periventricular white matter is highly susceptible to injury. In addition to motor dysfunction, visual impairment may occur, as the optic radiations pass through periventricular white matter en route to occipital cortex(87). Following third trimester periventricular white matter injury (PVWMI), DTI has demonstrated re-routing of developing thalamocortical axons

around damaged areas, reaching their intended occipital cortex target(88). These neuroimaging findings were associated with normal visual field examination, despite a large lesion in the left periventricular white matter where the optic radiations are normally located. This finding supports similar work which has demonstrated re-routing in the somatosensory cortex in children with PVWM injury, allowing projections to reach the post-central gyrus(87). The critical window for plasticity in the visual system remains unknown, however plasticity potential appears to exist until at least 3 months of age. Evidence of this is provided by the longitudinal assessment of an infant following perinatal left arterial stroke affecting the optic radiations but sparing primary visual cortex, demonstrated by fMRI and DTI. At 3 months, fMRI visual cortical activation was demonstrated in the unaffected hemisphere only. Diffusion tractography failed to demonstrate the optic radiations. By 20 months, fMRI showed clear activation with structural changes on DTI in the affected hemisphere(89), providing indirect evidence of re-organisation within the damaged visual system. In one case report, visual event related potentials (VERPs) and fMRI provide evidence that primary visual functions can be re-located to contiguous parietal and temporal cortex outside the normal boundary of primary visual processing, resulting in normal visual fields on testing with Goldmann perimetry(90).

Speculative therapies which support processes of synaptogenesis and selective synaptic pruning may be beneficial in circumstances of synaptic loss or injury. Furthermore, the plastic abilities of the developing brain may depend on adequate amounts of the substrates necessary to synapse formation and connectivity.

9.4 Sequelae of perinatal brain injury

The sequelae of perinatal brain injury (PBI) are far-reaching and exert varying effect throughout an individual's life. A discussion of all of the potential consequences of PBI is beyond the scope of this thesis, but include disorders of motor development, cognitive and learning difficulties (general and specific), speech and language disorders, hearing and visual impairment, epilepsy, neurodevelopmental disorders including autism spectrum disorders and attention deficit hyperactivity disorder, and behavioural difficulties. The following sections focus on the impact of PBI on infant and childhood cognitive, visual and motor development, including CP as these are common consequences of PBI and are outcomes which relate directly to the Dolphin trial hypotheses (see Results sections 15.1 and 15.2).

9.5 The cerebral palsies

9.5.1 Definition of cerebral palsy

Since 2006 the accepted definition of the cerebral palsies (CP) has been "a group of permanent disorders of movement and posture, causing activity limitation, that are attributed to non-progressive disturbances that occurred in the developing fetal or infant brain. The motor disorders of CP are often accompanied by disturbances of sensation, perception, cognition, communication, and behaviour, by epilepsy, and by secondary musculoskeletal problems"(91). Definitions of CP have evolved over the last 150 years. Previous definitions focused primarily on the motor deficits associated with CP, however motor disability is only one component of a constellation of domains experienced by a child with CP. Inclusion of commonly

occurring non-motor disorders in the current definition highlights that these disorders are all manifestations of an insult to the developing brain. The definition also acknowledges the high level of morbidity associated with co-occurring disorders. Certain manifestations of disordered brain development may be more apparent in some individuals with CP than in others, and may vary in impact across an individual's lifespan. Consideration of all the common features of CP also prompts consideration of the impact of all components of CP on everyday function and participation, in line with the frameworks conceptualised within the World Health Organization International Classification of Function, Disability and Health (ICF)(92).

9.5.2 Epidemiology of the cerebral palsies

CP results from events occurring in the antenatal, perinatal, postnatal period, and in early infancy. Approximately 50% of cases of CP are caused by antenatal events(93). Perinatal events account for approximately 33%, and postnatal events for 10-18%(93, 94).

The overall prevalence of CP is around 2 per 1000 live births, making it a common cause of childhood disability(95, 96). CP incidence has remained stable over the last few decades despite increasing numbers of preterm infants surviving neonatal intensive care (NICU)(9). The prevalence of CP is higher in children born preterm than in children born at term. Pooled prevalence data shows that prevalence is highest in infants born before 28 weeks, being 111.8 per 1000 live births. In children born after 36 weeks, pooled prevalence was 1.35 per 1000 live births(96). When stratified by gestational age, the prevalence is highest in children whose birth weight was 1000-1499g, being 59.18 per 1000 live births(96). This information is important for the identification of infants at high risk of CP for inclusion in early intervention trials.

9.5.3 Classification of cerebral palsy

The classification of CP is important for clinical and research purposes, and is discussed in the following sections in relation to its importance for intervention trials in children with risk factors for CP, and with established CP.

Clinically, classification provides a description of an individual child's problems, and their severity. Classification also facilitates comparison of an individual's difficulties and function over time, and enables prediction of the nature and level of current and

future service provision need. From a research perspective, classification allows comparison across broad groups of children with CP, as well as correlation of clinical presentation with neuropathology(91). Detailed classification of the nature and aetiology of CP for individual children allows greater homogeneity within groups of children eligible for future CP interventional trials.

The CP classification proposed alongside the 2006 definition of CP covers four domains:

- Motor abnormalities
    - Nature and typology of the motor impairment
    - Functional motor abilities
- Accompanying impairments
- Anatomical and neuroimaging findings
    - Anatomic distribution
    - Neuroimaging findings
- Causation and timing

The current classification of CP acknowledges a number of challenges, which are of particular interest in the context of developing CP intervention trial protocols.

9.5.3.1 Nature and typology of the motor impairment

The first challenge results from attempts to describe predominant motor patterns, the current terminology is not uniformly applied(97). For example, the term diplegia is variously used to mean involvement of both lower limbs without upper limb involvement, or alternatively, involvement of all four limbs but with greater involvement of the lower limbs. As a result, the current classification recommends

avoidance of the term diplegia, and quadriplegia for similar reasons, and advocates the use of unilateral and bilateral motor involvement e.g. affecting both legs, affecting the arm and leg on the right, or all four limbs. If the term is used the guideline recommends a full description of the anatomical areas involved, alongside description of the identified tonal abnormalities and any accompanying movement disorder(97). Universal application of the same terminology is imperative to allow comparison of groups of children with CP between studies.

9.5.3.2 Functional motor abilities

Inclusion of a child's functional motor abilities in the current classification is helpful, as is the recommendation to use functional classification scales for gross motor function (Gross Motor Function Classification Scale – GMFCS)(98, 99) and the upper limb Manual Ability Classification System (97). These functional scales can be used to help describe the impact of motor impairments on daily living, plan functional goals of therapy and assess individual change over time. Functional outcomes may be appropriate "real life" outcome measures for CP intervention trials.

9.5.3.3 Accompanying impairments

Comorbid conditions contribute to the classification, in recognition that these may have a greater impact on activity limitation and participation than motor impairment alone. The 2006 classification references the ICF as a framework for considering and documenting a child's participation, and so is aligned with current social models of health and disability(100). The ICF also places emphasis on Quality of Life (QoL) and there are now a number of paediatric QoL tools available. QoL assessment and

description is not included in the current classification of CP, although some argue that it should be included to provide as full a profile of CP as possible(100). Standard detailing of comorbid conditions allows identification of groups of children with CP with specific comorbidities of interest, and also allows research teams to control for commonly identified comorbid conditions during trial design.

A recent systematic review outlined the rates of comorbidities and functional limitations in children with CP(101). The systematic review drew mainly on population based studies and concluded: “Among children with CP 1 in 3 cannot walk; 1 in 4 cannot talk; 1 in 4 had epilepsy; and 1 in 25 were deaf. There is moderate-quality evidence that 3 in 4 were in pain; 1 in 2 had an ID (intellectual disability); 1 in 3 had a hip displacement; 1 in 4 had a behaviour disorder; 1 in 4 had bladder control problems; 1 in 5 dribbled ; 1 in 10 were blind; 1 in 15 were tube-fed. There is low quality evidence that 1 in 5 had a sleep disorder. Children and adults unable to walk are more likely to experience these accompanying impairments. The risk for pain and behavioural problems occurs equally at all levels of physical disability. There is insufficient evidence to be certain about the rates of sleep disorders, and more research is warranted”(102). This information may be useful to families at the time of diagnosis, and emphasises the wider clinical picture of CP. Information regarding the rates of comorbid conditions will also help set research priorities and identify interventions capable of improving the lives of children with CP. The neurocognitive and visual impairments associated with perinatal brain injury will be discussed in sections 9.6 and 9.7.

9.5.3.4 Correlation of neuroimaging and cerebral palsy subtype

Neuroimaging has the potential to improve understanding of the pathogenesis, aetiology and timing of the brain injury underlying CP, and is recommended for all children with CP(103). Neuroimaging may also be useful for early prognostication, identification of appropriate subgroups for early intervention trials, thus reducing heterogeneity within and across studies, and therapeutic targeting of novel interventions(104).

A number of studies correlating neuroimaging findings to CP are now available(104-106). Magnetic resonance imaging (MRI) is the imaging modality of choice in CP (compared to computed tomography (CT) or cranial ultrasound (cUSS)), as it is better able to identify white matter abnormalities, the commonest brain injury pattern in children with CP(104-106). Population studies that report combined CT and MRI imaging findings are likely to have under-reported the association of white matter injury (WMI) and CP(104, 106). The European Cerebral Palsy Study compared clinical and MRI correlates of CP (n=351)(105). The study identified WMI in 42.5%, basal ganglia lesions in 12.8%, cortical/subcortical lesions in 9.4%, malformations in 9.1% and miscellaneous lesions in in 7.1%. Normal MRI findings were identified in 11.7% of children with CP(105). WMI was the commonest lesion in children with diplegia (71.3%), followed by children with quadriplegia (35.1%) and children with hemiplegia. Although WMI is predominantly a preterm pattern of brain injury, 25% of children with this pattern on MRI were born at term(105). BG damage was predominantly associated with dystonic CP (75.6%). Focal infarction was almost exclusively associated with hemiplegia. Injury patterns described as

cortical/subcortical incorporated multicystic encephalomalacia and other cortical lesions. There was no specific motor distribution associated with this neuroimaging category (n=33)(105). Thirty two (9.1%) children had a cortical malformation. Hemiplegia is the most commonly associated motor pattern (n=12). Twenty five children (7.1%) had miscellaneous findings which did not fit the aforementioned categories. All motor patterns were represented in this category. Forty one children (11.7%) had a normal MRI. Half of the children with ataxia were in this group, however all CP subtypes were represented(105). Similar findings were reported in a recent study which reviewed neuroimaging findings in relation to gestational age at birth, CP subtype and GMFCS(107). Neuroimaging findings were combined across five regions (Western Sweden, South-West Germany, Quebec, California and Victoria, Australia). The authors report neuroimaging abnormalities in 86% of 1065 children with CP, similar to previous studies(104, 106). White mater injury (WMI) was the predominant neuroimaging pattern identified in children born below 37 weeks gestation. As in Bax et al(105), a significant proportion of term born children (≥ 37 weeks) also had predominant WMI (12-32%). Term children were more likely to have Grey Matter Injury (GMI) (21% vs 4-20% in preterm), focal vascular events(12% vs 5%) or cerebral malformations (13% vs 7%)(107). In relation to MRI pattern by CP subtype, children with spastic diplegia most commonly had WMI (31-60%). Spastic quadriplegia and dyskinesia was most commonly associated with GMI (34% and 21% respectively). Focal vascular events were most common in children with hemiplegia (24%) and cerebral malformations were identified in ataxic (18%), quadriplegic (16%) and hemiplegic CP (13%). Normal neuroimaging was most often associated with

ataxic sub-types (24-57%). WMI was the commonest neuroimaging finding across all GMFCS levels, with the exception of the most severely affected children (level V), who more commonly had GMI (36% vs 7-22%) or malformations (18% vs 8-12%). Children with GMFCS level I-II were most likely to have normal neuroimaging(107).

9.5.3.5 Neuroimaging to predict neurodevelopmental outcome

Recent research efforts have tried to establish the predictive value of abnormal neuroimaging on neurodevelopmental outcomes. A meta-analysis of neurodevelopmental outcome predicted by cranial ultrasound and MRI in preterm infants(108) used overall background risk of CP from the EPIPAGE Study(109) to calculate the positive predictive value (PPV) of abnormal neuromotor function for specific brain injuries. Using cranial ultrasound, the highest risk of CP was associated with Grade 4 IVH and cystic PVL, whilst Grade 3 IVH(110), ventricular dilatation and post haemorrhagic hydrocephalus conveyed a smaller risk of developing CP. Fewer studies assessing the relationship between MRI findings and neurodevelopmental outcome were available. Moderate to severe WMI predicted abnormal motor outcome with a pooled probability of 35% (95% CI 19-55%)(111, 112) and cognitive impairment with a pooled probability of 52% (95% CI 36%-67%)(112, 113). It was not possible to combine studies assessing the relationship between ventricular enlargement due to variation in diagnostic categorisation. In one study ventricular diameter of greater than 8 mm gave a PPV of abnormal neurodevelopmental outcome of 86% (95% CI 42-99%)(114). A second study showed that combined ventriculomegaly and WMI caused abnormal motor development with PPV of 55% (95% CI 23-85%)(113). One study suggested that with concurrent PVL, posterior limb

of the internal capsule (PLIC) abnormality predicts abnormal motor development with PPV 90% (95% CI 54-96%)(115), and with IVH a PPV of 78% (95% CI 40-96%)(111). A recent systematic review and meta-analysis concluded that the presence of moderate-severe WMI on MRI performed around term equivalent age best predicts CP and motor function in very preterm (<32 weeks) or low birth weight (<1500g) infants with sensitivity of 67% (95% CI 38-87%) and specificity of 92% (95% CI 85-96%)(116). In the future, improved ability to predict abnormal neurodevelopmental outcome through neuroimaging will enable researchers to best target appropriate groups of infants for early intervention trials.

9.5.3.6 Cause and timing

The cause of CP in a child is likely to be multifactorial. Emerging understanding regarding the interplay of risk factors and of the influence of timing of brain injury on the development of CP may help identify appropriate early intervention strategies. As knowledge of this area is still evolving, the current CP classification recommends that this domain is only assigned when there is firm evidence of causality and timing(91).

9.5.4 Making a diagnosis of cerebral palsy

A diagnosis of CP is based on careful clinical history and examination findings. Neuroimaging contributes useful information on aetiology and extent of brain injury. The typical clinical picture of CP becomes clear over time, early abnormal neurological signs suggestive of CP can be transient(117). Non-specific motor signs such as hypotonia may evolve into spasticity, dystonia or athetosis during the first 2

years of life as the brain myelinates(118). This changing pattern of neurological presentation during infancy has led some authors to recommend that a firm diagnosis of CP is delayed until after the age of 2 years(119, 120). The diagnosis of CP in some children is clear, however diagnostic uncertainty during infancy can make the identification and entry of children into early intervention trials extremely challenging.

9.5.5 Intervention for children with cerebral palsy

Currently, interventions for children with CP focus on maintaining mobility and optimising function through services such as physiotherapy, orthotics, occupational therapy, speech and language therapy and specialist education, symptom reduction (e.g. interventions for drooling, pain), and the prevention of secondary problems such as contractures, scoliosis and constipation. With the exception of therapeutic hypothermia for term infants with HIE(121), there are no currently identified treatments capable of limiting the extent of the brain lesion or of optimising brain repair and plasticity following perinatal brain injury. There is an urgent need to identify new therapies aimed at reducing the level of disability experienced by children with CP.

9.6 Neurocognitive impairment following perinatal brain injury

In children with perinatal brain injury who avoid severe motor impairment, neurocognitive deficits are well documented. Recent publications have focused on the medium to long term outcomes of children with perinatal brain insults, with particular interest given to the impact of neurocognitive ability on school attainment

and social functioning. The following sections will concentrate on the neurocognitive consequences of specific groups of children with risk factors for neurodevelopmental disability as included in the Dolphin study, namely children born very preterm, with preterm intra-uterine growth restriction (IUGR), or HIE.

9.6.1 Neurodevelopmental outcomes following preterm birth

Severe disability is particularly common amongst extremely preterm infants (infants born less than 28 weeks). The EPIcure study group reported the neurodevelopmental outcomes of infants born before 26 weeks gestation at 30 months; 49% were disabled, of which 23% had a severe disability at 30 months of age(122). On follow up at 11 years 17 % had CP. Moderate or severe impairment of neuromotor function was present in 10%, vision impairment in 9% and hearing impairment was identified in 2%. Forty per cent had serious cognitive impairment, compared to 1.3% of classmates, highlighting the serious lasting consequences of perinatal brain injury(123). Global cognitive deficits are present in as many as 45% of survivors of extreme prematurity (EP) (gestational age at birth less than 28 weeks)(123). Babies born at less than 32 weeks gestational age (very preterm) appear to have a specific neurocognitive profile(124, 125), including deficits in attention(126-129), global visual motion processing, visuomotor control, and spatial cognition(124, 125). ADHD is more common in infants born extremely preterm, however infants born moderately preterm are also more likely to develop ADHD. Risk is proportional to degree of immaturity and in the moderately preterm group is modified by social adversity as measured by maternal education level(130). Deficits in wider, executive function(127, 131), working memory(132), processing

speed(133), visuomotor skills(134), and mathematical skills(135) have also been identified. Deficits in these modalities are particularly relevant to the more subtle impairments affecting school achievement and everyday function and may be over and above any general intellectual disability.

9.6.2 Neurodevelopmental outcome and intrauterine growth restriction

IUGR is defined as a reduced fetal growth rate that results in birth weight below the 10th percentile for gestational age(136). IUGR is most commonly associated with placental insufficiency, which can be caused by a variety of placental pathologies, and is associated with abnormal umbilical artery flow on antenatal Doppler ultrasound studies. Placental insufficiency results in chronic fetal hypoxaemia, hypoglycaemia and elevated blood lactate levels(137), and is associated with increased morbidity and mortality(138). The majority of infants who suffer IUGR achieve catch-up growth by age 2 to 3 years(139), however approximately 10% do not achieve this(140). A number of observational studies have associated IUGR with adverse neurodevelopmental outcome. A recent systematic review examined neurodevelopmental outcomes of children aged 6 months to 3 years across 16 studies(141). The review concluded that there is indication of poorer neurodevelopmental outcome at ages 6 months-3 years in children following IUGR, however interpretation of the available data is hampered by heterogeneity in study design, outcome measures, in IUGR definition, with failure to distinguish between IUGR, small for gestational age (SGA) and extremely low birth weight (ELBW) infants, and lack of appropriate comparison groups. Levine et al (2015) recommended

further follow up studies employing standardised definitions, study designs and outcome measures, with the addition of neuroimaging(141).

It appears that the neurodevelopmental consequences of IUGR extend at least into later childhood, and likely beyond(141). Longer term neurodevelopmental follow-up of IUGR cohorts have identified lower verbal IQ and full scale IQ scores at 5-8 years(142), lower neurodevelopmental scores and IQ at 6 and 9 years(143, 144), lower academic achievement at 9 to 10 years(145), and higher levels of neuropsychological difficulties(144). Specific difficulties with verbal knowledge, reading and arithmetic are also reported in children with IUGR(144).

Recent brain imaging studies undertaken in IUGR infants have identified specific deficits in gray matter development at 12 months corrected gestational age (CGA) affecting the amygdala, basal ganglia, thalamus, and insula bilaterally, left occipital and parietal lobes, and right perirolandic area. Gray matter volume showed a positive correlation with birth weight. Aberrant white matter development was also identified, but appears to be a result of IUGR and prematurity combined, not IUGR alone(146). In a further study, regional brain volume catch-up growth was demonstrated in IUGR preterm infants who achieved body weight catch up by term equivalent age(147). Infants who did not achieve catch up growth had significant reductions in regional brain volumes, as well as maturational immaturities of white matter development in areas associated with attention, language, cognition, memory and executive functioning, Body weight and head circumference at term equivalent age (TEA) correlates with white matter development as assessed by FA(147).

9.6.3 Neurodevelopmental outcomes following term hypoxic ischaemic encephalopathy

In the absence of significant neuromotor problems, cognitive deficits, particularly in attention/executive functions, are also found in children following moderate-severe term hypoxic ischaemic encephalopathy(148-150). The impact of these deficits on school attainment and social functioning is likely to be significant.

9.7 Visual impairment following perinatal brain injury

Visual impairment is common in children with perinatal brain lesions(151). The literature has traditionally been divided into descriptions of visual impairment in children with CP, or description of the visual consequences of specific brain lesions occurring in the antenatal and perinatal periods. As with deficits occurring in other modalities, the specific pattern of visual impairment relates to the timing, nature and extent of insult.

Exploration of visual impairments following perinatal brain injury needs the context of typical infant visual development, as outlined in Section 9.9, with particular focus on the development of brain areas related to the visual outcome measures used in the Dolphin study.

9.8 Intervention for perinatal brain injury

Therapeutic hypothermia is the only known therapy that can lead to reduced mortality and morbidity in term infants with neonatal HIE(152, 153). Therapeutic hypothermia takes advantage of a short delay prior to the onset of cell death due to secondary energy failure following hypoxic-ischaemic brain injury. A recent meta-

analysis of three trials examining the combined outcome of death or disability concludes that there is reduction in the combined rate of death and severe disability following therapeutic hypothermia for term infants with HIE, with numbers needed to treat of nine i.e. 9 infants need to be treated to achieve therapeutic benefit in 1 infant(152). However this reduction in the combined outcome of death or severe disability was restricted to babies with moderate HIE. The value of therapeutic hypothermia for babies with severe HIE is yet to be ascertained(152). When mortality data from the combined outcome trials was analysed with data from seven further trials whose primary outcome was mortality alone, reduced mortality rates were again demonstrated with number needed to treat of 14(152). These findings were corroborated by a subsequent Cochrane review including data from 1505 babies born at greater than 35 weeks gestation(153). Neurologic and developmental outcomes at 6-7 years of infants treated with therapeutic hypothermia in the TOBY trial(121, 154) have demonstrated lasting benefit in the treated group compared with controls in IQ score greater than 85 (RR 1.31; p=0.04), survival without neurological abnormality (RR 1.61), risk of CP (21% vs 36% p=0.03) and risk of moderate-severe learning impairment (authors use term "delay") (22 vs 37%; p=0.03) and motor function scores. There was no difference in morbidity between the two groups at 6-7 years(155).

Despite the advances in neuroprotection afforded by therapeutic hypothermia, HIE associated mortality and morbidity remains significant, and so current research efforts are focused on the identification of adjunctive therapies that may improve the efficacy of therapeutic hypothermia, and so improve mortality rates and

neurodevelopmental outcomes in survivors of HIE. A number of therapeutic adjuncts have been considered including anticonvulsant therapies, anti-inflammatory, anti-oxidant agents and growth factors(25).

A recent review of potential adjuncts to therapeutic hypothermia has considered how close each potential agent is to clinical application(156). Agents administered antenatally and postnatally were considered, taking into account factors such as placental transfer, ease of administration, dosing knowledge, side effect profile, teratogenicity and toxicity data, and overall effectiveness. Antenatally, the most promising adjuncts to therapeutic hypothermia seem to be tetrahydrobiopterin and melatonin(156). Postnatally, melatonin, erythropoietin and N-acetyl cysteine were appear promising. Much interest has been generated in the combination of xenon, an anaesthetic agent, with therapeutic hypothermia.

The use of xenon in infants undergoing therapeutic hypothermia for HIE has recently been shown to have an anticonvulsant and electroencephalogram (EEG) suppressant effect(157). Seizures that occur as a result of hypoxic ischaemic injury are often resistant to standard anticonvulsant therapy and the authors propose that the use of xenon may play a role in reducing additional cerebral injury caused by ongoing seizure activity(157). A small feasibility study in 14 infants combining xenon with therapeutic hypothermia confirms the suppressant effects of xenon on seizures, with similar outcomes on Bayley Scales of Infant Development (BSID) II mental Development Index (MDI) and Psychomotor Development Index (PDI) scores at 18 months in infants treated with combination therapy compared to therapeutic hypothermia alone. Approximately 50% of infants in both groups had MDI and PDI

scores greater than 70, indicating normal outcome or mild impairment only. Importantly, no adverse effects of xenon therapy were identified(158). The outcomes of two randomised trials therapeutic hypothermia combined with xenon are awaited (TOBYXeNCT00934700 and CoolXenon2-NCT01545271).

9.9 Visual development

It is estimated that approximately half of the adult human brain is involved in processing information necessary to normal everyday vision. Infants must develop these functional networks through the course of brain development. The majority of this development occurs during the first two years of life, but continues within certain networks into later childhood and the early teenage years(18). Vision underpins the development of other key neurodevelopmental domains, such as motor and attentional development(159, 160). The visual system is easily accessible via a number of tests designed to tap specific visual networks, and provides a "window" on the developing brain(159). The following sections will concentrate on the development of the visual brain, and its disorders. Visual disorders which primarily affect the eye are not included. Particular attention will be given to the brain systems underpinning the visual outcome measures used in the Dolphin study.

9.9.1 Functional organisation of visual pathways

Figure 2. Cortical and subcortical pathways for vision.

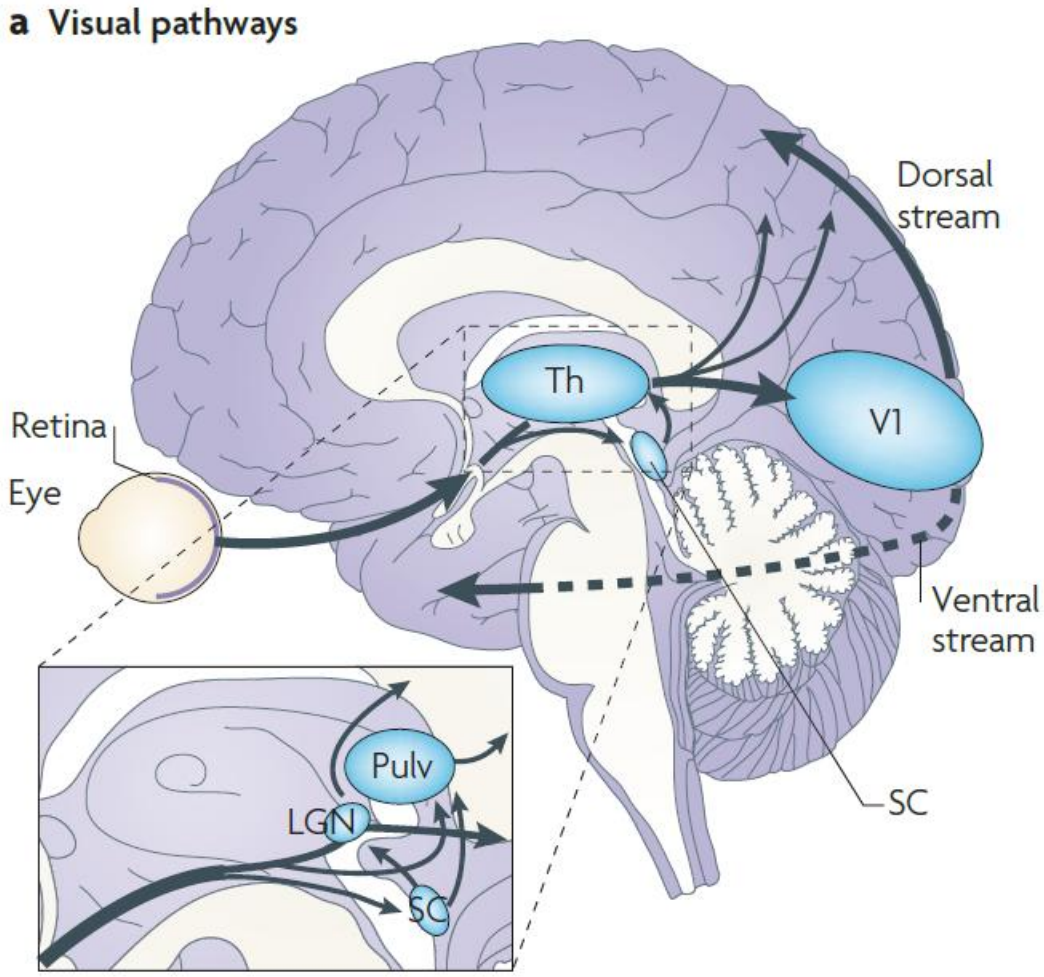


Figure 2. Cortical and subcortical pathways for vision. The primary visual pathway (shown by thick black arrows) originates from the retina and projects to the primary visual cortex (V1) in the occipital lobe via an intermediate station in the lateral geniculate nucleus(LGN) of the thalamus (Th). From V1, visual stream information reaches the extrastriate cortex along the ventral (occipitotemporal) and dorsal (occipitoparietal) stream. However, a minority of fibres originating in the retina take a secondary route (shown by thin arrows) and reach both the superior colliculus (SC) and the pulvinar (Pulv). These two subcortical sites are connected and also send direct projections to the extrastriate visual cortex, bypassing V1. Another V1 independent visual pathway consists of the direct projections between the superior colliculus and the LGN that in turn send efferents to extrastriate cortices in the dorsal stream(161). Reproduced with kind permission from Springer Nature.

The cornea and lens of the eye focus light rays on the retina. The retinal photoreceptors (rods and cones) produce an electrical response to the light falling on them, which is conducted to relevant brain areas by the optic nerves, via two distinct routes. Firstly, retinotectal pathways via the superior colliculus (SC) and other midbrain structures, control eye movements, especially saccades (sudden eye movements which shift gaze from one target to another, and smooth pursuit eye movements used when tracking moving targets. These pathways also control reflex optokinetic nystagmus (OKN), which becomes operational when a whole visual field is moving. In the mature visual system retinotectal pathways are integrated with more complex cortical visual systems, particularly the frontal eye fields (FEF) and posterior parietal cortex (PPC) via bidirectional connections. The geniculostriate route is via the lateral geniculate nucleus (LGN), a relay station in the thalamus. From the thalamus, signals reach the primary visual cortex (also known as V1, or striate cortex) in the occipital lobe. Functional distinctions between the two routes were identified by rodent studies showing that cortical injury impaired pattern discrimination whereas SC injury affected orienting responses(162, 163).
Newborn vision is under sub-cortical control for the first weeks and months of life, with cortical control starting at around 2 months of age(164). Newborn infants can orient to an appropriate visual or auditory stimulus using head and eye movements, suggesting a rudimentary “where” response, but show only the most rudimentary discrimination of pattern, for which later cortical input is required. The maturing cortex does not take over subcortical orienting responses, but rather modulates and refines them. Cortical functions do not emerge simultaneously, but as distinct

functional modules, served by distinct pools of neurons in V1 which encode responses to specific visual attributes such as slant or orientation, directional motion and binocular disparity(159, 165, 166). For example, in typically developing infants cortical orientation selectivity appears around 3 weeks to 3 months CGA (exact timing varies with stimulus properties), directional motion selectivity appears around 2-3 months CGA. Stereoscopic depth perception appears around 4-5 months(124). Pattern-reversal VERPs can be recorded from birth but do not necessarily indicate cortical processing of contrast change since pattern reversal stimuli also generate a neural response at the retinal ganglion level. However, the sequential appearance of specific neuronal signatures through the course of visual development has allowed the development of "marker tasks" to identify key early visual milestones. These marker tasks include, for example, identification of a neural response to orientation reversal using visual event related potentials (OR-VERP). OR-VERP and additional measures of visual cortical function used in the Dolphin Study will be further discussed in Section 9.11.

Evidence for cortical modulation of subcortical responses is derived from a number of sources. OKN is elicited by large field movement. In the newborn there is asymmetry of monocular OKN responses; OKN only occurs to motion in the temporal-nasal direction(167, 168). The nasal-temporal response does not emerge until 2-3 months. This asymmetry is a result of directional bias in the nucleus of the optic tract (NOT). In the neonate, direct pathways from each retina to the contralateral NOT can only drive the temporal-nasal OKN response. Later in development, descending cortical binocular neurons connect with the ipsilateral

NOT facilitating the nasal-temporal OKN response. The emergence of symmetrical OKN is considered evidence of cortical input to this subcortical sensory-motor loop(169). Indeed, infants undergoing very early hemispherectomy for intractable epilepsy caused by cerebral malformations never develop the nasal-temporal response that should have developed in the missing hemisphere(170, 171).

Further evidence of cortical modulation of subcortical processing is derived from studies of infant visual fixation shift behaviour. Newborn infants are able to make switches of visual fixation, provided that there is no competing target(172, 173). This process is mediated by the superior colliculus. When there is competition of visual stimuli then fixation shifts under "competition" (i.e. the original target persists whilst a novel target appears) require the infant to disengage attention from the original target in order to fixate that which newly appears, and does not appear until around the age of 3 months. This ability is mediated by inhibitory drive from the frontal eye fields, and indicates the development of a cortical system and/or its connection to the superior colliculus. Fixation Shift testing in the infants described above who underwent hemispherectomy at a young age can make shifts under competition to targets in the half-field of their intact hemisphere, but not in the other half-field served by the missing hemisphere(170).

9.9.1.1 Dorsal and ventral streams

Theoretical frameworks, supported by neuropsychological and neuroimaging studies, have been developed to test the hypothesis that beyond primary visual cortex (V1), visual processing divides into two anatomical and functional streams. Visual information carried in the *ventral* stream projects to the inferior temporal lobe,

whilst the *dorsal* stream projects to the posterior parietal lobe. The original model suggested that the ventral stream processes information regarding "who or what" is being looked at, whereas the dorsal stream interprets "where" that person or object lies in the environment and in relation to self(174). In planning and executing a goal based task, both systems must be operational. The ventral stream allows selection and planning of the goal based task, whereas task execution depends on the manipulation of spatial information and on-line navigation through the environment afforded by the dorsal stream.

A complementary model suggested two anatomically defined streams, distinct at retinal ganglion cell and LGN levels, which map onto the cortical divisions between the dorsal and ventral streams(175-177). Magnocellular (M) cells and Parvocellular (P) cells are morphologically and functionally distinct(178, 179). The fast responding M cells process low spatial frequency, high temporal frequency information, e.g. relating to course-scale structure of the image. They respond intermittently to visual stimuli and provide input for motion perception, spatial relations and the direction of actions. P cells process high spatial frequency information and have a sustained response to visual stimuli providing input to the discrimination of colour, texture, fine shape and pattern. Magnocellular and parvocellular projections to visual cortex remain initially distinct, and are referred to as magnocellular and parvocellular streams respectively. The magnocellular stream has projections to the primary visual cortex (V1) and to accessory visual cortex V2, V3, V5 (MT), which together form the dorsal stream. The magnocellular stream is closely related to the dorsal stream, and the parvocellular stream to the ventral stream. A number of studies have attempted

to isolate magnocellular and parvocellular responses using sine wave grating phase reversal VERP stimuli varying in temporal and spatial frequency(180, 181). As the magnocellular pathway responds to lower luminance contrasts and is most sensitive to higher temporal and lower spatial frequencies, and the parvocellular pathway is most sensitive to lower temporal and higher spatial frequencies, specific VERP stimuli can theoretically isolate magnocellular and parvocellular pathway responses. These methods have been used to assess the effects of preterm delivery on early visual development using VERP amplitude and latency as outcome measures. These studies consistently identify magnocellular (dorsal stream) deficits in preterm infants compared to term infants(180, 181). Although the differing contrast-response functions of magno- and parvocellular neurons may provide a means of isolating dorsal and ventral stream responses Skottun et al have demonstrated that similar contrast response functions, which in some cases give a better fit than magno- and parvocellular contrast-response functions, arise from other cortical structures, or a combination of these, concluding that it is very difficult to ascribe particular contrast response functions to isolated magno- or parvocellular activity(182).

The most widely accepted model of visual processing was described by Goodale and Milner (1992 and 2006) and proposes a ventral stream which processes perceptual information, and a dorsal stream which controls actions: the "perception-action" model (PAM)(183, 184). According to the PAM, real time information concerning an objects location and disposition which is required for manipulation and interaction with the object is processed in the dorsal stream. Information concerning the identification of objects and events, and the attachment of meaning to them is the

domain of the ventral stream. Much of the evidence in support of the PAM model comes from a patient, DF, who suffered bilateral anoxic damage to areas of the visual brain associated with ventral stream function, with resultant visual form agnosia, but intact dorsal stream function(185-189). Although there is broad acceptance of the principle of two distinct cortical visual streams, there remains discussion as to the extent to which these are autonomous. Many believe that whilst the two system model has served well as a framework for investigating visual processing, the specialisations of the two streams are relative, not absolute(190). Proponents of this theory provide evidence of ventral stream involvement at all levels of action planning and programming, with the dorsal stream responsible mainly for the fast online updating of movements and perhaps some forms of implicit object avoidance, as reviewed in Schenk et al 2010(190). Milner and Goodale accept that the functional independence of the two streams may in the past have been over-emphasized, but uphold that the two streams are distinct, being able to work together to produce adaptive behaviour(191). Further review of the literature relating to the degree of independence of the two visual streams is out with the scope of this thesis. Detailed review of the subject is provided by Schenk and McIntosh (2010)(190) and Goodale (2014)(191).

9.9.1.2 Dorsal and ventral stream development in infancy

In infancy, the dorsal and ventral stream and magno- and parvo- pathways which feed them appear to undergo differential maturation. The developmental relationship between the two streams is complex and differs in functional onset, rate of development and vulnerability(169). Initial development of the dorsal stream may

be slower than that of the ventral stream; direction sensitivity and binocularity associated with magnocellular-dorsal pathways develop later than orientation selectivity and colour discrimination mediated via parvocellular-ventral pathways(192). Later on in infancy, tests of global form and motion provide direct comparison between the two streams. Young infants are more proficient at detecting coherent motion patterns (dorsal stream) than global form (ventral stream) as identified by preferential looking techniques to global form and motion stimuli(193). These findings are corroborated by VERP showing reliable responses for motion but not form coherence in a majority of 4-5 month olds(194). Similarly, high density VERP studies mapping the spatial distribution of VERP responses showed that amplitude response to form coherence was approximately half that for motion coherence in 5 month old infants, and reached statistical significance in only 50% of infants(194). Considered together these data suggest that whilst local directional motion sensitivity develops later than for local orientation, global motion perception (dorsal stream) occurs earlier than global form perception (ventral stream)(169). By age 4-5 years, the relative maturity of the dorsal and ventral stream appear to reverse again, as demonstrated by higher and more variable motion coherence thresholds relative to adult values, than for form coherence as demonstrated using computer generated “ball in the grass” or “road in the snowstorm” stimuli(160). Thresholds for form coherence reach adult values slightly ahead of motion coherence; both have reached adult thresholds by approximately 10 years of age(195). Thresholds for global motion coherence also appear more susceptible to disruption than for global form coherence, as identified in a number of

acquired and genetic neurodevelopmental disorders, such as Williams and Fragile X syndromes, and in unilateral CP(196, 197). The relative dissociation of visuomotor and perceptual vision deficits in these groups of children has led to the proposal of "dorsal stream vulnerability" as a common feature of neurodevelopmental disorders(125).

A randomised trial of dietary enrichment (including increased DHA intake) in very low birth weight infants has identified stronger motion VERP responses obtained close to the posterior midline region in f1 and f3 VERP components in the treatment group compared to controls(198). These findings support the theory that dorsal stream development may be particularly sensitive to DHA supply(198). Given the higher prevalence of dorsal stream vulnerabilities amongst preterm infants, and the increased risk of DHA deficiency in those infants born before or early in the third trimester of pregnancy, it will be imperative to establish the contribution of DHA insufficiency to dorsal stream vulnerability, whilst assessing the neurodevelopmental impact of dietary provision of nutrients required for healthy brain development.

9.10 The development of attentional systems

The development of attention underpins a child's ability to achieve in everyday and academic life. Problems of directing and maintaining attention are more common in children following early brain injury, preterm birth, and in some developmental disorders of genetic origin e.g. Williams syndrome(129). Attention harnesses high level cortical networks, which can influence numerous brain processes including early sensory systems, such as vision(199). Neuropsychological models provide evidence for three distinct attentional subsystems: selective attention, sustained

attention, and attentional (executive) control(200, 201). Processes of selective attention are mediated by the parietal lobes, connecting with frontal eye fields and the superior colliculus, and are responsible for orienting in space. Sustained attention is the domain of the parietal lobes, right frontal cortex and locus coeruleus. Attentional control or executive function is governed by bilateral frontal lobes and the anterior cingulate cortex(129). Attentional networks are closely related to the extended dorsal stream of cortical visual processing(129, 202). Children who display dorsal stream mediated visual processing difficulties, as a result of perinatal brain injury or certain developmental disorders, often have accompanying attentional deficits(129).

9.11 Measures of visual cortical function in the newborn and infant

The following sections outline neurophysiological and behavioural tests of cortical visual function in infancy, as used in the Dolphin study. None of these tests depend upon good acuity or contrast sensitivity, however children participating in the trial were screened for normal acuity, measured binocularly with Teller Acuity cards as part of the Atkinson Battery of Child Development for Examining Functional Vision (ABCDEFV)(203).

9.11.1 Visual event related potentials

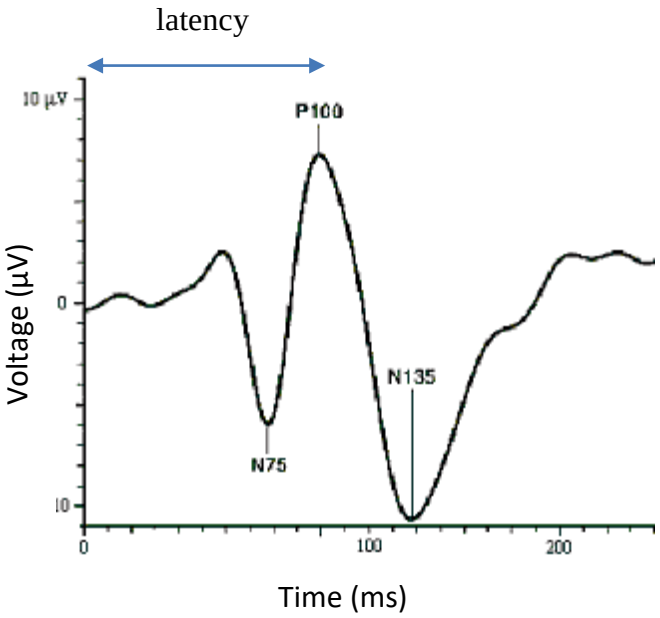
VERPs are used clinically to assess the integrity and maturity of the visual system in infants(204). They can be measured from the occipital scalp using a simple montage of 3-4 electrodes. Pattern or phase reversal (PR) stimuli are typically used to assess cortical response to contrast change using alternating luminance checkerboard, sine

or square wave stimuli. Unique VERP stimuli have also been developed to test the emergence of specific cortical functions such as orientation and direction of motion. The following sections will outline detail about the VERP measures used in the Dolphin study.

9.11.2 Pattern (phase-reversal) Visual Event Related Potentials

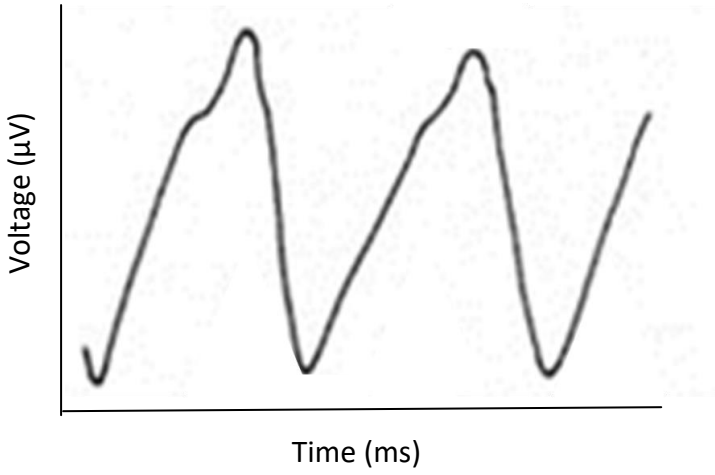
The VERP waveform matures rapidly during the first months of life(204-207). The most consistent feature is the major positive peak P_1. The time interval between VERP signal onset and appearance of the P_1 peak is referred to as *transient* latency, and reflects the latency between VERP signal onset and activation of the relevant cortical neuronal pool in occipital cortex i.e. the time taken for initial retinal processing, transmission via the optic nerve, optic tracts and radiation leading to activation of visual cortical cells in V1 (Figure 3).

Figure 3. Measurement of transient pattern-reversal visual event related latency using P_1 peak



Whilst a PR-VERP response indicates that contrast change has been detected at cortical level it may not reflect cortical processing per se(208). Transient latency therefore primarily reflects the time required for subcortical visual processing, with some additional more limited cortical component. Transient latency is around 260 ms in newborn term infants, falling towards an adult latency of around 100 ms by the age of 6 months(204-206). This rapid fall in latency is likely to result from a combination of retinal development(209), progressive myelination of the optic nerve and radiation(210, 211), exuberant cortical synaptogenesis and maturation of synaptic transmission within the visual pathways(22). The study of Dubois et al showed a direct, and strong correlation between MRI measures of myelination of the optic radiation, but not of other cerebral tracts, and the developing P_1 latency, suggesting a substantial pre-cortical contribution to transient latency(210). The P_1 peak is only identifiable at slow reversal rates, typically 1-2 reversals per second (r/s). At faster reversal rates (above 4 r/s) the P_1 peak is no longer visible and overlapping responses produce a *steady- state* waveform (Figure 4).

Figure 4. Steady-state waveform



Studies measuring transient latency report the time taken to produce the first prominent peak in the VERP waveform. This method is vulnerable to age-related variation in the shape, number and latency of the P_1 peak, which can make interpretation problematic(206, 212). An alternative latency measure can be obtained by plotting the phase of two or more temporal frequencies (TF) against TF. This method can utilise faster reversal rates which have the advantage of a much shorter recording time. The slope of the phase-TF plot provides a *calculated* latency(208, 213, 214). Phase values cycle every 360° therefore any given value of phase could also correspond to that value plus or minus 360°. Accepted practice is to "unwrap" subsequent phase values by multiples of 360° to achieve "maximum orderliness" or minimise the distance to the preceding point, although there is no rigorous published criterion for this procedure(215, 216). Calculated latency is thought to reflect the timing of the whole VERP waveform, unlike the P_1 peak which reflects arrival of the VERP stimulus signal at occipital cortex. Calculated latency is prolonged compared to transient latency in young infants, and takes longer to reach adult calculated latency values (around 30 weeks), compared to transient latency (around 15 weeks)(217). Delay in reaching adult calculated latency values may reflect maturation of cortical processing networks beyond V1(217). Calculated latency better reflects cortical visual processing time than transient latency(217). Since the phase of the steady-state waveform is determined by the overall temporal properties of the evoked response, including components later than P_1, it will reflect the timing of the whole cortical response, not just the initial neural barrage reflected in P_1. Thus, compared to transient P_1 latency, the calculated latency from steady-

state phase will show a stronger contribution of the course of cortical processing after the arrival of impulses at the visual cortex. The difference in the developmental time course for the two measures(217), and indeed the different effects of perinatal brain injury found in the Dolphin cohort, support the idea that transient and calculated latency reflect, at least in part, different underlying neural processes. Comparison of the two methods may better expose any differences in the maturation of different levels of visual pathway processing(217).

9.11.3 Orientation reversal Visual Event Related Potentials

Orientation reversal VERPs (OR-VERP) reflect cortical processing of slant (orientation) and first emerge in typically developing and preterm infants without overt brain injury between 3-6 weeks of age depending upon the stimulus reversal rate used(218, 219). As orientation selectivity is known to arise in cortical cells, but not sub-cortical structures, OR-VERP responses can be used as a marker of cortical functioning(169). Abnormal OR-VERP responses have been identified in infants with generalised brain injury following HIE tested in the first 5 months of life(220, 221), and in infants with preterm (less than 33 weeks gestation at birth) brain injury (218). In a series of 46 term infants with HIE and/or lesions on brain MRI, PR- and OR-VERP (8 or 4 r/s) responses correlated with MRI brain injury pattern and were predictive of neurodevelopmental outcomes up to age 3 years (221, 222). Identified brain lesions were focal (e.g. focal infarction or haemorrhage) or generalised, as typically seen in moderate to severe HIE. Involvement of the basal ganglia was consistently associated with abnormal VERP responses, however lesions affecting the optic radiation and occipital cortex was variably associated with VERP abnormalities.

Children with normal VERP responses had normal neurodevelopmental outcomes. Persistently abnormal VERP responses across serial testing was associated with moderate or severe neurodevelopmental impairments and cerebral palsy, with abnormal visual function in the majority with abnormal VERPs(221). In infants born preterm (less than 32 weeks) brain MRI lesions were graded as normal/mild, moderate or severe according to the extent of injury to the white matter, basal gangila/thalami and brain parenchyma. Brain injury severity correlated with significant OR-VERP, far fewer children in the moderate and severe group (43 and 30% respectively) had normal OR-VERP responses. OR-VERP response predicted a Griffiths developmental quotient of less than 80 with sensitivity 86%, specificity 65%, positive predictive value 50% and negative predictive value 92% age 2 years (223). OR-VERP was measured as a marker of cortical function in infants participating in the Dolphin study.

By 5 months of age, typically developing term-born infants can detect global motion and form pattern(194). Infants born very preterm have identified global motion processing deficits(224). This finding is consistent with the identified dorsal stream vulnerabilities following very preterm birth (see section 9.9.1.2). However, VERP responses to global form and motion were not included in the Dolphin study visual assessment schedule as it was felt this would place too high an assessment burden on participants.

9.11.4 The Fixation Shift test

The procedure used in the FS test was Forced Choice Preferential Looking, where the 'blind' observer has to choose from the infant's eye and/or head movements

whether the infant makes eye and/or head movements to the peripheral target when it appears. The Fixation Shift (FS) test assesses the integrity and maturation of networks in frontal and parietal brain regions concerned with attention switching(225). The FS test assesses an infant's ability to shift their gaze from a central target to a novel peripheral target. Under conditions of non-competition (FSNC) the central target disappears prior to onset of the novel target. Under conditions of competition (FSC) the central target remains whilst the peripheral target appears. The assessment of direction of eye movements and the measurement of latency is objective, since the observer does not know on which side the computer has generated the target. Figure 5 shows a diagrammatic representation of the FS stimulus used in the Dolphin study. Figure 6 shows a study participant undergoing FS testing.

Figure 5. Diagrammatic representation of the Fixation Shift stimulus.

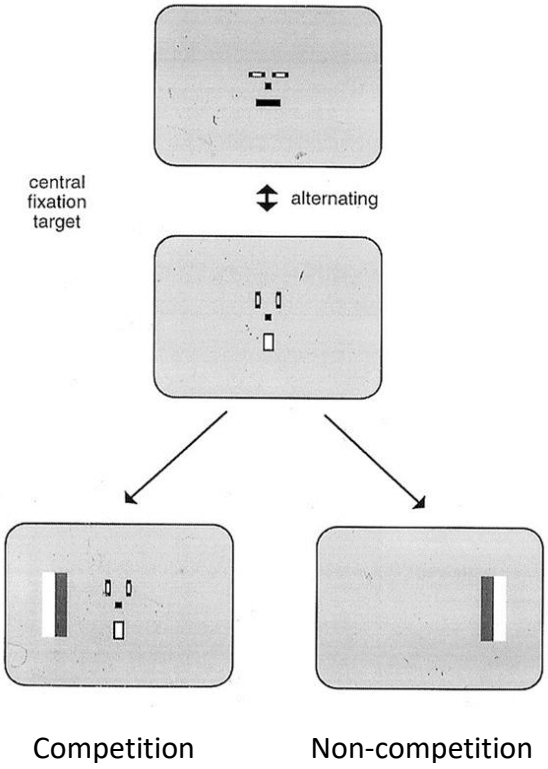


Figure 6. A study participant undergoing Fixation Shift testing.

FSNC are predominantly controlled by sub-cortical structures, largely the superior colliculus. Healthy term infants successfully make these switches days after birth, and with increasing speed over the first few weeks of life. FSC are mediated by cortical networks involving the primary and supplemental visual cortex, posterior parietal cortex and the frontal eye fields, networks closely related to the dorsal stream (Figure 7).

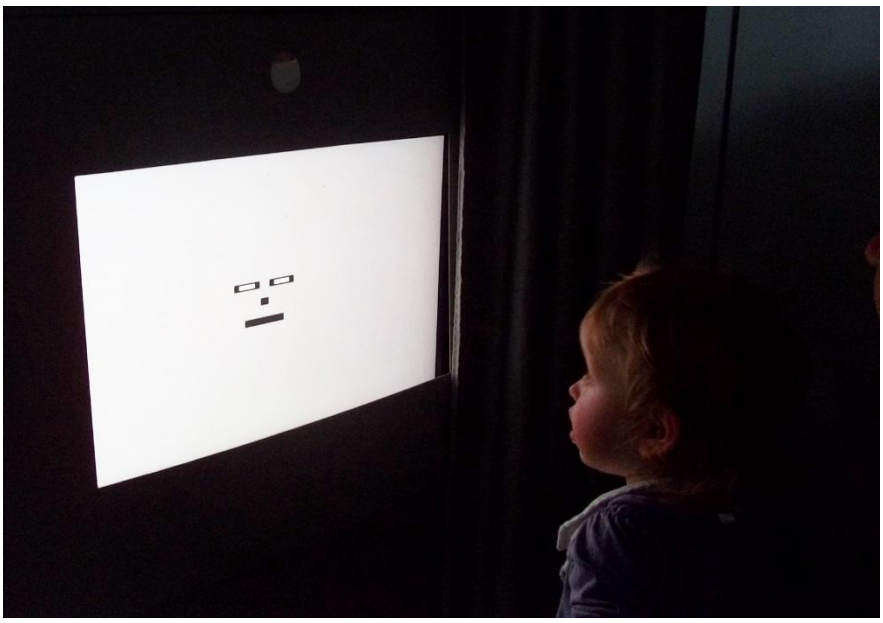


Figure 7. Simplified diagram of the pathways involved in the control of fixation shifts.

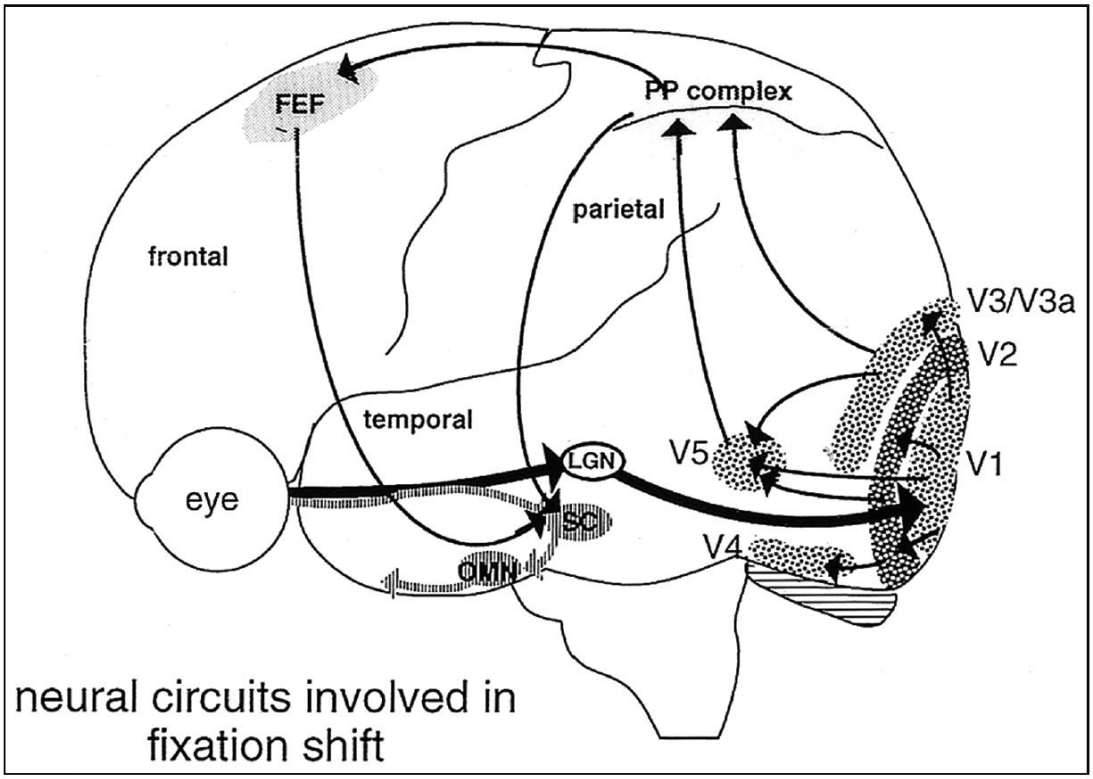


Figure 7. The direct route from the retina to the superior colliculus (SC) and oculomotor nuclei (OMN) allows young infants to orient towards a salient stimulus and maintain fixation upon it. The cortical visual network develops (areas V1, V2, V3/V3a, V5 and others not shown) and provides inputs to the posterior parietal (PP) complex and frontal eye fields (FEF), which allow the subcortical pathway to be modulated (e.g. by inhibition via the caudate nucleus and substantia nigra (not shown)). Reproduced with kind permission by Oxford University Press in Chapter 8, Janette Atkinson (Ed) 2000. The Developing Visual Brain. Oxford University Press (159). Oxford University Press is exempted from any Creative Commons-style license that would allow onward reuse of their content, permission for re-use must be sought from OUP: www.oup.com.

Healthy term born infants confidently make fixation shifts under competition by 3 months of age. Infants with PBI perform less well on FS testing than typically developing infants (TDI), with worst performance in FSC, identifying early attentional deficits in infants with PBI. FS performance predicts neurocognitive outcome in

infants with perinatal brain injury including focal brain injury(226), HIE(220, 222), and PWMI(223). FS performance measured as the percentage of correct re-fixations under non-competition and competition, and FS latency under non-competition and competition were used to compare sub-cortical and cortical function of infants in the Dolphin control group to infants in the Dolphin treatment group.

Recently, new infant friendly eye-tracking technologies have become available. Eye tracking is well suited to infant studies as it is non-invasive, can be used in non-verbal groups and has high test-retest reliability for individual differences in the infant age range(227, 228). Eye tracking techniques have been used in a number of infant studies including in the exploration of social orienting in preterm infants and emotion processing(227, 229, 230). Recently, eye tracking was combined with the Fixation Shift test to assess infant fixation latency(231). Combining eye tracking with this established assessment of early attention disengagement removes observer response time from recorded latency measures, improving latency accuracy. Recording time is reduced and so a greater number of trials can be delivered in a shorter time improving test efficiency and yield. Furthermore, it is possible to assess accuracy of infant saccades to the target, which improves with age. This is not possible with observational techniques(231). Although eye tracking techniques produce shorter latencies than observational methods, the overall pattern of longer latencies under competition compared to non-competition remains the same, confirming that observational methods remain valid. Eye tracking was not available to the group at the onset of the Dolphin study, and a consistent methodology had to be used over the period that new infants were recruited into the study. Although eye

tracking would introduce higher precision, latency comparisons between competition and non-competition, and between infants, are valid given the use of a common, objective observational method throughout the study.

9.11.5 Assessment of early functional visual development

Integration of dorsal/ventral stream and attentional processes are necessary for the acquisition of typical infant developmental milestones, such as reaching and grasping and locomotion, which are visually guided. Atkinson has developed a model describing how these systems may overlap through early development, underpinning the acquisition of key developmental milestones (Figure 8).

Figure 8. Model of visual spatial development in the first year.

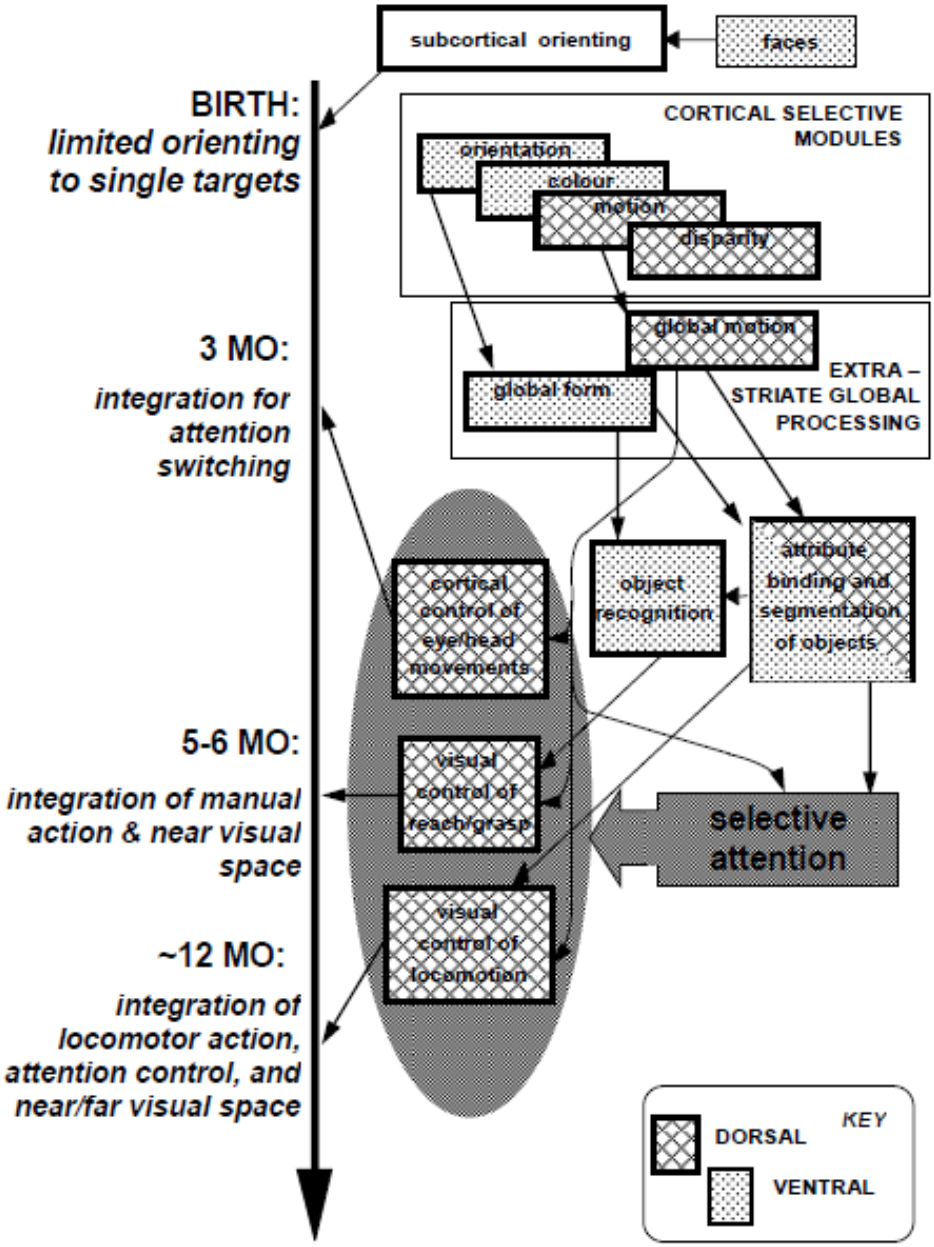


Figure 8. Model of visual spatial development in the first year, with behavioural milestones and brain processes in dorsal and ventral streams which become functional at different stages of development. Developed from Atkinson 2000(159) and Atkinson & Braddick 2003(160). Reproduced with kind permission from Oxford University Press in Chapter 12 in P. D. Zelazo (Ed) 2013. Oxford Handbook of

As well as using neurophysiological markers of visual cortical development, infant visual function can also be assessed using a behavioural approach. The Atkinson Battery of Child Development for Examining Functional Vision (ABCDEFV) assesses perceptual, motor, spatial and cognitive skills and has been standardised for children from birth to five years developmental age(203). The battery is divided into core vision tests (requiring no verbal or motor response beyond saccadic eye movements), and subsequent age-specific tests of visuomotor, cognitive and spatial functional visual behaviour, and are underpinned by the visual developmental processes summarised in Figure 8. Core tests include assessment of strabismus, pupillary responses, nystagmus, tracking eye movements, attention at distance, visual fields and screening visual acuity using a shortened version of the Teller Acuity Cards(232). The age-specific tests of visuomotor, cognitive and spatial function specifically assess visual functions associated with the dorsal and ventral streams and their interactions e.g. picking up fine cotton thread, copying block construction, and detection of embedded figures. An outline of the tests involved are briefly outlined in Appendix 1. Readers are directed to the original manuscript for more detailed information(203).

