Biochemical correlates of cognition: exploring the relationships between blood, brain and behaviour

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Doctor of Philosophy

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This multi-modal investigation aimed to refine analytic tools including proton magnetic resonance spectroscopy (^{1}H-MRS) and fatty acid gas chromatography-mass spectrometry (GC-MS) analysis, for use with adult and paediatric populations, to investigate potential biochemical underpinnings of cognition (Chapter 1). Essential fatty acids (EFAs) are vital for the normal development and function of neural cells. There is increasing evidence of behavioural impairments arising from dietary deprivation of EFAs and their long-chain fatty acid metabolites (Chapter 2). Paediatric liver disease was used as a deficiency model to examine the relationships between EFA status and cognitive outcomes. Age-appropriate Wechsler assessments measured Full-scale IQ (FSIQ) and Information Processing Speed (IPS) in clinical and healthy cohorts; GC-MS quantified surrogate markers of EFA status in erythrocyte membranes; and ^{1}H-MRS quantified neurometabolite markers of neuronal viability and function in cortical tissue (Chapter 3). Post-transplant children with early-onset liver disease demonstrated specific deficits in IPS compared to age-matched acute liver failure transplant patients and sibling controls, suggesting that the time-course of the illness is a key factor (Chapter 4). No signs of EFA deficiency were observed in the clinical cohort, suggesting that EFA metabolism was not significantly impacted by liver disease. A strong, negative correlation was observed between omega-6 fatty acids and FSIQ, independent of disease diagnosis (Chapter 5). In a study of healthy adults, effect sizes for the relationship between ^{1}H-MRS- detectable neurometabolites and cognition fell within the range of previous work, but were not statistically significant. Based on these findings, recommendations are made emphasising the need for hypothesis-driven enquiry and greater subtlety of data analysis (Chapter 6). Consistency of metabolite values between paediatric clinical cohorts and controls indicate normal neurodevelopment, but the lack of normative, age-matched data makes it difficult to assess the true strength of liver disease-associated metabolite changes (Chapter 7). Converging methods offer a challenging but promising and novel approach to exploring brain-behaviour relationships from micro- to macroscopic levels of analysis (Chapter 8).

Key words: Magnetic Resonance Spectroscopy; Gas chromatography-mass spectrometry; Cognition; Essential fatty acids; Liver disease

To my wife and family

Mae, mum, dad, Kisna and Dip

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Abbreviations and terminology

^{1}H-MRS	proton magnetic resonance spectroscopy
^{13}C-MRS	carbon-magnetic resonance spectroscopy
^{31}P-MRS	phosphorous-magnetic resonance spectroscopy
AA	arachidonic acid
ADHD	attention deficit hyperactivity disorder
ALA	α-linolenic acid
Bo	magnetic field strength
BCH	Birmingham Children's Hospital
Cho	choline
CNS	central nervous system
Cr	creatine
CSF	cerebrospinal fluid
DHA	docosahexaenoic acid
DHGLA	dihomo-γ-linolenic acid
EDTA	ethylene diamine tetra-acetic acid
EFA	essential fatty acid
EPA	eicosapentaenoic acid
FA	fatty acid
FAME	fatty acid methyl ester
FID	flame ionisation detection
FSIQ	full-scale intelligence quotient
GC	gas chromatography
GC-FID	gas chromatography-flame ionised detection
GC-MS	gas chromatography-mass spectrometry
GM	grey matter
Gln	glutamine
Glx	glutamate/glutamine
HE	hepatic encephalopathy
HUFA	highly unsaturated fatty acid
HPLC	high performance liquid chromatography
IPS	information processing speed
LA	linolenic acid
LC-MS	liquid chromatography-mass spectrometry
LCPUFA	long-chain polyunsaturated fatty acid
LCT	long-chain triacylglycerol
mI	myo-Inositol
MRI	magnetic resonance imaging
MS	mass spectrometry
n-3	omega-3
n-6	omega-6
NAA	N-actyl aspartate
NAAG	N-acetyl aspartyl glutamate
PBS	phosphate buffered saline
PCP	phosphatidylcholine
PDE	phosphodiester

PE	phosphatidylethanolamine
pHi	intracellular pH
PME	phosphomonoester
PI	phosphatidylinositol
PS	phosphatidylserine
PCr	phosphocreatine
PIQ	performance intelligence quotient
Ppm	parts per million
PSI	processing speed index
PUFA	polyunsaturated fatty acid
RBC	red blood cell
RF	radio frequency
SHE	subclinical hepatic encephalopathy
SNR	signal-to-noise ratio
SVS	single-voxel scan
TE	echo time
TLC	thin layer chromatography
TR	relaxation time
Tx	transplant
VIQ	verbal intelligence quotient
VOI	volume of interest
WAIS-III	Wechsler Adult Intelligence Scale – 3rd edition
WASI-III	Wechsler Abbreviated Scale of Intelligence – 3rd edition
WISC-IV	Wechsler Intelligence Scale for Children – 4th edition
WM	white matter
WPPSI-III	Wechsler Preschool and Primary of intelligence – 3rd edition

1 Exploring the biological basis of individual variation in cognitive ability

1.1 Approaches to studying human intelligence differences

This chapter provides a brief introduction to some of the approaches to studying the individual variation in cognitive ability and outlines the aims of this thesis.

The wide ranging variability in cognitive ability found in human beings results from differences in brain functioning, differences in the exploitation of learning opportunities provided by the environment, and from interactions between the brain and the environment (Angoff, 1988; Ceci and Williams, 1999; Sternberg, Lautrey et al., 2003). Individual differences in intelligence are typically measured using psychometric tests, which cover cognitive domains such as reasoning, processing speed, executive function, memory and spatial ability. The term 'general intelligence' or 'g' (interchangeable with IQ) (Spearman, 1927), recognises that people who perform well in one domain of psychometric assessment also tend to perform well in the others (Chabris, 2006; Deary, 2001; Deary, Penke et al., 2010; Neisser, Boodoo et al., 1996). Figure 1-1, adapted from Deary, Penke et al. (2010) illustrates the high associations between g and five principal cognitive domains putatively assessed with psychometric measures.

Figure 1-1 The hierarchy of intelligence differences, adapted from Deary, Penke et al. (2010)
The diagram shows the high inter-relatedness of different cognitive domains putatively assessed with psychometric measures. Data is based on 33 studies conducted by (Salthouse, 2004) comprising 7,000 participants from age 18–95.

Maclullich, Seckl et al. (2003) suggested that validating the construct of intelligence and cognitive abilities involves, in part, discovering associations between more basic cognitive constructs and individual differences in psychometric test scores. This type of reductionism is realised in two broad approaches: (1) the relationships between tests of

psychometric intelligence and basic information processing tests, such as reaction times and inspection time, which are used to discover the extent to which putatively fundamental cognitive components account for variance in broader IQ-type measures (Vernon, 1983); and (2), more biological approaches, such as the relationships between tests of psychometric intelligence and measures of brain structure and function (Garlick, 2002; Gray and Thompson, 2004; McDaniel, 2005; Thompson, Cannon et al., 2001; Toga and Thompson, 2005).

Investigations into the bases of human intelligence differences can thus be separated into two broad groups: cognitive correlates and biological bases (Figure 1-2, page 19, adapted from Deary and Caryl (1997)). 'Differential psychology' is the general term for research that seeks to accurately describe cognitive and personality traits and discover the real-life impact of trait differences, whilst 'differential neuroscience' is the term more specifically concerned with research investigating the biological bases of quantitative intelligence differences (Deary, Penke et al., 2010).

These two approaches can be considered as looking at intelligence on different levels. The cognitive (differential psychology) approach is a factor-driven strategy, which identifies the cognitive components that give rise to differences in human abilities and how these are related to one another, without necessarily revealing the causes or consequences of these differences. The biological (differential neuroscience) approach uses more of a constructivist account by understanding the underlying differences in brain structure and function that provide a scaffold for variation in cognitive skills across the population. In Figure 1-2 the asterisks indicate the normally assumed (reductionistic) direction of causation of intelligence differences, but Deary and Caryl (1997) stressed that these relationships are not unidirectional; the direction of causation could be reversed, or individual differences in both variables might be caused by a third variable.

Figure 1-2 Research on human intelligence differences, adapted from Deary and Caryl (1997). Approaches to investigations of intelligence can be divided into those seeking cognitive correlates and those seeking the biological bases of variation in cognitive abilities. The asterisks indicates the normally assumed (reductionistic) direction of causation of intelligence differences.

Cognitive and biological approaches can be used together in a mutually informative way. Developments in the accuracy, resolution, versatility and accessibility of psychological assessment methods and neuroimaging technologies are advancing the study of the neurophysiological basis of individual variation in cognitive ability in normal individuals, with increasing precision (Deary, 2001; Deary, Penke et al., 2010; Haier, 2009; Jung and Haier, 2007; Matarazzo, 1992).

The challenge, however, is to specify the appropriate functional outcome to be measured. In the interests of hypothesis-driven inquiry, the most productive approach is to measure an outcome known to be associated with a particular biochemical pathway or mechanism. The investigation of information processing speed (IPS) has, for example, been integral to the study of individual differences in cognitive ability and goes back to early notions by Galton (1883) (in Sternberg, Lautrey et al., 2003), who attempted to measure reaction time and diverse sensory and motor variables in relation to independent indicators of accomplishment or intelligence.

Salthouse (1996) suggested that a basic parameter such as speed is directly related to biological factors and is essential for higher order cognitive processing. Vernon (1983) asserted that a large part of the variance in g is attributable to variance in speed and efficiency of execution of a small number of basic cognitive processes. The existence of a unitary intelligence factor is in itself, however, a matter of some debate (Kranzler and Jensen, 1997). The strength of the relationship between processing speed and intelligence is also debateable (Neisser, Boodoo et al., 1996); processing speed cannot substitute psychometric intelligence or g as it is not identical with intelligence (Chabris, 2006; Deary and Caryl, 1997). Reed and Jensen (1992) suggest that in theory, inter-individual differences in speed of information processing could be due to differences in brain (cerebral cortex) structure, average cortical nerve conduction velocity (NCV), average cortical speed of synaptic transmission, or most likely, to differences in all of these.

Whilst the functional organisation of neurons is vital to the study of cognitive function (van den Heuvel, Stam et al., 2009), an investigation of inter-individual variation in the biological properties of neurons and the physiology of neuronal cell membranes is likely to provide information about the mechanisms of information processing in the brain, and provide some suggestions as to the cause of individual differences in the ability to process this information.

1.2 Applying an integrative multi-modal approach

The process of effectively describing complex phenomena such as cognitive ability and putative biological bases poses a considerable challenge. A multitude of terms have been used to describe combinatorial approaches, including integration, synthesis, multi-method and mixed-methods, but these can be generally described as the integration of more than one method or data source to investigate a phenomenon (Creswell, 2003). Combinations of methods can provide advantages at different levels of analysis in the spectrum from molecule to man, and triangulation can be used as means to gain multiple perspectives on complex phenomena and produce a more complete picture.

1.3 Aims

1.3.1 General aims

Using a multi-modal approach, the present set of studies aimed to develop and refine analytic tools for biochemical assays, for use with both adult and paediatric populations. The main aim was to investigate some of the potential biochemical underpinnings of cognition, relating neural, systemic and behavioural levels of analysis.

At the systemic level, quantification of fatty acid biomarkers in erythrocyte membranes with gas chromatography-mass spectrometry (GC-MS) provides surrogate markers for cortical fatty acid levels, allowing the study of the cognitive effects of variation in phospholipid cell membrane composition. At the neural level, proton magnetic resonance spectroscopy (^{1}H-MRS) enables non-invasive, *in vivo* analysis of brain metabolism and offers a way of investigating the strength of relationships between biochemical markers of neuronal viability and cognitive ability. Figure 1-3, page 22 provides an overview of the converging methods approach of the current work investigating potential biochemical correlates of cognition.

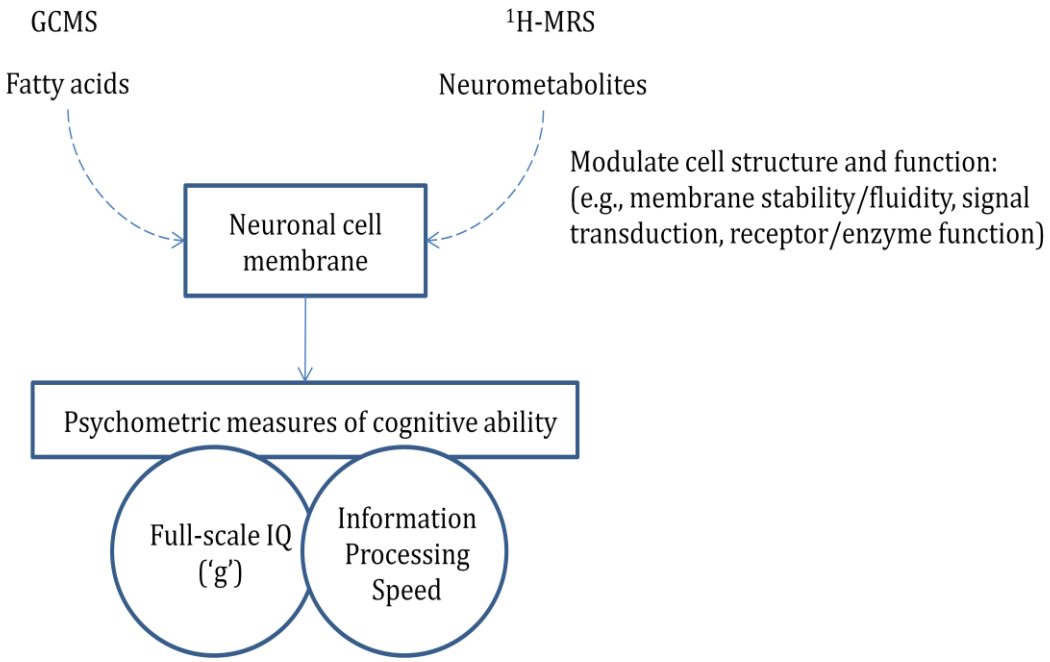

Figure 1-3 The converging methods approach used in the present study
Quantification of fatty acid biomarkers and ^{1}H-MRS-detectable neurometabolites enables the investigation of specific biochemical properties of neuronal function which may underlie variability in performance of psychometric measures of intelligence and processing speed.

1.3.2 Specific aims

A deficiency of essential fatty acids (EFAs), which are crucial for normal neural function and development, is common in children with liver disease. Liver disease and subsequent liver transplantation provide models to answer questions of whether:

1. Sub-optimal concentrations of essential fatty acids, as a result of fat malabsorption or dependence on inadequate dietary sources, is associated with deficits in cognitive ability.
2. ^{1}H-MRS-detectable metabolites can provide surrogate markers of sub-clinical changes in neuronal viability.

To answer these questions, the aims were to:

1. Measure and evaluate the range of fatty acid concentrations in cross-sectional cohorts of children with a variable onset of liver disease, pre- and post-liver transplant.
2. Provide a set of convergent measures for assessing the impact of potential EFA deficiency in paediatric liver disease.
3. Determine if/how cognitive function is related to fatty acid status.
4. Characterise surrogate measures of neural viability and health using ^{1}H-MRS and assess the relationships between ^{1}H-MRS-detectable metabolites and cognitive ability in both a healthy and clinical population.

1.4 Summary of chapters

Chapter 1 introduced the framework for investigating the biological bases of cognitive ability and outlined the approach and aims of the present study.

Chapter 2 provides an overview of the physiological importance of EFAs and the potential mechanisms that may impact cognitive function.

Chapter 3 provides descriptive data for the clinical patient population and outlines the three converging analytical methods used: psychometric assessments, GC-MS for assessment of fatty acid status, and ^{1}H-MRS for analysis of *in vivo* brain biochemistry.

Chapter 4 investigates the impact of the time-course of liver disease and subsequent liver transplantation on cognitive ability.

Chapter 5 investigates the effect of paediatric liver disease on EFA status and the relationships between levels of omega-6 and omega-3 fatty acids and cognitive ability.

Chapter 6 examines the use of ^{1}H-MRS as a tool for assessing brain tissue composition in vivo and the relationships between ^{1}H-MRS-detectable neurometabolites and cognitive ability in a healthy adult cohort.

Chapter 7 explores the ability of ^{1}H-MRS-detectable metabolites to provide surrogate markers of subclinical changes in neuronal viability in paediatric liver disease, and whether levels of these markers can be related to cognitive function.

Chapter 8 summarises the main findings and their implications, offers suggestions of future directions of work motivated by this study, and concluding remarks.

2 Essential fatty acids and the brain

The adult brain contains approximately 50–60% of its dry weight as lipid and is exceeded only by adipose tissue in its concentration of fatty acids (Yehuda, Rabinovitz et al., 1999). High levels of fatty acids are found in cellular membranes and the myelin sheath of the cortex, which are composed of 50% and 70% of fatty acids respectively (Yehuda, Rabinovitz et al., 2005) and they appear to be vital to the structure and function of neural tissue. The nature of essential fatty acid (EFA) metabolites, and their importance and function in the brain, are described in this chapter.

2.1 Common fatty acids and nomenclature

Fatty acids are major components of brain lipids. They consist of hydrocarbon chains of different lengths, terminating in a methyl group at one end and a carboxyl group at the other (see Figure 2-1). Most naturally occurring fatty acids consist of an even number of carbon atoms. The number of intermediate carbon atoms vary and as such, fatty acids chains can be classified as short (2–4 carbon atoms), medium (4–6) or long (6–10 and greater).

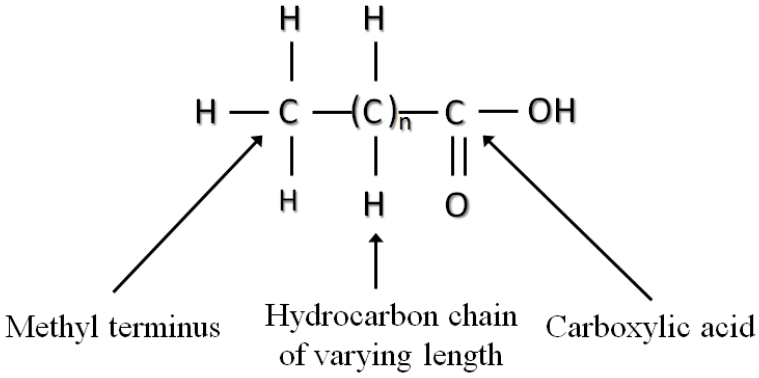

Figure 2-1 The basic structure of a fatty acid

The term ‘saturation’ refers to a chemical structure in which each carbon atom in the fatty acyl chain is bound to (‘saturated with’) four other atoms. Saturated fatty acids have no double bonds, mono-unsaturated fatty acids have one double bond and polyunsaturated fatty acids have two or more (see Figure 2-2, page 26). For example,

oleic acid (18:1) has 18 carbon atoms and one double bond. The location of the first carbon atom, where the first double bond appears when counting from the methyl end of the molecule, is designated by the omega or n number. The main types of polyunsaturated fatty acyl chains found in mammalian membrane lipids can be categorised into three main families based on the location of the first double bond, namely n-3, n-6 and n-9 (see Figure 2-2).

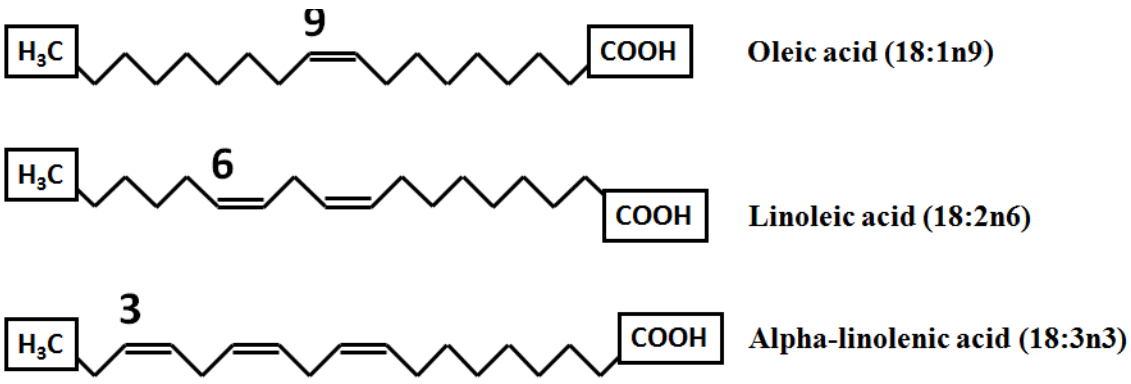

Figure 2-2 Examples of structural formulas for omega-9, -6 and -3 fatty acids

2.2 Fatty acid biosynthesis

The availability of EFA precursors and the action of metabolic enzymes are crucial to the synthesis of highly polyunsaturated fatty acids (PUFAs). The mechanisms of fatty acid metabolism have been reviewed in depth by Sprecher (2000) and will be briefly summarised here.

Higher animals are unable to synthesise *de novo* neither omega-6 or omega-3 long-chain PUFAs as they lack the capacity to introduce double bonds at the n-6 and n-3 positions from the carbonyl end of oleic acid (Calvani and Benatti, 2003). As such, they are dependent on dietary sources of EFA precursors, such as linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3), to meet their physiological needs for these families of fatty acid (Simopoulos, 2000; Sprecher, 2000). Figure 2-3, page 27, adapted from de Groot (2003), is a schematic representation of the major pathways of fatty acid metabolism and illustrates the synthesis of long-chain PUFAs. Table 2-1, page 28, lists the principal fatty acids with their common, chemical and abbreviated designations.

Figure 2-3 Schematic representation of the major pathways of fatty acid metabolism, adapted from de Groot (2003)

Table 2-1 Fatty acids with their common, chemical and abbreviated designations

Common name	Abbreviation	Systematic name	Omega-reference
Omega-9			
Mead		all-cis -5,8,11-eicosatrienoic	20:3n-9
Omega-6			
Linoleic	LA	cis,cis-9,12-octadecadienoic	18:2n-6
γ-linolenic	GLA	all-cis-6,9,12-octadecatrienoic	18:3n-6
dihomo-γ-linolenic	DGLA	cis,cis,cis--8,11,14-eicosatrienoic	20:3n-6
arachidonic	AA	all-cis-5,8,11,14-eicosatetraenoic	20:4n-6
Osbond		all-cis-4,7,10,13,16-docosapentaenoic acid	22:5n-6
Omega-3			
α-linolenic	ALA	all-cis-9,12,15-octadecatrienoic	18:3n-3
eicosapentaenoic	EPA	all-cis-5,8,11,14,17-eicosapentaenoic	20:5n-3
clupanodonic	DPA	all-cis-7,10,13,16,19-dicosapentaenoic	22:5 n-3
docosahexaenoic	DHA	all-cis-4,7,10,13,16,19-docosahexaenoic	22:6n-3

Saturated fatty acids can be synthesised *de novo* from Acetyl coenzyme A. The liver is the most important organ for such synthesis, which takes place both in mitochondria and in the endoplasmic reticulum. Reactions are catalysed by several enzymes that form a multi-enzyme complex known as fatty acid synthetase. The end product of these reactions would normally be palmitic acid (16:0). Double bonds are formed in desaturation reactions catalysed by a site-specific enzyme, represented by Δ. The numbering for the site of action of these enzymes is taken from the position of the double bond in relation to the carboxyl end of the molecule.

LA and oleic acid compete for the same Δ6-desaturase in the metabolic cascade. The desaturase enzymes show a preference for the different fatty acid series, in descending order from n-3 to n-6 to n-9. Oleic acid is not an EFA, as in animals, including humans, there is the capacity to introduce a double bond, or desaturate at the Δ9 position in saturated stearic acid. There is little requirement for saturated fatty acid synthesis in humans because the dietary supply is usually adequate.

Omega-6 and omega-3 fatty acids share metabolic pathways (see Figure 2-3, page 27) and thus interact with each other through a complex system involving several factors: dietary substrate availability, competition for the same metabolic enzymes for synthesis and membrane incorporation, and negative feedback of the end products.

2.2.1 Omega-6 fatty acid synthesis

In the omega-6 pathway, LA is converted to γ-linolenic acid (GLA, 18:3n-6), a positional isomer of ALA. GLA, in turn, can be converted to the longer chain arachidonic acid (AA, 20:4n-6). The activity of the desaturation/elongation pathway in the liver is the most important in terms of supply of long-chain omega-3 PUFAs to other tissues. The initial conversion of ALA to stearidonic acid by the action of Δ6 desaturase is the rate-limiting reaction of the n-3 pathway. The activity of the Δ6-desaturase is slow and can be further compromised by nutritional deficiencies and inflammatory conditions. Therefore, the maximal capacity for synthesis of AA occurs with GLA, the product of the Δ6-desaturase. GLA is converted to dihomo-γ-linolenic acid (DGLA) and then to AA. Like the Δ6-desaturase, the activity of the Δ5-desaturase is limiting in AA synthesis and its activity is also influenced by diet and genetic factors (Simopoulos, 2010). The metabolic pathway of omega-6 fatty acid synthesis is illustrated in Figure 2-4.

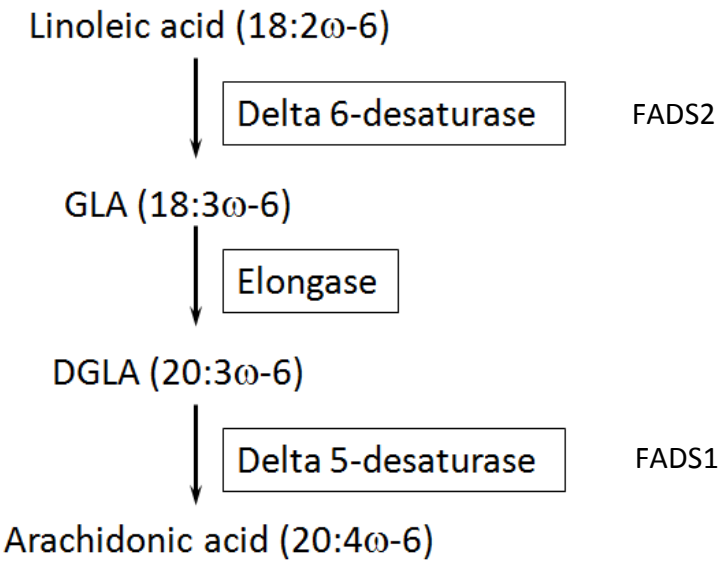

Figure 2-4 The metabolic pathway of omega-6 fatty acid synthesis.
FADS: Fatty acid desaturase enzymes (1 and 2) which are responsible for catalysing the conversion of fatty acids.

2.2.2 Omega-3 fatty acid synthesis

The introduction of a double bond at the Δ6 position is followed by the addition of two carbons by elongation and then by desaturation at the Δ5 position by Δ5 desaturase to form EPA. DPA (22:5n-3) is synthesised from EPA by the further addition of two carbons, and is then converted into DHA (22:6n-3) by further chain elongation, Δ6-desaturation and peroxisomal β-oxidation. The metabolic pathway of omega-3 fatty acid synthesis is illustrated in Figure 2-5. All reactions occur in the endoplasmic reticulum, with the exception of the final reaction, which results in the formation of DHA. Although ALA can serve as the precursor for EPA and DHA synthesis in humans, the rate of conversion of dietary ALA to DHA in the body is very low (Pawlosky, Hibbeln et al., 2001), and varies between individuals. Therefore, direct dietary intake of omega-3 fats rich in EPA and DHA is of the most benefit to compensate for the potentially suboptimal levels derived solely from endogenous metabolism of ALA precursors.

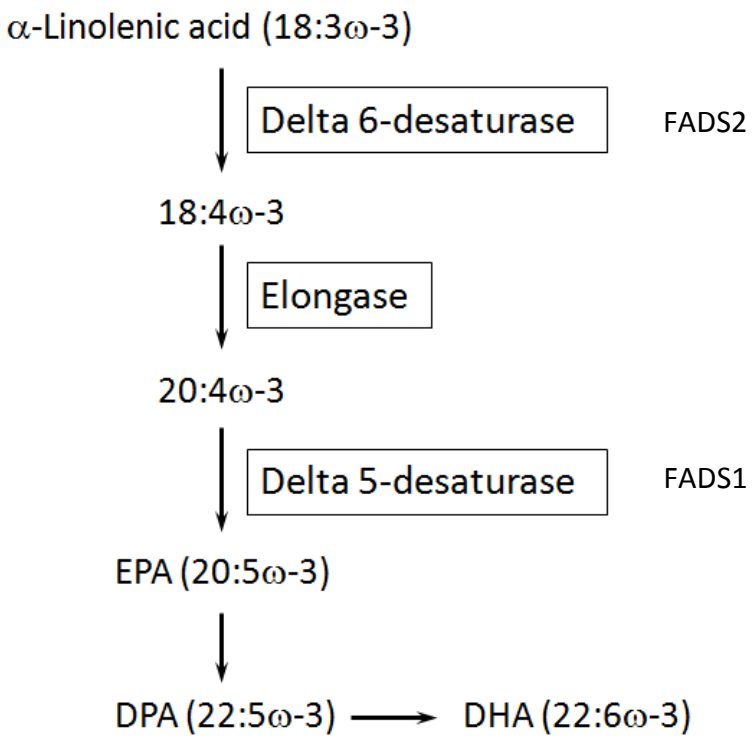

Figure 2-5 The metabolic pathway of omega-3 fatty acid synthesis.
FADS: Fatty acid desaturase enzymes (1 and 2) which are responsible for catalysing the conversion of fatty acids.

2.2.3 Recommended and actual dietary intake of essential fatty acids

It is now accepted that it is important to consider the functions of the different types of fatty acids (omega-3, -6, and -9) rather than simply the amount of total fat or polyunsaturated fat. Fatty acids other than the EFAs can be synthesised endogenously, however, the major source is from dietary fat, which accounts for 25–50% of the energy content of most diets (Heird and Lapillonne, 2005).

Healthy adults have approximately 1kg of LA stored in adipose tissue, yet adipocytes contain hardly any ALA or long-chain PUFAs (LCPUFA). Recommendations regarding adequate dietary intake of EFA and LCPUFA (generally expressed as g/day–1 or as a percentage of total energy intake), are highly variable between countries, and vary with age (i.e. with adipose tissue stores and growth rate). The International Society for the Study of Fatty Acids and Lipids (ISSFAL) has produced a number of statements which deal with recommendations for dietary intake of PUFAs throughout life, which are summarised in Figure 2-6, adapted from Alessandri, Guesnet et al. (2004).

Figure 2-6 Recommended dietary intakes for omega-3 fatty acids (g/day), adapted from Alessandri, Guesnet et al. (2004).

The estimated daily intake of omega-3 PUFA in western countries varies greatly, ranging between an intake of 15/1 to 16.7/1 omega-6 to omega-3, compared to a ratio of closer to one based the diets humans evolved eating (Simopoulos, 2000). DHA intakes largely depend on fish consumption, which can differ greatly between countries (Welch, Lund et al., 2002), and has been related to the quality and length of education and degree of attention paid to maintenance of a healthy diet (Johansson, Solvoll et al., 1998).

The nutritional demands for DHA in healthy adults are likely to be modest, as they reflect principally the need to supply DHA to support turnover and re-synthesis of cell membranes. The actual intake is often under the recommended amount, for example, the mean intake of ALA rarely reaches that which is recommended (Johansson, Solvoll et al., 1998). Table 2-2 summarises the various international recommendations on dietary PUFA intake (ISSFAL, 2010), which include an intake of 2% of total energy intake for LA and 0.7% for ALA for adults.

Difficulty in estimating recommended intakes, particularly for infants, arises from three principal issues identified by Gibson and Makrides (2000). Firstly, LCPUFAs can be synthesised from precursor fatty acids; secondly, plasma omega-3 LCPUFA concentrations representing deficiency and sufficiency are not clearly defined; thirdly, there are currently no recognised clinical tests for omega-3 LCPUFA deficiency and sufficiency.

The importance of breast milk as the sole natural source of omega-3 fatty acids, as well as the omega-6 fatty acids to support the growth and development of the breastfed infant, is well documented (Innis, 2000; Innis, 2004; Innis, 2007; Koletzko, Agostoni et al., 2001; Koletzko, Lien et al., 2008). Breast milk from women following western diets generally contains 10% to 17% LA, 0.8% to 1.4% ALA, 0.3% to 0.7% AA, and 0.1% to 0.5% DHA (Innis, 2003). Concentrations are largely dependent on maternal dietary intake (Innis, 2004) and varies with dietary habits and geographic region (Yuhas, Pramuk et al., 2006). For example, DHA can be up to 1% of total milk lipids in cases where fish is a major food source.

The IFFSAL report summarises the general consensus that the total fat content of infant formulas falls within a range of 4.4–6.0 g/100 kcal (equivalent to about 40–54% of energy content), which is a value consistent with that typically found in breast milk, and which is taken as the gold standard. A summary of recommendations for EFA intake for full-term infants is found in Table 2-2, page 34, adapted from (ISSFAL, 2008). For LA, the level accepted in formulas ranges from around 6% to as much as 25–30% of total fatty acids. In general, the minimum acceptable level of LA is around 3% of total energy. The low level of ALA in breast milk (usually less than 0.2% of total fat) has prompted agencies to make recommendations for minimum requirements.

Table 2-2 Summary of the international recommendations on dietary polyunsaturate intake, adapted from ISSFAL (2010)

Source	Date	n-6:n-3 ratio	Other specific recommendations (%en = percentage of daily energy intake)
National Nutrition Council of Norway	1989	none	0.5%en n-3 LCPUFA (1-2g /day)
NATO workshop of -3/-6	1989	none	0.8g/day EPA/DHA (0.27%en)
Scientific Review Committee of Canada	1990	5:1-6:1	n-3 at least 0.5%en
British Nutrition Foundation Task-force	1992	6:1	EPA 0.2-0.5%en; DHA 0.5%en
FAO/WHO Expert Committee on Fats and Oils in Human Nutrition	1994	5:1-10:1	Consider pre-formed DHA in pregnancy
UK Committee on Medical Aspects of Food Policy (COMA)	1994	none	Minimum intake EPA/DHA 200mg/day
Ad Hoc Expert Workshop	2000	none	EPA+DHA -.03%en; 0.65g/day minimum
France: AFFSA, CNERNA & CNRS	2001	5:1	500mg n-3 LCPUFA/day; DHA 120mg minimum
US National Academy of Science/ Institute of Medicine	2002	none	130-260mg EPA+DHA/day
American Heart Association	2002	none	If no coronary heart disease, eat oily fish twice a week
UK Scientific Advisory Committee on Nutrition (SACN)	2004	none	Minimum intake EPA/DHA 450mg/day
ISSFAL	2004	none	500mg n-3 LCPUFA/day
Australia and New Zealand Government Recommendations	2005	none	LC n-3 for men: 160mg/day; for women: 90/mg/day
Superior Health Council of Belgium	2006	none	Minimum 0.3%en EPA+DHA for adults
Health Council of the Netherlands	2006	none	450mg n-3/day

2.3 Physiological functions of EFAs

The cell membrane provides structure for cells and organelles. For neuronal cells, it maintains the internal and external physiochemical properties fundamental to the propagation of action potentials and the general functioning of the cell (Alberts, 2002). Mammalian cell membranes consist of a bilayer composed primarily of lipids (phospholipids and cholesterol) embedded with protein receptors, transporters, and enzymes. Figure 2-7, adapted from Sum (2005), provides a schematic representation of the phospholipid membrane that encapsulates all mammalian neurons.

Figure 2-7 Schematic representation of a phospholipid cell membrane, adapted from Sum (2005)

Phospholipids are one of the principal lipid components of the membrane. Each phospholipid has a glycerol 3-carbon backbone with a phosphorous attached at the 3 position, to which one of five possible 'head' groups are attached (choline, ethanolamine, inositol, glycerol and serine). These five types of phospholipid make up over 80% of total phospholipids (Reddy, Keshavan et al., 2004). A more detailed account of the structure of the various phospholipid species is provided by Christie (1982). Each phospholipid type consists of a large number of fatty acid chains, attached to the 1- and 2-carbon atoms in the glycerol backbone, which governs the functional properties of the phospholipid, such as its conformational shape (see Figure 2-8).

Figure 2-8 Schematic illustrating phospholipid membrane fluidity conferred by LCPUFAs, adapted from Nelson and Cox (2000)
Red squares signify double bonds which result in a 'kinked' structure

As an integral component of cell membranes, fatty acids exert a multitude of effects not only on phospholipid membrane structure and function, but also on enzymes and receptors embedded in the membrane and through localised action as precursors for localised hormone-like factors. Some of these effects are briefly described below.

2.3.1 Membrane fluidity

Both the chain length and the number of double bonds of the fatty acyl chains that constitute membrane phospholipids have substantial and significant effects on the dynamic properties of the membrane, such as its fluidity, permeability and rigidity (Hac-Wydro and Wydro, 2007). Saturated fatty acids, for example stearic acid (18:0), have straight carbon chains that cause relatively solid regions in the membrane. In contrast, PUFAs, such as oleic acid (18:1n-6), LA (18:2n-6) and ALA (18:3n-3), have a cis configuration at each double bond that produces 'coiling' of the hydrocarbon backbone resulting in a reduction in fatty acid length and a more curved, or 'kinked' structure that increases the fluid properties of the membrane (Figure 2-8, adapted from Nelson and Cox (2000)). DHA (six double bonds) and EPA (five double bonds) confer the greatest level of fluidity from the major membrane fatty acids (Feller, Gawrisch et al., 2002; Gawrisch, Eldho et al., 2003). As a rule, the more fluid a membrane, the more efficient its biochemical performance (Else and Hulbert, 2003).

Alterations in the fluid properties of the lipid membrane can affect the conformation of integral membrane proteins which in turn modulates their function. The most detailed characterisation of direct PUFA–protein interactions comes from studies on rhodopsin (Grossfield, Feller et al., 2006), which show that DHA acyl chains specifically bind to the protein and directly modifies its conformational state.

2.3.2 Myelination

The myelin sheaths surrounding axons of the central and peripheral nervous systems are specialised extensions of glial cells, with unique morphological and biochemical properties related to axon protection and impulse conduction. The integrity of the myelin is of the utmost importance for the proper functions of axons in the nervous system. Myelination improves the connectivity of the brain, facilitating the synchronous integration of information across the many spatially segregated associative neocortical regions involved in higher cognitive functions (Nicholls, Martin et al., 2001).

Lipids constitute around 70% of the myelin sheath, and dietary fatty acids are positively involved in the control of myelinogenesis (Di Biase and Salvati, 1997). During maturation, a shift from short chain saturated fatty acids to the long chain unsaturated forms has been observed (O'Brien and Sampson, 1965; Svennerholm, Vanter et al., 1978). In rats fed with diets rich in polyunsaturated omega-3 fatty acids, decreases in the relative amount of myelin basic protein (MBP) and a CNPase activity indicate a delay in myelin deposition and/or an instability of its structure (Di Biase and Salvati, 1997), whilst acceleration of myelinogenesis can also be induced by dietary lipids (Salvati, Sanchez et al., 1996).

2.3.3 Ion Channels

Fatty acids are important direct modulators of ion function, both through modulation of membrane parameters such as fluidity (Leaf, Xiao et al., 2002), but also through direct action on channel proteins (Ordway, Singer et al., 1991; Tillman and Cascio, 2003). It is not only the presence of specific lipids, but the particular combination of lipids which

are necessary for ion channels to exhibit native properties (le Maire, Champeil et al., 2000).

For example, Ehringer, Belcher et al. (1990) have shown that DHA has a more pronounced effect on membrane ion permeability than LA, despite largely similar effects of both fatty acids on membrane fluidity. In terms of direct action on ion channels, DHA has been shown to have a facilitatory effect on NMDA-glutamate receptors in rat pyramidal neurons (Nishikawa, Kimura et al., 1994), whilst in CA1 neurons isolated from the rat hippocampus, both EPA and DHA had an inhibitory effect on sodium and calcium currents by inducing a shift in the inactivation response observed in more negative potential membranes (Verlengia, Gorjao et al., 2004). AA has been shown to modulate secretory chloride channels (Hwang, Guggino et al., 1990) and specific classes of K^+ channels have also been shown to be reversibly opened by AA, with a dependence on PUFA carbonyl chain length (Fink, Lesage et al., 1998).

Whilst there is a wealth of evidence suggesting the modulatory role of PUFAs in ion channel function, Tillman and Cascio (2003) stress the difficulty of assigning the causes of the observed effects of fatty acids due to the multiple, inter-dependant and complex factors which govern the interaction ion channels and their lipid environment.

2.3.4 Neurotransmitter function

The decreased DHA in the brain of animals fed an ALA-deficient diet during development is accompanied by altered metabolism of several neurotransmitters, including dopamine and serotonin, and membrane-associated enzyme and receptor activities (Innis, 2007). Chalon recently summarised investigations of chronic omega-3 fatty acid deficiency and neurotransmission in rodent models (Chalon, 2006) (where the these effects best described), and report complex, multi-factorial modulations, including synthesis, storage, release, and receptor-mediated uptake, with effects also differing between regions of the cortex.

2.3.5 Eicosanoids and immune function

Whilst PUFAs are widely understood for their role in cell membrane structure and function as described above, they have an important secondary role as eicosonoid precursors. Eicosanoids are hormone-like autocrine or paracrine factors such as leukotrienes, prostaglandins, and thromboxanes that exert local effects by mediating processes such as constriction or relaxation of endothelial cells, platelet aggregation, leukocyte activation and chemotaxis, and consequently modulate the immune response (Shaikh and Edidin, 2008).

AA is the precursor of 2-series prostaglandins and 4-series leukotrienes, which are highly-active mediators of inflammation and generally pro-inflammatory and pro-aggretory. Factors derived from EPA, however, perform competitive functions that decreases the production of several substances, including cytokines, interleukin 1β (IL-1β) and tumour necrosis factor α (TNF-α), leading to a predominantly anti-inflammatory state (Calder, 2002; Calder, 2006; Simopoulos, 2002b). EFA-derived eicosonoid precursors are also involved in the brain in oxidative stress, memory and learning, and potentially in neuropsychiatric disorders such as depression (Tassoni, Kaur et al., 2008). Figure 2-9 summarises some of the physiological effects of essential fatty acids.

Figure 2-9 Summary of physiological functions modulated by EFAs, adapted from Horrocks (2003)

In summary, the effects of EFAs are multifactoral and are also specific to individual classes of fatty acid. EFAs and their PUFA metabolites have important roles not only at the membrane level, but also through their involvement in inflammatory processes,

both of which may be important in understanding the modulatory role of fatty acids on cognition in health and in disease.

2.4 Essential fatty acids and cognitive ability

2.4.1 Essential fatty acids and brain composition

Within the brain, four EFAs are particularly important: DHGLA (23:3n-6) and AA of the omega-6 series, and EPA (20:5n-3) and DHA (22:6n-3) of the omega-3 series (Sprecher, 2000). Between them, these four fatty acids make up 15–30% of the dry weight of neuronal tissue, with AA and DHA amounting to approximately 80–90% of that total (Horrobin, 1998). Figure 2-10 illustrates the routes whereby the EFAs required for normal brain function reach brain phospholipids.

Figure 2-10 The routes whereby the EFAs required for normal brain function reach brain phospholipids, adapted from Horrobin (1998)

Individually, DHA constitutes 10–20% of total fatty acid composition, compared ALA, EPA and DPA, which comprise <1% (McNamara and Carlson, 2006). DHA has also been reported

to constitute as much as 15–25 mol% of the lipids of the grey matter of the human brain (Huber, Rajamoorthi et al., 2002). The presence of DHA chains in mixed-chain phospholipids leads to a marked increase in the fluidity of the saturated chain (see section 2.3.1), with the 'kinked' structure of the fatty acid chains able to influence permeability properties of the bilayer (Huber, Rajamoorthi et al., 2002),

DHA accumulates specifically in phosphatidylserine in brain cortex and hippocampus and plays a regulatory role in membrane phospholipid homeostasis (Kim, 2007). This relatively specific tissue distribution of DHA gives some indication of a possible important role in the membranes of these tissues. Figure 2-11, adapted from Stillwell and Wassall (2003), illustrates the incorporation of DHA into the phospholipid membrane and its association with cholesterol and trans-membrane proteins.

Figure 2-11 The incorporation of DHA (22:6n-3) into the membrane phospholipid bilayer, adapted from Stillwell and Wassall (2003)

2.4.2 The impact of EFA deficiency on cognitive ability

The plasma membrane is a primary interface between exogenous influences (for example, diet) and endogenous control over the biosynthesis or utilisation of varied substrates. The physical properties of the individual lipid components in the membrane are integral to the characteristics of the lipid matrix. The specific organisation and lipid composition of membranes affect their physicochemical properties and determine their proper functioning. It is therefore of interest to consider whether dietary fatty acid balance modulates the lipid composition of the plasma membrane, thereby having potential to modify control functions at this cellular interface, which have identifiable cognitive outcomes.

One of the best ways of identifying the importance and functions of EFAs is to study the consequences of EFA intervention. There are three principal methods typically employed: (1) animal studies, which involve controlled dietary deprivation of EFAs with comparison to untreated offspring; (2) observational breast feeding studies comparing breastfed vs non-breastfed children (EFA-deficient formula milks); and (3) randomised controlled trials comparing children fed formulas either supplemented or unsupplemented with EFAs such as DHA.

EFAs and their LCPUFA metabolites, omega-6 fatty acids (n-6) and the omega-3 fatty acids (n-3), are crucial for the normal function and development of human and animal cells. The functional importance and mechanisms of action of omega-3 fatty acids on brain function have been extensively reviewed (Alessandri, Guesnet et al., 2004; Benatti, Peluso et al., 2004; Salem, Litman et al., 2001; Simopoulos, 2000; Sinclair and Wesinger, 2004; Wainwright, 2002; Wurtman, 2008; Yehuda, Rabinovitz et al., 1999; Yehuda, Rabinovitz et al., 2005). A summary of findings relating the importance of EFAs to cognitive outcomes, using the three methods outlined above, will be briefly described here.

2.4.2.1 Animal models of essential fatty acid deficiency

A main advantage of animal studies is that they afford the opportunity for more flexibility in design and in the ability to control experimental variables than can be achieved in human studies. Animal studies with different proportions of PUFAs in the diet have proved fruitful in identifying broad dietary requirements for maintaining optimal brain function (discussed in section 2.2.3, page 31) and have demonstrated that metabolic and behavioural defects arise from severe long-term omega-3 PUFA dietary deprivation. This allows the physiological effects of these fatty acids to be magnified and the behavioural consequences to be more easily identified. Feeding the newborn animal with milk formulas low in ALA reduces DHA and increases AA and osbond acid (DPA; 22:5n-6) in the brain, brain synaptic membranes and retina (Ward, Huang et al., 1998).

Levels of DHA and EFA precursors seem to be crucial for normal cognitive function as deviation from its physiological level is associated with cognitive impairment, including impaired visual evoked potential and disturbances of cognition, including deficits in frontal cortex-dependent working memory, hippocampus-dependent spatial learning and elevated indices of anxiety and depression (reviewed by McNamara (2006)).

The latest work in rat models of omega-3 deficiency have found deficits in spatial reversal learning that may be related to changes in dopamine transmission in critical brain circuits such as frontal cortex and hypothalamus (Fedorova, Hussein et al., 2009) and delay in the development of parameters related to glutamate transmission, which manifests as increases in memory impairment and anxious behaviour in adulthood (Moreira, Knorr et al., 2010).

Compared to a DHA and EPA diet, omega-3 deficient and low LA diets also caused a substantial deficit in prepulse inhibition (PPI) in rats, whereas the high LA diet induced a less pronounced, but significant reduction of PPI (Fedorova, Alvheim et al., 2009). Deficits of PPI manifest in the inability to filter out the unnecessary environmental information and have been linked to abnormalities of sensorimotor gating.

Regionally specific effects of DHA have also been observed with relation to learning performance in rats. Hippocampal DHA levels were observed to increase from 12 to 15% in supplemented rats, accompanied by improved water-maze learning memory performance (Chung, Chen et al., 2008), whilst others have observed that chronic administration of DHA may be conducive to the improvement of reference memory-related learning ability, which may be related to the ratio of DHA to AA in hippocampal tissues (Gamoh, Hashimoto et al., 1999).

Whilst there is an abundance of research with rodent models, there have been few investigations of non-human primate models of omega-3 deficiency and behaviour. In rhesus monkeys, chronic dietary deficiency in omega-3 fatty acids has been associated with deficits in visual acuity and electroretinogram abnormalities, which represent defects in photoreceptor function in rod and cone cells (Anderson, Neuringer et al., 2005), as well as deficits in visual attention processes, related to attention to novel stimuli (Reisbick, Neuringer et al., 1997), and increased stereotype behaviour (Reisbick, Neuringer et al., 1994). The extrapolation of animal models, particularly from rats to human infants, should be done with caution as omega-3 fatty acid depletion is typically more severe and the animals have often been depleted for more than one generation.

2.4.2.2 Human infant models of essential fatty acid deficiency

Results of intervention trials assessing the cognitive effects of omega-3 deficiency in humans have been variable, and the experimental protocols less rigorous, because of ethical considerations such as the potential of long-term dietary restriction, and indeed supplementation, for inducing long-term irreversible damage.

Much of the support for the importance of EFA intake in early life comes from observational studies of breastfeeding data. The importance of EFAs in infant nutrition is confirmed by the rapid accumulation of these fatty acids in the brain during the first postnatal year (Martinez, 1992) and last intrauterine trimester. After birth, infants are reliant on maternal breast milk (or formula) as the sole source of DHA, with substantial accumulations of DHA and AA in the human brain during the first postnatal months (Heird and Lapillonne, 2005).

Although infants are able to synthesise DHA *de novo*, the amount produced may be inadequate to support the DHA levels observed in breastfed infants. The cerebral and overall DHA status of breastfed babies is better than that of infants fed formula lacking DHA (Cunnane, 2000), with the accumulation of DHA and phosphatidylserine (PS) during development required to prevent inappropriate cell death and to support neuronal differentiation (Kim, 2007). Breast-fed infants are also uniquely provided with an additional digestive enzymes which are essential for the complete hydrolysis of triacylglycerols containing arachidonic acid or DHA (Chen, Blackberg et al., 1994).

Feeding infant formulas devoid of preformed LCPUFA has been associated with decreased brain DHA and AA contents and with transiently impaired neurological maturation (Lauritzen, Hansen et al., 2001). The effect of postnatal omega-3 fatty acid deficiency on neurocognitive development of full term infants has previously been reviewed in detail (Carlson and Neuringer, 1999; Gibson, Neumann et al., 1996; McCann and Ames, 2005; Uauy, Hoffman et al., 2001), and include for example, delayed VEPs for retinal function, preferential looking activity, and means-end problem solving Findings from animal studies have been extended to understanding of the impact of EFA deficiency in human infancy (Innis, 2000), with findings replicated in infants fed formulas deficient in omega-3 fatty acids (Hoffman, Birch et al., 2000; Hornstra, 2000), see Lauritzen, Hansen et al. (2001) and Uauy, Mena et al. (2000) for reviews.

Because of the high concentrations of omega-3 and -6 fatty acids in neural tissue membranes, all domains of neural and cognitive function are potentially influenced by LCPUFA status. The hypothesis that EFA deprivation should manifest as changes in broad-based measures of cognitive function, such as FSIQ is supported by a meta-analysis of breastfeeding studies conducted by Anderson, Johnstone et al. (1999) which suggests that, after adjustment for appropriate key cofactors, including duration of breastfeeding, socio-economic status and age at gestation, breastfeeding was associated with significantly higher cognitive development scores (3.2 points; $p < .001$) for breastfed compared to formula-fed infants.

More recent evidence from a randomised trial of nearly 14,000 children assessed at 6.5 years of age with the Wechsler Abbreviated Scales of Intelligence (WASI) measures

found that that prolonged and exclusive breastfeeding improves cognitive development (Kramer, Aboud et al., 2008). Kramer et al. concede, however, that the variability of WASI scores and wide confidence intervals in both the experimental and controls groups means that the true magnitude of observed effects is uncertain.

McNamara and Carlson (2006) summarised some of the more specific neurocognitive impairments in formula-fed versus breastfed children: lower visual acuity, slower processing speed on tests of visual recognition memory, deficits in problem-solving and more mature motor movement, problem-solving, and psychomotor function.

A recent review of randomised control trials concluded that those studies with experimental formulas providing DHA close to the worldwide breast milk average of 0.32% were more likely to yield functional benefits, such as improvements in visual function and cognitive ability, than those formulas with less than this putatively optimal amount (Hoffman, Boettcher et al., 2009). For example, in a study considered to be of high-quality in a recent Cochrane review of cognitive outcomes in full-term infants supplemented with LCPUFAs (Simmer, Patole et al., 2008), Birch et al. found that visual acuity in the PUFA-supplemented group was significantly better than in the control group at ages 6, 17, 26, and 52 weeks (Birch, Castaneda et al., 2005).

Tests of human infants in general use standardised global tests that screen broadly for cognitive-related functions, usually the Bayley Scales of Infant Development, which has scales for Mental and Psychomotor Development Index (MDI; PDI). Supplementation of term infant formula milk with 0.36% DHA and 0.72% AA during the first 4 months of life was associated with a mean increase of 7 points on the MDI at 18 months of age over the control formula group (Birch, Garfield et al., 2000). Improvements in MDI have also been observed over shorter periods, with Gibson et al. seeing an improvement of MDI in exclusively breastfed infants at 12 months of age following just 3 months postpartum maternal dietary supplementation (Gibson, Neumann et al., 1997). Improvements on Bayley indices following supplementation have not always been observed when followed up over a similar time period (Ben, Zhou et al., 2004; Makrides, Neumann et al., 2000; Scott, Janowsky et al., 1998).

Taken together, findings from animal models, observational studies of breastfeeding effects and supplementation trials suggest that interference in the accumulation of EFAs by nutritional deprivation has lasting effects on neural functions and developmental processes which influence later cognitive outcomes.

Chapter 3 outlines the converging methods used to investigate the effects of EFAs on cognitive outcomes using a paediatric liver disease model.

3 Methods

The following chapter provides descriptive data for the primary clinical patient population and outlines the three converging analytical methods used: psychometric assessments, GC-MS for assessment of fatty acid status, and ^{1}H-MRS for analysis of *in vivo* brain biochemistry. A brief summary of the background and utility of each technique is followed by details of the procedures and data acquisition parameters used in this study.

3.1 Ethical considerations

Informed consent was obtained from all participants and guardians under a protocol consistent with the tenets of the Declaration of Helsinki and with the approval of the Black Country Research Ethics Committee (08/H1202/38) and the Aston University's Human Subjects Ethics committee (REG/00/175). Written consent was obtained from the participant's parent or guardian. Verbal assent was obtained from children prior to testing. Participants were permitted to withdraw at any stage and were reassured that their withdrawal would not affect the level of clinical care they subsequently received. Oral and written debriefing was given after each session and procedure. Principal researchers had previously obtained Criminal Bureau Enhanced Disclosure for working with children and vulnerable adults. All blood samples were treated in compliance with the Human Tissues Act 2004.

3.2 Participants

Over a two and a half year period (2007–2010) all children under the age of 18 with a diagnosis of liver disease and their healthy siblings were recruited into study from the Liver Unit at the Birmingham Children's Hospital.

3.2.1 Patient exclusion criteria

- Children who cannot undergo neurophysiological testing before liver transplantation (for example fulminant liver failure)
- Recipients of multi-organ transplants and re-transplants
- Children of families who do not consent to the procedure
- Participants with any contraindications for the MR procedure (ferrous metal implants, bone pins etc.) were specifically excluded from the ^{1}H-MRS.

3.2.2 Sibling control exclusion criteria

Sibling control participants were screened prior to testing to exclude the presence of probable neurological dysfunction, including previous serious brain injury, history of learning disability, neurological disease, psychiatric diagnosis or current use of psychoactive medication. Sibling controls with any contraindications for the MR procedure (ferrous metal implants, bone pins etc.) were also excluded from the ^{1}H-MRS portion of the study.

3.2.3 Liver disease categorisation

The liver disease patients were divided into three principal categories, which are outlined in Table 3-1. Descriptive data for the patient and control populations is provided in Table 3-2, page 50.

Table 3-1 Categorisation of liver disease patients

Disease category	Criteria for classification
Early-onset, pre-transplant	Patients with stable, well compensated congenital liver disease, who did not require transplantation, or were on the transplant waiting list at the time of participation in the study.
Early-onset, post-transplant	Patients with severe congenital liver disease who had undergone liver transplantation prior to participation in the study.
Acute liver failure, post-transplant	Patients who developed postnatal liver disease after two years of age, usually presenting with acute liver failure, who had undergone liver transplantation prior to participation in the study.

3.2.4 Descriptive data for the sibling control and liver disease groups

Table 3-2 Descriptive data for the sibling control and liver disease groups

Group	Total n	Mean age (years)	SD age (years)	M:F	Mean onset age (years)	n	Diagnoses
Sibling controls	11	12.2	5.08	5:6			
Early-onset liver disease, pre-transplant	17	11.8	5.08	6:11	-	9	Extra-hepatic biliary atresia
						1	Alpha 1-antitrypsin (A1AT) deficiency
						5	Progressive familial intra-hepatic cholestasis
						1	Neonatal haemochromatosis
						1	Alagille's syndrome
Early-onset liver disease, post-transplant	8	15.5	3.2	3:5	-	2	Progressive familial intra-hepatic cholestasis
						2	Extra-hepatic biliary atresia
						1	Aegeneas Syndrome
						2	Neonatal liver failure
						1	Alpha 1-antitrypsin (A1AT) deficiency
Acute liver failure, post-transplant	6	13.7	3.7	2:4	5.4	1	Autoimmune hepatitis
						1	Fulminant hepatitis A infection
						1	Wilson's disease
						3	Sero-negative hepatitis

The three principal methods employed in this study (psychometric assessment, blood sampling for GC-MS analysis of erythrocyte EFA status and ^{1}H-MRS) were routinely administered in a single day. Due to the young age and nature of the patient's conditions, successful completion of all three modes of assessment was not always possible. Specific descriptive data of the number and composition of each sample group with the appropriate data available is provided in each of the separate analyses in Chapters 5 and 7.

3.3 Psychometric assessments

Cognitive ability was assessed using the following age-appropriate Wechsler scales, depending upon the age of child at testing:

- Wechsler Preschool and Primary Scale of Intelligence for Children – 3rd Edition (WPPSI-III), for children aged between 2 years 6 months and 7 years 3 months (Wechsler, 2002).
- Wechsler Intelligence Scale for Children – 4th Edition (WISC-IV), for children aged between 6 years 0 months and 16 years (Wechsler, 2003).
- Wechsler Adult Intelligence Scale – 3rd Edition (WAIS-III) or the Wechsler Abbreviated Scale of Intelligence (WASI), for those aged 16 years and over (Wechsler, 1997a; Wechsler, 1997b).

The Wechsler scales provide a measure of general cognitive ability across both verbal and non-verbal dimensions. FSIQ is an estimate of overall IQ based on performance across 11 subtests. Separate estimates of verbal and performance IQ contribute to FSIQ; the Verbal dimension reflects language-mediated skills and the Performance dimension reflects non-verbal, visual-spatial and visuo-motor skills. In addition to the IQ scores, the Wechsler scales also yield sub-scores across a number of processing dimensions, such as the Information Processing Speed (IPS) index (aka Processing Speed Index), which is derived from the Symbol Search and Coding subscales. The IPS index is taken as an indicator of the mental and motor speed required to solve visuo-spatial problems as contributions of higher cognitive functions to task performance are minimised (Groth-Marnat, Gallagher et al., 2000).

Each of the Wechsler tests yields IQ indices with a population mean of 100 and standard deviation of 15. Performance on each of the individual subscales has a mean of 10 and standard deviation of 3. All standard scores can be converted to z-scores to enable comparison between standard scores with different population means and standard deviations.