The ABCDEFV has been used to identify visual deficits in a number of clinical groups; preterm infants who showed abnormal OR-VERP responses also demonstrated failures on components of ABCDEFV between ages 1-5 years, which correlated with neonatal MRI brain injury severity(124). Failure on multiple subtests of the ABCDEFV

were also identified in a study of the functional visual abilities of 73 children with Williams syndrome(233).

Even in typically developing infants functional visual assessment is challenging due to variability in state of alertness, especially in newborns and young infants, interest in the task and sustained attention skills. Attentional difficulties are common following very preterm delivery and perinatal brain injury and may make it difficult to establish whether or not poor performance is due to functional visual impairment or is secondary to emergent attentional difficulties.

9.12 Cerebral visual impairments in children with perinatal brain injury

Cerebral visual impairment (CVI) describes a wide range of visual deficits resulting from abnormalities of the retro-chiasmal visual pathways and visual association pathways (extra striate occipital areas and functionally identified parietal and temporal areas), in the absence of ocular causes. As such, it includes diverse deficits of visual processing. Such difficulties may occur in isolation, or be accompanied by refractive errors or anterior visual pathway abnormalities(234). CVI is now the commonest cause of visual impairment in developed countries(235). CVI is common in children with PBI. Despite this, perceptual visual impairments are under-recognised(236, 237). The nature of the visual impairments experienced depends upon the aetiology of the underlying brain lesion and its anatomical distribution. The typical visual phenotypes presenting in children with PBI are considered below, in relation to aetiology and lesional anatomy. It is worth noting however, that functional deficits are often seen in the absence of specific neuroimaging abnormalities, presumably reflecting insensitivity of current neuroimaging protocols

to microstructural neuronal disruption. Functional neuroimaging and tractography may be better able to help define the functional anatomical correlates of visual impairments in children following PBI(238, 239).

9.12.1 Visual impairment in cerebral palsy

Visual impairment is common in children with CP(240-242), by virtue of the fact that the same brain injury which results in the motor deficits of CP also commonly involves the visual pathways. The range of visual impairments seen in children with CP is broad and can encompass any of the components of the visual pathway(240). Peripheral problems may be related to abnormalities of the anterior visual system resulting in strabismus, errors of refraction or retinopathies/fundoscopic abnormalities, while central involvement leads to problems such as amblyopia, delayed visual maturation or cerebral visual impairment (CVI), as summarised by Fazzi et al(240). It is important to note that although strabismus is often manifest peripherally in abnormalities of eye alignment, its causes are often central in origin. CVI refers to “a visual deficit caused by damage to, or malfunctioning of the retrogeniculate visual pathways”(243). Visual information is processed at different cortical levels and so CVI may result from damage to the optic radiations, occipital cortex (primary visual cortex), or visual association cortices(240), leading to more complex cognitive visual disorders.

In a population based cohort of children with CP taken from the Quebec Cerebral Palsy Registry, almost half of the children had some form of visual impairment; the severity of visual impairment directly correlated with severity of motor impairment(241). The specific motor patterns of CP have been linked to specific

patterns of visual deficit(240). In a study of 129 patients with spastic CP and CVI children with diplegic CP were found to have a high prevalence of refractive errors (75%), strabismus (90%), saccadic abnormalities (86%), and impaired visual acuity (82%). Almost 40% of this group of children had abnormalities of visuocognitive function. In children with hemiplegia, whilst strabismus and refractive abnormalities remained common (71% and 88% respectively), oculomotor involvement was less common (59%) than in diplegic children. Strikingly, visuocognitive dysfunction was very rare (6.6%). Children with tetraplegia had the most severe visual phenotype; ocular abnormalities (98%), oculomotor dysfunction (100%) and low visual acuity (98%) were typical. Visuocognitive testing could not be performed in this group due to the severity of their neurological and visual abnormalities(240).

9.12.2 Visual impairment in preterm white matter disease

Children born preterm without overt brain injury have a potential advantage over infants born at term but tested at the same corrected gestational age, as the preterm born infants have had more time in a visually stimulating environment. There is some evidence that this may be true from VERP acuity studies(244), however this is not backed up by studies using behavioural acuity measures(245). Maturation of OR-VERP responses in preterm infants are comparable to that of term infants, however direction-selective VERP responses develop several weeks later than in term born infants tested at the same corrected gestational age(246) and may be early evidence of dorsal stream vulnerability(169).

Prematurity is a risk factor for both ophthalmological disorders and cerebral visual impairment, in addition to wider deficits of cognition and attention. Visual acuity has

historically been the most studied measure of visual function in prematurity; the incidence of low acuity in infants with periventricular leucomalacia (PVL) is as high as 60%, although the measures of acuity which have been used may also involve visual attentional factors(151). Acuity is most abnormal in children with PVL grades 3 and 4, according to the classification of de Vries et al(247). The severity of visual impairment in children with PVL also relates to lesions of the peritrigonal white matter, optic radiations and occipital cortex involvement(248, 249). Thalamic atrophy also contributes to the severity of visual impairment in children with PVL(250). In recent years, neuropsychological testing has identified a specific profile of dorsal stream visuocognitive deficits, giving rise to visual -spatial and visual-motor integration difficulties(124, 251). Although abnormalities of dorsal stream function predominate in preterm children with PVL, a number of recent studies in older preterm born children with PVL have also identified a number of visual perceptual difficulties associated with functions of the ventral stream(251, 252). These visual perceptual difficulties manifest as difficulties in object recognition, and as an inability to identify objects presented in a difficult-to decode manner. In contrast, letter and face recognition and gestalt perception were relatively unimpaired(252). Ventral stream deficits were unrelated to attentional deficits, demonstrated by performance on a sustained attention task. These findings are in contrast to earlier reports of a predominantly dorsal stream vulnerability following preterm birth(124, 125, 252). These discrepancies can in part be explained by the older age at which visual perceptual deficits were identified; dorsal stream functions are easier to assess in younger children than are the more complex perceptual functions of the ventral

stream(252). Furthermore attentional deficits may be related to particular components of attention such as executive function and selective attention rather than sustained attention per se. Many ventral stream functions emerge later in childhood and so deficits can only be identified at an age at which these functions should be established(253). Co-existing dorsal and ventral stream difficulties may also indicate a higher degree of co-operation between the two streams than previously thought, with deficits in one stream contributing to deficits in the other(124). This possibility is substantiated by the identification of disordered visual imagery processing in the cohort reported by Fazzi et al, as visual imagery processing is thought to require a high level of integration of visual brain areas(252). A classic PVL phenotype was identified in the cohort of Fazzi et al, with more severe involvement of peritrigonal white matter, and frequent involvement of the optic radiations(252). Parietal lobe involvement (consistent with dorsal stream involvement) was identified in a number of the children. Interestingly, no temporal lobe abnormalities were identified, despite the ventral stream difficulties demonstrated. This is consistent with the findings of Atkinson et al(124). The lack of positive imaging findings despite identified ventral stream deficits may be a result of limitations to current imaging techniques, or may be better explained by a loss of integrity of complete brain networks serving these visual functions, not necessarily visible on structural brain imaging(252). Indeed, DTI studies have identified abnormalities of white matter organisation in preterm children(254, 255), and related FA of white matter in the optic radiations to performance on visual testing

independent of the identification of structural brain lesions on conventional MRI(256).

9.12.3 Visual impairment in term hypoxic ischaemic encephalopathy

HIE commonly causes visual impairment, although the occurrence of visual impairment is difficult to predict from the severity of HIE alone(220). Visual outcome is most difficult to predict in those children with moderate (grade 2) HIE, whereas severe (Grade 3) HIE is commonly associated with severe visual impairment. Children with mild (Grade 1) HIE often have normal visual function as tested in infancy(220). MRI studies have shown that severity of visual impairment is more closely associated with involvement of the BG than to lesions of occipital cortex or optic radiations alone. Severe visual impairment is particularly associated with involvement of the posterior putamen(257). These findings suggest that the integrity of connections between the BG and visual cortical areas are necessary to the development of normal visual function(257). Isolated BG lesions may be associated with abnormalities of vision shortly after birth, which tend to resolve by one year of age(257).

9.12.4 Visual impairment in perinatal stroke

The visual outcome of children who sustain neonatal cerebral infarction is far harder to predict than ischaemic stroke sustained later in childhood or adulthood(257, 258). Ocular movements and visual acuity are usually normal, however tests of visual attention (Fixation Shift test) and visual fields may be abnormal. When children were tested at school age many of the earlier identified visual deficits had normalised,

consistent with theories of early brain plasticity. Persistent visual abnormalities were more common in children with concomitant hemiplegia, indicating more extensive brain injury in the territory of the middle cerebral artery(258).

9.13 Clinical assessment of cerebral vision in children with neurodisabilities

The assessment of vision in children with neurodisabilities is complex and accurate profiling of impairments and functional consequences requires experienced multidisciplinary assessment(259). Assessment may be complicated by other features of the child's condition, such as motor, communication and learning difficulty. An appreciation of the child's overall developmental level is key to contextualising the findings of visual examination, as is an understanding of non-visual aspects which may impact on visual behaviours. Optimising factors such as comfort and positioning to enable optimal responses is also important.

Much important information can be gained from careful history taking, including current parental concerns and expectations, and current visual skills. Understanding parental expectation can help to clarify report of current skills, as these may be reported according to parental hope, or to misunderstanding of a report of "normal eyes" following ophthalmological assessment. Salt et al outline a useful framework for establishing current visual skills by parent history(259). A number of teams assessing visual ability in children with complex neurodisability have created structured questionnaires to aid the identification of visual problems in children with neurological disability(260, 261). These are perhaps most usefully considered as a starting point in the history taking process with clarification and elaboration as appropriate during the consultation.

Assessment should always include a detailed ophthalmological examination by an experienced orthoptist or ophthalmologist. However, children with a normal eye examination may still have significant visual impairment as a result of disruption of the visual pathways beyond the optic chiasm, and it is important to communicate this to the child's parents. Measurement of visual acuity assessing the child's ability to resolve detail necessitates use of age appropriate tools. Careful behavioural observation is important to aid understanding of how a child is using their vision to engage with their environment. Electrophysiological assessment using flash or PR-VERP can also provide useful information regarding the integrity of the visual system, and visual acuity. Brain MRI may contribute useful anatomical correlates of visual function. Children who are identified to have severe visual impairment (SVI) require immediate referral to the Specialist Teaching team for children with VI(259).

Once a child's visual profile has been described it is important to share this information with parents and educational professionals, with the inclusion of advice on ways to promote each child's developmental progress. Evidence suggests that appropriate visual promotion improves developmental outcome in children with severe VI(262).

9.14 Effect of visual impairment on general development

Visual impairment (VI) has a substantial impact on early neurodevelopment with constraints across all domains. Specific neurodevelopmental scales for children who are blind or partially sighted have been developed, and are currently being updated(263).

Children with VI are a heterogeneous group, and up to two thirds have additional impairments which may contribute to their developmental trajectories(264). Separating the contribution of VI and additional impairments to a child's developmental progress is challenging.

In an attempt to define the specific impact of VI on neurodevelopmental outcome Cass et al studied only children with disorders of the peripheral visual system (globe, retina, or anterior optic pathway). They excluded children with peripheral visual disorders associated with more widespread CNS involvement (e.g. retinal dystrophy associated with Joubert's syndrome; a brain malformation syndrome including cerebellar hypoplasia). This provided a cohort of children with "potentially simple" peripheral visual disorders, free from the confounders associated with VI of cerebral origin(265).

Level of VI was also quantified. Traditionally visual acuity assessed using Snellen charts or logMAR standards has been used to categorise children and adults as "blind" or "partially sighted". Preferential looking has been used in infants too young to match optotypes. For infants and preschool children with visual acuity below the lower limits of these scales the Near Detection Vision (NDV) scale can be used(266). The presence or absence of "form" vision discriminates between severe VI (SVI) and profound VI (PVI) respectively. Children with PVI (PVI- awareness of light, or light reflecting objects, no form vision) are most at risk of profound developmental impairments, and a sub-group experience developmental stasis or regression during their second year of life(267). In particular, autism spectrum disorders are prevalent in this group(259).

9.15 The role of nutrition in brain growth and neurodevelopment

9.15.1 Nutrition and the brain

The second and third trimester of pregnancy and first 2 years of life represent a period of rapid brain growth and development, involving complex processes of neuronal proliferation, axon and dendrite growth, synapse formation and pruning, myelination and apoptosis. These processes form the bedrock of later cognitive, social-emotional and motor functioning. Following birth brain development is further refined by experience, sensory stimulation plays an important role in shaping neuronal connectivity and functioning(268). Adequate nutrition, both at macronutrient and micronutrient level is essential for the progression of normal brain development.

Head growth is a proxy measure of brain growth, but does not indicate whether growth in all areas is symmetrical. Cheong et al have established the link between head circumference and developmental outcome(269). Adequate brain growth and development relies upon the combination of appropriate macronutrient and micronutrient supply.

9.15.1.1 Macronutrient intake and brain growth

Brain development is an extremely energy costly process. Whereas the adult brain consumes 20-25% of resting metabolic rate (RMR), the newborn brain consumes 87% of RMR, falling to around 44% by the age of 5 years(19, 270). The achievement of adequate brain growth depends upon the provision of adequate dietary energy, initially via maternal diet and later via infant diet.

A small number of randomised control trials have examined the effects of protein-energy supplementation on the developmental outcomes of malnourished children, as reviewed by Prado et al(271). These studies have shown conflicting results most probably due to variations in timing and duration of supplementation, age at follow up and method of developmental assessment used. Two of these trials(272-279) and one other(280) have reported long term follow up data. The first of these randomised pregnant Guatemalan women to a high protein and energy drink with micronutrients or low protein and energy drink with micronutrients. Offspring were supplemented until 7 years according to randomisation group. Follow up was reported at 11-18 years, 22-29 years (women) and 26-42 years (men). At 11-18 years those in the high energy and protein group had higher scores in maths and knowledge tests than those in the low protein and energy group. Beneficial effects on vocabulary and reading were identified in those supplemented before 2 years of age(272). Women in the high energy protein group had better reading and IQ scores at 22-29 years of age, whilst men in the high energy protein group had a 46% increase in average wage at 26-42 years compared to men in the low energy protein group(273, 274). A trial conducted in Jamaica randomised growth retarded children aged 9-24 months to supplementation with high protein and energy milk, psychosocial stimulation or both supplementation and stimulation for a two year intervention period. Follow up was reported at 33-48 months, 7-8 years, 11-12 years and 17-18 years. At 33-48 months the combined intervention group had a higher Griffith's Developmental Quotient score and higher scores on the locomotor and performance subscales(275). Disappointingly these developmental advantages were

not visible upon testing in the later age groups, only the psychosocial stimulation group demonstrated sustained benefit on IQ, language and reading ability across age groups(278). Most recently, the impact of high energy-protein supplementation from 20 weeks gestation until delivery versus supplementation through lactation until 20 weeks post-partum in rural Gambia has been reported. Supplementation in pregnancy and supplementation during lactation gave comparable outcomes for educational ability and cognitive performance at 16-22 years, however there was no non-intervention group for comparison(280). In recognition of the failure to thrive commonly seen in children with perinatal brain injury, Dabydeen et al conducted a small randomised control trial of energy and protein supplementation (120% recommended average intake (RAI)) versus average intake (100% RAI) in children with perinatal brain injury. Supplementation was daily for 1 year. The study was terminated after 16 participants had completed the protocol as the predetermined stopping criteria of change in head circumference greater than 1 SD in the 120% RDI group was met. Those in the high protein energy group also had increased axonal diameters of the cortico-spinal tract as measured by transcranial magnetic stimulation. Unfortunately developmental outcome data have not been reported for this study group(281).

9.15.1.2 Nutritional challenges following pre-term delivery

Very preterm infants are at increased risk of developmental disability, and are also particularly vulnerable to postnatal growth failure associated with inadequate nutrition, which may further compound neurocognitive impairment. The third

trimester of pregnancy is characterised by important processes of brain growth and development, as outlined in section 9.1.

Very preterm infants born during this time of rapid brain growth are at high risk of postnatal growth restriction(282), coinciding with nutritional deficits which develop in very preterm infants in the first weeks of life(283, 284). These deficits develop in part due to the inadequate provision of protein and energy sufficient to mimic fetal growth rates in addition to the higher metabolic demands created by major intercurrent illness(285). Postnatal growth restriction is a particular problem in extremely low birth weight (<1000g) (ELBW) preterm infants, many of whom drop 1 or 2 standard deviations for weight, length and head circumference between birth and hospital discharge(286, 287). Poor head growth is a particular concern, as head growth is a good proxy of brain growth(288), overall brain volume assessed using MRI(269), and neurodevelopmental outcome(289).

It is possible to ameliorate early growth faltering, including that of head growth, amongst ELBW and very preterm infants by early provision of adequate protein-energy nutrition(290). The relationship between high protein nutrition and neurodevelopmental outcome is less clear. Several observational studies have investigated this relationship(291-293). Stephens et al found a positive association between protein intake in the first postnatal week and Bayley II MDI score at 18 months, reporting a 4.6 point increase for each 10kcal/kg per day and an 8.2 point increase for each gram per kilo per day in protein intake(291) whereas Blanco et al reported poorer growth associated with lower Bayley II MDI scores at 18 months CGA in an extremely preterm population receiving 2g/kg/day intravenous protein

increasing to 4g/kg/day, compared to a group receiving 0.5g/kg/day increasing up to 3.5g/kg/day intravenous protein. These differences were not sustained at 2 years CGA(293). These differing results may reflect failure to achieve protein intakes adequate to sustain optimal growth in this cohort of extremely preterm infants, but supports the relationship between early growth and neurodevelopmental outcomes(294). Maintaining adequate growth remains important beyond hospital discharge. Franz et al assessed the neurodevelopmental outcomes of a group of 219 children born at gestational age less than 30 weeks at age of school entry (median 5.4 years), using standardised neurological examination, the Kauffmann Assessment Battery for Children and the GMFCS(292). Increasing standard deviation (SD) scores for weight and head circumference from birth to discharge were associated with reduced risk of abnormal neurological examination, and catch up head growth was associated with a reduced risk of impairments in gross motor function. Furthermore, increasing head circumference SD score from discharge to follow up was associated with a higher mental processing composite score(292).

A small number of randomised control trials have sought to establish whether or not provision of a nutrient enriched formula milk at term equivalent age improves developmental outcome in ex-preterm infants(295, 296). In both studies investigators analysed small for gestational age (SGA) and appropriate for gestational age (AGA) infants separately. In the first of these studies(295) infants fed with the nutrient enriched preterm formula performed better on the Griffith's Developmental Scale at 6 months CGA, as did male infants at 6 and 9 months CGA. In contrast Gianni et al found no benefit in neurodevelopmental outcome at 24 months CGA in either

SGA or AGA infants following provision of a nutrient rich formula up until 6 months CGA(296). Perhaps the most striking reports of the beneficial effects of early nutritional support on long term neurocognition are provided by the long term follow up trials of preterm dietary supplementation by Lucas et al(297). This work reports cognitive advantage in preterm infants born less than 30 weeks gestation who were fed a "high-nutrient" or "standard-nutrient" formula milk. The intervention was for a short period (median 4 weeks supplementation), between 26 and 34 weeks. Despite this, results showed improved performance in BSID II motor and psychomotor scales at 18 months CGA, with the largest effect sizes seen in those with highest intake of the "high-nutrient" formula, in babies who were SGA, and in male infants(298). When participants were assessed at 7.5-8 years significantly higher IQ scores were identified in the "high-nutrient" group, with the largest effect sizes seen in males. There was also a higher incidence of CP in the group receiving the "standard-nutrient" milk(299). Most recently improved verbal IQ was reported in a sub-group tested around 16 years of age (300). Higher verbal IQ in the "high-nutrient" group was also associated with larger caudate nucleus volumes in males, providing evidence that early diet affects brain structure as well as function(301). The "high-nutrient" milk contained supplemented micronutrients as well as a higher protein and energy content. It is not possible to conclude whether the neurodevelopmental advantages conferred were as a result of improved macronutrient intake, improved micronutrient intake, or the combination of both.

9.15.1.3 Micronutrients important to normal brain development

The roles of individual micronutrients to CNS function are summarised in Table 1 below.

Table 1. The role of micronutrients in central nervous system function

Micronutrient	Role in central nervous system(271, 302-316)
Iron	Catecholamine metabolism, myelin production, CNS cell division(271)
Zinc	DNA and RNA synthesis, dendritic arborisation, modulation of synaptic function
Copper	Brain-energy metabolism, dopamine metabolism, antioxidant activity, iron uptake in fetal and neonatal brain
Iodine	Production of thyroid hormones necessary to neurogenesis, neuronal migration, axon and dendrite growth, synaptogenesis and myelination
B Vitamins	Neural tube formation, carbohydrate metabolism (providing energy), membrane structure and function, synapse formation and function.
Selenium	Protection against free-radical damage through antioxidant enzyme function, conversion of thyroid hormone T4 to its active form triiodothyronine (T3).
Choline	Source of methyl groups (methylation of DNA and RNA), component of phosphatidylcholine and sphingomyelin, production of acetylcholine.
Uridine	Synapse membrane production
Docosahexaenoic acid (DHA)	Phospholipid membrane production and stability, neurogenesis, neurite outgrowth, synaptic plasticity, axonal elimination and gene expression

9.15.1.3.1 Iron

The developmental consequences of iron deficiency have been investigated in both animal and human studies. The hippocampus is a brain structure important to memory and learning. In rodents, iron deficiency in the prenatal and neonatal period causes reduced hippocampal volume, dendritic branching and synaptic

maturity, which are persistent despite iron repletion(302, 303). Prenatal and early neonatal iron deficiency anaemia (IDA) reduces myelin synthesis and changes its composition in animal models. These changes are uncorrected despite later iron sufficiency(304).

In humans IDA, particularly in the first two years of life is a risk factor for long term cognitive and learning problems(317). Longitudinal studies demonstrate that children who had IDA in infancy continue to have poorer IQ scores and higher levels of social problems and inattention in their teenage years despite iron supplementation in infancy(318). Prenatal iron supplementation trials have demonstrated mixed results, with only one trial demonstrating improved cognitive outcome on tests of non-verbal intelligence, executive function and motor ability at 7-9 years following antenatal supplementation with iron, folic acid and vitamin A compared to vitamin A alone(319). Prenatal supplementation trials which used BSID scores at 3, 6, and 12 months and IQ at 4 years as outcome measures did not show benefit to supplementation(320, 321). These discrepancies may well be methodological. Trials of iron supplementation in low and middle income countries where IDA is prevalent have been associated with a number of improved developmental outcomes including improved motor, language/cognitive, and social emotional development(318). In these trials not all subjects were iron deficient and the supplementation dose was lower than would be necessary for the treatment of IDA. Similar outcomes were not observed at 3.5 years in a Swedish iron supplementation study(322), nor at 7-9 years in a Nepalese study(323).

9.15.1.3.2 Zinc

Gestational zinc deficiency in rodents can cause reduced dendritic arborisation, reduced total brain DNA(324) and reduced regional brain volumes in the cerebellum, limbic system and cortex(325). Functionally, zinc deficient animals have deficits in activity, attention, learning and memory(326). Decreased preferential looking behaviour suggestive of altered hippocampal function has been reported in infants born to zinc deficient mothers(305). Human antenatal zinc supplementation studies have not shown beneficial effect to child development(271, 319, 327, 328). Zinc supplementation in infancy supports the normal reduction in look duration in habituation tasks in 6-12 month infants, and ability to shift between objects during a free play attentional task in 12-18 month infants. These findings suggest that zinc has a role in information processing and active attention systems(329). Studies of zinc supplementation in infancy have shown mixed effects on motor development(271). A meta-analysis of randomised control trials of zinc supplementation in infancy conducted in 2009 showed no change in BSID mental or motor scores. The authors acknowledge that there are very few trials in this area, many of which may have supplemented for too short a time to exert effect(330). Since this meta-analysis was published a study conducted in Zanzibari children aged 5-9 months and 10-14 months at baseline, lower age at independent walking was associated with combination zinc and iron supplementation in the 5-9 month group, but not when either nutrient was provided alone. In this group earlier age at independent walking predicted higher motor activity levels following 9 months of supplementation, which was associated with improved object manipulation, and better reported language

acquisition following 12 months of supplementation. In the 10-14 month group earlier age at independent walking was associated with iron monotherapy, although increased motor activity was associated with combination iron and zinc therapy. In this age group iron or zinc monotherapy was associated with poorer language scores than those receiving placebo or iron or zinc in combination(331). A Nepalese study randomised 4-17 month old infants to iron-folic acid and zinc, iron-folic acid or zinc alone, or placebo. The supplementation period was one year. Maternally reported motor and language milestones were recorded every 3 months for 1 year by the research team. There was no statistically significant difference in motor or language outcomes at any time point between supplementation groups(332).

9.15.1.3.3 Copper

Clinical copper deficiency syndromes are uncommon in human neonates, however copper deficiency has important neurodevelopmental consequences in rodents, leading to abnormal cerebellar development and lasting abnormalities in motor function, balance and co-ordination, even after copper repletion(333).

9.15.1.3.4 Iodine

Iodine deficiency during pregnancy results in maternal hypothyroidism and the clinical syndrome of cretinism in offspring. Cretinism is characterised by severe learning difficulties, facial abnormalities, and deaf-mutism and growth retardation. Cretinism cannot be reversed following birth and so prevention by ensuring adequate iodine intake and euthyroidism during pregnancy is essential(334). It appears that even mild iodine deficiency in pregnancy can affect cognitive

development. In a study of 8 year old children, those whose mothers were iodine deficient in the first trimester had lower scores on tests of verbal IQ and reading comprehension(335). Only two small randomised control trials of iodine supplementation in pregnancy have considered neurodevelopmental outcomes in childhood. The two studies show an average effect size of 10.2 IQ points between 0-5 years of age(336). In the absence of overt cretinism individuals living in iodine deficient areas have an IQ which is on average 12.5-13.5 point lower than those living in iodine sufficient areas(337, 338). The limitations to observational studies of this nature are that the IQ of individuals from these populations may be confounded by many factors not considered in the analyses. A meta-analysis of cohort and intervention studies which assessed the effect size of iodine status on IQ in children of 5 years of age and under reported a more conservative though still striking effect size of 6.9-8.1 IQ points(336). Further large well designed trials are required to more fully quantify the effect of iodine status on brain development(336).

Preterm infants are likely to be particularly at risk of developing iodine deficiency as they are entirely dependent on iodine provision via parenteral feeding regimes which may be iodine deficient. The I2S2 RCT will examine the neurodevelopmental effect of adequate iodine provision to preterm infants(339).

9.15.1.3.5 B vitamins

Deficiency of the B vitamin Thiamine is associated with neurological symptoms. Thiamine deficiency is rare in infants in developed countries as everyday foods are supplemented with thiamine, but is more common in some developing countries where routine food supplementation does not occur. In the absence of overt

neurological signs, thiamine deficiency in infancy has recently been shown to result in language deficits in 5-7 year old Israeli children fed with an infant proprietary milk feed, later found to be thiamine deficient(340). Although observational studies have reported associations between infant development and maternal B-vitamin levels during pregnancy(341) and lactation(342), and infant cobalamin and folate status(343), randomised control trials of B-vitamin supplementation are lacking. The only such trial randomised infants under 8 months old with feeding difficulties, subtle neurological symptoms or delayed psychomotor development and biochemical evidence of low serum cobalamin to an intramuscular injection of hydroxycobalamin or sham injection. Infants who received hydroxycobalamin injection had higher scores on the Alberta Infant Motor Scale, and a reduction in parentally reported feeding symptoms at 1 month post intervention, suggesting that adequate cobalamin is important during infant brain development(344). Cobalamin supplementation may be beneficial in infants with early signs of neurological impairment, however additional large RCTs are required in order to confirm these initial findings(345).

9.15.1.3.6 Selenium

As described in table 1 selenium is incorporated into deiodinases which convert the thyroid hormone T4 to its active form triiodothyronine (T3). Low thyroid hormone levels during pregnancy causes permanent severe delays in neurodevelopment in offspring. Animal studies have identified neurodevelopmental deficits associated with gestational selenium deficiency, however the role of this trace element in child development has been little studied. One large study in Bangladesh has studied the

relationship between maternal selenium levels in pregnancy and neurodevelopmental outcomes at 18 months. Maternal selenium levels were associated with performance on the psychomotor scale of the BSID II, particularly in girls, and in language comprehension assessed using the MacArthur's Communicative Developmental Inventory in boys and girls(346).

9.15.1.3.7 Choline

Choline (2-hydroxy-*N,N,N*-trimethylethanaminium) is obtained through the consumption of choline-rich foods (eggs, milk and meat), and by endogenous production in the liver.

There is indirect evidence that adequate choline is important to the developing fetus, as the placenta preferentially transports choline resulting in fetal choline levels which are much higher than maternal choline levels(347). This is also true during lactation(348). Gestational choline deficiency causes permanent changes to the hippocampus with resultant defects in cognition and memory(349). Despite this, human studies of gestational choline levels and neurodevelopmental outcomes have shown mixed results. One study found no association between cord blood choline levels and intelligence quotient scores using the Wechsler Preschool and Primary Scale of Intelligence-Revised at age 5 years in 400 mother-child pairs(350). Wu et al found that maternal choline levels at 16 weeks gestation correlated with cognitive outcome using the BSID III at 18 months of age(307). As part of the Seychelles Child Development Nutrition Study the relationship between child plasma levels of choline and its metabolites and developmental status age 5 years was assessed. Plasma choline level was moderately associated with plasma betaine level, which was

positively related to the total language score of the Preschool Language Scale, but not to any other cognitive outcome measure(351). Another study identified a positive association between maternal dietary choline intake during the second trimester of pregnancy (as opposed to biochemical choline levels) and performance on a test of visual memory (a function of the hippocampus) in the children at age 7 years, but not on any other cognitive measures assessed(352). Variation in findings of these studies may be due in part to the outcome measures chosen, some of which did not assess hippocampal function, and the age of the children at testing. Very few trials have assessed the effects of choline supplementation in humans. One double-blind randomised control trial supplemented pregnant women from 18 weeks gestation through 90 days post-partum. They found no association between supplemented and placebo groups on any measure of infant cognition (specifically assessing hippocampal function) at 10 and 12 months, however women in this study group were already consuming moderate amounts of choline in their diet and were not deemed to be choline deficient(353). It is also possible that infants were tested too early to detect any benefit of supplementation, as the hippocampus reaches peak synaptic development and functional maturity around 12-15 months of age(354). Maternal choline supplementation may also have beneficial effect in developmental disorders and CNS congenital anomalies. In a study which examined mid-pregnancy metabolite levels in pregnancies affected by neural tube disorders (NTDs), higher total choline levels were associated with a lower risk of NTDs. This finding was independent of maternal folate status(355, 356). A randomised control trial of maternal phosphatidylcholine supplementation in the second trimester of

pregnancy, and continuing in infants to three months of age was associated with higher numbers of infants with normal cerebral inhibition at 5 weeks of age compared to controls(357). Cerebral inhibition is a measure of sensory gating which is reduced in schizophrenia and other disorders. These effects may be mediated via the CHRNA7 gene, a candidate gene for schizophrenia encoding the α7-nicotinic acetylcholine receptor necessary to hippocampal acetylcholine signalling and the development of cerebral inhibition(358). In the study by Ross et al the CHRNA7rs3087454 genotype was correlated with diminished cerebral inhibition in the placebo, but not the treatment group suggesting that phosphatidylcholine supplementation may facilitate the development of normal cerebral inhibition even in the presence of a genotype normally associated with its inhibition(357). In Down syndrome mouse models maternal choline supplementation in pregnancy and lactation improved offspring performance on tasks of attention, spatial cognition and emotional regulation compared to controls(359, 360). Furthermore, maternal choline supplementation was associated with increased neurogenesis in adult offspring hippocampus(360), and increased number and size of neurons in the medial septum. Neuronal degeneration is associated with the development of Alzheimer type pathology in Down syndrome(361). Studies in rodents have also shown that the developmental consequences of fetal alcohol syndrome can be attenuated by maternal choline supplementation(362). A pilot study investigating the neurodevelopmental effects of choline supplementation in children age 2-5 years with fetal alcohol spectrum disorders concluded that daily choline supplementation is feasible, and reports beneficial effect on one memory

measure(363). The authors conclude that additional evaluation of the neurodevelopmental effects of choline supplementation in children with FAS should be undertaken.

9.15.1.3.8 Uridine

Most of an infant's uridine is provided in bioavailable form by breast milk or uridine containing formula milks(364, 365). Although deemed a "conditionally essential" nutrient, there has been little exploration of the effects of uridine sufficiency or insufficiency states on cognitive outcomes in childhood.

9.15.1.3.9 Multiple micronutrients

Children who are lacking in any one micronutrient are most likely to be at risk of multiple micronutrient deficiency. It seems less likely that the cognitive effects of undernutrition occur as a result of a single micronutrient deficiency, and a number of trials have looked to multiple micronutrient supplementation in an attempt to improve the neurodevelopmental outcomes of children at risk of undernutrition, such as those living in resource poor settings. A number of trials have shown some beneficial effects on various measures of infant development following multiple micronutrient supplementation in pregnancy. Two trials demonstrated marginal benefits in motor development(366, 367). One of these trials only showed benefit to motor development and activity levels in offspring of mothers with a low BMI in pregnancy, however the effect size was small. There were no overall group effects in motor or cognitive outcomes(366). Conversely a study in Western China demonstrated improved cognitive but not motor development in infants up to 1

year(320), with yet another trial showing benefits to cognitive and motor outcomes at 3.5 years in children of anaemic or undernourished mothers(368). A trial conducted in Nepal showed improved performance on one test of executive function in 7-9 year old children whose mothers received multinutrient supplementation in pregnancy compared to those mothers who received vitamin A alone(319). However, test results across five other tests of executive function, a test of intellectual function and of motor function were no different in the multinutrient compared to placebo group. The largest differences in cognitive functioning were seen in the iron/folic acid versus placebo group(319). A small number of trials have investigated the neurodevelopmental consequences of infant multiple micronutrient fortification. Randomised trials conducted in resource poor settings have shown benefits in motor development(369-371), and activity levels(372). One of these trials also showed a positive effect on developmental quotient(370). In contrast, a randomised control trial providing 6 month old infants with weekly iron, zinc, iron plus zinc or a micronutrient mix containing 16 micronutrients found no additional advantage to motor development than conferred by supplementation with iron plus zinc alone. No change in cognitive development was identified in any group(373).

9.15.1.4 Long chain polyunsaturated fatty acids

9.15.1.4.1 The omega-3 and omega-6 fatty acids

The omega-3 (n-3) and omega-6 (n-6) fatty acids (FA) are polyunsaturated fatty acids (PUFAs) with distinct and specific functions throughout the body, and are important to health and development. All n-3 PUFA's are derived from the parent molecule

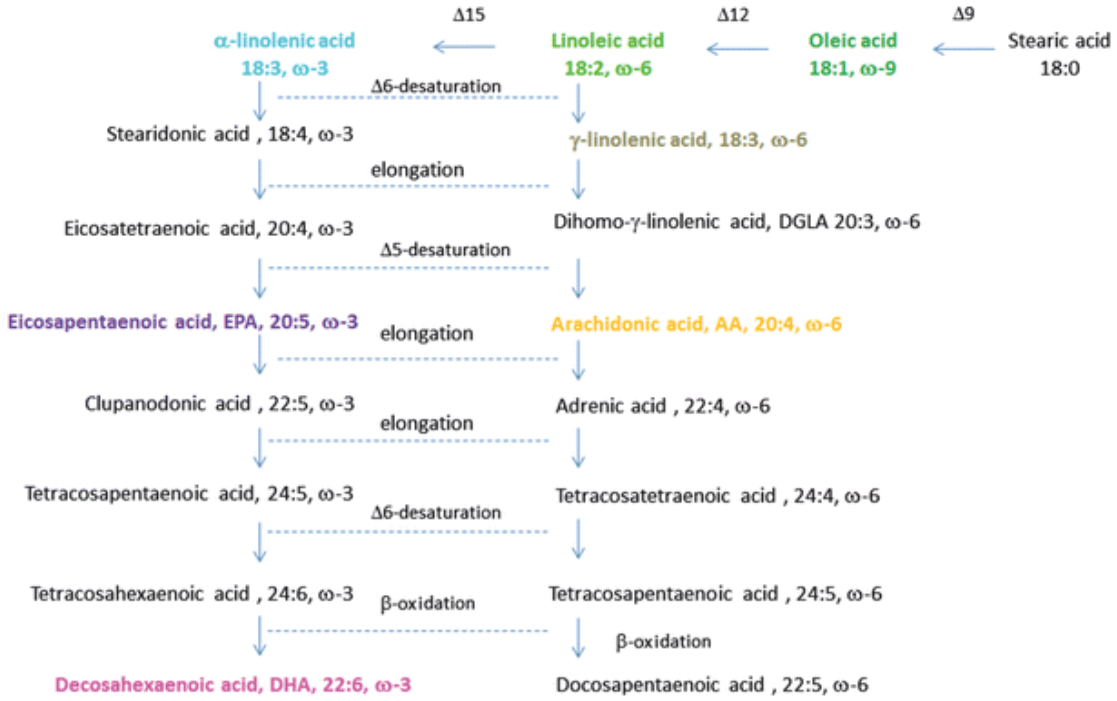
alpha linolenic acid (α-LA; 18:3 n-3). All n-6 PUFA's are derived from linoleic acid (LA; 18:2 n-6). These parent molecules are "conditionally essential" as they cannot be sufficiently produced endogenously and must be obtained from the diet. These short chain parent molecules then undergo a series of enzymatic conversions via desaturase and elongase enzymes in the liver to produce the biologically active long chain polyunsaturated fatty acids (LCPUFAs) docosahexaenoic acid (DHA; 20:6 n-3), eicosapentaenoic acid (EPA; 20:5 n-3) and arachidonic acid (AA; 20:4 n-6) (Figure 9). The desaturase and elongase enzyme pathways are encoded by the FADS gene cluster and ELOVL gene family. Fatty acid desaturases are encoded by the FADS1 and FADS2 genes which form a cluster with FADS3. FADS2 is the rate limiting step in the endogenous production of DHA and AA. Polymorphisms in these two gene clusters are associated with alterations in LCPUFA levels(374-376). Endogenous conversion of α-LA accounts for 0.2 to 8% of EPA and for 0.05 to 4% DHA. Less than 0.1% of LA is converted to AA. Conversion levels also depend upon the amount and ratio of n-3: n-6 PUFA's consumed in the diet as these precursor molecules share the same enzymatic conversion pathways. Diets high in n-6 PUFAs appear to result in higher rates of conversion down the n-6 pathway, potentially worsening any existing n-3 deficiency(377). Individuals who produce lower amounts of endogenous LCPUFA will require higher dietary intakes of LCPUFA's to meet physiological demand.

The main sources of dietary DHA and EPA are from oily, cold-water fish. There are very few plant sources of DHA and EPA although algal sources have been identified. AA is predominantly derived from red meat and vegetable oil sources. Western diets are lacking in EPA and DHA, and are high in AA due to typically low oily fish intakes

and high levels of red meat and vegetable oil consumption. This results in a diet approximately 16 times higher in n-6 than n-3 PUFA intakes. The optimal n-6:n-3 ratio required to support high levels of DHA in the developing brain remains to be established, however are likely to be far closer to 2:1(377-379). Current ratios may compound dietary n-3 deficiency, with potentially adverse effects on neurodevelopment.

The majority of fatty acids in human breast milk are supplied as triacylglycerols. The carrier molecule of supplemental dietary sources varies depending upon the source of the fatty acids, and may be provided as free fatty acids (FFA) free fatty acids bound in ethyl esters (EE), triacylglycerol (TAG), or phospholipid (PL). The carrier molecule used may affect supplemental fatty acid bioavailability. Overall, evidence suggests that FFA are more bioavailable than TAG, which have better bioavailability than EE. Concerning TAG versus PL formulations, animal studies have shown that omega-3 LCPUFA provided as PL was more bioavailable than as TAG, however there is insufficient evidence of improved bioavailability of PL compared to TAG from human studies conducted to date. For additional information on this subject the reader is referred to the comprehensive reviews of Schuchardt and Hahn(380), Michalski et al(381) and Ghasemifard et al(382).

Figure 9. Schematic presentation of fatty acid metabolism(383).



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9.15.1.4.2 The role of LCPUFA's in normal brain development

It is now widely accepted that adequate maternal nutrition during pregnancy and breastfeeding is necessary to support normal brain development. The polyunsaturated fatty acids arachidonic acid (AA; 20:4 n-6) and docosahexaenoic acid (DHA; 22:6 n-3) appear to be particularly important; low levels of DHA in plasma and blood cell lipid are associated with increased risk of visual and neurodevelopmental impairment in childhood(384-387). DHA is present in high levels in the central nervous system, particularly in the membrane lipids of brain grey matter and retina(388), where it contributes to structural integrity and function, as described in Table 1.

9.15.1.4.3 The role of LCPUFA's in visual development

DHA is present in high concentrations in the retina and cerebral cortex. In the retina DHA comprises around 50% of the total fatty acids of the retinal membrane bilayer; the highest concentration of DHA in the human body(389). The DHA content of photoreceptor cells, maintains a high degree of membrane fluidity, necessary to photoreceptor functioning(390). DHA is required for photoreceptor differentiation, and for the processing of rhodopsin in rod cells, the light sensitive retinal pigment necessary for conversion of light into an electrophysiological signal (photo transduction)(391). Rod photoreceptors are less mature at birth than cone photoreceptors and continue to undergo significant postnatal development(392); inadequate postnatal DHA intake may compromise ongoing photoreceptor development. In laboratory studies cultured fatty acid free photoreceptor cells degenerate and undergo apoptosis, which is prevented by the addition of DHA(393). DHA also appears to be necessary to the optimal functioning of specific populations of retinal ganglion cells, particularly the M retinal ganglion cells which project to the magnocellular layers of the lateral geniculate nucleus, as distinct from P retinal ganglion cells which project to the parvocellular layers of the LGN.

M cells require high levels of DHA to function effectively, and so functions which are M cell derived may be particularly vulnerable to DHA deficiency(394). Indeed, preterm infants who miss out on third trimester transplacental DHA transfer typically exhibit difficulties in the domains subserved by the magnocellular or "dorsal" stream, such as in contrast sensitivity, stereopsis (depth perception), motion processing, and spatial perception in addition to the wider identified cognitive and visuocognitive

deficits in attention, learning and visuomotor integration(394). DHA is necessary for myelination and so DHA deficiency states may result in poor myelination of the complex neural networks subserving vision and visuocognitive functions. The atypical early postnatal visual experience of preterm infants may further contribute to abnormal visual development, as sensory input refines cortical circuitry. DHA deficiency augments pre-existing visual processing difficulties in the very preterm population(394).

9.15.1.4.4 Preterm and IUGR babies are particularly vulnerable to DHA deficiency

DHA cannot be synthesised in sufficient quantity via endogenous processes, and the developing fetus is reliant upon a maternal source. During the third trimester of pregnancy the placenta preferentially transports DHA to the fetus, by up-regulating fatty acid (FA) transport proteins, alongside processes of passive FA transport(395). Very preterm infants (<28 weeks) are at particular risk of developing a DHA "gap" as they miss out on third trimester transfer of DHA, have poor adipose tissue stores, particularly if of VLBW, and have very limited capability for endogenous conversion from precursor fatty acids(396-398). Although in vivo labelled isotope studies demonstrate endogenous conversion of LA and ALA to AA and EPA/DHA in the liver in term and preterm infants, the absolute amount of LCPUFA produced by endogenous methods has not been quantified(399, 400). The rate of endogenous conversion is inadequate to meet preterm LCPUFA demand(399, 401). Preterm infants are therefore dependent upon adequate exogenous DHA supply, however nutritional provision is often inadequate to meet the needs of the rapidly growing brain. Very preterm infants may be dependent upon parenteral nutrition for a

number of weeks prior to the introduction of enteral feeds. Current TPN formulations do not provide preformed DHA, supplying precursor fatty acids only(398). Even when enteral feeds are commenced, these are often started cautiously and increased slowly due to fears of necrotising enterocolitis in this population. Furthermore, feeds are often interrupted during periods of serious intercurrent illness. Once enteral feeds are established, they are unlikely to provide adequate DHA to match third trimester DHA accrual (approximately 70mg/kg/day). Maternal breast milk is considered to be the best option for preterm infants; the milk produced by mothers of preterm infants has higher DHA content than that produced by mothers of term infants. Despite this regulation, the DHA content of human milk is subject to huge variance (0.06 to 1.4% wt/wt %)(402, 403) and may also be reduced by factors such as expressing (fats adhere to tubing), natural variation by time of day, and storage. Some infants may need supplemental donor breast milk or infant formula milk. The overall fat content of donor milk is typically lower than that of mother's own milk. Formula milk preparations typically provide 0.2-0.35 wt/wt%, matching the average content of human breast milk. Available feed combinations typically provide 3 to 23mg/day DHA, which is far below the estimated in utero accretion of 42 to 75mg/day DHA(404-406). Continuous drip or gavage feed delivery systems for infants unable to suck feeds may further compromise DHA delivery due to fat adherence to tubing.

The small intestine only absorbs around 80% of delivered DHA; over the first month of life, a very preterm infant receives approximately 50% of that which would be received in utero(404). Given the rapid brain development occurring during this time

it is essential that efforts to close this "gap" are made, in order to support optimal brain development. DHA provision equating to 1-1.5wt/wt% may be more appropriate for preterm infants(404). The development of TPN solutions containing preformed DHA, or of methods of direct DHA administration independent of milk feeds will help deliver timely appropriate DHA quantities. The results of trials of such TPN products are awaited(398).

9.15.1.4.5 Docosahexaenoic acid and cognition - intervention trials

Many studies have examined the potential neurocognitive or visual benefits of DHA supplementation during pregnancy, lactation, and early infancy. These studies have produced variable results, perhaps explained by variations in the population studied, dose and duration of supplementation, LCPUFA source and composition, and outcome measures used(372). A recent Cochrane review including 6 randomised controlled trials (RCTs) of LCPUFA supplementation of breastfeeding mothers concluded there was insufficient evidence of improved infant development or visual acuity(407). Previous Cochrane reviews showed no neurodevelopmental advantage of LCPUFA supplementation of formula milk for term(408) or preterm infants(409). An individual patient data meta-analysis combining data from four studies of term and preterm infants showed no effect on Bayley developmental scores in either group(410). This meta-analysis did not include data from two relevant trials demonstrating improved cognitive performance in preterm(411) and term infants(412), as data from these two trials were not made available to the authors. However, all the trials included supplemented with relatively low dose DHA (0.17-0.5% total fatty acids) for relatively short durations (3 weeks-6 months). The DINO

trial is the largest, well conducted randomised placebo controlled trial of high dose DHA (1% total fatty acids) supplementation in preterm neonates, and reported improvements in Bayley II Mental Development Index (MDI) score in supplemented girls, but not in boys. There was a significantly lower proportion of children with impaired neurocognitive development in the DHA supplementation group compared to the placebo group(413). The group also reported higher MDI scores in infants weighing less than 1250g at birth in unadjusted but not adjusted analysis. Visual acuity was improved in the supplemented group at 4 months compared to controls in both sexes. Sex differences in outcome may be related to more efficient conversion of endogenous ALA in females compared to males(414). Fewer studies have examined the long term effects of DHA supplementation in pregnancy or infancy, however this is important as the assessment tools available for use in infancy may be too blunt to detect more subtle effects. Specific cognitive functions, such as those subserved by the hippocampus, can be hard to test in pre-school children; appropriate measures may be unavailable until school age. Longer term or more subtle benefits may therefore be missed if adequate follow up does not take place. The DINO study group recently published the results of follow up at 4 years of age, however there were no differences in the cognitive outcomes of the DHA treated group compared to the control group(415), or in full scale IQ or any other cognitive measure at age 7 years(416). Similarly, a RCT trial of high dose DHA supplementation (0.86% total fatty acids) in preterm VLBW infants showed no cognitive benefit in the treatment group compared to the control group at 8 years.

Neuroimaging (brain MRI) did not show any between group structural brain differences(417).

Lucas et al undertook a range of cognitive assessments on a group of 9 year old ex-preterm children randomly assigned to receive DHA supplemented formula (in addition to breast milk if breast fed, or as sole feed if formula fed). The supplemented formula contained 0.5% DHA. Although there were no significant differences between randomisation groups on any cognitive measure, DHA supplemented girls, but not boys, showed improved performance in tests of literacy. Furthermore, verbal IQ, full scale IQ, and memory scores were higher in LCPUFA supplemented groups who received formula alone. This was not the case in those who had received a combination of formula and breast milk suggesting that the DHA intake of breast fed infants was sufficiently high that no additional benefit of DHA supplementation was seen using the chosen cognitive measures(418).

9.15.1.4.6 Docosahexaenoic acid and vision – intervention trials

A number of studies have examined the effect of prenatal and postnatal DHA supplementation on visual outcomes. The majority of studies have concentrated on visual acuity measured by VEP or behavioural methods. Like the trials investigating cognitive effects of supplementation, trials assessing visual acuity outcomes are diverse in study design, population, dose and duration of supplementation, assessment method and timing. The most recent meta-analysis of published and unpublished infant DHA supplementation trials reported improved visual acuity as measured by VEP at 2, 4, and 12 months and at 2 months when measured by behavioural methods(419). Trials of prenatal DHA supplementation have

demonstrated variable results, and the most comprehensive systematic review conducted was unable to support or refute benefit to visual development following DHA supplementation. Meta-analysis was not possible due to large variation in assessment method and age(420). Four of the seven trials reviewed reported no difference between treatment and control groups(421-427). Two trials reporting improved visual outcome in the treatment group compared to the control group (when combined treatment groups were compared to combined control groups) had attrition rates of 56% and 65%(424, 427).

9.15.1.4.7 Docosahexaenoic acid and attention

Low blood omega-3 fatty acid levels and increased omega-6:omega-3 fatty acid ratios have been identified amongst children with a range of neurodevelopmental disorders including Attention Deficit Hyperactivity Disorder (ADHD)(428-430). Low blood omega-3 fatty acid levels have also been identified in children with Autism Spectrum Disorders (ASD), although data are conflicting(431-434). Furthermore, low DHA/EPA and AA and higher AA/EPA ratios in children with ADHD and ASD correlate significantly with symptoms in a large cohort study(435). These findings have led investigators to suggest that dietary supplementation with DHA and/or EPA may improve neurodevelopmental outcomes for children with ADHD or ASD. As with intervention trials of DHA supplementation and infant neurodevelopmental outcome, interpretation of trials of LCPUFA supplementation in children with ADHD is limited by variation in design including LCPUFA dose, duration of supplementation and outcome measures used. Despite this, children with ADHD appear to be benefit from supplementation(436). This is corroborated by findings from a randomised

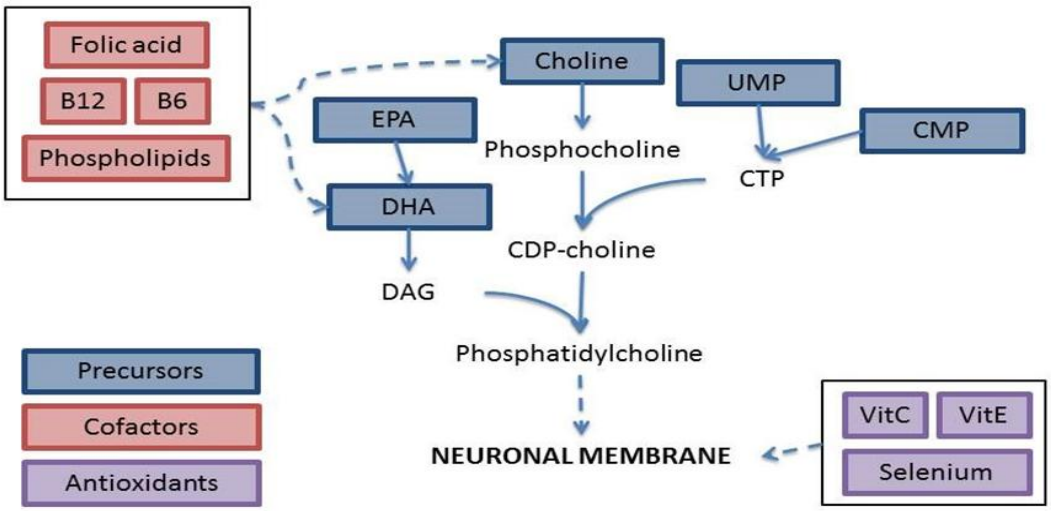
control trial of DHA supplementation of children meeting DSM-IV criteria for ADHD diagnosis, which employed a number of cognitive and behavioural as well as biochemical measures of the effects of intervention. The study confirmed that omega-3 mix supplementation increased DHA and EPA levels in erythrocyte membranes and improved working memory function in the treatment group. No other cognitive advantage or improvement in parent and teacher behaviour rating scales was identified(437).

9.16 The rationale for combination neurotrophic supplementation in infants at risk of neurodevelopmental impairment

Appropriate macro- and micro-nutrient intake is required for healthy brain growth and development. Given their diverse CNS functions the biological rationale for DHA and choline supplementation during periods of maximal brain growth and development is sound. However, studies to date which have provided single nutrient supplementation have shown inconclusive results(410).

Phosphatidylcholine (PC) is an essential component of new phospholipid nerve cell membrane. During the Kennedy Cycle, the essential omega-3 fatty acid docosahexaenoic acid (DHA) combines with choline, an amine, and uridine-5-monophosphate (UMP), a pyrimidine, to form PC (Figure 10).

Figure 10. The Kennedy reaction for membrane biosynthesis



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Animal research work shows that dietary supplementation with DHA, choline and UMP in combination increases brain phosphatide levels(1). Supplementation with all three precursors results in greater increases in brain phosphatide levels compared to supplementation with UMP or DHA alone, suggesting synergistic effect. Supplementation also produces increases in pre and post-synaptic elements(1), and dendrite spine density(2). Increased synaptic element and dendritic spine densities are associated with improved performance on cognitive tasks using serial maze tests in gerbils (438). Preliminary data from a randomised placebo controlled trial of DHA, choline and UMP supplementation in adults with early Alzheimer's disease has shown improvements in verbal recall on the Wechsler Memory Scale-revised(439).

9.17 The Dolphin study

This novel pilot study is the first to supplement infants with risk factors for neurodevelopmental impairments with adequate amounts of combination neurotrophic precursors. Provision of adequate neurotrophic nutrients during the period of maximal brain growth may support mechanisms underlying normal brain growth and repair in infants at risk of neurodevelopmental impairment (ARNI). If successful, this intervention has the capacity to ameliorate the level of disability experienced by children at risk of neurodevelopmental impairment, with important potential implications for quality of life and participation.

10 GENERAL METHODS

As visual neurophysiology and blood fatty acid measures were Dolphin trial secondary outcome measures, the methods relevant to these measures are discussed within the context of the overall Dolphin trial methodology.

10.1 Dolphin study design

The full published trial protocol is available in Appendix 2(440). Appendix 2 also includes protocol information relating to Dolphin 2, which was a randomised control trial of neurotrophic dietary supplementation in infants age 1-18 months with early clinical evidence of cerebral palsy. Dolphin 2 data are not included in this thesis. Full treatment of the Dolphin 2 data would make this thesis unwieldy and reduce its focus, so are not further discussed. A summary of the Dolphin 1 methods are presented below.

This pilot study was a double-blind randomised placebo-controlled trial of DHA, choline and uridine in babies and infants with risk factors for neurodevelopmental impairments. Neonates were recruited from the John Radcliffe Hospital, Oxford, Royal Berkshire Hospital (RBH), Reading, and Wexham Park Hospital (WPH), Slough. Ethical approval for the study was granted by Oxford Research Ethics Committee B, Research Ethics Committee number: 08/H0605/70. Trial consent and parent information sheets are shown in Appendix 3.

10.1.1 Inclusion criteria

Dolphin trial inclusion criteria were as follows:

Birth ≤30^{+6} weeks gestation:

- Small for Gestational Age – weight less than 9th percentile (according to UK-WHO Neonatal and Infant Close Monitoring Growth Chart 2009) OR
- Grade IIa, IIb, III or IV Germinal Matrix Haemorrhage (GMH) –IVH or pathological periventricular flare/ leucomalacia

Birth 31-40 weeks plus 28 days gestation(i.e.up to 4 weeks post - term age):

- HIE Sarnat Grade II and III OR
- Grade IIa, IIb, III or IV GMH-IVH or pathological periventricular flare/ leucomalacia OR
- MRI abnormalities: PLIC, basal ganglia, thalami, white matter and cortex.

Exclusion criteria were infants with major congenital malformation, underlying progressive neurological, genetic or metabolic conditions, severe hearing loss, malabsorption, cow's milk protein intolerance or egg allergy.

10.1.2 Randomisation and trial entry

Families of eligible babies were identified by senior clinical staff on participating neonatal units (NNU), supported by regular research team visits to the NNU in Oxford and WPH, and regular telephone contact with two NNU research nurses at RBH. Following identification of an eligible baby, initial approach to the family was either made by the baby's clinician, or by MA or CMJ following family agreement for them to do so. After giving the parent information sheet MA or CMJ contacted the family after 48 hours to answer any questions the family had regarding the study. For

babies in WPH or RBH parent information sheets were distributed by the clinical team and then CMJ travelled to meet the families on the NNU or at their home to answer queries and take consent where appropriate. Following written informed consent trial participants were randomised by the trial statistician to intervention group X or Y. Randomisation forms were completed by the research team and faxed to the trial statistician at the Centre for Statistics in Medicine who performed the randomisation using minimisation by brain injury severity (normal/mild, moderate or severe – see Table 2), gestational age and sex, to ensure equal distribution between intervention groups. Completed forms detailing infant allocation were then returned by fax to the study dietitian and kept in the trial master file (TMF), separate to the participant case report form (CRF) in order to help maintain blinding of those involved in participant neurodevelopmental assessment.

10.1.3 Neuroimaging severity grading system

In order to stratify participants according to severity of brain injury a neuroimaging severity grading system was devised for the trial (Table 2). The literature pertaining to neuroimaging predictors of cerebral palsy was reviewed up to and including 2010, when the neuroimaging stratification system was devised(43, 112, 441-447). The stratification system used in the trial was broadly modelled on the system used by Leisjer et al (2010), which split neuro-imaging findings into normal/mild, moderate or severe brain injury(444). Not all recruited babies had a brain MRI scan available at the point of randomisation. Brain injury severity categorisation was made on cranial ultrasound imaging in those where magnetic resonance imaging was unavailable at

randomisation. Section 9.5.3.5 further discusses the use of neuroimaging in the prediction of neurodevelopmental outcome.

Table 2. Dolphin trial neuroimaging grading of brain injury severity

	Normal/Mild	Moderate	Severe
Preterm injury **Cranial ultrasound scan (cUSS)**	• Normal • Grade I/II Intraventricular haemorrhage (IVH) • Ventricular index (VIn)<13mm at term equivalent age (TEA) OR • VIn < 97th percentile for corrected gestational age (CGA)	• Grade III IVH • Non-cystic Periventricular leucomalacia (PVL) • VIn 13-15mm TEA OR • VIn >97th percentile but < 4mm above 97th percentile for CGA	• Grade IV IVH • Periventricular haemorrhage infarction (PVHI) • Cystic PVL • Subcortical leucomalacia • VIn at TEA >15mm OR • VIn >4mm above 97th percentile for CGA • Basal ganglia (BG) lesions • Focal infarction
Term hypoxic ischaemic **encephalopathy** **Magnetic resonance imaging (MRI)** **cUSS where MRI unavailable**	• Focal subtle abnormalities of BG with normal appearance of the PLIC • Periventricular white matter changes difficult to differentiate from normal appearances and therefore not classified as abnormal • Changes confined to cerebral cortex and subcortical white matter (WM)	• Multi-focal lesions in BG with equivocal or abnormal signal intensity within PLIC • Small focal lesions of without loss of grey matter (GM)/WM differentiation.	• Widespread abnormalities involving all Basal ganglia-Thalamus (BGT) structures and PLIC • Larger areas of abnormality with loss of GM/WM differentiation, consistent with infarction • Central grey matter hyperechogenicity +/- more extensive cortical and subcortical hyperechogenicity
Term infarction **MRI** **(cUSS where MRI unavailable)**	•	• Focal, non-territorial infarct	• Territorial infarct

10.1.4 Dietary interventions

The treatment supplement contained DHA (1% total fatty acids), EPA, AA, choline, UMP, cytosine monophosphate, Vitamin B12, zinc and iodine. Both treatment and control supplement were produced on a background of infant formula, and therefore the control supplement contains a small amount of choline, vitamins, minerals and trace elements. The amounts of active ingredient in the treatment and control supplement are listed in Appendix 4.The supplement was deodorised to minimise smell and taste. Supplement sachets were packaged in light reflecting silver foil sachets labelled X or Y and were available in 2g, 3g and 12g quantities.