The WAIS, which consists of 6 subtests, was administered solely in the healthy adult population studied in Chapter 6. The WASI has high reliability (.98 for FSIQ in adult samples) and high validity characteristics (r=.92 with WAIS-III FSIQ).

There is some overlap between these tests, with children aged 7 being able to complete the WPPSI or the WISC, and children aged 16 being able to complete the WISC or the WAIS. In cases where the patient fell in the intersecting age range, it was at the discretion of the patient's referring Clinical Psychologist, who had knowledge of the patient's case history and background, which assessment was administered. Different floor and ceiling effects can be achieved using the different tests, allowing for a greater understanding of the child's abilities or deficits. In the current patient sample, the lower age limit was used in all cases: all patients above 6 years 0 months performed the WISC-IV and all patients above 16 years 0 months performed the WAIS-III.

All versions of tests were administered according to the standardised test protocol, in a single session, and within one week of completing the neuroimaging and blood sampling procedures.

3.4 Essential fatty acid status

3.4.1 Erythrocyte biomarkers of fatty acid status

Biochemical analysis of biopsy samples is the traditional method for the determination of metabolite concentrations in tissue. Whilst histological evidence from animal and post-mortem data has revealed a great deal about the basic metabolic processes of the brain, the research has been hampered by the need make inferences of processes occurring in a highly dynamic tissue from either static or remote investigative tools,

revealing two distinct disadvantages: (1) labile metabolites may be altered or destroyed during the tissue extraction procedure and the decomposition products may become more abundant; and (2) tissue biopsies cannot be obtained from healthy, living human brain tissue.

Histological evidence is certainly vital for the development of theory, but *in vivo* or surrogate biomarker techniques have important wider applications in the study of living tissues and individuals. A functional marker may reflect a biochemical (e.g. micronutrient-dependent enzyme activity) or physiological (e.g. cognitive ability) response upon current or imminent micronutrient deficiency. Kuratko and Salem (2009) outline the properties of acceptable biomarkers for fatty acid status: (1) the method of measurement is standardised, specific and sensitive; (2) the biological material used for biomarker determination is easily obtainable; (3) a correlation between the nutrient biomarker and intake of the nutrient is established; (4) the relationship of the biomarker status and nutrient intake is sensitive and specific; and (5) the biomarker status shows an association with important clinical outcome.

Erythrocyte EFA levels are commonly used as an *in vivo* measure of EFA status in human studies, as samples are easily accessible through phlebotomy. Such measures provide a reliable estimate of cellular EFA status, reflecting bone marrow fatty acid availability and the plasma–RBC phospholipid exchange aggregated over the lifespan of the cell, or its 120-day half-life. Erythrocyte EFA has some clear advantages over plasma for EFA assessment. The fatty acid profile of plasma derives from at least four different lipid classes, which are located in various lipoproteins with different functions, origins, targets, turnover rates and inter-individual compositions. In erythrocytes, however, the fatty acids derive solely from plasma membrane phospholipids, which contain the full range of long-chain PUFAs and can be analysed using standard lipid quantification techniques (Arab and Akbar, 2002).

Erythrocyte biomarkers are well-defined with respect to their dietary dependence and correlate with the fatty acid composition of brain. The strength of correlation between dietary intake and biomarker values, however, may vary considerably between individual fatty acids. Factors such as affinities to particular enzymatic pathways and

varying inter-conversion rates means that fatty acids are incorporated with different efficiencies (Katan, Deslypere et al., 1997). It is predicted that biomarkers of the omega-3 and omega-6 PUFAs, such as LA and ALA, would have the strongest association with intake since the inability to generate double-bonds more than nine carbons from the carboxyl or delta end of the fatty acid ensure these PUFA may be derived from diet alone.

EPA levels in cholesteryl esters reflect intake over the past week or two, in erythrocytes over the past month or two, and in adipose tissue over a period of years (Katan, Deslypere et al., 1997). Justification of the use of erythrocyte DHA as a marker for neural DHA comes primarily from the work by Carlson et al., which demonstrated that weanling rats fed a diet with a 240:1 ratio of LA to ALA (where ALA is the precursor to DHA) had lower concentrations of DHA in the phosphatidylethanolamine fraction of erythrocytes, brain cortex and cerebellum, compared to those fed a diet with a ratio of LA to ALA of 7:1 (Carlson, Carver et al., 1986).

In humans, Makrides et al. have shown that erythrocyte DHA is a significant predictor of DHA levels in the cortex by demonstrating that infants who were breastfed had a greater proportion of DHA in both their erythrocytes and cortical tissue relative to those fed formula (Makrides, Neumann et al., 1994). A dietary intervention study of human adults showed that erythrocyte DHA was correlated with dietary intake over a period as short as 3 weeks (r= ~.4) (Poppitt, Kilmartin et al., 2005), confirming its potential as a reliable biomarker. In their summary of DHA biomarkers, Kuratko and Salem (2009) found that circulating DHA appears to show the strongest correlation with brain tissue when assessed in the context of background diet and when correlations are made with DHA as a percentage of total dietary fatty acids instead of absolute values.

In addition to values of individual PUFAs of interest such as LA, ALA, DHA and EPA, biomarkers of EFA deficiency can potentially provide a more subtle analysis of fatty acid metabolism and utilisation. The body compensates for EFA deficiency in two ways: first through enhanced conversion of omega-3, -6, -9 fatty acids to their derivatives, and second through the accumulation of mono-unsaturated fatty acids.

Mead acid (20:3n-9) synthesis (see Figure 2-3, page 27) is ordinarily inhibited by adequate concentrations of ALA and LA. The presence of mead acid, therefore, acts as a functional marker for suboptimal levels of fatty acids of the omega-3 and -6 families (Fokkema, Smit et al., 2002). Similarly, if there is a functional shortage of DHA, the body begins to synthesise the analogous long-chain polyene of the omega-6 family: osbond acid (22:5n-6) (de Groot, 2003). Therefore, under steady state conditions, the ratio between DHA and osbond acid is a reliable indicator of the functional DHA status (Hornstra, 2000). Table 3-4 on page 61 summarises the various biomarkers in erythrocyte membranes used to assess fatty acid status.

3.4.2 Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) is an instrumental technique, comprising of a gas chromatograph (GC) coupled to a mass spectrometer (MS), by which complex mixtures of chemicals may be separated, identified and quantified. The information provided by a GC-MS analysis of fatty acyl chains consists of GC retention times, which can subsequently be compared with known structural properties of various compounds, and the mass spectral data of the corresponding compounds derived from the mass spectrometer. Details of the GC-MS technique have been described by Harris (1999). A detailed discussion of the various chromatographic methods for the assessment of phospholipids in biological samples has been provided by Peterson and Cummings (2006).

3.4.3 Lipid extraction and fatty acid methyl ester derivitisation

3.4.3.1 Reagents

All reagents and solvents (HPLC grade unless otherwise stated in Table 3-3, page 56) were purchased from Fisher Chemicals (Leicester, UK) and Sigma-Aldrich (Dorset, UK). Consumables for the Agilent 6890 GC were purchased from Agilent Technologies (West Lothian, UK).

Polyunsaturated fatty acids autoxidise rapidly when exposed to air; the more double bonds present, the more rapid the rate of oxidisation. Tissues will contain natural antioxidants such as a-tocopherol (Wang and Quinn, 1999), but the levels of these may not be sufficient to prevent deterioration on storage. The synthetic antioxidant butylated hydroxytoluene (BHT; 2,6-di-tert-butyl-*p*-cresol) was therefore added to all solvents used in the extraction and transmethylation process. Addition of BHT does not interfere with chromatographic analysis as it is relatively volatile and can be removed together with the solvents when evaporated under nitrogen. Any residual BHT elutes just after the isohexane peak and ahead of the fatty acid methyl esters (FAMEs). All vials were additionally flushed with nitrogen at various stages in the extraction procedure and before storage at -80°C.

Table 3-3 List of reagents for fatty acid extraction and derivitisation

Reagent	Specific grade
Butylated hydroxytoluene (BHT; 2,6-di-tert-butyl-*p*-cresol).	Technical grade
Chloroform	>99% HPLC certified, Fischer Scientific (+ 50 mg of BHT/litre)
Deionised water	HPLC certified, Fischer Scientific
Ethanol	>99.8% HPLC certified, Fischer Scientific
Hydrochloric acid	Optima Ultra Pure grade, Fischer
Isohexane	>99% HPLC certified, Fischer Scientific
Methanol	99.8+% HPLC certified, Fischer Scientific (+ 50 mg of BHT/litre)
Phosphate buffered saline (PBS)	Molecular Biology grade, 1X solution (0.137M sodium chloride; 0.0027M potassium chloride; 0.0119M phosphates)
Sodium chloride solution	Technical grade; 5% in deionised water

3.4.3.2 Phlebotomy and sample storage

5ml of venous blood was obtained from each participant and dispensed into tubes containing 0.10ml of 15% anticoagulant solution (ethylene diamine tetra-acetic acid EDTA; 15 mg). The sample was centrifuged at 3,000 rpm for 10 minutes with the resulting plasma supernatant and buffy coat discarded. The sample was then washed twice with 5ml PBS at 3,000 rpm for 10 minutes. The RBC precipitate was then

resuspended in 5ml PBS, aliquoted into 2ml glass vials, and sealed under nitrogen and stored at -80 °C until required.

3.4.3.3 Lipid extraction

Prior to any analysis of fatty acids by GC-MS, the compounds of interest must be extracted and split into their constituent fatty acid methyl esters (FAMEs). First, the membrane lipids must be separated from the biological milieu of the blood sample to remove any other constituents such as proteins, sugars or other small molecules that would interfere with the chromatographic analysis. Secondly, before the fatty acid components of lipids can be analysed by GC, it is necessary to convert them to low molecular weight non-polar derivatives, such as methyl esters, through the process of derivitisation.

Various solvents or solvent combinations have been suggested as optimal extractants of lipids from biological samples, but the most widely used are the Folch (Folch, Lees et al., 1957) and the Bligh and Dyer (Bligh and Dyer, 1959) methods. Both methods were trialled and evaluated for efficacy based efficiency and quantity of fatty acid mass extracted from 1ml of RBC sample. Extractions using the Bligh and Dyer method resulted in approximately 3.5±0.5mg of fatty acid per 1ml sample, but by contrast the Folch method was able to extract 5.0±0.5mg per sample. A modified version of the Folch method (described in more detail below) was subsequently adopted as the standard procedure for lipid extraction from whole RBC samples.

After removing the tissue from storage at -80°C the 1 ml suspension of RBC in PBS was brought to room temperature. The sample was transferred to a glass homogeniser and 6ml chloroform containing BHT (0.1% w/v) was added. The mixture was homogenised for 60 seconds before adding 3ml methanol containing BHT (0.1% w/v) and the homogenisation was then repeated. 2ml sodium chloride was added to the extract to aid removal of any non-lipid contaminants and vortexed for 30 seconds. The mixture was centrifuged for five minutes at 3,000 rpm to aid phase separation. The lower chloroform phase contains the lipids, while the upper acidified methanol/water phase contains and

most of the non-lipid contaminants. Protein precipitates are present at the interface between the two layers.

To maximise the removal of contaminants, the upper aqueous methanol layer was discarded and additional wash of 4.5ml methanol/water (1:1 v/v) was added to the remaining chloroform phase, followed by gentle mixing and centrifugation as previously described. The lower chloroform layer containing the purified lipid was transferred using a glass Pasteur pipette to a 10ml glass tube and evaporated to dryness under nitrogen at 25°C using a Techne "Dri-Block" sample concentrator. Evaporation of the final 1ml of solvent was carried out in a pre-weighed 2ml glass vial, which was re-weighed on completion. The weight difference was taken as total weight of lipid extracted. The lipid sample was resuspended in 1ml chloroform, sealed under nitrogen and stored at -80°C.

3.4.3.4 Fatty acid methyl ester derivitisation

The determination of the fatty acids involves their derivitisation to give their corresponding FAMEs. The fatty acids undergo a reflux reaction with excess anhydrous methanol in the presence of an acidic catalyst, leading to the trans-esterification of the ester linked fatty acids, and producing a mixture of fatty acid methyl esters (Christie, 1989).

Purified lipid fractions were trans-methylated by reflux with methylating reagent (2.5% sulphuric acid: 97.5% methanol) at 80°C for 70 minutes. FAMEs were extracted into isohexane (1.5ml) partitioned against water (1ml) following thorough mixing of the phases. The isohexane phase containing the FAMEs was transferred to a 1.5ml glass vial and evaporated to dryness under nitrogen. The FAME were subsequently re-suspended in 100µl isohexane in a glass capillary insert and stored at -80°C, pending GC analysis.

3.4.4 Gas chromatography methods

Gas chromatography is used to separate volatile organic compounds (Harris, 1999). All systems of chromatography consist of a stationary and a mobile phase. The compounds to be separated are placed on a stationary phase, which for lipids is liquid, and then

volatilised and passed through a mobile phase where a stream of inert gas (helium) is allowed to pass through the system. Substances are separated according to their partition coefficients, yielded by the varying physical and chemical properties that impart different affinities for the two phases. The time point from the injection of the sample to that when the maximum amount of each component emerges (i.e. when the peak has reached its maximum height) is known as the retention time of the substance.

The method of gas chromatography is both specific and sensitive for the determination of individual fatty acids, including DHA. Modern capillary columns are highly efficient and provide complete separation of longer-chain fatty acids DHA from other fatty acids (Masood, Stark et al., 2005), for measurement of values as low as 0.01 percent weight.
In the present study, conventional GC analyses were performed on an Agilent 6890N with a DB-23 30m capillary column, coupled with an Agilent Technologies 5973 Network Mass Selective Detector.

Operating conditions were as follows: the split-splitless injector was used in split mode with a split ratio of 1:50. The injection volume of the sample was 1μl. The injector and detector temperatures were kept at 250°C and 270°C respectively. Total run time was 29 minutes per sample. Helium was used as the carrier gas, with a linear velocity of 36cm/sec (average at 160°C). Pressure: 7.64psi; detector gas flows: H_2: 1ml/min; air: 350ml/min; make-up Gas (N_2): 53.8 ml/min. Fatty acid methyl esters were separated by thermo gradient elution 180–240°C. The temperature program is illustrated in Figure 3-1, total run time 29 minutes per sample.

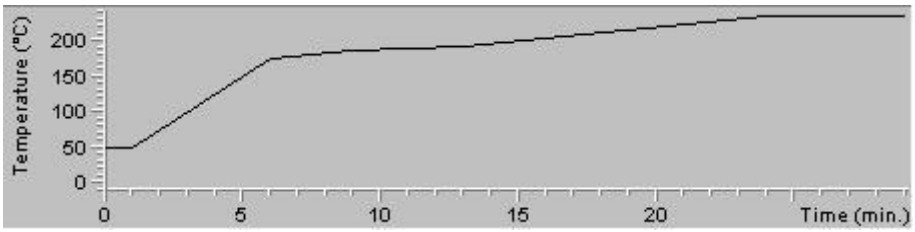

Figure 3-1 GC-MS temperature sequence

3.4.5 Mass spectrometry methods

Mass spectrometry allows qualitative identification of the compounds extracted through chromatography (Harris, 1999). In the mass spectrometer, organic compounds in the vapour phase are bombarded with electrons and form positively charged ions, which can fragment in a number of different ways to give smaller ionised entities. The resulting ions are then passed through a powerful magnetic field and are separated according to their mass to charge ratio (m/z). The resulting mass spectrum displays the relative abundance of each fragment striking the detector of the mass spectrometer. As discussed previously, fatty acids differ not only in chain length, but also in the position of double bonds; the longer the chain length and the greater the number of double bonds, the longer the retention time (Christie, 1982).

Data acquisition and processing were performed on Agilent Technologies 5973 Network Mass Selective Detector standard software for the Agilent GC system. Peak identification was based upon comparison of spectra with known standards in the NIST library software. Having identified the fatty acid components using this method, samples were routinely run on under identical chromatographic conditions, but using a flame ionisation detector (FID), with fatty acid identification made based on retention times.

3.4.6 Fatty acid biomarkers

Fatty acid analysis was limited to a number of individual fatty acids and the four main fatty acid families: saturated (SFAs), monounsaturated (MUFAs), omega-3 PUFAs and omega-6 PUFAs.

Table 3-5 summarises the biomarkers used to evaluate fatty acid status. Values in analyses are reported as percentages of the 12 fatty acids quantified in total.

Table 3-4 The major fatty acids detected in erythrocyte samples with conventional GC-MS

Common name	Carbon number
Saturated fats	
myristic	14:0
palmitic	16:0
stearic	18:0
Monounsaturated fats	
oleic	18:1
Omega-9	
mead	20:3
Omega-6	
linoleic (LA)	18:2
dihomo-γ-linolenic (DHGLA)	20:3
arachidonic (AA)	20:4
Adrenic	22:4
osbond	22:5
Omega-3	
eicosapentaenoic (EPA)	20:5
docosahexaenoic (DHA)	22:6

Table 3-5 Summary of erythrocyte biomarkers of fatty acid status.

Fatty acid biomarkers	Fatty acids
SFA	stearic (18:0n-6) + palmitic (16:0n-6) + myristic (14:0n-6)
MUFA	oleic (18:1n-6)
Omega-3 index	DHA (22:6n-3) + EPA (20:5n-3)
Omega-6 index	arachidonic (20:4n-6) + linoleic (18:2n-6)
Omega-3:Omega-6	Omega-3 index/Omega-6 index
EFA shortage marker	mead acid (20:3n-9)
Functional DHA shortage marker	DHA/osbond acid (22:5n-6)

3.4.7 Fatty acid biomarker reliability analyses

The accuracy and reliability of fatty acid measurements are affected by a number of factors including intrinsic sample variability, sample handling and the extraction and quantification methodology employed (Arab and Akbar, 2002).

To ensure maximum reliability for fatty acid identification, only fatty acids that matched with 98% or greater accuracy with known standards in the NIST library software were included in analyses. To maximise quantification accuracy, all samples were run in duplicate, with the averaged value used in subsequent analyses. In cases where values for individual fatty acids exceeded 10% variance between runs, the sample was run a third time.

3.5 Proton magnetic resonance spectroscopy (^{1}H-MRS)

Modern imaging paradigms, including conventional radiography, computerised tomography (CT), positron emission tomography (PET) and single photon emission computerised tomography (SPECT), now make it possible to study normal brain biochemistry *in vivo*. However, while these techniques may be used with paediatric patient populations when expressly clinically warranted, the ethics of exposing children to the radioactive isotopes required for these techniques solely for the advancement of science are less than clear (Casey and Cohen, 1996).

By taking advantage of the unique electronic structure of molecules to characterise a wide variety of different chemical compounds (Govindaraju, Young et al., 2000), magnetic resonance spectroscopy (MRS) addresses the shortfall of histological methods by virtue of being a non-destructive procedure that does not require parenteral injections nor use radioactive materials evidence, to provide relatively region specific, quantitative, *biochemical* brain data *in vivo*. The physical properties of MRI facilitate investigations of the structure, metabolism and function of the brain in normal healthy individuals, and allow monitoring of progression and recovery in abnormal development.

3.5.1 The magnetic resonance phenomenon

The physics behind the magnetic resonance technique has been described in detail by Freeman (2003) and practical applications discussed by Ross and Bluml (2001). Nuclei that have an odd number of nucleons (protons and neutrons) possess both a magnetic moment and angular momentum (or spin). In the presence of a homogeneous external

magnetic field these nuclei precess around their axis at a rate proportional to the strength of the magnetic field, emitting electromagnetic energy in the process.

In ^{1}H-MRS, a large static magnetic field (B_0) preferentially aligns hydrogen nuclei along the direction of the applied field. In clinical scanners, the strength of B_0 is most often 1.5 or 3 Tesla (T) and is oriented horizontally from head to toe along the long axis of the cylindrical magnet. A pulse of electromagnetic energy is applied at a specific radiofrequency (RF) with an RF coil placed around (or near) the head. The frequency is selected to be the same as the frequency of precession of the imaged nuclei at a given strength of B_0; for hydrogen, this 'resonant' frequency is approximately 127.7 MHz at 3T. The RF pulse rotates the precessing nuclei away from their axes and a receiver coil measures the time it takes for the nuclei to 'relax' back to their original position pointing along B_0. The spatial origin of the signal is determined using subtle position-related changes in B_0 induced by gradient coils.

The signals generated are subsequently amplified and displayed as a sinusoidal wave that decays with time, termed free-induction decay, and it is this transient signal that is amplified electronically, detected and then converted into a spectrum for high-resolution MRS, or an image for MRI (see Figure 3-2, page 65 adapted from Chan (1985)).

3.5.2 ^{1}H-MRS metabolite detection and quantification

A brief overview of the MRS technique is provided below. Refer to Lambert and Mazzola (2004) for a comprehensive description of the physical basis of MRS, Ross and Bluml (2001) and Soares and Law (2009) for a broad discussion of the current understanding and applications, and Rosen and Lenkinski (2007) for a discussion of the most recent technical advances in the technique.

Chemical bonding within a given molecule modifies the shape and density of its outer valence electrons, effectively creating a 'magnetic shield' that dictates the magnetic field of the nucleus. The corresponding change in the nuclear precession frequency is called the chemical shift and it is this parameter that permits identification of the chemical

environment of a given atom and gives rise to the individual peaks seen in MRS spectra. The chemical shift is measured using the dimensionless unit parts per million (ppm) and is the position on the δ scale where the peak occurs. It is defined in absolute terms by the frequency of the resonance expressed with reference to a standard compound, which is defined to be at 0 ppm.

Most atoms have at least one isotope that possesses a magnetic moment, but the most widely used nuclei in biomedical MRS are hydrogen (^{1}H), phosphorous (^{31}P), carbon (^{13}C) and sodium (^{3}Na). ^{31}P-MRS was the first to be applied to medicine in vivo and can be used to evaluate brain metabolic energy (Argov and Chance, 1991). The major motivation for using the proton nucleus (^{1}H-MRS) is that hydrogen has the highest natural abundance in the body, which results in greater sensitivity of MR methods to this particular nuclei, compared with either ^{31}P or ^{13}C.

Observing nuclei other than protons requires the development of radio-frequency coils and other specialised hardware tuned to their specific frequencies; ^{1}H-MRS uses the same hardware, such as head coils, as standard MRI. The boundaries between MRI and MRS are becoming blurred as developments in MRI underline developments in MRS and vice versa. Cox (1996) suggests that to some extent, the proton-derived spectrum can be considered as another contrast parameter in the MRI examination, but adds that this does injustice to the wealth of chemical information available, and motivates the use of MRS as an MR paradigm with specific utility in the present study. Since Frahm, Bruhn et al. (1989) published the first reports of *in vivo* ^{1}H-MRS, in which the methodology used in the detection and measurement of metabolite concentrations in the human brain was described, ^{1}H-MRS has become the dominant tool in cognitive spectroscopy.

Figure 3-2 Simplified explanation of the magnetic resonance phenomenon, adapted from Chan (1985)

The initial intensity of the ^{1}H-MRS signal is proportional to the number of nuclei giving rise to the signal. Protons attached to large macromolecules have short T2 values and are observed as broad, short peaks, whereas protons attached to small metabolites have longer T2 values and are observed as narrow, tall peaks in the proton spectrum. In principle it is the area under the individual peaks of the spectra which is indicative of the concentration of the particular chemical. In practice, however, there are so many variables that influence the signal intensity that absolute quantification is still relatively difficult to achieve (Freeman, 2003; Lambert and Mazzola, 2004).

MRS is generally less sensitive than MRI because the concentrations of atoms measured by MRS are orders of magnitude less than the concentration of hydrogen used in anatomical MRI; only compounds in or near the millimolar range can be detected *in vivo*. Compounds of interest in biological systems are typically in the order of 1–10 mmol/L and are masked by the presence of a large background signal arising from water, which can have a concentration approaching 90 mol/L in protons (Rosen and Lenkinski, 2007). Techniques such as water suppression are therefore employed to minimise these intense proton signals, which would otherwise interfere with detection of the signals of interest (Freeman, 2003).

In the brain, the strongest and most reliable metabolite signals in ^{1}H-MRS are generated by N-acetyl aspartate (NAA), creatine and phosphocreatine (Cre), choline (Cho), and myo-Inositol (mI). These four metabolites, and other reasonably well-resolved compounds such as glutamate/glutamine (Glx) and lactate, form the principal focus of ^{1}H-MRS research (Imamura, 2003; Inder and Huppi, 2000; Lambert and Mazzola, 2004; Rosen and Lenkinski, 2007; Ross and Bluml, 2001). Hunter's Angle is the nominal 45 degree ratio between the major metabolites, deviation from which is taken as an indication of abnormality (Danielsen and Ross, 1999). A typical, representative MRS spectrum is illustrated in Figure 3-3, page 67.

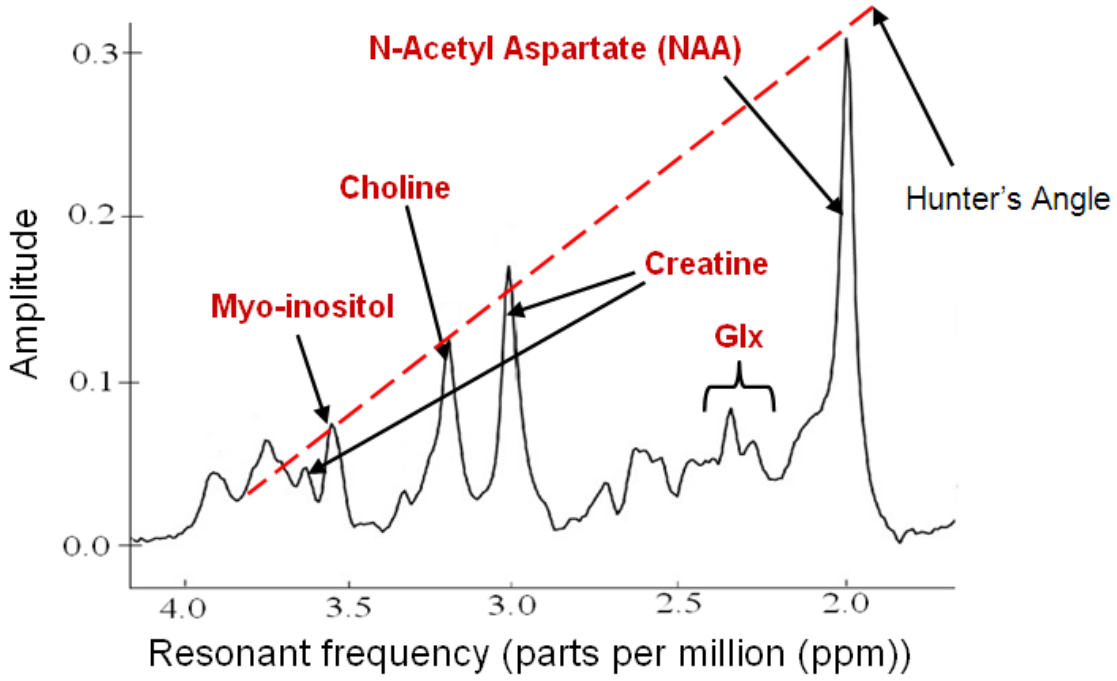

Figure 3-3 Representative ^{1}H-MRS spectrum of normal human brain
The five major metabolite peaks (choline, myo-Inositol, creatine, N acetyl aspartate and Glx) and Hunter's Angle, the putative 45° ratio signifying 'normal' adult spectra, identified.

3.5.2.1 Metabolite quantification as ratios

All analyses reported in the present work adopt the standard convention of expressing metabolite ratios to an internal creatine standard. The creatine peak is thought to be relatively constant between individuals and in most brain areas, and is therefore used as an internal reference, where creatine equals 1 in the expression of all ratios (Danielsen and Ross, 1999). The explicit rationale for this approach is that the use of ratios will correct for several unknown, difficult to obtain or uncontrollable experimental conditions. These include static (B_0) and radiofrequency (RF, B_1) field inhomogenieties, instrumental gain drifts, imager and localisation method differences, and voxel partial volume contamination with metabolite-free cerebral-spinal fluid (CSF) (Li, Wang et al., 2003).

3.5.3 Single-voxel ^{1}H-MRS acquisition parameters

All MR acquisitions were carried out on a Siemens ^{1}H-Magnetom 3T (Siemens Medical Solution, Berkshire, UK) using standard software and quadrature head coil.

Scout images for localising the volume of interest (VOI) were acquired using a 5-plane localiser (Relaxation time (TR); 20msec, Echo time (TE); 5msec, 10 slices at 5mm thickness).

^{1}H-MRS data were acquired in a 2cm^{3} volume using a stimulated echo acquisition mode (STEAM) pulse sequence (TR; 2000msec, TE; 30msec, 96 averages). The choice of TE and TR reflects a compromise between a number of factors including scan time, patient tolerance and obtaining optimal information. With increasing TE, those neurochemical compounds with the shorter spin-spin (T2) relaxation times (mI and Glx) disappear, while those with longer T2 values (NA, Cr, Cho) become more prominent. With TEs shorter than 30ms, the mI and Glx region of the spectrum become even more prominent; however, water and lipids become more difficult to suppress adequately and the pulse sequences become more prone to artefacts. Voxel size is limited by a trade off between the signal-to-noise ratio (SNR) and tissue specificity; with smaller voxel sizes, specificity of voxel localisation is increased but SNR is reduced (Freeman, 2003).
The acquisition parameters summarised in Table 3-6 consistently produce a high-quality spectra containing all the major MR-visible compounds within the brain in an examination time of approximately 3 minutes per voxel.

Table 3-6 ^{1}H-MRS acquisition parameters

Repetition time (TR)	2000 msec
Echo time (TE)	30 msec
Number of averages	96
Flip angle	90^{o}
Voxel size	2 x 2 x 2 cm^{3}
Pixel resolution	1024

Water suppression was achieved by using three chemical shift-selective radio-frequency pulses followed by a dephasing gradient applied on each of the three axes. Localiser scans were performed after each acquisition to compensate for potential participant movement between scans. Scan sequences and duration are summarised in Table 3-7, page 69.