The supplement was produced and quality control checked by Nutricia, the Netherlands, and shipped by courier to the John Radcliffe Hospital, Oxford. There were three productions of supplement over the period of the study. The study dietitian took receipt of the supplement and stored it in a dedicated supplement cupboard in the Dolphin assessment lab in The Women's Centre, on the John Radcliffe Hospital site. Supplement was stored away from a direct heat source.

The study dietician delivered a 3 monthly supply of supplement to participating families at the time of each participant growth assessment. Parents were given written and verbal instructions on the storage, preparation and delivery of the supplement. Parents and neurodevelopmental assessors were blinded to participant group. The study dietitian was not blinded for reasons of safety.

Each infant's diet was supplemented with 2g/kg/day, to a maximum of 24g/day, of study product for the duration of their compliance with the study protocol. Those completing the protocol were supplemented daily for 2 years. For babies, the

supplement was mixed with their normal feed. Breast feeding mothers were asked to express a sufficient quantity of expressed breast milk (EBM) to which to add the supplement. So as not to exceed recommended daily carbohydrate and fat intakes and osmolality a minimum volume of 35ml of EBM or term formula milk per 2g supplement was required for mixing the supplement. The resultant mix was the same thickness of a standard formula milk. There was no detectable difference in the way that the control and treatment supplement dissolved in milk. Breastfeeding mothers were given the option of delivering the supplement by cup, syringe or bottle depending upon preference. On introduction, the supplement was started at a low dose and built up to the prescribed dose over a period of 1-2 weeks as tolerated. Parents received phone calls twice weekly as the supplement was introduced and thereafter fortnightly, or as required, telephone contact from the trial dietitian to discuss progress, trouble-shoot any dietary or supplement related concerns and advise on optimising macro- and micro-nutrient intake.

10.1.1 Outcome assessments

Corrected gestational age was used for all participants born before 37 weeks gestation, across all outcome measures.

10.1.2 Growth monitoring

Head circumference, weight, height, mid-arm circumference and skinfold thickness (triceps, biceps, supra-iliac and sub-scapular) were measured 3 monthly. Occipito-frontal (head) circumference was measured using a Lasso-o™ tape measure. Three measurements were made, if possible, and a mean head circumference calculated. If

an infant was not compliant with measurement then one confidently obtained measurement was accepted. Weight was measured using the SECA baby scales 717, and the SECA Flat scale 875 in older children. Length was measured using the SECA Measuring rod 231 and SECA Portable stadiometer 213. Linear limb length measurements were used to estimate height in children unable to stand(448).

10.1.3 Participant whole blood fatty acid levels

Participant whole blood fatty acid levels were measured by heel- or finger-prick at study entry pre-supplement and at the end of supplementation. Maternal whole blood fatty acid level was measured at baseline. Whole blood spot samples were collected using a Guthrie card. Once dry, the samples were sealed in a foil envelope containing desiccant and stored at -20°C in a dedicated freezer within the Weatherall Institute of molecular Medicine on the John Radcliffe Hospital site. At the end of the study the samples were couriered on dry ice to Nutricia, the Netherlands, where they were analysed according to the methods of Marangoni et al(449).

10.1.3.1 Developmental assessment

The Bayley Scales of Infant Development (BSID) II(450) have been widely used to determine rates of developmental delay(451, 452), and as an outcome measure in randomised controlled trials (RCTs)(453, 454). Bayley Scales of Infant Development III (BSIDIII)(455) reports separate composite scores for cognitive, language and motor scales and is its main advantage over its predecessor. BSID III was administered in the child's home by one of two trained administrators (MA and CMJ). In one or two children who moved out of area during the course of the study BSID III was

conducted in the Dolphin vision assessment lab on the day of vision assessment. Assessments were video-recorded for scoring verification as required. The Vineland Adaptive Behaviour Scales II (VABS-II)(456) is a standardised parental interview, administered by MA or CMJ following Bayley assessment, as a subjective measure of child development. For a small number of children it was not practical to complete the VABS-II at the time of BSID III assessment and so was completed by telephone interview as close to BSID III assessment as possible, usually within a couple of days.

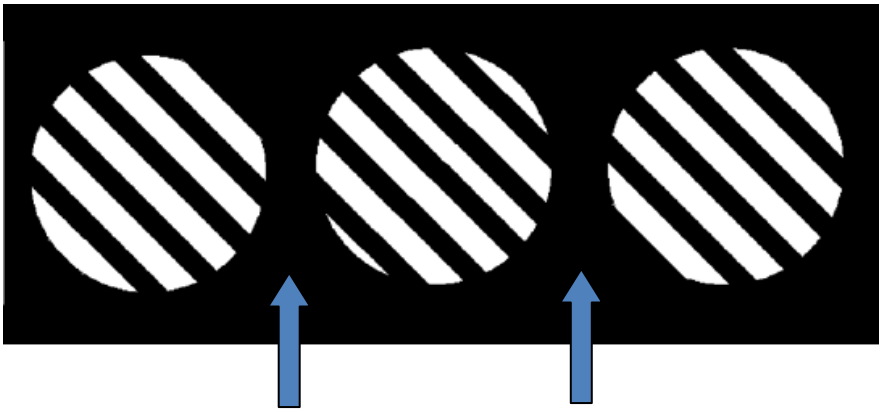
10.1.3.2 Functional vision assessment

Vision assessment was performed by MA or CMJ under the supervision of Professors OB and JA. Assessments were conducted in the Dolphin vision assessment lab, a purpose designed facility in the Women's Centre, John Radcliffe Hospital, Oxford. Infants sat beside parents or on the caregiver's lap during testing, as appropriate to age. The Atkinson Battery of Child Development for Examining Functional Vision (ABCDEFV)(203) assesses perceptual, motor, spatial and cognitive skills and has been standardised for children from birth to five years developmental age. ABCDEFV has been used to assess functional vision in a number of clinical populations(124, 233). Core vision tests include orthoptic assessment (ocular movements, pupil response), refractive errors, binocular optokinetic nystagmus (OKN), acuity (Teller acuity cards), and attention at distance, visual fields (Stycar balls), defensive blink and fixation shift. Age-specific tests to identify problems in perceptual, visuomotor and spatio-cognitive domains are included. A summary of ABCDEFV assessments is provided in Appendix 1.

10.1.3.3 Phase and orientation reversal visual event related potentials (VERP)

During VERP recording infants sat in a darkened room on their caregiver's lap, 40cm from the stimulus screen. Three electrodes were placed on the infant's scalp, over the vertex, occiput at 1cm above the inion, and a ground electrode placed on the forehead at the hairline. The electrodes were placed using a saline gel to improve contact and secured with micropore tape. Electrodes were connected to a low voltage pre-amplifier. Stimuli comprised a sine wave grating of oblique black and white stripes with a spatial frequency (SF) of 0.24 cycles per degree (cpd) (comparable to 81' of arc checks) and mean luminance of 31cd/m2 at about 93% contrast. For PR-VERP the stripe orientation is unchanged at 45° but alternated with periodic 180° phase shifts (Figure 11).

Figure 11. Successive views of Pattern-Reversal Visual Event Related Potential stimulus showing 180° phase shift (i.e. replacement of black bars by white).

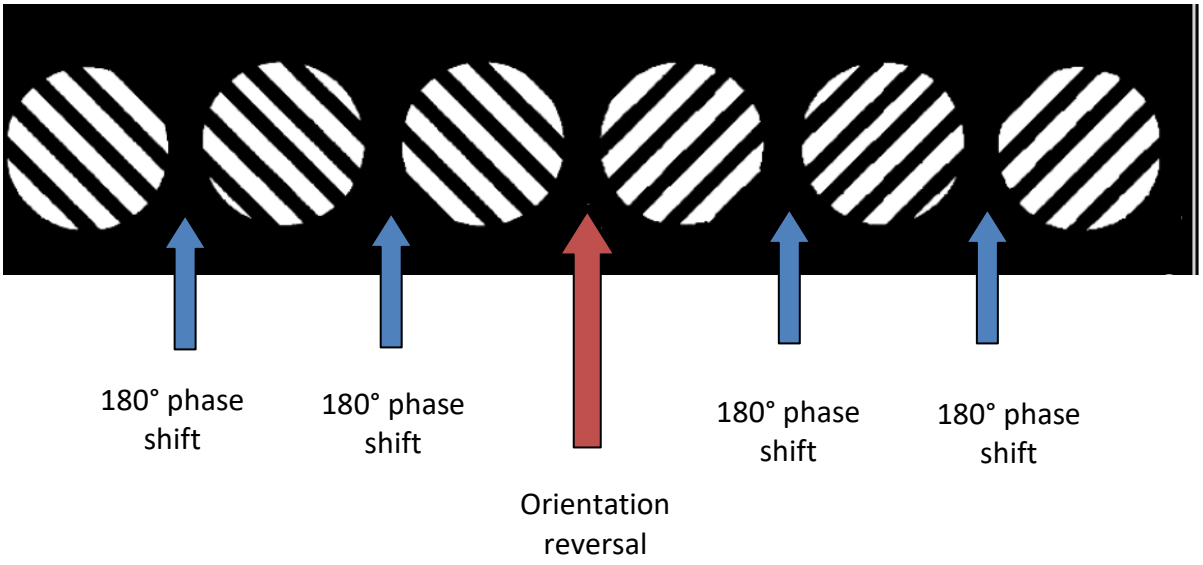


A photograph of the VERP set-up is shown in Figure 12.

Figure 12. Photograph of the Visual Event Related Potential recording set-up showing use of adhesive tape game on stimulus monitor to maintain infant attention

For OR-VERP stripe orientation changes between 45 and 135 degrees. Between orientation changes, random phase shifts (jitter) at a rate of 25 per second with no overall change in the luminance of the screen were introduced in order to ensure that VERP responses obtained were in response to orientation reversal rather than contrast change(457), see Figure 13.

Figure 13. Successive views of Orientation Reversal Visual Event Related Potential stimulus showing random phase shifts in the range 0- 180°, and orientation reversal



VERPs were recorded using a computer-based acquisition system (Espion; Diagnosys, Cambridge, UK). Control of the averaging process, and analysis of the results, was performed by software written by Dr John Wattam-Bell (UCL). Impedance was measured with an applied voltage at 1000 Hz and electrodes were adjusted until this was < 15 kΩ. Signals were amplified (20,000x), band pass filtered between 0.5 and 30 Hz, and sampled at 1000 Hz. Measurement using a photoelectric photometer demonstrated a systematic software delay of 45 ms between the stimulus onset in the centre of the display monitor and the recording cycle for the PR-VERP and OR-VERP stimuli. This value (45ms) was subtracted from measured transient and calculated latency values. One hundred epochs (2 cycles per epoch) were averaged on the computer. Epochs containing signals greater than 200 µV in amplitude were considered artefactual and were automatically rejected from the signal averaging. VERP recording was commenced a few seconds after the stimuli appeared to limit

any onset effect. PR-VERPs were measured at transient (2 reversals/second) and steady state (4-8 reversals/second). The order of PR-VERP stimuli at different temporal frequencies (TFs) was random in order to avoid any systematic adaptation effects. OR-VERPs were measured at 8 reversals/second (r/s). 100 sweeps were recorded at each reversal rate, then averaged. Recording was interrupted using an observer operated gating switch if the infant became inattentive. A small noisy toy was shaken in the centre of computer screen to attract and maintain attention as necessary. For older infants a game using micropore tape applied to the monitor screen was devised to help maintain attention.

10.1.3.3.1 Visual Event Related Potential analysis

Fourier analysis was used to extract relevant VERP response frequencies. Moore's test of circular variance(458, 459) was used to identify the presence of a statistically significant component at the stimulus frequency and its harmonics. The test yields a value of the Mann-Whitney U statistic reflecting the consistency of the signal phase across epochs within a run. Signal/noise ratio (SNR) was calculated using measurements of noise power in a band 1 Hz either side of the stimulus frequency (Braddick et al., 2005). Runs with $p > 0.05$ on the Mann Whitney U test or an SNR < 1.5 were discarded.

VERP waveforms were analysed using Matlab 2011. Transient PR-VERP latency was obtained from VERP runs at 2r/s by identification of the first most positive peak of the waveform by application of a manual cursor. Transient latency measures were corrected for the identified systematic software delay of 45ms. Examples of the waveforms generated are shown in Figure 35.

The phase (0 to 360°) of averaged signal components of the second harmonic frequency, F2, as separated using Fourier analysis, was measured for VERP runs at each temporal frequency 2-8r/s. Phase was calculated as the arctangent of the ratio between amplitudes of sine and cosine components at this frequency. Arctangent is bounded between ± 180°, and so an infinite series of phase values separated by 360° exist with the same tangent. Because the slope of the phase introduced by a delay, plotted against TF, is proportional to delay(208, 213), phase values should reduce linearly with TF. To identify the appropriate phase, the difference between two sequential frequencies' phase obtained from adjacent TFs was considered. If the phase value of the subsequent TF was positive relative to the preceding phase value then multiples of 360° were subtracted from that phase value until the phase value was negative relative to the preceding phase value, and so on for all sequential TF phase values. This process is referred to as "unwrapping" the phase(212, 215). A minimum of 2 and maximum of 4 "unwrapped "phases were then plotted against TF and the slope derived by linear regression. The slope of this plot was then converted into a calculated latency using the formula:

Latency (ms) = - (Phase Δ / TF Δ) * 1000 / 360°- 45 ms

where 45ms represents the identified inherent software delay as described above. Evaluation of the OR-VERP response did not involve a latency measure, but was simply the identification of a statistically significant OR-VERP response at 8r/s ($p < 0.05$) as identified at the second harmonic (F2) or the fourth harmonic (F4) of the epoch frequency. OR-VERP response was recorded as either present or absent for analysis.

10.1.3.4 Fixation Shift test

The procedure used in the FS test was Forced Choice Preferential Looking, where the 'blind' observer has to choose from the infant's eye and head movements whether the infant makes a FS to the peripheral target when it appears . During the FS test(225) infants sat on their caregiver's knee in front of a 28 inch (70cm) wide screen LED computer monitor. A central target (a computer generated face) was used as a fixation stimulus before the appearance of a peripheral target (alternating black and white stripes) to the left or right of the screen. The central face either disappeared simultaneously to the appearance of the peripheral target (non-competition), or remained (competition). In total 20 randomly generated FS sequences were generated; 5 under non-competition and competition, to left and right. A hidden observer unaware of the random FS sequence recorded the child's eye or head movement using keyboard cursors to indicate left or right. Unsuccessful trials (an infant made no shift after a reasonable time interval) were aborted and testing resumed. The programme continued to generate FS sequences until 20 responses had been recorded. Regular breaks were given to prevent habituation and declines in sustained attention to the central target, by turning children away from the screen for a brief period before testing was resumed. Performance was quantified as the number of correctly observed fixation shifts under each condition, and the mean latency to fixation shift.

A detailed schedule of study assessments is shown in Table 2, Appendix 2.

10.2 Dolphin Study statistical analyses

10.2.1 Power calculation

This is a proof of principle and pilot study using a novel intervention. Power calculations were performed for primary outcome measure BSID-III score, assuming power of 80%, 5% significance level, use of two-sided statistical tests throughout and equal allocation to each arm. Recruitment of 60 patients to each arm, assuming 20% loss to follow-up, provides 80% power to detect a 12.5 point difference in BSID-III score assuming SD of 15 points.

Power calculations have also been performed for VERP latency. Thirty infants per group provide 90% power to detect a latency difference of 25ms, assuming standard deviation (SD) of 25ms and significance level of 5%(217).

10.2.2 Analysis of primary outcome

All analyses were carried out using R version 3.2.1 (R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria).

The primary outcome measure was the composite score on the cognitive scale of the Bayley Scales of Infant Development III (CCS-BSID III) at 24 months of supplementation. The composite score is a normalised transformation of a distribution of scores and is derived from normative tables of standardised scaled scores, composite scores and percentile ranks provided in the BSID III manual. The composite score has a range of 40 – 160.

BSID III data was normally distributed. Change in BSID III score from baseline was analysed at 12 and 24 months of supplementation using mixed effects linear regression to account for the repeated measures over time. Baseline BSID III score was entered as a covariate in the model. The mixed effects model included BSID III score at 12 and 24 months of supplementation as the response variable, time point (12 months or 24 months), treatment group and baseline BSID III score as fixed effects and a patient specific random intercept. An interaction between time and treatment group was fitted as a fixed effect to allow estimation of treatment effect at both time points. The minimisation (design) factors severity of neurological damage (normal-mild/moderate/severe), gestation (≤31 weeks/31-42 weeks), and sex (male/female) were also included as fixed effects.

For primary outcome, CCS-BSID III score following 24 months of supplementation, mean difference in CCS-BSID III between intervention groups at 24 months of supplementation are presented along with 95% CI and associated 2 sided p-value. No adjustment of p-values to account for multiple endpoints has been made, as there was only one primary analysis.

A sensitivity analysis for the primary outcome was also conducted per-protocol (PP) to examine the robustness of conclusions to different assumptions about departures from randomised policies. The PP analysis included participants who were randomised and received sufficient trial treatment, where sufficient was defined to be supplementation for at least 8 weeks.

10.2.3 Analysis of secondary outcome measures

All secondary analyses were conducted on the ITT population.

10.2.3.1 BSID III secondary outcomes

Secondary outcomes included CCS-BSID III at 12 months, BSID III composite language scale score (LCS-BSID III) and BSID III composite motor scale score (MCS-BSID III). These analyses follow the procedure described for the primary outcome analysis.

10.2.3.2 Vineland Adaptive Behaviour Scales II

The analysis of between group differences in the composite standard score on the Vineland II questionnaire at 12 and 24 months of supplementation followed the procedure described for the primary outcome analysis.

10.2.3.3 PR-VERP latency

Analysis of between group phase reversal transient and calculated Visual Event Related Potential (VERP) latencies at 6, 12, and 24 months of supplementation follows the procedure described for the primary outcome analysis. Missing data is not missing at random.

10.2.3.4 Fixation Shift test: proportion of correct responses

Between-group differences in the proportion of correct re-fixations for non-competition and competition at 6, 12 and 24 months of supplementation were analysed at 6, 12 and 24 months of supplementation using mixed effects logistic regression to account for the repeated measures over time. Logistic regression was used as the log binomial model with and without the interaction term did not converge. The mixed effects model included the proportion of correct responses (i.e. correct refixations over total number of trials delivered) at 6, 12 and 24 months of supplementation as the response variable, time points (6, 12 and 24 months), and

treatment group as fixed effects, and a patient specific random intercept. An interaction between time and treatment group was fitted as a fixed effect to allow estimation of treatment effect at both time points. The minimisation (design) factors of neurological damage (normal-mild/moderate/severe), gestation (≤31 weeks/31-42 weeks), and sex (male/female) could not be adjusted for as the models with and without interaction did not converge. Competition and non-competition were analysed separately.

10.2.3.5 Fixation Shift latency

Between group differences in FS latency for non-competition and competition at 6, 12 and 24 months of supplementation were analysed using mixed effects Cox proportional hazards regression. The mixed effects model included the times taken to respond at 6, 12 and 24 months of supplementation as the response variable, time points (6 months, 12 months, 24 months), and treatment group as fixed effects, and a patient specific random intercept. An interaction between time and treatment group was fitted as a fixed effect to allow estimation of treatment effect at both time points. The minimisation factors of neurological damage (normal-mild/moderate/severe), gestation (≤31 weeks/31-42 weeks), and sex were included as fixed effects. Time to re-fixation was limited to 10 seconds to account for "sticky fixation", where a child failed to make any fixation shift following appearance of the peripheral target, but remained fixed on the central stimulus. Competition and non-competition were analysed separately.

10.2.3.6 A Test Battery of Child Developmental for Examining Functional Vision

For analysis, ABCDEFV data were divided into core vision, attention and visuo-cognitive domains. Core vision comprised test items pupillary responses, eye movements, tracking, nystagmus, strabismus, convergence and defensive blink, as age appropriate. Attention included test items attention at distance, visual fields, preferential looking and a pass/fail score for fixation shift under non-competition and competition, as age appropriate. The visuocognitive domain included reaching, watching fallen toy, partially hidden and totally hidden toy, invisible displacement of hidden toy, black and white threads, bimanual box, shape matching, Griffith's boxes, embedded animals, block construction, as age appropriate (See Table 1.1-1.3 Appendix 1). Completed age appropriate items were given a score of 1, failed items were given a score of 0. Appropriate negatives e.g. absence of nystagmus were given a score of 1. A Fixation Shift pass was allocated a score of 1 and was awarded if the proportion of correct re-fixations ≥ 88% for non-competition, or ≥ 75% competition, as based on 85th percentile norms (personal communication Oliver Braddick October 2013). Domain scores were the sum of items passed (score =1) divided by the number of test items which should have been administered for age at time of testing. Binomial mixed effects logistic regression was used to analyse ABCDEFV domain and overall score at 6, 12 and 24 months of supplementation.

10.2.3.7 Orientation-reversal Visual Event Related Potentials

Between group differences in the presence of a statistically significant OR8 response at 6, 12 and 24 months of supplementation was analysed using mixed effects logistic

regression to account for the repeated measures over time; a log binomial model would not converge. The mixed effects model included responses at 6, 12 and 24 months of supplementation as the response variable, time points (6 months, 12 and 24 months), and treatment group as fixed effects, and a patient specific random intercept. An interaction between time and treatment group was fitted as a fixed effect to allow estimation of treatment effect at all time points.

The model including an interaction term between visit and treatment did not converge, and the model with no interaction term but adjusting for minimisation factors did not converge, therefore the model was fitted without adjusting for the minimisation factors, but is adjusted for treatment and visit to allow estimation of treatment effect at all time points. The odds ratios generated were adjusted for treatment and visit.

10.2.3.8 Anthropometry

Head circumference data was collected every 3 months, therefore data from each time point was analysed using mixed effects linear regression to take account of the repeated measures over time. For analysis all measurements were converted to a z-score. Baseline head circumference was entered as a covariate. The mixed effects model included all head circumference measurements as the response variable, time as a continuous covariate and treatment group as a fixed effect, with a patient specific random intercept. An interaction between time and treatment group was also included as a fixed effect along with the minimisation factors.

Analysis of weight, height, head circumference, mid-upper arm circumference and triceps skinfold thickness followed the same procedure as head circumference.

10.3 Blood Fatty Acid analyses

Blood fatty acid analyses were conducted using IBM SPSS Statistics 22.

10.3.1 Cohort Blood fatty acid analyses

Baseline blood fatty acid data was normally distributed. Participant mean blood fatty acid levels at baseline according to gestational age at birth and sex were compared using independent samples t-tests. Mean blood fatty acid levels (relative %) are presented alongside standard deviation, and 2 sided p-values, unless otherwise stated.

Correlation was used to identify any relationship between infant baseline blood fatty acid level and maternal blood fatty acid level at baseline. Multiple regression analysis was used to assess how well maternal blood fatty acid level, gestational age at birth and sex predicted infant baseline blood fatty acid level.

10.3.2 Dolphin trial blood fatty acid analyses

Mean blood fatty acid levels in the treatment and control group were assessed at baseline and 24 months using independent samples t-tests. Mean blood fatty acid levels are presented alongside standard deviation, and 2 sided p-values, unless otherwise stated.

Correlation was used to identify any relationship between mean blood fatty acid level at 24 months and BSID-III composite scores.

10.4 Cohort Visual Event Related Potential latency analyses

All statistical analyses were carried out using Stata 14 (StataCorp. 2015. *Stata Statistical Software: Release 14*. College Station, TX: StataCorp LP.). Latencies were plotted against gestational age to observe patterns, and fractional polynomial curves were fitted to separate groups (e.g. males and females), to observe differences in trajectories. These fractional polynomial curves used the two best fitting powers from the set (-2, -1, -0.5, 0, .5, 1, 2, 3). In order to be able to statistically compare two groups (e.g. males versus females) fractional polynomial curves were again fitted. One power was chosen from the set (-2, -1, -0.5, 0, .5, 1, 2, 3), and all groups (e.g. male and female) were fitted using this same power, to enable a comparison between groups. An interaction term was fitted for group and gestational age, and a random effect was added to account for multiple observations within the same patient. Variation in the number of observations per child was accounted for within the mixed effects regression model, which assumes that measurements from any same individual are correlated. The interpretation of these models is that the main effect assesses any separation between the groups, regardless of corrected gestational age at time of assessment; and the interaction term assesses whether the curves follow different trajectories over time (for example, they may cross over). The predicted latencies from the model were plotted to aid interpretation. In order to compare latencies for ARNI versus TDI, the mean, 10^{th} and 90^{th} centiles of TDIs were plotted over gestational age. This was calculated using fractional polynomials for the mean and for the standard deviation, each with 1 power. Thus, the proportion of ARNI children falling above the 90^{th} centile was calculated.

11 RESULTS – COHORT BLOOD FATTY ACID ANALYSES

The following section relates to cohort baseline fatty acid levels, and explores potential differences in baseline fatty acid levels between cohort subgroups. Fatty acid analyses relating to trial neurodevelopmental outcomes are presented separately in Section 15.3.

11.1 Exploratory analyses

Hypotheses:

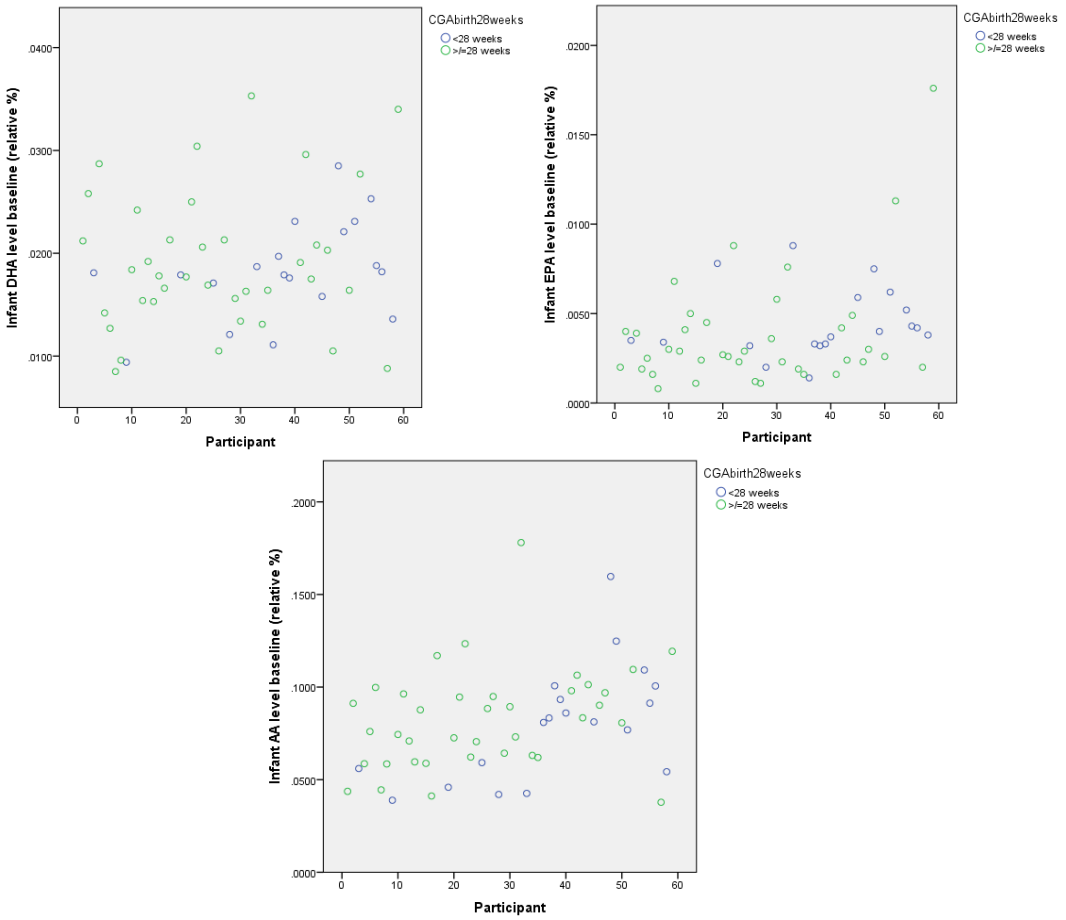
- Mean blood DHA and EPA levels are lower in infants born preterm compared to infants born at term.
- There is a direct positive correlation between maternal DHA and EPA level at baseline and infant baseline DHA and EPA level respectively.
- There is no sex difference in infant baseline DHA, EPA or AA at baseline.

11.1.1 Baseline blood fatty acid level and corrected gestational age at birth

Endogenous production of DHA is limited and the developing fetus is dependent upon third trimester transplacental transfer of DHA. Infants born preterm are at risk of DHA insufficiency. In order to investigate whether gestational age at birth affected LCPUFA levels we compared baseline EPA, DHA, and AA blood levels in those born less than 28 weeks gestational age compared to those born at or above 28 weeks gestational age. Figure 14 shows the distribution of baseline infant blood DHA level by gestational age at birth group (<28 weeks and ≥ 28 weeks gestational age). There

does not appear to be any difference in the spread of baseline fatty acid levels for DHA, EPA or AA by gestational age at birth group (Figure 14).

Figure 14. Baseline infant blood DHA, EPA and AA levels in infants less than or equal to 28 weeks gestation or above



Using an independent samples T-test, there was no difference in the mean baseline DHA (t= -0.453, df 55, p= 0.65), EPA (t = 0.871, df = 55, p = 0.39) or AA (t= -0.276, df= 55, p= 0.39) level of those infants born before 28 weeks and those infants born at or after 28 weeks (Table 3).

Table 3. Mean baseline infant blood fatty acid levels in infants less than or equal to 28 weeks gestation or above.

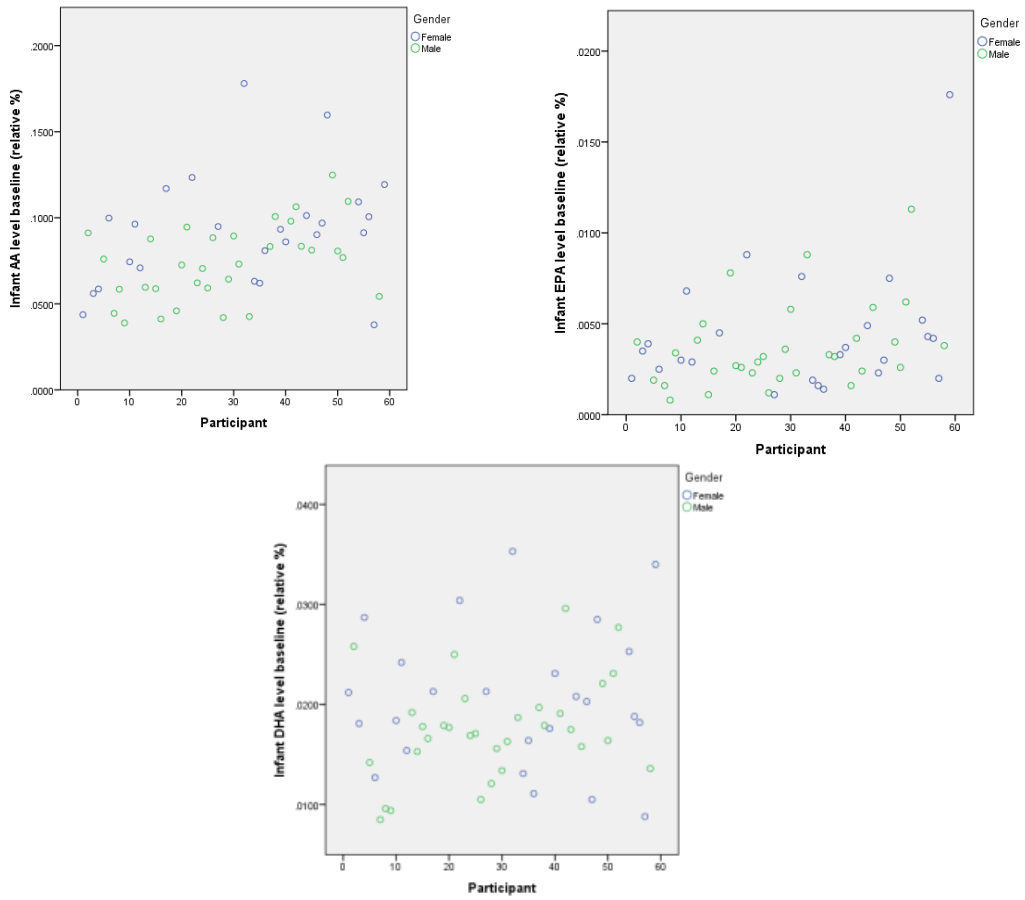
	Gestational age at birth		
Mean infant baseline blood fatty acid level (relative %)	Less than 28 weeks (n=19) (SD)	Greater than 28 weeks (n=38) (SD)	p-value*
DHA	0.183 (0.005)	0.191(0.007)	0.33
EPA	0.004 (0.002)	0.004 (0.003)	0.2
AA	0.08 (0.031)	0.08 (0.027)	0.39

*one sided p-value

11.1.2 Baseline blood fatty acid level and sex

Scatter plots of baseline infant DHA, EPA and AA blood levels does not suggest any sex difference in the spread of baseline fatty acid levels (Figure 15).

Figure 15. Baseline infant DHA, EPA and AA levels at baseline by sex.



Comparison of mean baseline DHA, EPA and AA levels using an independent samples t-test shows that there was no statistically significant difference in baseline blood DHA (t=1.884, df 55, p=0.06), or EPA level (t=0.908, df 55, p=0.368) between females and males, however mean blood AA level (t=2.54, df 55, p=0.014) was significantly higher in females than in males (Table 4).

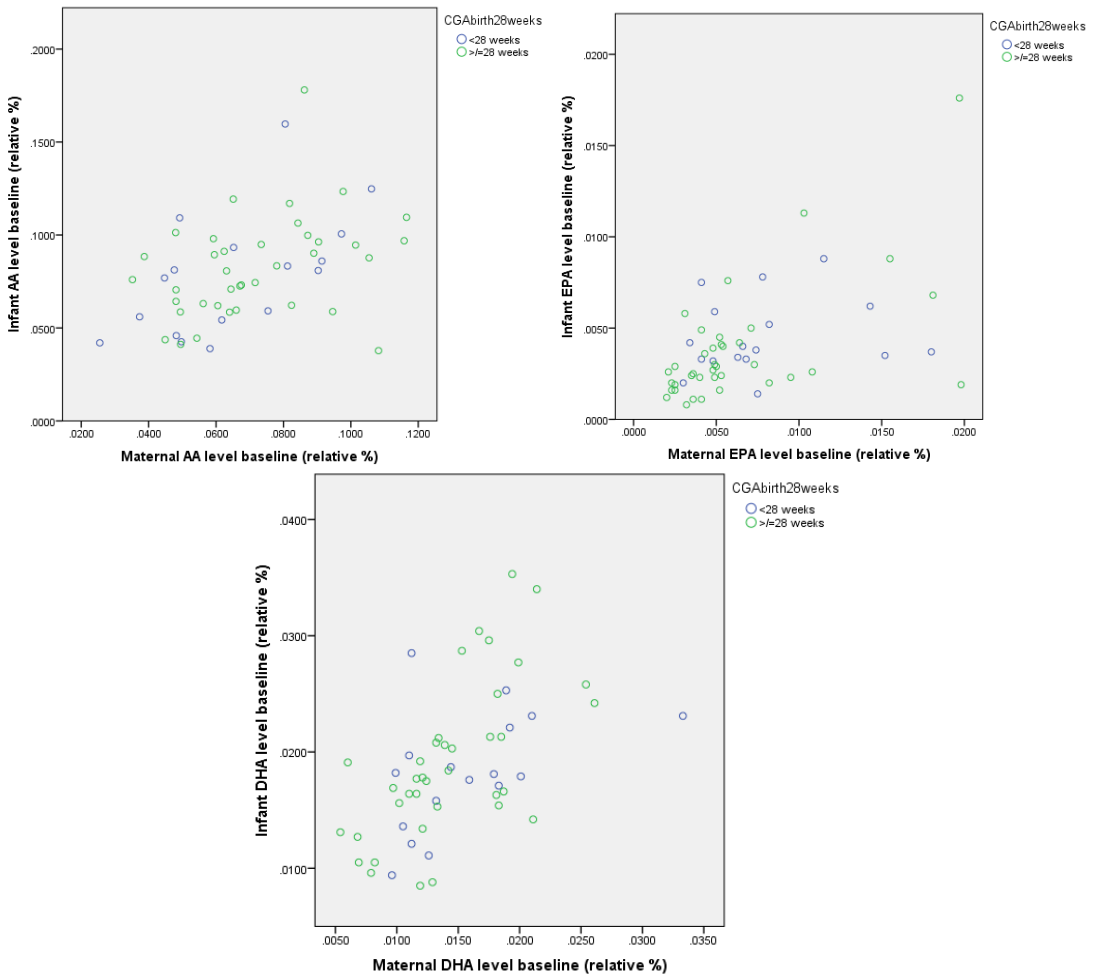
Table 4. Mean infant DHA, EPA and AA level in males and females

	Sex		
Mean infant baseline fatty acid level (relative %)	Male (n=25) (SD)	Female (n=32) (SD)	p-value
DHA	0.18 (0.005)	0.21 (0.007)	0.06
EPA	0.04 (0.002)	0.04 (0.003)	0.368
AA	0.074 (0.022)	0.092 (0.032)	0.014

11.1.3 Maternal baseline blood fatty acid levels and infant baseline blood fatty acid levels

Figure 16 shows the relationship of maternal baseline fatty acid level and infant baseline fatty acid level for DHA, EPA and AA. There is a direct positive relationship between maternal and infant blood fatty acid levels for DHA, EPA and AA (Figure 16).

Figure 16. Relationship between maternal and infant baseline DHA, EPA and AA levels in infants less than or equal to 28 weeks gestation or above



Correlation analyses show that overall there is a moderate correlation between maternal baseline DHA level and infant baseline DHA level (0.56); maternal DHA level accounts for 56% of the variation in infant baseline DHA level. The correlation between maternal DHA level at baseline and infant DHA level at baseline is stronger for infants born at or after 28 weeks gestational age (0.623) than for those born before 28 weeks gestational age (0.458). There is moderate correlation between maternal and infant EPA levels (0.518); maternal EPA level accounts for 52% of the variation in infant baseline EPA level. The correlation between maternal and infant

baseline EPA level is much stronger for infants born at or after 28 weeks gestation (0.606) compared to those born at less than 28 weeks (0.158). For AA there is moderate correlation between maternal blood AA level at baseline and infant blood AA level at baseline (0.437); maternal baseline AA level accounts for 44% of the variability in infant baseline AA level. The correlation between maternal and infant baseline AA level is stronger for those infants born at less than 28 weeks gestation than for those born at or greater than 28 weeks gestation (Table 5).

Table 5. Correlation coefficients for baseline maternal blood DHA level and baseline infant blood DHA level by gestational age at birth

Maternal baseline blood fatty acid level	**Infant baseline DHA level**	
	Gestational age at birth < 28 weeks	Gestational age at birth ≥ 28 weeks
DHA	0.458	0.623
EPA	0.158	0.606
AA	0.58	0.35

A multiple regression model including baseline maternal DHA level, gestational age at birth and sex, show that neither gestational age at birth nor sex significantly improve the predictability of infant baseline DHA, EPA or AA levels compared to maternal baseline DHA, EPA and AA level alone (Table 6).

Table 6. Contribution of maternal baseline DHA, EPA and AA level, gestational age at birth and sex to prediction of infant baseline blood fatty acid levels

Step	Fatty acid	Variable	B	SE B	β (constant)
1	DHA	Constant	0.009	0.002	
		Maternal DHA	0.645	0.133	.553
	EPA	Constant	0.002	0.001	
		Maternal EPA	0.324	0.074	.518
	AA	Constant	0.041	0.012	
		Maternal AA	0.572	0.162	.437
2	DHA	Constant	0.002	0.001	
		Maternal DHA	0.664	0.132	.57
		Gestational age at birth	0.000	0.000	.191
	EPA	Constant	0.002	0.001	
		Maternal EPA	0.320	0.075	.511
		Gestational age at birth	0.000	0.000	-0.039
	AA	Constant	0.039	0.013	
		Maternal AA	0.587	0.166	.449
		Gestational age at birth	0.000	0.001	-0.065
3	DHA	Constant	0.012	0.002	
		Maternal DHA	0.638	0.131	.547
		Gestational age at birth	0.000	0.000	.177
		Sex	-.002	0.001	-0.166
	EPA	Constant	0.002	0.001	
		Maternal EPA	0.316	0.078	.511
		Gestational age at birth	0.000	0.000	-0.042
		Sex	0.000	0.001	-0.028
	AA	Constant	0.051	0.014	
		Maternal AA	0.519	0.164	.397
		Gestational age at birth	0.000	0.001	-0.073
		Sex	-0.015	0.007	-0.259

Note: For DHA R^2 = 0.306 (p=0.000) for Step 1, ΔR^2 = 0.036 for Step 2 (p =0.097), ΔR^2= 0.027 for Step 3 (p = 0.146).
For EPA R^2 = 0.268 (p=0.000) for Step 1, ΔR^2 = 0.001 for Step 2 (p=0.749), ΔR^2= 0.001 for Step 3 (p=819).

For AA R^2 = 0.19 (p=0.001) for Step 1, ΔR^2 = 0.004 for Step 2 (p=0.612), ΔR^2= 0.064 for Step 3 (p=0.041).

11.2 Summary of cohort blood fatty analyses

Mean blood DHA, EPA and AA levels were similar in infants born before 28 weeks and at or greater than 28 weeks. No sex differences in infant baseline DHA or EPA levels were identified. Infant baseline AA level was higher in girls than in boys. There was moderate correlation between infant baseline DHA level and maternal baseline DHA, which was strongest in infants born at or after 28 week's gestation. In infants born at or after 28 week's gestation there was also a moderate correlation between infant EPA and maternal EPA blood levels at baseline, but not for those infants born less than 28 week's gestation. Infant AA level at baseline was less well correlated with maternal AA level in those born at or after 28 weeks gestation than for infants born before 28 week's gestation. Multiple regression analysis shows that maternal baseline blood DHA level explains 30.6% of the variance of infant DHA level, with gestational age at birth and sex contributing 3.6% and 2.7% of the variance in infant baseline DHA level. For EPA, maternal baseline blood EPA level explains 26.8% of the variance in infant baseline EPA level, with gestational age and sex contributing 0.1% each. For AA, only 19% of the variance in infant baseline level is explained by maternal AA level; gestational age at birth contributes only 0.4% and sex 6.4%.

12 RESULTS – PATTERN REVERSAL –VISUAL EVENT RELATED POTENTIAL LATENCY MEASURES IN TRIAL COHORT CHILDREN AT RISK OF NEURODEVELOPMENTAL IMPAIRMENT COMPARED TO TYPICALLY DEVELOPING CHILDREN

The following section includes analyses using the whole Dolphin study cohort (control and treatment children). Given there were no between group treatment differences identified for PR-VERP measures, analyses comparing the whole Dolphin study cohort to a cohort of 101 *typically developing infants* (TDI) are also presented. The typically developing infants were recruited by Dr Jin Lee as part of her doctoral work (217, 460, 461).

Hypotheses:

- Transient PR-VERP latency in trial children with risk factors for neurodevelopmental impairment will be prolonged compared to transient PR-VERP latency in *typically developing children* (Section 12.1)
- There will be a direct relationship between brain injury severity and PR-VERP transient latency in children at risk of neurodevelopmental disability (Section 12.1.1)
- There will be no difference in PR-VERP transient latency between boys and girls in children at risk of neurodevelopmental disability (Section 12.1.2)

- There will be no difference in PR-VERP transient latency between children born preterm compared to children born at term (Section 12.1.3)
- Calculated PR-VERP latency in trial children with risk factors for neurodevelopmental impairment will be prolonged compared to calculated PR-VERP latency in *typically developing children* (Section 12.2)
- There will be a direct positive relationship between brain injury severity and calculated PR-VERP latency in children at risk of neurodevelopmental disability (Section 12.2.1)
- There will be no difference in PR-VERP calculated latency between boys and girls in children at risk of neurodevelopmental disability (Section 12.2.2)
- There will be no difference in calculated PR-VERP latency between children born preterm compared to children born at term (Section 12.2.3)

PR-VERP can be used to assess the integrity and maturity of the visual system in infants. PR-VERP latency falls rapidly following birth, as a result of retinal development, progressive myelination of the optic nerve and radiation, exuberant cortical synaptogenesis, and maturation of synaptic transmission within the visual pathways. Transient PR-VERP latency is believed to reflect the time between VERP signal onset and activation of the relevant cortical neuronal pool in occipital cortex.

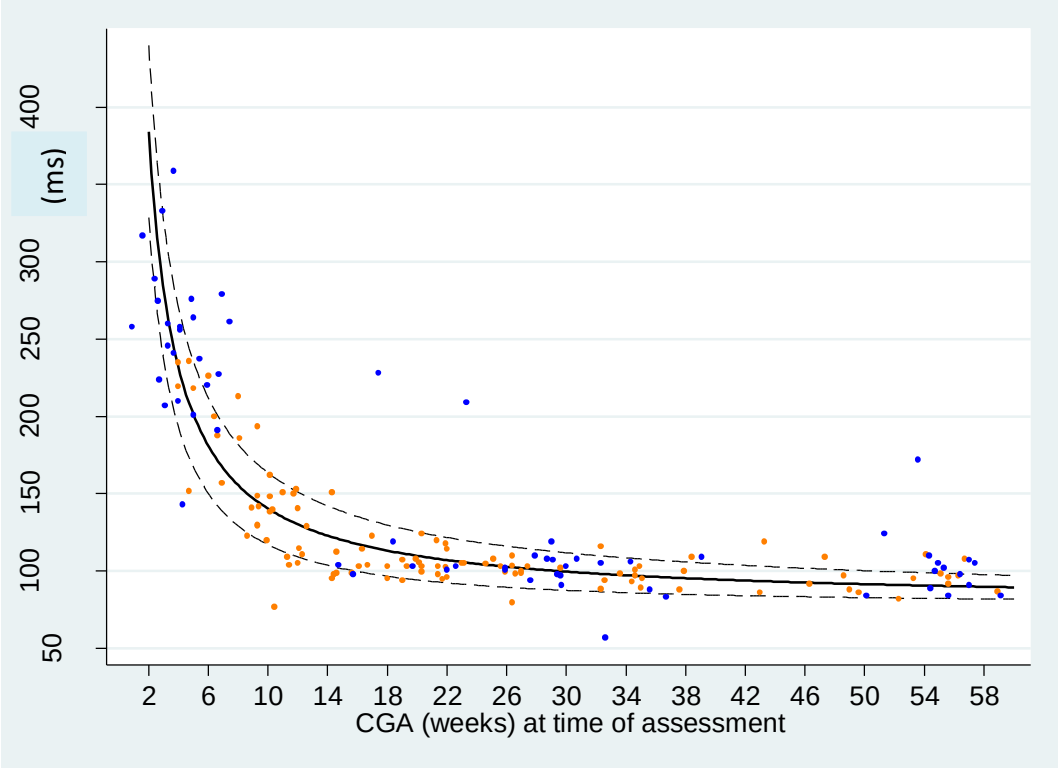
In contrast, calculated latency is thought to reflect the timing of the whole VERP waveform and is prolonged compared to transient latency in young infants, taking longer to reach adult calculated latency values (around 30 weeks), compared to transient latency (around 15 weeks). Delay in reaching adult calculated latency values may reflect maturation of cortical processing networks beyond V1 (217). Please refer to Introduction Section 9.11.2 for a fuller discussion of the relation between these two PR-VERP latency measures. All figures presented in this section depict all observations, rather than mean or median values per child. Raw PR-VERP data is shown in Appendix 6.

12.1 Transient Pattern Reversal –Visual Event Related Potential latency in trial participants compared to typically developing infants

In order to assess whether or not transient PR-VERP latency is prolonged in children with risk factors for neurodevelopmental impairment, as defined by the Dolphin Trial entry criteria (see General Methods Section 10.1.1), PR-VERP transient latency for trial participants was first plotted alongside PR-VERP transient latency for a cohort of 101 typically developing infants recruited by Dr Jin Lee as part of her doctoral work examining the validity of calculated PR-VERP latency as an alternative to PR-VERP transient latency in adults and infants(217, 460, 461). One hundred and ninety eight observations were available from 138 children (101 TDI and 37 ARNI). The TDI group consisted of 49 boys and 52 girls, born within 2 weeks of term (40 weeks), whose median age at testing was 23.6 weeks (range 3.6 to 79 weeks CGA). The median number of observations per child was 1 (range 1-4) observations. Figure 17 shows the raw transient PR2 VERP latency values. Mean transient PR2 VERP latency for

typically developing infants (TDI) is denoted by the solid black line, with 10th and 90th percentiles denoted by dashed black lines. Orange dots correspond to individual observations for TDI, and blue dots to trial individual observations. This figure shows that 31% of observations for children ARNI fall above the TDI 90th percentile.

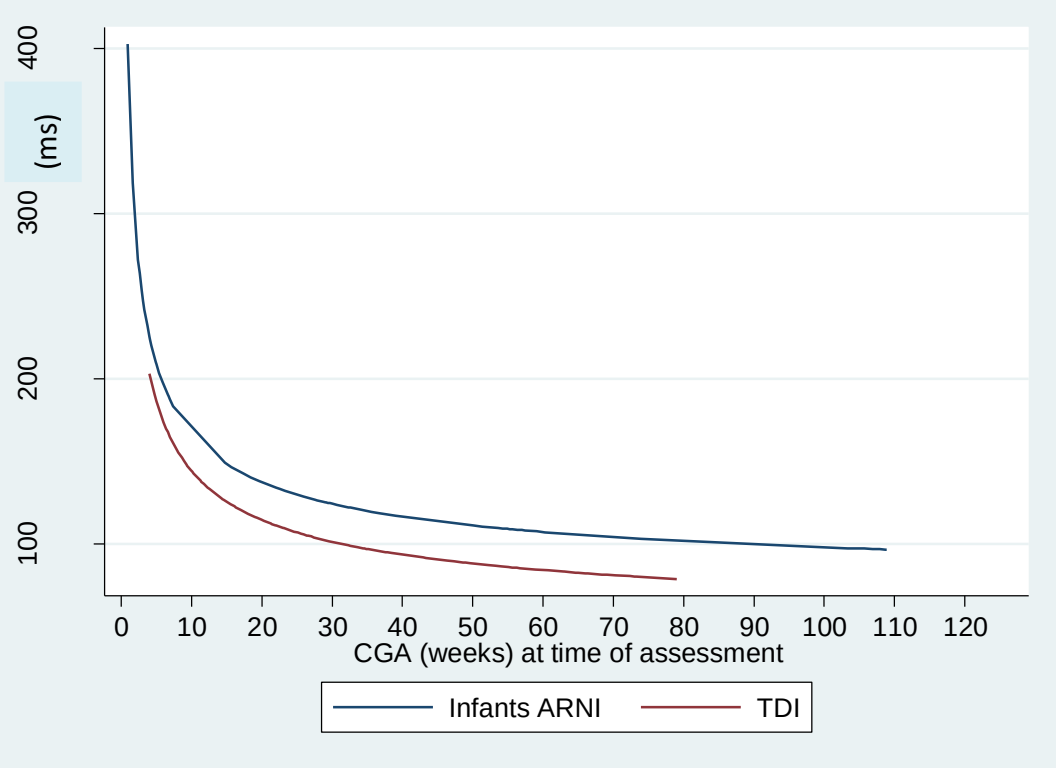
Figure 17. Raw Pattern Reversal-Visual Event Related Potential transient latency values for children at risk of neurodevelopmental impairment and typically developing infants, showing 10th and 90th percentiles for typically developing infants



Mean transient PR-VERP latency was prolonged in infants ARNI compared to TDI. There was a statistically significant effect for group, with TDI having shorter latencies than infants ARNI (mean difference = -23.3ms, 95% CI (-42.10 - -4.54) p=0.015). There was no significant interaction between corrected gestational age and

treatment (mean difference per week GA =1.63ms, (CI -67.94 – 77.89) p=0.96). Figure 18 shows the modelled data. These data confirm the hypothesis that as a group, infants ARNI have prolonged transient PR-VERP latency compared to TDI.

Figure 18. Modelled transient Pattern Reversal-Visual Event Related Potential latency in Dolphin trial participants and typically developing infants



12.1.1 Transient Pattern Reversal –Visual Event Related Potential latency by brain injury severity

To assess the effect of brain injury severity grading as defined by the Dolphin trial minimisation criteria (See section 10.1.2) transient PR-VERP latency was compared across brain injury severity groups normal/mild vs moderate and normal/mild vs severe groups. Seventy seven observations from 37 infants contributed to the analysis. A median of 2 (range 1-4) observations were made. Transient PR-VERP

latency did not differ significantly between children from the normal/mild and moderate brain injury severity groups (mean difference = -24.82ms, 95% CI (- 63.25 – 13.61), p=0.21) or between children from the normal/mild and severe brain injury severity groups (mean difference = -5.98ms, 95% CI (-48.11 – 36.16), p=0.78. The interaction of brain injury severity with corrected gestational age (CGA) at time of assessment was not significant in either brain injury severity group. These data show that transient PR-VERP latency does not differ according to brain injury severity, despite the observed separation between children with risk factors for neurodevelopmental impairment and TDI.

Figure 19 shows the raw data for TDI and brain injury severity groups with individual fractional polynomial curves overlaid. Figure 20 shows that there is little separation of transient PR-VERP latency between brain injury severity groups.

Figure 19. Transient Pattern Reversal-Visual Event Related Potential latency by trial participant brain injury severity grouping compared to typically developing infants

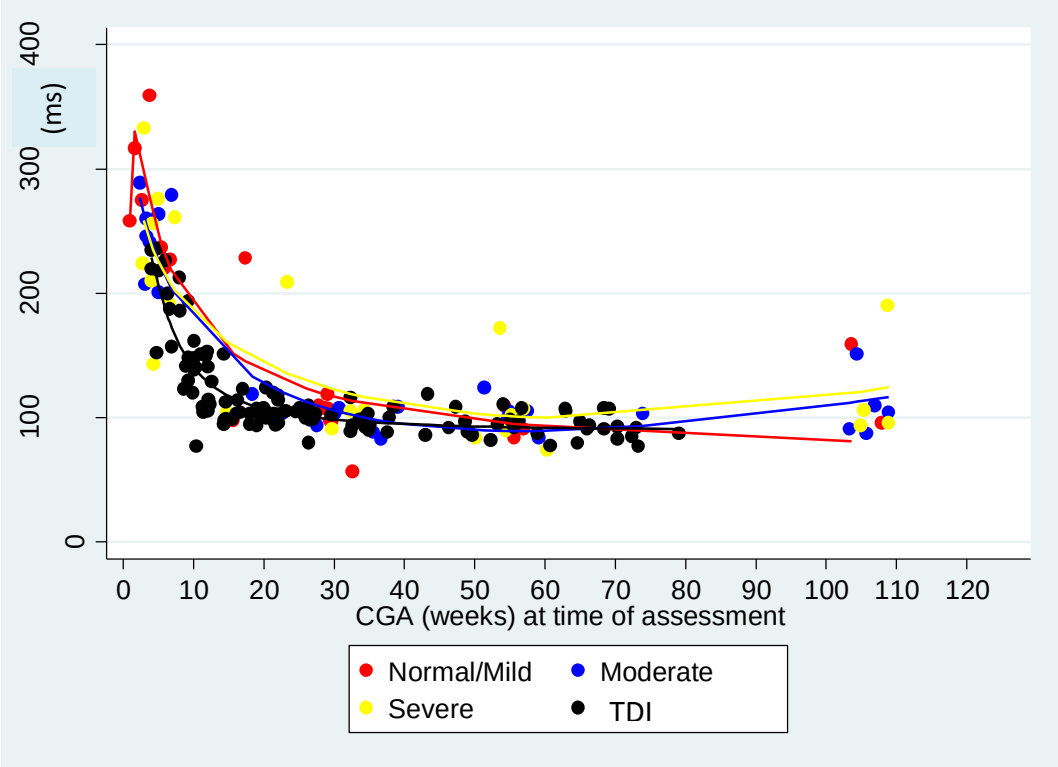
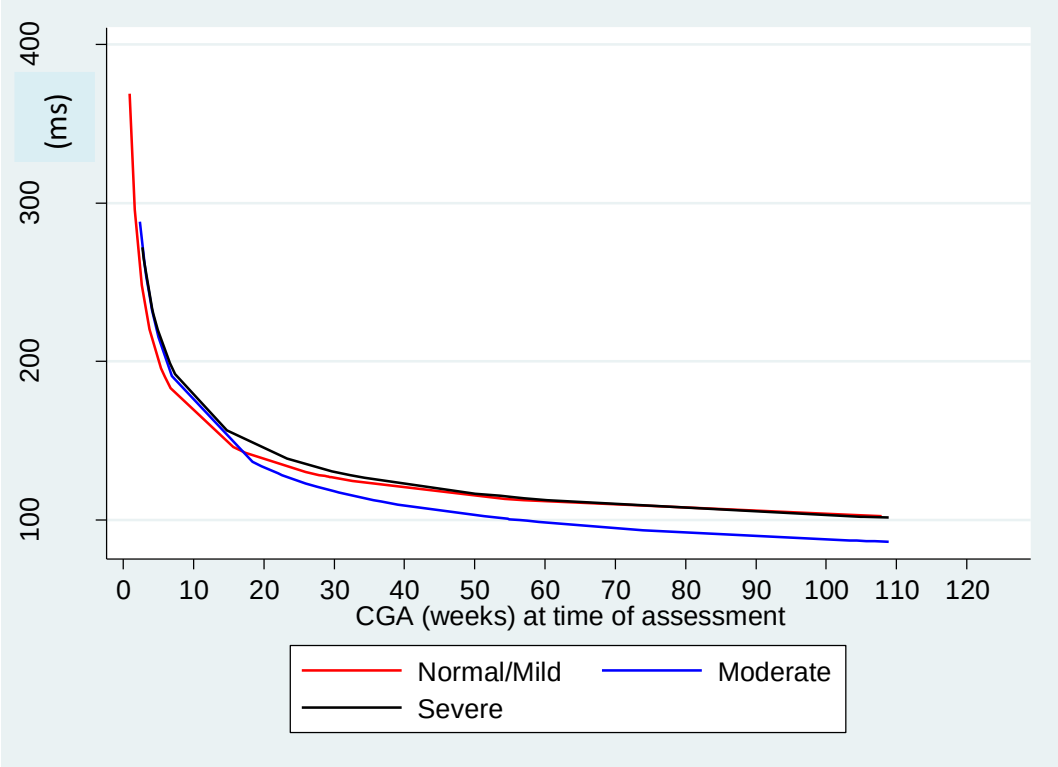


Figure 20. Modelled transient Pattern Reversal-Visual Event Related Potential latency by trial participant brain injury severity group



Modelled figures will only be shown from this point forward, if they aid data interpretation alongside the raw data figures shown.

Table 7 shows that there was a high percentage of missing PR-VERP transient latency observations across brain injury severity categories. 76% of missing observations were from infants with severe brain injury. The high proportion of missing data makes interpretation of these results challenging, and may be influencing the lack of difference in PR-VERP transient latency between brain injury severity groups, in particular between children with severe brain injury compared to children with no/mild brain injury.

Table 7. Number and percentage of missing transient Pattern Reversal-Visual Event Related Potential latency values for infants at risk of neurodevelopmental impairment by brain injury severity

Brain injury severity grading	Missing data
	Number of observations, n (%)
Normal-Mild	44 (66)
Moderate	33 (65)
Severe	53 (76)
Total	130 (63)

12.1.2 Transient Pattern Reversal –Visual Event Related Potential latency by sex

To establish whether or not there was any sex effect on transient PR-VERP latency in infants with ARNI, mean transient latency values were compared between boys and girls. Thirty seven infants (77 observations) contributed to the analysis. A median of 2 (range 1-4) observations were made per child. There was no statistically significant difference in transient PR-VERP latency between boys and girls (mean difference = 9.83ms, 95% CI -23.3-43.0, p=0.56), or when CGA at time of assessment was included in the model (mean difference = -37.39ms, 95% CI 34.95-85.68, p=0.46).

Figure 21 shows the raw data with independent fractional polynomial curves overlaid. This figure demonstrates that there is no statistically significant difference in transient PR-VERP latency between boys and girls.

Figure 21. Transient Pattern Reversal-Visual Event Related Potential latency in boys and girls at risk of neurodevelopmental impairment

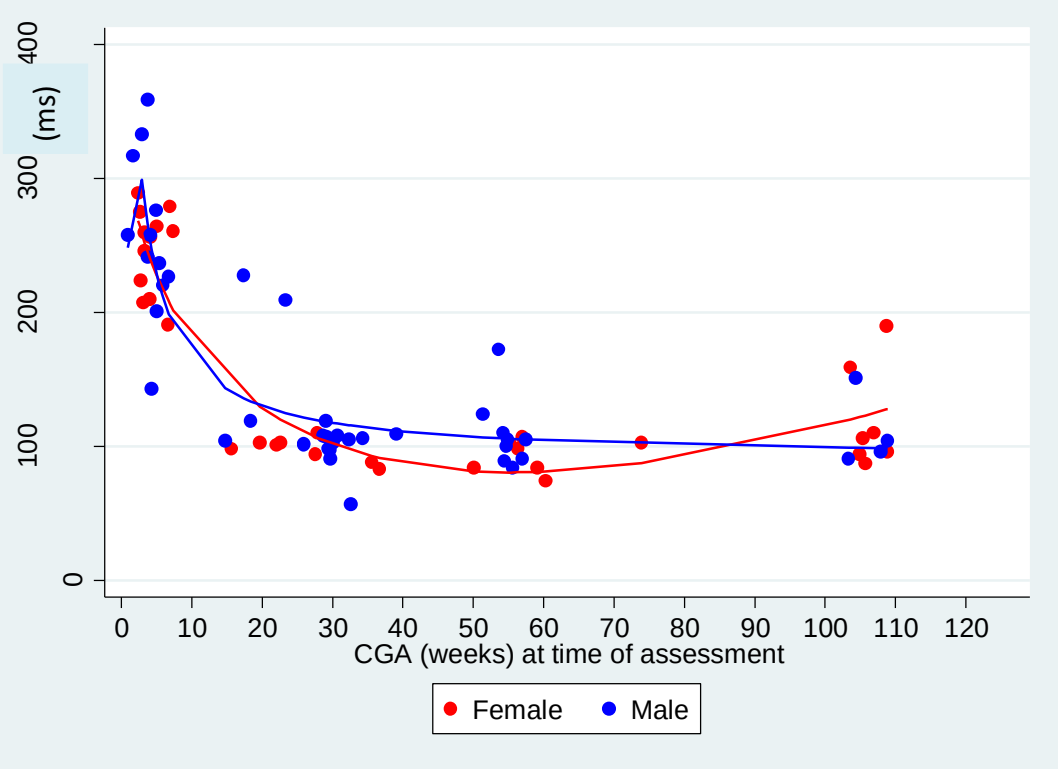


Table 8 shows the brain injury severity grading of boys and girls contributing transient PR2 data. These data show that there are equal numbers of boys and girls with severe brain injury (n=6). There are more boys than girls with normal/mild brain injury (9 boys compared to 2 girls), and fewer boys than girls with moderate brain injury (6 boys compared to 8 girls). It is possible that the lack of a statistically significant difference in PR transient latency is being influenced by the milder overall brain injury profile of the boys.

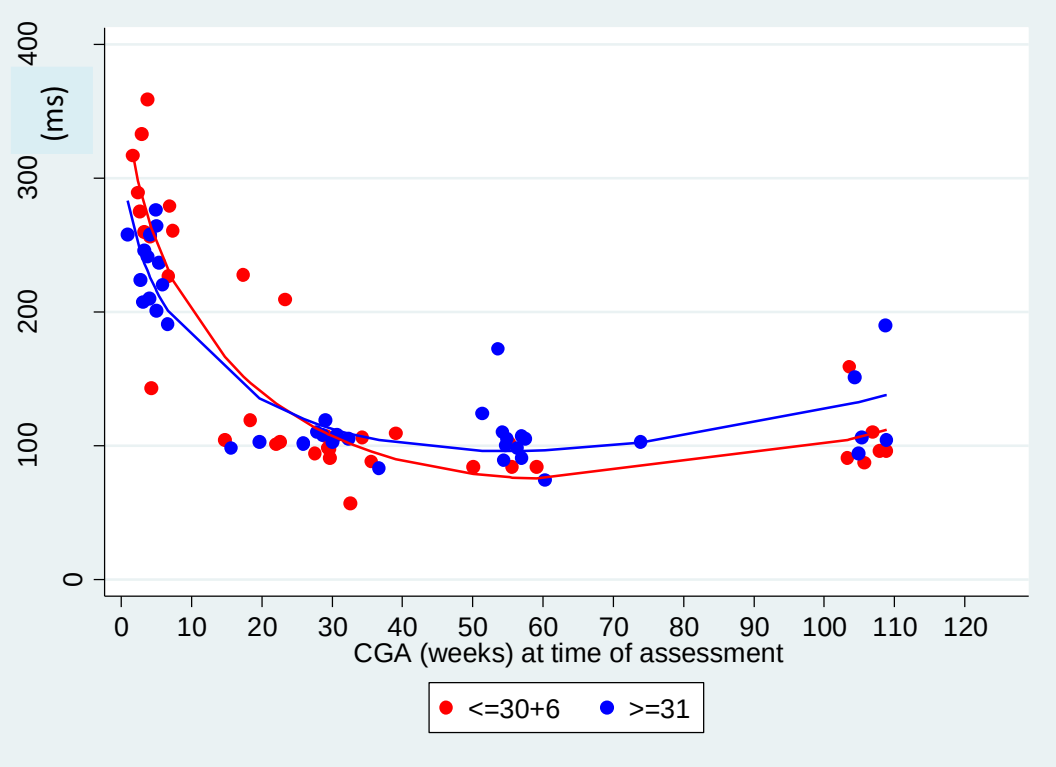
Table 8. Brain injury severity grading of males and females contributing transient Pattern Reversal-Visual Event Related Potential data

Brain injury severity grading	Number of children (n)		
	Sex		
	Boys	Girls	Total
Normal-Mild	9	2	11
Moderate	6	8	14
Severe	6	6	12
Total	21	16	37

12.1.3 Transient Pattern Reversal –Visual Event Related Potential latency by corrected gestational age at birth

To assess the effect of gestational age at birth on transient PR-VERP latency mean transient PR-VERP latency was compared in gestational age groups as defined by the Dolphin Trial minimisation criteria (gestational age at birth ≤30^{+6} weeks vs ≥ 31 weeks) and by gestational age at birth ≤36^{+9} weeks vs ≥ 37 weeks. Seventy seven observations were made in 37 infants. A median of 2 observations were made per child (range 1-4 observations). Transient PR-VERP latency was shorter in those infants born at 30^{+6} weeks or below compared to those born at 31 weeks or above (mean difference = 32.59ms, 95% CI 1.64-63.53, p = 0.04). The interaction term gestational age at birth greater than 31 weeks and CGA at time of assessment was also significant (mean difference = - 149.11ms, 95% CI (-241.63 - -56.6), p=0.002), suggesting that the effect of gestational age at birth varies with time. Figure 22 shows the PR2 transient latency plotted against CGA, with independent fractional polynomial curves overlaid.

Figure 22. Transient Pattern Reversal-Visual Event Related Potential latency in trial infants born at 30^{+6} weeks or below compared to those born at 31 weeks or above



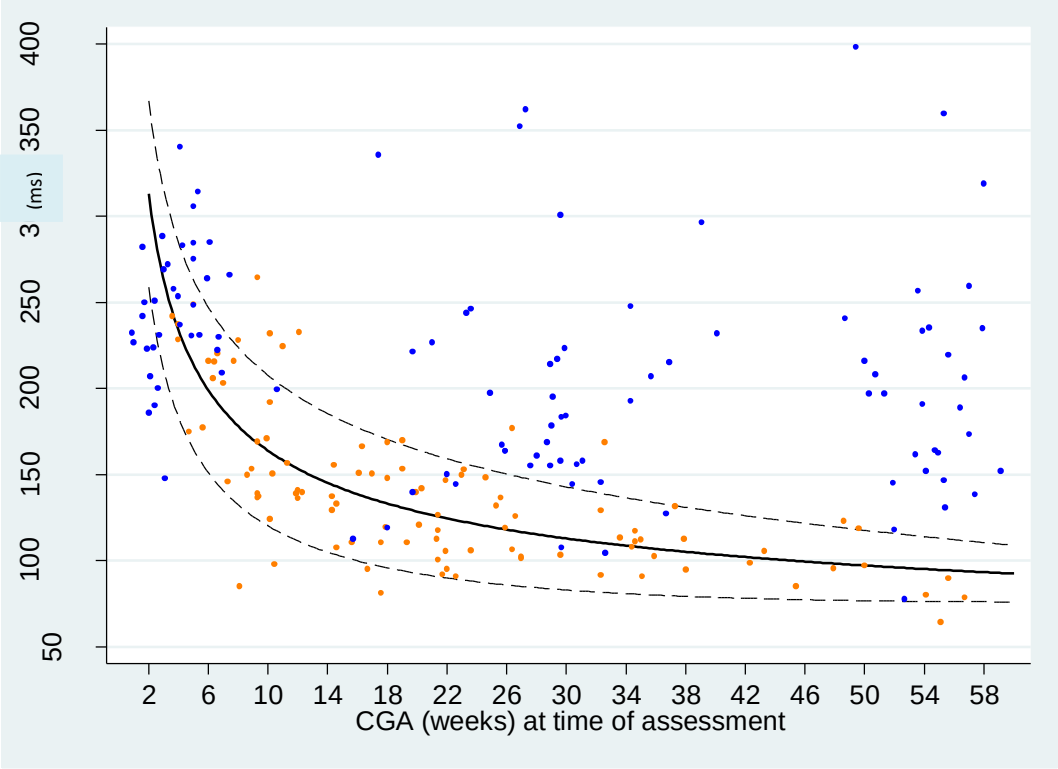
Re-analysis of the data according to re-grouping into gestational age at birth ≤36^{+9} weeks vs ≥ 37 weeks did not identify any differences between groups (mean difference = -5.3, 95% CI (-39.2-28.6), p=0.76) (data not shown) suggesting that the observed differences applied only to the more premature infants.

12.2 Calculated Pattern Reversal –Visual Event Related Potential latency in trial participants compared to typically developing infants

In order to ascertain if there were any differences in calculated PR-VERP latency between infants ARNI and TDI, raw calculated PR-VERP as defined by the Dolphin Trial entry criteria (see Methods Section 10.1.1), calculated PR-VERP latency for trial

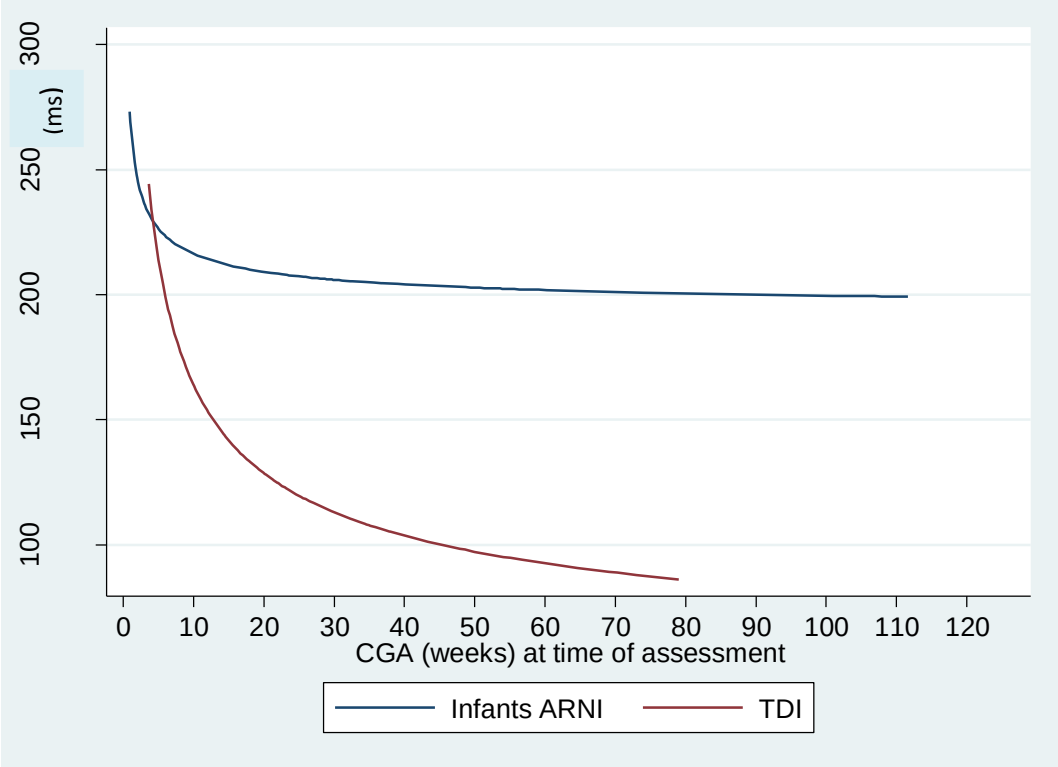
participants was first plotted alongside calculated PR-VERP latency for a cohort of 101 typically developing infants(460) . Two hundred and forty three observations were available for 139 children. The median number of observations per child was 1 (range 1-5) observations. Figure 23 shows the raw calculated PR-VERP data. Mean calculated PR-VERP latency for TDI is denoted by the solid black line, with 10th and 90th percentiles denoted by dashed black lines. Orange dots correspond to individual data for TDI, and blue dots to trial participants. This figure shows that 66% of values obtained from children ARNI fall above the 90th percentile for TDI.

Figure 23. Raw calculated Pattern Reversal-Visual Event Related Potential latency values for children at risk of neurodevelopmental impairment and typically developing infants, showing 10th and 90th percentiles for typically developing infants



Comparison of mean calculated PR-VERP latency in infants ARNI compared to TDI showed that calculated PR-VERP was prolonged in infants with risk factors for neurodevelopmental disability compared to normally developing infants. There was a statistically significant effect for both group (mean difference = -148.60ms 95% CI -179.77—117.43, p=0.00) and the interaction of group with time (mean difference = 304.29ms, 95% CI 189.85-418.72, p=0.00) in favour of the TDI group. These data show calculated PR-VERP latency is prolonged in infants ARNI compared to TDI and remains elevated across the testing period. Figure 24 provides graphical illustration of the modelled data.

Figure 24. Modelled calculated Pattern Reversal-Visual Event Related Potential latency in trial participants and typically developing infants

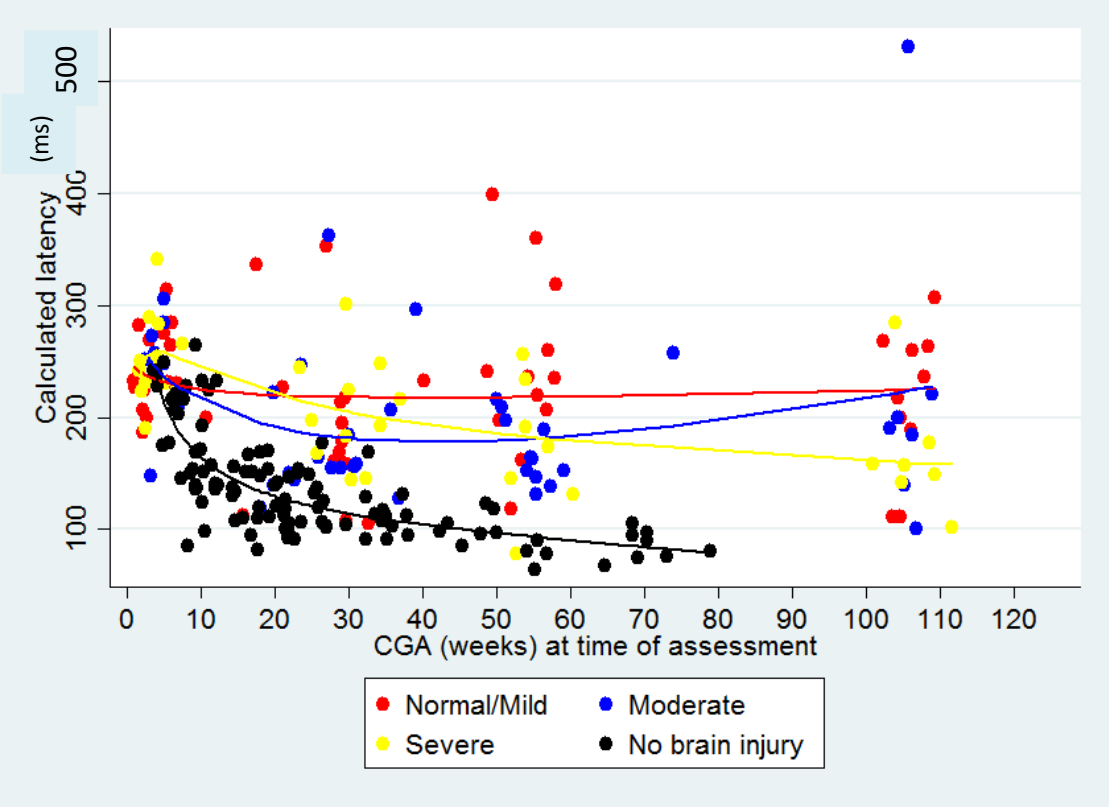


12.2.1 Calculated Pattern Reversal –Visual Event Related Potential latency by brain injury severity

To investigate whether or not there was a direct relationship between brain injury severity and calculated PR-VERP calculated latency was compared for normal/mild and moderate, and normal/mild and severe brain injury severity groups. Fifty one children (133 observations) contribute to the mixed effects multiple linear regression analysis (median 2 observations per child, range 1-5 observations). Calculated PR-VERP latency between normal/mild and moderate brain injury severity groups (mean difference = - 5.77ms, 95% CI (-68.7-80.23), p=0.88), and between normal/mild and severe brain injury severity groups (mean difference = - 39.69ms, 95% CI (-26.54-105.93), p=0.24) were not significantly different. The interaction of brain injury

severity with corrected gestational age (CGA) at time of assessment was also statistically insignificant, although the interaction between normal/mild compared to severe brain injury and CGA at time of assessment is close to statistical significance (mean difference -18.88ms, 95% CI (-38.59 - 0.835), p=0.061) . Figure 25 provides graphical illustration of the raw data.

Figure 25. Calculated Pattern Reversal-Visual Event Related Potential latency in trial participants by brain injury severity group



There was a high proportion of missing data for calculated PR-VERP latency. Table 9 shows the brain injury severity grading of the children for whom calculated PR-VERP latency was unavailable; the highest proportion of missing observations were in children with moderate and severe brain injury. It is possible that the high

proportion of missing data, in particular from children with severe brain injury has led to a conservative estimate of between brain injury severity group differences in calculated latency. A more complete data set may have identified greater differences in calculated PR-VERP latency between children with severe brain injury compared to children with normal/mild brain injury.

Table 9. Brain injury severity grading for children for whom it was not possible to produce a calculated Pattern Reversal-Visual Event Related Potential latency

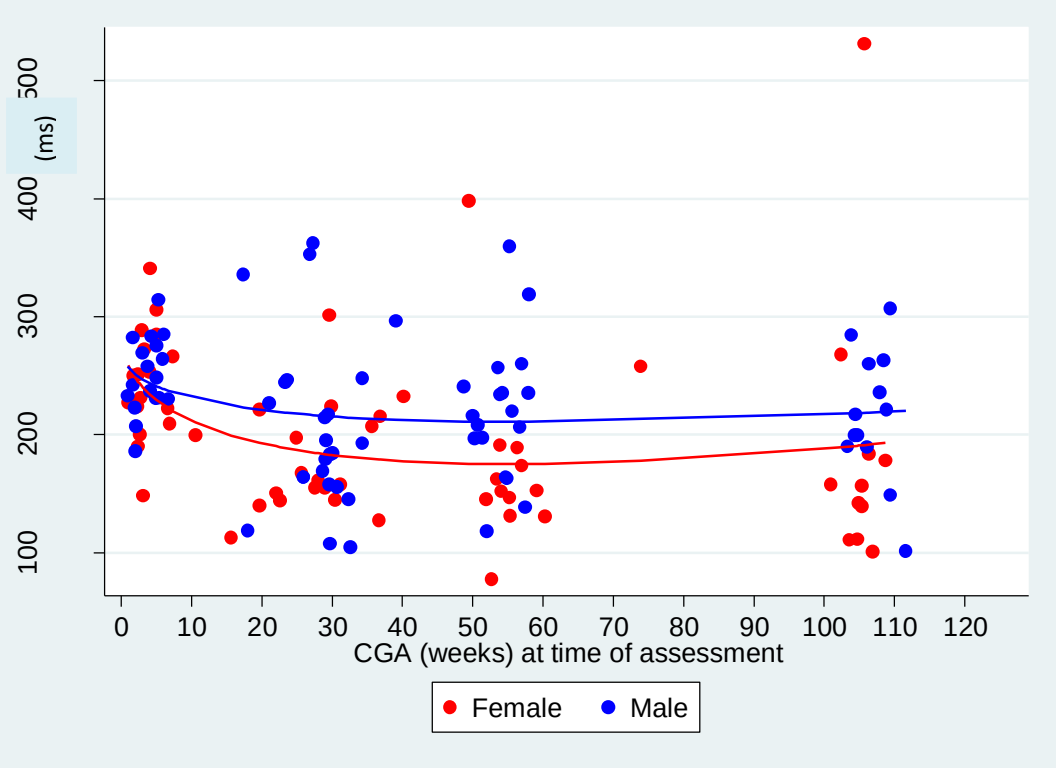
Brain injury severity grading	Missing data	
	Number of missing observations (n)	Percentage of missing observations (%)
Normal-Mild	14	21
Moderate	21	65
Severe	39	76
Total	74	36

12.2.2 Calculated Pattern Reversal –Visual Event Related Potential latency by sex

In order to test whether or not there was a sex effect on calculated latency, calculated PR-VERP latency was compared between boys and girls. One hundred and thirty three observations were made on 51 infants. The median number of observations per child was 2 (range 1-5) observations. There was no statistically significant sex difference in calculated PR-VERP latency (mean difference = 0.04ms, 95% CI (-57.76-57.84), p=0.99), or with the interaction term sex by CGA at time of assessment (mean difference = 7.81ms, 95% CI (-9.15-24.77), p=0.37). Figure 26

shows the raw data by sex. Curves have been fitted independently for each group using fractional polynomials. Although not statistically significant, this figure shows a trend of higher calculated latency in boys than in girls.

Figure 26. Calculated Pattern Reversal-Visual Event Related Potential latency in males and females with risk factors for neurodisability



Re-analysis of the data following removal of the outlier visible in Figure 26 identifies a trend towards higher calculated PR-VERP latency in male infants which approaches statistical significance (mean difference = 13.3ms, 95% CI (1.64 -28.23), p=0.081), data not shown.

It is possible that any apparent sex differences in calculated PR-VERP are being driven by brain injury severity. To consider this possibility, data on brain injury

severity is compared for males and females contributing calculated latency data. Table 10 shows the brain injury severity of males and females contributing calculated latency data.

Table 10. Brain injury severity for males and females with calculated PR-VERP latency data

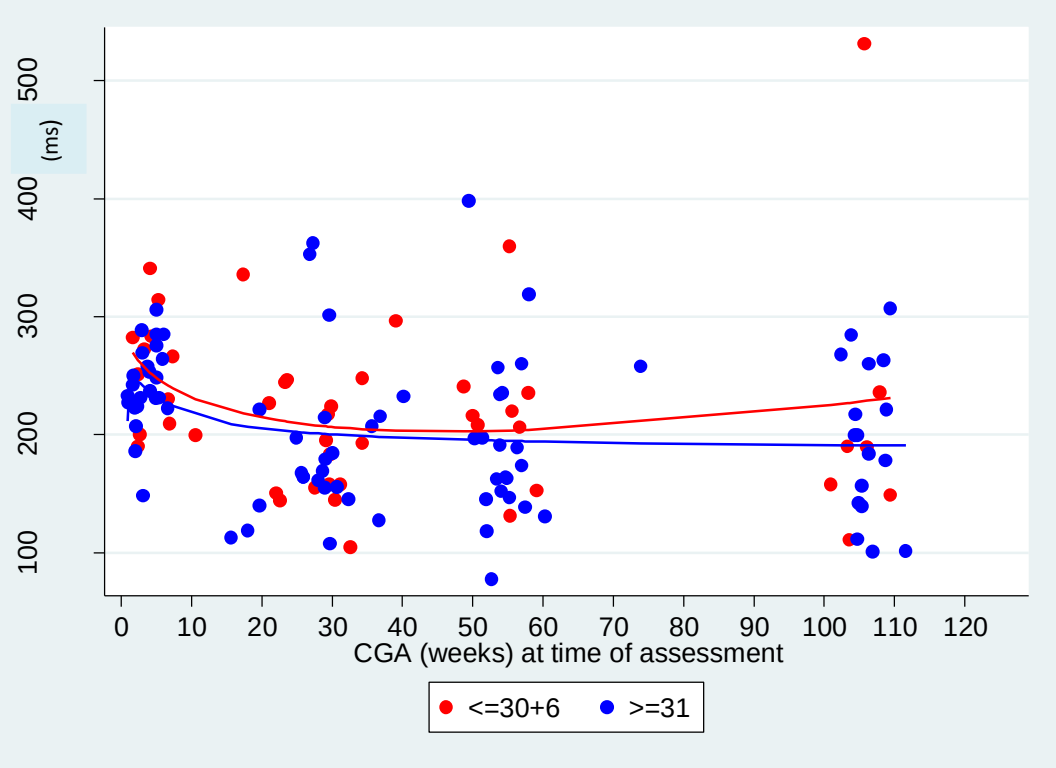
Brain injury severity grading	Sex		
	Female	Male	Total
Normal-Mild	3	15	18
Moderate	9	6	15
Severe	11	7	18
Total	23	28	51

12.2.3 Calculated Pattern Reversal –Visual Event Related Potential latency by gestational age at birth

Calculated PR-VERP latency may vary with gestational age at birth, and so calculated PR-VERP latency was compared for gestational age at birth groups ≤30^{+6} weeks vs ≥ 31 weeks, as per the Dolphin Study minimisation criteria, and by clinically defined groups ≤36^{+9} weeks (pre-term) vs ≥ 37 weeks (term). Fifty one infants (26 preterm ≤36^{+9} weeks) contributed a total of 133 observations to the mixed effects multiple linear regression analysis, with a median number of observations per child of 2 (range 1-5 observations). Calculated PR-VERP latency did not differ significantly between those born ≤ 30+6 weeks and those born ≥ 31 weeks gestation, either by group (mean difference = -15.02ms , 95% CI (-79.85-49.81), p= 0.65) or by the interaction of group with CGA at time of assessment (mean difference = 0.13ms, 95% CI (-19.01 – 19.26), p= 0.989). Figure 27 shows the raw data by gestational age

at birth group. Curves have been fitted independently for each group using fractional polynomials. This figure shows that there is no difference in calculated latency for those children born ≤36^{+9} weeks compared to those born ≥ 31 weeks gestation.

Figure 27. Calculated Pattern Reversal-Visual Event Related Potential latency by gestational age at birth in infants with risk factors for neurodisability



Re-analysis of the data following removal of the outlier visible in Figure 27 did not identify any significant difference between those born ≤ 30+6 weeks and those born ≥ 31 weeks gestation (mean difference = -35.12ms, 95% CI (-93.44 – 23.19), p=0.23). The interaction term gestational age at birth group and CGA at time of assessment was also non-significant (mean difference = 8.92ms, 95% CI (-8.37-26.2), p=0.31), showing that corrected gestational age at the time of the assessment visit did not

alter the fact that there was no difference in calculated latency between the two groups.

12.2.3.1 Post-hoc analysis

Calculated PR-VERP data was re-analysed using revised gestational age cut offs ≤ 36+9 weeks (preterm) and ≥ 37 weeks (term). There was no statistically significant difference in calculated PR-VERP latency between groups (mean difference = -5.84ms, 95% CI (-65.23 – 53.56), p=0.85) or for the interaction of gestational age with time (mean difference = - 1.05ms, 95% CI (-18.44-16.35), p= 0.91), showing that re-defining gestational age groups according to clinical definitions of preterm and term birth did not identify any between group differences in calculated latency.

12.3 Summary of findings in Pattern Reversal –Visual Event Related Potential latency measures in trial cohort children at risk of neurodevelopmental impairment compared to typically developing infants

Transient and calculated PR-VERP latency are prolonged in infants with risk factors for neurodisability compared to TDI. However calculated latency was prolonged to a greater degree, and remained prolonged across the first year of life compared to TDI. This was not the case for transient latency in infants ARNI, where transient latency followed a similar trajectory as for TDI, reaching adult latency of 100ms at 89 weeks compared to 29.5 weeks in TDI (Fig 18). Neither transient nor calculated latency discriminated between brain injury severity groups. Calculated latency, but not

transient latency identified a trend towards higher PR-VERP latencies in boys compared to girls. Transient PR-VERP latency identified shorter latencies in infants born at 30^{+6} weeks or below, compared to more mature infants.

13 RESULTS - FIXATION SHIFT PERFORMANCE IN CHILDREN AT RISK OF NEURODEVELOPMENTAL IMPAIRMENT

13.1 Fixation Shift performance in infants at risk of neurodevelopmental disability

Hypotheses:

- Fixation Shift performance, particularly under competition, will be poorer in children with more severe brain injury compared to children with normal/mild brain injury
- Fixation Shift performance will be comparable in male and female children
- Fixation Shift performance will be poorer in children born at ≤ 30^{+6} weeks compared to children born at ≥ 31 weeks
- Fixation Shift latency, particularly under competition, is prolonged in children with more severe brain injury compared to children with normal/mild brain injury

Raw FS data for the Dolphin cohort is shown in Appendix 7. To establish whether or not FS performance varies with brain injury severity, the FS performance of Dolphin cohort children with moderate and severe brain injury, as defined by the Dolphin Study criteria, were separately compared to children with normal/mild brain injury severity. FS performance for boys compared to girls, and for children born at ≤ 30^{+6} weeks compared to children born at ≥ 31 weeks was also investigated. Forty seven

children contribute to the following analyses. Table 11 gives odds ratios for the number of correct re-fixations by trial minimisation factors, brain injury severity, sex and gestational age at birth. Odds ratios are provided for children with moderate brain injury compared to normal/mild brain injury and severe brain injury compared to normal/mild brain injury, for children born at gestational age ≥ 31 weeks compared to ≤ $30^{+6,}$ and for males compared to females. There is no significant difference in the number of correct re-fixations across any of the brain injury severity groups under non-competition (p=0.54) or competition (p=0.66). There is a non-significant trend of poorer FS competition performance in children with severe brain injury compared to children with normal/mild brain injury. There are no significant differences in the number of correct re-fixations according to CGA at birth, however there is a non-significant trend of poorer competition performance in children born ≥ 31 weeks compared to children ≤ 30^{+6} weeks. FS performance under non-competition and competition is similar in males and females.

Table 11. Odds ratios for the likelihood of correct re-fixation by trial minimisation factors brain injury severity, gestational age at birth and sex

	Non-competition*		**Competition****	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Moderate neurological damage	1.3 (0.67, 2.54)		1.01 (0.66, 1.55)	
Severe neurological damage	0.92 (0.49, 1.75)	0.54	0.86 (0.56, 1.31)	0.66
Gestational age ≥31 weeks	1.01 (0.62, 1.66)	0.97	0.86 (0.63, 1.19)	0.37
Male	1.1 (0.65, 1.86)	0.73	1.13 (0.8, 1.59)	0.50

*Adjusted for treatment, visit, treatment visit interaction, neurological damage, gestational age and sex

**Adjusted for treatment, neurological damage, gestational age and sex

To further investigate the influence of brain injury severity on FS performance the median percentage of correct re-fixations was calculated for each brain injury severity group. Table 12 shows the medians and interquartile ranges for the percentage of correct re-fixations for FS non-competition and FS competition by brain injury severity group. These data show that there is a trend of falling median percentage correct re-fixations under competition with increasing brain injury severity at the 6 month assessment. By the 24 month assessment FS competition performance is identical in normal/mild and moderate brain injury severity groups, but reduced in infants with severe brain injury, suggesting that severe brain injury severity is associated with poorer FS competition performance in the first 2 years of life.

Table 12. Median and interquartile ranges for Fixation Shift percentage correct re-fixations across brain injury severity groups by visit

Neurological damage	Non-competition Median (interquartile range)	Competition Median (interquartile range)
6 months		
Normal/mild	0.9 (0.8, 1)	0.8 (0.7, 0.9)
Moderate	0.9 (0.8, 1)	0.7 (0.6, 0.9)
Severe	0.8 (0.7, 0.9)	0.6 (0.5, 0.7)
12 months		
Normal/mild	0.8 (0.8, 0.9)	0.7 (0.5, 0.8)
Moderate	1 (0.9, 1)	0.8 (0.6, 0.8)
Severe	0.9 (0.8, 1)	0.8 (0.6, 1)
24 months		
Normal/mild	0.9 (0.8, 1)	0.8 (0.6, 0.8)
Moderate	0.9 (0.7, 1)	0.8 (0.6, 0.8)
Severe	0.9 (0.8, 1)	0.6 (0.4, 0.8)

13.2 Fixation Shift latency in infants at risk of neurodevelopmental disability

Fixation Shift latencies have been established for typically developing infants (unpublished data from Atkinson, Braddick and Wuensche appearing in Atkinson and Braddick 2012(129)). There are no published data on FS latency in children with risk factors for neurological impairment, or for infants with perinatal brain injury, however, the proportion of infants whose latency was longer than a 'passing' criterion has been studied for HIE, focal lesions and premature groups(222, 223, 226).

We aimed to investigate whether or not FS latency was prolonged in the Dolphin Study children with moderate and severe brain injury as separately compared to the children with normal/mild brain injury in order to establish the suitability of this measure as an indicator of cortical function in children with risk factors for neurodevelopmental impairments. Forty seven children contribute to the FS latency analyses. Table 13 shows the FS latency Hazard Ratios for FS latency in children with moderate brain injury compared to children with normal/mild brain injury, and for children with severe brain injury as compared to children with normal/mild brain injury. The data is adjusted for treatment, visit, interaction between treatment and visit, and the minimisation factors sex, neurological damage and length of gestation. There was no statistically significant difference in FS latency between normal-mild and moderate brain injury severity groups under non-competition or competition. However there is a trend towards prolonged FS latency for children in the normal-mild brain injury severity group compared to children in the severe brain injury group, under non-competition and competition, although this does not reach statistical significance.

Table 13. Fixation Shift latency Hazard Ratios for children with moderate and severe brain injury compared to children with normal/mild brain injury severity

Neurological damage	Non-competition, HR (95% CI)	p-value	Competition, HR (95% CI)	p-value
Moderate	1.05 (0.69, 1.60)		0.92 (0.67, 1.27)	
Severe	0.70 (0.47, 1.05)	0.08	0.70 (0.51, 0.96)	0.06

Hazard Ratios are typically presented alongside a median statistic. Table 14 presents the time taken for an infant response to be observed in 50% of trials according to brain injury severity group per study visit. At 6 months the time taken for an infant response to be observed in 50% of FS non-competition and competition trials is longer in children with moderate and severe brain injury than in children with normal/mild brain injury, and is longest in those children with severe brain injury. This trend is no longer visible at 12 months. By 24 months the time taken for an infant response to be observed in 50% of FS competition trials is prolonged in children with severe brain injury compared to children with normal-mild and moderate brain injury.

Table 14. Time taken for an infant response to be observed in 50% of Fixation Shift trials by brain injury severity and visit

Time taken for an infant response to be observed in 50% of trials (seconds)						
Visit (months)	Non-competition			Competition		
	Brain injury severity					
	Normal/mild	Moderate	Severe	Normal/mild	Moderate	Severe
6	1.23	1.33	1.43	1.96	2.60	2.71
12	1.43	1.18	1.30	2.26	1.96	2.16
24	1.28	1.13	1.22	1.95	1.95	2.88

13.3 Summary of findings for Fixation Shift performance in children at risk of neurodevelopmental impairment

Fixation Shift cohort data identify a weak trend of poorer FS competition performance in the Dolphin trial cohort children with severe brain injury compared to the children with normal/mild brain injury, however these differences do not reach statistical significance, making it difficult to draw any firm conclusions from the data. Data concerning the effects of brain injury severity on FS latency give conflicting results. Hazard Ratios show prolonged FS latency in children with normal/mild brain injury compared to children with moderate and severe brain injury. However, at 6 months there is a direct relationship between time taken for an infant response to be observed on 50% of trials and brain injury severity under non-competition and competition. By 24 months, the time taken for a response to be observed on 50% of FS *competition* trials to be made is prolonged in children with

severe brain injury compared to children with normal/mild and moderate brain injury. These results do not reach statistical significance.

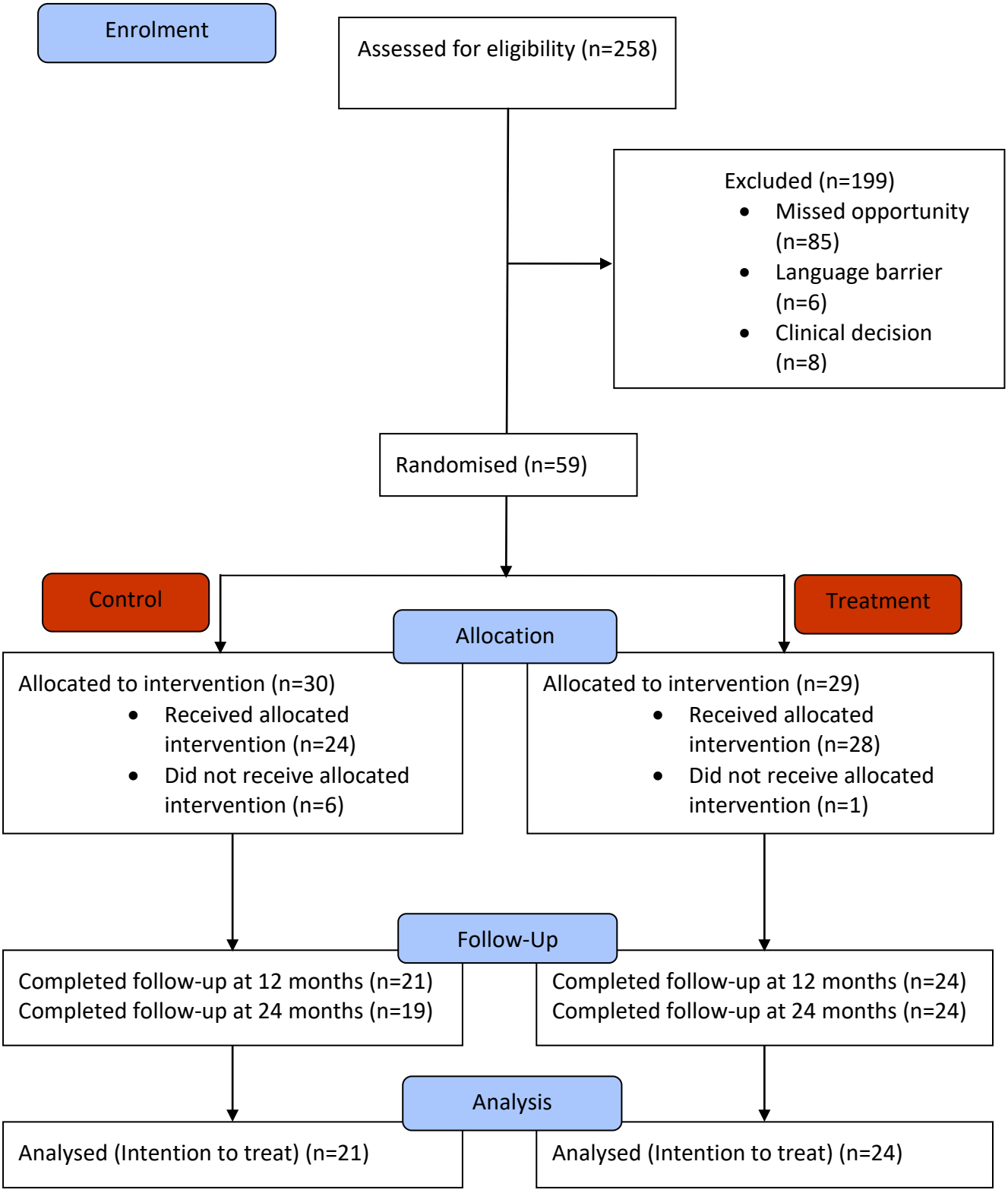
14 RESULTS – FEASIBILITY OF DAILY NUTRITIONAL SUPPLEMENTATION IN INFANTS WITH RISK FACTORS FOR NEURODEVELOPMENTAL IMPAIRMENT

14.1 The Dolphin cohort

14.1.1 Recruitment and retention

Trial recruitment and allocation is outlined in a consort diagram below (Figure 28). Demographics were not collected on those who were missed or who declined to participate. This would provide useful information regarding selection bias and should be collected as part of any future trial.

Figure 28. Trial consort diagram



Sixteen children withdrew over the course of the study. The reasons for participant withdrawal are depicted in Table 15.

Table 15. Reasons for trial participant withdrawal

Reason for withdrawal	Number of children
Loss of contact before baseline	4
Poor feed tolerance post necrotising	2
Family circumstance	3
Moved region	2
Possible Cow's Milk Protein Intolerance	2
Other appointments	2
Comorbidity	1
Total	16

Baseline comparability of the cohort is outlined in Table 16. At baseline, there were no statistically significant differences between the treatment and control groups on composite BSID III scores, nor on transient or calculated PR-VERP latency. There was good comparability across minimisation factors neurological injury severity, sex and gestational age at birth. Children in both groups were a similar corrected age at baseline. There were more mothers who had attended tertiary level education in the intervention group. Birthweight was slightly higher in the placebo group, as was head circumference.

Table 16. Baseline comparability of randomised groups

	Control group Number or mean (SD) or median (LQ,UQ)	**Treatment group Number or mean (SD) or median (LQ,UQ)**
Numbers of children	30	29
Neurological injury: Mild/Moderate/Severe	11/9/10	12/7/10
Gestation: ≤ 30^{+6} weeks/31-40^{+28} weeks	14/16	14/15
Sex: Male/Female	17/13	17/12
Birthweight (g)	2278.0 (947.8, 3718.0)	2095 (950, 3040)
Head circumference (cm)[1]	36.34 (3.09)	35.58 (2.52)
Maternal educational qualification: GCSE/A level/College+	1/8/9	3/4/17
Corrected age at baseline (weeks)[1]	3.32 (4.37)	2.25 (2.67)
Bayley cognitive score[2]	77.78 (12.81)	74.29 (13.86)
Bayley language score[2]	68.48 (10.79)	66.39 (9.50)
Bayley motor score[2]	82.93 (11.46)	82.00 (9.20)
Transient VERP latency (ms)[3]	258.8 (38.68)	255.2 (58.25)
Calculated VERP latency (ms)[4]	260.03 (62.11)	253.28 (37.14)

[1] Based on the 28 patients on placebo and 28 patients on supplement with information
[2] Based on the 27 patients on placebo and 28 patients on supplement with information
[3] Based on the 10 patients on placebo and 15 patients on supplement with information
[4] Based on the 19 patients on placebo and 23 patients on supplement with information

Following baseline, there was differential withdrawal across the brain injury severity groups between the control and treatment groups. There were more withdrawals in the control group (n=11) compared to the treatment group (n=5). There were more children in the control group with normal/mild brain injury (n=8) compared to the treatment group (n=2). There were similar numbers of children with severe brain injury in the control (n=3) and treatment (n=2) groups. There were no children with moderate brain injury in the control group, and only 1 child with moderate brain injury in the treatment group (Table 17). The impact of differential brain injury severity amongst children who withdrew is discussed in the relevant results sections.

Table 17. Brain injury severity of children who withdrew from the study

	Numbers of children (n)	
Brain injury severity	Control group	Treatment group
Normal/mild	8	2
Moderate	0	1
Severe	3	2

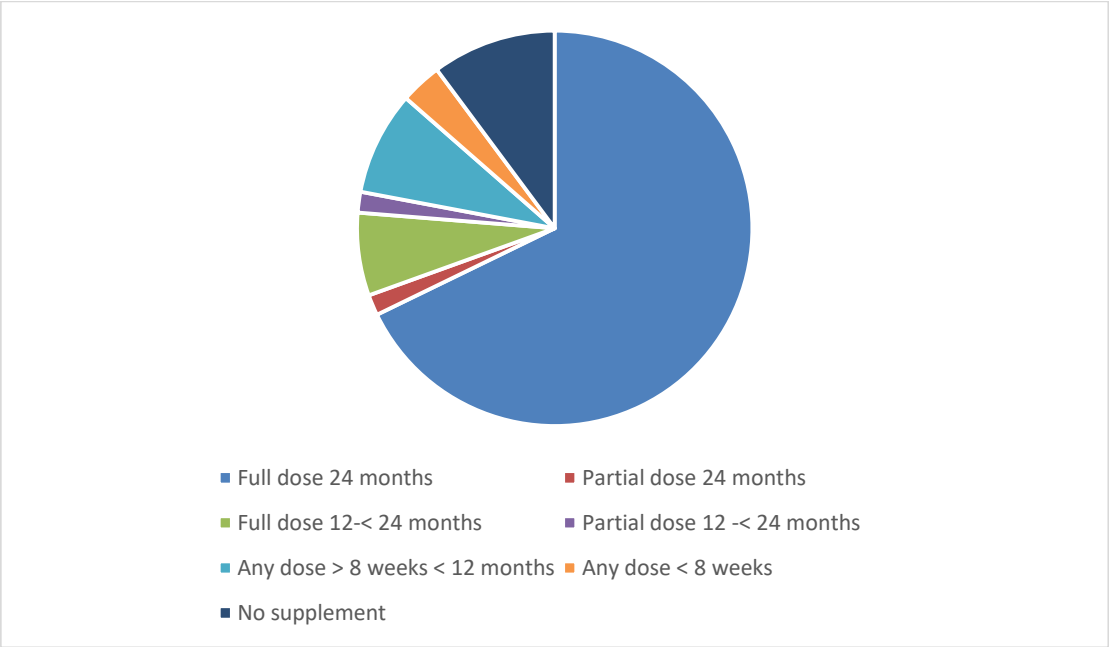
14.1.2 Feasibility of nutritional supplementation in children with perinatal brain injury.

Sixty two children were recruited to the study. Fifty nine were randomised. Withdrawals are defined to be children who discontinued supplementation during the course of the trial. Only one child who stopped supplementation before the end of the trial (following 15 months supplementation) continued with study

assessments, the others did not. The majority of children who withdrew during the study did so before 6 months. Of the 11 children who withdrew before 6 months, 6 withdrew post randomisation and prior to starting supplementation. By 12 months 14 children had withdrawn. Between 13 and 24 months only 2 further children withdrew. The reasons for parents withdrawing their child from the study are shown in table 15. There were equal numbers of breast fed (exclusive or mixed) and formula fed infants amongst withdrawals. More children in the control than treatment group withdrew from the study (11 vs 5 children). Of control group withdrawals, the majority were in the normal/ mild brain injury group (8 children in the control group vs 2 children in the treatment group).

Of the children remaining in the study, 40 (68%) completed 24 months of full dose supplementation and 1 child completed partial dose supplementation for 24 months. Four children (7%) completed full dose supplementation for at least 12 months (12/18/15/18 months) and 1 child completed partial supplementation for 16 months. Of the children who completed less than 12 months supplementation, five completed between 8 weeks and 11 months of supplementation (7/3/6/2/5 months). Two children were supplemented for less than 8 weeks before withdrawing (see Figure 29).

Figure 29. Duration of supplementation



Overall, the supplement was well tolerated. Two children had ongoing problems establishing feeds following necrotising enterocolitis and so the decision to stop supplementation was taken in conjunction with the baby's clinical team. A further 2 infants developed symptoms suggestive of cow's milk protein intolerance. The carrier protein for the supplement was cow's milk protein and so these families were advised to take a break from supplementation whilst trialling a dairy free diet. Both families opted to leave the trial.

Families found it easiest to deliver the supplement whilst the infants were predominantly milk fed and found that the supplement mixed well with both breast and formula milk. For breast fed infants there was the added inconvenience of expressing sufficient volumes to which to add the supplement. Once infants were established on a solid diet the trial dietitian advised families to mix the supplement

with wet foods. More families reported difficulties with delivering the full supplement allocation once their child was fully weaned onto solids and no longer having large milk feeds. Families reported that the supplement altered the taste and texture of the food which some children found off-putting. A small number of families told the research dietitian, who was unblinded, that they thought they had "guessed" their supplement allocation, based on a smell, which became stronger if supplemented milk or food was not finished in the normal time. The families were not told their allocation, and remained blinded to group allocation. Only a small number of families reported that their child was aware of the supplement in their feed. Table 18 outlines infant awareness of the supplement by intervention group and the effect of awareness on intake. This shows that at each time point the majority of infants were unaware of the supplement in their milk or feed. In those children who were reported to be aware of the supplement, intake was similarly reduced in the control (19%) and treatment groups (17%). Estimated participant daily fatty acid intakes from diet and supplement are shown in Appendix 8.

Table 18. Infant supplement awareness and effect on supplement intake

	Control group/treatment group (n)							
	Months of taking supplement							
Infant awareness	3	6	9	12	15	18	21	24
Not aware	20/24	18/23	18/25	18/22	16/21	18/18	18/18	17/19
Aware, no change in intake	1/2	1/2	1/2	1/1	3/2	0/2	0/1	0/0
Aware, reduced intake	0/0	0/1	0/0	0/2	1/2	1/2	1/3	0/1
Aware, not tolerating	0/1	1/0	0/0	0/0	0/0	0/0	0/1	0/1

Data regarding potential symptoms which could feasibly be associated with a dietary intervention were routinely collected at each of the 3 monthly dietetic reviews. These symptoms are listed in Table 19 with the numbers of children in the control and treatment group who experienced each symptom. Vomiting was reported in 2 child in the treatment group, 1 at 18 and 1 at 21 months, neither child was vomiting at 24 months. One child in the treatment group had an episode of diarrhoea in the first 3 months of supplementation, and another had an episode of diarrhoea in the final 3 months of supplementation, however it is likely that at this time the diarrhoea was unrelated to supplementation, as the supplement had been well tolerated at full dose for a number of months. A maximum of 2 (12%) children in the control group and 3 (14%) children in the treatment group were constipated for short periods during the study. Constipation is very common in young children, and particularly in

children with neurodisability; the prevalence in the study cohort is similar to that of the general population (10-20% depending on diagnostic criteria used)(462).

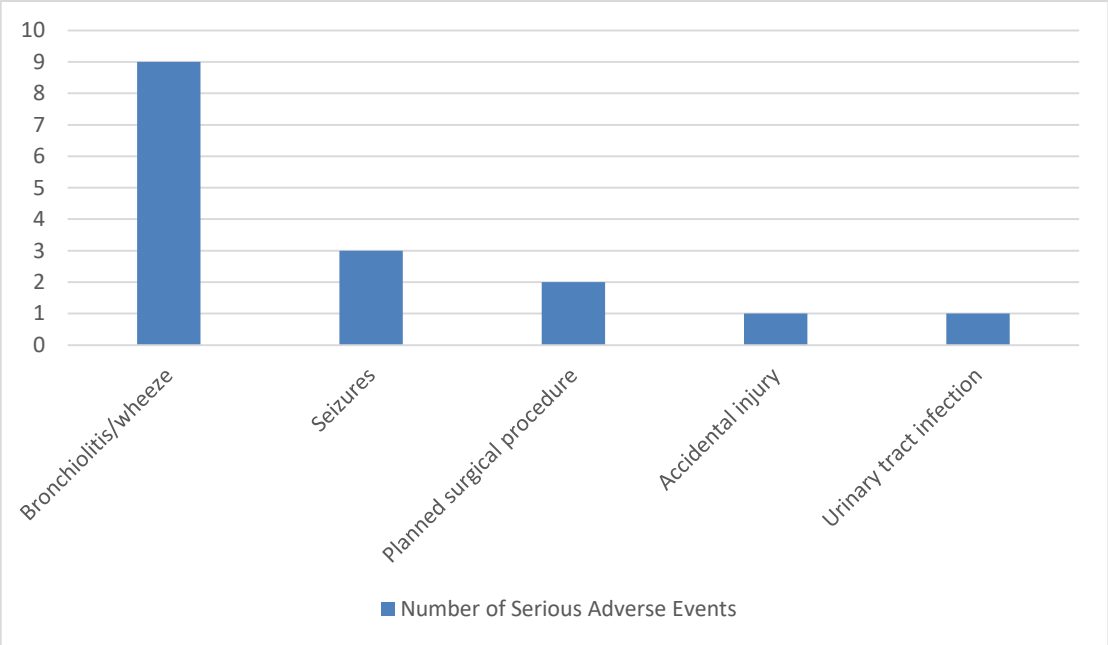
Table 19. Systematic symptom enquiry during study supplementation as reported for infants in the control and treatment groups

	Months of taking supplement							
	3	6	9	12	15	18	21	24
	Number of children yes/no							
Proportion of nausea								
Control	0/21	0/20	0/19	0/19	0/20	0/19	0/19	0/17
Treatment	0/27	0/26	0/27	0/25	0/25	0/23	0/23	0/21
Proportion of vomiting								
Control	1/20	1/19	1/18	0/19	0/20	0/19	0/19	0/17
Treatment	1/26	0/26	0/27	0/25	0/25	1/22	1/22	0/21
Proportion of diarrhoea								
Control	0/21	0/20	0/19	0/19	0/20	0/19	0/19	0/17
Treatment	1/26	0/26	0/27	0/25	0/25	0/23	0/23	1/20
Proportion of constipation								
Control	0/21	0/20	2/17	1/18	0/20	0/19	0/19	0/17
Treatment	0/27	0/26	0/27	1/24	1/24	0/23	0/23	3/21

There were 16 serious adverse events (SAE) over the course of the study; none of these were attributed to the product. Some children had more than one SAE during the study. Admission to hospital for bronchiolitis or wheeze was the commonest (n=9 admissions) SAE. Five of these episodes were for confirmed bronchiolitis; one

child had two episodes aged 13.1 and 33.1 weeks. The median age at first episode was 16.35 weeks (range 7.1-21.6 weeks). All episodes of confirmed bronchiolitis occurred in the treatment group. Seizures were the next commonest cause of admission to hospital (n=3 admissions) (Figure 30).

Figure 30. Number and causes of Serious Adverse Events in study population



14.1.3 Completeness of outcome data

Tables 20-22 describe the number of children for whom data was obtained for each of the trial neurocognitive and visual outcome measures.

Data were most complete for the BSID III (Table 20); Bayley composite cognitive score data were obtained for 100% of children at each visit. Bayley language score data were obtained for 100% of children at baseline and 12 months and 98% of children at 24 months. The Bayley motor scale was the last assessment scale to be completed. The scale was completed in 100% of children at baseline, and 98% of

children at 12 and 24 months. Vineland Adaptive Behaviour Scale II (VABS II) data were obtained in 93, 91 and 86% of children at baseline, 12 and 24 months. It was not always practical to complete the VABS II at the time of Bayley assessment due to the needs of the child. If this was the case, the person completing the Bayley assessment sought permission to collect the VABS II data by telephone at a time convenient to the child's parent, however not all parents could then be contacted as arranged resulting in loss of data.

Table 20. Bayley Scales of Infant Development III and Vineland Adaptive Behaviour Scale II data acquisition per study visit

	Data obtained/data collection attempted, n (%)		
	Trial visit (months)		
Assessment	Baseline (n=55)	12 (n=45)	24 (n=43)
BSID III			
CCS-BSID III	55/55, (100)	45/45, (100)	43/43, (100)
LCS-BSID III	55/55, (100)	45/45, (100)	43/43, (100)
MCS-BSID III	55/55, (100)	44/45, (98)	42/43, (98)
VABS-II	51/55, (93)	41/45, (91)	37/43, (86)

Table 21 shows the number and percentage of children for whom VERP data were obtained. Calculated VERP latency data were obtained in approximately 70% of attempts, whereas PR2 transient latency data were obtained in approximately 50% of attempts. PR2 was the slowest VERP run to complete, making it more vulnerable to variation in infant state, hence the lower PR2 data acquisition level. OR-VERP data were obtained in 70-80% of attempts, but less than 50% of attempts at 24 months;

by 24 months participants were less co-operative with VERP testing. As OR-VERPs were assessed at the end of VERP testing, more infants had stopped attending to the VERP stimulus, resulting in lower levels of data acquisition at 24 months.

Table 21. Visual Event Related Potential data acquisition per study visit

	Data obtained/data collection attempted, n (%)			
	Trial visit (months)			
Assessment	Baseline (n=55)	6 (n=48)	12 (n=45)	24 (n=43)
Calculated VERP latency	42/53, (79)	34/47, (72)	31/42, (74)	24/34, (71)
Transient PR2 VERP latency	25/42, (60)	21/44, (48)	16/22, (73)	11/25, (44)
OR VERP response obtained	N/A	34/42, (81)	26/36, (72)	13/29, (45)

Fixation Shift data acquisition is shown in Table 22. Fixation Shift data (≥ 10 runs) were obtained in approximately 70% of children at each visit. Incomplete FS data were obtained in 12 and 13% of children at 6 months, rising to 21 and 23% of children at 24 months. FS assessment was not attempted if the child was so severely brain injured as to be unable to respond to the stimulus, or was non-compliant with vision testing.

Table 22. Fixation Shift test data acquisition per study visit

	Number of children ≥ 10 runs obtained/<10 runs obtained/assessment not attempted, n (%)		
	Trial visit (months)		
Assessment	6 (n=48)	12 (n=45)	24 (n=43)
FS non-competition	34/6/8 (70/13/17)	32/8/5 (71/18/11)	32/9/2 (74/21/5)
FS competition	33/6/9 (69/12/19)	30/9/6 (67/20/13)	31/10/2 (72/23/5)

For ABCDEFV, only core vision is assessed at baseline, as per the assessment schedule. At 6 months, core vision/ attention/ visuocognitive data were obtained for 98/98/42% of children. At 12 and 24 months core vision/ attention/ visuocognitive data were obtained for 100/100/98% of children and 100/100/98% of children respectively. The ABCDEFV is structured so that, as with BSID-III, items previously passed at the age appropriate time were not re-tested on subsequent assessments to ensure completion of the then age appropriate items within the allocated testing time. For the purposes of data analysis, previously passed items were carried forward to the subsequent study assessment, so that once a child had passed a test item there was no need to repeat it on subsequent visits, thereby keeping testing time as short as possible.

15 RESULTS – DOLPHIN TRIAL NEURODEVELOPMENTAL OUTCOMES

Pre-specified hypotheses will be presented alongside the sections that they relate to.

15.1 Primary outcome: Composite cognitive score Bayley Scales of Infant Development III

Hypothesis: Cognitive performance as measured by the Bayley Scales of Infant development III will be higher in infants who received neurotrophic dietary supplementation compared to those who received control supplementation.

CCS-BSID III data at baseline were available for 55 participants. Forty five participants had CCS-BSID III data at baseline and at least one other time point. All participants who had CCS-BSID III data at either 12 or 24 months also had CCS-BSID III data at baseline.

The brain injury severity of infants contributing data to CCS and LCS BSID III analyses is shown in Table 23. This shows that at all assessment visits there more children in the treatment group with a normal/mild brain injury severity grading than in the control group (42 vs 19% at baseline and 12 months and 15% at 24 months). There were fewer children in the treatment group with moderate brain injury (25% vs 42%). There were equal numbers of children with severe brain injury in treatment and control groups, although proportionally fewer children with severe brain injury in the treatment group compared to the control group (33 vs 38%).

Table 23. Brain injury severity grading of children contributing Bayley Scales of Infant Development III composite cognitive score and language composite score data in control and treatment group

Visit	Control group brain injury severity			Treatment group brain injury severity		
	Normal/ mild	Moderate	Severe	Normal/ mild	Moderate	Severe
Number of children, n (%)						
Baseline	4 (19)	9 (43)	8 (38)	10 (42)	6 (25)	8 (33)
12 month	4 (19)	9 (43)	8 (38)	10 (42)	6 (25)	8 (33)
24 month	3 (16)	9 (47)	7 (37)	10 (42)	6 (25)	8 (33)

In order to investigate whether control or treatment group children had better cognitive outcomes, BSID III composite scores were compared for the two groups. As outlined in section 10.2.2, a mixed effects linear regression model was fitted adjusting for baseline score, treatment, visit, interaction between treatment and visit, and the minimisation factors neurological severity, gender and length of gestation. Intention to treat analysis showed that CCS-BSID III was higher in the treatment group than in the control group, although this difference in mean score did not reach statistical significance (mean difference 9.0 points, 95% CI (-0.2, 18.2), p= 0.13). There was no statistically significant difference in treatment effect by visit (no interaction) ($\chi 2(1) = 1.64$, $p = 0.20$), however there was a trend of increasing CCS-BSID III score with study visit (see Table 24). This treatment group advantage equates to an effect size of 0.31 (Cohen's d).

Table 24. Intention to treat analysis: Mean Bayley Scales of Infant Development III composite cognitive score in control and treatment groups by visit

Visit	Numbers of children		Mean (SD)		Difference in means (95% CI)
	Control	Treatment	Control	Treatment	
Baseline	21	24	77.4 (14.2)	75.8 (13.5)	
12 month	21	24	82.4 (20.1)	85.2 (18.1)	4.4 (-4.7, 13.4)
24 month	19	24	81.6 (18.5)	87.7 (20.4)	9.0 (-0.2, 18.2)

15.1.1 Per protocol analysis

A per protocol analysis was conducted for the primary outcome. The analysis was adjusted for baseline measurement, treatment, visit, interaction between treatment and visit, and the minimisation factors neurological severity, sex and length of gestation. The per protocol analysis showed that mean CCS-BSID III was 10.6 points higher in the treatment group than in the control group, and that this difference approached statistical significance (p=0.08) (See Table 25). There was a trend of increasing CCS-BSID III score with study visit, although this did not reach statistical significance (no interaction) ($\chi 2(1) = 2.02$, $p = 0.16$). This trend suggests that treatment effect may have increased with time, or that a longer duration of supplementation may have been associated with higher CCS-BSID III.

Table 25. Per protocol analysis: Mean Bayley Scales of Infant Development III composite cognitive score in control and treatment groups

Visit	Numbers of children		Mean (SD)		Difference in means (95% CI)
	Control	Treatment	Control	Treatment	
Baseline	20	25	77.0 (14.5)	76.2 (13.3)	
12 month	20	25	82.0 (20.5)	85.4 (17.8)	5.4 (-3.7, 14.6)
24 month	18	25	80.8 (18.7)	88.0 (20.1)	10.6 (1.3, 19.9)

15.1.1.1 Exploratory analyses

Following the exclusion of infants with severe neurological damage the observed trend of higher CCS-BSID III in the treatment group remained (mean difference between groups 8.7 points, 95% CI (-2.7, 20.0), suggesting that treatment improved cognitive outcome across all brain injury severity groups, albeit this difference did not achieve statistical significance (Table 26).

Table 26. Mean Bayley Scales of Infant Development III composite cognitive score in control and treatment groups following exclusion of infants with severe brain injury

Visit	Numbers of children		Mean (SD)		Difference in means (95% CI)
	Control	Treatment	Control	Treatment	
Baseline	13	16	80.8 (12.6)	77.2 (13.8)	
12 month	13	16	90.0 (18.6)	89.7 (17.5)	3.4 (-7.6, 14.5)
24 month	12	16	88.8 (14.0)	92.5 (19.9)	8.7 (-2.7, 20.0)

Cognitive advantage in infants who were breastfed compared to those who were not has been reported in previous studies, as reviewed in Belfort et al, 2013 (463). This advantage is independent of maternal DHA levels. To investigate whether the observed trend of cognitive advantage in the treatment group may be the result of

higher breastfeeding levels in this group, further subgroup analysis was performed according to whether infants were breastfed or not. An infant was defined as breastfed at baseline if they were breastfed exclusively or partially for at least two weeks after birth. All data were analysed together. Analysis included a covariate for breastfed and an interaction term for breastfed and treatment, as well as adjusting for baseline Bayley cognitive score, treatment group, visit, treatment and visit interaction, and the minimisation factors neurological severity, sex and length of gestation. There were 12 babies who were either exclusively or partially breastfed. Results from fitting a mixed effects linear regression, including a main effect for breastfeeding and an interaction term for breastfeeding and treatment, as well as adjusting for baseline Bayley cognitive score, treatment group, visit, treatment and visit interaction, and the minimisation factors neurological severity, gender and length of gestation showed was no statistically significant treatment effect on CCS-BSID III at 24 months between those who were breastfed and those who were not (p-value = 0.87) (Table 27).