Table 3-7 ^{1}H-MRS scan sequences and duration

Scan sequence	Duration (min:secs)
Localiser	0:09
5-plane localiser	0:40
STEAM occipitoparietal cortex	3:20
STEAM frontal cortex	3:20

The technical limits of magnet construction, coupled with the presence of the participant in the scanner, introduces inhomogenieties in the magnetic fields over the whole of the brain volume located within the detection coil. This results in distorted lineshape of the spectra, leading to poor resolution and sensitivity. Small gradients in B_0 are compensated for by 'shimming', which is the application of a small DC current through x, y, z gradient shim coils. An active, automated first-order shim was performed prior to each acquisition for each participant introduced in the scanner. During the shim process, a 3D gradient echo sequence acquires volumetric data of the region to be shimmed. The Siemens ^{1}H Magnetom 3T scanner software analyzes the collected data to estimate the current needed in each shim coil to compensate for deficiencies in field homogeneity and optimize the magnetic field homogeneity

3.5.3.1 Voxel localisation

Whilst differences in grey/white tissue composition affect the metabolite values obtained (Wang and Li, 1998), the brain region that is measured may be of more importance with respect to the cognitive domain that is tested. In the present study, voxels in the frontal cortex were selected because of the association between this region and executive cognitive function (Baddeley, 1996; Duncan, Seitz et al., 2000). A volume in the occipitoparietal cortex was selected as a control region to assess the functional specificity of the brain-behaviour relationship, and the comparatively homogenous nature of the cortical tissue in this region aids in providing consistent high-quality comparison spectra. Inter-hemispheric metabolite differences in metabolite values have been observed (Jayasundar, 2002; Jayasundar and Raghunathan, 1997). In the present study, a single hemisphere was focused on in the interest of parsimony, as reductions in

the number of variables and statistical comparisons required, decreases the probabilities of Type 1 error.

Positioning of the voxel in the region of interest was achieved using anatomical references and ventricle landmarks from the 5-plane scout images. For the left frontal VOI, the voxel was placed as close possible to the anterior of the brain, centred superior to the lateral ventricles and avoiding overlap into the skull and corpus callosum. For the left occipitoparietal VOI, the voxel was placed in the region encompassing both occipital cortex and the inferior parietal lobule, with placement preferentially positioned in the anterior of the brain to avoid overlap into the skull or cerebellum. In all cases, manual positioning ensured that the voxel did not encroach upon non-neural sources such as skull, meninges, ventricles and major blood vessels and avoided as many of the cerebral sulci as possible.

Figure 3-4 shows the location of the voxels in occipital and frontal cortex. Using a rule-based approach to identifying anatomical landmarks for head positioning and voxel placement provided a straightforward method for effective and consistent voxel localisation.

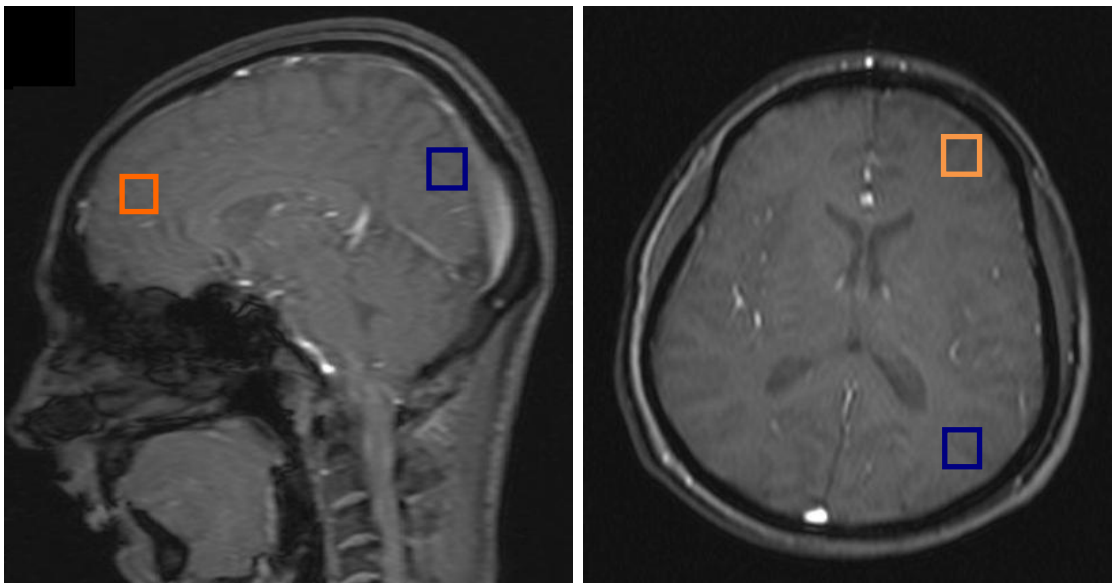

Figure 3-4 Localisation of ^{1}H-MRS voxels shown in the saggital and transverse planes
Voxel in frontal cortex is shown in orange and occipitoparietal cortex in blue.

3.5.4 LCModel

The metabolite values obtained were subsequently modelled with LCModel software (Provencher, 2001), a user-independent fitting routine which provides spectral quantification and metabolite detection optimised for short echo time spectroscopy by fitting spectra in the frequency domain using a basis set of spectra of *in vitro* metabolite solutions acquired under conditions identical to those under which *in vivo* data are acquired. By using a nearly model-free constrained regularisation method to automatically estimate the smoothest lineshape and baseline consistent with the data, the model has the potential to improve the reliability of the metabolite values obtained (see below) and specifically addresses the problems of spectra complexity and baseline formation, in addition to providing results with a broader basis of comparability.

3.5.5 ^{1}H-MRS reliability analyses

In order for ^{1}H-MRS to be a valid technique in metabolite quantification, data needs to be accurate and reliable. Accuracy refers to the degree of conformity of the measured quantity to its actual or true value. In living tissue it is difficult to quantify, as many standard measurement techniques, being invasive, are impractical. Reliability and precision refers to the variation in replicated readings, for example, as a result of variance introduced by scanner-related factors such as that caused by field inhomogenieties.

To validate data quality and assess reliability, data from two successive scans of the same voxel were obtained and averaged at each voxel placement. Standard software with the Siemens ^{1}H Magnetom 3T scanner produced metabolite values which were reliable when intra-voxel values are compared; all metabolites both in frontal and occipital regions were significantly correlated ($p < .05$). These values were, however, susceptible to large and unexpected distortions such as residual water signal, macromolecules and lipid signals, which may account for the range of correlation coefficients (.29 to .82) observed. The coefficients of variance were approximately 9% for frontal and 8% for occipitoparietal voxels, values which demonstrate acceptable

precision of the technique and which are consistent with data from previous reports (Ross et al., 2005).

The LCModel (Provencher, 2001) specifically addresses the problems of spectra complexity and baseline formation. By accounting for unexpected distortions in the baseline, correlations between values for the first and second scan improved to a range between .773 and 0.976 ($p < .01$), and produced mean metabolic concentrations consistent with literature values (Danielsen and Ross, 1999). Coefficients of variance with LCModel were now between 6 and 8%. Chard, McLean et al. (2002) contend that with use of the LCModel, biological factors contribute a greater proportion to measurement variability than ^{1}H-MRS measurement errors. In comparison with the AMARES (Advanced Method for Accurate, Robust and Efficient Spectral fitting) tool (Vanhamme, van den Boogaart et al., 1997), Kanowski, Kaufmann et al. (2004) found that metabolite-to-creatine ratios, estimated by LCModel with extended prior knowledge, are more accurate than absolute concentrations, and are nearly independent of SNR and line broadening.

4 Cognitive outcomes in paediatric liver disease

4.1 Summary

The aim of the present cross-sectional study was to evaluate neuropsychological function in children with liver disease and to investigate the impact of the disease on cognitive ability compared to sibling controls, in the context of covariates such as the age of onset and transplant intervention.

Chronic liver disease appears to have significant negative effects on cognitive development, with age at the onset of disease an important moderator of this effect. Significant differences were observed between the early onset, post-transplant group and age-matched sibling controls on a measure of IPS (p= .017; (d)= 1.36), with 31% of the variance in IPS accounted for by the early onset of liver disease coupled with transplantation.

There is a general pattern in the literature that supports the theory that early-onset liver disease has a significant impact on cognitive outcomes, possibly through the disruption of early neurodevelopment processes. Whilst greater numbers of participants are required to achieve the statistical power required to genuinely probe the effects of disease onset and some of the cofactors which may contribute to the cognitive deficits observed, the results of the present study show that the effects in this sample of this rare population is considerable. Enrolling children with liver disease in a large-scale, longitudinal study would help illuminate the extent and persistence of the cognitive deficits observed in paediatric liver disease.

4.2 Introduction

Liver transplantation (LTx) is now a standard treatment for children with end-stage liver disease (Muiesan, Vergani et al., 2007). With improving survival rates, resulting from innovations in operative techniques and the development of more effective immunosuppressant drugs (Kelly, 2008; Kelly, 1998), the life-expectancy of children with end-stage liver disease has significantly improved. One-year survival rates now

exceed 90% across most centres, and long-term survival figures for 10–15 years are greater than 80% (Soltys, Mazariegos et al., 2007). With a rapidly growing population of children who are long-term survivors of liver disease, there is an increased focus on variables associated with longer-term survival and cognitive and psychosocial outcomes (Alonso, 2008; Andrews, Sommerauer et al., 1996; Fouquet, Alves et al., 2005; Park, Rim et al., 2005).

Some of the earliest investigations of children who had undergone LTx compared their neurocognitive outcomes to those of disease control groups, for example cystic fibrosis. Compared to cystic fibrosis patients, Stewart, Hiltebeitel et al. (1991) and Stewart, Silver et al. (1991) identified deficits specific to post-liver transplant patients in a variety of cognitive domains, including learning and memory, abstraction and concept formation, visual-spatial function, and motor function. However, a more recent study with the same experimental and control groups found no statistically significant differences between them on measures of visual-perceptual and visual-motor skills, but found deficits in language abilities in the liver disease group (Krull, Fuchs et al., 2003).

A group of studies conducted in the late-1980s through early 1990s by Stewart et al. found that the child's age at the onset of disease correlates with levels of physical and cognitive impairment. Children who developed liver disease in infancy had significantly lower IQ scores compared with children who became symptomatic later in life (Stewart, Campbell et al., 1992; Stewart, Uauy et al., 1988; Stewart, Uauy et al., 1989). In addition, the developmental delay which appears to be a frequent problem in infants awaiting liver transplantation does not reverse quickly after transplant (Wayman, Cox et al., 1997).

Given these age of onset related effects, the majority of children receiving liver transplants may be at risk of cognitive impairment, since the median age of paediatric liver transplant recipients is less than 2 years (Krull, Fuchs et al., 2003). Although specific cognitive deficits have been identified previously in liver disease patients, there have been few additional studies since the early work of Stewart et al.

4.2.1 Aims

The aims of this cross-sectional study were to:

1 Examine neuropsychological function in children with liver disease in order to investigate the impact of the disease on cognitive ability in comparison to sibling controls.
2 Assess the effects of age of onset and transplant intervention on cognitive outcomes, specifically, FSIQ and IPS.

4.2.2 Hypotheses

1 Children with liver disease would score lower than the comparison control group on global measures of cognitive ability, specifically FSIQ and IPS.
2 Children with early-onset liver disease would show greater deficits in cognitive ability than sibling controls and those who developed the disease later in childhood.

4.3 Methods

4.3.1 Participants

Psychometric data was available for a total of 32 participants, 23 patients with a liver disease diagnosis and 9 healthy sibling controls (16 females, 16 males; mean age 13.1, SD: 5.0), from the larger sample described in Chapter 3 (Table 3-2, page 50). Psychometric data was not collected for two sibling controls and four patients in the early onset, post-transplant group who had consented into the study because of lack of availability at the time of testing. One patient in the early onset, pre-transplant group and one in the early onset, pre-transplant group were not available to complete the psychometric assessment battery. Table 4-1, page 76 shows the descriptive data of the patients consented into the study and the mean and standard deviation of psychometric scores.

Table 4-1 Descriptive data for the sibling control and liver disease groups with available psychometric data

Group	Total n	Mean age (years)	SD age (years)	M:F	Mean onset age (years)	n	Diagnoses
Sibling controls	9	13.2	4.5	6:3			
Early-onset liver disease, pre-transplant	10	14.9	4.0	4:6	-	7	Extra-hepatic biliary atresia
						1	Alpha 1-antitrypsin (A1AT) deficiency
						1	Progressive familial intrahepatic cholestasis
						1	Neonatal haemochromatosis
Early-onset liver disease, post-transplant	7	16.3	2.4	3:4	-	2	Progressive familial intrahepatic cholestasis
						2	Extra-hepatic biliary atresia
						2	Neonatal liver failure
						1	Alpha 1-antitrypsin (A1AT) deficiency
Acute liver failure (late onset), post-transplant	6	13.7	3.7	3:3	5.4	1	Autoimmune hepatitis
						1	Fulminant hepatitis A
						1	Wilson's disease + acute liver failure
						3	Sero-negative hepatitis

Dash indicates that liver disease was diagnosed from birth

4.3.2 Psychometric assessments

The standardised protocol for administration of psychometric assessments is described in Chapter 3 (section 3.3, page 51).

An age-appropriate battery of the Wechsler Preschool and Primary Scale of Intelligence-III, Wechsler Intelligence Scale for Children-IV or Wechsler Adult Intelligence Scale-III was administered for each child to derive scores for Verbal, Performance, Working Memory, FSIQ, and IPS.

4.4 Results

4.4.1 Descriptive data and psychometric scores

Table 4-2 shows the mean and standard deviation of psychometric scores of sibling controls and liver disease groups. Table 4-3, page 78 provides descriptive data for the patients consented into the study.

Table 4-2 Summary of psychometric scores for sibling control and liver disease groups

Psychometric measure	Sibling controls	EOLD pre-Tx	EOLD post-Tx	ALF post-Tx
FSIQ	103.8 (21.9)	91.6 (19.2)	80.9 (21.3)	102.3 (21.4)
IPS	104.7 (12.8)	91.7 (10.7)	81.6 (21.2)	102.5 (12.0)
Verbal IQ	104.2 (23.3)	91.0 (20.1)	81.4 (22.0)	101.2 (22.2)
Performance IQ	99.1 (20.7)	94.7 (15.8)	83.7 (22.4)	105 (20.2)
Working Memory*	102.6 (12.0)[a]	89.0 (16.1)[b]	89.3 (20.9)[c]	97.3 (15.7)[d]

EOLD: Early-onset liver disease, ALF: Acute liver failure, Tx: liver transplant.
Mean z scores and standard deviations are shown. Wechsler assessments have a population mean of 100 and standard deviation of 15.
* n= 17; [a]n=7, [b]n=3, [c]n=3, [d]n=4

4.4.2 Cognitive outcomes in paediatric liver disease

A one-way ANOVA was conducted to assess the differences in performance on the various psychometric indices based on diagnostic grouping. The results are shown in Table 4-3.

Table 4-3 Differences in psychometric scores between sibling controls and liver disease groups

Psychometric measure	ANOVA		
	F	df	P
FSIQ	1.949	3,28	.145
IPS	4.124	3,28	.015*
Verbal IQ	1.708	3,28	.188
Performance IQ	1.423	3,28	.257
Working Memory†	1.181	3,13	.355

*p<.05, †n=17

The ANOVA showed no significant difference between groups for FSIQ (p>.5). IPS was, however, identified as significantly different between groups (Table 4-3). Descriptive statistics (Table 4-1, page 76) show that mean IPS score was lower in the early onset, post-transplant group than the other two patient groups and the sibling controls. A one-way ANOVA showed that this difference was statistically significant (F= 4.124 (p .015)), which represented an effect size Eta^2 of .31, showing that 31% of the variance in IPS can be accounted for by the early onset of liver disease coupled with transplantation.

A *post hoc* Tukey's Honestly Significant Difference test, which corrects for Type 1 error, confirmed that the difference between the early onset, post-transplant group and sibling controls was unlikely to have arisen due to sampling error (p= .017, effect size (d)= 1.36). The Tukey test also showed that difference between the early onset, post-transplant group and acute liver failure (late onset), post-transplant group approached statistical significance p= .06; effect size (d)= 1.26) (see Figure 4-1, page 79).

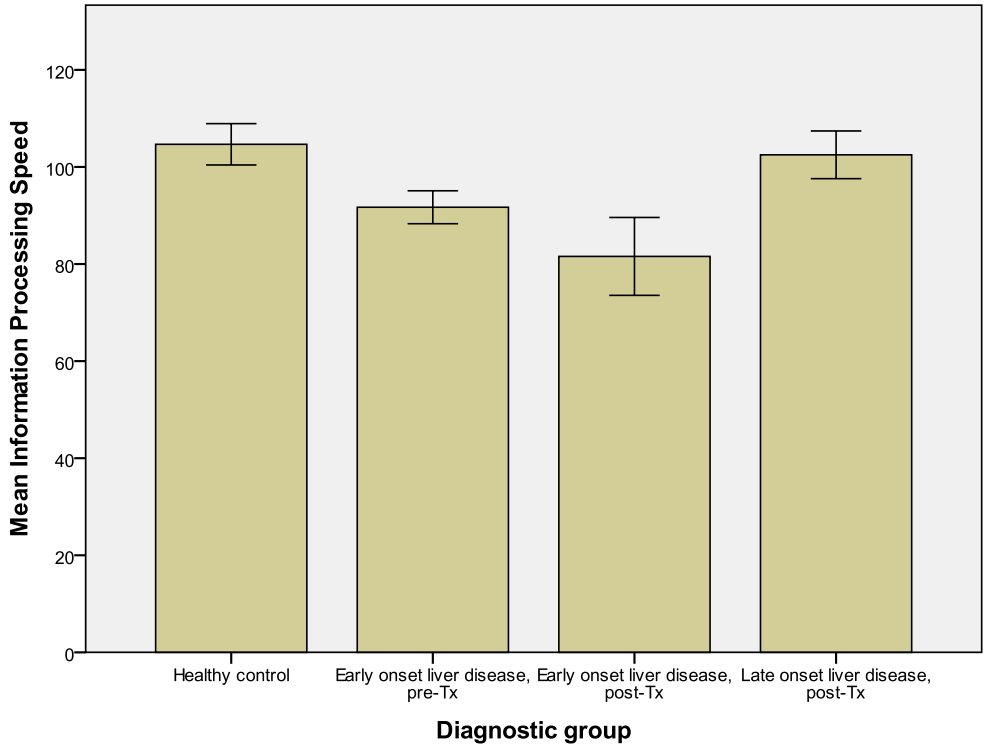

Figure 4-1 Comparison of IPS performance between control and liver disease groups
Error bars represent ±1 standard error mean. The early onset, post-transplant cohort had significantly lower IPS scores compared to sibling controls (p= .017, effect size (d) = 1.36).

4.5 Discussion

The present study presents cross-sectional psychometric data from a total of 23 patients with liver disease who were categorised into three independent groups: early onset pre-transplant, early-onset post transplant and acute liver failure, post-transplant. Performance on tests of cognitive ability was compared between the liver disease patient groups and with nine age-matched healthy sibling controls.

As hypothesised, chronic liver disease appears to be associated with impaired cognitive development and the age of liver disease onset appears to be important. A statistically significant difference between the early onset, post-transplant group and age-matched sibling controls on IPS scores (p= .02, (d)= 1.36 was observed. The difference between the early onset, post-transplant group and acute liver failure, post-transplant group for this measure also approached statistical significance (p=. 06; (d)= 1.26). Cognitive deficits appear to be specific to processing speed; no statistically significant difference was observed for FSIQ scores, although the pattern of results between groups was the same.

It has been previously demonstrated that the cognitive effects of liver disease cannot be accounted for by the general effects of chronic, life-threatening disease. Compared to a group of cystic fibrosis (CF) disease controls, Stewart, Hiltebeitel et al. (1991) found that children studied at one-year post-LTx showed deficits in the areas of learning and memory, abstraction and concept formation, visual-spatial function, and motor function. They did not demonstrate differences in Verbal IQ, alertness and concentration, perceptual-motor, and sensory-perceptual areas.

Building on this work, Stewart, Silver et al. (1991) narrowed the age range of the cohort and concentrated on a more selective neuropsychological test battery to study potential lateralisation effects associated with vocabulary performance. They found that children between the ages of 4 and 9 years showed similar deficits to the previous older age group studied by Stewart, Hiltebeitel et al. (1991), with no differences in lateralisation, but with the addition of deficits in Verbal IQ.

Krull, Fuchs et al. (2003) also found that post-transplant children tended to have lower scores, particularly on receptive language tasks, but observed no significant difference between 15 liver and 15 CF patients on measures of academic achievement or visual-spatial performance. As with Stewart, Hiltebeitel et al. (1991), Krull, Fuchs et al. (2003) found specific effects on verbal ability, something which was not seen in the cohort in the present study.

Whilst significant difference for IPS between the early and late-onset groups were observed, the significant difference in FSIQ seen in the studies conducted by Stewart et al. was not replicated. Despite variations in the cognitive abilities assessed, the present findings are in accordance with previous evidence that the onset of liver disease may interfere with important early stages of cognitive development. In a heterogeneous group of 36 children who were 4 years old or older when they were referred for liver transplant, cognitive assessments pre- and one year post-transplant showed that children who developed liver disease in infancy had significantly lower IQ scores than children who became symptomatic later in life (mean ± SD for FSIQ; 85 ± 8.8 versus 99.5 ± 13.8) (Stewart, Uauy et al., 1988).

A subsequent study of 43 school-age children with advanced liver disease confirmed the findings that the onset of symptoms of liver disease in the first year of life had a stronger negative impact on cognitive function than those who were older at the time of transplantation, independent of diagnosis (Stewart, Campbell et al. 1992). In the present study, the acute liver failure transplantation group had comparable psychometric scores to the age-matched sibling controls (Table 4-2, page 77, and Figure 4-1, page 79), lending support to the idea that time of onset is a key factor.

Stewart, Silver et al. (1991) suggested that deficits found on performance tasks, rather than reflecting decreased visual-spatial function, may be indicative of diminished ability for other skills that underlie many of the visual-spatial tasks, such as *timed* performance. Given the systemic nature of liver disease, effects on global, rather than domain-specific, measures of cognitive ability would be anticipated. In the present study, a specific deficit it processing speed measures was observed in the early onset, post-transplant cohort, which may be related to myelination in so much that the primary function of myelin is the acceleration of neural impulses. The degree of and integrity of myelination is strongly related to processing speed measures, including the Wechsler measures of IPS employed in this study (Turken, Whitfield-Gabrieli et al., 2008), and processing speed is thought to underlie, but is not synonymous with, general cognitive ability (Miller, 1994; Salthouse, 1996; Vernon, 1983). Myelination begins in the second trimester of gestation and continues into adulthood (Lenroot and Giedd, 2007). Early onset of liver disease may specifically interfere with key neurophysiological developmental mechanisms such as myelination, resulting the deficits observed in this study. One of the potential mechanisms by which this may occur, related to EFA status, is discussed in the following chapter.

As with the present work, the studies conducted by Stewart et al. used a cross-sectional design, which has the limitation of introducing heterogeneity (i.e. inter-individual confounders) into the population and data. However, when Stewart et al. studied children longitudinally, both before and after transplantation, the same pattern of results was observed: children with persistent cognitive deficits after transplant were

more likely to have had onset of disease in the first year of life (Stewart, Uauy et al., 1989).

Another finding of note in the present study was that deficits in cognitive performance were not uniform across both early-onset groups, but were largely limited to the post-transplant patients. Whilst this non-uniformity may be a result at least in part from the small sizes typically employed in studies of this kind, there is also evidence that transplantation coupled with early onset has specific detrimental effects, which is to be anticipated as transplanted patients are usually the most severely ill. Kennard, Stewart et al. (1999) suggest that developmental delay, secondary to chronic liver disease in infancy, and acute or chronic hepatic encephalopathy in older children may result in enduring cognitive deficits and learning disabilities even after successful liver transplantation. This may explain the findings observed in early onset, post-transplant patient group.

Wayman, Cox et al. (1997) found that infants who received LTx before 2 years of age had standardised scores of mental and motor developmental that dropped below pre-transplant levels at 3 months post-transplant, and recovered to pre-transplant levels only at 12 months after the procedure. Similar deterioration preceding recovery in both cognitive and physiological outcomes has also been reported by van Mourik, Beath et al. (2000). The study by Wayman et al. was conducted in a young population 12 months post-transplant. These results cannot be easily compared to findings from the present study, which studied children at around 12 years of age, in some cases years after having received a transplant. Whilst the cognitive deficits observed in these children could improve over time, Wayman et al. suggest that post-operative effects, lasting up to one year post-transplant, are likely to persist in the longer term.

The results from Wayman et al. and the present study demonstrates the need for a longitudinal investigation with a considerable follow-up period in order to probe the longer-term, potentially persistent effects of transplantation on cognitive development. The children in the current study were recruited post-transplant and therefore pre-transplant evaluations were unavailable. It is possible that some of the deficits are related to the major surgery of liver transplantation and/or immunosuppressant

medication following transplantation, which may also explain the post-operative effects identified by Wayman et al.

Krull, Fuchs et al. (2003) also highlight a number of cofactors which need to be taken into consideration. In a group of 15 liver transplant patients, they observed that the number of days in the intensive care unit, the total number of days spent in the hospital during the first year following the transplant, and elevated pre-transplant bilirubin levels, significantly predicted the speech and language delays. Only data for bilirubin levels at the time of study were available in the present study, and no correlation was observed between this marker of disease severity and IQ performance ($p < .05$). Hyperbililirubinemia (>342 μmol/L (20mg/dL)) in infancy is, however, associated with a higher risk of for low IQ scores (<85) at age 17 (Seidman, Paz et al., 1991), which again supports the theory that greater disease severity in early infancy is associated with poorer cognitive outcome in later childhood. Data for a number of co-factors, including length of hospitalisation and periodic measures of bilirubin levels was collected in a number of the patients in the current study (see Appendix A), but analysis was considered beyond the scope of this thesis.

In the present study, age-matched healthy children were used as a control group. Employing an intra-subject design would alleviate the problem of identifying an appropriate comparison group. Given the ongoing complications associated with liver failure and post-transplant recovery, an age-matched chronic disease group, such as cystic fibrosis patients used in previous studies, has greater utility in controlling for co-factors such as number of days missed from school, hospital visits and similar potentially confounding effects.

4.6 Conclusion

The most important finding of the present study is that post-transplant children with an early onset of liver disease show deficits in cognitive ability, specifically in Information Processing Speed, compared to age-matched acute liver failure transplant patients and sibling controls. This highlights the need to assess the developmental and cognitive status in children with liver disease and the importance of the timing of the onset of

illness. Whilst the results of the present study are in keeping with the literature demonstrating age of onset-related cognitive deficits in children with liver disease, the limited number of participants in each group, and the heterogeneity in specific diagnoses, is a significant limitation and the findings of the present study must be interpreted with caution, despite the large effect sizes.

The numerous activities of the liver that are disturbed as a consequence of liver disease may each contribute in some small degree to the observed deficits in cognitive functioning. One possible mechanism that may explain the results is the reduced exposure to molecules important in development of brain, such as EFAs, which is investigated Chapter 5.

Greater numbers of participants are needed to achieve the statistical power required to genuinely probe the effects of disease onset and some of the cofactors identified which may contribute to the cognitive deficits observed. Longitudinal studies would help illuminate the extent and persistence of the cognitive deficits observed in paediatric liver disease. The availability of patients is, however, a significant limiting factor to large-scale studies with this group, which means that in general there is a shortage of studies assessing the impact of liver disease, and treatment-related variables, on neuropsychological function.

5 Polyunsaturated fatty acids and cognitive outcomes in paediatric liver disease

5.1 Summary

Using a paediatric liver disease model, the aim of this study was to evaluate whether sub-optimal concentrations of EFAs, as a result of fat malabsorption or dependence on inadequate dietary sources, is associated with deficits in cognitive ability, particularly as specific deficits in processing speed measures were observed in children with early-onset congenital liver disease who had received a liver transplant (see Chapter 4). Specifically, the aim was to measure and evaluate the range of EFA biomarker concentrations in cross-sectional cohorts of children with a variable onset of liver disease, some of whom with more severe manifestations of liver disease had received a liver transplant, and determine if cognitive ability in these children is related to their EFA status.

GC-MS analysis of erythrocytes was used to quantify biomarkers of fatty acid status, including the major omega-6 fatty acids (LA and AA), LCPUFA omega-3 fatty acids (DHA and EPA), and specific functional deficiency markers (osbond and mead acid). Fatty acid status was compared between groups of patients with liver disease and age-matched sibling controls and relationships between EFAs and the cognitive outcomes described in Chapter 4 were investigated.

Compared to sibling controls, no signs of fatty acid deficiency were observed in any of the cohorts of patients with liver disease. This suggests that: (1) these patients were not deficient in their dietary intake of EFAs, LA and AA and (2) these patients are able to sufficiently metabolise these precursor lipids to synthesise LCPUFAs, DHA and EPA, to levels comparable to sibling controls. Duration of breastfeeding was not correlated with later cognitive outcomes in this cohort, but a strong correlation was observed between omega-6 (LA and AA) status and FSIQ and IPS ($r = -.62$ and $-.39$; $p < .001$), independent of disease diagnosis.

5.2 Introduction

The advent of paediatric liver transplantation has placed additional emphasis on the importance of optimum nutritional management of children with chronic liver disease, as improvement of nutritional status in the pre-transplantation period maximises success of the liver transplant itself. There is also a growing awareness of active nutritional support for children with end-stage liver disease in order to maintain a state of health that supports normal physical and psychological development (Alonso, 2008; Bavdekar, Bhave et al., 2002; Protheroe, 1998).

Whatever its original cause, chronic liver disease in children is characterised by an on-going inflammatory process that injures the liver tissues causing fibrosis and in severe cases, cirrhosis. Some children require a liver transplant to survive, although many can be managed with medical treatments such as drugs to regulate the immune system and food supplements to compensate for fat malabsorption (Kelly, 2008; Kelly, 1997; Kelly, 2006; Kelly and Sibal, 2006).

The liver has a diverse range of functions including the production of bile, constituents of which are required for efficient intestinal fat absorption. Additionally, biliary secretion of cholesterol (as such, or after incorporation into bile salts) and phospholipids from the liver into the intestine is of major importance in body lipid homeostasis. Most importantly, the liver is the site of active synthesis, metabolism and/or oxidation of a wide range of proteins and carbohydrates, as well as EFAs and LCPUFA, which are the subject of this study. For a more detailed discussion of lipid absorption and metabolism in cholestasis refer to Werner, Kuipers et al. (2003).

As a result of the importance and functions of EFA, which were described in Chapter 2, interest in the role of EFAs in liver disease has been growing rapidly in recent years (de Meijer, Le et al., 2010; Diamond, Sterescu et al., 2008; Lee, Gura et al., 2007). Isolated omega-3 deficiency has attracted attention since its recognition in a 6-year-old girl who received long-term total parenteral nutrition that lacked adequate omega-3 fatty acids (Holman, Johnson et al., 1982).

5.2.1 Dietary EFA deficiency in paediatric liver disease

Malnutrition and consequent EFA and PUFA deficiency can be a result of multiple factors, such as increased energy requirements, malabsorption and abnormal hepatic metabolism (Motta, Sterling et al., 1999; Protheroe, 1998). Patients are at risk of malnutrition if under 2 years of age, or having severe cholestasis, or progressive liver disease, such as biliary atresia or severe neonatal hepatitis, and if awaiting liver transplantation intervention (Protheroe, 1998). Malnutrition is however recognised as a risk factor that is potentially reversible, making nutritional support a cornerstone of therapeutic management of these children.

Malabsorption of lipids is a critical nutritional issue, particularly in cholestasis, because a fundamental energy source is lost. Inadequate energy intake due to malabsorption of fat can be compensated for by using soluble medium-chain triglycerides (MCTs), which are fatty acids with carbon chain lengths between 6 and 12 (primarily octanoic (C8) and decanoic (C10) acids). Most infant formulas used by children with liver disease contain MCTs (for examples of feeds, see Table 5-1, page 88). MCTs do not require extensive biochemical transformation or incorporation into chylomicrons during intestinal absorption and MCT oil supplemented diets have been successfully used in reducing steatorrhea, improving energy balance, and promoting growth in liver disease patients (Cohen and Gartner, 1971; Kaufmann, Murray et al., 1987).

Concerns have been raised that MCT-based nutritional feeds, lacking adequate levels of EFAs lead to deficiency states (Hirono, Suzuki et al., 1977; Pettei, Daftary et al., 1991). Furthermore, EFA deficiencies in patients on parenteral feeds are not easily reversed, at least in the short term (Deurksen, Nehra et al., 1999). Table 5-1, page 89, shows the fatty acid content of Pregestimil and Peptisorb, two feeds used by the Birmingham Children's Hospital to treat children with cholestasis and which contain no AA, EPA or DHA. Table 5-2, page 89, shows the fatty acid content of Pepti Junior, an alternative feed that contains appreciable levels of LCPUFAs.

Table 5-1 Fatty acid content of two feeds used by the Birmingham Children's Hospital to treat children with cholestasis

Brand of feed	MCT (%)	Fatty acid content (mg/100ml)		
		18:1	18:2	18:3n-3
Pregestimil	54	179	220	28.9
Peptisorb	47	408	1010	92.7

Data retrieved from http://nutritiondata.self.com/facts/baby-foods/440/2

Table 5-2 Fatty acid content of Pepti Junior, an MCT-based feed with appreciable levels of LCPUFAs

Brand of feed	MCT (%)	Fatty acid content (%/100g FA)						
		18:1	18:2	18:3n-3	18:3n-6	20:4	20:5	22:6
Pepti Junior	50	22.7	14.4	2.7	0.02	0.2	0.05	0.2

5.2.1.1 EFA deficiency due to fat malabsorption

Even with adequate intake, up to half of dietary fat, along with fat-soluble vitamins and the essential PUFAs, may be malabsorbed due to reduced intraluminal bile concentration (Beath, Hooley et al., 1993; Glasgow, Hamilton et al., 1973). Fat malabsorption has also been shown to decrease EFA serum concentration, whilst total unsaturated fatty acid concentration is maintained through increases in nonessential fatty acid endogenous production (Jeppesen, Christensen et al., 1997). Abnormalities in the hepatic omega-6:omega-3 PUFA ratio impacts hepatic lipid homeostasis through modulation of transcription factors, and also impacts fatty acid desaturase (FADS) enzymes which have a major role in fatty acid metabolism and fat accumulation in the liver (El-Badry, Graf et al., 2007).

In summary, infants with cholestatic liver disease are at risk of DHA deficiency as a result of malabsorption of LCTs, prescription of diets rich in MCT, or suboptimal liver desaturase enzyme activity. The critical role of PUFAs such as DHA in neuronal development (Chapter 2) suggests that adverse effects of inadequate levels of these nutrients may manifest as deficits in cognitive ability in later life and may be one of the mechanisms that explains, at least in some part, the specific deficit in processing speed measures observed in the early-onset liver disease patients in Chapter 4.

5.2.2 Aims

Using a paediatric liver disease model, the aim of this study was to evaluate whether sub-optimal concentrations of EFAs, as a result of fat malabsorption or dependence on inadequate dietary sources, is associated with deficits in cognitive ability. Specifically, the aims were to:

1. Measure and evaluate the range of EFA biomarker concentrations in cross-sectional cohorts of children with a variable onset of liver disease who may also have had a liver transplant.
2. Determine if cognitive ability in these children is related to their current EFA status.

5.2.3 Hypotheses

1. Patients with liver disease would show biomarkers of EFA and PUFA deficiency compared to the sibling control group.
2. Omega-3 fatty acids (particularly DHA) would be positively correlated with broad-based measures of cognitive ability.

5.3 Methods

5.3.1 Participants

Blood sample data was available for a total of 39 participants, of which 28 patients had a liver disease diagnosis and 11 were sibling controls (24 females, 15 males; mean age 13.8, SD: 4.2), from the larger sample described in Chapter 3 (Table 3-2, page 50). EFA data was not available for three of the early onset, pre-transplant patients. One patient did not provide a blood sample on the study day and blood samples from two patients were not of sufficient quantity to permit adequate lipid analysis. Descriptive details of the subsample included in this study are provided in Table 5-3, page 90.

Table 5-3 Descriptive data for the sibling control and liver disease groups with available psychometric and EFA data

Group	Total n	Mean age (years)	SD age (years)	M:F	Mean onset age (years)	n	Diagnoses
Sibling controls	11	12.2	5.1	5:6			
Early-onset liver disease, pre-transplant (EOLD pre-Tx)	14	13.6	4.6	5:9	-	8	Extra-hepatic biliary atresia
						1	Alpha 1-antitrypsin (A1AT) deficiency
						4	Progressive familial intrahepatic cholestasis
						1	Neonatal haemochromatosis
Early-onset liver disease, post-transplant (EOLD post-Tx)	8	15.5	3.2	3:5	-	2	Progressive familial intrahepatic cholestasis
						2	Extra-hepatic biliary atresia
						1	Aegeneas Syndrome
						2	Neonatal liver failure
						1	Alpha 1-antitrypsin (A1AT) deficiency
Acute liver failure, post-transplant (ALF post-Tx)	6	13.7	3.7	2:4	5.4	1	Autoimmune hepatitis
						1	Fulminant hepatitis A infection
						1	Wilson's disease
						3	Sero-negative hepatitis

The sample provides the study with statistical power in excess of 80% to detect moderate correlations of .4 and above, with statistical significance evaluated at an α level of .05 (Friedman, 1968).

5.3.2 Quantification of fatty acid biomarkers in erythrocyte membranes

The total lipids were extracted from the erythrocytes, transesterified to the methyl esters and analysed with conventional GS-MS. Details of the procedures and reagents are described in Chapter 3 (section 3.4, page 52). Fatty acid analyses were limited to a number of individual fatty acids and the four main fatty acid families: saturated (SFAs), monounsaturated (MUFAs), omega-3 and -6 PUFAs (refer to Chapter 3, page 60 for details). Table 5-4 summarises the biomarkers used to evaluate fatty acid status.

Table 5-4 Summary of erythrocyte biomarkers of fatty acid status

Fatty acid biomarkers	Fatty acids
SFA	stearic (18:0n-6) + palmitic (16:0n-6) + myristic (14:0n-6)
MUFA	oleic (18:1n-6)
Omega-3 index	DHA (22:6n-3) + EPA (20:5n-3)
Omega-6 index	arachidonic (20:4n-6) + linoleic (18:2n-6)
Omega-3:omega-6	omega-3 index/omega-6 index
EFA shortage marker	mead acid (20:3n-9)
Functional DHA shortage marker	DHA/osbond acid (22:5n-6)

5.3.3 Psychometric assessments

An age-appropriate battery of the either the Wechsler Preschool and Primary Scale of Intelligence-III, Wechsler Intelligence Scale for Children-IV or Wechsler Adult Intelligence Scale-III, was administered to derive scores for Verbal IQ, Performance IQ, Working Memory and FSIQ, and IPS. The standardised protocol for administration of psychometric assessments was described in Chapter 3. Psychometric test scores were converted to scaled scores to standardise the data across age groups according to the standard Wechsler administration instructions (refer to section 3.3, page 51).

5.3.4 Breastfeeding data

Breastfeeding data was collected by a physician who interviewed the mothers about both the timing and duration of breastfeeding.

5.4 Results

5.4.1 Summary of erythrocyte fatty acid biomarker data

Figure 5-1 provides an example of a high-quality GC-MS spectrum of fatty acids extracted from erythrocyte membranes. Table 5-4, page 91, summarises the fatty acid data for each of the four participant groups.

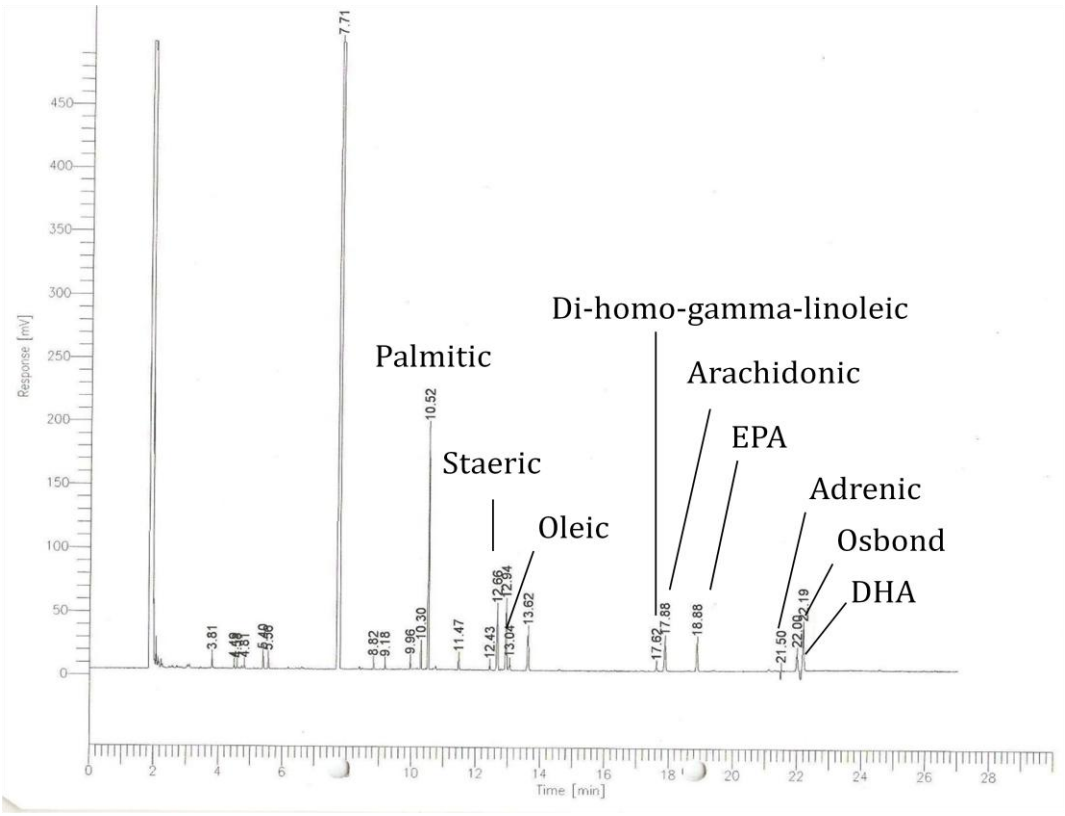

Figure 5-1 Example of high-quality GC-MS spectra of erythrocyte EFAs
Spectra shown is from one sibling control participant. Fatty acids are identified by their relative retention times and are labelled on the spectra. The integral of the peak indicates relative quantity of each fatty acid.

Data for each of the biomarkers assessed consistently satisfied distributional assumptions of normality (evaluated with the Shapiro-Wilk statistic at an α level of .05), therefore justifying the use of parametric statistics in subsequent analyses.

Psychometric data was available for 32 of the 39 participants, permitting correlational analyses of the relationship between cognitive outcomes and current fatty acid status. Mean and SD psychometric scores of the participants included in this study have been described previously in Table 4-2, page 77).

Table 5-5 The distribution of erythrocyte EFAs in sibling controls and liver disease groups

	Sibling controls	**EOLD pre-Tx**	**EOLD post-Tx**	**ALF post-Tx**
N	11	14	8	6
Fatty acid		% Total (SD)		
SFA	27.95 (5.26)	27.93 (6.21)	30.21 (5.0)	27.76 (5.54)
palmitic (16:0n-6)	7.02 (1.07)	6.86 (.93)	6.64 (.65)	6.64 (.49)
stearic (18:0n-6)	9.21 (.80)	9.49 (1.02)	8.92 (1.57)	8.85 (1.03)
MUFA	8.64 (.63)	8.82 (1.15)	9.16 (1.71)	8.98 (.55)
oleic (18:1n-6)				
Total omega-6	19.28 (1.86)	16.66 (2.71)	20.10 (3.11)	19.29 (1.74)
linoleic (18:2n-6)	9.43 (.97)	9.58 (1.39)	9.89 (1.78)	9.16 (1.14)
arachidonic (20:4n-6)	9.85 (1.02)	10.06 (1.49)	10.22 (1.60)	10.13 (.76)
Total omega-3	17.64 (1.70)	17.03 (3.40)	15.59 (2.68)	18.29 (2.37)
EPA (20:5n-3)	8.68 (.87)	7.61 (3.51)	7.28 (3.13)	8.55 (.94)
DHA (22:6n-3)	8.96 (.97)	9.41 (1.59)	8.63 (1.49)	9.73 (1.50)
Omega-3:omega-6	.92 (.05)	.91 (.17)	.86 (.14)	.95 (.13)
DHA/osbond acid (22:5n-6)	1.06 (.08)	1.07 (.25)	1.0 (.08)	1.14 (.20)

Mean percentage total and standard deviation of fatty acids quantified in erythrocyte membranes are shown. Percentage values do not total 100% as only selected fatty acids were included in analyses (see Chapter 3).

5.4.2 Inter-diagnostic differences of EFA and PUFA and biomarkers

Table 5-6 Differences in levels of fatty acid erythrocyte biomarkers between sibling controls and liver disease groups

Fatty acid biomarkers	ANOVA		
	F	df	p
SFA	.355	3, 35	.79
MUFA	.369	3, 35	.78
Omega-3 index	1.030	3, 35	.39
Omega-6 index	.202	3, 35	.89
Omega-3:omega-6	1.005	3, 35	.40
Functional DHA shortage marker	.585	3, 27*	.63

*Osbond acid (the denominator in this ratio) was below the detection threshold, in six of the participants

No significant differences were observed between diagnostic groups and controls for any of the six EFA status biomarkers assessed ($p > .05$).

5.4.3 Relationships between fatty acids and cognitive ability in a paediatric sample

Findings from Chapter 4 showed that there was no statistically significant difference between diagnostic groups for FSIQ. This justified the investigation of correlations between this broad-based measure of cognitive ability and fatty acid levels across the entire cohort, including both sibling controls and clinical groups. Whilst group difference in IPS were observed (see section 4.3.2, page 77) analysis was also conducted to test the relationship between fatty acid levels and this measure, independent of disease diagnosis.

To minimise susceptibility to Type I error, a Bonferroni correction was performed and the alpha level was set at .0014. No significant correlation was observed between total SFA (a composite of myristic, palmitic and stearic acid) for VIQ and PIQ ($p > .03$) or FSIQ and IPS ($p > .05$).

Table 5-7 Correlations between erythrocyte fatty biomarkers and cognitive ability

FA biomarkers	Correlation with cognitive indices; r (p value)				
	FSIQ	**IPS**	**PIQ**	**VIQ**	**WM**
N	32	32	32	32	17
SFA	.33 (.07)	-.01 (.97)	.39 (.03)	.38 (.03)	.13 (.61)
MUFA	-.29 (.10)	-.05 (.98)	.23 (.20)	-.34 (.06)	-.20 (.45)
Omega-3 index	.03 (.87)	.20 (.26)	-.71 (.70)	.13 (.95)	.44 (.08)
DHA	-.35 (.05)	-.28 (.12)	-.42 (.02)	-.16 (.37)	-.17 (.50)
Omega-6 index	-.62 (.00)*	-.39 (.03)	-.60 (.00)*	-.66 (.00)*	-.48 (.05)
Omega-3: omega-6	.40 (.02)	.32 (.08)	.32 (.08)	.39 (.03)	.60 (.01)
Functional DHA shortage marker	-.24 (.90)	.16 (.42)	.03 (.86)	.16 (.42)	.21 (.44)

FSIQ= Full-scale IQ, IPS= Information Processing Speed, VIQ= Verbal IQ, PIQ=Performance IQ, WM= Working memory, SFA= saturated fatty acids, MUFA = monounsaturated fatty acids, Omega-3 Index= EPA+DHA, omega-6 index= LA + AA
EFA data were normally distributed justifying the use of parametric Pearson's correlation coefficients
*p<.0014 (Bonferroni-corrected α value)

No significant correlation was observed between the omega-3 index (comprised of the percentage total of EPA and DHA) and any of the cognitive indices measured (p>.05). As DHA is putatively the most important long-chain PUFA, analyses were also targeted at this fatty acid specifically. However, no statistically significant correlation was observed between DHA values and FSIQ performance (r= -.35; p=.05, see Table 5-7).

The omega-6 index (comprised of the percentage total of LA and AA) showed a strong, negative correlation with FSIQ (r= -.62; p< .001, see Figure 5-2 , page 96), and a trend towards moderate negative correlation with IPS (r= -.39; p= .03, see Figure 5-3, page 96), although this relationship was not statistically significant at the Bonferroni-corrected α level of .0014.

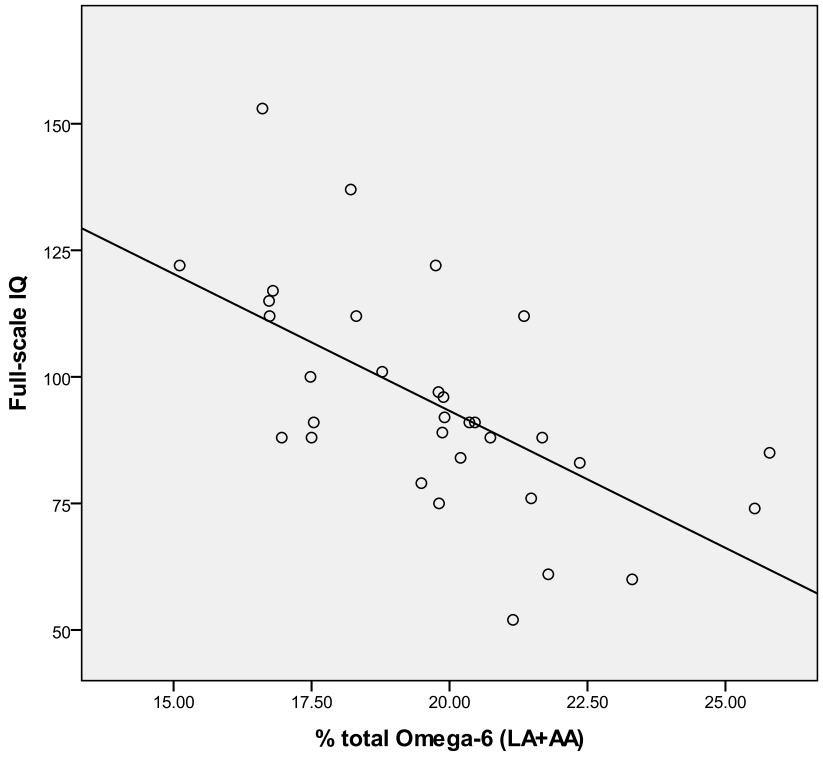

Figure 5-2 Correlation between % total omega-6 (LA and AA) and FSIQ
r= -.62 p< .001; n= 32

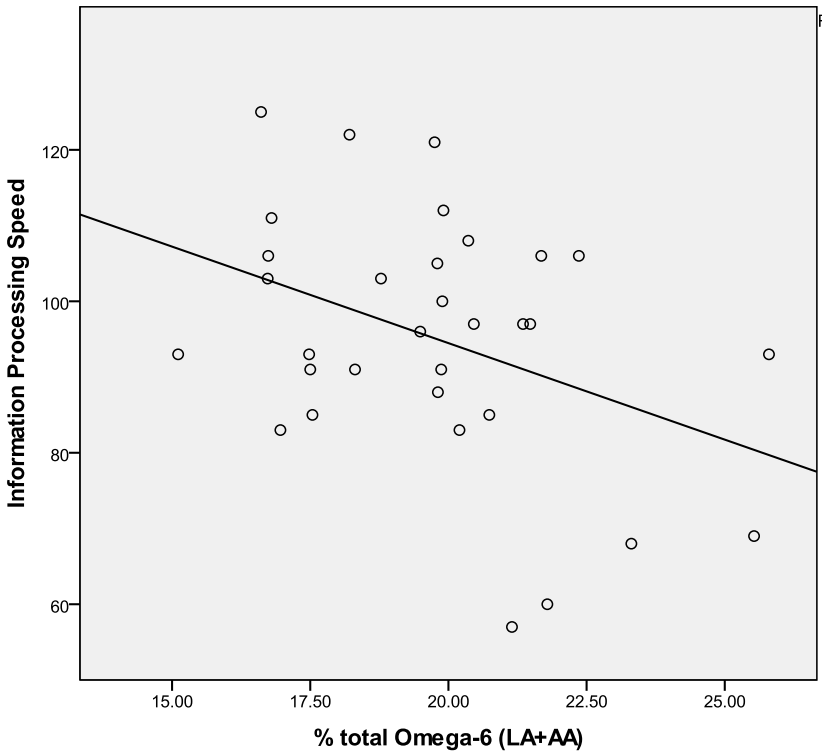

Figure 5-3 Correlation between % total omega-6 (LA and AA) and IPS
r= -.39 p= .03; n= 32

The omega-3:omega-6 ratio showed a trend towards a moderate positive correlation with FSIQ (r= .40; p= .02, see 5-4), and a strong, positive correlation with Working Memory (r= .60; p= .01, n= 17). The functional DHA index (the ratio of DHA to osbond acid) was weakly and not statistically significantly correlated with cognitive indices (p>.05).

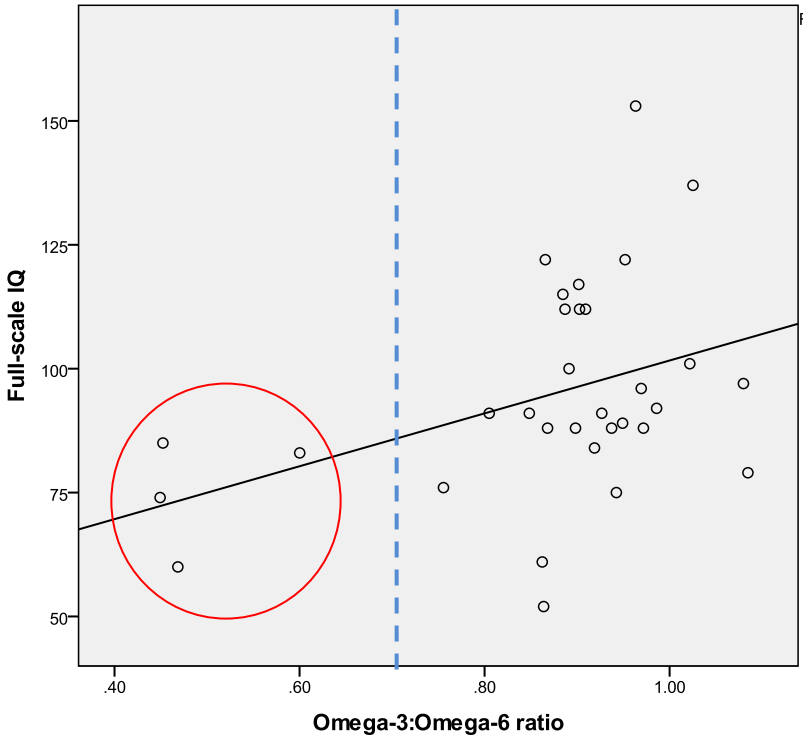

Figure 5-4 Correlation between the omega-3-omega-6 ratio and FSIQ
r= -.40, p= .02; n= 32.
A small subset of participants (circled in red) appear to be qualitatively different to the rest of the group, falling well below an omega-3omega-6 ratio of .70(blue dashed line)

Analysis of Figure 5-4, page 97, shows that there may be a sub-group of four participants (circled in red) that are qualitatively distinct from the rest of the group, with an omega-3omega-6 ratio below an arbitrary threshold of .70 (blue dashed line). Removal of these four outliers negates the previously significant linear relationship between the omega-3:omega-6 ratio and FSIQ (r= .31, p= .27; n= 28). The potential for categorical associations, rather than traditionally assumed linear relationships between EFA levels and cognitive variables is discussed in section 5.5.2.3.

5.4.4 Effect of breastfeeding on cognitive outcomes

Breastfeeding data was collected for 34 of the 39 patients and controls. Data from one patient and their sibling control was removed as duration of breastfeeding exceeded three standard deviations from mean duration. 26 of the remaining 32 participants had a full set of psychometric data. The numbers in each group are summarised in Table 5-8.

Table 5-8 The number of breastfed children in each diagnostic group

Diagnostic group	Breastfed		
	Yes	No	Total
Sibling controls	3	6	9
EOLD pre-Tx	5	7	12
EOLD post-Tx	4	3	7
ALF post-Tx	1	3	4

Participants were first grouped into breastfed/non-breastfed categories to assess the overall impact of breastfeeding on later cognitive outcomes. The results are shown in Table 5-9.

Table 5-9 Cognitive outcomes in breastfed vs non-breastfed children

	Breastfed vs non-breastfed		
	t	Df	p
FSIQ	-2.19	26	.04*
IPS	-1.43	26	.17
VIQ	-2.17	26	.04*
PIQ	-1.65	26	.11
WM	-1.97	15	.07

FSIQ= Full-scale IQ, IPS= Information Processing Speed, VIQ= Verbal IQ, PIQ= Performance IQ, WM= Working memory
Analyses were performed for the aggregated cohort of 26 children to assess the impact of breastfeeding on IQ, independent of disease diagnosis
*p<.05

Independent-samples t tests showed that FSIQ was higher in the non-breastfed groups than the breastfed group (t= -2.19 (26); p= .04), largely as a result of differences in the VIQ performance (t= -2.17 (26); p= .04).

Correlations between duration of breastfeeding (in weeks) and later cognitive outcomes in the 17 children with data available are shown in Table 5-10.

Table 5-10 Correlation between duration of breastfeeding and cognitive outcomes

	Correlation with cognitive ability; r (p value)				
	FSIQ	**IPS**	**VIQ**	**PIQ**	**WM**
n	17	17	17	17	7
Duration of breastfeeding	.18 (.49)	.37 (.15)	.15 (.57)	.40 (.12)	-.49 (.26)

FSIQ= Full-scale IQ, IPS= Information Processing Speed, VIQ= Verbal IQ, PIQ= Performance IQ, WM= Working memory
Data were analysed with non-parametric statistics, Spearman's Rho due to the small sample size

Non-parametric correlations showed that duration of breastfeeding (number of weeks) was not significantly correlated with FSIQ (r= .18; p=.49).

5.5 Discussion

5.5.1 EFA deficiency in paediatric liver disease

The risk of developing EFA deficiency depends on the amounts of different EFAs in the diet, the ability to absorb ingested fat, the demand for EFAs, and the amount and availability of endogenous stores. A poor EFA intake in the setting of a generally inadequate food intake has been considered as one of the major factors responsible for potential EFA deficiency in patients with liver disease. A lack of essential precursors certainly would be expected to lead to a deficit in LCPUFAs. The severity of the deficiency in the EFAs and LCPUFAs is related to the degree of overall malnutrition in these patients (Cabre and Gassull, 1996).

The evidence from this study, however, suggests that these patients do not show signs of EFA deficiency (see Table 5-5, page 93) as they had EFA levels comparable with the sibling control group. To prevent EFA deficiency, the diet requires only approximately .5% of the total caloric requirement as .7% ALA and 2% as LA (ISSFAL, 2010). Given that the average western diet provides 10–20 times more than these required amounts (Simopoulos, 2000), it is probable that levels of EFAs in the diets of the present cohorts was sufficiently high to prevent EFA deficiency. Examples of dietary data collected from a small number of patients involved in the current study (and additional disease control patients) are provided in Appendix A, Table A, page 206. Analysis of this small set of data is beyond the scope of the current study, but dietary data in future studies would be useful for providing greater context for investigations of deficiency and the erythrocyte biomarker data.