Table 27. Mean Bayley Scales of Infant Development III composite cognitive score in control and treatment groups by breastfeeding group

Visit	Control		Treatment	
	No breastfeeding	Breastfeeding	No breastfeeding	Breastfeeding
Baseline	74.67 (15.86)	84.17 (4.92)	80.00 (10.98)	63.33 (13.29)
12 month	76.67 (18.87)	96.67 (16.63)	85.56 (18.70)	84.17 (18.00)
24 month	76.92 (18.88)	91.67 (14.02)	85.00 (18.47)	95.83 (25.58)

15.2 Trial neurodevelopmental secondary outcome measures

15.2.1 Language composite score Bayley Scales of Infant Development III (LCS-BSID III)

Hypothesis: Language performance as measured by the Bayley Scales of Infant development III will be higher in infants who received neurotrophic dietary supplementation compared with those who received control supplementation.

LCS-BSID III data at baseline was available for 55 participants. Forty five participants had LCS-BSID III data at baseline and at least one other time point. All participants who had LCS-BSID III data at either 12 or 24 months also had LCS-BSID III data at baseline.

In order to investigate whether control or treatment group children had better language outcomes, BSID III composite scores were compared for the two groups. The analysis was adjusted for baseline measurement, treatment, visit, interaction between treatment and visit, and the minimisation factors neurological severity, sex and length of gestation as outlined above. LCS-BSID III was higher in the treatment group than in the control group, although this difference in mean score did not reach statistical significance (mean difference 8.6 points, 95% CI (-1.1, 18.2), p= 0.21). There was no statistically significant difference in treatment effect by visit (no interaction) ($\chi2(1) = 1.78$, $p = 0.18$), although the data show a trend of increasing LCS-BSID III at 24 months compared with 12 months, suggesting that treatment effect may increase with time, or that a longer duration of supplementation is associated with higher LCS-BSID III. See Table 28. The treatment group advantage equates to an effect size of 0.42 (Cohen's d).

Table 28. Mean Bayley Scales of Infant Development III composite language score in control and treatment groups by visit

Visit	Numbers of children (n)		Mean (SD)		Difference in means (95% CI)
	Control	Treatment	Control	Treatment	
Baseline	21	24	67.5 (11.9)	67.0 (9.6)	
12 month	21	24	74.6 (12.0)	77.6 (14.6)	2.7 (-6.7, 12.1)
24 month	19	24	83.2 (19.6)	91.5 (20.1)	8.6 (-1.1, 18.2)

15.2.1.1 Sensitivity (post hoc) analyses

To investigate the influence of severe brain injury on Bayley language outcome, children with severe brain injury were excluded from the analysis. Analyses were adjusted for baseline measurement, treatment, visit, and minimisation factors neurological severity, sex and length of gestation.

Following exclusion, there was a smaller difference in mean LCS-BSID III between the control and treatment groups (mean difference 2.7, 95% CI (-11.0-16.5)) (Table 29), suggesting that there was less advantage to language outcome for children with no/mild or moderate brain injury.

Table 29. Mean Bayley Scales of Infant Development III composite language score in control and treatment groups following exclusion of infants with severe brain injury

Visit	Numbers of children		Mean (SD)		Difference in means (95% CI)
	Control	Treatment	Control	Treatment	
Baseline	13	16	70.7 (12.6)	65.6 (9.9)	
12 month	13	16	77.1 (13.0)	73.1 (21.6)	-5.2 (-18.6, 8.1)
24 month	12	16	88.8 (17.7)	92.5 (21.5)	2.7 (-11.0, 16.5)

15.2.2 Motor composite score Bayley Scales of Infant Development III (MCS-BSID III)

Hypothesis: Motor performance as measured by the Bayley Scales of Infant development III will be the same in infants who received neurotrophic dietary supplementation compared to those who received control supplementation.

MCS-BSID III data at baseline was available for 55 participants. Forty five participants had MCS-BSID III data at baseline and at least one other time point. All participants who had MCS-BSID III data at either 12 or 24 months also had MCS-BSID III data at baseline.

Table 30 shows that at all assessment visits there more children in the treatment group with a normal/mild brain injury severity grading than in the control group (42 vs 19% at baseline and 12 months and 43 vs 16% by 24 months). There were fewer children in the treatment group with moderate brain injury (25 vs 42% at baseline and 22 vs 47% by 24 months). There were similar numbers of children with severe brain injury in treatment and control groups, although proportionally fewer children

with severe brain injury in the treatment group compared to the control group (33 vs 37%).

Table 30. Brain injury severity of participants contributing Bayley Scales of Infant Development III composite cognitive data in control and treatment groups

Visit	Brain injury severity of control infants			Brain injury severity of treatment infants		
	Mild	Moderate	Severe	Mild	Moderate	Severe
Number of children (n)						
Baseline	4	9	8	10	6	8
12 month	4	9	7	10	6	8
24 month	3	9	7	10	5	8

In order to investigate whether or not motor performance differed between control and treatment groups, the mean MCS-BSID III score for each group was compared. The analysis was adjusted for baseline measurement, treatment, visit, interaction between treatment and visit, and the minimisation factors neurological severity, sex and length of gestation. These data show that MCS-BSID III scores were similar for the control and treatment group (mean difference = -0.2 points, 95% CI -10.6, 10.3, p = 0.89), across visits (mean difference = -1.2 points (-11.9, 9.5), p= 0.85) (Table 31).

Table 31. Mean Bayley Scales of Infant Development III composite motor score in control and treatment groups by visit

Visit	Numbers of children		Mean (SD)		Difference in means (95% CI)
	Control	Treatment	Control	Treatment	
Baseline	21	24	82.2 (12.5)	83.3 (8.8)	
12 month	20	24	80.0 (21.4)	79.6 (18.3)	-0.2 (-10.6, 10.3)
24 month	19	23	78.8 (21.6)	77.8 (21.8)	-1.2 (-11.9, 9.5)

15.2.2.1 Sensitivity (post hoc analyses)

In order to investigate the effect of severe brain injury on the motor performance of children in the control and treatment groups, a sensitivity analysis was conducted excluding children with severe brain injury. This analysis showed that MCS-BSID III was lower in the intervention group compared to the placebo group, however this difference was not statistically significant and did not persist at 24 months. Analyses were adjusted for baseline measurement, treatment, visit, and minimisation factors neurological severity, sex and length of gestation (Table 32).

Table 32. Mean Bayley Scales of Infant Development III composite motor score in infants with normal-mild and moderate brain injury

Visit	Numbers included		Mean (SD)		Difference in means (95% CI)
	Control	Treatment	Control	Treatment	
Baseline	13	16	83.4 (13.9)	82.9 (8.9)	
12 month	13	16	84.5 (20.8)	79.6 (18.3)	-8.1 (-21.2, 4.9)
24 month	12	15	85.2 (19.3)	84.2 (15.9)	-1.9 (-15.5, 11.7)

15.2.3 Vineland Adaptive Behaviour Rating Scale (VABS-II)

Hypothesis: Parent reported adaptive behaviour scores as measured by the Vineland Adaptive Behaviour Rating Scale II (VABS-II) at 24 months will be higher in children who received neurotrophic dietary supplementation compared with those who received the control supplement.

Baseline VABS-II data were available for 51 participants. Forty one participants had VABS-II data at baseline and at least one other time point. To investigate whether VABS-II performance was improved in the treatment group compared to the control group, we compared the VABS-II domain standard score between the two groups. The analysis was adjusted for baseline measurement, treatment, visit, interaction between treatment and visit, and the minimisation factors neurological severity, sex and length of gestation as outlined above. There was a trend of improved performance on VABS-II in the treatment compared to the control group at 24 months, however this difference did not reach statistical significance. (mean difference = 4.4 points, 95% CI (-3.1, 12.0), p = 0.40). The interaction of treatment effect by visit was not significant ($\chi 2(1) = 0.71$ points, p = 0.40) (Table 33).

Table 33. Mean Vineland Adaptive Behaviour Scale II domain standard score in control and treatment groups

Visit	Numbers of children		Mean (SD)		Difference in means (95% CI)
	Control	Treatment	Control	Treatment	
Baseline	20	21	83.4 (8.4)	84.1(11.3)	
12 month	20	21	84.6 (13.6)	86.9 (16.0)	1.6 (-5.7, 8.8)
24 month	17	20	87.4 (15.1)	92.3 (10.8)	4.4 (-3.1, 12.0)

15.2.4 Summary of findings in trial neurodevelopmental outcomes

Neurodevelopmental hypotheses stated that daily neurotrophic supplementation would improve cognitive and language performance in treated children compared to controls. The data show that children in the treatment group had non statistically significantly improved cognitive and language performance, as measured by BSID III composite scores, compared to children in the control group. Although not statistically significant the effect size for both cognitive and language outcomes are clinically significant (see section 15.1). The data show that for cognitive performance this effect occurs across brain injury severity groups, whereas for language performance the greatest benefit was observed in children with severe brain injury. A smaller effect was observed in children with normal/mild or moderate brain injury. No between group differences in motor performance were identified. Vineland Adaptive Behaviour Scales II domain standard scores were non statistically significantly higher in the treatment group compared with the control group. These

effects are not as a result of increased head circumference (brain size) amongst children in the treatment group. The data show a weak correlation between omega-3 fatty acid DHA and EPA whole blood levels and Bayley cognitive and language composite scores.

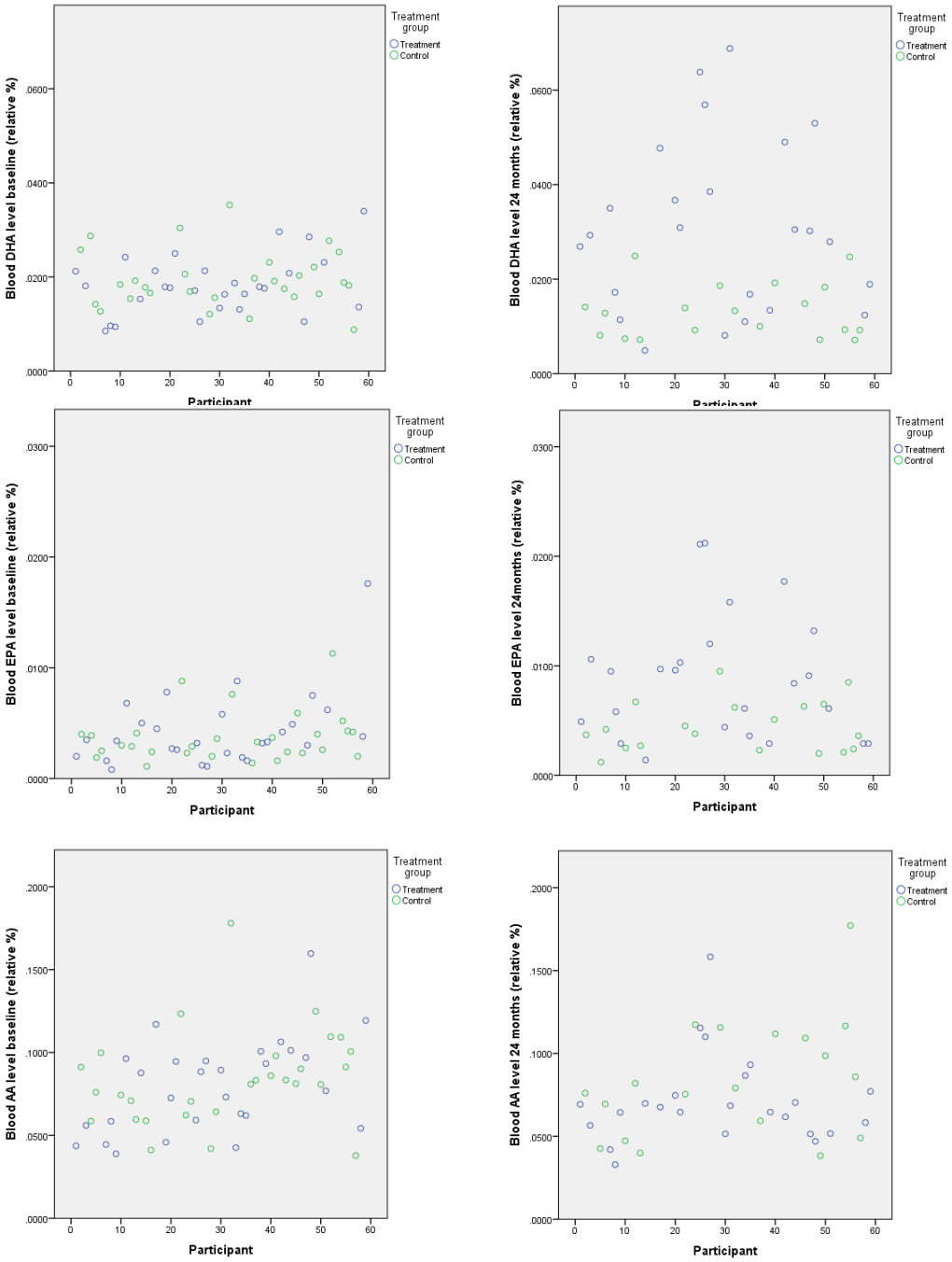
15.3 Bayley Scales of Infant Development III performance and participant long chain polyunsaturated fatty acid levels

Hypotheses:

- Infant cognitive outcomes at 24 months will be directly correlated to infant whole blood DHA and EPA levels following dietary supplementation for 24 months.
- Infants who showed greatest improvement in CCS-BSID III will have higher mean blood DHA and EPA levels than infants who showed no improvement in CCS-BSID III.
- Infants who showed greatest improvement in LCS-BSID III will have higher mean blood DHA and EPA levels than infants who showed no improvement in LCS-BSID III.
- There will be no difference in the mean DHA and EPA level of infants who showed the greatest improvement in motor performance compared to those infants who showed no improvement in motor performance.

Participant blood fatty acid levels for DHA, EPA and AA levels at baseline and 24 months supplementation are shown in Figure 31. Raw blood fatty acid levels are presented in Appendix 5.

Figure 31. Participant blood DHA, EPA and AA levels at baseline and 24 months



Mean DHA, EPA and AA levels for the treatment and control group at baseline and 24 months are shown in Table 34. Independent samples t-tests were performed for DHA, EPA and AA to assess the difference in means at baseline and 24 months' supplementation in treatment and control children. There were no differences in mean blood fatty acid levels between treatment and control children at baseline. Statistically significant differences in mean blood acid levels between treatment and control group children were identified at 24 months for DHA (t=4.496, df 28.57, p=<0.001) and EPA (t=3.453, df 31.88, p=0.002) but not for AA (t=-1.323, df 41, p=0.19), demonstrating that treatment was effective in increasing blood DHA and EPA levels compared to baseline. Treatment did not significantly affect blood AA levels.

Table 34. Mean participant blood DHA, EPA and AA levels in control and treatment group at baseline and 24 months

	Mean blood fatty acid level at baseline (relative %) (SD)			**Mean blood fatty acid level at 24 months (relative %) (SD)**		
	Control (n=29)	Treatment (n=28)	p-value	Control (n=19)	Treatment (n=24)	p-value
DHA	0.019 (0.006)	0.018 (0.006)	0.47	0.013 (0.006)	0.031 (0.018)	<0.001
EPA	0.004 (0.002)	0.004 (0.003)	0.43	0.004 (0.002)	0.009 (0.006)	0.002
AA	0.084 (0.029)	0.080 (0.028)	0.62	0.084 (0.036)	0.071 (0.027)	0.19

In order to assess whether there was an effect of breastfeeding on blood fatty acid levels mean DHA, EPA and AA blood level was compared for no breastfeeding and

breastfeeding infants at baseline and 24 months, where breastfeeding was defined as partial or exclusive breastfeeding for greater than 2 weeks. There were no differences in any mean blood fatty acid level in breast fed and non-breast-fed infants (Table 35).

Table 35. Mean participant blood DHA, EPA and AA levels by breastfeeding group at baseline and 24 months

	Mean blood FA level baseline (relative%)(SD)		**Mean blood FA level 24 months (relative %)(SD)**	
	No breastfeeding (n=43)	Breastfed for at least 2 weeks (n=14)	No breastfeeding (n=31)	Breastfed for at least 2 weeks (n=12)
C22_6	0.018 (0.006)	0.020 (0.006)	0.023 (0.017)	0.024 (0.017)
C20_5	0.004 (0.002)	0.004 (0.004)	0.007 (0.005)	0.007 (0.007)
C20_4	0.082 (0.031)	0.082 (0.021)	0.077 (0.034)	0.076 (0.022)

Thirty nine of 42 subjects with blood fatty acid level data took full supplement for 24 months. Forty of 43 subjects with blood fatty acid level data were still taking some supplement at 24 months (1 child was receiving less than full dose supplementation). It was not therefore possible to examine any correlation between duration of supplementation and BSID III outcomes. In order to investigate whether there was an association between Bayley cognitive, language and motor scores and whole blood DHA and EPA and AA levels, blood fatty acid levels were compared for treatment and control groups and correlated with CCS-BSID III, LCS-BSID III and MCS-BSID III outcomes. There was a weak correlation between participant whole blood DHA level (0.14) and EPA level (0.24) at 24 months and CCS-BSID III at 24 months.

There was no correlation between participant whole blood arachidonic acid (AA) levels at 24 months and CCS-BSID III at 24 months(-0.09). There was also a weak correlation between participant whole blood DHA level (0.24) and moderate correlation between EPA level (0.31) at 24 months and LCS-BSID III at 24 months. There was no correlation between participant whole blood AA levels at 24 months and LCS-BSID III at 24 months (-0.005). There was a weak correlation between participant whole blood DHA (0.15) and EPA (0.19) level and MCS-BSID III. There was no correlation between participant whole blood AA level and MCS-BSID III (Table 36).

Table 36. Correlation of infant long chain polyunsaturated whole blood level at 24 months and Bayley Scales of Infant Development III composite cognitive score and language scores at 24 months

	Infant LCPUFA levels at 24 months		
BSID III composite score at 24 months	Docosahexaenoic acid (DHA)	Eicosapentaenoic acid (EPA)	Arachidonic acid (AA)
CCS-BSID III	0.14	0.24	-0.09
LCS-BSID III	0.24	0.31	-0.005
MCS-BSID III	0.15	0.19	-0.16

Change in BSID III cognitive and language composite score from baseline to 24 months provides an alternative assessment of the relationship between participant blood fatty acid levels and Bayley cognitive, language and motor performance. Change in CCS- and LCS-BSID III composite score between baseline and 24 months were divided into 4 groups dependent upon the range of change in score in each domain between baseline and 24 months. A no improvement category included children who had shown no change in composite score between baseline and 24

months, or whose 24 months composite score was lower than the baseline composite score. Figure 32 shows boxplots of the change in Bayley III composite cognitive, language and motor score and mean blood DHA level at 24 months. These figures show that at 24 months the greatest improvements in BSID III composite cognitive and language scores were associated with the highest mean blood DHA level. The same pattern is identified for change in BSID III composite cognitive and language scores and mean blood EPA level; the greatest improvements in BSID III composite cognitive and language scores were associated with the highest mean blood EPA level (Figure 33). There are no differences in mean blood AA level and change in BSID III composite score Figure 34). Due to the variation in number of children per group and the small numbers in each group formal statistical analysis of any difference in mean blood fatty acid level is inappropriate and has not been performed.

Figure 32. Change in Bayley III composite cognitive, language and motor score and mean blood DHA level

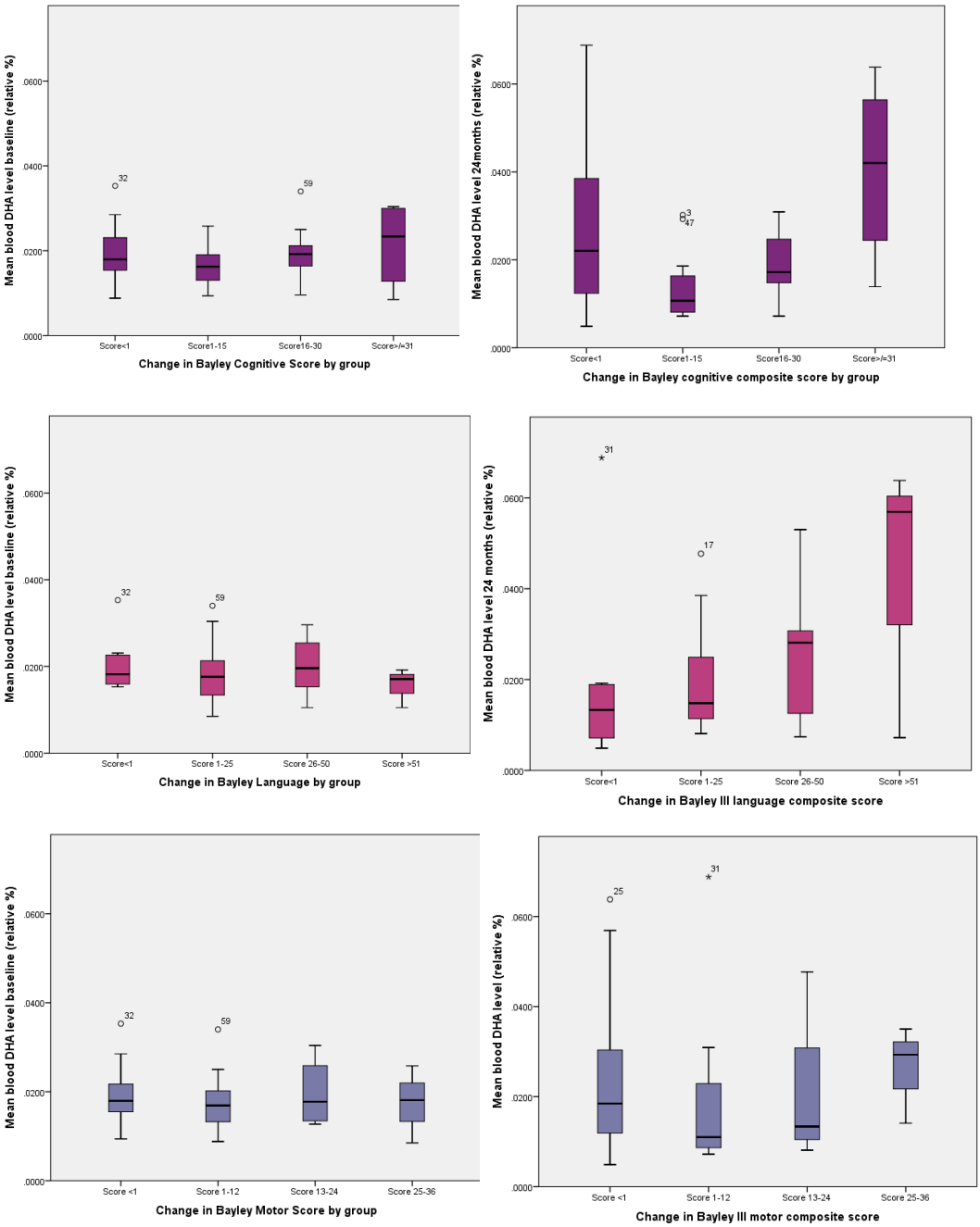
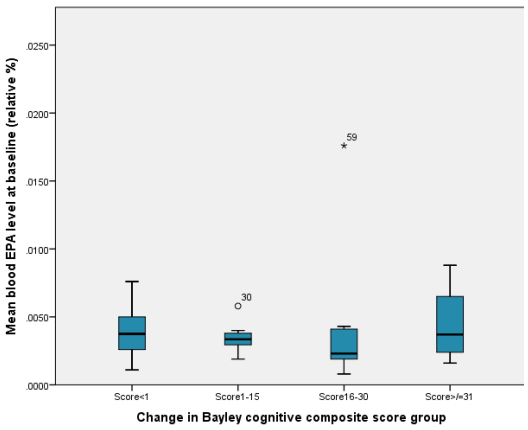
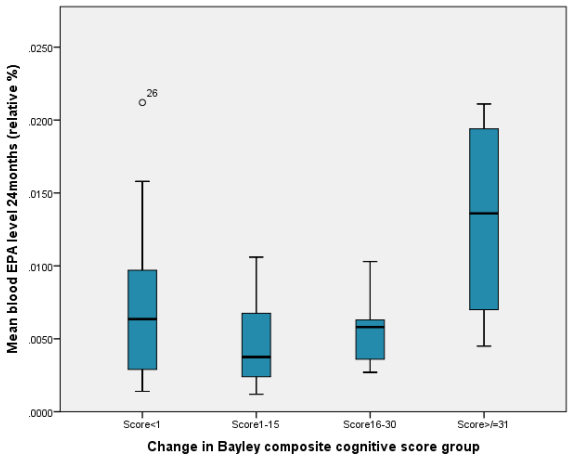
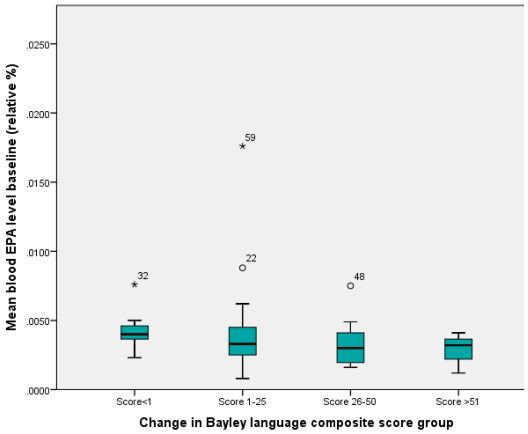
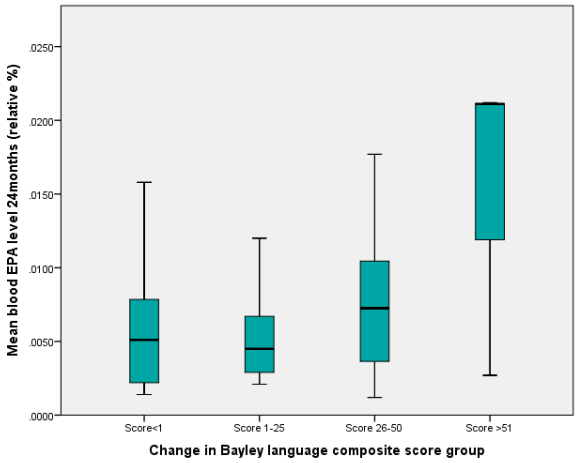


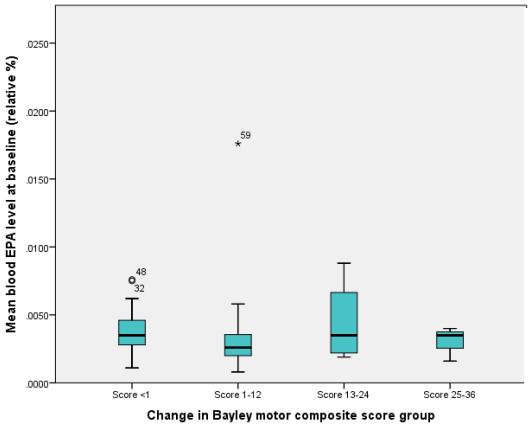
Figure 33. Change in Bayley III composite cognitive, language and motor score and mean blood EPA level











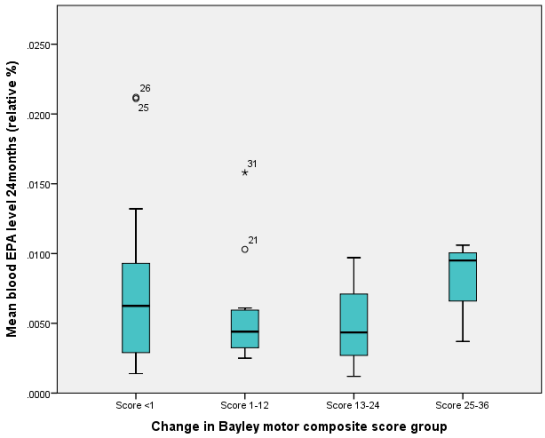
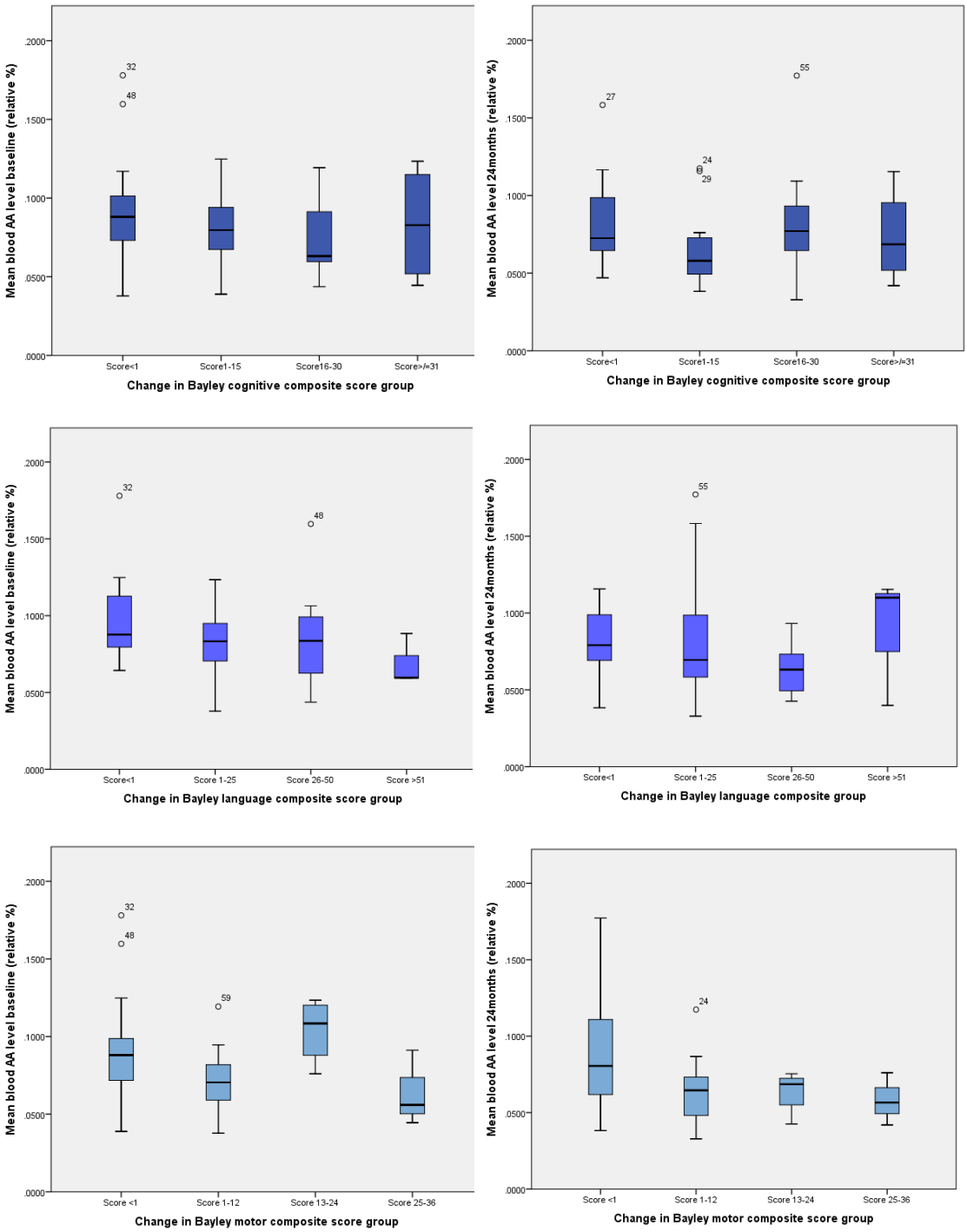


Figure 34 Change in Bayley III composite cognitive, language and motor score and mean blood AA level



Three of the 4 children in the highest change CCS-BSID III group were in the treatment group, and 3 of the 4 were male. Two of the 3 children in the highest

change in LCS-BSID III group were in the treatment group, all 3 were male. There was no relationship between CGA at birth and change in CCS- or LCS-BSID III. Due to the small numbers in both change in CCS-BSID III (n=4) and LCS-BSID III (n=3) groups no conclusions can be drawn from these data.

15.3.1 Summary of trial fatty acid analyses

DHA and EPA levels, but not AA levels were significantly higher in the treatment group than in the control group at 24 months. There were no blood fatty acid level differences between intervention groups at baseline. There were no significant differences in FA levels between breast fed and non-breast fed infants at baseline or 24 months. Weak correlations between infant whole blood DHA and EPA level, but not AA level and CCS-BSID III were identified. A similar correlation was identified for infant whole blood DHA level and LCS-BSID III, and a moderate correlation for infant whole blood EPA level and LCS-BSID III score at 24 months. A weak correlation between participant whole blood DHA and EPA level and MCS-BSID III was also identified. There was no correlation between participant whole blood AA level and MCS-BSID III.

At 24 months the greatest improvements in BSID III composite cognitive and language scores were associated with the highest mean blood DHA and mean blood EPA levels. There were no differences in mean blood AA level and change in BSID III composite score.

15.4 Anthropometry

Head circumference, as a proxy for brain size, correlates with childhood cognitive outcomes(269). In order to explore whether or not the trend of improved cognitive and language performance in the treatment group could be explained by improved growth amongst treatment group children, height and weight and head circumference z-scores were compared between control and treatment groups. Table 37 shows the fitted difference in means (mixed effects linear regression) between treatment groups for 12 and 24 months, as well as the raw means and the numbers of subjects with data. There were no significant difference between treatment groups for any of the anthropometry variables.

There was no significant difference in weight (p= 0.68), height (p=0.56), or head circumference (p=0.19) between the control and treatment group at any visit (Table 37), refuting the possibility that improved growth was responsible for the higher cognitive scores in the treatment compared to the control group.

Table 37. Mean weight, height and head circumference z-score in control and treatment groups by visit

Visit	Numbers of children (n)		Mean (SD) (z-scores)		Difference in means (95% CI)[$]
	Control	Treatment	Control	Treatment	
Weight					
Baseline	28	28	-0.77 (1.69)	-1.28 (1.43)	
12 month	20	24	-0.01 (1.33)	-0.36 (1.22)	0.1 (-0.5, 0.7)
24 month	18	23	-0.18 (1.48)	-0.51 (1.31)	0.2 (-0.4, 0.8)
p-value for interaction					0.32
p-value for treatment effect					0.68
Height					
Baseline	25	23	-1.30 (2.86)	-0.15 (2.04)	
12 month	20	24	-0.19 (1.57)	-0.04 (1.29)	0.2 (-0.5, 0.9)
24 month	18	23	-0.74 (1.64)	-0.48 (1.12)	0.1 (-0.6, 0.8)
p-value for interaction					0.49
p-value for treatment effect					0.56
Head circumference					
Baseline	28	28	0.13 (1.67)	-0.27 (1.55)	
12 month	20	24	0.57 (1.21)	0.21 (1.53)	0.3 (-0.2, 0.8)
24 month	18	23	0.32 (1.73)	0.08 (1.55)	0.4 (-0.1, 0.9)
p-value for interaction					0.33
p-value for treatment effect					0.19

[$] Adjusted for treatment, visit and the interaction between treatment and visit, and the minimisation factors neurological severity, gender and length of gestation

It was not possible to convert mid-upper arm circumference and triceps skinfold thickness into z-scores for preterm babies and so no formal analyses of these data were performed. Trajectory plots for weight, height, head circumference, mid-upper arm circumference and triceps skin fold thickness are shown in Appendix 9.

16 RESULTS – DOLPHIN TRIAL VISION OUTCOMES

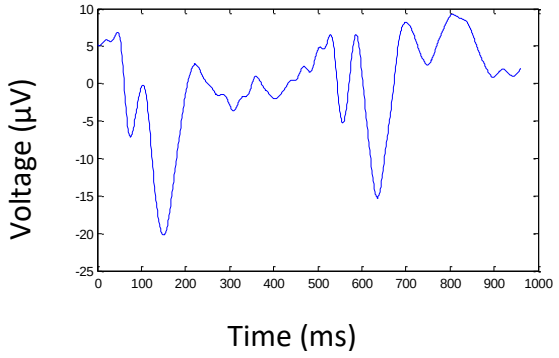
16.1 Phase Reversal Visual Event Related Potential latency

Hypotheses: Phase Reversal Visual Event Related Potential (PR-VERP) transient latency (PR2) and calculated latency will be shorter in treated than control children. In order to investigate whether or not this hypothesis can be accepted PR-VERP latencies, using transient and calculated methods, for treatment and control groups were compared.

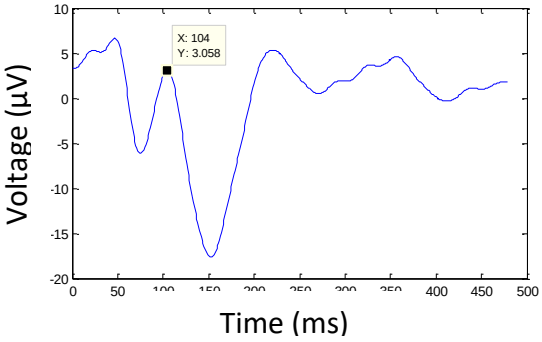
16.1.1 Phase Reversal Visual Event Related Potential (PR-VERP) transient latency

PR-VERP waveforms showed typical responses with a prominent identifiable peak at P_1 (see Figure 35).

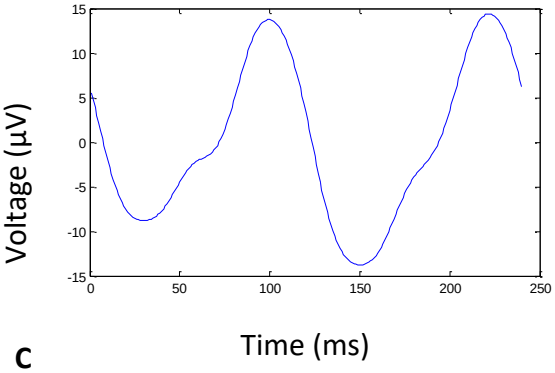
Figure 35. Example of two Pattern-Reversal Visual Event Related Potential waveforms for and infant aged 12 months: (A) Two cycles of PR2 waveform; (B) Average of two cycles of PR2 obtained by splitting waveform (A), showing P_1 peak at 104ms; (C) PR8 steady state waveform from same infant on same visit.



A



B



C

Baseline PR-VERP transient latency (PR2) data was available for 25 children; 22 children had data at baseline and at least one other time point (8 children in the control group and 14 children in the treatment group). Data were available for 9/22 children at 12 and 24 months therefore a comparison of PR-VERP transient latency between groups was not feasible (data not shown).

16.1.2 Pattern Reversal –Visual Event Related Potential calculated latency in intervention and placebo groups

To establish whether calculated PR-VERP latency is shorter in children who received treatment supplementation compared to control calculated PR-VERP latency between groups was compared. Baseline PR-VERP calculated latency data were available for 42 participants; 35 had data at baseline and at least one other time point. Table 38 shows the brain injury severity grading of children contributing data to the analysis. There were higher numbers of children with normal/mild brain injury and severe injury in the treatment group at all time points except 12 months, when the numbers of children with severe brain injury across in both groups was approximately equal.

Table 38. Neurological severity of infants contributing Pattern Reversal-Visual Event Related Potential calculated latency data

Visit	Control, neurological severity			Treatment, neurological severity		
	Normal/ mild	Moderate	Severe	Normal/ mild	Moderate	Severe
Number of children						
Baseline	3 (21)	8 (58)	3 (21)	9 (43)	6 (28.5)	6 (21)
6 month	2 (22)	6 (66)	1 (11)	7 (39)	5 (28)	6 (33)
12 month	3 (16.5)	8 (57)	3 (16.5)	7 (58)	3 (25)	2 (17)
24 month	2 (33)	3 (50)	1 (17)	7 (47)	4 (26.5)	4 (26.5)

In order to test the hypothesis that calculated PR-VERP latency will be shorter in treated than control children, calculated PR-VERP was compared between groups. The analysis was adjusted for baseline measurement, treatment, visit, interaction

between treatment and visit, and the minimisation factors neurological severity, sex and length of gestation as outlined above. Calculated VERP latency did not differ between intervention groups ($\chi2(1) = 0.94$ms, $p = 0.33$), or by visit ($\chi2(1) = 1.13$ points, $p = 0.57$) (Table 39).

Table 39. Mean calculated Pattern Reversal-Visual Event Related Potential latency in control and treatment groups

Visit	Numbers of children (n)		Mean (SD)		Difference in means (95% CI)
	Control	Treatment	Control	Treatment	
Baseline	14	21	245.3 (45.2)	248.7 (35.3)	
6 month	9	18	195.7 (65.2)	192.2 (63.4)	-7.3 (-65.8, 51.3)
12 month	14	12	181.4 (48.8)	217.3 (80.5)	29.8 (-26.4, 86.1)
24 month	6	15	187.0 (40.0)	221.7 (108.3)	33.4 (-33.8, 100.6)

16.1.2.1 Sensitivity (post hoc analyses)

It is feasible that the lack of difference in calculated PR-VERP between groups may have been influenced by higher numbers of children with severe brain injury in the treatment group. To establish if this was the case, the data were re-analysed following exclusion of children with severe brain injury. Table 40 shows the mean calculated latency and difference in mean calculated latency between the two groups. The data show that there was no significant difference in mean PR-VERP calculated latency between intervention groups following exclusion of participants with severe neurological injury.

Table 40. Mean calculated Pattern Reversal-Visual Event Related Potential latency in control and treatment groups excluding subjects with severe neurological damage

Visit	Numbers of children		Mean (SD)		Difference in means (95% CI)
	Control	Treatment	Control	Treatment	
Baseline	11	15	248.2 (50.8)	242.8 (31.6)	
6 month	8	12	195.4 (69.7)	183.1 (68.6)	-20.7 (-88.4, 47.0)
12 month	11	10	185.2 (41.5)	230.4 (81.7)	38.1 (-28.5, 104.8)
24 month	5	11	196.0 (37.3)	232.6 (121.4)	35.4 (-43.4, 114.1)

16.2 Orientation Reversal - Event Related Potential latency

Hypothesis: More children in the treatment group will have a positive OR-VERP response than in the control group.

Raw OR-VERP data are shown in Appendix 10. OR-VERP is a robust measure of cortical orientation processing in infants and has been shown to be a sensitive measure of cortical function in infants with perinatal brain injury. In order to establish whether or not more children who received the treatment have evidence of cortical orientation processing than controls, the number of children who produced a statistically significant OR-VERP response at 8 r/s was compared in the treatment compared to control group.

Table 41 shows the neurological severity grading of the children included in the analysis.

Table 41. Neurological severity grading of participants contributing Orientation Reversal-Visual Event Related Potential data

Visit	Control group, neurological severity			Treatment group, neurological severity		
	Normal/ mild	Moderate	Severe	Normal/ mild	Moderate	Severe
6 month	4 (23)	8 (44)	6 (33)	10 (42)	5 (21)	9(37)
12 month	4 (24)	8 (47)	5 (29)	8 (42)	4 (21)	7 (37)
24 month	2 (20)	5 (50)	3 (30)	8 (44)	3 (17)	7 (39)

OR-VERP data for at least one time point (6, 12 or 24 months) were available for 46 participants. Odds ratios were adjusted for visit, treatment, neurological damage, sex and length of gestation. More children in the placebo group had a statistically significant OR-VERP response than in the intervention group (p=0.03) (Table 42) showing that OR-VERP performance was poorer in the treatment group compared with the control group.

Table 42. Orientation Reversal-Visual Event Related Potential performance in control and treatment group

Visit	Numbers of children		Number of children (%)with significant response		Odds ratio (95% CI)
	Control	Treatment	Control	Treatment	
Total	20	26			0.16* (0.03, 0.85)
6 month	18	24	17 (94.4)	17 (70.8)	
12 month	17	19	14 (82.4)	12 (63.2)	
24 month	10	18	6 (60.0)	6 (33.3)	

*The odds ratio for treatment effect is estimated to be the same at each visit as it was not possible to fit a model with an interaction.

16.3 Fixation Shift Test

Hypothesis: Children in the treatment group will make fewer errors of re-fixation and will have shorter Fixation shift latencies than children in the control group.

The Fixation Shift (FS) test assesses an infant's ability to make shifts of visual attention from a previously fixated target to a newly appearing one. Fixation shifts under non-competition (original target disappears before new target appears) is mediated by sub-cortical structures. Fixation shifts under competition require cortical modulation of this sub-cortical loop controlling non-competition responses and so provides a measure of cortical visual function. The FS test has been used to identify deficits of cortical function in infants with perinatal brain injury.

Table 43 shows the numbers of children contributing to the following Fixation Shift analyses at each visit. Data were available for 47 children. Baseline FS data was not collected as in typically developing infants, fixation shifts are not consistently made under competition until 3-4 months of age.

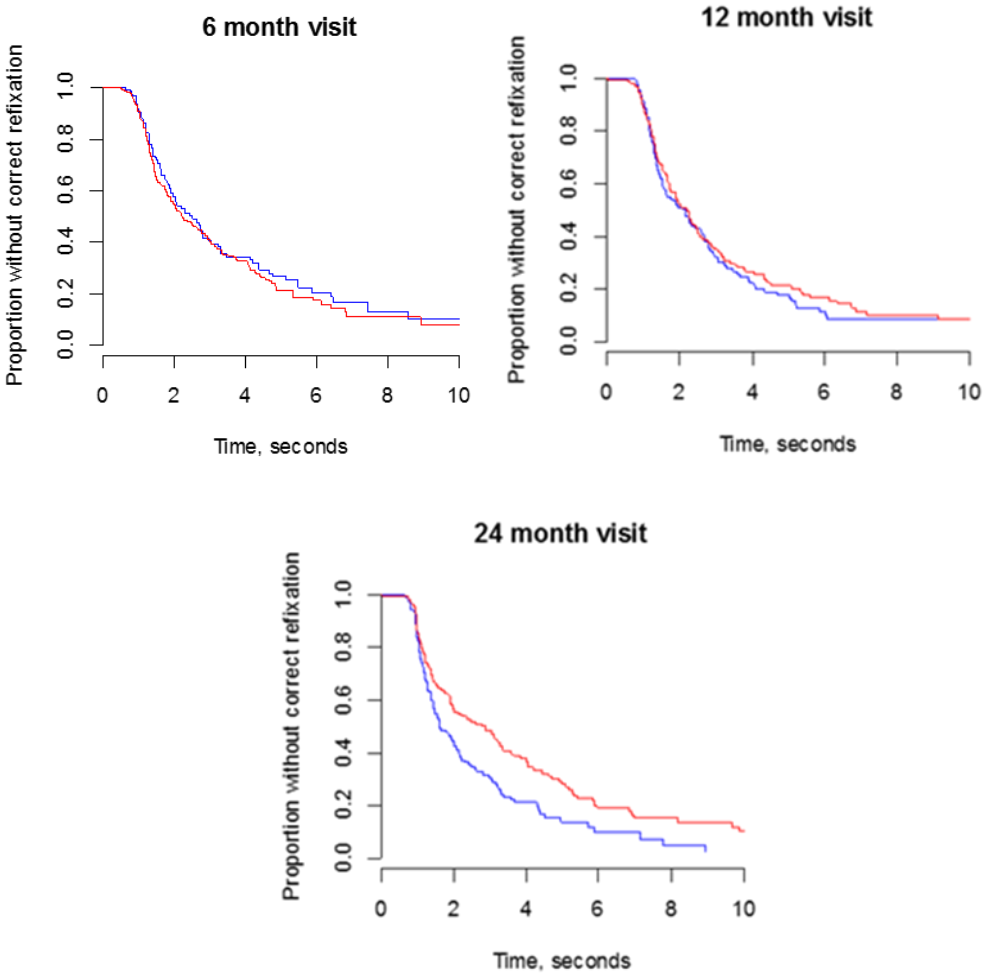
Table 43. Numbers of children contributing Fixation Shift data in control and treatment group per visit

Visit	Number of children (number of tests) contributing data			
	Non-competition		Competition	
	Control	Treatment	Control	Treatment
6 month	17 (162)	23 (221)	15 (147)	22 (211)
12 month	18 (171)	22 (196)	17 (152)	22 (201)
24 month	19 (172)	22 (211)	19 (170)	22 (204)

16.3.1.1 Time to correct re-fixation

Studies show that infants with perinatal brain injury make more Fixation Shift errors than typically developing infants. Fixation shift latency normative data are available. Previous work using the Dolphin cohort suggests that FS latency is prolonged in infants with perinatal brain injury compared to typically developing infants(464). To identify whether FS latency was shorter in children in the treatment group compared to control children, FS latency for non-competition and competition was plotted for the two groups using Kaplan-Meier curves. Data from FS competition at 6, 12 and 24 month visits are shown in Figure 36. Control responses are in blue, treatment responses are in red. Each plot shows the mean probability that a child had not yet made a fixation shift in the correct direction at time t, as a function of t; thus a more steeply descending curve indicates a shorter average latency. Kaplan-Meier plots for FS non-competition show no difference between control and treatment group performance and are not shown.

Figure 36. Kaplan-Meier plots of proportion of Fixation Shift competition responses without correct re-fixation for control and treatment groups at 6, 12 and 24 months



Hazard ratios and 95% confidence intervals for FS latency non-competition and competition per intervention are given in Table 44. Analyses are adjusted for treatment, visit, interaction between treatment and visit, and the minimisation factors sex, neurological damage and length of gestation. There were no differences in the time to correct re-fixation (FS latency) between the control and treatment group. There was a statistically significant interaction between treatment and visit for competition (p=0.01) and a near statistically significant interaction for non-

competition (p=0.08) that favours the control group. This shows that although there is no overall treatment effect, with time, FS latency under competition becomes significantly shorter in the control group compared to the treatment group. A similar trend is seen for FS latency non-competition, although the difference does not reach statistical significance.

Table 44. Fixation Shift latency Hazard Ratios for non-competition and competition by visit

Visit	Non-competition Hazard ratio (95% confidence interval)	Competition Hazard ratio (95% confidence interval)
6 month	0.99 (0.68, 1.43)	1.12 (0.80, 1.58)
12 month	0.67 (0.46, 0.98)	0.91 (0.65, 1.27)
24 month	0.80 (0.55, 1.17)	0.64 (0.46, 0.89)
p-value for interaction*	0.08	0.01
p-value for treatment$	0.21	0.26

* p-value for interaction between treatment and visit
$ p-value for overall treatment effect

Hazard ratios are typically presented alongside a median value. In the case of the presented FS latency data this is the time taken for an infant FS response to be observed in 50% of trials, providing an alternative summary statistic and means of comparing FS latency between control and treatment groups. The repeat measurement structure of the data is not taken into account. These data show that under competition at 6 months, the time taken for half of observations to be made is

shorter in the treatment group than in the control group. The time taken for half of non-competition observations to made is the same in the control and treatment groups (Table 45).

Table 45. Time taken for an infant Fixation Shift response to be observed in 50% of trials under non-competition and competition in control and treatment group

Visit	Non-competition		Competition	
	Control	Treatment	Control	Treatment
Time taken for an infant FS response to be observed in 50% of trials (seconds)				
6 months	1.33	1.33	2.51	2.20
12 months	1.27	1.35	2.15	2.21
24 months	1.15	1.25	1.60	2.85

Sensitivity analyses censoring FS latency data at 5 seconds did not alter the results of this analysis.

Table 46 shows the neurological severity grading of children contributing Fixation Shift latency data. This shows that there are fewer children with no/mild brain injury in the control group compared to the treatment group, but similar numbers of children with severe brain injury in both groups. It is possible that the higher numbers of children with no/mild brain injury in the treatment group could be contributing to the shorter time taken for an infant FS response to be observed in 50% of trials under competition in the treatment group at 6 months.

Table 46. Neurological severity grading of children contributing Fixation Shift latency data

Visit	Control group, neurological severity			Treatment group, neurological severity		
	Normal/ mild	Moderate	Severe	Normal/ mild	Moderate	Severe
6 month	4	8	5	10	6	7
12 month	3	7	8	9	6	7
24 month	3	9	7	9	5	8

16.3.2 Fixation Shift performance in control and treatment groups

To test the hypothesis that children in the treatment group will make fewer errors than children in the control group, the proportion of correct FS under non-competition and competition was compared between the groups. The definition of correct re-fixation is that the child responded correctly with no time limit for response. Table 47 shows odds ratios for the proportion of correct re-fixations in placebo and intervention groups. There was no overall effect of treatment on the proportion of correct FS responses under non-competition (p=0.61) or competition (p=0.76). There was a significant interaction of treatment and visit for both FS non-competition (p=0.03) and FS competition (p=0.04). These data show that although there is no overall treatment effect on FS performance, by visit, there is a statistically significant treatment effect favouring the control group. This effect varies by visit. At 6 months, there are a higher proportion of correct re-fixations under non-competition and competition amongst children in the treatment group compared to controls. This effect is not sustained beyond the 6 month visit, and by 24 months

there are a higher proportion of correct re-fixations under competition in the control group, with a similar proportion of correct re-fixations between groups under non-competition i.e. as time proceeds, children in the control group make a higher proportion of correct refixations than children in the treatment group.

Table 47. Odds ratios for the proportion of correct re-fixations under Fixation Shift non-competition and competition in control and treatment group by visit

Visit	Odds ratios (95% CI)	
	Non-competition	Competition*
6 month	1.22 (0.60, 2.47)	1.56 (0.93, 2.60)
12 month	0.41 (0.19, 0.90)	0.79 (0.46, 1.34)
24 month	1.19 (0.59, 2.44)	0.71 (0.43, 1.16)
p-value for interaction	0.03	0.04
p-value for treatment	0.61	0.76

*Models with and without interaction will not converge, therefore fitted without adjusting for minimisation factors

16.4 A Test Battery of Child Development for Examining Functional Vision (ABCDEFV)

Hypothesis: Children in the treatment group will have better functional vision as measured by performance on ABCDEFV than children in the control group.

The ABCDEFV test battery assesses how children use their vision to perform everyday functions. In order to assess whether or not there were performance differences between control and treatment groups (on ABCDEFV subtests, or on the battery as a whole) test scores were compared for treatment and control groups. ABCDEFV data were available for 48 children. There were no performance differences between the control and treatment groups on any ABCDEFV subtest

(core vision, attention, visuo-cognitive) or the assessment battery as a whole (Table 48).

Table 48. A test Battery of Child Development for Examining Everyday Vision performance of children in the control and treatment group

	Median percentage of tests passed Control /Treatment	OR (95% CI)*	OR (95% CI)[$]
Core Vision			
Baseline	75/50		
6 months	100/86	1.60 (0.33, 7.85)	1.24 (0.24, 6.50)
12 months	100/100	1.07 (0.17, 6.98)	0.84 (0.12, 5.83)
24 months	100/100	0.86 (0.11, 6.72)	0.68 (0.08, 5.63)
Attention			
6 months	40/60	1.74 (0.66, 4.63)	1.78 (0.67, 4.75)
12 months	80/70	1.20 (0.43, 3.30)	1.22 (0.44, 3.38)
24 months	80/80	0.95 (0.32, 2.85)	0.97 (0.32, 2.92)
Visuo-cognitive			
6 months	100/67		0.80 (0.17, 3.70)
12 months	67/75		1.20 (0.49, 2.95)
24 months	71/80		1.43 (0.67, 3.04)
All vision tests			
Baseline	60/40		
6 months	70/75	1.34 (0.71, 2.54)	1.29 (0.68, 2.44)
12 months	82/82	1.10 (0.57, 2.12)	1.05 (0.54, 2.04)
24 months	89/86	1.22 (0.64, 2.33)	1.17 (0.62, 2.24)

* adjusted for baseline, visit, treatment and interaction of visit and treatment
[$] adjusted for visit, treatment and interaction of visit and treatment

16.5 Summary of findings in trial vision outcomes

This section has tested a number of hypotheses which proposed that performance on a number of visual measures, including tests of cortical visual function, would be improved in treatment group children compared to control group children. The first of these examined the hypothesis that PR-VERP transient and calculated latency was shorter in treatment group children as compared to control group children, however the data showed no statistically significant difference in calculated latency between the two groups. There was a trend towards higher calculated latency in the treatment group. There was insufficient transient PR-VERP data to allow analyses. We hypothesised that more children in the treatment group would demonstrate an OR-VERP response than control group children, however the reverse was shown to be true, more children in the control group made an OR-VERP response than children in the treatment group. For Fixation Shift data, latency was non-significantly shorter in the treatment group for competition at 6 months, however this effect was not sustained beyond 6 months and there was no overall effect of treatment. Fixation Shift performance measured as the proportion of correct re-fixations under non-competition and competition was improved in the treatment group relative to the control group at 6 months only. There were no significant differences between groups in performance on ABCDEFV subtests or on the battery as a whole, however the data suggest a non-significant improvement in treatment group performance compared to controls (Table 48). Taken as a whole, the data do not support improved visual or visuocognitive performance amongst children in the treatment

group as compared to the control group. Discussion of these findings is made in section 17.

17 DISCUSSION

17.1 Cohort blood fatty acid analyses

Mean blood DHA, EPA and AA levels were similar in infants born before 28 weeks and at or greater than 28 weeks. This is perhaps surprising given that the majority of transplacental DHA transfer occurs during the third trimester. However, this finding may be explained by the fact that supplementation did not begin until infants were on full milk feeds due to concerns about supplement osmolality and a theoretical risk of necrotising enterocolitis with the use of hyperosmolar feeds. In practice this means that baseline infant blood DHA level does not provide a true reflection of infant DHA status at birth. Maternal baseline DHA level may more accurately reflect infant DHA status at birth. Some evidence of an effect of gestational age at birth on infant LCPUFA status is provided by a moderate correlation between infant baseline DHA and EPA level and maternal baseline DHA and EPA levels, which were strongest in infants born at or after 28 week's gestation.

No sex differences in infant baseline DHA or EPA levels were identified. Some studies suggest that endogenous conversion of FA precursors to DHA and EPA is less efficient in boys than in girls, however there is no evidence of this amongst infants participating in the Dolphin trial. It is possible that any sex differences in endogenous conversion are over-shadowed by other factors such as gestational age at birth and maternal DHA status, or by the influence of postnatal nutrition in the time between birth and baseline FA measurement.

Multiple regression analysis shows that, of the variables tested, maternal baseline blood DHA and EPA levels are the strongest predictors of infant baseline DHA and EPA levels respectively, explaining 30.6% of the variance of infant DHA level and 26.8% of the variance in infant EPA level. Gestational age at birth and sex contributing 3.6% and 2.7% of the variance in infant baseline DHA level, and only 0.1% and 0.1% of the variance in infant EPA level. The small contribution of gestational age at birth and sex may again be influenced by the fact that infant baseline LCPUFA levels were not a true reflection of the infant's fatty acid status at birth.

17.2 Cohort pattern reversal visual event related potential (VERP) latency measures

17.2.1 Transient and calculated VERP latencies as measures of cortical visual function in infants at risk of neurodevelopmental disability

There was a higher degree of variability within the VERP latency data collected from infants at risk of neurodevelopmental disability compared to typically developing infants (see Figures 17 and 23). Both transient and calculated latency were prolonged in infants at risk of neurodevelopmental disability compared to typically developing infants. Calculated latency was prolonged to a greater degree than transient latency (mean difference of 148.6ms compared to 23.3ms). TDI transient latency was an average latency of 3 reversal rates (2, 3 and 4 r/s) and contained very few latencies recorded at 2 r/s, which was the reversal rate used to generate transient latency in the study cohort. Although reversal rates of 4 r/s were recorded

in the trial cohort participants, in general, it was harder to identify the P_1 peak due to merging of a proportion of waveforms at this faster reversal rate and there was poor agreement between transient latencies recorded at 2 and 4r/s. It is possible that a greater difference in transient latencies between the TDI and study cohort would be identified if only transient latencies obtained at 2r/s were available, however good comparability was reported for transient latencies obtained across 2/3/ and 4 r/s for the TDI group(217). In general, the transient latencies in the infants at risk of neurodevelopmental disability followed the same trajectory as those collected from typically developing infants, but took longer to reach adult levels (89 weeks for ARNI compared to 29.5 weeks in TDI). In contrast, calculated latency values remained elevated across the age-range tested, failing to fall to typical adult values across the first year of life as occurs in TDI(217). Neither transient nor calculated VERP latency varied by neurological severity grading. Failure to identify any statistically significant variation in VERP latency may be a result of the very small numbers in each neurological severity group. There is also substantial heterogeneity in the nature of brain injury across the cohort, which may mask any differences between the brain injury severity groups. MRI brain scans were not available for all trial participants. In these circumstances cranial ultrasound scans (cUSS) were used to allocate brain injury severity group according to the neurological severity grading system devised for the study. It is possible that variation in the imaging modality used resulted in underestimation of brain injury severity in infants who only had a cUSS, resulting in less clear separation of brain injury severity groups.

These data suggest that calculated VERP latency may be a more appropriate measure in infants at risk of neurodevelopmental impairment. Calculated latency is reported to reflect the timing of the whole VERP wave-form rather than simply reflecting the detection of contrast in primary visual cortex as with transient latency(217). Calculated latency may be prolonged in children at risk of neurodevelopmental impairments as a result of disruption to wider visual cortical networks.