No evidence was found of LCPUFA deficiency in the patient groups, suggesting that these patients are able to adequately synthesise long-chain fats from the levels of EFAs that are present. It has been suggested that PUFA deficiency may be more closely related to rates of very long-chain PUFA biosynthesis rather than inadequate intakes of LA and ALA (Burke, Ling et al., 1999), but only in cases where diets are not entirely fat-free, as is the case with present cohort. It has been observed that children with liver disease may have a specific problem with malabsorption that is characterised by decreased linoleic and arachidonic acids and increased non-essential fatty acids (Gourley, Farrell et al., 1982; Jeppesen, Christensen et al., 1997; Pettei, Daftary et al., 1991). Socha et al., also reported decreased plasma AA levels in paediatric cholestatic patients, which they attributed to impaired hepatic microsomal desaturase and/or elongase activity (Socha, Koletzko et al., 1998). In the current study, however, no decreases in either linoleic or arachidonic acid were observed in the patients compared to the sibling controls (see Table 5-4).

In the absence of dietary DHA, Rapoport et al found that a normal brain DHA content can be maintained by liver conversion of ALA to circulating DHA, provided

sufficient ALA is present in the diet (Rapoport, Rao et al., 2007). In the present study, the question of whether DHA can be synthesised by older children in the absence of dietary sources of DHA was followed up in a clinical case study of a patient who was given an oral diet consisting of a specialised feed devoid of DHA for 12 months (see Appendix B, page 212). This individual (aged 11) was able to maintain erythrocyte DHA concentrations comparable to sibling controls, supporting the idea that dietary absence may not cause DHA deficiency, at least not detectable via erythrocyte biomarkers, provided EFA precursors are available for endogenous synthesis.

It has been suggested that PUFA deficiency may be closely related to genetic factors. The conversion of EFAs to LCPUFAs is dependent on fatty acid desaturases (FADs), which show phenotypic variability within the human population (Simopoulos, 2010). In the context of end-stage liver disease and profoundly cholestatic conditions such as Alagille's Syndrome, genetic-based variations in biosynthesis may be as important as inadequate intakes of LA and ALA (Burke, Ling et al., 1999), as these gene variants may be exacerbating the potentially higher than normal requirements for these nutrients in these patients. The genetic contribution to fatty acid metabolism is discussed as a future direction of research in Chapter 8, section 8.5.2.

5.5.1.1 Functional biomarkers of PUFA deficiency: osbond and mead acid

In addition to percentage values of the various lipids, biomarkers which provide a more subtle measure of EFA metabolism were also assessed. Osbond acid (22:5n-6) is synthesised specifically in response to DHA shortage. For example, dietary ALA deficiency (the DHA precursor) promotes accumulation of osbond acid in brain tissue (Rapoport, Rao et al., 2007). Under steady state conditions, the ratio between DHA and osbond acid (22:5n26) is a reliable indicator of functional DHA status (Hornstra, 2000), that is, the amount of DHA that is available for use. No difference between the disease groups for levels of functional DHA marker

(DHA/osbond) were observed, which provides further support to the finding of a specific lack of deficiency in the liver disease cohorts.

However, the near uniform presence of osbond acid in the patient and control groups (see Table 5-4) may indicate that, according to biomarkers representing three months dietary intake, even the sibling control group showed signs of DHA deficiency, which is in keeping with the hypothesis that modern western diets generally contain excessive amounts of omega-6 fatty acids and have a high ratio of omega-6 fats to omega-3 (Benatti, Peluso et al., 2004; Simopoulos, 2002a).

However, the use of osbond acid as a functional DHA shortage marker is not without question. In the present study, a moderate, but not statistically significant, correlation was observed between osbond and DHA levels ($r = -.30$ $p > .05$). Innis, Vaghri et al. (2004) found no evidence that low DHA concentrations in humans are accompanied by high osbond acid concentrations when erythrocyte fatty acids are used as a measure of fatty acid status, which they propose is suggestive of the fact that metabolic markers of deficiency traditionally seen in animals may not be easily translated to humans fatty acid metabolic processes.

Elevations in the triene:tetraene (arachidonic:mead) ratio have also been observed in patients with suboptimal lipid absorption (Pettei, Daftary et al., 1991). The failure to identify appreciable amounts of mead acid in any of the participants, a lipid that is produced in classic EFA deficiency by elongation and desaturation of oleic acid (18:1n-9) (see Figure 2-3, page 27), when LA and ALA are limited, may be a further explanation why the liver disease cohort did not present with EFA deficiency despite their illness. As with osbond acid, however, the use of mead acid as a deficiency biomarker is not unequivocal. Lands (2008) argued that detectable levels of mead acid may actually be a biomarker of the potentially beneficial restricted intake of omega-6 PUFAs, and may become a valid surrogate endpoint in future efforts to avoid excessive PUFA intakes and excessive omega-6 eicosonoid actions (see section 8.2.1).

5.5.2 EFA status and cognitive ability

There is increasing evidence that EFAs have a central role in cognitive development, with EFA deficiency linked to cognitive impairment; The fundamental importance of EFAs, and their LCPUFA metabolites, particularly DHA, for brain development and cognitive function is beyond dispute (McCann and Ames, 2005).

Results from Chapter 4 showed that early onset, post-transplant patients showed significant deficits in processing speed compared to sibling controls ($p = .017$, $(d) = 1.36$; see Table 4-3 and Figure 4-1, page 78). Analysis of current fatty acid status, measured using erythrocyte biomarkers, showed that this patient group was not significantly different in EFA levels compared to controls, which means that deficits in processing speed in these individuals cannot be attributed to their current fatty acid status, or at least that the effects are negligible.

The need for liver transplantation is an important indicator of disease severity so the finding that deficits were specific to the early onset, post-transplant group is in keeping with the hypothesis that EFAs may have a crucial role in later cognitive outcomes, given the relative importance of EFAs in early infancy (discussed in Chapter 2) and potential for EFA intake and metabolism to be disturbed in patients with liver disease (Beath, Hooley et al., 1993; Glasgow, Hamilton et al., 1973). That the acute liver failure group (with a mean age of onset of 5.4 years) did not show significant deficits compared to controls may be indicative of the fact that fatty acid intake and metabolism, as a result of liver disease, was undisturbed until after the crucial period of perinatal development, which is when EFAs are deemed especially important (Innis, 2007; Innis, 2008).

One explanation for the specific deficit in processing speed may be the action of EFAs on the myelination process (section 2.3.1, page 37) and ion channel function and (section 2.3.2, page 37). Rat models have shown that EFA deficiency during early development can give rise to aberrations in the process of myelination and

myelin composition (McKenna and Campagnoni, 1979; Trapp and Bernsohn, 1977). The integrity of the myelin is of utmost importance for the proper functions of axons in the nervous system and for facilitating the synchronous integration of information across the many spatially segregated associative regions of the brain that are involved in higher cognitive functions (Nicholls, Martin et al., 2001).

During maturation in humans, a shift from short chain saturated fatty acids to the long-chain unsaturated forms has been observed (O'Brien and Sampson, 1965; Svennerholm, Vanter et al., 1978). If myelin formation is compromised, particularly by a deficiency in LCPUFAs, nerve conduction velocity (NVC) may be suboptimal, resulting in a deficit in the ability to perform timed tasks of cognitive ability. NVC in brain nerve axons has been suggested to underlie general cognitive performance (McRorie and Cooper, 2004; Reed and Jensen, 1992). One way to probe the potential effects of liver disease on axons and myelination is to use ^{1}H-MRS which is able to detect NAA, a metabolite found exclusively in the brain that has a role in myelination and is regarded as a marker of neuronal viability. The findings from the ^{1}H-MRS study of the cohort of liver disease patients are presented in Chapter 7.

The current study retrospectively recruited patients with liver disease and as a result, what is necessarily lacking are measures of EFA status at birth/infancy that would have allowed us to directly probe the effects of liver disease on EFA status at a time when intake may be most crucial. A limited number of prospective studies have investigated the direct effects of early PUFA exposure on later cognitive outcomes by assessing LCPUFA content of umbilical plasma and erythrocytes as a surrogate measure of the LCPUFA availability during late gestation, but the association does not appear robust. Neither plasma nor erythrocyte phospholipid DHA and AA levels showed any significant correlation with cognitive development at four years of age (Ghys, Bakker et al., 2002) and in a similar study performed in a Dutch cohort, B Bakker, Ghys et al. (2003) found no evidence of a positive relationship between cognitive performance at age seven and LCPUFAs measured both at birth (in umbilical plasma samples) and also at seven years of age (in venous plasma).

The studies described above show no relationship between perinatal EFA status and cognitive outcomes in later childhood in healthy individuals, but the disturbances to EFA metabolism in liver disease are considerable (Dupont, Ameedee-Manesme et al., 1990) and therefore a more significant impact on cognitive outcomes may be anticipated. Conclusions from the current study remain speculative without a prospective study collecting periodic measures of EFA status and cognitive outcomes from birth.

5.5.2.1 Relationship between current omega-3 fatty acid status and cognitive ability

The finding that FSIQ and EFA status was not significantly different between groups allowed aggregation of the cohort of 39 in order to assess the association between current levels of the various lipids and cognitive performance. The hypothesis that current levels of omega-3 fatty acids would have a positive relationship with cognitive performance was not confirmed when a strict α level of .0014 was applied to the data. The biomarker for omega-3 status (comprised of the sum of EPA and DHA, putatively the most important LCPUFAs), did not correlate with FSIQ or IPS performance ($p > .05$).

Across the cohort of 39 participants, DHA was moderately, negatively correlated specifically with Performance IQ ($r = .42$; $p = .02$). Whilst the result is surprising, given the common consensus in the literature regarding the 'positive' effects of DHA such as increasing membrane fluidity and efficiency (Chapter 2), other studies have also found negative correlations between DHA levels and cognitive performance. For example, plasma DHA levels were associated with a slower learning curve on general speed of information processing as measured by the Stroop Colour Word Interference Test in a group of healthy adult women (de Groot, Hornstra et al., 2007). This suggests that the IQ relationship, at least for omega-3 fatty acids, is possibly not clear cut and this is certainly a reason for further research.

5.5.2.2 Relationship between current omega-6 fatty acid status and cognitive ability

The most striking finding from the current results is the strong correlation between omega-6 fatty acids and cognitive measures. The omega-6 index (comprised of the percentage total of LA and AA) was negatively correlated with FSIQ ($r = .62$; $p < .001$; Figure 5-2), which appears to be a robust result given the strict Bonferroni-corrected α level employed. Trend results were also observed for IPS ($r = -.39$; $p = .03$; see Figure 5-3, page 96), and Working Memory ($r = -.48$ =; $p = .05$). Whilst this negative relationship between omega-6 fatty acids and IQ measures was not predicted, it is in keeping with the present understanding of the functions of this group of fatty acids and their potential physiological effects.

The difficulty in placing these findings in sufficient context, however, is that the vast majority of the literature has focused almost exclusively on the positive and potentially ameliorative effects of omega-3 on cognitive outcomes, at the expense of considering the effects of omega-6 fatty acids (negative or otherwise), a problem also identified by Eilander, Hundscheid et al. (2007). Furthermore, there has been far less research on the role of brain AA specifically, partly because it is difficult to deplete brain AA levels by dietary deficiency studies.

5.5.2.3 The omega-3:omega-6 ratio and cognitive ability

As discussed in Chapter 2, omega-6 and omega-3 fatty acids share metabolic pathways and thus interact with each other through a complex system involving several factors including dietary substrate availability, competition for the same metabolic enzymes for synthesis and membrane incorporation (see section 2.2, page 26). An improved understanding of the role of free EFA in mediating cognitive and biochemical functions was derived from a series of findings that indicate that not only are the levels of EFAs or PUFAs critical, but also the ratio between the omega-3 and omega-6 fatty acids, both in terms of cognition (Simopoulos, 2002a) and chronic disease (Simopoulos, 2008).

Generally, higher ratios of omega-3 to omega-6 fatty acids are associated with improved cognitive status (Yehuda, 2003). The effects of omega-3 deprivation were discussed in detail in Chapter 2, section 2.4, page 40. In the majority of cases, the ratio between omega-3 and omega-6 fats is not studied directly, but chronic supplementation or deprivation of fatty acids, such as DHA, is regarded as manipulating the physiologically active ratio through changes in bioavailability. For example, in rats, administration of ALA and LA preparations with ratios of fatty acids ranging between 1:3.5-1.5 led to a significant improvement in learning, measured by performance on a Morris Water Tank task (Yehuda and Carasso, 1993), and reduced reference memory errors on a radial tasks was associated with cerebral DHA/AA ratios, after chronic administration DHA supplementation (Gamoh, Hashimoto et al., 1999).

Studies of humans, using erythrocyte biomarkers specifically relevant to the present study, appear to reinforce the importance of a higher omega-3:omega-6 ratio. In children with attention deficit hyperactivity disorder (ADHD) for example, it has been negatively related with oppositional, restlessness and problematic behaviour scales (Colter, Cutler et al., 2008), and eight weeks of EFA supplementation has been shown to reduce the AA/EPA ratio, which was significantly higher in ADHD children than controls at baselinel, with changes in the AA/EPA ratio linked with significant improvements in Inattention and Hyperactivity using Connor's short-term inventory (Germano, Meleleo et al., 2007).

Studies of adults report associations between an imbalance in the erythrocyte omega-3:omega-6 ratio in psychiatric disorders such as depression (Maes, Smith et al., 1996), schizophrenia (Yao, van Kammen et al., 1994). Higher total omega-3 fatty acids and DHA/AA ratios have been linked with better cognitive performance at the age of 64, even after accounting for IQ at age 11 (Whalley, Fox et al., 2004), and ratios of omega-3 to omega-6 fatty acids have also been inversely associated with mild cognitive decline (Heude, Ducimetiere et al., 2003).

In the present study, initial analysis with a bivariate, linear Pearson correlation showed a moderate, positive correlation between the omega-3:omega-6 ratio and FSIQ (r= .40; p=.02) and working memory (r= .60; p= .01, n= 17) . More careful analysis of the data, however, showed that this regression was skewed by four sets of outliers (see Figure 5-4, page 97). Removal of these data resulted in a non-significant linear relationships between the omega-3:omega-6 ratio and FSIQ and WM but also highlighted the need for a more subtle approach to analysing these complex relationships.

It may not be as simple as treating these four outliers as anomalies, as they point to an important alternative method of approaching the data. Given the competitive metabolism and function of omega-3 and omega-6 fatty acids, and the robust nature of the cellular membrane, it is not unreasonable to speculate that effects of variations in omega-3:omega-6 ratios may not manifest until a critical threshold has been exceeded (in the current data, an arbitrary value of ~.70), revealing a categorical, as opposed to the traditionally assumed linear, association between EFAs and cognitive outcomes.

Confined to data from only 39 participants, the idea of categorical dissociations must be treated with caution, but it is at least an idea worthy of consideration for future studies, particularly as much of the work in this field is still largely exploratory in nature.

5.5.2.4 Mechanisms by which EFA levels can effect cognitive function

The physiological roles that may explain the effects of omega-3 and omega-6 fatty acids on physiological function and therefore cognition were introduced in Chapter 2. Yehuda, Rabinovitz et al. (2005) have summarised the potential effects of PUFAs on membrane function into six nominal categories: (1) modifications of membrane fluidity; (2) modifications of the activity of membrane bound enzymes; (3) modifications of the number and affinity of receptors; (4) modifications of the function of ion channels; (5) modifications of the production and activity of

neurotransmitters; and (6) signal transduction, which controls the activity of neurotransmitters and neuronal growth factors. There are two mechanisms of action: (1) a long-term action on the composition and functioning of the membranes; and (2) a short-term action that would involve the metabolism of phospholipids (with subsequent modulation of signal transduction) and the action of EFA-derived metabolites such as eicosanoids.

The changes of greatest potential significance to the human infants and adults are those related to the physical properties of the membrane that impact membrane excitability. Both the chain length and the number of double bonds of the fatty acyl chains that constitute membrane phospholipids have substantial and significant effects on the dynamic properties of the membrane such as fluidity, permeability and rigidity (Hac-Wydro and Wydro, 2007).

Membrane fluidity may be one of the most salient mechanisms of effect that may explain the negative relationship between omega-6 levels and cognitive performance. The cis configuration at each double bond produces 'coiling' of the hydrocarbon backbone, resulting in a reduction in fatty acid length and a more curved, or 'kinked' structure. This results in longer-chain poly-cis unsaturated fatty acids (PUFAs; which have greater numbers of double bonds) occupying increased space in the membrane, resulting in bilayers that are thinner and more flexible than saturated/ monounsaturated chain bilayers (Rawicz, Olbrich et al., 2000). DHA (six double bonds) and EPA (five double bonds) confer the greatest level of fluidity from the major membrane fatty acids (Feller, Gawrisch et al., 2002; Gawrisch, Eldho et al., 2003). Arachidonic acid-containing phospholipid bilayers are, for example, more disordered and deformable than DHA-containing phospholipid bilayers (Rajamoorthi, Petrache et al., 2005).

The ever-changing mobility and proximity relationships of lipid and protein molecules in the plasma membrane also has a significant impact on essential cellular processes, including signal transduction, carrier mediated cellular transport, membrane bound enzyme activity and receptor function (McNamara

and Carlson, 2006). The capacity for omega-6 fatty acids to regulate neuronal excitability, for example, has been shown by Tsutsumi, Yamauchi et al. (1995), who demonstrated that rats fed high LA sunflower oil exhibited decreased Na+, K+ ATPase activity in myelin, together with 5'-nucleotidase activity in the rat cortex and hippocampus following a decline in DHA levels. Numerous animal models have also shown how PUFA levels my may influence several pathways with different neurotransmitters such as serotonin, noradrenalin, dopamine and acetylcholine (Chalon, 2006; Yehuda, Rabinovitz et al., 1999).

The finding that erythrocyte omega-6 fatty acids are inversely related to cognitive ability has also been observed in the elderly. Higher omega-6 PUFAs are associated with greater risk of cognitive decline, whilst omega-3 fatty acids may exert a neuroprotective effect (Heude, Ducimetiere et al., 2003). The competitive immunological functions of the two groups of PUFAs, may provide another explanation for their downstream cognitive effects, as cognitive decline, and psychiatric disorders including Alzheimer's, have been linked to neuroinflammation (Gorelick, 2010).

EFA in plasma membranes serve as substrates for a number of important, very active, short-lived, hormone-like compounds referred to as eicosanoids, which have numerous metabolic activities including platelet aggregation, inflammation, haemorrhage, vasoconstriction and vasodilatation, blood pressure and immune functions (Calder, 2007; Shaikh and Edidin, 2008; Simopoulos, 2002b). AA is the precursor of 2-series prostaglandins and 4-series leukotrienes, which are highly-active mediators of inflammation, generally pro-inflammatory and pro-aggretory, leading to a predominantly inflammatory state (Calder, 2002; Calder, 2006; Simopoulos, 2002b). EPA and DHA serve as the precursors for potent neuroprotective and anti-inflammatory lipids termed resolvins and neuroprotectins (Serhan, 2005), which exert their anti-inflammatory actions by blocking transendothelial migration, reduce dendritic cell function and regulate IL-12, thereby promoting the resolution of the inflammatory cycle.

PET studies have demonstrated increased brain AA incorporation in patients with Alzheimer's disease compared with healthy age-matched controls, particularly in neocortical regions with high levels of inflammatory cytokines (Esposito, Giovacchini et al., 2008) and EFA-derived eicosanoid precursors are also involved in the brain in oxidative stress, memory and learning (Tassoni, Kaur et al., 2008). Again, studies in psychiatric disorders and inflammation have largely concentrated on the damaging effects of potential omega-3 deficiency or ameliorative effects of omega-3 supplementation (Maclean, Issa et al., 2005), making it difficult to draw strong conclusions about the effects of omega-6 specifically. How well results from studies investigating cognitive decline and inflammatory status can be translated to studies of variation in cognitive ability in an ostensibly cognitively 'normal' population is still a matter for investigation.

5.5.2.5 Evidence from supplementation studies

The cross-sectional correlational analysis performed here means that firm conclusions about the causality of the associations observed cannot be made. Aside from disease models, such as the one adopted in the present study, intervention studies offer the strongest model to study the potential dose-dependent linear effects of EFA status on cognitive performance. Studies into the effects of variations in EFA levels on cognition in humans have largely focused on the effects of EFA supplementation in pre and peri-natal development in infants (Simmer, Patole et al., 2008; Simmer, Schulzke et al., 2008), with mixed results. Although, there is evidence for a relationship between omega-3 intake and cognitive and visual development in infants, to date, there have not been enough randomised, controlled trials investigating the effects of fatty acid supplementation as a model for studying the effects of EFAs on cognition in older children (Eilander, Hundscheid et al., 2007).

In contrast with the limited data in healthy children, there is more potential evidence for a beneficial effect of PUFA supplementation in children with neurodevelopmental disorders such as ADHD (Germano, Meleleo et al., 2007;

Richardson, 2006). Double-blind randomised controlled trials have shown promising results of supplementation with a combination of omega-3 and omega-6 fatty acids on diminishing some behavioural symptoms, including inattention and impulsivity, in school-aged children with ADHD (Hirayama, Hamazaki et al., 2004; Richardson and Puri, 2002; Stevens, Zhang et al., 2003; Voigt, Llorente et al., 2001). The potential mechanisms accounting these for effects, however, remain to be explained.

5.5.3 Breastfeeding

Breastfeeding is a vital source of EFAs in infancy and is therefore and important factor for consideration in the wider context of the study. The importance of EFAs in infant nutrition was suggested by the rapid accretion of these fatty acids in the brain during the first postnatal year (Martinez, 1992) and last intrauterine trimester. After birth, infants are reliant on maternal breast milk (or formula) as the sole source of DHA, with substantial accumulations of DHA and AA in the human brain during the first postnatal months (Heird and Lapillonne, 2005). Neuronal accretion of DHA and phsophatidylserine (PS) during development is required to prevent inappropriate cell death and to support neuronal differentiation (Kim, 2007). Interference in their accumulation by nutritional deprivation or in pathological states may diminish protective capacity in the central nervous system, with significant implications for neuronal dysfunction.

Although infants are able to synthesise DHA, the amount produced may be inadequate to support the DHA levels observed in breast-fed infants. In terms of fatty acid physiology, the cerebral and overall DHA status of breast-fed babies is better than that of infants fed formula lacking DHA (Cunnane, 2000). Breast-fed infants are also uniquely provided with an additional digestive enzyme known as bile-salt stimulated lipase, which Chen, Blackberg et al. (1994) demonstrated is essential for the complete hydrolysis of triacylglycerols containing AA or DHA.

The effect of breastfeeding on cognitive outcomes in the present sample, independent of disease diagnosis, was also investigated. When comparing breastfed vs non-breastfed participants, the breastfed group had significantly lower FSIQ scores ($t(26)= -2.19$, $p= .04$). This effect was not observed for IPS or Working Memory. Whilst this surprising finding may be a result of the small sizes of each group, split across the four diagnostic categories, the data is confounded by the fact that manufacturers introduced PUFAs to infant formulas (for example Pepti Junior, see Table 5-2, page 88) from around 1997 and almost as standard since 2000.

In this group of 17 participants with available dietary and psychometric data, the duration of breastfeeding was not significantly correlated with FSIQ in later childhood ($r= .18$; $p= .49$). Although the majority of studies suggest that breastfeeding promotes intelligence, (Anderson, Johnstone et al., 1999; Innis, 2008), with the positive effects of increased duration of breastfeeding potentially persisting into adulthood (Mortensen, Michaelsen et al., 2002), they have not always yielded consistent results.

The interpretation of findings from breastfeeding studies must be made with caution, particularly because of strong confounders such as maternal IQ and socioeconomic status (SES), as well as physiological mechanisms such as the variability in foetal brain DHA accrual during gestation, variability in maternal breast milk DHA concentrations (discussed in Chapter 2) and differences in the test instruments used, testing procedures, or outcomes studied (McCann and Ames, 2005). A meta-analysis of all randomised trials in which full-term infants were fed with formula supplemented with omega-3 and -6 PUFA declared that 'there is little evidence' that such supplementation is beneficial for visual or general development of full-term healthy infants (Simmer and Patole, 2004).

A systematic review of the literature, conducted by Jain, Concato et al. (2002), concluded that the evidence from higher-quality studies, which had stringent control of susceptibility bias (for example controlling for SES) and used

appropriate outcome measures, was less persuasive. Jacobson, Chiodo et al. (1999) found that after adjusting for maternal IQ and parenting skills the child's IQ was related to genetic and socio-environmental factors, rather than to the nutritional benefits of breastfeeding on neurodevelopment. The potential genetic contribution to EFA metabolism is discussed in greater detail in section 8.5.2, page 180.

5.6 Conclusion

There are two important findings from the present study, which was undertaken to assess the EFA status of patients with liver disease compared to sibling controls, and to investigate potential relationships between fatty acid status and cognitive ability.

First, compared to sibling controls, no signs of fatty acid deficiency were observed in any of the cohorts of patients with liver disease. This suggests that: (1) these patients were not deficient in their dietary intake of the EFAs LA and ALA; and (2) these patients are able to sufficiently metabolise these precursor lipids to synthesise LCPUFAs, DHA and EPA, to levels comparable to sibling controls.

Second, omega-6 (LA and AA) status was observed to have a strong inverse relationship with FSIQ and IPS, independent of disease diagnosis. EFA-derived LC-PUFAs in particular, have significant direct and indirect actions on cerebral function, not only through their function as membrane phospholipid components, but their function as active substances such as eicosanoids. The influence of fatty acids on the biological mechanisms that explain the co-variation of omega-6 lipids with broad-based measures of cognitive ability require further investigation, particularly in light of the fact that the majority of the research has focused on the positive effects of omega-3 fatty acids. The potential for categorical, as opposed to linear relationships between EFA measures and cognitive outcomes, particularly with regards to the omega-3:omega-6 ratio, is also worthy of consideration.

This study has shown that the onset of liver disease does not appear to have long-term effects on the ability of patients with liver disease to synthesise vital LCPUFAS. Without specific information about EFA status and dietary intake data in infancy to provide context, however, cross-sectional studies of retrospectively recruited patients are limited in what they are able to reveal about the cognitive consequences of liver disease in infancy with relation to fatty acid metabolism. Prospective studies collecting periodic measures of EFA status and cognitive outcomes from birth are required to directly probe the early effects of liver disease on EFA status and later cognitive outcomes.

6 Investigating biomarkers of cognitive ability with ^{1}H-MRS

6.1 Summary

^{1}H-MRS is a non-invasive imaging technique that enables quantification of neurochemicals *in vivo* and thereby facilitates investigation of the neurochemical underpinnings of human cognitive variability. Studies in the field of cognitive spectroscopy have typically focused on relationships between measures of N-acetylaspartate (NAA) and choline (cho) on broad measures of cognitive performance. In the present study, ^{1}H-MRS was used to interrogate single-voxels in occipitoparietal and frontal cortical white matter in parallel with assessments of psychometric intelligence (FSIQ) in a sample of 38 healthy, adult participants.

Correlations between ^{1}H-MRS detectable neurometabolites and IQ were observed that were within the range reported in previous studies. However, the magnitude of these effects was dependent upon the extent to which outlying values were accounted for in statistical analyses. Coupled with the wide range of effect sizes reported in the literature, the substantial methodological variability between studies poses a significant challenge for drawing inferences about the strength of the relationship between neurometabolites obtained with proton spectroscopy and IQ variables at the population level.

While ^{1}H-MRS offers a sensitive tool for assessing neurochemistry non-invasively, the relationships between brain metabolites and broad aspects of human behaviour are subtle. In a field of research that is still largely exploratory, there is a need to develop an increasingly rigorous analytical and interpretive framework for reporting data obtained from studies of this kind.

6.2 Introduction

Parallel refinements in neuropsychological assessment and neuroimaging techniques now make it possible to probe the neurophysiological basis of individual variation in cognitive ability with increasing methodological precision (Haier, 2009; Jung and Haier, 2007). ^{1}H-MRS provides a neuroimaging paradigm that enables non-invasive quantification of neurochemicals and their metabolites in a pre-defined region of tissue, with a typical spatial resolution on the order of cubic centimetres (see Chapter 3).

In the brain, the strongest and most reliable metabolite signals are generated by N-acetyl aspartate (NAA), creatine and phosphocreatine (Cre), choline (Cho; predominantly glycerophosphocholine and phosphocholine), and myo-Inositol (mI). These four metabolites, and other reasonably well-resolved compounds such as glutamate/glutamine (Glx) and lactate, form the principal focus of ^{1}H-MRS research (Ross and Sachdev, 2004; Soares and Law, 2009). Figure 3-3 on page 67 illustrates a representative proton MRS spectrum that shows these main metabolite peaks.

6.2.1 N acetyl aspartate (NAA)

In human brain, the most prominent and stable signal obtained with ^{1}H-MRS is that of N-acetyl aspartate (NAA), particularly beyond the age of three years (Danielsen and Ross, 1999). The three hydrogen atoms of the acetate group resonate a single sharp peak, with a chemical shift of 2.02 ppm relative to the tetramethylsilaneb standard (Moffett, Ross et al. 2007). While this peak at 2.02 ppm is mainly attributable to NAA, this signal includes smaller contributions from other acetylated compounds, such as the neuron-specific dipeptide, N-acetyl aspartyl glutamate (NAAG) (Caramanos, Narayanan et al., 2005), and underlying coupled resonances of glutamate and glutamine.

NAA is one of the most highly concentrated free amino acids in the brain, second only to glutamate. It is almost exclusively present in the central nervous system, where it is predominantly located in the soma of pyramidal cells, dendrites and axons (Simmons, Frondoza et al., 1991), oligodendrocyte type 2 astrocyte progenitor cells, and in immature (Urenjak, Williams et al., 1992) and mature oligodendrocytes (Bhakoo and Pearce, 2000). As an osmolyte, NAA constitutes 1% of the dry weight of the brain and 3-4% of total brain osmolarity (Baslow, 2000).