17.2.2 The effect of sex on Visual Event Related Potential VERP latency in infants at risk of neurodevelopmental disability

There was no statistically significant sex effect on transient VERP latency recorded at 2 r/s, or on calculated latency. However, when an extreme outlier was removed from the calculated latency data set there was a trend of prolonged calculated latency in males compared to females which approaches statistical significance (p=0.081). This sex difference was not identified on either VERP latency measure in the TDI group (personal communication with Professors Oliver Braddick and Janette Atkinson, September 2015). Sex differences in PR-VERP latency have been identified by one other research group. The study identified prolongation of P_1 transient latency in males compared to females, in a cohort of healthy term born infants assessed at 50 or 66 weeks post conceptual age(465). These findings were not explained by the larger head circumferences in males compared to females. Several VERP studies in older children and adults have also identified longer PR-VERP latencies in males compared to females, as reviewed by Malcolm et al(465). Interestingly, our data do not show prolongation of P_1 transient latency in males, which might be related to the

use of a PR-VERP checkerboard stimulus in the Malcolm et al study and a sine wave grating stimulus in the Dolphin study. Maturation of VERP latency to contrast change is dependent upon the stimulus used, however McCulloch et al have shown good inter-laboratory agreement on VERP latency maturation by combining studies which used a combination of checkerboard and contrast grating stimuli(204). It is also possible that sex effects on PR-VERP transient latency are overshadowed by the effects of any brain injury. Malcolm et al suggested that prolongation of PR-VERP latency in typically developing male infants may reflect a sex difference in the rate of visual maturation(465), however in the context of infants with risk factors for neurodevelopmental impairment it is also possible that prolongation of calculated latency in males compared to females reflects a greater vulnerability of the developing male brain to adverse environmental factors. Supporting this suggestion, male sex was a pervasive risk factor for poor outcome in preterm infant participants of the EPICURE study(122). These findings are not explained by a greater number of male compared to female infants with severe brain injury in the cohort (see Table 8). Additional studies on the effect of sex on VERP latency in infants with risk factors for neurodisability are required. Prolongation of calculated but not transient PR-VERP latency in males may also support the suggestion that calculated PR-VERP latency is a more appropriate measure of cortical functioning than transient PR-VERP latency in infants at risk of neurological impairment.

17.2.3 The effect of prematurity on Visual Event Related Potential latency in infants at risk of neurodevelopmental disability

Children who are delivered preterm are born with relatively immature brains. However, preterm infants who avoid apparent brain injury may have some developmental advantage over term born counterparts as they have been exposed to a longer period of visual stimulation. Preterm PR-VERP latency studies have shown conflicting results. Shorter PR-VERP latencies were reported in two studies of preterm infants(207, 466) whilst a number of other studies found no differences between PR-VERP latency in preterm and term children(467, 468). Variation in findings may be explained by the heterogeneous neurological status of the preterm groups studied. O'Reilly et al proposed that the PR-VERP latency differences identified in their cohort may indicate very subtle abnormalities of cortical processing in the preterm group despite the absence of overt pathology on neuroimaging(466). More recently, Jando et al showed that PR-VERP latency is not affected by preterm birth, suggesting that this process follows a pre-determined process, whereas the onset of binocularity (stereopsis), demonstrated by age of onset of dynamic random dot correlogram VERPs is accelerated in preterm infants, suggesting that this process is experience dependent and therefore occurs at a younger corrected age in infants who have a longer period of visual stimulation following preterm birth than their term counterparts(469). Our data show that transient PR-VERP latency was shorter in infants born at 30^{+6} weeks or below as compared to more mature infants. There was no difference in calculated PR-VERP latency between those born at 30^{+6} weeks or below compared to more mature

infants. Our findings are not directly comparable to previously published work as the stimulus used in our study differs from those studies reporting shorter latencies in preterm cohorts(207, 466). Each of the previous studies reporting transient PR-VERP latency in preterm infants have very small cohort sizes (6-15 and 24 infants)(207, 466-469). Our cohort of preterm infants is one of the largest described (n=26). Differences in PR-VERP latency identified using transient latency were not corroborated using calculated latency. However, all of our infants had risk factors for neurodisability and it is possible that any small effect of gestational age at birth may be overshadowed by the larger effect of brain injury on calculated VERP latency.

17.3 The Fixation Shift test as a marker of cortical visual function in infants with risk factors for neurodevelopmental disability

The Fixation Shift test (FS) is a sensitive indicator of cortical function in infancy, and predicts neurocognitive outcome in infants with perinatal brain injury including focal brain injury(226), HIE(220, 222), and preterm white matter disease(223). Previously published work from a subset of the Dolphin cohort confirmed that FS competition is a more sensitive indicator of disruption to cortical attention networks than FS non-competition(464). FS competition performance measured by percentage correct re-fixations demonstrated performance below the 15th percentile for TDI in infants with moderate and severe brain injury severity. FS latencies under competition were prolonged across all neurological severity grading groups, including normal-mild, compared to 15th percentile norms for TDI. It is difficult to draw direct comparisons with this and other previously published work using the FS test due to differences in the method used to analyse the Dolphin study FS data. In order to gain maximal

information from the data individual trial FS latencies contributed to a time to event analysis. In previously published work means of the 5 trials making up each FS condition were calculated, and a between groups comparison of means made. Individual trial data for the TDI was not available for re-analysis using a time to event analysis and so FS data from the Dolphin study cohort cannot be compared to FS performance TDI.

The direction of the Dolphin cohort data suggests poorer FS competition performance in infants with severe compared to normal/mild or moderate brain injury, although these differences did not reached statistical significance. For FS latency, time for half of FS observations to respond increased with increasing brain injury severity at 6 months under non-competition and competition, however this effect is lost by 12 months. At 24 months, the time for half of FS observations to respond is prolonged in infants with severe brain injury under competition alone. These results did not reach statistical significance. The FS latency findings at 6 months may reflect the relative ease of test engagement at this time, compared to at 12 and 24 months when it was harder to maintain infant attention on the FS target. This was particularly true of those children whose behaviour was typical for chronological age. The FS latency analyses only included data from the infants who were tested. A small number of children were not tested at each visit, either because of poor compliance or severity of brain injury. However, of 12 children who were excluded from the FS analyses due to having no data, only 1 had moderate brain injury and 3 had severe brain injury (data not shown). Due to the small numbers of

children included in the FS analyses it is likely that these results provide a conservative estimate of the effects of brain injury severity on fixation shift latency. It is not possible to comment on the effect of sex or gestational age at birth, or interactions between these factors and brain injury severity, on time for half of FS observations to respond, as it was not possible to control for sex or gestational age at birth in this analysis. Earlier studies relating FS performance to brain injury have been based on simple pass-fail criteria(220, 222, 223, 226). It would be desirable to have further studies designed to address the quantitative influence of brain injury severity on FS latency, to establish the value quantitative latency measures as a marker of function of the cortical attention network.

17.4 The Dolphin Trial

An intention to treat (ITT) analysis did not identify any *statistically significant* difference in trial primary outcome measure composite cognitive score on the Bayley Scales of Infant Development III (CCS-BSID III) in the intervention compared to placebo group. However, despite having a slightly lower mean CCS-BSID III at baseline, following 24 months of supplementation, the treatment group had a 9 point advantage compared to the control group (mean score 87.7 vs 81.6). This difference in mean CCS-BSID III represents a *clinically* significant effect size, such that treated infants are lifted out of the moderate/severe developmental "delay" category as defined in the BSID III. One child who was randomised to receive control erroneously received the intervention for 24 months. Repeating the analysis of CCS-BSID III per protocol increased the intervention group CCS-BSID III advantage to 10.6

points (mean score 88.0 vs 80.8); with the difference between groups approaching statistical significance (p=0.08).

A statistically insignificant advantage of 8.6 points in secondary outcome measure LCS-BSID III was seen in the intervention group (mean score 91.5 vs 83.2). Early language ability, particularly receptive language ability, is considered a good indicator of cognitive ability in infants(470), and so the observed improvements in both cognitive and language performance of the intervention group are as expected. There was no statistically or clinically significant difference between control and treatment groups on Bayley motor score.

The identified Dolphin treatment advantage equates to an effect size of 0.31 for primary outcome CCS-BSID III and 0.42 for secondary outcome LCS-BSID III. Direct comparisons with other intervention trials where neurodevelopment was a primary or secondary outcome is made more difficult by the fact that to date the majority of intervention trials have used the previous (2nd) version of the Bayley Scales, which gave a combined cognitive and language score represented by the Mental Development Index (MDI). In order to compare outcomes from trials using BSID II and BSID III as their primary outcome measure, a mean of the combined BSD III cognitive and language scores (CB III) can be used to approximate the BSID II MDI(471). The available literature suggest CB III scores are on average 7 points higher than Bayley II MDI scores, however as we are interested in comparing the *change* in mean CB III scores in infants participating in the Dolphin trial with change in mean MDI scores from infants participating in other dietary intervention trials, no further manipulation of CB III scores is necessary. The unadjusted difference in mean

CB III for the Dolphin 1 cohort is 7.2 points. The DINO randomised control trial of high dose DHA (1% total fatty acids) supplementation in preterm neonates reported a mean Bayley II MDI advantage of 4·5 points in supplemented girls, but not boys, and mean Bayley II MDI advantage of 4.7 in infants born less than 1250g in unadjusted analyses but not adjusted analyses(413). Lucas et al report cognitive advantage in preterm infants born less than 30 weeks gestation fed a "high-nutrient" or "standard-nutrient" formula milk (median of 4 weeks), with a mean treatment advantage of 8·6 points in BSID II mental development index (Bayley II) at 18 months corrected gestational age. Bayley II performance was best in those infants with the highest intake of the "high-nutrient" formula, in babies who were small for gestational age (SGA), and in male infants. The “high nutrient” formula supplemented macro and micro nutrient intake; it is not possible to ascertain whether micro or macronutrient supplementation singly or in combination resulted in the observed treatment group cognitive advantage. Long term follow up of the Lucas cohort confirms lasting neurodevelopmental benefit in the treatment group at school age(300, 301, 418). These comparisons show that the treatment advantage identified in the Dolphin study are similar to those demonstrated by Lucas et al, and of a higher magnitude than seen in sub-groups of the infants participating in the DINO trial(413). Caution must be applied in drawing any definite conclusions from comparisons between the Dolphin data and that of Makrides et al and Lucas et al, as these data relate only to preterm infants, whereas the Dolphin cohort included both term and preterm infants.

There are a number of challenges to the interpretation of the observed differences in mean CCS-BSID III and LCS-BSID III. Firstly, this feasibility study had an 80% power to detect a 12.5 point difference in mean composite scores across domains of BSID III. This is a very large difference in BSID III composite score between placebo and intervention groups. The study recruitment target of 60 children was exceeded (n=62), however due to withdrawal before randomisation only 59 children were randomised. Drop-out was slightly higher than anticipated (27% of those recruited vs 20% anticipated) resulting in a smaller number of children contributing a complete data set than anticipated at trial outset. The trial was unable to adjust recruitment for the high dropout rate, as the trial had already taken a year longer to recruit to than anticipated, necessitating a no-cost extension from the funders.

As expected, there was significant heterogeneity within the cohort, in particular in relation to gestational age at birth and aetiology, anatomical location and severity of brain injury. Stratification by brain injury severity, gestational age at birth and sex at randomisation was performed to allow reasonable comparison between intervention groups, however the categories devised are relatively crude and continue to contain substantial heterogeneity. Much larger numbers of children would be required to reduce the level of heterogeneity between groups. Importantly, there were more withdrawals from the control group than from the treatment group, and the majority of control group withdrawals had normal/mild brain injury, resulting in proportionately fewer children with severe brain injury in the treatment group. Although analysis of the primary outcome measure were adjusted for brain injury severity, it is possible that the benefits in cognitive and language outcomes identified

in children in the treatment group are being influenced by the milder brain injury phenotype of the treatment group compared to the control group.

There are a number of important confounding factors which were not controlled for in the analysis of our data, in order to minimise the risk of over-controlling with its associated risk of Type II error. Maternal education level, which is known to affect cognitive performance in infancy, was not a minimisation factor at randomisation. Table 16 shows that higher numbers of mothers of children in the intervention group attended tertiary level education, and this may have contributed to the higher CCS-BSID III and LCS-BSID III scores in the intervention group. In the 2 years following birth substantial variation in the levels of environmental stimulation across the cohort is likely to have developed, and could explain between group differences. This study did not control for variation in environmental stimulation between participants. Variation in level and quality of environmental stimulation presents a challenge to the interpretation of any long term intervention study where neurodevelopmental outcomes are of interest. The Home Observation for Measurement of the Environment (HOME) Inventory combines observation of the child and parent/caregiver at home and a semi-structured interview. Versions are available for different age groups: Infant-Toddler (0-3 years), Early Childhood (3-6 years), Middle Childhood (6-10 years) and Early Adolescent (10-15 years)(472-474). This tool has been used in a number of intervention studies, including nutritional intervention studies where neurodevelopmental measures are primary or secondary outcomes(475). Validation of the tool has predominantly been performed in the USA, however the tool has been validated in a British population aged 10-13 years

and found to effectively identify children at risk from adverse family circumstances and suboptimal home environments(476). This tool could be used within a larger multicentre trial to stratify participant infants according to the sufficiency of environmental stimulation. As the number of confounders to be controlled for, and covariates, is likely to be higher within a larger trial than in this trial, multivariate analysis is likely to provide an appropriate statistical approach(477).

One potential environmental factor is nutrition (beyond that introduced by the intervention). Observed differences in BSID III outcomes cannot be related to higher protein and energy intake in the intervention group, as in previous studies(271-280), as dietary analysis confirms parity between intervention groups on these measures. It is also possible that participation in the trial may have altered the dietary habits of participant families towards increasing the omega-3 fatty acid content of their diet. However, standard advice was offered by the study dietitian which included advice on currently recommended weekly oily fish intakes. If the oily fish intake of placebo and intervention groups was higher than that of the general population, this may have limited any beneficial effect of the supplement if the greatest benefits were to infants who were DHA deficient. In fact, dietary data collected during the study shows that during the first 12 months of the study daily DHA intake from milk feed and food was higher in the supplement group than in the placebo group. At 12 months, by which time a majority of typically developing infants would be expected to be fully weaned onto solids, there is a reversal in this trend and daily DHA intake from milk feed and food is higher in the placebo than the intervention group (see Appendix 8). This trend was also observed for EPA intake, but not for choline intake.

The higher dietary intake of DHA and EPA in the placebo group may have narrowed the between group differences observed on Bayley III cognitive and language measures.

A further limitation was that no formal measures of compliance with supplementation were made throughout the intervention period; estimates of DHA/EPA, choline and UMP from the supplement relied upon accurate parental reporting. This was a demanding trial for parents and it was felt that formal compliance monitoring would be an additional burden to families. However participant blood fatty acid levels at trial end showed that blood DHA levels were significantly higher in the treatment group compared to the control group, implying that compliance was sufficient to produce a measurable effect (see Table 34). Formal compliance measures would be desirable within a larger multicentre trial.

The choice of outcome measure and age at testing may be relevant to study outcomes. The BSID III has become the gold standard infant neurodevelopmental assessment outcome measure for early intervention neurodisability trials, and allows comparability of outcomes across trials. Creation of BSID III involved a major re-structuring of the assessment tool to include separate gross and fine motor, and receptive and expressive domains, as well as a cognitive domain. Despite having strong correlations between BSID II MDI and BSID III cognitive and language scores several recent studies have reported under-estimation of developmental impairments using the reported Bayley III norms(478, 479). This has occurred as a result of a different approach to normalisation(480), producing normative scores which were on average 7 points higher than in BSID II. If using perinatally recruited

comparison groups the differences may be even higher(480). A study investigating the agreement between classifications of developmental "delay" using the BSID II and BSID III recommends that BSID III composite cognitive and language scores below 85 should be considered indicative of moderate to severe neurodevelopmental "delay"(478). Application of this cut off to our study population raises the group mean CCS-BSID III above this threshold. Similar trends are seen with LCS-BSID III. A non-significant trend of improved performance on VABS-II was also identified in the intervention group at 24 months, however this difference did not result in the crossing of performance categories as seen with BSID III cognitive and language outcomes.

There is some debate about the best time to assess infant neurocognitive performance. Conventionally performance is assessed at 18-22 months CGA in North America or at 24 months in Europe and Australasia(480). In this study, assessment at baseline, 12 and 24 months following intervention aimed to capture the trajectory of individual participant performance. However, the ability to predict cognitive outcome around the age of 24 months is limited, and there is substantial merit in maintaining cohorts such as this one in order to re-assess performance in later childhood when more detailed and probing neurocognitive testing can be applied. A recent meta-analysis of the predictive validity of neurodevelopmental assessment conducted between the ages of 1-3 years identified poor sensitivity but reasonable specificity and negative predictive value for school age cognitive outcome for infants born extremely preterm(481). Post-hoc meta-analysis of 11 studies using BSID II as the neurodevelopmental outcome measure had pooled sensitivity of 54.9% (39.5-

69.3%), specificity 84. 3% (70.1%-92.5%) for any impairment, and sensitivity 43.6% (23.5%-66%), specificity 96.4% (90.0%-98.8%) for severe impairment. These sensitivities and specificities were similar to those given for pooled sensitivities and specificities across all included neurodevelopmental assessment tools. It should be noted that this meta-analysis included studies using BSID II, not BSID III and so caution should be exerted in extrapolating the reported predicted validity data to the current version of the test, especially given the higher scores achieved with BSID III(481). Few studies reporting the predictive validity of BSID III for later cognitive performance are as yet available. One such study reported the predictive validity of BSID for cognitive outcome aged 4 years as measured using the Differential Ability Scale, Second Edition. In this cohort of preterm children BSID III predicted mild/moderate cognitive impairment with sensitivity 29.4%-38.5%, specificity 92.3%-95.5%, and mild/moderate language delay sensitivity 40%-46.7%, specificity 81.1%-85.7%(479). Conversely another group has reported strong predictive validity of BSID III for cognitive and language outcome aged 4 years as measured using the Wechsler Preschool and Primary Scale of Intelligence-III (WPPSI-III) (correlations .81 and .78 respectively)(482). Further studies are required to clarify how well performance on the BSID III cognitive and language scales predicts performance in the preschool and school years amongst infants born preterm, and in other groups of infants at risk of neurodevelopmental impairment.

Despite the relatively crude cognitive measures available in infancy, clinically but not statistically significant differences were detected using CCS-BSID III as the primary

outcome measure. Detailed neurodevelopmental assessment of the Dolphin cohort children at preschool age is now underway.

17.5 Trial vision outcome measures

Participant performance on the visual measures used in the study did not differ between the placebo and intervention groups, except in OR-VERP where performance was better in the placebo group compared to the intervention group (p=0.03). This raises the possibility of intervention toxicity, although this seems very unlikely as DHA is known to have strong antioxidant properties in brain and other tissues, thereby reducing superoxide induced neurodegeneration(483). OR-VERP was the only visual outcome measure, of several, where performance in the intervention group was significantly poorer than in the placebo group. Performance was comparable between groups PR-VERP measures, however unlike OR-VERP responses which are cortically mediated(457, 484), PR- VERP responses can be generated at retinal and cortical level, with no way of separating the relative contribution of retinal and cortical components(208). There are more children with severe brain injury contributing data in the treatment group, and although the odds ratios are adjusted for the minimisation factors which include brain injury severity, it remains possible that the higher numbers of children with severe brain injury in the intervention group contribute to the finding of poorer OR-VERP performance in the intervention group. VERPs were typically recorded at the end of the visual assessment, which took approximately one hour to complete. The assessment was conducted in this order to allow time for the participants to become comfortable with the testing setting and the research team. OR-VERPs were performed at the end

of the VERP testing session. In practice, many of the children were tired and had difficulty in maintaining attention on the VERP stimulus by the end of the VERP testing session, increasing the chances of a negative (statistically insignificant) OR-VERP response. In view of the burden of testing, infants who had shown a positive OR-VERP response on a previous visit were not always re-tested. A Fisher's exact test comparing the number of children in placebo and intervention groups who gave a response *at any visit* did not however identify any between group differences (p=0.12).

The absence of improved performance on any visual measure in the intervention group is perhaps surprising, given the importance of DHA to normal visual development. DHA is specifically concentrated in the retina and cerebral cortex(388). Studies performed in the 1980s showed that preterm infant visual performance was improved in infants supplemented with DHA compared to those who received no DHA in milk(485-487), leading to the introduction of DHA to all infant milks at the level of 0.2-0.3% of total fatty acids, mimicking DHA levels of breast milk in women consuming Western-style diets. DHA is required for retinal development, therefore preterm infants who miss out on third trimester transplacental transfer of DHA may be at increased risk of visual and visuocognitive difficulties and require higher levels of DHA than delivered by currently available milk formulations.

DHA exerts differential effect upon particular aspects of the visual system, and appears to be particularly important for the functioning of M ganglion cells which project to the magnocellular layers of the lateral geniculate nucleus, and are important to functions such as motion perception, spatial relationships and directing

actions(488-490). Anatomical, physiological and behavioural studies demonstrate close associations between the functions of the magnocellular pathways and the dorsal stream, and so it may be that dorsal stream functions are particularly vulnerable to inadequate levels of DHA(394). A number of authors have identified specific dorsal stream deficits in preterm infants and in children with neurodevelopmental difficulties(160, 196, 197, 491-493), leading to the proposal of a general dorsal stream vulnerability during development(125).

The FS test(225) provides a measure of dorsal stream functioning in infancy, however no improvements in FS performance were seen in the intervention group either on measures of percentage correct re-fixations or latency. Although there was no overall treatment effect on FS performance or latency, with time, both measures showed improved performance of the control group compared to the treatment group. Typically developing infants can make accurate fixation shifts under non-competition at birth, and under competition by around 3 months of age(225). As the study assessments fell at 6, 12 and 24 months following initiation of supplementation it was not possible to ascertain whether or not individual infants who had established this skill at the 6 months assessment had in fact been late to develop it. Assessment at 4 months CGA would have been more instructive and may have identified between group differences in time of attainment of FS competence. Compliance with testing became a real challenge with advancing age and may have reduced the sensitivity of the test at later ages. Beyond 6 months of age, it was much harder to maintain infant attention on the monitor screen. This may explain the inconsistent findings in FS performance by treatment group, as outlined in Table 47,

which show a trend of improved treatment group FS performance at 6 months, but not at 12 or 24 months, and the apparent control group advantage in performance and latency measures with time. FS latency analyses were unable to take into account the non-independent (multiple measure) structure of the data. Furthermore, there were a small number of children at each visit who could not participate in testing at all, either because they were too severely affected, or because of poor compliance on the day and so these children are unrepresented in the data set, which may be skewing the results. There was no imputation for missing data.

It is also possible that any between group differences in FS performance were diluted by the effects of brain injury or by the effects of variation in external environmental influences by the 12 and 24 month assessments. There were similar numbers of children with moderate and severe brain injury in control and treatment groups, and so imbalance in brain injury severity in children contributing FS data is not skewing the data. Assessment of other aspects of dorsal stream function such as motion sensitivity, which has consistently identified dorsal stream deficits in clinical populations(125, 246, 491, 492), was not made in the Dolphin study. There was a limit to how many assessments could be performed within a testing period acceptable to infants and parents. This measure may better identify between group differences in dorsal stream functioning in early childhood and could be incorporated into follow on studies assessing the effect of the Dolphin study intervention on visuocognitive functions in later childhood(494). Such measures are incorporated into the Dolphin pre-school study.

The ABCDEFV is a more specific visuocognitive assessment battery compared to BSID-III, and comprises more specifically visuospatial tasks. No overall group differences in performance on ABCDEFV were observed, however the data suggest some improvement in visuocognitive performance at 24 months in the treatment group compared to controls. These differences were not statistically significant. Our analyses did not isolate dorsal and ventral stream tasks. If dorsal stream networks are differentially sensitive to DHA supplementation, then it may be that analysis of between group performance on specific dorsal stream tasks, and those requiring dorsal and ventral stream integration would be informative in a larger cohort. Tests of motion and form coherence have been devised as signatures of dorsal and ventral stream functioning(125, 193, 194). These tests are included in the current follow-up of the Dolphin cohort, when these children are 4-6 years of age.

Our findings are in contrast to previous work reporting short-term benefits to DHA supplementation in term and preterm infants. These studies predominantly used VERPs or grating acuity as outcome measures, both of which assess relatively low level visual function(419). The Dolphin study did not determine the limit of participant visual acuity, rather we ascertained that children achieved the minimum visual acuity expected for age, and so our data is not directly comparable with studies which determined visual acuity accurately. However, our findings are in line with a study of the visual outcomes of participants in the DINO trial at school age, which does not suggest any lasting benefit to high dose DHA supplementation in preterm infants(494). This study used a wide range of visual measures, but did not include measures which specifically taps dorsal stream networks e.g. form and

motion coherence. Although not statistically significant, the direction of effect actually favoured the placebo group on the majority of visual outcome measures used(494). Although the original DINO trial supplemented preterm infants with DHA approaching 1% of total fatty acids (as in Dolphin), infants were not supplemented with other crucial brain nutrients choline and UMP known to increase brain phospholipids, synaptic components and functional brain connectivity. The DINO trial supplemented participants for an average time of around 8 weeks, compared to the 2 year supplementation period of the Dolphin trial(413). It remains possible that re-assessment of specific dorsal stream functions in later childhood will identify performance differences between Dolphin intervention and placebo groups.

17.6 Supplementation may benefit some more than others

It is possible that certain sub-groups of infants are more likely to benefit from neurotrophic supplementation than others. Following 2 years of daily supplementation, children in the treatment group had higher Bayley cognitive and language scores than children in the control group. Although these findings did not reach statistical significance, the effect size was such that the cognitive and language scores of treatment children were lifted out of the moderate/severe neurodevelopmental “delay” group. At randomisation, infants were stratified according to severity of brain injury, gestational age at birth and sex, however due to the small numbers in the cohort it has not been possible to tease out the effect of each of these minimisation factors individually; this might be possible in a larger future trial.

17.6.1 Brain injury severity

It may be anticipated that greater neurocognitive benefits would be demonstrated following neurotrophic supplementation in infants with mild or moderate brain injury, as the potential for effective neuroplasticity may be limited by destructive brain lesions associated with loss of large amounts of brain tissue. The differences in trial primary outcome measure CCS-BSID III remained following removal of the children with severe brain injury from the analysis, demonstrating that the reported benefits apply across all brain injury severity groups. Differences in LCS-BSID III did not remain following exclusion of infants with severe brain injury, suggesting that there may be differential advantage across brain injury severity groups, with the greatest advantage seen in those with severe brain injury. These differences may simply reflect variation in the cortical circuitry involved in the processing of cognitive and language functions(470). The small numbers of children in each brain injury severity group makes it difficult to recognise any associations between more specific brain injury characteristics and performance on BSID III subscales, and requires clarification in a larger trial.

17.6.2 Gestational age at birth

Preterm infants are particularly at risk of DHA insufficiency as they do not receive third trimester trans-placental DHA transfer. Preterm infants have limited adipose tissue reserves, and are unable to synthesise useful amounts of endogenous DHA due to the inefficiency of the enzyme systems necessary to LCPUFA precursor conversion in humans(396). Preterm infants are then further subjected to conditions of depletion during neonatal intensive care stays, which may involve use of total

parenteral nutrition (TPN). Currently used TPN solutions do not contain a source of pre-formed DHA(398). As a result of these combined factors it is estimated that in the first month of life, preterm infants receive only 50% of that which would be received in utero(404). Numbers of pre-term children were equally distributed across placebo and intervention groups at randomisation. However, it is possible that greater benefits to supplementation would be seen in the preterm group compared to term born participants. The small numbers in this study make it difficult to make reasonable comment on this.

17.6.3 Sex

Sex may also play a role in DHA sufficiency and response to DHA supplementation. Males appear to be less efficient converters of endogenous ALA to DHA(414). In addition, the male brain has greater apparent vulnerability to adverse factors, including prematurity(122). The observed trend of longer (slower) calculated PR-VERP latency in boys compared to girls in the Dolphin Study cohort also suggests that males may be more adversely affected by injury to the developing brain. In combination, these factors may imply potential greater advantage to DHA supplementation in males compared to females(397). However, in the DINO trial which was specifically powered to look at sex differences in response to DHA supplementation, Bayley II MDI score was higher in supplemented preterm girls, but not boys(413). No cognitive advantages were identified upon cohort follow up at age 7 years, in either girls or boys(416). Sex was one of three minimisation factors in this study, however the study was not powered to look at the differential effects of sex on neurodevelopmental outcome following neurotrophic supplementation.

17.6.4 Birth weight

IUGR (birth weight < 10th percentile for gestational age) has been associated with adverse neurodevelopmental outcomes, which extend into later childhood(141). IUGR babies lack fat stores and so are likely to be at risk of DHA deficiency; these risks will likely be compounded if prematurity co-exists. The DINO trial reported higher MDI scores in DHA supplemented infants weighing less than 1250g at birth in unadjusted but not adjusted analyses (gestational age, sex, maternal education, and birth order). Decreased risk of mild neurodevelopmental impairments in the DHA supplementation group, as compared to controls, was also reported(413). The Dolphin cohort did not contain any term growth restricted infants. In recognition of the neurodevelopmental risks posed by preterm growth restriction Dolphin inclusion criteria included babies whose gestational age at birth was less than 31 weeks, combined with a birth weight below the 9th percentile. Conducted analyses did not control for birth weight, due to the risk of type 2 errors with over-controlling in small sample sizes. However, mean birthweight was higher in the placebo group than in the intervention group and so differences in birth weight between the groups is unlikely to account for any observed between group differences in outcome measures.

17.6.5 Docosahexaenoic acid status

Infants in the treatment group had higher mean blood DHA levels than infants in the control group. Weak correlations were identified between infant DHA and EPA level at 24 months and CCS-BSID III and between infant blood DHA levels and LCS-BSID III score at 24 months, and a moderate correlation between infant blood EPA level and

LCS-BSID III score at 24 months. Exploratory analyses suggest that infants showing the greatest change in BSID III cognitive and language scores between baseline and 24 months had higher mean blood DHA and EPA levels than infants who showed less or no improvement in BSID III scores. The numbers included in these analyses are too small to allow meaningful conclusions to be drawn. Further work is necessary to establish any links between DHA levels achieved in blood and BSID III performance. It is possible that greater benefits from dietary supplementation can be gained in children who are deficient in DHA, choline or UMP, individually or in combination. Maternal DHA deficiency may lead to DHA insufficiency in offspring, despite preferential transfer of DHA across the placenta(395). Maternal DHA deficiency can evolve through a number of mechanisms, including insufficient intake of preformed DHA. High intakes of n-6 fatty acids in Western diets may exacerbate n-3 insufficiency as n-3 and -6 precursor molecules share enzymatic conversion pathways. Diets high in n-6 PUFAs encourage conversion down the n-6 pathway, exacerbating n-3 deficiency(377).

Epigenetic mechanisms also contribute to an individual's dietary requirement for nutrients important to neurodevelopment(495-497). LCPUFA alter the epigenome, with subsequent effect on endogenous LCPUFA metabolism, mediated via FADS1 and FADS2 gene clusters(498). A small number of studies have established a relationship between fatty acid status, FADS genotype and cognitive outcome in children(30, 31). One study identified a sex specific association between infant minor FADS polymorphisms, red blood cell DHA status and language and cognitive outcomes(31). These findings were corroborated by another large cohort study

which reports associations between specific maternal minor FADS polymorphisms and IQ at 8 years of age(30). We did not ascertain maternal or infant genotype in this study and so are unable to make comment on the impact of genotype on our results.

17.7 Trial design and feasibility of neurotrophic nutritional supplementation in neonates at risk of neurodevelopmental disability

Identification of infants eligible for the study depended upon the daily presence on the neonatal unit (NNU) of either the research fellow (MA) or research nurse (CMJ). Daily visits to the NNU and direct discussion with clinical staff reminded clinicians about the study and its inclusion criteria and prompted approaches to families with information regarding the study. This high level visibility was more difficult in the RBH, Reading and WPH, Slough. The NNU at RBH had two research nurses, who were tasked with identifying eligible babies. There were no neonatal research nurses at WPH, and so CMJ made weekly visits to the NNU there to boost recruitment. The NNU at the John Radcliffe Hospital in Oxford is a tertiary unit taking referrals from across the Thames Valley. One particular challenge to recruitment on the JRH NNU was in making a timely approach to families transferred to the JRH from a local district general hospital (DGH) before their transfer back there. On occasion babies born at the JRH were also transferred out to local DGHs according to clinical priority. This was the reason for a number of the missed opportunities outlined in the trial Consort diagram (Figure 28). Once recruited into the study, infants were randomised according to severity of brain injury severity, gestational age at birth and sex. This method of randomisation resulted in reasonable comparability across minimisation

factors between groups, suggesting that the randomisation process was feasible for this study.

Regarding feasibility of supplementation, 75% of children achieved full supplementation for at least 1 year, and 68% of children completed 24 months of supplementation at full dose. This is a very high retention rate for a trial that demands so much parent time and reflects firstly the dedication of participating families, and secondly the benefits of the support provided by the trial dietitian. Despite parental reports that supplementation became more challenging once the infants were weaned off large volume milk feeds the majority of children who left the study did so before this time. These data suggest that a larger multi-centre RCT of neurotrophic supplementation in neonates at risk of neurodevelopmental disability is feasible. Family feedback indicates that product alteration to address issues of taste and texture is likely to aid compliance.

There were more withdrawals in the placebo group than in the intervention group. Children in the placebo group who were withdrawn from the study tended to have normal-mild brain injury. This may have occurred by chance, however families who believed their child to be at lower risk of neurodevelopmental disability or to be making good developmental progress may have felt less motivated to continue with this demanding study. The higher withdrawal rate amongst the normal/mild brain injury severity participants in the placebo group limits comparison of performance on outcome measures in children with normal-mild brain injury between intervention groups, and raises difficulties for overall group comparisons, particularly

if there are interactions between severity and supplementation. These would be more readily overcome in a larger future trial.

17.8 Feasibility of trial neurodevelopmental outcome measures

A particular strength of this study is the range of outcome measures being employed, utilising behavioural and neurophysiological measures to assess the impact of supplementation on neurodevelopmental outcome. CCS-BSID III primary outcome data was completed in all infants in whom assessment was attempted, identifying BSID III as an appropriate primary outcome measure for any future large multi-centre trial of neurotrophic supplementation in infancy. As with many developmental assessment tools performance on the cognitive domain depends in part on motor ability, as a number of tasks had a motor component. This limits the value of the assessment tool in infants and children with motor difficulties. There are a limited number of neurodevelopmental assessment tools specifically developed for children with motor difficulties, however as our cohort was recruited in the neonatal period before motor difficulties could be confidently identified, it was necessary to use an assessment tool which could be applied to all infants. Later application of a different assessment tool to infants with motor problems gives rise to issues with comparability of findings between participants. BSID III has the advantage of being widely available and can be administered by any appropriately trained clinician(122, 480).

Collection of trial secondary visual outcome measure data was less complete, particularly beyond the 6 month visit. The vision assessment which infants underwent was demanding, particularly for infants with neurodevelopmental

impairments, and many infants found the session tiring. This is likely to have negatively affected data ascertainment and performance on a number of the tasks. As the infants grew older, the VERP stimulus used was unable to consistently hold their attention, despite the development of a number of games using sticky tape on the screen to maintain gaze on the stimulus screen. This is likely to have contributed to the lower proportion of successful VERPs obtained at latter study visits, and in particular of the slow 2 r/s stimulus required to produce a transient PR-VERP latency. Similarly, the alternating facial image used to elicit Fixation Shifts became less interesting as the infants matured, decreasing test compliance and data reliability by the 24 month visit. Infants with the most severe brain injuries were unable to comply with testing. Both PR-VERPs and the FS test require specialist training and equipment and would be less suitable for use in a large multi-centre trial.

In general, infants were easily engaged with the ABCDEFV assessment. As with BSID III, some visuocognitive tasks demand a motor response and so have limited application in children with motor impairments. Data ascertainment for this assessment was excellent by 12 and 24 months. Like BSID III, ABCDEFV although not currently widely available, can be administered by appropriately trained clinicians and has been used to describe functional visual performance in clinical groups, so that it could be deployed in a large multi-centre trial(124, 226, 257, 499).

17.9 Strengths and limitations

The Dolphin Study has three main advantages over previous trials examining the effects of DHA supplementation on infant neurodevelopment(407-413). Firstly, this study provides DHA at 1% total fatty acids, mimicking maximal levels found during

third trimester transplacental fatty acid transfer and in human breast milk. With the exception of the DINO trial(413), previous fatty acid supplementation trials have given DHA at much lower doses(410). Despite delivering high dose DHA, the DINO study supplemented infants for a mean of only 8 weeks(413). No trial has provided the additional substrates UMP and choline that are required for normal neural development. The combination of DHA with choline and UMP has been shown to have positive synergistic effects on brain phosphatide levels, synaptic elements and dendritic spine density compared to these nutrients given singly(1). This trial is the first to provide high dose DHA in combination with choline and UMP. In contrast with other studies, this study supplements infants daily from close to birth for 2 years, throughout the period of maximal brain growth and synaptogenesis. The high DHA dose delivered to trial participants throughout the period of maximal brain growth minimise the likelihood of type 2 errors resulting from insufficient dose or duration of supplementation. No other study has supplemented infants with this dose, in combination with other neurotrophic nutrients, for this long.

A major challenge to the interpretation of the findings of this study come from the lack of statistical power in relation to primary outcome, and the higher withdrawal rate than anticipated. The cohort was heterogeneous and included a wide range of risk factors for neurodevelopmental impairment, of varying aetiology. Brain injury severity, gestational age at birth and sex were minimisation factors during randomisation, achieving reasonable parity between the control and treatment group at baseline, however following withdrawals, there were proportionally more severely brain injured children in the control group, as higher numbers of children

with normal/mild brain injury were withdrawn from the study. This raises the question as to whether or not observed trend in cognitive and language benefit seen in the treatment group may simply be a result of disparity in the distribution of brain injury severity between the treatment and control groups. Due to the risks of over-controlling, we did not control for other factors relevant to neurodevelopmental outcome, such as head circumference, maternal education and effect of environmental stimulation. A further limitation comes from the fact that the trial did not undertake formal compliance monitoring e.g. recording how many unused sachets were returned at supplement re-stocking or end of trial, and it is possible that some children were not supplemented according to their prescription. It is also possible that some families may have increased their child's omega-3 intake via over the counter nutritional supplements e.g. multivitamins with added omega-3. In relation to supplementation, it is also worth noting that children in the treatment group had a mean corrected gestational age of 2.25 weeks at baseline. As DHA is important to brain development throughout gestation and at least the first 2 years of life, it is possible that additional neurodevelopmental benefits could be identified if supplementation had started closer to birth. This was not feasible with the current product formulation, as infants could not be supplemented before they achieved full milk feeds.

18 GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

This novel trial is the first to provide all three phosphatide precursors DHA, choline and UMP to infants with or at risk of CP. There are very few interventions that have been shown to improve the neurodevelopmental outcome of babies and infants with perinatal brain injury, and there is urgent need to identify such therapies. The findings of this study demonstrate that daily nutritional supplementation of neonates at risk of neurodevelopmental impairment is feasible and acceptable to parents, and is associated with clinically significant but not statistically significant benefits to infant cognitive and language development. The findings of this study warrant exploration in a larger multi-centre RCT. If these between group differences are replicated in an adequately powered study, this intervention has the potential to improve the long term neurodevelopmental outcomes and quality of life of thousands of infants with risk factors for neurodisability.

Significant heterogeneity within the Dolphin cohort hampers interpretation of findings. In any future larger trial it would be important to reduce heterogeneity in order to allow a more valid between groups comparison. This could be done, for example, by only including very preterm children e.g. all those born at less than 28 weeks gestation, and stratifying according to brain injury severity, gestation and sex as in the current trial. Power calculations assuming power of 90% to detect a 9 point difference in CCS-BSID III, with 5% significance level, and the same attrition rate as the Dolphin indicate that 158 children would need to be recruited. In order to recruit this number of babies, it would be necessary to run this trial as a multicentre trial, including at least five tertiary neonatal units. Controlling for potential confounding

factors such as neonatal comorbidity, maternal education level and level of environmental stimulation, which could not be controlled for in the Dolphin study would be desirable in order to interpret outcome data appropriately. The findings of the Dolphin study aid in the selection of outcome measures. As BSID-III cognitive and language outcomes were the only measures which identified a trend of improved treatment group outcome, BSID-III should remain the primary outcome measure. As vision testing did not identify any between group treatment advantage, and requires specialist equipment and training, inclusion of these measures across multiple trial sites would be inappropriate. It would be desirable to include detailed magnetic resonance neuroimaging in the study protocol in order to better define and compare brain injuries between babies. Follow up neuroimaging including tractography to assess any structural effects of supplementation would also be desirable, but may be off-putting to potential participants, as this would involve a general anaesthetic. Any large scale future trial should also consider epigenetic influences on nutritional status and neurodevelopmental outcome; this could be done simply by collection and genotyping of salivary DNA samples. The Dolphin trial did not include any formal measures of treatment compliance, although blood fatty acid level comparison between the treatment groups showed higher blood DHA and EPA levels in treatment children compared to controls (see Table 34). More detailed information regarding individual supplement intake would help in establishing fidelity and the nature of any dose-response relationship. This could be done through the provision of hand-held devices loaded with data collection apps from which data could be uploaded on a weekly basis, or by the documentation of returned unused product at

the point of re-supply. The nutritional supplement product may require further development ahead of a large multi-centre trial, as some infants were aware of altered taste and texture of milk and food following addition of the product. Several families had to give the supplement in a number of feeds/meals per day to achieve the prescribed daily dose. Focus group work involving the parents and caregivers of participants in the study is currently underway to help inform product development. Due to the osmolality of the supplement, and neonatal feeding practices at the time, the average age on starting supplementation was 3.32 weeks in the control and 2.25 weeks in the treatment group. Improvements to product formulation combined with the much earlier introduction of milk feeds under current feeding practice is likely to result in earlier commencement of supplementation. Finally, any future trial should include Health Economics, to establish the potential cost implications of supplementation; improved neurodevelopmental outcome may result in reduced utilisation of health and education resources.

Despite these challenges to interpretation of the trial primary outcome findings, if the observed improvement in CCS-BSID III for children in the treatment group compared to children in the control group was replicated in a larger trial, then this will have important implication for our understanding of the role of DHA, choline and uridine to neurodevelopment and will help generate new hypotheses in order to test the mechanisms by which the observed effects on cognition occur. The implication for families of children with risk factors for neurodevelopmental impairment, and not least for the children themselves are also significant; this nutrient combination has the potential to improve neurodevelopmental outcome for children at risk of

neurodevelopmental impairment worldwide, and may have knock on effects for other important short, medium and long term outcomes including quality of life through, for example, improved participation resulting from better communication skills. Participation is a strong determinant of quality of life in children with CP(500). Finally, current work investigating the longer term impact of neurotrophic intervention in our original Dolphin cohort will help ascertain whether the neurocognitive and language benefits identified around age 2 years persist into later childhood, and may identify new impairments which become clearer with age, or to identify benefits which may only become apparent as participants reach an age at which more detailed neurocognitive assessment can be conducted. An important aspect of work with the original cohort data will be to establish which outcome measures best identify any neurocognitive advantage identified in later childhood.

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20 APPENDICES

20.1 Appendix 1: Summary of ABCDEFV (2008) components as used in the Dolphin study

Table 1.1: Core Vision Tests

	Purpose of the Test
1. Pupil responses	Responsiveness of pupils to light. Failure of pupil constriction in one/both eyes is likely to indicate severe neurological problems.
2. Diffuse light reaction	Responsiveness to light and dark, but not a measure of pattern vision. Only appropriate for very young infants and in cases of suspected total blindness. Failure is likely to indicate severe ophthalmological and/or neurological problems.
3. Lateral tracking	Assessment of eye movements (saccadic and/or smooth pursuit) and visual attention. Inability to track (appropriately for age) indicates attentional/neurological problems.
4. Peripheral refixation - lateral field testing	Assessment of visual attention (and the extent of visual fields). Failure can indicate ophthalmological and/ or visual neurological problems (hemianopia; visual neglect). Age norms need to be applied to the estimated size of field to decide abnormality.
5. Symmetrical corneal reflections	Alignment of the eyes. Eyes constantly mis-aligned indicate manifest, convergent or divergent strabismus. May be associated with marked refractive errors, amblyopia /ocular/ neural pathology.
6. Convergence of eyes to approaching object	Assessment of convergence of the eyes. Failure to converge may relate to eye pathology and/or neurological problems.
7. Attention at distance	Assessment of visual attention at moderate distance. Failure (beyond 6 months) can indicate attentional problems and/or ophthalmological/ neurological problems.
8. Defensive blink to object approaching face	Assessment of visual attention. Failure beyond 6 months indicates neurological/ophthalmological problems.
9. Visually follows falling toy	To assess understanding of *object permanence* i.e. The understanding that an object goes on existing when it disappears from view

Table 1.2 Optional Additional Core Vision Test

Test	Purpose of the Test
10. Teller Acuity Cards	Assessment of visual acuity.

Table 1.3 Age Specific Tests

Test	Min age	Purpose of the test
11. Batting/ reaching	4 months	Assessment of visuomotor development. Failure to reach in infants over 6 months suggests visual/ neurological and/or visuo-cognitive problems.
12. Pick up black and white cotton thread	12 months	Assessment of visual control of fine hand and finger movement (including pincer grasp). Also crude test of contrast sensitivity, when white cotton is used on a white table top.
13. Retrieval of partially covered object	6 months	Assessment of later stage of *object permanence* i.e. Understanding that an object partially hidden from view still exists and can be retrieved. Failure beyond 12 months may indicate visuo-cognitive problems.
14. Retrieval of totally covered object	6 months	Assessment of later stage of *object permanence* i.e. Understanding that an object fully hidden from view still exists and can be retrieved. Consistent failure beyond 15 months may indicate visuo-cognitive problems.
15. Shape matching	18 months	To measure ability to match simple shapes (a test of spatio-cognitive visual function - shape recognition and manipulation). Failure may represent a general delay or specific visual spatial problems.
16. Overlapping figures	2 years	Assessment of figure-ground segmentation together with shape recognition. Failure may represent general delay or specific visual perceptuo-cognitive problems.
17 Placing letter in envelope	2 years	A spatial test combining of visuo- cognitive and visuomotor visual abilities. The task involves relative orientation matching in space, and use of this for planning and control of manual actions
18. Block construction - free play	13 months	A spatial test combining visuo-cognitive and visuomotor abilities. Requires recognition of spatial relations, and use

Test	Min age	Purpose of the test
		of these for planning and control of manual actions.
19. Copying block constructions	18 months	To test a combination of spatial, cognitive and motor aspects of vision. The block constructions are graded in difficulty for ages 18 months to 5 years. In adults, failure on such tests is called 'constructional apraxia'.

Tables 1.1-1.3 Developed Atkinson 2002(203). Reproduced with the kind permission of Professors Janette Atkinson, Oliver Braddick, and Taylor & Francis.

20.2 Appendix 2: BMC Pediatrics Dolphin study protocol paper

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BMC Pediatrics

STUDY PROTOCOL Open Access

Optimising nutrition to improve growth and reduce neurodisabilities in neonates at risk of neurological impairment, and children with suspected or confirmed cerebral palsy

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Abstract

Background: Neurological impairment is a common sequelae of perinatal brain injury. Plasticity of the developing brain is due to a rich substrate of developing neurones, synaptic elements and extracellular matrix. Interventions supporting this inherent capacity for plasticity may improve the developmental outcome of infants following brain injury. Nutritional supplementation with combination docosahexaenoic acid, uridine and choline has been shown to increase synaptic elements, dendritic density and neurotransmitter release in rodents, improving performance on cognitive tests. It remains elusive whether such specific 'neurotrophic' supplementation enhances brain plasticity and repair after perinatal brain injury.

Methods/Design: This is a two year double-blind, randomised placebo controlled study with two cohorts to investigate whether nutritional intervention with a neurotrophic dietary supplement improves growth and neurodevelopmental outcomes in neonates at significant risk of neurological impairment (the D1 cohort), and infants with suspected or confirmed cerebral palsy (the D2 cohort).
120 children will be randomised to receive dietetic and nutritional intervention, and either active supplement or placebo. Eligible D1 neonates are those born $<30^{+6}$ weeks gestation with weight $<9^{th}$ centile, $\leq 30^{+6}$ weeks gestation and Grade II, III or IV Intra-Ventricular Haemorrhage or periventricular white matter injury, or those born at $31\text{-}40^{+28}$ weeks gestation, with Sarnat grade I or II or III Hypoxic Ischaemic Encephalopathy or neuroimaging changes compatible with perinatal brain injury. Eligible D2 infants are those aged 1-18 months with a suspected or confirmed clinical diagnosis of cerebral palsy. The primary outcome measure is composite cognitive score on the Bayley Scales of Infant and Toddler Development III at 24 months. Secondary outcomes include visuobehavioural and visual neurophysiological assessments, and growth parameters including weight, height, and head circumference.

Discussion: This is the first study to supplement neonates and infants with perinatal brain injury with the combination of factors required for healthy brain development, throughout the period of maximal brain growth. A further study strength is the comprehensive range of outcome measures employed. If beneficial, supplementation with brain phosphatide precursors could improve the quality of life of thousands of children with perinatal brain injury.

Trial registration: Current Controlled trials: ISRCTN39264076 (registration assigned 09/11/2012), ISRCTN15239951 (registration assigned 23/04/2010).

Keywords: Docosahexaenoic acid, Choline, Uridine-5-monophosphate, Neonates, Infant, Neurodisability, Neurodevelopment, Cerebral palsy, Brain plasticity, Growth

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Background

Perinatal brain injury (PBI) has a range of consequences dependent upon the exact timing, location and extent of the brain insult. Brain white matter appears particularly vulnerable to injury in the preterm period [1], whereas grey matter injury is typically the consequence of acute injury sustained around term [2]. It is now recognised, however, that white and grey matter injury occur concurrently in pre-term and term brain injury [3,4]. Cerebral palsy (CP) is a common sequelae of brain injury in the antenatal, perinatal or postnatal period. CP describes "a group of permanent disorders of movement and posture, causing activity limitation that is attributed to non-progressive disturbances that occurred in the developing fetal or infant brain. The motor disorders of CP are often accompanied by disturbances of sensation, perception, cognition, communication, and behaviour, by epilepsy, and by secondary musculoskeletal problems" [5]. Although the brain injury resulting in CP is non-progressive, its manifestations vary throughout development. The incidence of CP worldwide is between 2.0-2.5 per 1000 live births [6,7], and CP is more common in infants born preterm, and in growth restricted infants [7]. Perinatal brain injury does not inevitably result in CP; however a range of cognitive and visual impairments are common sequelae, even in those who do not have severe motor impairment [8-10].

Brain plasticity and functional recovery

Brain plasticity describes a process whereby the brain is functionally altered [11]. Studies of brain growth and development suggest that, as a result of an abundance of developing neurons and synaptic connections, the brain's capacity for plasticity is probably highest through mid-gestation to early childhood [12]. It may also be true that the substrate richness that underlies the developing brain's potential for plasticity also underlies its' early vulnerability. Moreover, the outcome of perinatal brain injury may depend on the precise timing of injury; insults occurring during a "critical period" of development are more likely to cause significant impairment [12,13]. By contrast, insults occurring toward the end of specific developmental windows when critical neural connections have been made are less likely to result in as severe impairment [14].

The advent of more advanced neurophysiological and neuroimaging techniques has provided direct evidence of neuronal plasticity in infants and children [15]. For example, in children with congenital hemiparesis developing axons have been shown to re-route around areas of damage to reach their intended cortical target [16]. Furthermore, in children with congenital hemiparesis intensive therapy programmes can increase the size of primary hand motor cortex motor contralateral to the paretic hand, with associated functional improvement in the paretic hand [17-20]. Similar evidence for plasticity has also been demonstrated in the visual system where diffusion tractography imaging clearly identifies thalamo-cortical axon circumnavigation of white matter damage, to reach their predetermined destination in the occipital cortex [21].

Evidence supporting the plasticity potential of the developing brain raises the question of whether interventions that support the regeneration of developing neuronal projections might improve function as well as structure, and reduce neurodisability.

Nutrition and brain development

The integrity and function of the brain depends in large measure upon a specific profile of membrane lipids and their fatty acids. In the brain, many enzymes involved in neurotransmitter metabolism are lipid-dependant, as are functions involved in the synthesis of brain structural lipids. Phosphatidylcholine is the primary component of neuronal cell membranes. The synthesis of brain phosphatidylcholine utilises three circulating precursors: choline; a pyrimidine e.g. uridine-5^1-monophosphate (UMP); and a polyunsaturated fatty acid (PUFA) e.g. docosahexaenoic acid (DHA). Phosphatidylethanolamine, another component of neuronal cell membranes, may utilise two of these precursors, a pyrimidine and a PUFA. There is evidence in animals and humans that dietary supplementation of the precursors of neuronal cell membranes improves function. Dietary supplementation of rodents with a combination of DHA, choline and UMP increases brain phosphatide levels [22], pre and post-synaptic elements [22,23], and dendrite spine density [24], and is associated with improved performance on cognitive tasks [25]. Dietary supplementation of rats with UMP increase striatal levels of the neurotransmitter acetylcholine, relevant to cognitive processes in humans and other animals [26]. The first human randomised placebo controlled trial of DHA, choline and UMP has shown improvements in verbal recall on the Weschler Memory Scale-revised in adults with early Alzheimer's disease [27].

Nutrition and infant development

Infant milk formulae and normal feeding recommendations have been developed considering growth in weight and length as the main outcomes. Recently there been more consideration of neural development as an additional outcome when determining the optimal nutrition for neonates and infants.

Studies of improved early nutrition in preterm infants have shown improved neurodevelopmental outcomes in intervention groups [28,29]. Whether increased nutritional intake in the first years of life in children with CP improves growth or neurodevelopmental outcome, however, has not been adequately studied. Recently, a small non randomized

trial of children with neurological impairments aged 1 – 14 years showed that those who received 6 months of additional nasogastric tube feeds, had improved growth and gross motor functioning compared with a group who did not receive supplements [30]. A further study identified increased head circumference and corticospinal tract diameter in infants fed with 120% of the recommended daily allowance, however neurodevelopmental outcome data was not reported [31].

The crucial role of long chain polyunsaturated fatty acids (LCPUFA) in infant brain development has been extensively investigated over the last decade. Studies have provided inconclusive results of the potential neurodevelopmental benefits of LCPUFA supplementation, in preterm and term infants. This may be a result of variation between conducted studies in design, maturity of supplemented infants, supplementation dose and duration, and outcome measures used. A recent individual patient data meta-analysis failed to show neurodevelopmental advantage of LCPUFA supplementation [32], but did not include data from two relevant studies which demonstrated improved performance on the Bayley Scales of Infant Development (BSID) in pre-term [33] and term infants [34]. The DINO study was the first trial to supplement preterm infants with levels of DHA approaching maximal concentrations found in human breast milk (around 1% total fatty acids) [35]. Infants were supplemented until discharge from hospital. The results of this randomised control trial (RCT) did not show any difference in the incidence of CP, but did demonstrate improved cognitive performance in preterm girls (but not boys), and in infants with birth weight less than 1250g in unadjusted analyses. Visual acuity was also improved in cases at 4 months compared to controls [35].

Whether or not the provision of adequate supplies of the biochemical precursors of neuronal cell membranes such as choline, uridine and DHA can improve neurological function in brain damaged infants remains unknown and has not been studied in infancy and childhood.

Methods/Design

The overall aim of this study is to investigate whether nutritional intervention providing adequate choline, uridine and DHA supports the plasticity potential and regeneration of neuronal projections in the developing brain, to improve function and structure, and reduce neurodisability.

Primary objective

To investigate whether nutritional intervention, with supplementation of choline, uridine and DHA improves neurodevelopmental outcomes in neonates at significant risk of neurological impairment, and infants with suspected or confirmed CP.

Secondary objectives

To investigate whether nutritional intervention improves growth, visual outcomes and improved indices of general health status, including prevalence of epilepsy, feeding difficulties, clinically significant gastro-oesophageal reflux, constipation, chest infections (requiring antibiotics) and hospital admissions.

Primary outcome measure

The primary outcome measure is performance on the composite cognitive scale of the BSD- III following 24 months of supplementation.

Secondary outcome measures

- BSID-III composite cognitive scale at 12 months
- BSID-III composite language score at 12 and 24 months
- BSID-III composite motor score at 12 and 24 months
- Visual function, including Pattern Reversal Visual Event Related Potential (PR-VERP) latency and behavioural vision assessment score at 12 and 24 months
- Growth
  - Weight for age z-score at 12 and 24 months
  - Length for age z-score at 12 and 24 months
  - Triceps skinfold thickness for age z-score at 12 and 24 months
  - Mid-upper arm circumference for age z-score at 12 and 24 months
  - Head circumference for age z-score at 12 and 24 months

Study design

The Dolphin studies are double-blind randomised placebo-controlled trials of supplementation with a combination of DHA, choline and uridine. Two cohorts will be recruited:

Dolphin 1 (D1): Optimising nutrition to improve growth and reduce neurodisabilities in neonates at risk of neurological impairment and
Dolphin 2 (D2): Optimising nutrition to improve growth and reduce neurodisabilities in children with suspected or confirmed cerebral palsy.

Subject population

Term and pre-term neonates who are at significant risk of neurological impairment, either as the result of a perinatal

neurological event or by their extreme prematurity and low birth weight (<9th centile).

Infants up to 18 months of age with a suspected or confirmed diagnosis of CP, made by their consultant paediatrician.

Neonatal inclusion criteria

Birth ≤30+6 weeks gestation and:

- Small for Gestational Age – weight less than 9th centile OR
- Sarnat Grade IIa, IIb, III or IV Germinal Matrix Haemorrhage (GMH) – Intra Ventricular Haemorrhage (IVH) or pathological periventricular flare/ leucomalacia

Birth 31-40+28 weeks gestation:

- Hypoxic Ischaemic Encephalopathy Sarnat Grade II and III OR
- Grade IIa, IIb, III or IV GMH-IVH or pathological periventricular flare/ leucomalacia OR
- Magnetic resonance imaging (MRI) abnormalities: Posterior limb of the internal capsule (PLIC), basal ganglia, thalami, white matter and cortex.

Consent to enter the trial will be obtained before the infant is 4 weeks post term corrected age.

Infant inclusion criteria

Aged 1–18 months with a suspected or confirmed clinical diagnosis of cerebral palsy according to their consultant Paediatrician, using the following definition:

> *"A group of permanent disorders of the development of movement and posture, causing activity limitations that are attributed to non-progressive disturbances that occurred in the developing fetal or infant brain. The motor disorders of cerebral palsy are often accompanied by disturbances of sensation, perception, cognition, communication, and behaviour, by epilepsy, and by secondary musculoskeletal problems"* [5].

Exclusion criteria

- Progressive neurological conditions
- Gastrointestinal disease which significantly impairs absorption
- Multiple congenital abnormalities or syndromic associations
- Such low hearing that assessment with the Bayley scales cannot be completed
- Parents considered by clinicians to be unable to follow study protocol

And for infants born at <36 weeks gestation:

- Grade I GMH-IVH

Identification and recruitment of participants

This is a multi-centre trial to be carried out at the Oxford University Department of Paediatrics with 6 collaborating regional hospitals. Participants will be recruited following referral from Neonatologists, Community Paediatricians and Neurologists at these collaborating sites.

Neonatal identification

Term and pre-term neonates will be recruited from the neonatal units at the Oxford University Hospitals NHS Trust, Oxford, the Royal Berkshire Hospital, Reading, and Wexham Park Hospital, Slough. Eligible neonates will be identified by the neonatal clinical team who make the initial parental approach, with support from the research team. The trial will only be discussed with the parents when their baby is fully enterally fed and no longer receiving neonatal intensive care. With parental agreement the research nurse (CMJ) or clinical research fellow (MA) will then approach the family to discuss the trial in more detail and give them the parent written information.

Infant identification

Paediatricians and Neurologists with knowledge of the infant's previous clinical history, presenting clinical condition, and study protocol will identify infants with a clinical diagnosis of suspected or confirmed cerebral palsy at the collaborating research sites. These sites are at Stoke Mandeville Hospital and Wycombe General Hospitals (Buckinghamshire Hospitals NHS Trust), Northampton General Hospital (Northampton General Hospital NHS Trust), Department of Child Health (Milton Keynes Hospital NHS Foundation Trust), The Child Development Centres at Upton Hospital, and St Marks Hospital, (Heatherwood and Wexham Park Hospitals NHS Foundation Trust), and the Dingley Specialist Children's Centre at the Royal Berkshire NHS Foundation Trust. With the lead clinicians' agreement, the clinical research fellow (MA) or research nurse (CMJ) will contact the parents by telephone to explain the trial in more detail and if they are interested, send them written information.

Consent procedures

Neonates

The research nurse or clinical research fellow will take written consent, either on the neonatal unit or, if the baby had been discharged subsequent to the written information being given, at the family home.

Infants

Written consent will be taken by the research nurse or clinical research fellow during a pre-arranged visit to the family home.

Randomisation and blinding

Randomisation will be carried out by the study statistician (NW) in the University of Oxford Centre for Statistics in Medicine, Oxford (CSM). Randomisation will occur following consent, at least 24 hours before start of supplementation. Both neonates and infants will be randomised to intervention group X or Y, to receive dietetic and nutritional intervention, with either active or placebo supplementation. The randomisation programme will include a minimisation algorithm which will be used to ensure balanced distribution of children with different severities of brain injury between intervention groups. The research team will be notified by CSM of randomisation group by return fax of the completed randomisation form.

Neonate stratification

Neonates will be allocated across the 2 treatment groups by the following prognostic factors: gender, gestational age ($\leq 30^{+6}$ weeks, ≥ 31 weeks) and severity of brain injury (normal/mild, moderate, severe – see Table 1).

Infant stratification

Infants will be stratified by gender, age at recruitment (1–5 months, 6–12 months, and 13–18 months), known visual impairment at trial entry and severity of motor disorder (4 limb involvement or other).

The research team will be blinded to treatment group allocation with the exception of the trial dietician (KL) and trial assistant (BB). KL will responsible for the delivery of supplement to participating families and will be aware of group allocation (X or Y), as will be participating families. Unblinding at the end of the trial will be undertaken by CSM.

Intervention

Parents will receive fortnightly input from the trial dietitian (KL) to optimise macro- and micro-nutrient intake. Each infant's diet will be supplemented with 2g/kg/day of study product for 2 years. The supplement will be added to the infant's milk or mixed with solids depending on the age and diet of individual participants. Detailed advice on dose

Table 1 Neuroimaging severity categorisation [2,49-57]

	Normal/Mild	Moderate	Severe
Preterm injury			
• Cranial ultrasound scan (cUSS)	• Normal	• Grade III IVH	• Grade IV IVH
	• Grade I/II Intraventricular haemorrhage (IVH)	• Non-cystic Periventricular leucomalacia (PVL)	• Periventricular haemorrhage infarction (PVHI)
	• Ventricular index (VI) < 13 mm Term equivalent age (TEA) OR	• VI 13-15 mm TEA OR	• Cystic PVL
	• VI < 97th percentile for corrected gestational age (CGA)	• VI >97th percentile but < 4 mm above 97th percentile for CGA	• Subcortical leucomalacia
			• VI at TEA >15 mm OR
			• VI >4 mm above 97th percentile for CGA
			• Basal ganglia (BG) lesions
			• Focal infarction
Term hypoxic ischaemic encephalopathy			
• Magnetic resonance imaging (MRI)	• Focal subtle abnormalities of BG with normal appearance of the posterior limb of the internal capsule (PLIC)	• Multi-focal lesions in BG with equivocal or abnormal signal intensity within PLIC	• Widespread abnormalities involving all Basal ganglia-Thalamus (BGT) structures and PLIC
• cUSS where MRI unavailable	• Periventricular white matter changes difficult to differentiate from normal appearances and therefore not classified as abnormal	• Small focal lesions of without loss of grey matter (GM)/WM differentiation.	• Larger areas of abnormality with loss of GM/WM differentiation, consistent with infarction
	• Changes confined to cerebral cortex and subcortical white matter (WM)		• Central grey matter hyperechogenicity +/– more extensive cortical and subcortical hyperechogenicity
Term infarction			
• MRI (cUSS where MRI unavailable)		• Focal, non-territorial infarct	• Territorial infarct

incrementation and supplement mixing with food or fluid will be provided by the trial dietician (KL). The active supplement contains DHA (1% total fatty acids), arachidonic acid (AA), choline, UMP, multivitamins, trace elements and minerals. The placebo contains multivitamins, minerals and trace elements only. Supplement sachets are labelled X or Y and are available in 2g, 3g and 12g quantities. The supplements are produced under strict Good Manufacturing Practice (GMP) conditions and donated by Nutricia®, Netherlands, and have been deodorised to minimise smell and taste. Parents and assessors will be blinded as to whether X or Y is the active supplement.

Study assessments

A detailed schedule of the study assessments is shown in Table 2.

Treatment monitoring and growth outcomes

Head circumference, weight, length, height, mid-arm circumference and skinfold thickness (triceps, biceps, suprailiac and sub-scapular) will be measured 3 monthly [36].

Participant whole blood fatty acid levels will be measured by heel or finger-prick [37] at trial entry and at the end of supplementation. In the neonatal trial maternal whole blood fatty acid levels will be measured. Blood fatty acid levels at baseline and trial end will be correlated with neurodevelopmental outcome.

Developmental assessment

The BSID II [38] has been widely used to determine rates of developmental disability or disordered development [39,40], and as an outcome measure in RCTs [41,42]. The BSIDIII [43] has superseded the BSID II and includes separate composite scores for cognitive, language and motor scales. The BSID III will be administered in the child's home by a trained administrator (MA or CMJ). Assessments will be video-recorded for verification of scoring.

The Vineland Adaptive Behaviour Scales II (VABS-II) [44] is a standardised parental interview, and will be administered by MA or CMJ following Bayley assessment, as a parent report measure of child development.

Functional vision assessment

Vision assessment will be performed by MA and CMJ under the supervision of Professors O Braddick and J Atkinson. Assessments will be conducted in vision testing facilities in the Women's Centre, Oxford University Hospitals NHS Trust, Oxford. Infants will sit on the caregiver's lap during testing. The Atkinson Battery of Child Development for Examining Functional Vision (ABCDEFV) [45] will be used to assess perceptual, motor, spatial and cognitive skills, and published normative data are available [45].

Core vision tests will include orthoptic assessment (ocular movements, pupil response), refractive errors, binocular optokinetic nystagmus (OKN), acuity (Teller acuity cards), and attention at distance, visual fields (Stycar balls), defensive blink and fixation shift. Age-specific tests to identify problems in perceptual, visuo-motor and spatio-cognitive domains will be included.

Neurophysiological measures

Phase and orientation reversal visual event related potentials (PR- and OR-VERP) During VERP recording infants sit in a darkened room on their caregiver's lap,

Table 2 Schedule of trial assessments

Procedure	Frequency	Details
Feed supplementation	Daily	Both groups will receive a measured feed supplement (active or placebo) to add to a milk feed daily
Dietetic review	Every 2 weeks or as required	Dietetic review will take place in person or by telephone every two weeks or as required for the duration of the trial
Anthropometry	Every 3 months	Measurements will be taken using calipers and anthropometer at baseline and then every 3 months to monitor growth.
MRI/MRS	0 and 3 months	This scan will be performed at baseline and then three months later to assess brain chemistry and choline uptake.
Visual Event Related Potential and behavioural vision testing	Baseline, term, 6 m, 12 m, 24 m	During this test the child will be positioned on the parents lap or in a chair to view a monitor where moving black and white stripes were shown. For the test 3 adhesive electrodes will be placed on the head and connected to a computer by fine cables. The child will also be observed for reactions to moving stimuli and given simple tasks to perform
Bayley Assessment	Baseline, 12 and 24 months	The child will be asked to do a number of activities to see if their thinking, language, and moving (sitting, walking) skills are similar to children his or her own age.
Vineland Assessment	12 and 24 months	During a semi-structured interview the parents will be given a questionnaire to fill in about their child's personal and social skills.
Fatty acid profile analysis	Baseline and 24 months	0.05mls of blood will be taken using a finger prick test.
Maternal fatty acid profile analysis	Baseline	0.05mls of blood will be taken using a finger prick test.

40 cm from the stimulus screen. Three electrodes are placed on the infants scalp, over the occiput, forehead, and vertex. Electrodes are connected to a low voltage pre-amplifier. Stimuli are oblique black and white stripes. For phase reversal VERPs (PR-VERP) the stripe orientation is unchanged but contrast reverses periodically. For orientation reversal VERPs (OR-VERP) stripe orientation changes between 45 and 135 degrees. Both PR- and OR-VERPs are measured at transient (2 reversals/second) and steady state (4–8 reversals/second). 100 sweeps are recorded at each reversal rate.

Neuro-imaging In the neonatal (D1) trial, brain proton magnetic resonance spectroscopy (1H-MRS) will be used to assess brain choline uptake in cases versus controls, at baseline and following 3 months of supplementation. MR studies cannot be performed beyond this age without sedation.

1H-MRS and MRI studies will be performed using a Philips 1.5 T Achieva machine, with an 8-channel SENSE adult head coil. 1H-MRS will be performed in natural sleep following a milk feed. In neonates and infants 1H-MRS will also be performed at the end of the intervention if MRI under general anaesthetic is performed for clinical reasons. Axial T1 and T2 weighted images will be obtained. T2 weighted images will be used to place two 1H-MRS slices, one at basal ganglia level, the other through the cerebral hemispheres above the lateral ventricles. 1H-MRS acquisitions will be performed using a manufacturer standard two-dimensional CSI technique. The volume of interest will be sized so that suppression bands (REST slabs) lie well within the inner table of the calvarium. After automated shimming and water suppression steps, spectra will be acquired for each of the 25 voxels of the 5 × 5 matrix within the sampled volume at each level. 1H-MRS data will be analysed using a Philips Extended Workspace with SpectroView software, which generates spectra for each voxel, performs metabolite peak fitting for choline, creatine, N-acetyl aspartate (NAA) and lactate, and calculation of corresponding metabolite ratios.

The infant (D2) trial participants will not undergo MRI/MRS as part of the protocol.

Data collection, storage and record keeping

Each participant will be allocated a unique study number at the time of randomisation and this will be used throughout the study to identify all data relating to the participant. All assessment data collected by the research nurse, clinical research fellow or dietician will be entered on paper then transferred to a data protection compliant drive in the research team's office. All data will be double entered and data entry errors corrected. Data stored on the database will be updated by the University's Information Management Services Unit every night. All patient identifiable information will be kept on encrypted computers in a secure office, with access limited to authorised members of the research team. In accordance with the requirements of the University of Oxford, all data will be archived in secure archive facilities within the University until three years after the last participant reaches the age of eighteen. After this time all documentation will be destroyed and all data deleted securely. The final dataset will initially be available only to the study team. After the results of the trials have been reported, anonymous trial data will be made publicly available.

Sample size calculation

No previous studies have supplemented children with this combination of micronutrients. Power calculations were performed for primary outcome measure BSID-III score, assuming power of 80%, 5% significance level, use of two-sided statistical tests throughout and equal allocation to each arm. Recruitment of 60 participants to each trial (30 to each arm within each trial), assuming 20% loss to follow-up, provided 80% power to detect a 12.5 point difference in BSID-III score assuming standard deviation (SD) of 15 points. Power calculations were also performed for secondary outcome VERP latency. 30 infants per group provided 90% power to detect a latency difference of 25 ms, assuming SD of 25 ms and significance level of 5%.

Statistical analysis

Data analysis will be undertaken by the study statistician at CSM using appropriate statistical software.

Continuous variables at baseline will be presented using means and standard deviations (unless not normally distributed, in which case medians and percentiles will be used).

Categorical/binary variables at baseline will be presented using proportions and percentages.

The primary outcome (change in Bayley score from baseline) will be analysed at 12 and 24 months using mixed effects linear regression to account for the repeated measures over time. Baseline Bayley score will be entered as a covariate in the model. The mixed effects model will include Bayley score at 12 and 24 months as the response variable, time point (12 or 24 months), treatment group and baseline Bayley score as fixed effects and a patient specific random intercept. An interaction between time and treatment group will be fitted as a fixed effect to allow estimation of treatment effect at both time points. The minimisation (design) factors will also be included as fixed effects. The primary outcome is Bayley score at 24 months. Mean difference in cognitive score between the 2 groups at 24 months will be presented along with 95% CI and associated 2 sided p-value.

If the Bayley score has severe departure from normality, thus invalidating the linear regression model, the first approach will be transformation. If the data cannot be transformed to normality, a Mann–Whitney test will be adopted. The difference in median change from baseline will be presented alongside the 95% CI for the difference in medians. There will be no adjustment for covariates if the data cannot be transformed to allow parametric analyses. Primary analysis will be conducted on the Intention To Treat population.

All analyses of continuous outcomes will follow the same procedure as the primary outcome analysis. Analysis of proportions will use binomial regression. Odds ratios will be presented.

If missing data are substantial, multiple imputation will be used to assess the impact of missing data in a sensitivity analysis.

Withdrawal of participants

Parents will have the right to withdraw their child from the study at any time, for any reason, and without giving a reason. The Chief Investigator will also have the right to withdraw participants from the study in the event of adverse events, serious adverse events, suspected unexpected serious adverse reactions or protocol violations. Where parents withdraw before randomisation, completion of baseline assessments and without starting supplementation, they will be replaced. If a parent decides to withdraw their child from the study every effort will be made to report the reason for withdrawal as thoroughly as possible. Parents will be informed, prior to consenting into the study, that should they subsequently withdraw all data collected prior to their withdrawal will be maintained and used for the purposes of the study.

Management of the study, quality control and assurance

The study will be managed through the University of Oxford's Clinical Trials Research Group and the research team of the Paediatric Gastroenterology and Nutrition Group in the Department of Paediatrics. Quality control will be maintained through adherence to the study protocols, standard operating procedures, research governance and clinical trial regulations.

An independent Data Monitoring and Ethics Committee, consisting of a senior medical statistician and two independent paediatric clinicians, will meet part-way through the study to provide independent review. It's purpose will be to safeguard the interests of trial participants, assess the safety and efficacy of the interventions, and monitor the overall conduct of the clinical study. The committee will be chaired by one of the independent clinicians and will have access to unblinded study data.

Plans to communicate study results

No publications containing results from this study have been published or submitted to any journal. Results will be submitted to peer-reviewed journals for publication when all statistical analyses have been completed and the study unblinded. This should be in the summer of 2015.

The research team will contact all the participants when the results of the study are available. Results will be sent to all participants who expressed interest in receiving them.

Ethics committee and regulatory approval

The study will be conducted in accordance with ethical principles as listed in the Declaration of Helsinki. Ethics approval was granted by Oxfordshire REC B for both trials. Approval for the neonatal trial was granted on 08 May 2008, number 08/H0605/70. Approval for the infant trial 12 January 2009, number 08/H0605/155. A total of 6 substantial amendments were submitted to the REC and approved as follows:

1st amendment - 19 February 2009
2nd amendment - 21 July 2009
3rd amendment - 7 January 2010
4th amendment - 13 May 2010
5th amendment - 10 August 2010
6thamendment - 13 November 2012.

Discussion

This novel project will be the first to provide all three phosphatide precursors (DHA, choline and UMP) to infants at risk of neurodisability. There are very few interventions that have been shown to improve the neurodevelopmental outcome of babies and infants with PBI. To date therapeutic hypothermia for term hypoxic encephalopathy is the only therapy which improves mortality and morbidity outcomes [46]. There is a clear need to identify other treatments capable of reducing morbidity in infants who sustain perinatal brain injury. If successful, dietary supplementation with this combination of phosphatide precursors will ameliorate the level of disability experienced by these children and their families.

This study has several advantages over previous studies examining the effects of DHA supplementation on infant neurodevelopment. Firstly, it provides DHA at 1% total fatty acids, mimicking maximal levels found during third trimester transplacental fatty acid transfer [47] and human breast milk [48]. With the exception of the DINO trial [35], previous fatty acid supplementation studies have given DHA at much lower doses, and have not provided the additional substrates required for normal neural development. However, the combination of DHA with choline and UMP has been shown to have positive synergistic effects on brain phosphatide levels, synaptic elements and dendritic spine density compared to when these nutrients are given alone.

This study will be the first to provide high dose DHA in combination with choline and UMP. Furthermore, in contrast with other studies, most of which had a short intervention period, this study will supplement infants for 2 years, throughout the period of maximal brain growth and synaptogenesis. This will be a longer period of supplementation than any previous DHA intervention trial, thus minimising the likelihood of type 2 errors resulting from an insufficient supplementation period. A further study strength will be the range of outcome measures being employed, utilising behavioural, neurophysiological and neuroimaging measures.

If this study demonstrates a neurodevelopmental advantage to supplementation with phosphatide precursors, the supplement could be provided as part of routine clinical care for babies with PBI, or the supplement's effectiveness could be tested in a larger multi-centre trial.

Study status

The study is in progress and will continue until the last infant recruited completes their 2 year participation. This is anticipated to be in March 2015. A period of statistical analysis will follow and it is anticipated that this will be completed in the summer of 2015.

Abbreviations

PBI: Perinatal brain injury; CP: Cerebral palsy; PUFA: Polyunsaturated fatty acid; DHA: Docosahexaenoic acid; UMP: Uridine-5'-monphosphate; LCPUFA: Long chain polyunsaturated fatty acid; BSID-III: Bayley scales of infant development III; RCT: Randomised control trial; PR-VERP: Pattern reversal visual event related potential; GMH: Germinal matrix haemorrhage; IVH: Intra ventricular haemorrhage; MRI: Magnetic resonance imaging; PLIC: Posterior limb of the internal capsule; CSM: Centre for statistics in medicine; GMP: Good manufacturing practice; VABS-II: Vineland adaptive behaviour scales II; ABCDEFV: Atkinson battery of child development for examining functional vision; OKN: Optokinetic nystagmus; VERP: Visual event related potential; OR-VERP: Orientation reversal visual event related potential; MRI: Magnetic resonance imaging; 1H-MRS: magnetic resonance spectroscopy; NAA: N-acetyl aspartate; SD: Standard deviation; cUSS: Cranial ultrasound scan; VI: Ventricular index; TEA: Term equivalent age; CGA: Corrected gestational age; PVL: Periventricular leucomalacia; PVHI: Periventricular haemorrhagic infarction; BG: Basal ganglia; WM: White matter; GM: Grey matter; BGT: Basal ganglia- Thalamus.

Competing interests

No potential conflicts of interest have been identified for any of the investigating team. PBS has received lecture fees and consultancy fees from Nestec Ltd, and Danone Ltd.

Authors' contributions

PBS and JRP are co-Principal investigators, developed the trial protocol and obtained funding. OB helped develop, and provided expertise in, the elements of trial design relating to vision assessment. NW provided statistical expertise throughout the trial design and will perform the statistical analyses. KL will perform the growth measurements, dietetic review and provide dietetic advice to the cohort. MA helped refine the trial protocol. MA and CMJ will perform the neurodevelopmental assessments, Visual Evoked Potential and Behavioural Vision assessments. GQ will perform the MRI/MRS assessments and report on their findings. This manuscript was written by MA and CMJ, with PBS and JRP.
All authors have read and approved the final manuscript.

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The University of Oxford, Centre for Statistics in Medicine provided advice on the statistical tests to be carried out on the trial data and will undertake the statistical analysis. The randomisation of participants into each trial and the unblinding of the trials at the end of the Study will conducted by the Centre. BB will be responsible for all data management relating to the trial and assist MA and CMJ with the trial assessments. Professor Wilkinson, former Director of Neonatal Medicine, John Radcliffe Hospital, facilitated the collaboration with the Neonatal Unit. Gerardine Quaghebeur helped develop, and provided expertise in, the elements of the trial design relating to MRI/MRS assessment and reporting. Janette Atkinson, Emeritus professor of Psychology, UCL, designed the Atkinson Battery of Child Development for Examining Functional Vision (ABCDEFV) which will be used as part of the assessment of visual function, and has provided expertise in the trial design relating to visual assessment. Bridget Lambert (former dietician) and Angharad Vernon-Roberts (former research nurse) assisted in the early stages of the trial's development.

Funding

Funding for the study is from The Castang Foundation and SPARKS. Both funders completed independent, external review of the study. Richard Stevenson, Professor of Paediatrics at the University Of Virginia School Of Medicine, provided the following critique:
'The scientific basis of the protocol appears sound and is logical. The idea of the proposed intervention is and the rationale behind its use is excellent. Overall, this is a very important line of research that needs to be pursued.'
These funders have had no part in the study design, and will have no part in the collection, management, analysis and interpretation of the data, or decisions to submit report(s) for publication.

Trial sponsor

The sponsor for this study is The University of Oxford. The sponsor had no part in study design, and will have no part in the collection, management, analysis and interpretation of data or decisions to submit report(s) for publication. Trust management Approval and Indemnity was granted by Oxford University Hospitals NHS Trust.

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20.3 Appendix 3: Dolphin Trial consent forms and parent information sheets

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2nd August 2010
Version 6

Parent/Guardian Information Sheet

Dear Parent/Guardian,

NRES study number: Oxford Research Ethics Committee B 08/H0605/70
Study Title: Optimising nutrition to improve growth and reduce neurodisabilities in neonates at risk of neurological impairment

Invitation paragraph
You and your child are being invited to take part in our research study. Before you decide it is important for you to understand why the research is being done and what it will involve.
Please take time to read the following letter carefully. Talk to others about the study if it would help.

- Part 1 tells you why we are doing the study and what will happen to you if you take part.
- Part 2 gives you more information about the conduct of the study.

Ask us if you would like more information or if there is anything that you do not understand. Please take time to decide whether you wish your child to take part.

Part 1
What is the purpose of the research project?
The research project is looking at ways to improve growth and brain development in young children who are at risk of neurological (brain) impairment. The research means that children who take part will have extra input from a dietitian (feed specialist), and may be given a special feed supplement.

Why has my child been chosen?
Your child has been identified by their hospital doctor as being suitable to take part in this research study. This means that they have risk factors for neurological impairment, which your hospital doctor will have talked to you about. About 60 other families will be approached to take part in this study.

Does my child have to take part?
No. It is up to you to decide whether you want your child to take part. We will describe the study and go through this information sheet, which will be given to you. If you agree to take part we will ask you to sign a consent form. You are free to withdraw your child from the research at any time and without giving a reason. Your decisions about this study will not affect the standard of care your child will receive.

What will happen to my child if we agree to take part?
The study will begin as soon as your child is on full oral feeds and will continue until they are 2 years old. The research is going to be a randomized controlled trial. This means that because we do not know which way of treating patients is best, we need to make comparisons. Patients in this study will be put into two groups which will be called the 'intervention' and 'control' groups. Each group has a different treatment and the results are then compared at the end to see which one is better.
The groups are selected by chance using a computer which has no information about the individual patients. Parents will not be able to choose which group their child goes in to, and the parents and researchers will not know which group each child has been allocated to. The chance of your child getting in either group is 50% and once they have been allocated they will not be able to change groups.

Both groups will receive:

- A review once every two weeks, or as needed, with a paediatric dietitian (in person or by telephone).
- Advice about feeding and optimizing both dietary and nutrient intake.
- The 'intervention' group will receive a neurotrophic feed supplement. *Neurotrophic factors are substances that are responsible for the growth and survival of neurons (nervous system cells) during development.*
- The 'control' group will receive a placebo supplement. *A placebo is a harmless substance with no healing effect used as a control in testing new treatments- i.e. a blank sample in a test*

All children will undergo the following measurements:

What is the name of the test?	**What does it involve?**	**How often is it done?**	**How long will it last?**	**Where will it be done?**
Anthropometry	Measuring growth using a tape measure and callipers	Every 3 months	15 mins	At home
MRI/MRS scans	Once your child is asleep following a milk feed they will have a scan which takes place in the MRI scanner. This is a large machine shaped like a tunnel which uses magnets, not radiation, to look at soft tissues. If your child wakes up during the MRI/MRS, the scan will be paused. If they can not settle back to sleep the scan will be stopped and your child removed from the machine.	Baseline and 4 months	30 mins	At hospital
Visual Evoked Potential and behavioural vision testing	Your child will be positioned on your lap or in a chair to view a monitor where moving black and white stripes will be shown. For the test 5 small metal discs, about the size of the tip of the little finger, are placed on the head. The discs are connected to a computer by fine cables. The discs are held in place by a paste and are easily removed. Your child will also be observed for reactions to moving stimuli and be given some simple tasks to perform	Baseline, term and 6, 12 and 24 months post term.	45 mins	At hospital
Bayley Scale of Infant and Toddler Development	Your child will be asked to do a number of activities to see if their thinking, language, and moving (sitting, walking) skills are similar to children his or her own age	Baseline, 12 and 24 months	45 mins	At home
Vineland Adaptive Behaviour Scales	As the parent you will have a semi-structured interview and be given a questionnaire to fill in about your child's personal and social skills.	Baseline, 12 and 24 months	30 mins	At home
Blood fatty acid profile analysis(child)	We will take a very small sample of blood (0.05ml) using a finger prick test or we will collect the sample if your child is having blood tests for any other reason in the hospital.	Twice	5 mins	At hospital
Blood fatty acid profile analysis(mother)	We will take a very small sample of blood (0.05ml) from the child's mother using a finger prick test at the beginning of the study.	Once	5 mins	At hospital

The results of these tests will not be given straight to the parents but will be passed on to your child's GP and hospital doctor who will explain the result to you. A number of the Bayley assessments will be video recorded so that a second researcher can study the test to make sure that the testing is reliable. As soon as the research is finished, the video tape will be erased; none of the films will be shown publicly or used for teaching.
Once your child has finished the study the neurotrophic or placebo feed supplements will stop. Follow up with a paediatric dietitian will carry on as often as your hospital doctor thinks is needed.

Expenses and Payments
Travel expenses for any study visits will be refunded once you have given us your receipts. We will try to match up visits with other hospital appointments to ease the amount of extra travelling for you. We expect that no more than 3 extra visits to the hospital will be necessary.

What does my child have to do if we agree to take part?
If you agree to let your child take part in the study they will have the tests that were shown in the table above. Your child will also have a supplement added to their feed at least once every day for the whole of the study. This supplement will either have the neurotrophic factors in it, or it could have the placebo substance. This will be given to you by the paediatric dietitian with instructions of the amount to give and any measuring device that you may need. Their progress will be followed up every two weeks by a paediatric dietitian.

What is the supplement being tested?
The supplement being tested is made up of different neurotrophic factors. Neurotrophic factors are substances that are responsible for the growth and survival of neurons (nervous system cells) during development. The ones being included in your child's feed supplement are called choline, uridine and docosahexanoic acid (DHA). These substances all occur in nature and are found in everyday foods. All of them work in different ways in the developing brain. We are testing whether these chemicals given together improve development in children who are at risk of brain impairment. The supplement is being provided by Nutricia Research.

What are the alternatives for treatment?
If you did not want your child to take part in the study then the other option for treatment would be the usual clinical care provided by your specialist. This means that your child would not have the feed supplements and would not have the same level of dietetic follow up.

What are the possible disadvantages and risks of taking part?
The disadvantages of taking part are that there will be more assessments and tests involved both in hospital and at home than if you were not taking part in the study.

What are the side effects of any treatment received when taking part?
Your child is unlikely to experience side effects from the supplements as we are following strict international guidelines on the amounts that we can feed children. Possible effects could be an unusual taste, a mild rash or slightly looser stools than they normally have. Any side effects should resolve as your baby gets used to the supplement.

What are the possible benefits of taking part?
Your child may experience improved growth, general health and neurodevelopmental outcome if they take part in this study.

What happens when the research stops?
When the research study is finished you will be told by the research team and given the chance to contact the lead researcher for further details.

What if there is a problem?
Any complaint about how the research has been done, or any possible harm you might suffer, will be looked in to. Contact details are included in Part 2 of this information sheet.
An independent review of the data will be done at set intervals. This will show the researchers if there have been any early dramatic effects or possible harmful effects from the supplements.

Will my child's taking part in the research project be kept confidential?
Yes. All the information about your child taking part in this study will be kept confidential in agreement with the Data Protection Legislation. The details are included in Part 2.

This completes Part 1 of the Information Sheet.
If the information in Part 1 has interested you and you would like your child to take part, please continue to read the additional information in Part 2 before deciding.

Part 2

What if relevant new information becomes available?
Sometimes during the course of a research project, new information is found about the treatment/drug that is being studied. If this information could make a difference to the way we are doing the study then the researchers will check the research and decide if it is suitable to continue.

What will happen if my child or I don't want to carry on with the research?
If you wish your child to leave the study this will not make any difference to the care they will be given, but we will need to use the information collected up to that point.

What if there is a problem?
Complaints
If you wish to complain about any part of the way you have been approached or treated during the study, you can either contact the research team directly (01865 234249) or contact the University of Oxford Clinical Trials and Research Governance office on 01865 743005.

Harm
Compensation for harm caused by an accidental injury which happened because of your child taking part in the study will be covered by the University of Oxford. If you are harmed and it is caused by someone being careless then you may have reason for legal action for compensation against the University of Oxford (caused by harm from taking part in the study) or the NHS (caused by harm from having a procedure done).

Will my child's taking part in this study be kept confidential?
Yes. There will be no personal data kept for this research; no-one will be able to identify your child from any of the information we take.

- Information will be collected by the research nurse, clinician or dietitian and looked at by the research team only.
- Information will be stored securely by the researchers and will be kept nameless, no-one will be able to identify your child from any of the information we take
- The information will be used for the research study and will be stored on a Data Protection Act compliant computer in a locked room at the University Department of Paediatrics.
- The information will be kept for ten years and it will then be destroyed and deleted

Involvement of the General Practitioner/Family doctor (GP)
Your child's GP will be told of them taking part in the study, with your consent. Your child's GP will not be contacted for any extra medical details.

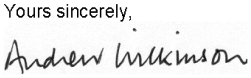
What will happen to the results of the research study?
The research team will publish the results of the study in a medical journal. No personal information will be used for the research, which means that no-one will be able to identify your child from any of the information we take. The results of the study will be made available to you if you contact the team once they have been published.

Who is organising and funding the research?
The research is being organised and carried out by Dr Peter Sullivan and Dr Jeremy Parr at the University Department of Paediatrics at the Oxford Children's Hospital. The research is being funded by the Castang Foundation, a charity that funds research into the causes of neurological impairment and other neurodevelopmental disorders.
The University of Oxford will be acting as Sponsor to the study.

Who has reviewed the study?
This study was reviewed by the Oxfordshire Research Ethics Committee and was given a favourable ethical opinion for conduct in the NHS.
You and your child will be given a copy of the signed consent form to keep.

Thank you for giving us the opportunity to explain our research. Please ask us if there is anything that is not clear or if you would like some more information. We hope that you will agree for your child to participate.

Yours sincerely,



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Version 2

CONSENT FORM

NRES Number: Oxford Research Ethics Committee B **08/H0605/70**

Optimising nutrition to improve growth and reduce Neurodisabilities in neonates at risk of neurological impairment

Please initial in box

I have read the information sheet [version . dated ……] …… ☐

I have had the opportunity to ask questions and discuss the study …… ☐

I have received satisfactory answers to all of my questions …… ☐

I have received enough information about this study …… ☐

I understand that I am free to withdraw my consent for the study:
- at any time
- without having to give a reason, and
- without affecting my/my child's future medical care or legal rights …… ☐

I understand that my child's GP will be informed of my participation in this study …. ☐

I understand that sections of any of my child's medical notes may be looked at by responsible individuals from the Oxford University, Oxford Radcliffe Trust or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my child's records …… ☐

I understand that a number of the Bayley assessments will be video recorded and that as soon as the research is finished, the video tape will be erased; none of the films will be shown publicly or used for teaching …… ☐

Legally authorised representative:

Name of patient……

Parent / guardian signature ……

Name of parent/guardian (block letters) ……

Name of researcher taking consent ……

Researcher signature ……

Date of consent ……

20.4 Appendix 4: Nutritional composition of Dolphin supplement

Table 1: Nutritional composition of the active ingredients in the study supplements (in 2g powder).

Component	Amount per 2g powder*	
	Active	Control
Docosahexaenoic acid	37.8 mg	0.4 mg
Eicosapentaenoic acid	7.8 mg	0.08 mg
Arachidonic acid	4.4 mg	0.02 mg
Uridine monophosphate	1.8 mg	-
Cytidine monophosphate	1.8 mg	-
Choline	10.5 mg	1.38 mg
Vitamin B12	0.12 mcg	0.02mcg
Zinc	0.76 mg	0.06mg
Iodine	15 mcg	0.6 mcg

20.5 Appendix 5: Participant raw blood fatty acid levels

	Docosahexaenoic acid (relative %)			Eicosapentaenoic acid (relative %)			Arachidonic acid (relative %)		
Participant	Baseline	24 months	Maternal	Baseline	24 months	Maternal	Baseline	24 months	Maternal
1	0.0212	0.0269	0.0134	0.002	0.0049	0.0023	0.0437	0.0693	0.0449
2	0.0258	0.0141	0.0254	0.004	0.0037	0.0054	0.0912	0.0761	0.0624
3	0.0181	0.0293	0.0179	0.0035	0.0106	0.0152	0.056	0.0566	0.0373
4	0.0287		0.0153	0.0039		0.0048	0.0586		0.0494
5	0.0142	0.0081	0.0211	0.0019	0.0012	0.0198	0.076	0.0426	0.0352
6	0.0127	0.0128	0.0068	0.0025	0.0042	0.0036	0.0998	0.0695	0.0872
7	0.0085	0.035	0.0119	0.0016	0.0095	0.0025	0.0445	0.042	0.0543
8	0.0096	0.0172	0.0079	8.00E-04	0.0058	0.0032	0.0585	0.0329	0.064
9	0.0094	0.0114	0.0096	0.0034	0.0029	0.0063	0.0389	0.0644	0.0582
10	0.0184	0.0074	0.0142	0.003	0.0025	0.0049	0.0744	0.0473	0.0716
11	0.0242		0.0261	0.0068		0.0181	0.0963		0.0904
12	0.0154	0.0249	0.0183	0.0029	0.0067	0.005	0.0709	0.082	0.0644
13	0.0192	0.0072	0.0119	0.0041	0.0027	0.0053	0.0596	0.0399	0.066
14	0.0153	0.0049	0.0133	0.005	0.0014	0.0071	0.0877	0.0698	0.1054
15	0.0178		0.0121	0.0011		0.0041	0.0588		0.0946
16	0.0166		0.0187	0.0024		0.0035	0.0412		0.0495
17	0.0213	0.0477	0.0176	0.0045	0.0097	0.0052	0.117	0.0676	0.0818
18									
19	0.0179		0.0201	0.0078		0.0078	0.0459		0.0482
20	0.0177	0.0367	0.0116	0.0027	0.0096	0.0048	0.0726	0.0747	0.0671
21	0.025	0.0309	0.0182	0.0026	0.0103	0.0108	0.0946	0.0646	0.1014

	Docosahexaenoic acid (relative %)			Eicosapentaenoic acid (relative %)			Arachidonic acid (relative %)		
Participant	Baseline	24 months	Maternal	Baseline	24 months	Maternal	Baseline	24 months	Maternal
22	0.0304	0.0139	0.0167	0.0088	0.0045	0.0155	0.1234	0.0754	0.0977
23	0.0206		0.0139	0.0023		0.004	0.0622		0.0824
24	0.0169	0.0092	0.0097	0.0029	0.0038	0.0025	0.0705	0.1174	0.0481
25	0.0171	0.0638	0.0183	0.0032	0.0211	0.0048	0.0592	0.1154	0.0754
26	0.0105	0.0569	0.0069	0.0012	0.0212	0.002	0.0884	0.11	0.0387
27	0.0213	0.0385	0.0185	0.0011	0.012	0.0036	0.0949	0.1583	0.0734
28	0.0121		0.0112	0.002		0.003	0.042		0.0255
29	0.0156	0.0186	0.0102	0.0036	0.0095	0.0043	0.0643	0.1157	0.0481
30	0.0134	0.0081	0.0121	0.0058	0.0044	0.0031	0.0894	0.0515	0.0595
31	0.0163	0.0688	0.0181	0.0023	0.0158	0.0049	0.0731	0.0685	0.0675
32	0.0353	0.0133	0.0194	0.0076	0.0062	0.0057	0.178	0.0791	0.0862
33	0.0187		0.0144	0.0088		0.0115	0.0426		0.0497
34	0.0131	0.011	0.0054	0.0019	0.0061	0.0025	0.0631	0.0867	0.0562
35	0.0164	0.0168	0.011	0.0016	0.0036	0.0023	0.062	0.0932	0.0605
36	0.0111		0.0126	0.0014		0.0075	0.0809		0.0903
37	0.0197	0.01	0.011	0.0033	0.0023	0.0041	0.0833	0.0593	0.0812
38	0.0179			0.0032			0.1007		
39	0.0176	0.0134	0.0159	0.0033	0.0029	0.0068	0.0933	0.0646	0.0652
40	0.0231	0.0192	0.0333	0.0037	0.0051	0.018	0.086	0.1118	0.0914
41	0.0191		0.006	0.0016		0.0052	0.098		0.0592
42	0.0296	0.049	0.0175	0.0042	0.0177	0.0064	0.1064	0.0617	0.0843
43	0.0175		0.0124	0.0024		0.0053	0.0834		0.078
44	0.0208	0.0305	0.0132	0.0049	0.0084	0.0041	0.1013	0.0704	0.048
45	0.0158		0.0132	0.0059		0.0049	0.0812		0.0476

	Docosahexaenoic acid (relative %)			Eicosapentaenoic acid (relative %)			Arachidonic acid (relative %)		
Participant	Baseline	24 months	Maternal	Baseline	24 months	Maternal	Baseline	24 months	Maternal
46	0.0203	0.0148	0.0145	0.0023	0.0063	0.0095	0.0902	0.1093	0.089
47	0.0105	0.0302	0.0082	0.003	0.0091	0.0073	0.0969	0.0514	0.1158
48	0.0285	0.053	0.0112	0.0075	0.0132	0.0041	0.1597	0.047	0.0805
49	0.0221	0.0072	0.0192	0.004	0.002	0.0066	0.1248	0.0383	0.1061
50	0.0164	0.0183	0.0116	0.0026	0.0065	0.0021	0.0807	0.0986	0.0631
51	0.0231	0.0279	0.021	0.0062	0.0061	0.0143	0.0769	0.0517	0.0447
52	0.0277		0.0199	0.0113		0.0103	0.1095		0.1165
53									
54	0.0253	0.0093	0.0189	0.0052	0.0021	0.0082	0.1092	0.1166	0.0492
55	0.0188	0.0247		0.0043	0.0085		0.0913	0.1772	
56	0.0182	0.0071	0.0099	0.0042	0.0024	0.0034	0.1006	0.0859	0.0972
57	0.0088	0.0092	0.0129	0.002	0.0036	0.0082	0.0378	0.049	0.1082
58	0.0136	0.0124	0.0105	0.0038	0.0029	0.0074	0.0543	0.0583	0.0617
59	0.034	0.0189	0.0214	0.0176	0.0029	0.0197	0.1193	0.0771	0.0651

20.6 Appendix 6: Raw Pattern Reversal Visual Event Related Potential data

Table 1: Raw transient pattern reversal visual event related potential latency (2 reversals/second) data at baseline, 6, 12 and 24 months

Participant Code	Visit* (months)	CGA (weeks) at time of assessment	Brain injury severity grading	Calculated latency**	PR2 transient latency**
D1-01v1	0	1	1	227.08	
D1-01v2		15.7	1	112.64	98
D1-01v3	6	27.9	1		110
D1-01v4		40.1	1	232.22	
D1-01v5	12	49.4	1	398.19	
D1-01v6	24	102.4	1	267.78	
D1-02v1	0	4.1	2	237.07	258
D1-02v2		18	2	119.028	
D1-02v3	6	25.9	2	163.88	102
D1-02v4	12	54.7	2	164.05	100
D1-02v5	24	108.9	2	221.11	104
D1-03v1	0	4.1	3	340.42	256
D1-03v2	6	29.9	3	223.75	
D1-03v3	12	55.3	3		102
D1-03v4	24	108.9	3		96
D1-04	0		1		
D1-05v1a	0	-4.1	2	273.06	305
D1-05v1b		9.3	2		
D1-05v2	6	18.4	2		119
D1-05v3		39.1	2	296.33	109
D1-05v4	12	50.7	2	208.40	
D1-05v5	24	105	2		
D1-06v1a	0	5	2	305.69	
D1-06v2		18.7	2		
D1-06v3	6	35.7	2	207.08	
D1-06v4	12	54.1	2	152.08	
D1-06v5	24	105.3	2	139.57	
D1-07v1	0	-1.9	2	236.67	
D1-07v2		13	2		
D1-07v3	6	27.3	2	362.22	
D1-07v4	12	51.3	2	197.29	124
D1-07v5	24	104.3	2	199.79	151
D1-08v1	0	0.9	1	232.5	258
D1-08v2	6	25.9	1	163.71	101
D1-08v3	12	52	1	117.99	
D1-08v4	24	104.4	1	216.94	

Participant Code	Visit* (months)	CGA (weeks) at time of assessment	Brain injury severity grading	Calculated latency**	PR2 transient latency**
D1-09v1		-4.6	1		
D1-09v2	0	3.7	1		359
D1-09v3	6	29.6	1	158.06	97
D1-09v4	12	55.3	1	359.72	
D1-09v5	24	106.1	1	189.17	
D1-10v1	0	2.7	3	231.02	224
D1-10v2	6	25.7	3		
D1-10v3	12	53.9	3	190.76	
D1-10v4	24	104.9	3	141.94	94
D1-11v1	0	2.9	3	288.61	
D1-11v2		15.7	3		
D1-11v3	6	29	3		
D1-13v1	0	2.7	3		
D1-13v2		13.4	3		
D1-13v3	6	25.7	3	167.5	
D1-13v4	12	51.9	3	145.21	
D1-13v5	24	107.1	3		
D1-14v1	0	2.1	1	207.08	
D1-14v2	6	26.9	1	352.36	
D1-14v3	12	50.3	1	197.08	
D1-14v4	24	104.7	1	199.65	
D1-15v1	0	2.3	3		
D1-15v2		13.1	3		
D1-15v3	6	27	3		
D1-15v4	12	53.6	3	256.60	172
D1-15v5	24	111.6	3	101.18	
D1-16v1	0	3	1	269.31	
D1-17v1	0	-2.1	1	325.64	
D1-17v2	6	21	1	226.94	
D1-17v3	12	48.7	1	240.69	
D1-18v1	0	-4.3	2	308	291
D1-18v2	6	22	2	150.33	101
D1-18v3	12	47.1	2		
D1-18v4	24	101.9	2		
D1-19	0		1		
D1-20v1	0	5.3	1	314.28	
D1-21v1	0	5	1	275.28	
D1-21v2	6	29.7	1	107.57	
D1-21v3	12	56	1		
D1-21v4	24	108.4	1	262.86	
D1-22v1	0	5.9	1	264.22	220
D1-22v2	6	29	1	178.72	119

Participant Code	Visit* (months)	CGA (weeks) at time of assessment	Brain injury severity grading	Calculated latency**	PR2 transient latency**
D1-22v3	12	58	1	319.03	
D1-22v4	24	109.4	1	307.08	
D1-23v1	0	1.7	3	250.07	
D1-23v2	6	24.9	3	197.5	
D1-23v3	12	52.7	3	77.78	
D1-23v4	24	108	3		
D1-24v1	0	1.9	3	223.19	
D1-24v2	6	26.7	3		
D1-24v3	12	53.9	3	233.61	
D1-25v1	0	1.6	1	282.17	317
D1-25v2	6	26.1	1		
D1-25v3	12	56.7	1	206.32	
D1-25v4	24	107.9	1	235.92	96
D1-26v1b	0	-2.1	2	233.40	
D1-26v2	6	23.6	2	246.11	
D1-26v3	12	50	2	215.97	
D1-26v4	24	103.3	2	189.65	91
D1-27v1	0	2	1	186.11	
D1-27v2	6	28.7	1	169.10	108
D1-27v3	12	54.3	1	235.56	110
D1-27v4	24	106.3	1	260.06	
D1-28v1c	0	6.6	3	222.61	191
D1-28v2	6	36.9	3	215.56	
D1-28v3	12	60.3	3	130.83	74
D1-28v4	24	108.7	3	177.64	190
D1-29 v1	0	-1.9	1	455.56	
D1-30 v1	0	5	2	248.30	201
D1-30v2	6	30.7	2	155.83	108
D1-30v3	12	57.4	2	138.42	105
D1-30v4	24	107.4	2		
D1-31v1	0	1.6	3	242.16	
D1-31v2	6	32.3	3	145.42	105
D1-31v3	12	54.4	3		89
D1-31v4	24	103.9	3	284.39	
D1-32v1	0	5.4	1	230.90	237
D1-32v2	6	28.9	1	214.17	
D1-32v3	12	57	1	259.72	91
D1-32v4	24	107.9	1		
D1-33v1a	0	3.1	2	147.90	207
D1-33v1b		3.3	2		246
D1-33v2	6	28.9	2	155.14	
D1-33v3	12	56.4	2	188.96	98

Participant Code	Visit* (months)	CGA (weeks) at time of assessment	Brain injury severity grading	Calculated latency**	PR2 transient latency**
D1-33v4	24	106.3	2	183.61	
D1-34v1	0	-1.3	3	238.86	367
D1-34v2		2.9	3		333
D1-34v3		14.7	3		104
D1-34v4	6	34.3	3	192.78	106
D1-36v1	0	5	2	284.67	264
D1-36v2	6	40.3	2		
D1-36v3	12	55.1	2		
D1-36v4	24	106.9	2	100.97	
D1-37v1	0	4	3	253.53	210
D1-37v2	6	29.6	3	300.92	
D1-37v3	12	57	3	173.54	107
D1-37v4	24	105.3	3	156.60	106
D1-38v1	0	7.4	3	266.07	261
D1-39v1	0	2.6	1		
D1-39v2		17.4	1	335.78	228
D1-39v3	6	29.4	1	217.08	98
D1-39v4	12	55.6	1	219.65	84
D1-39v5	24	105.7	1		
D1-40v1	0	2.9	1		
D1-40v2	6	29.1	1	195.14	107
D1-41v1	0	2.4	2	251.25	289
D1-41v2	6	27.6	2	155.15	94
D1-41v3	12	52.4	2		
D1-41v4	24	105.7	2	530.83	87
D1-42v1	0	6.9	2	209.10	279
D1-42v2	6	22.6	2	144.21	103
D1-42v3		35.6	2		88
D1-42v4	12	59.1	2	152.26	84
D1-42v5	24	113.3	2		
D1-43v1	0	-1.4	1		
D1-44v1	0	3.7	2	257.79	241
D1-44v2	6	30	2	184.33	103
D1-44v3	12	54.9	2	162.80	105
D1-44v4	24	107.1	2		
D1-45v1	0	6.1	1	284.72	
D1-46v1	0	-0.3	3		
D1-46v2		2.4	3	190	
D1-46v3	6	27.3	3		
D1-46v4	12	53.6	3		
D1-46v5	24	102.7	3		
D1-47	0		1		

Participant Code	Visit* (months)	CGA (weeks) at time of assessment	Brain injury severity grading	Calculated latency**	PR2 transient latency**
D1-48v1	0	4.1	2		
D1-48v2		19.7	2	139.75	103
D1-48v3	6	36.7	2	127.28	83
D1-48v4	12	55.3	2	146.67	
D1-48v5	24	105.1	2		
D1-49v1	0	2.6	1	200.04	275
D1-49v2		10.6	1	199.44	
D1-49v3	6	34.7	1		
D1-49v4	12	57.7	1		
D1-49v5	24	103.6	1	110.61	159
D1-50v1	0	8.6	3		
D1-50v2	6	25.1	3		
D1-50v3	12	50.1	3		84
D1-50v4	24	101	3	158.06	
D1-51v1	0	9.9	3		
D1-51v2		23.3	3	243.86	209
D1-51v3	6	34.3	3	247.86	
D1-51v4	12	62	3		
D1-51v5	24	113.6	3		
D1-52v1	0	4.9	3	230.71	276
D1-52v2	6	30	3		
D1-52v3	12	54.7	3		
D1-52v4	24	107.1	3		
D1-53v1	0	4.3	3	282.92	143
D1-53v2	6	29.7	3	183.47	91
D1-53v3	12	55.6	3		
D1-53v4	24	109.4	3	148.89	
D1-54v1	0	0.3	3		
D1-55	0		2		
D1-56v1	0	3.3	3		
D1-56v2		11.1	3		
D1-56v3	6	31.1	3		
D1-56v4	12	55.4	3		
D1-56v5	24	106.9	3		
D1-57v1	0	3.3	2	272.36	260
D1-57v2	6	31.1	2	158.06	
D1-57v3	12	55.4	2	131.04	
D1-57v4	24	106.9	2		110
D1-58v1	0	5.7	3		
D1-58v2		18.6	3		
D1-58v3	6	30.4	3	144.58	
D1-58v4	12	59.1	3		

Participant Code	Visit* (months)	CGA (weeks) at time of assessment	Brain injury severity grading	Calculated latency**	PR2 transient latency**
D1-58v5	24	109.3	3		
D1-59v1	0	19.7	2	221.48	
D1-59v2	6	47.7	2		
D1-59v3	12	73.9	2	257.81	103
D1-59v4	24	126.7	2		
D1-61v1	0	6.7	1	230.07	227
D1-61v2	6	32.6	1	104.80	57
D1-61v3	12	57.9	1	235.22	
D1-61v4	24	107.1	1		
D1-62v1	0	2.3	1	224.10	
D1-62v2	6	28	1	161.04	
D1-62v3	12	53.4	1	161.94	
D1-62v4	24	104.7	1	111.32	

*A small number of infants attended for additional VERP testing if their behavioural state was not conducive to testing on their planned study visit.

**Blank spaces indicate data not obtained.

Table 2: Raw calculated pattern reversal visual event related potential latency data at baseline, 6, 12 and 24 months

		F2 Phase				F2 Unwrapped phase				Slope F2	F2 latency (ms)
		Reversals/second (r/s)				Reversals/second (r/s)					
Code	CGA (weeks) at testing	2	4	6	8	2	4	6	8		
D1-01v1	1		-118.3		-92.5		-118.3		-452.5	83.55	227.08
D1-01v2	15.7	14.5		-154.9		14.5		-154.9		42.35	112.63
D1-01v3	27.9	M	M	M	81.5				81.5		
D1-01v4	40.1	M	-109.6	NS	98	-109.6			-262	25.4	65.55
D1-01v5	49.4	M	-107	-37.3	M		-107	-397.3		145.15	398.19
D1-01v6	102.4	22.9	23.5	-99.7	129.3	22.9	-336.5	-459.7	-590.7	98.2	267.77
D1-02v1	4.1	122	-39.8	160.7	-45.8	122	-39.8	-199.3	-405.8	87.14	237.06
D1-02v2	18	M	M	103.5	14.1			103.4	14.1	44.65	119.02
D1-02v3	25.9	22.8	-97.2	136.2	19.7	22.8	-97.2	-223.8	-340.3	60.79	163.87
D1-02v4	54.7	71.9	-26.1	M	71.5	71.9	-26.1		-288.5	60.85	164.04
D1-02v5	108.9		31.7	-165.6	66.1		31.7	-165.6	-293.9	81.4	221.11
D1-03v1	4.1	162	-107.1	24.6	M	162	-107.1	-335.4	M	124.35	340.41
D1-03v2	29.9	M	-13.6	161	53	M	-13.6	-199	-343	82.35	223.75
D1-03v3	55.3	M	M	M	93.4	M	M	M	93.4		
D1-03v4	108.9	NS	41	M	M						
D1-05v1a	-4	70.6	-129.6	M	M	70.6	-129.6		M	100.1	273.05

		F2 Phase				F2 Unwrapped phase				Slope F2	F2 latency (ms)
		Reversals/second (r/s)				Reversals/second (r/s)					
Code	**CGA (weeks) at testing**	**2**	**4**	**6**	**8**	**2**	**4**	**6**	**8**		
D1-05v1b	9.3	M	M	M	M	M	M	M	M		
D1-05v2	18.4	M	2.7	M	M	M	M	2.7	M		
D1-05v3	39.1	-134.7	86.2	-130	-65.8	-134.7	-273.8	-490	-785.8	108.48	296.33
D1-05v4	50.7	M	32.5	-97.8	85.2	M	32.5	-97.8	-274.8	76.82	208.40
D1-05v5	105	M	M	M	M	M	M	M	M	M	
D1-06v1a	5	M	-7.1	129.2	M	M	-7.1	-230.8	M	111.85	305.69
D1-06v2	18.7	M	M	119.7	M	M	M	119.7	M		
D1-06v3	35.7	M	M	-106	101.3	M	M	-106	-258.7	76.35	207.08
D1-06v4	54.1	M	10.5	-102.6	M	M	10.5	-102.6		56.55	152.08
D1-06v5	105.3	127.1	24.9	-78.8	174.7	127.1	24.9	-78.8	-185.3	52.04	139.56
D1-07v1	-1.9	M	-169	16.7	-159.2	M	-169.3	-343.3		87	236.66
D1-07v2	13	M	M	M	33.1						
D1-07v3	27.3	M	-140	-143.9	50.9		-140.1	-503.9	-669.1	132.2	362.22
D1-07v4	51.3	NS	153.6	-11.3	-137.7	M	153.6	-11.3	-137.7	72.82	197.29
D1-07v5	104.3	M	146.2	-5	-148.7	M	146.2	-5	-148.7	73.72	199.79
D1-08v1	0.9	M	-60.7	125.6	-42.7	M	-60.7	-234.4	-402.7	85.5	232.5
D1-08v2	25.9	49.4	-44.9		50.4	49.4	-44.9		-309.6	60.73	163.72
D1-08v3	52		107.2	66	-69.9		107.2	66	-69.9	44.27	117.98
D1-	104.4		91.2	-38.4	131.6		91.2	-38.4	-228.4	79.9	216.94

		F2 Phase				F2 Unwrapped phase				Slope F2	F2 latency (ms)
		Reversals/second (r/s)				Reversals/second (r/s)					
Code	**CGA (weeks) at testing**	**2**	**4**	**6**	**8**	**2**	**4**	**6**	**8**		
08v4											
D1-09v1	-4.6	NS	NS	NS	M						
D1-09v2	3.7	52.3	M	M	M						
D1-09v3	29.6	103.5		-131.3		103.5		-131.3		58.7	158.05
D1-09v4	55.3	M	M	-94.6	2.8			-94.6	-357.2	131.3	359.72
D1-10v1	2.7	158	M	-177.5	7.1	158		-177.5	-352.9	84.96	231.02
D1-10v2	25.7	NS	-15.8			39.9	-15.8			27.85	
D1-10v3	53.9		-1.6	-114.8	76.5		-1.6	-114.8	-283.5	70.47	190.76
D1-10v4	104.9	60.2	-14.6		109	60.2	-14.6		-251	52.9	141.94
D1-11v1	2.9	M	-66.8	81.8	M	M	-66.8	-278.2	M	105.7	288.61
D1-11v2	15.7	NS	NS	NS	NS						
D1-11v3	29	NS	NS	NS	NS						
D1-13v1	2.7	-140	NS	NS	NS	NS					
D1-13v2	13.4				0						
D1-13v3	25.7	NS	-83.7	M	27.9		-83.7		-332.1	62.1	167.5
D1-13v4	51.9	M	-129	M	14.9		-128.8		-345.1	54.07	145.20
D1-13v5	107	M	M	M	M	M	M	M	M	M	M
D1-14v1	2.1			76.5	-76.2			76.5	-76.2	76.35	207.08
D1-14v2	26.9		-174	160.5	31.5		-173.9	-559.5	-688.5	128.65	352.36

		F2 Phase				F2 Unwrapped phase				Slope F2	F2 latency (ms)
		Reversals/second (r/s)				Reversals/second (r/s)					
Code	**CGA (weeks) at testing**	**2**	**4**	**6**	**8**	**2**	**4**	**6**	**8**		
D1-14v3	50.3	NS	-19.6	-165.1			-19.6	-165.1		72.75	197.08
D1-14v4	104.7	NS	52.4	-63.9	60.1		52.4	-63.9	-299.9	88.07	199.65
D1-15v2	13.1		166.3								
D1-15v3	27										
D1-15v4	53.6	103.9		87.2		103.9		-272.8		94.17	256.59
D1-15v5	111.6	M	102.2	-48.2	-108.3		102.2	-48.2	-108.3	52.62	101.18
D1-17v1	-2.1		-111		132.9		-111		-587.1	119.03	325.63
D1-17v2	21	41.1	30.2	-170.9	56.2	41.1	-329.8	-530.9	-663.8	115.79	316.63
D1-17v3	48.7		83.2	-76.3	89.4		83.2	-76.3	-270.6	88.45	240.69
D1-18v1	-4.3	115.5	-91.5	24.8	M	115.5	-91.5	-335.2		112.68	308
D1-18v2	22	28.8	-73.8	-29.8	3.2	28.2	-73.1	-389.8	-716.8	55.92	150.33
D1-18v3	47.1	M	M	M	M	M	M	M	M	M	
D1-18v4	101.9	NS	NS	M	-30						
D1-21v1	5	M	-135	23.2	M		-135	-336.8		100.9	275.27
D1-21v2	29.7		166.3	99.6	4.2		166.3	99.6	4.2	40.52	107.56
D1-21v3	56	NS			-48.5						
D1-21v4	108.4	NS	-63	-38.8	-146.3		-63	-398.8	-506.3	110.83	262.86
D1-22v2	29	74.6		-141.7	25.7	74.6		-141.7	-334.3	66.13	178.71
D1-	59.4			-56.9	69.8			-56.9	-290.2	116.65	319.02

		F2 Phase				F2 Unwrapped phase				Slope F2	F2 latency (ms)
		Reversals/second (r/s)				Reversals/second (r/s)					
Code	**CGA (weeks) at testing**	**2**	**4**	**6**	**8**	**2**	**4**	**6**	**8**		
22v3											
D1-22v4	109.4	NS	NS	-45.3	61		-45.5	-299		126.75	307.08
D1-23v2	24.9		-53.6	152.9	14.8		-53.6	-207.1	-345.2	72.9	197.5
D1-23v3	52.7		-66.1	-125.7	NS		-66.1	-125.7		29.8	77.77
D1-23v4	108	M	M	M	M	M	M	M	M		
D1-24v2	26.7	NS	NS		NS						
D1-24v3	53.9		-86.3	101.9	NS		-86.3	-258.1		85.9	233.61
D1-25v1	1.6	67.7	-117	14.2	M	67.7	-117.2	-345.8		103.38	282.16
D1-25v2	26.1	NS	145.7	NS	M	NS	145.7				
D1-25v3	56.7	M	57.4	-69.2	113.1		57.4	-69.2	-246.9	76.07	206.31
D1-25v4	107.9	NS	-6.9	-170.2	-51.4		-6.9	-170.2	-411.4	101.13	235.91
D1-26v2	23.6	-113.3	65.9	NS	NS	-113.3	-294.1	NS	NS	90.4	246.11
D1-26v3	50		159.3	-81.2	-158.9		159.3	-81.2	-158.9	79.55	215.97
D1-26v4	103.3	NS	9.4	-163.1	31.4		9.4	-163.1	-328.5	84.47	189.65
D1-27v2	28.7	32.4	-121	M	10.7	32.4	-121.2		-349.3	62.67	169.09
D1-27v3	54.3	NS	30.6	-111.9	44.2		30.6	-111.9	-315.8	86.6	235.55
D1-27v4	106.3	-48.3	-30.8	NS	-11.8	-48.3	-390.8		-731.8	109.82	260.05
D1-28v2	36.9	NS	M	-146.2	55			-146.2	-305	79.4	215.55
D1-28v3	60.3		63.5	-49.7	-132.1		63.5	-49.7	-132.1	48.9	130.83

		F2 Phase				F2 Unwrapped phase				Slope F2	F2 latency (ms)
		Reversals/second (r/s)				Reversals/second (r/s)					
Code	**CGA (weeks) at testing**	**2**	**4**	**6**	**8**	**2**	**4**	**6**	**8**		
D1-28v4	108.7	NS	NS	-90.5	109.2			-90.5	-250.8	80.15	177.63
D1-30 v1	5	-153.3	M	-148.2	17.1	-153.3		-508.2	-702.9	91.18	248.30
D1-30v2	30.7	14.7			27.3	14.7			-332.7	57.9	155.83
D1-30v3	57.4	26.7	-22.1	-114.5	73.3	26.7	-22.1	-114.5	-286.7	51.63	138.41
D1-30v4	107.4	M	-56.2	NS	NS		-56.2				
D1-31v2	32.3	38.7	NS	-177.9	M	38.7		-177.9		54.15	145.41
D1-31v3	54.1	12.4		NS	NS	12.4					
D1-31v4	103.9	NS	60.4	-130.2	53.9		60.4	-130.2	-413.9	118.58	284.38
D1-32v2	28.9		57.9		102.3		57.9		-257.7	78.9	214.16
D1-32v3	57	NS	-85.4	NS	84		-85.4		-276	95.3	259.72
D1-32v4	107.9		-70.4	NS	NS		-70.4				
D1-33v1b	3.3	129.5			101.3	129.5			-258.7	64.7	
D1-33v2	28.9	-158.3		35.1	-144.2	-158.3			-504.2	57.65	155.13
D1-33v3	56.4	88.7	-50.3	169.4		88.7	-50.3	-190.6		69.82	188.95
D1-33v4	106.3	M	-36.9	121.3	-6.1		-36.9	-238.7	-366.1	82.3	183.61
D1-34v2	2.9	NS	NS	NS	109						
D1-34v3	14.7	NS	-40.4	NS	NS						
D1-34v4	34.3	NS	74.2	-83.8	149.4		74.2	-83.8	-210.6	71.2	192.77
D1-	5	NS	-48	82.2	-105.1	NS	-48	-277.8	-465.1	104.28	284.66

		F2 Phase				F2 Unwrapped phase				Slope F2	F2 latency (ms)
		Reversals/second (r/s)				Reversals/second (r/s)					
Code	**CGA (weeks) at testing**	**2**	**4**	**6**	**8**	**2**	**4**	**6**	**8**		
36v1											
D1-36v2	40.3	NP	-160	M	NS						
D1-36v3	55.1	M	M	NS	NS						
D1-36v4	106.9	NS	29.3	-74.6	179.1		29.3	-74.6	-180.9	52.55	100.97
D1-37v2	29.6	NS	137.5	-139.5	57		137.5	-139.5	-303	110.13	300.91
D1-37v3	57	NS	-48.9	-85.2	54		-48.9	-85.2	-306	64.27	173.54
D1-37v4	105.3	-25.2	NS	105.1	-115.8	-25.2		-254.9	-475.8	72.57	156.59
D1-39v1	2.6	NS	NS	NS	NS	NS	NS	NS	NS		
D1-39v2	17.4	NS	67.5	NS	-63.2		67.5		-423.2	122.68	335.77
D1-39v3	29.4	NS	105.4	-54.5	NS		105.4	-54.5		79.95	217.08
D1-39v4	55.6	NS	6.5	-137	-14.6		6.5	-137	-374.6	95.27	219.65
D1-39v5	105.7	M	NS	NS	-30.2				-30.2		
D1-41v1	4	99.5	-85	NS	NS	99.5	-85	NS	NS	92.25	251.25
D1-41v2	27.6	47.5	NS	-176.8	60	47.5		-176.8	-300	57.65	155.15
D1-41v3	52.9		NS	NS	NS						
D1-41v4	105.7	-70.6	85	-125.2	NS	-70.6	-275	-485.2		207.3	530.83
D1-42v3	35.6	13	NS	NS	NS						
D1-42v4	59.1	4	-114		-57.3	4	-114.1		-417.3	71.01	152.26
D1-42v5	113.3	NS	NS	NS	NS	NS	NS	NS	NS		

		F2 Phase				F2 Unwrapped phase				Slope F2	F2 latency (ms)
		Reversals/second (r/s)				Reversals/second (r/s)					
Code	**CGA (weeks) at testing**	**2**	**4**	**6**	**8**	**2**	**4**	**6**	**8**		
D1-44v3	54.9	57.4	-37		-20.4	57.4	-37		-380.4	74.80	162.79
D1-44v4	107.1	M	M	M	M	M	M	M	M		
D1-46v2	2.4	46.2	NS	NS	-15	46.2			-375	70.2	190
D1-46v3	27.3	M	89.8	NS	NS	89.8				M	
D1-46v4	53.6	NS	NS	NS	NS						
D1-46v5	103	M	M	M	M	M	M	M	M		
D1-48v3	55.3		-80.8	155.7	3.2		-80.8	-204.3	-356.8	69	146.66
D1-48v4	105.1	M	NS	-168.8	M			-168.8			
D1-49v4	57.7	M	M	M	M	M	M	M	M		
D1-49v5	103.6	91.7	-6.3	-112.7	113.7	91.7	-6.3	-112.7	-246.3	56.02	110.61
D1-51v4	62		NS	90.1	NS			90.1			
D1-51v5	113.6	NS			18.5						
D1-52v2	30	M	M	NS	-37.1						
D1-52v3	54.7	M									
D1-52v4	107.1	M									
D1-53v2	29.7	95.2	-24.1	140.5	-28	95.2	-24.1	-219.5	-388	82.25	183.47
D1-53v3	55.6			NS							
D1-53v4	109.4	M	-13.3	-176.9	67.5		-13.3	-176.9	-292.5	69.8	148.88
D1-	0.3	M	M	M	M						