The functional role of NAA in the central nervous system and its metabolism has been extensively reviewed by Moffett, Ross et al. (2007). Amongst its many proposed roles, NAA is involved in osmoregulation and the control of cell volume (Davies, Gotoh et al., 1998). It is also linked with neuronal energy metabolism, as demonstrated by decreases in NAA that have been observed in a number of conditions of impaired energy metabolism in the brain (Clark, 1998). In addition, it has been proposed that NAA, NAAG and their intermediates are exchanged between neurons and glia as a mechanism of intercellular signalling (Baslow, 2000). NAA has also been implicated in enhancing mitochondrial energy production from glutamate (Moffett, Ross et al., 2007); functioning as a molecular water pump (Baslow (2002) cf. Moffett et al. (2007)) to increase the speed and efficiency of neuronal signalling; osmoregulation and the control of neuronal volume (Davies, Gotoh et al., 1998), and in intercellular signalling (Baslow, 2000).

NAA has been suggested to serve an important regulatory role within myelin lipid synthesis during postnatal axonal myelination (Namboodiri, Peethambaran et al., 2006). Abnormal NAA metabolism has been implicated in the pathophysiology of Canavan's disease, characterised by a progressive loss of myelin (Matalon, Michals et al., 1988; Namboodiri, Peethambaran et al., 2006). The NAA resonance within white matter regions is thought to reflect both the metabolic function of the neuronal axons as well as the extent and efficiency of myelination of those axons. The finding of approximately equal concentrations of NAA in white and grey matter of the human brain makes it clear that NAA is a component of the axon or the axonal sheath in man (Ross and Bluml, 2001).

Levels of NAA in various tissues of the brain have been found to correlate with neuronal health or integrity. Decreased levels of NAA have been interpreted to indicate neuronal/axonal loss, or compromised neuronal metabolism, leading to the idea that NAA is a neuronal marker (Moffett, Ross et al., 2007). This is, however, still a matter of debate, with some suggesting NAA is taken as a marker of functioning neurons rather than as a mere indicator of the presence of nerve cells (Pouwels, Brockmann et al., 1999); others refute the role of NAA as a marker altogether (Martin, Capone et al., 2001).

6.2.2 Choline

The major components of myelin and the cell membrane lipid bilayer (phosphatidylcholine (PCho), phosphatidyl -ethanolamine, -serine and -inositol; see Chapter 2) are most likely entirely immobile and MR-invisible (Ross and Bluml, 2001), but their putative breakdown products are a normal feature of cerebral proton spectra. The choline peak at 3.22 ppm is a narrow singlet originating from nine identical protons ($(CH3)^3$) and reflects combined total choline including choline containing compounds such as PCho, glycerophosphocholine (GPCho) and a comparatively small amount of free choline (<5%) (Miller, Chang et al., 1996). The ^{1}H-MRS choline peak is therefore thought to reflect the concentration of water-soluble choline-containing compounds and cellular density (Miller, Chang et al., 1996), as well as degradation of choline-containing phospholipids, which are abundant in cell membrane and in myelin (Alberts, 2002).

Choline (Cho) and its metabolites are needed for the structural integrity and signalling functions of cell membranes. In addition, they are precursors in the synthesis and breakdown products of membrane phospholipids such as phosphatidylcholine (PC) and sphingomyelin. They are central to the production of potent lipid mediators such as platelet-activating factor and lysophosphatidylcholine, and in the syntheses of acetylcholine (Zeisel, Da Costa et al., 1991). Quantitatively, PC is the most important metabolite of choline and accounts for approximately one-half of the total membrane lipid content of the

brain (Zeisel, Da Costa et al., 1991). A comprehensive review of choline metabolism and production is provided by Freeman (1996).

Free choline detectable by MRS is commonly associated with membrane turnover and inflammation, and negatively associated with cognitive ability (Danielsen and Ross, 1999). The signal intensity of choline may be increased by the acceleration of membrane synthesis and breakdown, or pathological conditions, where visible choline may be released from this pool. Further to its role in membrane composition, deprivation and supplementation studies of rats (Zeisel, 2004) have suggested that choline may have significant effects on gene regulation of cell division, apoptosis, migration and differentiation, through regulatory effects on DNA methylation.

6.2.3 myo-Inositol

The major nutritionally active form of inositol, myo-Inositol (mI), is vital to many biological processes of the body, participating in a diverse range of activities (Alberts, 2002). mI is a strongly coupled system and resonates at four chemical shift positions: 3.55, 3.61, 3.29 and 4.07ppm (Srinivasan, Vigneron et al., 2004). At 3T these resonances are resolved, but the C4/C6 peak will be partially overlapped with the glutamine-glutamate (Glx) C2 triplets. Due to its short T2 relaxation times, mI is detected better at shorter echo times.

mI plays an important role in the maintenance of osmotic equilibrium within the brain. For example, decreased brain mI has been measured in subjects with hypo-osmolarity. With normalisation of serum osmolarity, mI levels returned to the normal range (Häussinger, Kircheis et al., 2000). In addition to its chemically inert function as an osmolyte or cell marker, mI is at the centre of a complex metabolic pathway that contains, among other products, the inositol-polyphosphate messengers, inositol-1-phosphate, phosphatidyl inositol, glucose-6-phosphate and glucuronic acid (Ross and Bluml, 2001).

6.2.4 Creatine

Creatine is present in all neural cells, with *in vitro* work suggesting higher concentrations in oligodendrocytes (Urenjak, Williams et al., 1993). The creatine peak at 3.03 ppm, a narrow singlet peak originating from the CH_3 group of the molecule, indexes the sum of creatine and phosphocreatine (PCr), except at high magnetic fields where the two can be separated (Danielsen and Ross, 1999). The Cr/PCr system plays a number of roles in regulating cellular bioenergetics, including serving as a temporal energy buffer for the cell, and as an energy carrier, transporting high-energy phosphate groups from sites of synthesis to the specific subcellular compartments in which they are required (Saks, Ventura-Clapier et al., 1996).

Because ^{1}H-MRS sees both Cr and PCr as a single peak, the creatine signal cannot be strictly interpreted as either an index of current energy use or available energy reserves. The creatine peak is thought to be relatively constant between individuals and in most brain areas, and is therefore used as an internal reference, where creatine equals 1 in the denominator of expression of all neurometabolite ratios (Danielsen and Ross, 1999).

6.2.5 ^{1}H-MRS metabolites and cognition

The putatively important role of the metabolites most commonly resolvable at 1.5 and 3T in neural tissue has been extended to investigations at more macroscopic levels of analysis, for example toward developing an understanding of the potential biochemical correlates of cognitive ability. One general approach has been to obtain measures of the NAA, choline and mI in cortical tissue with concurrent psychometric measures of cognitive skills, such as those provided by standardised IQ tests.

The clinical utility of MRS in cases of aberrant neurometabolism is well established (Cox, 1996; Hollingworth, Medina et al., 2006; Ross and Bluml, 2001; Soher,

Doraiswamy et al., 2005; Steen, Hamer et al., 2005). Work in healthy individuals has largely concentrated on NAA in young adults, typically between the ages of 18 and 25. NAA in occipitoparietal white matter has been reported to correlate moderately with timed measures of neuropsychological performance, but not with metrics of ability derived from non-timed tests (Jung et al., 1999a; Jung et al., 1999b). NAA has also been demonstrated as a predictor of moderate effect for FSIQ, a construct derived from both timed and non-timed psychometric subscales (Jung et al., 2005). Choline has been positively correlated with vocabulary ability (Pfleiderer, Ohrmann et al., 2004) and, as with mI, negatively correlated with IPS in older, healthy adults (Ross, Sachdev et al., 2005).

Table 6-1 on page 125 summarises the results from the published literature in this area. The lack of consistency in the methodology demonstrates that work in this field is still largely exploratory. Correlations between neurometabolites and IQ variables vary according to the particular IQ subscale assessed and with the cortical region and tissue type in which the MRS voxel was located, an effect that has also been observed in children (Ozturk, Degaonkar et al., 2009; Yeo, Hill et al., 2000), adolescents (Gimenez, Junque et al., 2004) and in older populations (Charlton, McIntyre et al., 2007; Ferguson, MacLullich et al., 2002; Ross, Sachdev et al., 2005; Valenzuela, Sachdev et al., 2000).

6.2.6 Aim

The aim of this study was to build on previous work in an effort to examine the presence and strength of the relationships between ^{1}H-MRS detectable metabolites and cognitive ability in healthy individuals, with the eventual aim of clarifying its use in the paediatric liver disease cohort.

6.2.7 Hypotheses

1 Given its putative regulatory role in both myelination and in neurophysiological processing speed, positive associations between levels of NAA in frontal white matter and the IPS were predicted that would be stronger than those found for general cognitive ability (FSIQ).
2 As ^{1}H-MRS-detectable choline is associated with neuronal membrane turnover and breakdown, levels of this metabolite were predicted to negatively correlate with cognitive performance.

The role of mI in neural function is still largely unknown and no specific predictions regarding this metabolite were made. As a control condition, measures of NAA, choline and mI were obtained for voxels in occipitoparietal cortex, for which the same pattern of covariance with IQ measures was not predicted.

Table 6-1 Summary of published investigations into the relationships between common ^{1}H-MRS-detectable metabolites and cognitive abilities in healthy populations

Study	N	Age in years (SD)	Region(s)	Key result	Effect sizes (r^2)
Jung, Brooks et al. (1999)	26	22 (4.6)	Left OP WM	NAA, but not choline, positively correlated with FSIQ.	.45
Jung, Yeo et al. (1999)	45†	23 (4.9)	Left OP WM	NAA correlated with timed tasks to greater degree than general IQ. Choline was negatively correlated with FSIQ.	.43; .23
Yeo, Hill et al. (2000)	20	12.5	Right frontal WM	NAA positively correlated with Working Memory[a]. Cr[b] and Cho[c] negatively correlated with DI.	.45[a]; .31[b]; .30[c]
Jung, Yeo et al. (2002)	37	25 (5.8)	Left frontal WM	Individuals with``high' levels of Cho scored 10.55 points lower on the PIQ than those with 'low' levels. No group difference in FSIQ.	.22
Pfleiderer, Ohrmann et al. (2004)	62	38.5 (15.4)	DLPFC, left anterior cingulate cortex (ACC)	Choline correlated with vocabulary scores[d]. NAA positively correlated with Verbal Intelligence in women in left DLPF and left ACC[e].	.13[d]; .53[e]
Charlton, McIntyre et al. (2007)	78	58.2	Centrum semiovale WM	NAA positively correlated with general composite measure of cognitive performance[f]. Cr positively correlated with Executive Function[g] and long-term memory[h], but not after age is controlled for.	.33[f]; .45[g]; .47[h]
Jung, Gasparovic et al. (2009)	63	23.7 (4.2)	Left/right, posterior /anterior grey/white matter	Lower right anterior GM NAA predicted Verbal IQ[i]. Higher posterior GM NAA predicted Performance IQ[j].	.10[i]; .12[j]

Study	N	Age in years (SD)	Region(s)	Key result	Effect sizes (r^2)
Jung, Haier et al. (2005)	27	24.8 (5.9)	Left OP, left frontal and right frontal WM	Combination of higher left occipital WM and lower frontal WM NAA correlated with FSIQ in women.	.82
Valenzuela, Sachdev et al. (2000)	20	72	Left OP and left frontal and WM	Frontal WM NAA/Cr correlated with executive-attentional cognitive ability.	.61
(Ferguson, MacLullich et al. (2002)	88	65–70	Left parietal WM	Cho/Cr[k] and NAA/Cr[l] correlated with Logical Memory, but effect due to Creatine (Cr negatively correlated with Logical Memory).	.14[k]; .24[l]
Ozturk, Degaonkar et al. (2009)	51	12.3 (3.6)	Frontal WM, DLPFC, parietal WM, inferior parietal cortex, dorsal parietal cortex	Left frontal WM NAA/Cr correlated with Purdue Pegboard right-hand raw scores[m]. Right frontal WM NAA/Cr correlated with SB-IV "Bead Memory" raw scores[n].	.12[m]; .10[n]
Gimenez, Junque et al. (2004)	21	14 (2.3)	Left medial temporal cortex	NAA/Cho related to free recall[o] and recognition[p] memory abilities	.56[o]; .51[p]
Ross, Sachdev et al. (2005)	59	70.8	Left frontal WM, midline OP GM	Frontal WM NAA/H_2O correlated with a composite measure representing speed of information processing, attentional function and visual memory[q]. Cho/Cr[r], Cho/H_2O[s] and mI/H_2O[t] negatively correlated with speed of processing.	.32[q]; .07[r]; .08[s]; .08[t]

Key inter-study variables such as the size of the populations and their age are described as well as the primary cortical regions studied. The principal research findings from each study are presented with the wide range of associated effect sizes (r^2).
DLPFC: Dorsolateral prefrontal cortex; OP: Occipitoparietal; WM: White matter; GM: Grey matter
†26 participants' data previously published in Jung et al. (1999a)

6.3 Method

6.3.1 Participants

42 healthy volunteers (29 females, 13 males) were recruited from the local population and from the Aston University student body (mean age: 21.4, SD: 3.4). Informed consent was obtained from all participants under a protocol consistent with the tenets of the Declaration of Helsinki and with the approval of the University's Ethics committee (REG/00/175).

Participants were screened prior to testing to exclude the presence of probable neurological dysfunction, including previous serious brain injury, history of learning disability, neurological disease, psychiatric diagnosis or current use of psychoactive medication. The sample provides the study with statistical power in excess of 80% to detect moderate correlations of .4 and above, with statistical significance evaluated at an α level of .05 (Friedman, 1968).

6.3.2 Neuropsychological assessment

The Wechsler Abbreviated Scale of Intelligence (WASI) (Wechsler, 1997a) was administered to obtain scores for IPS and FSIQ, comprised of the Verbal and Performance IQ subtests). Refer to Chapter 3 for details.

6.3.3 Neuroimaging

Single-voxel ^{1}H-MRS (2cm^{3}) was performed following localisation of the volume of interest (VOI) using a 5-plane localiser (TR: 20 msec, TE: 5 msec, 10 slices at 5mm thickness). Automated shimming was followed by a stimulated echo acquisition mode (STEAM) pulse sequence (TR: 2,000 msec, TE: 30 msec, 96 averages), including water suppression, for voxels in frontal (Fro) and occipitoparietal (OP) cortex. Refer to Chapter 3 for details of the scanning procedures and acquisition parameters.

6.3.4 Data analysis

The metabolite values were estimated *post hoc* using LCModel (Provencher, 2001). This software provides spectral quantification and metabolite detection optimised for short echo time spectroscopy. All analyses reported here adopt the standard convention of expressing NAA and choline and mI as a ratio to creatine (/Cr).

6.3.5 ^{1}H-MRS data screening

Two levels of screening were applied to the spectroscopy data to ensure accurate and reliable data before statistical analyses. The first pass for data screening was the rejection of spectral data based on on-line visual inspection of the acquired spectra. High-quality spectra were identified by their narrow, well resolved peaks, a reasonably flat baseline and the presence of Hunter's Angle (in normal tissue). Poor quality spectra were recognised by a distorted baseline, reduced chemical shift dispersion and broadened linewidths (increased full-width half maximum peak height), see Figure 6-1. On-line data screening allowed reacquisition of data following necessary adjustments to the voxel location, maximising the possibility of acquiring high-quality data from every participant.

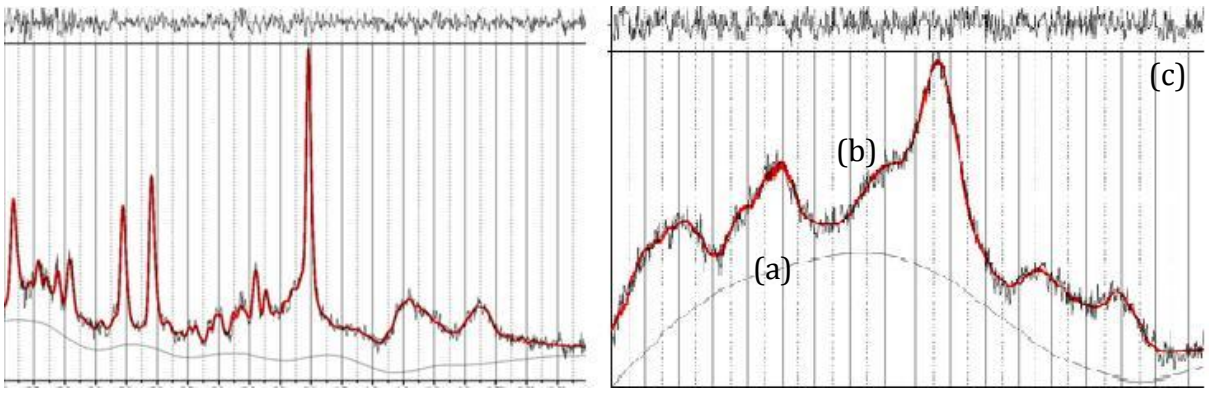

Figure 6-1 Examples of high-quality (left) and poor quality (right) ^{1}H-MRS spectra in frontal cortex, modelled with LCModel.
During on-line data screening, high-quality spectra were identified by their narrow, well resolved peaks, a reasonably flat baseline and the presence of Hunter's Angle (in normal tissue). Poor quality spectra were recognised by a distorted baseline (a), reduced chemical shift dispersion (b) and broadened linewidths (increased full-width half maximum peak height) (c)

All subsequent data was run through LCModel. The second pass of data screening was the exclusion of data if metabolite values had a %SD (the estimated standard deviations, expressed in percent of the estimated concentrations) exceeding 20%, the approximate criterion for acceptable reliability suggested by Provencher (2008). Based on visual inspection of the spectra and the 20%SD criteria, data from four participants were excluded due to unreliable, poor quality spectra, resulting from susceptibility or motion artefact (See Figure 6-1, page 127).

6.4 Results

38 participants (27 females, 11 males) from a total of 42 were included in the final analyses.

6.4.1 Summary of spectroscopy data

A summary of spectroscopy and psychometric data is provided in Table 6-2, page 129. Mean metabolite ratios to creatine were consistent with those reported previously. FSIQ scores were available for 29 participants, and IPS scores for 36 participants, out of the total 38 participants. MRS data for occipitoparietal cortex was obtained for all 38 participants and for 36 out of 38 for the frontal cortex volumes.

Table 6-2 Summary of psychometric and spectroscopy data

	Mean	SD	Min	Max
Age (years)	21.3	3.5	18	34
FSIQ†	111	16.2	52	153
IPS‡	108	15.1	57	140
VIQ	114	15.2	57	147
PIQ	110	17.0	54	150
Frontal voxel				
Cho/Cr	0.25	0.06	0.01	0.35
NAA/Cr	1.54	0.26	0.42	1.95
mI/Cr	0.67	0.12	0.34	0.86
Occipitoparietal voxel				
Cho/Cr	0.23	0.04	0.16	0.34
NAA/Cr	1.61	0.22	0.78	1.88
mI/Cr	0.65	0.08	0.46	0.85

FSIQ: Full-scale IQ; IPS: Information Processing Speed
OP: Occipitoparietal; Fro: Frontal
†n=29; ‡n=36

6.4.2 Correlations between neurometabolites and cognitive variables

Distributions of data prior to the removal of outliers did not consistently satisfy distributional assumptions of normality (evaluated with the Shapiro-Wilk statistic at an α level of .05) and were first analysed using non-parametric statistics. Following the removal of participants with outlying data points, the same data distributions did not deviate significantly from normality, thereby justifying the use of parametric statistics for these analyses. All correlations were evaluated for statistical significance at a Bonferroni-corrected α level of .002 (.05/24 correlations).

Table 6-3 and Table 6-4, page 130, present the correlations between the spectroscopic and IQ variables in two complementary ways. First, the metabolite values obtained from all participants were included in the analyses and assessed correlations using non-parametric Spearman rank-order correlation coefficients (Table 6-3). Second, three multivariate outliers whose NAA/Cr, Cho/Cr or mI/Cr values were ±2SD the overall sample mean were removed, justifying the use of Pearson correlation coefficients (Table

6-4) as the data were now normally distributed. All correlations were evaluated for statistical significance at a Bonferroni-corrected α level of .002 (.05/24 correlations).

Table 6-3 Non-parametric correlations between unscreened ^{1}H-MRS metabolite values and psychometric measures

	Unscreened data			
	Correlation with cognitive ability; r (p value)			
	FSIQ	**IPS**	**PIQ**	**VIQ**
Frontal (n)	27	36	27	27
Cho/Cr	.03 (.94)	-.15 (.37)	.11 (.57)	.04 (.85)
NAA/Cr	.09 (.65)	-.13 (.44)	.22 (.27)	.11 (.58)
mI/Cr	-.02 (.94)	-.07 (.70)	.09 (.66)	.12 (.54)
Occipitoparietal (n)	29	38	29	29
Cho/Cr	-.07 (.73)	-.07 (.67)	.08 (.70)	-.23 (.99)
NAA/Cr	.21 (.27)	-.16 (.71)	.34 (.07)	.05 (.78)
mI/Cr	-.09 (.63)	-.01 (.93)	.02 (.92)	.00 (.99)

Unscreened metabolite values were not normally distributed and analysed with non-parametric statistics (Spearman's Rho)
n=27

Table 6-4 Parametric correlations between screened ^{1}H-MRS metabolite values and psychometric measures

	Screened data			
	Correlation with cognitive ability; r (p value)			
	FSIQ	**IPS**	**PIQ**	**VIQ**
Frontal (n)	24	33	24	24
Cho/Cr	-.08 (.72)	.03 (.88)	-.08 (.72)	-.05 (.83)
NAA/Cr	.06 (.77)	-.24 (.18)	.06 (.78)	.02 (.42)
mI/Cr	-.03 (.89)	-.01 (.96)	.12 (.59)	-.10 (.63)
Occipitoparietal (n)	26	35	26	26
Cho/Cr	-.04 (.85)	.00 (.98)	.21 (.32)	-.17 (.40)
NAA/Cr	.11 (.60)	.09 (.60)	.21 (.31)	.05 (.80)
mI/Cr	-.17 (.40)	-.17 (.32)	-.18 (.390)	-.16 (.44)

Three outlier values (±2SD) excluded;
Normally distributed data analysed with parametric statistics (Pearson Product Moment Correlation)
n=24

Correlation coefficients for the unscreened data showed that the relationships between IPS or FSIQ and NAA/Cr, Cho/Cr and mI/Cr were neither strong, nor statistically

significant, a pattern that was consistent across both occipitoparietal and frontal voxels. This pattern of result was upheld with parametric analyses when multivariate outliers were removed.

A Related-samples Wilcoxon Signed-rank was used to assess inter-regional differences in metabolite values. No significant differences in mean NAA/Cr, Cho/Cr or mI/Cr were observed between frontal and occipitoparietal voxels ($p = .07$, .09 and .30, respectively). An Independent-samples Kruskall Wallis test showed no main effect of gender on metabolite values ($p > .05$).

6.5 Discussion

Neurometabolite changes, particularly for NAA, have been linked previously to psychometric and performance variables in a number of disorders of cognitive function, including traumatic brain injury, schizophrenia and Alzheimer's disease (Ross and Sachdev, 2004). Such demonstrations of the utility of MRS in tracking neural viability in cognitive neuropathology has motivated the study of normal populations in order to assess the validity of using this paradigm to identify biomarkers of human cognitive variability.

Several studies have demonstrated statistically significant correlations between ^{1}H-MRS detectable metabolites and broad measures of cognitive ability, with typically small to moderate effect sizes in samples of putatively normal populations (see Table 6-1, page 124). In the present study, measures of NAA, choline and mI were obtained with proton spectroscopy for a group of healthy adults. The principal finding is that quantitative estimates of these metabolites did not correlate strongly with standardised measures of IQ, including the IPS index, a construct of cognitive ability for which particularly strong relationships were predicted.

The small correlations obtained between metabolite concentrations and measures of cognitive function echo other findings that have reported poor strength of association between IQ and neurometabolite variables in healthy cohorts (Filippi et al., 2002; Friedman et al., 1998; Gimenez et al., 2004; Shim et al., 2001). However, they also contrast with other demonstrations of strong and significant relationships

between these variables obtained with similar research designs (Jung et al., 1999a; Jung et al., 1999b; Jung et al., 2005; Yeo, Hill et al., 2000).

6.5.1 NAA and cognition

The general theory in the literature is that if NAA is taken as a functional neuronal marker with natural variation in the population, reduced levels of NAA may be reflective of increased neuronal death, or decreased neuronal metabolism or myelination, which may then manifest as the subtle changes in general cognitive ability.

Weak to moderate positive and negative associations between NAA/Cr were observed in this cohort. These were within the range observed by others (Table 6-1, page 124), but were not statistically significant. Studies exclusively focusing on white matter have found associations between NAA and processing speed. NAA in occipitoparietal white matter has been reported to correlate moderately with timed measures of neuropsychological performance, but not with metrics of ability derived from non-timed tests (Jung, Brooks et al., 1999; Jung, Yeo et al., 1999).

NAA has also been demonstrated as a predictor of moderate effect for FSIQ (Jung, Haier et al., 2005). This association between occipitoparietal NAA with FSIQ was later replicated in a sample of 27 healthy volunteers, with a combination of left frontal and left occipitoparietal NAA strongly predicting performance in women, but not statistically significant in men (Jung, Haier et al., 2005).

Corroborating evidence for this potential gender difference is work by Pfleiderer, Ohrmann et al. (2004), who found that, in women only, NAA in the left dorsolateral prefrontal cortex and in the left anterior cingulated cortex was positively correlated with raw, not age-corrected and standardised vocabulary assessment scores. In contrast to these two studies, no differences in gender were observed in the present cohort for any of the metabolites assessed.

Valenzuela, Sachdev et al. (2000) observed a correlation between NAA/Cr in the left frontal subcortical white matter and attentional processing ability, which is functionally

associated with this region. In keeping with the findings of the present study, but unlike (Jung, Haier et al., 2005; Jung, Yeo et al., 1999), Valenzuela et al found no associations between measures in the occipitoparietal grey matter and cognition in these subjects. Ross, Sachdev et al. (2005) have shown a significant correlation between frontal white matter NAA/H_2O and a composite measure of neuropsychological performance representing speed of information processing, attentional function and visual memory, controlling for age and sex, in a population of 58–85 year old healthy individuals.

6.5.2 Choline and cognition

Weak, negative correlations, which were not statistically significant, were observed between choline and IQ measures (see Table 6-3, page 134). Choline concentration is reported to vary with the production and degradation of the choline-containing phospholipids, which are abundant both in cell membrane and myelin (Alberts, 2002). The free choline detectable by MRS is more commonly associated with membrane turnover and inflammation. An abnormality in membrane structure or myelination could potentially precipitate decreased synaptic strength and efficiency, manifesting in cognition as small, but significant, changes in ability.

For example, weak negative correlations between choline and cognitive ability have been observed by Ross, Sachdev et al. (2005), but this was in healthy, elderly men and with choline value expressed as ratios to both creatine and water. Jung, Yeo et al. (2002) split participants into 'high' and 'low' choline groups (details of cut-off criteria were not provided), and found that individuals in the 'high' group performed significantly higher on the Performance IQ subtests, and specifically the processing speed index. No differences between the groups were observed for Full-scale and Verbal IQ or Working Memory performance.

6.5.3 myo-Inositol and cognition

The relationships between mI and cognition are understudied in comparison to NAA and choline. Two studies from those listed in Table 6-1 reported data for mI in healthy populations. No relationship between mI concentrations and broad-based measures of

cognitive ability were observed in healthy young adults (Jung, Yeo et al., 1999), but in a healthy elderly population Ross, Sachdev et al. (2005), report correlations of -.33 between mI/Cr and mI/H_2O and executive function, and -.28 between mI/H_2O and speed of information processing. Studies of the role of mI in disease states and cognitive outcome in the elderly have been more fruitful, particularly in mild cognitive impairment and Alzheimer's disease. mI has, for example, been shown to be strongly negatively correlated with Mini Mental State (MME) scores (r^2= .54) (Salvan, Ceccaldi et al., 1998), and mI/H_2O correlates with language function in left prefrontal cortex (r= -.60) and visuoconstructional abilities in right prefrontal cortex (r= .68) in Alzheimer's disease patients (Chantal, Labelle et al., 2002). However, elevated mI is has not always been found to be related to the presence of MCI (Metastasio, Rinaldi et al., 2006; Ross and Sachdev, 2004), and the relationship between mI and cognitive abilities is still largely unknown. The role of mI as a potential clinical biomarker in the specific context of liver disease is discussed in Chapter 7.

6.5.4 Methodological issues in cognitive spectroscopy studies

Examination of the existing literature reveals a number of methodological or research design features that vary greatly between studies (see Table 6-5, page 135). Differences in the age of the participants, the cortical regions in which the MRS voxels were placed, the constructs of cognitive abilities assessed and the quality of data screening applied (see Table 6-1, page 124) may all be factors which explain the the inhomogeneous effects found across the population of published studies. In the following section the aim is to place the results of the present investigation within the context of these inter-study differences and outline recommendations for reporting future studies in cognitive spectroscopy.

Table 6-5 Summary of inter-study variables in cognitive spectroscopy studies as a result of the methodological approach and data analysis employed

Study	No. of comparisons				Outliers screened
	Metabolite quantification	No. of regions	No. of psychometric sub-tests	Total no. of comparisons	
Jung et al. (1999a)	Absolute values	1	11	9	N
Jung et al. (1999b)		1	10	9	N
Yeo et al. (2000)		1	7	9	N
Jung et al. (2002)		1	10+	10+	N
Pfleiderer et al. (2004)		3	1	12	Y
Charlton et al. (2007)		1	14	20	N
Jung et al. (2009)		8	5	24	N
Jung et al. (2005)		3	12	30	Y
Valenzuela et al. (2000)	Cr ratio	2	16	9	N
Ferguson et al. (2002)		1	11	33	Y
Ozturk et al. (2009)		6	4	20	N
Gimenez et al. (2004)	Cho ratio	1	18	7	N
Ross and Sachdev (2005)	H_2O and Cr ratio	2	15	8	N

Differences in the method of metabolite quantification and expression, the number of comparisons made in statistical analyses as a function of the number of regions and psychometric measures assessed, and whether data were appropriately screened, may contribute to the variable strength and size of effect sizes reported in the literature.