		F2 Phase				F2 Unwrapped phase				Slope F2	F2 latency (ms)
		Reversals/second (r/s)				Reversals/second (r/s)					
Code	**CGA (weeks) at testing**	**2**	**4**	**6**	**8**	**2**	**4**	**6**	**8**		
54v1											
D1-56v1	3.3	NS	NS	NS	NS						
D1-56v2	11.1		NS	NS							
D1-56v3	31.1		NS	NS	NS						
D1-56v4	55.4		NS	NS	NS						
D1-56v5	106.9	M									
D1-56.5	3.3		-107		-155.3		-106.9		-515.3	102.1	238.61
D1-57v1	3.3	NS	-124	7.6	NS		-123.9	-352.4		114.25	272.36
D1-57v2	31.1		-4.2	-150.4	NS		-4.2	-150.4		73.1	158.05
D1-57v3	55.4		21.3	-101.6	127.8		21.3	-101.6	-232.2	63.37	131.04
D1-57v4	106.9	NS	M	M	M						
D1-58v1	5.7	NS	NS	NS	NS	NS	NS	NS	NS		
D1-58v2	18.6	NS	NS	NS	NS	NS	NS	NS	NS		
D1-58v3	30.4	NS	39.9	173.9	126.9		39.9	-186.1	-233.1	68.25	144.58
D1-58v4	59.1	M	NS	M	99.5						
D1-58v5	109.3	M	M	M	NS						
D1-59v1	19.6	95.1	NS	10.6	-105.3	95.1		-349.4	-465.3	95.93	221.47
D1-59v2	47.7	NS	NS	-33.3	NS	NS	NS	NS	NS		
D1-59v3	74.3	0.8	-3.5	NS	37.8	0.8	-363.5		-682.5	109.01	257.80

		F2 Phase				F2 Unwrapped phase				Slope F2	F2 latency (ms)
		Reversals/second (r/s)				Reversals/second (r/s)					
Code	**CGA (weeks) at testing**	**2**	**4**	**6**	**8**	**2**	**4**	**6**	**8**		
D1-59v4	126.3	M	M	M	M						
D1-61v1	6.7	NS	-69.1	90.6	-105.2		-69.1	-269.4	-465.2	99.02	230.06
D1-61v2	32.6	NS	NS	133.2	NS			133.2		53.92	104.79
D1-61v3	57.9	M	0	-136.1	-43.5		0	-136.1	-403.5	100.88	235.22
D1-61v4	107.1	M	M	M	M	M	M	M	M		
D1-62v1	2.3		-53		-122.8		-53		-440.5	96.87	224.09
D1-62v2	28	NS	-92.4	155.3	-29.1		-92.4	-204.7	-389.1	74.17	161.04
D1-62v3	72.4	M	-58.3	-178.4	3.7		-58.3	-178.4	-356.3	74.5	161.94
D1-62v4	104.7	NS	-92.9	NS	42		-92.9		-318	56.27	111.31

M=missing data, not recorded
NS=Not statistically significant

20.7 Appendix 7: Raw Fixation Shift data at 6, 12 and 24 months

Table 1: Key for Fixation Shift data:

Fixation shift	Definition	Coding
nL+	under **n**on-competition, **L**eft shift – passed **+**	1
nL-	under **n**on-competition, **L**eft shift – failed **-**	2
nR+	under **n**on-competition, **R**ight shift – passed **+**	3
nR-	under **n**on-competition, **R**ight shift – failed **-**	4
cL+	under **c**ompetition, **L**eft shift – passed **+**	5
cL-	under **c**ompetition, **L**eft shift– failed **-**	6
cR+	under **c**ompetition, **R**ight shift – passed **+**	7
cR-	under **c**ompetition, **R**ight shift – failed **-**	8
	missing data (not tested)	m

Table 2: Raw 6 month Fixation Shift latency data

Code	Corrected age (weeks)	No. of trials delivered	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)
D1-01 v3	27.9	20	3	2.16	7	3.01	6	2.61	5	10.37	1	5.36	1	1.30	7	4.08	1	1.12	6	5.26	3	1.28	1	1.22	3	2.68	7	1.42	5	1.08	7	1.13	3	0.93	7	0.67	2	5.74	6	2.31	3	1.78
D1-02 v3	25.9	20	1	1.12	1	0.97	5	1.61	6	1.03	7	1.27	5	0.83	3	1.22	1	1.42	3	1.47	7	1.38	5	1.00	7	1.22	3	1.07	3	0.92	3	1.20	2	2.80	6	2.21	2	3.46	7	1.18	8	1.80
D1-03 v2	29.9	20	5	1.43	1	1.32	7	1.43	6	2.18	5	1.71	3	1.56	1	1.12	2	7.68	7	2.35	3	2.53	7	1.17	8	5.41	5	1.75	1	2.33	3	1.12	3	1.05	3	1.32	5	2.08	8	5.04	1	1.58
D1-05 v2	18.4	20	5	5.49	3	1.43	4	1.63	2	0.93	3	1.56	7	2.05	1	2.28	2	2.85	5	5.49	7	1.90	8	8.74	5	4.38	3	1.52	5	5.13	3	1.73	5	7.41	1	2.10	1	4.18	8	4.43	8	2.71
D1-06 v3	35.7	20	7	1.53	1	0.92	1	1.71	3	1.08	6	2.53	6	1.73	1	1.55	3	1.33	5	1.00	2	1.15	7	1.47	8	2.53	7	1.35	5	2.78	5	1.17	8	0.85	3	1.07	1	0.95	3	1.35	3	1.33
D1-07 v3	27.3	20	3	1.91	1	1.37	1	1.30	1	2.25	5	6.86	3	1.22	1	1.81	3	1.17	5	4.06	7	3.20	3	1.60	7	5.91	7	2.86	7	1.25	7	1.78	5	1.90	1	1.68	6	3.35	5	3.20	3	1.63
D1-08 v2	25.9		m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
D1-09 v2	29.6	20	1	1.28	5	1.18	1	1.91	8	3.60	8	7.86	3	1.32	3	2.11	8	3.20	1	1.68	7	3.00	6	2.18	1	1.90	3	1.33	3	4.58	5	4.13	3	1.38	7	2.18	5	6.81	5	0.52	1	0.33
D1-10 v2	25.7		m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
D1-11 v3	29.0		m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
D1-13 v3	25.7	20	3	1.02	3	1.68	2	1.95	5	5.89	5	2.28	5	6.43	1	1.25	5	7.41	7	1.78	3	1.37	2	1.20	8	1.81	8	2.35	1	1.43	4	1.38	3	1.25	7	2.05	8	1.30	1	0.87	6	5.39
D1-14 v2	26.9	20	7	1.96	5	1.85	3	1.55	1	1.28	5	1.83	1	1.37	1	1.12	1	1.60	7	2.28	3	0.95	5	1.50	3	1.42	7	2.68	8	1.98	5	2.60	3	1.88	8	2.35	4	1.96	6	6.78	1	1.38
D1-15 v3	27.0	9	2	7.13	2	4.50	1	4.48	3	1.42	4	3.91	3	3.78	2	2.68	3	4.86	1	2.85																						
D1-17 v2	21.0	20	5	6.44	1	1.15	7	1.28	8	1.50	3	1.20	7	1.30	3	0.82	1	0.72	1	1.25	2	1.10	5	15.58	5	3.05	3	1.15	6	2.35	1	0.98	7	0.93	7	1.13	7	1.10	3	1.03	3	0.83
D1-18 v2	22.0	20	3	1.15	1	1.25	6	5.14	1	2.10	3	1.08	1	1.07	7	3.36	4	1.16	5	4.56	7	0.93	7	2.05	5	4.11	3	1.23	7	4.89	5	1.90	3	0.95	5	2.93	7	0.95	3	1.25	1	1.05
D1-21 v2	29.7	20	4	1.20	1	1.15	8	4.03	7	1.27	3	1.25	4	1.90	1	1.23	1	1.53	7	1.03	5	2.51	8	1.12	3	1.53	5	1.73	5	1.42	1	1.68	7	3.73	1	1.08	7	5.36	5	2.26	3	2.25
D1-22 v2	29.0	20	1	1.37	5	1.60	7	4.73	3	1.15	1	1.22	7	0.88	7	4.28	1	1.90	3	0.98	5	1.40	1	1.35	5	1.32	7	1.05	1	1.12	6	3.60	3	1.35	3	1.33	6	5.36	7	1.53	3	0.95
D1-23 v2	24.9	11	1	1.25	5	2.43	2	2.88	7	2.71	1	2.00	3	1.13	6	1.96	7	1.61	5	0.95	3	3.48	6	1.23																		
D1-24 v2	26.7		m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
D1-25 v2	26.1	20	3	1.53	2	1.07	8	1.22	4	1.10	7	0.78	3	0.92	1	0.88	5	3.28	6	5.81	7	1.35	1	0.92	1	1.02	7	0.65	7	1.76	3	1.12	6	2.08	3	1.15	6	2.36	5	2.06	1	0.98
D1-26 v2	23.6	20	3	1.37	7	2.48	1	1.02	3	1.17	3	1.88	3	1.53	5	0.87	6	2.76	7	1.20	8	3.20	1	1.83	7	1.05	5	1.28	5	1.43	2	3.93	5	4.89	3	0.83	7	1.40	2	0.93	1	0.82
D1-27 v2	28.7	20	3	1.37	1	0.85	1	0.93	7	0.98	3	1.13	7	1.37	1	0.93	7	1.15	7	0.85	5	1.03	7	1.12	5	0.75	5	1.22	1	0.75	3	0.88	5	1.05	3	1.07	5	0.88	3	0.75	1	0.95
D1-28 v2	36.9	20	5	10.14	7	1.47	1	1.17	5	1.33	3	1.86	3	1.30	3	3.03	5	1.15	7	3.13	3	1.47	1	0.93	6	5.63	3	1.13	7	2.75	1	0.98	6	6.33	1	0.97	7	4.31	7	4.46	1	1.22
D1-30 v2	30.7	18	7	1.07	3	1.28	1	0.77	1	0.93	4	2.36	7	1.85	3	1.15	3	1.95	5	1.27	8	1.86	7	0.73	5	1.15	8	2.31	6	1.75	6	2.16	1	1.52	5	1.08	2	1.43				
D1-31 v2	32.3		m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
D1-32 v2	28.9	9	5	1.76	1	1.18	3	1.10	1	1.00	1	5.53	7	3.35	7	1.28	7	1.81	7	2.65																						
D1-33 v2	28.9	20	7	1.18	1	1.33	5	2.20	3	2.80	8	1.71	1	1.53	3	0.95	3	3.96	1	0.85	7	0.92	1	0.83	5	1.66	6	5.23	6	3.76	7	4.13	6	2.16	1	0.98	8	2.13	3	2.16	3	1.23
D1-34 v3	34.3	20	6	4.33	7	6.43	2	1.83	6	2.45	3	1.20	7	13.00	8	6.33	6	3.60	7	8.92	6	7.26	3	1.53	3	1.02	5	3.03	2	1.70	7	4.41	3	1.43	3	1.12	2	1.96	1	3.06	2	1.32
D1-36 v2	40.3	20	7	3.05	6	6.61	7	0.62	6	2.10	3	0.95	1	1.20	1	1.83	5	2.60	1	1.23	7	2.95	1	3.11	7	2.53	3	1.96	3	2.13	3	1.66	6	1.53	1	1.10	3	1.52	6	1.93	7	0.48
D1-37 v2	29.6	20	8	1.20	3	1.02	1	1.00	6	5.66	8	1.03	7	1.13	3	0.77	5	0.98	2	2.60	8	2.35	5	4.06	7	1.03	5	3.31	3	0.85	1	1.85	3	2.06	1	1.27	3	1.55	1	1.40	6	1.33
D1-39 v3	29.4	20	5	1.65	3	4.15	1	1.03	7	2.80	1	1.10	7	4.76	6	2.86	1	1.13	7	1.63	3	1.55	3	1.38	6	7.71	6	6.68	5	2.98	6	1.53	1	1.10	1	1.33	3	1.43	3	1.71	7	14.23
D1-40 v2	29.1	20	5	2.50	7	1.50	3	1.50	7	2.76	4	1.23	6	4.51	5	1.50	7	1.88	7	5.33	1	1.17	3	1.27	2	1.83	5	2.05	8	3.61	2	1.32	3	1.15	5	1.38	1	1.43	3	1.05	1	1.38
D1-41 v2	27.6	20	3	1.17	7	5.36	5	1.63	8	2.76	8	1.76	1	1.18	4	1.28	3	1.13	1	0.95	3	0.98	8	4.48	1	1.17	1	1.07	3	1.33	5	1.17	5	1.35	5	1.02	8	4.50	1	1.05	5	1.28
D1-42 v2	22.6	10	3	1.47	3	1.75	1	1.42	3	1.90	1	1.22	1	2.01	1	1.17	1	2.15	3	1.88	3	2.01																				
D1-44 v2	30.0	20	3	0.98	7	1.45	1	1.58	6	6.64	3	1.56	3	1.03	7	1.50	6	1.18	7	6.14	6	1.75	1	3.16	3	1.96	8	3.36	1	3.01	1	1.66	6	1.58	5	1.80	1	1.53	3	0.90	8	3.56
D1-46 v2	27.3		m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
D1-48 v2	36.7		m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
D1-49 v3	34.7	20	3	1.17	1	1.53	3	0.82	5	2.75	7	1.25	3	1.12	3	1.60	1	1.23	1	1.10	5	3.50	7	1.20	7	2.03	7	4.83	3	1.15	5	1.18	5	3.78	2	1.96	7	1.35	1	1.68	5	0.98
D1-50 v2	25.1	20	8	1.50	7	1.53	3	1.48	1	1.81	8	4.69	1	1.28	5	1.70	5	1.32	3	1.86	4	1.10	1	4.68	1	1.08	2	1.38	3	3.21	6	1.65	5	2.20	5	1.17	6	5.41	3	1.25	5	4.65
D1-51 v2	35.6	20	1	1.25	3	1.15	3	1.10	3	1.12	7	0.80	3	1.25	7	4.21	5	1.40	7	1.73	6	1.03	8	2.10	1	0.98	5	0.92	5	0.95	1	1.02	1	1.03	6	1.93	7	1.53	3	1.15	1	1.17
D1-52 v2	30.0	20	1	1.33	8	11.22	8	2.15	7	1.18	3	6.96	2	1.27	7	1.96	6	1.27	6	2.45	3	1.65	3	1.42	3	1.35	5	3.23	3	1.65	5	2.51	6	5.61	2	0.87	3	1.61	6	1.90	8	1.86

Code	Corrected age (weeks)	No. of trials delivered	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)
D1-53 v2	29.7	17	5	1.48	8	1.52	1	1.23	5	1.18	5	1.25	4	1.37	7	2.08	7	2.00	3	0.90	3	1.83	1	1.33	1	1.17	7	2.21	1	1.35	3	1.38	5	1.13	5	2.20						
D1-56 v3	31.1	10	1	2.13	3	4.18	2	3.56	1	4.98	4	8.71	2	2.06	1	1.18	4	2.38	4	4.08	3	4.01																		
D1-57 v2	31.1	20	7	2.08	1	0.92	7	1.71	5	1.38	3	1.37	7	0.98	5	0.93	6	1.75	5	1.18	3	1.15	7	1.08	3	1.12	1	0.93	7	1.38	3	1.25	3	0.87	1	0.83	5	1.27	1	0.88	1	0.92
D1-58 v3	30.4		m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
D1-59 v2	47.7	20	5	3.46	1	1.12	3	0.90	5	4.66	5	3.30	1	2.13	7	1.30	2	1.55	1	1.96	3	0.95	3	1.03	5	4.36	7	1.91	3	1.91	6	2.38	7	2.73	7	8.57	3	1.13	1	4.33	7	1.61
D1-61 v2	32.6	20	1	0.95	3	0.78	7	1.23	5	1.27	1	0.88	3	0.83	5	0.83	3	0.90	1	0.87	8	0.70	7	0.78	5	0.80	4	1.80	1	0.97	3	0.67	5	0.97	1	1.05	7	1.43	5	1.48	7	0.83
D1-62 v2	28.0	17	8	1.95	1	1.20	1	1.12	4	1.43	5	1.78	7	1.98	8	0.73	4	0.82	1	1.17	7	1.27	4	0.87	4	3.11	5	2.15	1	1.45	1	1.63	5	3.65	4	1.20						

Table 3: Raw 12 month Fixation Shift latency data

Code	Corrected age (weeks)	No. of trials delivered	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)
D1-01v5	49.4		m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
D1-02v4	54.7	20	1	1.17	3	1.53	1	1.22	3	1.18	5	3.26	1	1.42	3	1.96	7	1.60	5	2.15	7	3.46	3	4.18	5	2.58
D1-03v3	55.3	20	7	1.52	1	1.17	1	1.22	3	1.13	5	2.21	3	1.28	5	3.81	1	1.17	1	1.17	7	1.28	7	3.23	5	4.43
D1-05v4	50.7	20	1	1.32	3	1.18	7	2.30	6	1.45	7	1.52	1	2.86	3	5.74	8	5.33	3	2.11	5	12.37	1	1.27	3	1.70
D1-06v4	54.1	20	3	1.38	3	1.12	3	1.17	3	1.47	1	1.17	6	1.32	1	1.05	7	1.32	8	2.17	1	1.05	5	2.87	6	3.00
D1-07v4	51.3	12	5	1.33	1	5.23	1	0.98	6	1.43	1	0.93	7	1.00	4	4.36	3	0.82	1	1.35	3	0.95	7	0.87	7	1.00
D1-08v3	52.0	24	1	1.20	5	1.68	3	4.35	7	1.56	1	4.56	5	1.27	3	1.18	7	1.35	2	1.63	5	1.88	3	10.29	7	4.33
D1-09v3	55.3	20	5	1.32	3	0.87	5	1.53	3	0.87	1	0.80	8	1.60	1	1.20	1	1.66	7	1.53	5	1.28	4	1.61	1	1.05
D1-10v3	53.9	8	1	1.40	3	0.95	1	0.88	3	1.32	1	2.43	7	1.13	6	1.10	1	0.95								
D1-13v4	51.9	20	5	2.68	3	2.56	1	2.31	1	2.58	1	2.68	7	2.16	7	3.93	3	8.26	1	1.27	7	1.30	5	1.55	5	1.48
D1-14v3	50.3	20	3	1.18	4	0.95	3	1.03	8	1.85	5	1.75	1	1.48	3	0.95	7	2.16	1	0.97	5	1.18	1	0.87	1	0.95
D1-15v3	53.6	8	4	1.52	7	4.51	2	5.37	7	1.65	7	2.90	5	3.06	6	10.97	4	6.11								
D1-17v3	48.7	20	7	2.15	3	1.12	8	2.40	7	1.27	1	1.63	5	1.08	8	1.03	1	1.76	1	0.92	1	1.22	1	3.76	3	3.93
D1-18v3	47.1	20	7	1.32	7	1.33	1	0.88	2	1.33	3	1.12	5	1.76	5	1.90	3	1.32	3	1.83	1	0.90	1	0.87	6	2.33
D1-21v3	56.0	20	5	12.80	1	4.30	8	4.61	6	10.34	6	3.98	3	1.38	8	5.98	4	4.36	8	7.69	1	4.73	1	2.61	4	1.73
D1-22v3	58.0	20	7	2.55	3	3.31	8	1.78	4	3.81	1	1.22	5	1.22	1	1.38	1	2.15	5	6.13	5	2.05	5	2.35	7	1.71
D1-23v3	52.7	20	7	1.37	5	2.06	7	1.13	7	1.50	1	1.03	1	1.28	3	0.87	7	1.45	3	0.92	3	1.17	5	1.02	1	1.32
D1-24v3	53.9	16	6	3.98	7	4.69	6	2.41	3	1.20	1	1.30	6	1.17	7	0.97	7	1.55	1	1.58	8	3.56	3	1.17	3	2.16
D1-25v3	56.7		m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
D1-26v3	50.0	11	4	1.66	1	1.17	1	1.03	3	1.05	7	1.02	5	3.18	5	2.48	7	5.34	3	0.88	7	5.59	8	1.53		
D1-27v3	54.3	20	7	2.51	3	4.81	3	1.43	1	1.52	3	4.79	8	2.00	4	3.66	5	1.60	1	4.01	3	2.43	1	1.30	7	1.65
D1-28v3	60.3	20	2	1.60	8	6.39	1	1.15	3	1.55	7	10.77	6	1.17	3	1.07	6	2.10	5	1.37	1	1.55	3	4.50	7	1.70
D1-30v3	57.4	20	3	1.63	3	1.30	3	1.35	1	2.83	5	1.95	3	1.27	7	1.25	3	1.18	1	1.13	1	1.13	7	1.56	5	1.13
D1-31v3	54.4	20	3	2.40	4	0.80	5	4.40	7	6.86	3	6.68	5	9.16	5	2.31	7	2.56	5	1.88	7	7.19	8	4.03	1	12.52
D1-32v3	57.0	6	6	1.68	7	1.13	1	0.93	1	0.98	3	1.10	6	1.28												
D1-33v3	56.4		m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
D1-36v3	55.1	16	3	0.83	3	1.38	5	1.35	6	1.32	3	1.93	6	2.36	7	1.55	7	1.40	1	2.00	3	0.93	7	0.95	3	2.53
D1-37v3	57.0		m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
D1-39v4	55.6	19	3	1.17	1	1.00	1	1.13	7	0.97	5	3.03	7	3.23	1	1.02	1	2.18	7	1.33	3	2.28	5	3.90	1	2.30
D1-41v3	52.4	20	7	0.92	3	0.97	6	1.32	3	1.02	3	1.03	2	3.00	8	3.25	5	1.12	7	2.30	3	1.91	1	1.05	5	2.60
D1-42v4	59.1	20	1	0.98	1	0.85	3	1.03	7	1.10	7	0.87	7	3.03	5	1.10	5	0.80	5	1.88	1	0.82	3	1.52	1	0.83
D1-44v3	54.9	20	1	1.27	7	2.26	3	2.38	7	1.20	7	1.98	1	1.12	5	3.76	3	1.68	7	2.31	5	1.13	3	0.90	1	1.10
D1-46v3	53.6	20	7	2.18	3	1.48	1	1.17	3	1.00	6	2.36	1	1.13	1	2.48	2	2.41	3	1.12	7	4.05	3	1.30	3	1.93
D1-48v3	55.3		m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
D1-49v4	57.7	20	1	0.97	5	0.95	8	1.17	8	3.13	5	0.93	5	0.97	5	0.58	3	0.97	1	0.62	7	0.65	8	1.53	3	2.51
D1-50v3	50.1	20	1	0.85	5	1.70	5	1.10	7	1.03	3	1.13	7	1.22	8	9.79	1	0.88	5	1.27	3	0.80	5	6.34	7	0.75
D1-51v3	62.0	12	1	1.10	3	1.32	3	1.10	1	1.02	3	0.98	1	1.08	5	0.87	3	1.07	2	0.88	3	1.07	5	0.90	1	1.63
D1-52v3	54.7	20	1	1.10	1	1.61	3	1.48	4	1.33	7	2.25	8	3.36	6	0.98	7	1.22	1	2.13	5	3.65	1	1.12	1	1.32
D1-53v3	55.3	20	5	1.42	7	0.75	5	0.95	1	2.06	1	1.13	7	3.60	7	1.18	1	1.07	3	0.93	7	3.16	5	1.15	5	0.85
D1-56v4	55.4	20	4	2.28	2	6.39	1	10.29	2	5.36	8	10.01	5	2.80	6	1.60	1	6.09	5	2.58	3	3.70	7	2.96	3	7.89
D1-57v3	55.4	20	1	0.85	1	0.95	3	0.98	8	1.42	5	1.02	6	1.23	3	1.02	1	0.90	3	1.07	7	1.13	4	0.85	5	1.10
D1-58v4	59.1	8	1	1.50	3	0.93	1	1.55	2	2.96	4	1.96	1	1.00	3	1.35	3	1.12								
D1-59v3	73.9	20	4	0.77	5	1.53	3	2.46	6	0.43	2	0.02	7	1.42	7	1.00	3	0.83	3	0.90	5	1.03	8	0.97	6	2.93
D1-61v3	57.9	20	1	0.87	5	1.37	5	1.42	7	0.85	3	0.93	1	0.85	6	2.46	7	1.07	1	0.97	3	1.68	7	1.30	3	1.05
D1-62v3	53.4	20	1	1.35	1	2.91	3	2.35	5	1.91	1	7.26	3	1.03	6	3.35	4	3.06	3	3.21	8	1.12	7	4.33	7	2.50

Code	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)
D1-01v5	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m								
D1-02v4	1	1.05	5	1.96	7	1.22	1	1.18	3	1.13	7	1.83	5	1.18	7	1.27								
D1-03v3	3	1.02	1	1.20	5	1.00	3	0.90	5	1.40	3	1.15	7	2.25	7	1.63								
D1-05v4	7	4.35	1	1.17	7	1.61	3	3.31	1	1.43	5	6.04	5	16.40	5	11.39								
D1-06v4	7	1.30	1	1.35	6	9.88	5	1.40	1	1.33	7	2.67	7	0.92	3	1.88								
D1-07v4																								
D1-08v3	1	5.28	5	1.22	3	2.15	8	2.73	1	0.95	6	1.76	3	1.18	7	0.93	1	1.10	8	1.63	7	2.78	4	1.61
D1-09v3	4	2.08	5	1.10	5	1.71	7	1.07	3	0.95	1	0.90	7	1.22	7	1.27								
D1-10v3																								
D1-13v4	3	1.08	7	1.33	5	2.60	5	4.08	7	1.61	1	1.43	3	0.92	3	1.10								
D1-14v3	7	5.01	1	2.25	7	2.78	5	1.15	3	1.66	7	1.35	5	1.38	6	2.28								
D1-15v3																								
D1-17v3	3	1.15	5	0.83	6	1.07	7	6.06	3	1.28	6	2.26	3	1.38	5	3.58								
D1-18v3	7	2.71	5	6.76	7	0.90	7	1.93	6	1.28	6	4.80	3	1.45	3	1.25								
D1-21v3	7	5.39	6	5.14	2	3.21	4	1.81	6	3.81	3	4.98	8	5.43	2	2.36								
D1-22v3	7	1.45	1	1.05	3	1.50	1	0.97	7	1.65	3	1.58	4	0.85	5	2.26								
D1-23v3	7	1.10	1	0.90	3	0.95	5	1.22	5	1.13	3	1.02	1	1.07	5	4.05								
D1-24v3	3	4.68	6	0.78	3	1.66	5	0.85																
D1-25v3	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m								
D1-26v3																								
D1-27v3	6	2.16	6	2.55	6	1.28	2	1.23	5	0.93	7	2.88	2	1.30	7	2.98								
D1-28v3	1	1.25	3	1.18	7	1.60	1	1.45	5	2.05	7	1.40	5	0.02	3	1.98								
D1-30v3	7	1.42	8	2.85	1	1.30	5	0.92	5	2.83	1	1.13	5	1.35	7	0.83								
D1-31v3	4	1.38	7	1.18	1	11.00	1	5.58	1	0.90	3	1.50	1	1.90	6	0.55								
D1-32v3																								
D1-33v3	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m								
D1-36v3	8	1.58	5	1.22	1	1.61	8	1.07																
D1-37v3	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m								
D1-39v4	3	2.83	3	1.07	3	1.33	5	5.21	6	2.13	7	5.83	7	2.93										
D1-41v3	5	2.88	7	3.40	1	1.07	1	1.02	8	2.65	6	4.91	3	1.20	2	4.81								
D1-42v4	3	1.32	1	0.90	6	3.58	7	1.63	3	0.87	5	3.31	3	0.85	7	0.93								
D1-44v3	1	2.15	6	3.40	7	3.10	1	1.03	3	1.78	5	5.11	3	2.01	6	2.58								
D1-46v3	1	1.15	6	2.36	7	1.93	5	2.45	7	1.12	5	2.05	7	1.76	5	3.23								
D1-48v3	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m								
D1-49v4	6	3.65	1	1.42	8	0.70	3	1.88	3	0.95	2	1.00	4	0.42	2	0.82								
D1-50v3	5	2.26	3	0.83	1	1.23	3	0.85	3	1.20	2	1.75	7	0.83	1	1.48								
D1-51v3																								
D1-52v3	4	0.70	7	1.18	6	4.46	7	1.13	3	1.15	6	0.68	7	2.31	4	1.65								
D1-53v3	3	1.43	5	1.05	3	0.78	1	1.25	1	0.97	7	3.53	3	1.66	3	1.55								
D1-56v4	3	8.52	1	2.56	8	11.94	7	2.80	4	7.46	5	2.45	5	2.25	8	4.46								
D1-57v3	1	0.95	7	1.30	8	3.36	1	0.95	5	5.23	7	1.13	5	5.09	3	1.38								
D1-58v4																								
D1-59v3	3	1.55	5	2.68	8	1.33	1	1.23	1	1.20	1	1.68	7	1.37	1	1.10								
D1-61v3	1	0.82	5	2.51	4	1.58	3	1.55	7	0.87	5	1.03	1	0.88	8	1.58								
D1-62v3	8	3.60	6	1.50	7	1.32	1	1.38	6	1.30	6	0.97	2	10.06	3	4.16								

Table 4: Raw 24 month Fixation Shift latency data

Code	Corrected age (weeks)	No. of trials delivered	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)
D1-01 v6	102.4	20	5	2.26	3	1.70	1	0.98	7	1.10	5	1.33	1	1.53	3	0.72	1	0.82	7	1.42	3	1.25
D1-02 v5	108.9	20	1	1.20	3	1.18	1	0.88	5	1.35	7	1.43	1	0.90	3	1.47	1	3.33	2	4.11	7	1.10
D1-03 v4	108.9	20	7	1.10	3	1.32	7	10.92	8	3.43	5	3.85	3	2.98	1	1.20	3	0.83	5	8.19	1	2.63
D1-05 v6	105.0	8	5	2.00	3	1.40	7	0.93	6	1.70	5	1.05	7	0.80	1	0.88	7	1.08				
D1-06 v5	105.3	20	7	0.98	2	1.22	5	0.75	7	1.10	3	1.02	5	0.93	3	0.87	3	0.73	3	0.85	7	2.00
D1-07 v5	104.3		m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
D1-08 v4	104.4		m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
D1-09 v4	106.1	12	3	4.69	1	3.23	7	1.13	7	1.30	3	1.76	5	1.88	1	1.15	5	3.23	3	3.66	1	1.27
D1-10 v4	104.9	20	5	1.25	3	1.17	5	4.53	7	1.55	1	2.20	2	3.35	1	0.92	7	2.63	7	7.78	5	3.08
D1-13 v5	107.1	17	5	0.93	3	0.78	1	1.10	5	1.30	1	0.82	3	1.12	8	1.45	7	1.03	3	2.33	8	3.33
D1-14 v4	104.7	20	7	0.95	3	0.93	1	0.85	5	1.20	5	2.98	1	3.90	5	1.15	1	1.32	1	1.28	8	6.78
D1-15 v4	111.6	9	1	3.43	3	6.89	3	2.05	2	2.63	3	1.73	3	1.73	6	5.36	1	4.43	8	9.24		
D1-18 v4	101.9	30	8	2.16	3	1.35	1	0.90	5	1.85	1	1.02	1	2.58	7	0.93	7	0.98	1	1.15	7	1.91
D1-21 v4	108.4	20	1	1.43	3	2.60	3	2.41	5	3.20	5	4.01	8	2.93	4	2.10	4	1.55	1	2.75	8	3.21
D1-22 v4	109.4	20	1	1.13	5	1.02	3	1.18	7	1.52	1	0.88	7	0.97	6	2.66	3	1.56	3	2.26	5	4.20
D1-23 v4	108.0	16	5	1.17	1	0.93	1	1.60	1	2.11	7	1.65	3	0.83	5	0.97	6	0.60	5	1.60	4	0.85
D1-25 v4	107.9	20	5	0.93	3	0.87	7	1.43	5	0.82	1	0.85	6	2.23	7	2.23	3	1.08	8	3.11	1	0.87
D1-26 v4	103.3	20	3	0.85	3	0.88	8	0.95	1	0.88	5	1.03	3	0.80	6	0.78	7	1.42	1	0.90	7	1.56
D1-27 v4	106.3	20	6	4.16	1	1.18	3	1.08	3	0.93	4	1.23	1	0.93	5	1.02	5	1.50	7	0.75	6	2.36
D1-28 v4	108.7	20	3	1.12	1	0.92	6	1.15	5	9.87	7	0.98	7	1.42	1	1.23	3	1.13	3	0.98	1	0.87
D1-30 v4	107.4	20	6	1.07	7	0.68	3	0.92	5	1.33	3	1.00	1	0.97	1	1.13	7	0.93	1	0.90	7	1.42
D1-31 v4	103.9	20	5	1.22	1	0.90	7	0.92	7	0.75	5	1.02	7	1.12	5	0.98	6	0.55	3	1.07	7	1.35
D1-32 v4	107.9	20	1	2.06	1	0.87	6	3.98	2	2.21	3	1.07	1	1.37	5	0.83	2	1.50	3	1.68	7	2.85
D1-33 v4	106.3	14	1	0.80	8	1.80	7	0.82	3	1.10	4	3.30	1	1.25	5	1.43	6	3.70	7	1.48	3	1.00
D1-36 v4	106.9	20	1	1.81	8	1.28	7	2.00	8	1.91	5	1.37	3	1.18	5	3.60	1	1.68	7	9.69	4	2.11
D1-37 v4	105.3	20	5	1.88	8	2.65	7	1.15	7	1.23	3	1.17	8	4.91	1	1.12	3	1.83	5	4.63	1	1.80
D1-39 v5	105.7	13	7	1.05	1	0.73	5	1.22	3	1.12	3	0.92	2	1.27	7	1.13	5	1.15	5	0.95	1	1.27
D1-41 v4	105.7	20	3	0.80	5	1.22	6	3.35	5	1.17	1	1.00	7	0.98	7	4.68	3	0.70	8	1.93	1	0.93
D1-42 v5	113.3	20	3	1.20	6	0.65	6	2.25	1	2.35	2	0.93	7	2.18	8	2.05	6	3.98	1	1.17	5	1.08
D1-44 v4	107.1	20	7	1.10	6	3.36	1	1.25	6	5.28	1	2.46	6	1.83	3	1.08	3	1.27	3	6.41	7	2.10
D1-46 v5	102.7	20	7	3.35	8	2.31	3	3.21	1	0.92	8	1.83	1	1.13	3	1.95	5	1.00	5	3.25	7	3.13
D1-48 v5	105.1	20	3	1.48	5	1.61	7	1.45	5	2.46	3	0.98	1	1.50	7	2.01	7	1.30	3	1.25	2	0.93
D1-49 v5	103.6	20	7	0.88	7	1.68	1	1.20	7	5.18	3	1.66	3	1.47	6	2.50	1	1.63	8	3.85	7	5.43
D1-50 v6	101.0	20	6	4.16	2	2.48	1	1.22	5	1.90	7	2.60	2	0.97	3	0.63	6	20.15	7	0.95	2	1.48
D1-51 v5	113.6	20	1	0.98	7	1.95	3	1.58	3	0.95	7	3.38	5	1.86	1	1.00	3	0.77	5	3.58	7	1.60
D1-52 v4	107.1	20	8	2.86	1	1.00	3	2.31	1	1.10	8	1.52	5	1.81	5	1.07	1	2.50	3	0.82	6	0.62
D1-53 v4	109.4	20	1	1.86	6	0.72	7	1.07	7	5.94	6	10.27	3	4.35	6	2.10	8	0.47	5	3.06	5	2.20
D1-56 v5	106.9	14	3	1.47	5	3.18	5	2.80	3	1.22	4	14.45	2	1.17	8	1.81	6	2.58	3	1.17	1	4.00
D1-57 v4	106.9	20	1	0.93	7	1.08	5	1.00	5	1.05	7	1.17	1	0.88	7	1.27	5	0.92	7	0.97	3	1.00
D1-58 v5	109.3	20	7	1.60	5	2.08	5	2.58	3	1.10	3	5.36	3	7.89	7	7.13	5	1.43	3	10.34	2	1.53
D1-59 v4	126.7	20	3	0.77	7	0.93	2	0.55	7	0.65	6	1.08	6	4.18	3	1.00	3	2.73	8	1.70	7	4.96
D1-61 v4	107.1	4	3	0.72	6	1.90	7	0.98	1	1.03												
D1-62 v4	104.7	20	3	0.85	5	1.95	6	2.48	6	3.81	7	1.98	3	3.23	7	5.29	3	0.03	2	0.70	2	5.79

Code	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)
D1-01 v6	5	1.81	3	0.95	6	1.80	7	1.17	5	0.90	1	1.10	1	0.90	7	0.83	7	0.93	3	0.95
D1-02 v5	3	1.38	5	0.98	6	2.90	7	0.88	6	5.29	3	1.15	5	5.69	7	1.58	3	2.18	7	3.21
D1-03 v4	6	4.13	1	4.26	3	0.97	3	1.10	1	1.00	7	0.92	6	6.31	1	1.28	7	4.43	6	14.32
D1-05 v6																				
D1-06 v5	3	1.00	1	1.56	1	0.90	1	1.22	5	3.25	5	0.97	7	0.90	7	1.37	1	0.93	5	1.48
D1-07 v5	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
D1-08 v4	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
D1-09 v4	3	4.21	8	1.40																
D1-10 v4	3	1.12	5	2.16	3	0.92	3	1.18	3	1.13	8	2.51	7	8.92	5	3.30	1	1.20	1	0.88
D1-13 v5	7	5.88	5	0.83	1	1.17	7	0.97	5	1.02	1	2.16	3	1.63						
D1-14 v4	3	0.73	5	4.31	5	2.28	7	1.22	7	1.35	1	0.95	3	0.87	7	3.68	3	1.08	3	0.90
D1-15 v4																				
D1-18 v4	4	0.78	4	1.56	4	1.33	5	1.22	5	4.91	2	2.65	3	1.85	7	5.31	5	2.85	5	4.00
D1-21 v4	5	6.98	1	1.20	1	1.43	8	1.55	5	3.06	1	2.13	7	4.99	3	1.45	7	6.93	5	1.22
D1-22 v4	1	1.47	7	2.35	5	0.97	3	0.77	1	0.82	7	3.38	1	0.88	7	1.52	3	1.52	6	4.08
D1-23 v4	7	2.41	3	1.42	7	1.37	1	0.97	3	0.88	3	1.07								
D1-25 v4	3	0.88	7	1.00	1	0.87	2	1.17	1	0.92	7	1.20	5	0.83	5	1.27	3	0.87	3	0.97
D1-26 v4	7	0.97	1	0.90	7	3.26	3	0.93	1	1.07	6	1.10	3	0.98	5	1.96	1	0.90	5	0.87
D1-27 v4	8	1.08	5	1.40	8	2.16	3	2.08	1	1.71	1	1.50	3	1.07	7	4.06	7	2.46	1	1.53
D1-28 v4	3	2.28	8	4.60	8	1.53	5	1.02	1	0.85	3	1.00	5	2.46	1	0.85	7	1.33	5	1.35
D1-30 v4	1	1.03	5	2.21	3	0.83	1	0.98	7	1.17	6	1.25	5	1.98	7	3.03	3	1.08	3	2.23
D1-31 v4	1	0.60	1	1.68	3	0.82	7	1.35	6	0.55	1	1.38	3	0.90	3	0.68	1	1.03	3	0.98
D1-32 v4	7	3.35	7	1.63	5	1.90	4	1.48	3	1.78	5	5.89	7	0.95	3	0.97	6	3.26	7	1.40
D1-33 v4	1	1.60	5	2.06	2	0.50	2	0.62												
D1-36 v4	8	3.21	6	10.34	5	0.97	3	1.20	2	2.63	1	1.38	1	0.93	3	2.83	4	2.31	5	0.97
D1-37 v4	1	1.15	6	3.53	7	3.65	5	1.07	1	2.43	3	0.95	1	1.03	3	0.92	6	2.86	3	1.58
D1-39 v5	1	1.43	6	2.78	5	1.28														
D1-41 v4	2	1.22	3	0.87	3	1.08	1	1.22	1	0.98	7	1.08	3	1.12	6	1.73	7	0.83	5	4.06
D1-42 v5	8	1.07	3	1.42	3	2.31	7	1.37	1	0.95	2	1.10	3	0.88	6	1.23	8	8.94	3	2.11
D1-44 v4	7	4.43	1	1.10	2	2.26	3	1.61	3	1.81	2	3.31	7	5.08	7	6.86	6	5.89	5	5.83
D1-46 v5	3	2.53	1	1.32	5	2.88	1	1.42	6	6.03	1	0.97	6	2.63	8	2.41	3	1.12	3	2.51
D1-48 v5	5	2.78	7	1.95	1	2.13	2	2.31	8	2.66	6	2.80	5	2.16	3	5.83	3	1.17	1	1.38
D1-49 v5	4	4.36	2	1.08	6	1.42	6	5.08	6	1.70	3	1.37	1	1.42	1	1.15	3	5.34	6	11.17
D1-50 v6	3	1.12	1	1.13	6	2.78	3	0.87	3	1.23	3	1.22	6	9.34	8	0.83	7	3.10	8	0.97
D1-51 v5	8	2.23	7	1.22	1	0.93	6	3.96	1	0.92	3	1.00	4	1.65	5	4.36	6	2.03	1	1.28
D1-52 v4	3	4.63	6	4.21	7	1.60	3	4.50	1	1.28	6	0.03	8	4.68	7	1.80	2	4.26	4	3.63
D1-53 v4	3	3.31	1	8.84	7	0.93	1	1.40	2	0.75	7	1.76	3	1.22	3	1.38	1	1.15	3	1.05
D1-56 v5	3	3.43	1	1.55	2	1.98	8	2.81												
D1-57 v4	3	1.00	5	1.00	3	1.15	1	0.80	5	1.03	7	1.08	1	0.72	3	0.93	3	1.07	1	1.03
D1-58 v5	1	1.80	6	4.50	7	4.33	4	1.33	7	0.73	7	1.52	2	1.33	6	0.88	1	0.55	1	1.58
D1-59 v4	3	0.82	1	0.93	1	1.68	2	1.30	8	1.42	6	2.08	2	3.38	6	0.55	3	1.76	6	1.40
D1-61 v4																				
D1-62 v4	3	1.57	3	1.42	2	1.40	7	1.30	1	2.95	6	0.03	1	1.60	6	0.42	7	0.03	7	1.00

Code	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)	Direction of shift - coded	time to make shift (s)
D1-18 v4	7	1.22	3	0.83	7	2.75	7	1.98	3	1.02	1	1.12	1	1.05	3	1.35	5	2.01	7	1.35

20.8 Appendix 8: Dietary intakes for control and treatment groups

Table 1. Dietary intake of control and treatment groups based on normal diet plus prescribed supplement: intention to treat analysis

	Months of taking Treatment							
	3	**6**	**9**	**12**	**15**	**18**	**21**	**24**
Daily DHA intake (mg) from feed and food								
Control	73.2	40.5	32.5	111.5	92.4	83.5	55.5	82.8
Treatment	95.9	81.6	54.1	51.6	53.3	61.7	82.6	49.1
Daily EPA intake (mg) from feed and food								
Control	18.6	10.0	13.5	60.0	52.9	48.5	34.5	48.9
Treatment	41.9	26.0	17.8	36.8	34.6	40.9	68.7	39.1
Daily AA intake (mg) from feed and food								
Control	121.4	83.3	57.0	37.5	52.9	42.0	57.0	48.9
Treatment	116.8	100.7	75.4	53.6	38.3	33.9	34.8	31.3
Daily choline intake (mg) from feed and food								
Control	111.8	102.6	127.5	127.7	132.2	129.8	118.5	128.2
Treatment	98.7	106.8	101.3	118.0	125.0	118.9	116.0	121.0
Daily DHA intake (mg) from Treatment								
Control	9.5	11.0	11.5	14.0	16.2	19.0	18.0	20.0
Treatment	180.0	243.2	283.2	288	321.8	343.9	334.3	312.0
Daily EPA intake (mg) from Treatment								
Control	1.8	2.4	2.5	3.0	3.3	4.0	3.5	3.9
Treatment	33.8	52.4	58.9	59.6	66.3	70.4	68.3	62.8
Estimated and calculated daily AA intake (mg) from Treatment								
Control	0.9	1.4	1.5	1.5	1.9	2.0	2.0	2.2
Treatment	20.8	28.4	31.7	32.4	35.6	37.8	36.5	34.2
Daily choline intake (mg) from Treatment								
Control	9.0	12.0	13.2	15.8	17.0	18.6	18.6	19.9
Treatment	50.1	70.5	79.1	80.6	89.6	96.5	88.7	86.6
Daily DHA intake (mg)								
Control	82.7	51.4	44.0	125.5	108.6	102.5	73.5	102.8

	Months of taking Treatment							
	3	**6**	**9**	**12**	**15**	**18**	**21**	**24**
Treatment	275.9	324.8	337.3	339.6	375.1	405.7	417.4	361.1
Daily EPA intake (mg)								
Control	20.5	12.4	16.0	63.0	56.2	52.5	38.0	52.8
Treatment	75.8	78.4	76.7	96.4	100.9	111.3	137.0	102.0
Estimated and calculated daily AA intake (mg)								
Control	122.3	84.8	58.5	39.0	54.8	44.0	59.0	51.1
Treatment	137.6	129.1	107.1	86.0	73.9	71.7	71.3	65.5
Daily choline intake (mg)								
Control	120.8	114.5	140.7	143.4	149.2	148.3	137.2	148.1
Treatment	148.8	177.3	180.3	198.6	214.6	215.3	204.7	207.5

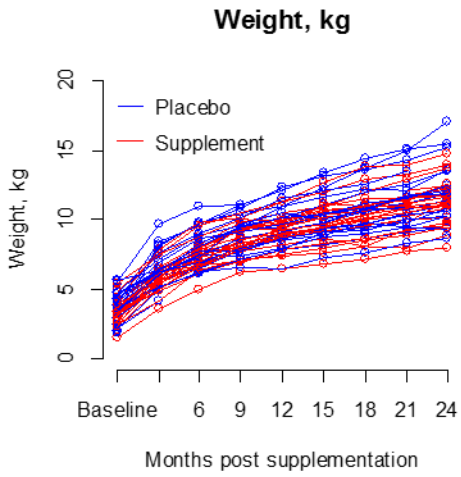
Table 2. Dietary intake of control and treatment groups based on normal diet plus prescribed supplement: per protocol analysis

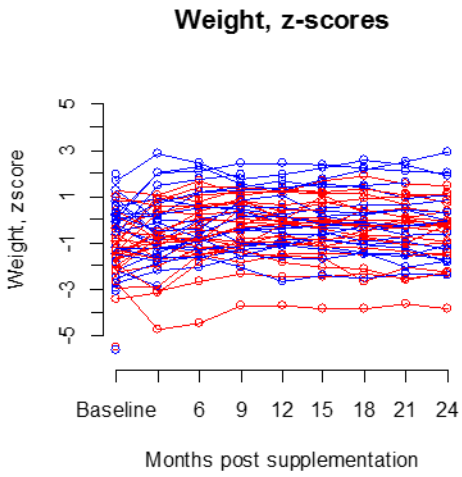
	Months of taking Treatment							
	3	6	9	12	15	18	21	24
Daily DHA intake (mg) from feed and food								
Control	71.0	42.5	33.2	110	97.0	79.5	53.2	83.5
Treatment	96.8	78.5	52.9	55	51.2	65.8	83.3	50.0
Daily EPA intake (mg) from feed and food								
Control	19.5	10.5	14.2	58.9	55.5	40.5	29.5	50.6
Treatment	40.4	25.0	17.1	38.5	33.2	47.5	71.2	38.3
Daily AA intake (mg) from feed and food								
Control	120	86.5	56.8	37.4	55.0	42.6	58.4	51.2
Treatment	118	97.6	74.8	53.1	37.2	33.8	34.6	30.4
Daily choline intake (mg) from feed and food								
Control	111.5	102.6	123.7	127.8	134.1	130.9	118.5	127.5
Treatment	99.3	106.6	104.8	118.3	123.8	118.4	116.1	121.7
Daily DHA intake (mg) from Treatment								
Control	0	0	0	0	0	0	0	0
Treatment	181.1	242.7	281.3	287.7	322.5	345.4	335.4	314
Daily EPA intake (mg) from Treatment								
Control	0	0	0	0	0	0	0	0
Treatment	34.1	52.3	58.6	59.6	66.5	70.8	68.3	63.1
Estimated and calculated daily AA intake (mg) from Treatment								
Control	0	0	0	0	0	0	0	0
Treatment	20.7	28.5	31.7	32.3	35.8	37.9	36.7	34.4
Daily choline intake (mg) from Treatment								
Control	6.7	9.4	10.5	12.4	13.1	14.0	14.4	15.2
Treatment	50.4	70.2	78.5	80.5	89.8	96.8	89.1	87.1
Daily DHA intake (mg)								
Control	71.0	42.5	33.2	110.0	97.0	79.5	53.2	83.5
Treatment	277.9	321.2	334.1	342.7	373.7	411.2	419.2	364.0
Daily EPA intake (mg)								
Control	19.5	10.5	14.2	58.9	55.5	40.5	29.5	50.6
Treatment	74.4	77.3	75.7	98.1	99.7	118.3	139.6	101.5

	Months of taking Treatment							
	3	6	9	12	15	18	21	24
Estimated and calculated daily AA intake (mg)								
Control	120.0	86.5	56.8	37.4	55.0	42.6	58.4	51.2
Treatment	138.8	126.0	106.5	85.4	73.0	71.7	71.2	64.8
Daily choline intake (mg)								
Control	118.2	112.0	134.2	140.2	147.2	144.9	132.9	142.7
Treatment	149.7	176.8	183.3	198.8	213.6	215.2	205.2	208.8

20.9 Appendix 9: Anthropometry trajectory plots for control and treatment group

Figure 1. Raw measurements and converted z-scores for weight, height and head circumference in control and treatment group


Weight, kg
Placebo
Supplement
Weight, kg
0
5
10
15
20
Baseline
6
9
12
15
18
21
24
Months post supplementation


Weight, z-scores
Weight, zscore
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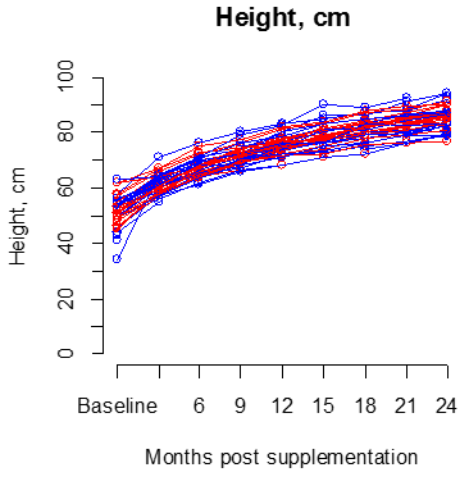
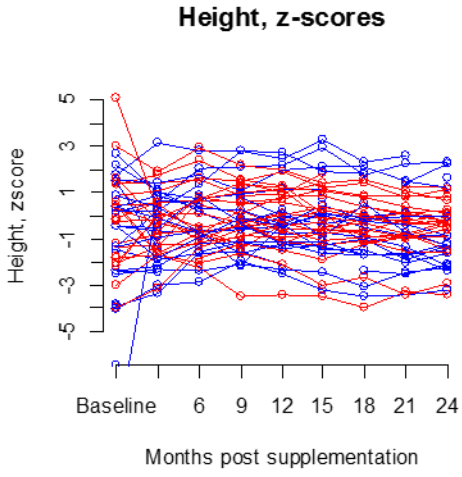
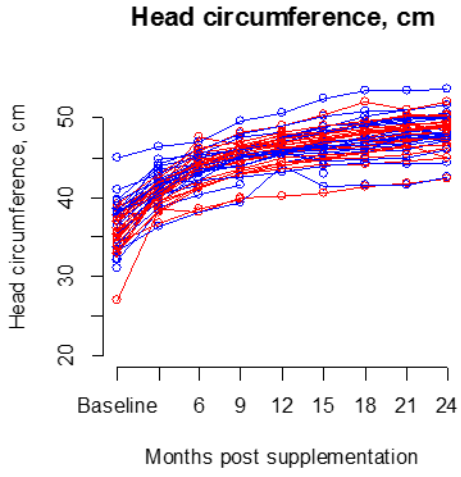
-3
-1
1
3
5
Baseline
6
9
12
15
18
21
24
Months post supplementation


Height, cm
Height, cm
0
20
40
60
80
100
Baseline
6
9
12
15
18
21
24
Months post supplementation


Height, z-scores
Height, zscore
-5
-3
-1
1
3
5
Baseline
6
9
12
15
18
21
24
Months post supplementation


Head circumference, cm
Head circumference, cm
20
30
40
50
Baseline
6
9
12
15
18
21
24
Months post supplementation

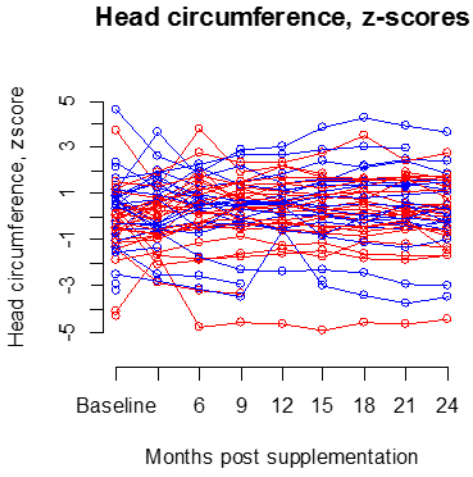
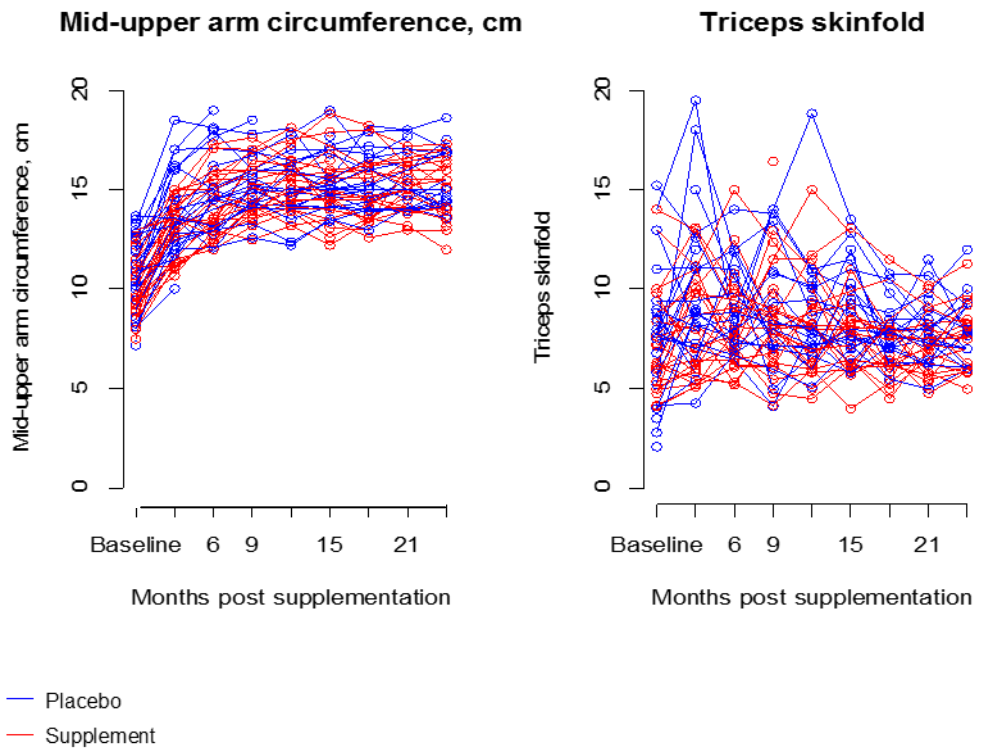

Head circumference, z-scores
Head circumference, zscore
-5
-3
-1
1
3
5
Baseline
6
9
12
15
18
21
24
Months post supplementation

Figure 2. Raw measurements for mid-upper arm circumference and triceps skinfold thickness in control and treatment group



20.10 Appendix 10: Raw Orientation - Reversal Visual Event Related Potential data

Table 1: Presence or absence of statistically significant Orientation - Reversal Visual Event Related Potential response at 6, 12 and 24 months

Code	Visit(months)	CGA at assessment	OR response obtained*
D1-01v3	6	27.9	1
D1-01v4		40.1	1
D1-01v5	12	49.4	1
D1-01v6	24	102.4	2
D1-02v2		18	2
D1-02v3	6	25.9	1
D1-02v4	12	54.7	1
D1-02v5	24	108.9	1
D1-03v2	6	29.9	1
D1-03v3	12	55.3	1
D1-03v4	24	108.9	1
D1-05v2	6	18.4	1
D1-05 v3		39.1	1
D1-05v4	12	50.7	1
D1-05v5	24	105	8
D1-06v3	6	35.7	1
D1-06v4	12	54.1	1
D1-06v5	24	105.3	1
D1-07v2		13	2
D1-07v3	6	27.3	1
D1-07v4	12	51.3	1
D1-07v5	24	104.3	8
D1-08v2	6	25.9	1
D1-08v3	12	52	1
D1-08v4	24	104.4	8
D1-09v2		3.7	8
D1-09v3	6	29.6	1
D1-09v4	12	55.3	2
D1-09v5	24	106.1	2
D1-10v2	6	25.7	1
D1-10v3	12	53.9	8
D1-10v4	24	104.9	1
D1-11v2		15.7	8
D1-11v3	6	29	8
D1-13v2		13.4	2

Code	Visit(months)	CGA at assessment	OR response obtained*
D1-13v3	6	25.7	1
D1-13v4	12	51.9	1
D1-13v5	24	107.1	8
D1-14v2	6	26.9	1
D1-14v3	12	50.3	1
D1-14v4	24	104.7	8
D1-15v2		13.1	8
D1-15v3	6	27	1
D1-15v4	12	53.6	1
D1-15v5	24	111.6	1
D1-17v2	6	21	1
D1-17v3	12	48.7	2
D1-18v2	6	22	1
D1-18v3	12	47.1	8
D1-18v4	24	101.9	2
D1-21v2	6	29.7	1
D1-21v3	12	56	2
D1-21v4	24	108.4	1
D1-22v2	6	29	1
D1-22v3	12	58	1
D1-22v4	24	109.4	2
D1-23v2	6	24.9	1
D1-23v3	12	52.7	1
D1-23v4	24	108	8
D1-24v2	6	26.7	2
D1-24v3	12	53.9	1
D1-25v2	6	26.1	1
D1-25v3	12	56.7	1
D1-25v4	24	107.9	1
D1-26v2	6	23.6	1
D1-26v3	12	50	1
D1-26v4	24	103.3	2
D1-27v2	6	28.7	1
D1-27v3	12	54.3	2
D1-27v4	24	106.3	2
D1-28v2	6	36.9	1
D1-28v3	12	60.3	1
D1-28v4	24	108.7	1
D1-30v2	6	30.7	1
D1-30v3	12	57.4	1
D1-30v4	24	107.4	8
D1-31v2	6	32.3	2

Code	Visit(months)	CGA at assessment	OR response obtained*
D1-31v3	12	54.4	2
D1-31v4	24	103.9	1
D1-32v2	6	28.9	2
D1-32v3	12	57	1
D1-32v4	24	107.9	2
D1-33v2	6	28.9	1
D1-33v3	12	56.4	8
D1-33v4	24	106.3	1
D1-34v3		14.7	2
D1-34v4	6	34.3	2
D1-36v2	6	40.3	8
D1-36v3	12	55.1	8
D1-36v4	24	106.9	2
D1-37v2	6	29.6	1
D1-37v3	12	57	1
D1-37v4	24	105.3	1
D1-39v2		17.4	1
D1-39v3	6	29.4	1
D1-39v4	12	55.6	1
D1-39v5	24	105.7	1
D1-40v2	6	29.1	2
D1-41v2	6	27.6	1
D1-41v3	12	52.4	1
D1-41v4	24	105.7	8
D1-42v2	6	22.6	1
D1-42v3		35.6	8
D1-42v4	12	59.1	1
D1-42v5	24	113.3	2
D1-44v2	6	30	1
D1-44v3	12	54.9	1
D1-44v4	24	107.1	8
D1-46v3	6	27.3	2
D1-46v4	12	53.6	2
D1-46v5	24	102.7	8
D1-48v1		4.1	8
D1-48v2		19.7	1
D1-48v3	6	36.7	1
D1-48v4	12	55.3	1
D1-49v2	24	10.6	1
D1-49v3	6	34.7	8
D1-49v4	12	57.7	8
D1-49v5	25	103.6	2

Code	Visit(months)	CGA at assessment	OR response obtained*
D1-50v2	6	25.1	1
D1-50v3	12	50.1	8
D1-50v4	24	101	2
D1-51v2	6	23.3	8
D1-51v3		34.3	1
D1-51v4	12	62	1
D1-51v5	24	113.6	2
D1-52v2	6	30	1
D1-52v3	12	54.7	8
D1-53v2	6	29.7	1
D1-53v3	12	55.6	2
D1-53v4	24	109.4	2
D1-56v2		11.1	8
D1-56v3	6	31.1	8
D1-56v4	12	55.4	8
D1-56v5	24	106.9	8
D1-57v2	6	31.1	8
D1-57v3	12	55.4	2
D1-57v4	24	106.9	2
D1-58v3	6	30.4	1
D1-58v4	12	59.1	2
D1-58v5	24	109.3	2
D1-59v1		19.9	1
D1-59v2	6	47.7	1
D1-59v3	12	73.9	1
D1-59v4	24	126.7	8
D1-61v2	6	32.6	2
D1-61v3	12	57.9	2
D1-61v4	24	107.1	8
D1-62v2	6	28	2
D1-62v3	12	53.4	8
D1-62v4	24	104.7	2

*1=Statistically significant response recorded

2= Statistically significant response not recorded

8= Not tested