6.5.4.1 Age

Previous studies of healthy populations varied substantially in the age of the population sampled, ranging from late childhood (Ozturk et al., 2009; Yeo et al., 2000) to older ages (Ferguson et al., 2002; Ross et al., 2005; Valenzuela et al., 2000) (see Table 6-1). In the present study of younger healthy adults with a mean age of 21 years, no significant

correlations were observed between the metabolites in either frontal or occipitoparietal voxels in the present study, yet the mean age and standard deviation of the participants was similar to that of other investigations of young, healthy adults, some of which have reported particularly large effects (Jung et al., 1999a; Jung et al., 2005; Jung et al., 1999b) (see Table 6-1; for example r^2= .45). Although previous reports suggest the presence of age-related metabolic changes across the lifespan (Angelie et al., 2001; Pouwels et al., 1999), there is no systematic relationship apparent between the age of the study sampled and the strength of the IQ/neurometabolite correlations (Table 6-1). The most reasonable interpretation of these data is that age does not account for the variability in effect size of the NAA/IQ relationship in this cohort of studies.

6.5.4.2 Metabolite quantification

Published studies vary according to whether absolute concentrations of NAA are reported or whether NAA values are expressed as a ratio to another neurometabolite (typically creatine, or alternatively choline). Expressing NAA as a ratio to creatine confers the advantage of correcting for potentially important unknown or uncontrollable, yet correlated, experimental factors, such as static (B_0) and radio frequency (RF, B_1) field inhomogeniety. However, the validity of using such ratios may be undermined by the potentially under-conservative assumption of the stability of the reference metabolite (Li et al., 2003). Alterations in creatine may be observed not only in disease states, but also in healthy aging (Haga, Khor et al., 2009; Maniega, Cvoro et al., 2008).

A study by Ferguson, MacLullich et al. (2002) provides an example of the cautionary approach required when using creatine ratios. In contrast to the negative relationships between choline and cognitive performance observed by others, Ferguson et al. observed that Cho/Cr values were moderately and positively correlated with tests of visual and logical memory in an elderly, healthy population. To account for the fact that creatine may not be constant, and therefore inappropriate in ratio analyses, Ferguson et al. regressed each metabolite value (NAA, Cho, Cr) against the remaining two, creating standardised residual or 'adjusted' metabolite value. When they accounted for the potential association between creatine and psychometric measures by including

'adjusted' creatine as a cofactor in the regression, choline levels did not correlate significantly with any cognitive variables. Elderkin-Thompson, Thomas et al. (2004) have shown that among normal participants, cognition was positively correlated with Ch/Cr and negatively correlated with PCh/Cr in the four domains of verbal learning, recognition, recall and hypothesis generation. By contrast, depressed patients showed no consistent relationships between Ch/Cr or PCh/Cr and cognition.

To remove issues of creatine as a confounder, a more optimal strategy may be to use one of a number of absolute quantification methods (Jansen, 2006), particularly using the water signal as reference. This may provide the most robust method for ensuring reliable results as the internal water standard technique can be readily implemented provides suitable precision and inter-laboratory reproducibility (Keevil et al., 1998). However, the differential water fractions of grey and white matter volumes necessitate precise tissue identification and is most reliably obtained with voxels of small spatial extent and verified following data acquisition. This in turn raises potential issues for the trade-off between ensuring sufficient sample sizes and tissue homogeneity across the voxels included in statistical analyses (see section 6.5.4.3 below).

Despite these issues, analysis of the studies in Table 6-1 (page 124) did not identify any systematic relationship between the method of metabolite quantification adopted and the size and strength of the reported findings between IQ and NAA. Improved methodology for ^{1}H-MRS data acquisition and analysis of absolute metabolite values is leading to the increased use of absolute metabolite values as a method of best practice (Jansen, 2006), but until such a standard referencing procedure is widely adopted, comparing data across studies that differ in this variable will remain difficult (De Beer et al., 1995; Knight-Scott et al., 2003). Investigating multivariate relationships using multiple metabolite expressions (for example ratios to water, choline and creatine (Ross, Sachdev et al., (2005)) compounds difficulties in interpretation, since this procedure increases greatly the number of potential statistical comparisons within a given study.

6.5.4.3 Tissue type

The typically large spatial extent of the voxel from which data is acquired in MRS studies makes it difficult to obtain measures from homogeneous tissue. However, decreasing the spatial extent of the voxel size leads to a corresponding decrease in the SNR (Freeman, 2003). The extent to which MRS samples grey or white matter can modulate the metabolite values obtained (McLean et al., 2000; Wang and Li, 1998; Wiedermann et al., 2001). Jung et al. (2009) have reported that the patterns of relationship between NAA and IQ variables may also vary according to the tissue sampled.

The precision gained from obtaining measures from homogeneous tissue, or after *post-hoc* correction for tissue inhomogeniety, something which was not implemented in the current study, is a reasonable aspiration for future studies, yet few studies in this area have applied such procedures to their data. As an exception, Charlton et al. (2007) excluded voxels containing less than 75% white matter in their study, but acknowledge the inevitable reduction in sample size (and correspondingly in nominal statistical power) that potentially results from applying such screening criteria *post hoc*.

6.5.4.4 Region

A volume in the occipital cortex was selected because of the homogenous nature of the cortical tissue (Swanson, 2003), which aids in providing consistent high-quality spectra. Furthermore, metabolites in this region have been shown to correlate with cognitive abilities in both diseased (Modrego, Fayed et al., 2005) and healthy (Jung, Yeo et al., 1999) cohorts.

No significant differences in neurometabolites were observed between frontal and occipitoparietal voxels in the present analyses, a result that is in accordance with some studies using single-voxel ^{1}H-MRS (for example Ozturk et al., 2009), but which contrasts with others that have observed significant inter-regional differences using both single (Minati et al., 2010) and simultaneous multiple voxel acquisitions (Angelie et al., 2001; Maudsley et al., 2009).

Assessments of metabolite concentrations in different tissues have not, however, always yielded the same pattern of effects (Wiederman et al., 2001). While the NAA/Cr and Cho, for the present study and for those listed in Table 6-1, are generally consistent with published normative data, it would not be surprising if the correlations between neurometabolites and cognitive function varied according to the cortical region investigated, given the functional specialisation of the cerebral cortex. Measures were obtained from voxels in frontal cortex, given the important role of this region in the higher cognitive functions assessed with the WAIS measures (Baddeley, 1996; Duncan et al., 2000), and from a control volume in occipitoparietal cortex, an approach that has also been adopted in other studies (Ozturk et al., 2009).

6.5.4.5 Assessments of cognitive ability

Most previous studies have used broad and non-specific measures of cognitive skill, such as those afforded by standardised assessments of general intelligence. As well as FSIQ, and its constituent Verbal and Performance subscales, other studies have adopted data reduction techniques to derive a composite score as a dependent variable (Ross et al., 2005; Valenzuela et al., 2000). Other studies have adopted a contrasting approach, measuring increasingly specific cognitive abilities such as working memory (Yeo et al., 2000) or IPS (Jung et al., 1999b).

In the present study a hybrid approach was adopted, assessing both FSIQ and the IPS subscale, measures that reflect both general cognitive ability and the mental and motor speed required to solve visuo-spatial problems respectively (Groth-Marnat et al., 2000). Given NAA's putative role in myelination and in neural efficiency, IPS may be more closely linked to neurophysiological functions for which NAA is a mediator than to broad-based IQ measures. It was hypothesised that NAA would co-vary with general cognitive ability, but specifically more strongly with IPS. However, no relationships of note between NAA/Cr and either FSIQ or IPS (see Table 6-4, page 130) were observed.

Whereas some studies have taken an exploratory approach to investigating relationships between neurometabolites and cognitive ability (Ferguson et al., 2002; Jung et al., 2009; Jung et al., 2005), others have either predicted a dissociation between

broad psychometric constructs (Jung et al., 1999b; Ross et al., 2005) or focused on increasingly specific cognitive attributes such as Executive Function (Charlton et al., 2007; Valenzuela et al., 2000) or Working Memory (Yeo et al., 2000). Studies that used broad-based measures such as FSIQ, or a composite score for IQ derived by factor analysis, showed an average effect size of .50, compared to .32 for those studies reporting findings for specific cognitive measures (Table 6-1, page 124).

A minority of studies have framed more specific hypotheses, for example regarding gender differences in NAA and verbal processing ability (Pfleiderer et al., 2004) or hemispheric specificity with relation to verbal working memory and motor speed (Ozturk et al., 2009). In the present study, broad-based FSIQ measure were used, but specific hypotheses relating to specific measures of IPS were made, with small effects observed in both instances.

The methodological approach to data analytic strategy should be directly informed by the *a priori* hypotheses adopted (or lack thereof) as this will govern the choice and range of psychometric assessments and how subscales are treated in statistical analysis. Given the potentially diffuse role of NAA in brain function, broad-based measures of cognitive function may be the most fruitful variables to consider for further investigation.

The relative merits of hypotheses that invoke brain plasticity, or subtle weak effects due to variations in neurometabolites, require considerable attention and are a reflection of the gap between molecules and manifest behaviour. The former hypothesis suggests that there may be no genuine consequence of relatively small variations in neurometabolites, whereas the latter suggests that these small natural variations could result in subtle performance deficits that are simply difficult to detect with current broad-based psychometric tools.

6.5.4.6 Data screening and analysis

Differences between studies are also apparent in their choice of analytical strategies, including procedures for data screening to ensure robust correlational data (Tabachnik

and Fidell, 2001). The validity of the data depends on several factors, including the assumption of univariate and multivariate normality and how outlying values are handled in statistical analyses. To date, most investigations have employed highly varying screening procedures, with little homogeneity of practice in the methods of analysis and presentation of data across studies. Levels of screening range from basic level removal of poor quality spectra (Jung et al., 1999b; Ross et al., 2005) to exclusion of metabolite values exceeding a critical statistical cut-off following *post hoc* quantification (Ferguson et al., 2002; Jung et al., 2005; Pfleiderer et al., 2004), or following screening for voxel tissue composition but not for the metabolite values obtained (Charlton et al., 2007). Other studies (Gimenez et al., 2004; Jung et al., 1999a; Valenzuela et al., 2000; Yeo et al., 2000) have neither reported the extent nor the methodology of data screening procedures.

The procedures used for data screening and the suitability of statistical analysis strategy may critically underpin the validity of the findings from a given study. Normality of spectroscopy data cannot be assumed, yet none of the studies reviewed here (see Table 6-1) included information on the normality or distributional assumptions of their data. However, all of the studies employed parametric statistics (Pearson correlation or linear regression analyses) in their data analyses.

Had the unscreened data in the present study been analysed with parametric measures (which were not justified in this case), significant correlations reported would have between NAA and IQ variables on the order of r= .4, a finding similar to those in the published literature (Table 6-1). Given the potential impact of outliers and non-Gaussian distributions of data on the validity of the results, a concerted effort should be made to report the extent of data screening employed in future studies.

6.5.4.7 Multiple comparisons and *a priori* hypotheses

Quantitative assessment of the data from healthy cohorts (Table 6-1) reveals a particularly strong negative correlation (r= -.68; p= .015) between the number of participants studied and the effect size for the main finding reported, indicating that the strongest relationships between NAA and cognitive abilities are typically found in

studies with smaller sample sizes. One interpretation of this effect is that insufficient screening of the data from relatively small samples, combined with the large number of variables investigated and a lack of specifically identified *a priori* research hypotheses, renders the typical study susceptible to inflated Type 1 error resulting from the large number of statistical comparisons employed.

Furthermore, a key consideration for future studies in cognitive spectroscopy is the distinction between statistical significance and statistical relevance, and whether the correlation between NAA (or any other metabolite) and a specific psychometric sub-test is meaningful for the development and refinement of theory, particularly in cases where effect sizes may not be large enough to survive with adjustments in α such as Bonferroni corrections or relate to results that were not hypothesised *a priori*.

The subtlety of the relationships that may exist between neurometabolites obtained with MRS and measures of cognitive ability motivates the collection of larger-sized samples of participants than have been typically obtained, to achieve the statistical power necessary to reliably detect multiple effects and their interactions (Jung et al., 2009; Ozturk et al., 2009). With an anticipated effect size in the range of 0.15, at an α level of .01, a study would require a sample of at least 82 participants to have an 80% chance of detecting a noteworthy relationship.

6.6 Conclusion

^{1}H-MRS provides a valuable and sensitive tool for quantification of brain tissue composition and viability *in vivo*, but the lack of a cohesive direction and consensus in the patterns of relationships across studies suggests that conclusions drawn from these types of studies need to be tempered by awareness of the methodological issues that complicate the work.

In the present study, where particular care was taken with data acquisition and screening in order to maximise the reliability of the data and strict and specifically appropriate statistical analyses were applied, no significant relationships were observed between ^{1}H-MRS detectable neurometabolites and measures of cognitive

ability. In future studies of this kind, emphasis must be placed on the need for specific, hypothesis-driven enquiry and awareness of the subtlety of the data analysis and psychometric measures being used and how they are reported. Despite its limitations, there is the potential for ^{1}H-MRS to make a significant contribution towards understanding some of the neurobiological correlates of cognitive ability.

7 ^{1}H-MRS-dectable metabolites as biomarkers in paediatric liver disease

7.1 Summary

The aim of this study was to evaluate the extent to which non-invasive ^{1}H-MRS can add information to the study of children with liver disease by revealing abnormalities in cerebral metabolism and to evaluate if performance on cognitive indices is related to concentrations of ^{1}H-MRS-detectable neurometabolites.

In a sample of 23 patients with liver disease and 11 sibling controls, single voxel ^{1}H-MRS in occipitoparietal and frontal cortical white matter was used to assay concentrations of 4 principal metabolites: NAA, choline, mI and Glx, in parallel with assessments of psychometric intelligence (FSIQ).

The most important finding of the present cross-sectional study, which used quantitative short echo-time proton spectroscopy, is that there were neither significant differences in metabolite concentrations among the three groups of children with different stages of liver disease, nor between these patients and sibling control children matched for age. The similarity of the metabolite values observed between the patient and control groups suggests that neurodevelopment, assayed by surrogate neurometabolite markers, is normal in this cohort.

When used in paediatric cohorts, ^{1}H-MRS may supplement neuropsychological test batteries to enable evaluation of the neurophysiological impact of liver disease and patient's response to clinical interventions designed to minimise disease progression. However, the use of ^{1}H-MRS is still largely exploratory and what is currently missing is careful quantification, age-specific, reproducible, regional studies in newborns, infants, children and adolescents to provide context for the clinical data.

7.2 Introduction

MRI studies have been used to probe the interplay between neuroanatomical and physiological changes in neural circuitry during cognitive maturation and development (Casey, Giedd et al., 2000; Casey, Tottenham et al., 2005; Paus, 2005). ^{1}H-MRS was identified as a technique of emerging importance in the investigation of paediatric brain development in a major review by Novotny et al. over a decade ago (Novotny, Ashwal et al., 1998). Since then, substantial progress has been made in understanding the relationship between ^{1}H-MRS-detectable metabolites and neurophysiological and neuropsychological development, both normatively and in abnormality.

When applied in a clinical environment, the biochemical profiles generated by ^{1}H-MRS reflect levels of endogenous metabolites involved in key cellular pathways. This data can therefore indicate the physiological status of neural tissue and potentially offer pointers to underlying pathophysiological processes to facilitate diagnosis of hereditary and acquired brain disorders in children (Grodd, Krageloh-Mann et al., 1991), and assist in the detection of functional abnormalities, potentially before the appearance of symptoms. This is discussed in greater detail below in the context of liver disease.

As a diagnostic tool, ^{1}H-MRS research has concentrated on the effects of diseased states such as metabolic (Zimmerman and Wang, 1997) and neurodegenerative (Tzika, Ball et al., 1993) disorders, hypoxic–ischaemic brain injury (Amess, Penrice et al., 1999), *in utero* drug exposure (Goncalves Rde, Vasconcelos et al., 2009; Smith, Chang et al., 2001) and epilepsy (Ranjeva, Confort-Gouny et al., 2000).

7.2.1 Subclinical hepatic encephalopathy

Hepatic encephalopathy (HE) is a syndrome encompassing a wide range of neuropsychiatric consequences of liver disease, which typically results from hepatocellular failure and/or portal-systemic shunts. The terms subclinical or 'minimal' hepatic encephalopathy (SHE) is used to describe the presence of cognitive impairment on psychometric testing and/or slowing of electroencephalographic (EEG) mean cycle frequency (for example reduced frequency of the α rhythm and disturbances by random

waves in the θ range over both hemispheres) in the absence of any clinically overt signs (Amodio, Montagnese et al., 2004; Ferenci, Lockwood et al., 2002). The broad definition of SHE reflects the existence of a spectrum of neuropsychiatric manifestations, which are typically diagnosed using neuropsychological or neurophysiological tools (Ortiz, Jacas et al., 2005), including composite measures such as the psychometric hepatic encephalopathy score (PHES) (Weissenborn, Ennen et al., 2001), which examines the motor speed and accuracy, visual perception and construction and attentional deficits which characterise the condition. The neuropsychological features of SHE are defined as a disorder of executive functioning, particularly selective attention and psychomotor speed (Amodio, Montagnese et al., 2004).

When evaluated using standard tools of psychometric assessment, the results from Chapter 4 indicated that liver disease is associated with good long-term cognitive outcome, as indexed by broad-based metrics such as FSIQ. However, whilst neuropsychological test batteries are the current gold standard measures for cognitive ability, a considerable problem in using these tests alone is that the results are not specific to the disorder and are limited in what they are able to reveal about the mechanisms underlying changes in cognitive behaviour. The term *subclinical* HE highlights the need for complementary tests to diagnose degrees of brain dysfunction that may not be detected through standard clinical examination or assessment.

7.2.2 ^{1}H-MRS biomarkers of subclinical hepatic encephalopathy

As a non-invasive, non-destructive procedure that does not require parenteral injections, or use of radioactive materials, ^{1}H-MRS is ideally suited for repeated measures of adult and paediatric populations where *in vivo* biochemical data is required. It has been shown repeatedly that for HE and SHE, a specific pattern of cerebral metabolic change exists in the brain, which even in the mildest form of HE can be detected using short echo-time MRS (Grover, Dresner et al., 2006). This typical pattern, largely observed in studies of adults, is a reduction in the mI and choline resonances and an increase in the glutamate/glutamine composite resonance (Glx) (Atkison, Ross et al., 2002; Kreis, Ross et al., 1992; Taylor-Robinson, Sargentoni et al.,

1994b). Figure 7-1 illustrates a representative ^{1}H-MRS spectra from a healthy individual.

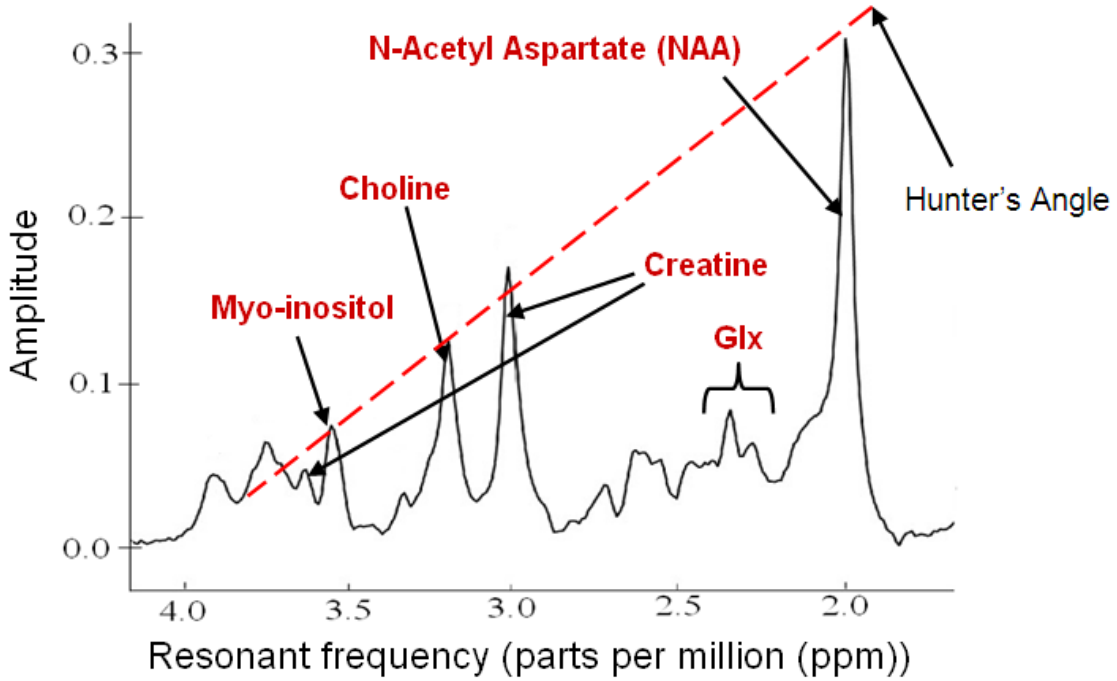

Figure 7-1 Representative ^{1}H-MRS spectra of normal human brain

In one of the few studies of metabolite changes in children, Tkac, Hamernick et al., (2004) observed that pre-transplant paediatric candidates had pronounced differences in neurometabolite levels, including increase of glutamine and the decrease of mI and NAA compared to controls, a pattern of results consistent with that from adult studies.

If liver disease causes the cerebral metabolite changes described for SHE, reversibility of metabolite levels after liver transplantation can be predicted if no persistent brain damage has already occurred. This effect has been observed in several studies with adult liver transplant patients (Naegele, Grodd et al., 2000; Ross, Jacobson et al., 1994; Shawcross, Balata et al., 2004; Thomas, Huda et al., 1998) and in paediatric patients studied pre- and post-transplant (Tkac, Hamernick et al., 2004). The finding by Tkac et al. that only four of the eight patients studied showed improvement of neurometabolic status post-transplant motivates studies using convergent measures to understand the variability of metabolite values in these populations compared to sibling controls.

7.2.3 ^{1}H-MRS and cognitive ability in children

^{1}H-MRS has also proved useful in terms of relating potential deficits in cognitive development with changes in neurometabolic concentrations, for example in traumatic brain injury (Brooks, Friedman et al., 2001; Friedman, Brooks et al., 1998) and developmental dyslexia (Rae, Lee et al., 1998). It has even been suggested that ^{1}H-MRS has the potential sensitivity to identify clinical subtypes of autism spectrum disorders (Gabis, Wei et al., 2008).

^{1}H-MRS has also been extended to assessment of developmental delay, whereby the metabolites provide supplementary information in cases where the cause of cognitive disturbance remains unknown. Filippi, Ulug et al. (2002) found that in children with normal MRIs, decreases in the NAA/Cr ratio and elevations of Cho/Cr in frontal and occipitoparietal subcortical white matter were associated with developmental delay in children over the age of two. This data in clinical populations of children point to the potential utility of ^{1}H-MRS as an adjunctive investigative technique to the traditional methods of psychometric tests typically used to assess for the presence of HE and cognitive deficits in liver disease.

^{1}H-MRS studies of healthy human cognition have mainly examined adults (see Chapter 6). Whilst methodological flaws are evident (see section 6.5.4), the findings from these studies suggest that NAA, measured across different cortical regions, co-varies with different constructs of cognitive ability, an effect that has also been observed in healthy younger populations. In healthy children, NAA/Cr ratios have been associated with measures of manual speed and dexterity, visual working memory (Ozturk, Degaonkar et al., 2009) and working memory (Ozturk et al., 2009; Yeo et al., 2000). In adolescents, Gimenez, Junque et al. (2004) have observed correlations between NAA and a composite measure of cognitive skill representing speed of information processing, attentional function and visual memory.

In a recent study, Lightsey et al. employed multi-voxel spectroscopy, sampling both white and grey matter, in a cohort of healthy six to 19 year olds (Lightsey, 2010). They observed that higher NAA/Cr mean and lower NAA/Cr standard deviations in grey

matter is associated with higher memory performance in boys, while lower NAA/Cr mean and higher NAA/Cr standard deviations in gray matter is associated with higher memory performance in girls. As with the studies of adults described in Chapter 6, the general approach has been to obtain in measures of ^{1}H-MRS metabolites in cortical tissue with concurrent psychometric measures of cognitive skills, such as those provided by standardised IQ tests.

7.2.4 Aim

The aim of the present study was to investigate whether children with liver disease have a neurometabolic pattern which differs from sibling controls and is indicative of SHE. Relationships between cognitive ability and neurometabolites were also investigated to determine if intellectual deficits associated with liver disease are reflected in reduced concentrations of NAA, or disturbances in other ^{1}H-MRS-detectable cerebral metabolites.

7.2.5 Hypotheses

1. If present, the characteristic combination of biomarkers of SHE, namely reduced choline and mI and increased Glx, would correlate with greater cognitive dysfunction and decreased FSIQ.
2. Post-transplant patients would show a pattern of metabolite values closer to that for sibling controls.
3. As a potential marker of neuronal viability and health, NAA would negatively correlate with measures of general cognitive ability.

7.3 Method

7.3.1 Participants

A total of 40 participants (15 females, 25 males; (mean age 13.1, SD: 5.0)) from the principal cohort described in Chapter 3 underwent the MR procedure. Twenty-nine participants from this cohort were patients with liver disease and 11 were healthy sibling controls.

34 participants (23 females, 11 males,) from a total of 40 participants were included in the final analyses. Descriptive data for the sibling control and patient groups are provided in Table 7-1, page 152. Three patients did not tolerate the scan procedure. Data from a further three participants was excluded due to poor quality spectra in both frontal and occipitoparietal voxels, as a result of artefact induced by motion or non-neural tissues.

7.3.2 Neuroimaging

The standardised neuroimaging protocol is described in Chapter 3 (section 3.5.3, page 67).

The ^{1}H-MRS data was obtained with a Siemens 3T Trio scanner (Siemens Medical Solutions, Berkshire, UK) using standard acquisition software and a quadrature head coil. Single-voxel ^{1}H-MRS (2 cm^{3}) was performed following localisation of the volume of interest (VOI) using a 5-plane localiser (TR: 20 msec, TE: 5 msec, 10 slices at 5 mm thickness). Manual voxel positioning maximised the white matter contribution and minimised the intrusion of grey matter, and ensured that the voxel did not encroach upon non-neural sources. Automated shimming was followed by a stimulated echo acquisition mode (STEAM) pulse sequence (TR: 2,000 msec, TE: 30 msec, 96 averages), including water suppression, for voxels in both frontal (Fro) and occipitoparietal (OP) cortex. To validate data quality, assess reliability and quantify error variance introduced by scanner-related factors such as that caused by field inhomogenieties, data from two successive scans of the same voxel were obtained and averaged for each participant.

7.3.3 Psychometric assessments

The standardised protocol for administration of psychometric assessments is described in Chapter 3, page 51.

An age-appropriate battery of the Wechsler Preschool and Primary Scale of Intelligence-III, Wechsler Intelligence Scale for Children-IV or Wechsler Adult Intelligence Scale-III was administered for each child to derive scores for Verbal, Performance, Working Memory and FSIQ and IPS.

7.3.4 Data analysis

Psychometric test scores were converted to scaled scores to standardise the data across age groups according to the standard Wechsler administration instructions.

The metabolite values were estimated *post-hoc* using LCModel (Provencher, 2001). This software provides spectral quantification and metabolite detection optimised for short echo time spectroscopy. All analyses reported here adopt the standard convention of expressing NAA as a ratio to creatine (NAA/Cr).

7.4 Results

7.4.1 Summary spectroscopy data and data screening

Ross, Jacobson et al. (1994) suggested quantitative criteria that enable prediction of SHE with MR spectroscopic data, whereby SHE is considered present when the mI/Cr and Cho/Cr ratios are less than 2SD below normal, with or without a Glx/Cr ratio more than 1SD above normal, where 'normal' was defined as metabolite values obtained in a sample of 12 sibling controls. Therefore, to adequately detect potential group differences in neurometabolite values, the stringent screening criteria applied to spectroscopic data in Chapter 6 (exclusion of data ±2SD) was not applied here.

Table 7-1 Descriptive data for the sibling control and liver disease groups with available psychometric and spectroscopy data

Group	Total n	Mean age (years)	SD age (years)	M:F	n	Diagnoses
Sibling controls	11	12.2	5.08	5:6		
Early-onset liver disease, pre-transplant	13	13.1	5.0	8:5	8	Extra-hepatic biliary atresia
					4	Progressive familial intrahepatic cholestasis
					1	Neonatal haemochromatosis
Early-onset liver disease, post-transplant	5	16.2	3.0	3:2	2	Progressive familial intrahepatic cholestasis
					2	Extra-hepatic biliary atresia
					1	Acute liver failure
Acute liver failure, post-transplant	5	14.0	4.1	4:1	1	Autoimmune hepatitis
					1	Fulminant hepatitis A infection
					3	Sero-negative hepatitis

Summary spectroscopy data for the sibling control group and the three individual patient groups (early-onset liver disease (EOLD) pre-transplant, EOLD post-transplant and acute liver failure (ALF) post-transplant) is shown in Table 7-2.

Table 7-2 Summary of spectroscopy data for paediatric liver disease patients vs sibling controls

	Sibling controls	**EOLD pre-Tx**	**EOLD post-Tx**	**ALF post-Tx**
Mean age	12.7	13.8	16.2	14.0
Mean age SD	4.90	4.97	2.95	4.01
Occipitoparietal voxel				
N	11	13	5	5
Mean Cho/Cr (SD)	.23 (.05)	.22 (.04)	.25 (.02)	.23 (.04)
Mean NAA/Cr (SD)	1.61 (.84)	1.34 (.58)	1.60 (.33)	1.67 (.15)
Mean mI/Cr (SD)	.71 (.60)	.69 (.21)	.52 (.23)	.70 (.07)
Mean Glx/Cr (SD)	1.85 (.23)	2.35 (1.33)	2.05 (.24)	1.69 (.14)
Frontal voxel				
N	10	11	5	5
Mean Cho/Cr (SD)	.25 (.07)	.25 (.08)	.18 (.13)	.28 (.03)
Mean NAA/Cr (SD)	1.58 (.13)	1.68 (.14)	1.02 (.71)	1.54 (.12)
Mean mI/Cr (SD)	.72 (.10)	.71 (.38)	2.18 (3.36)	.71 (.06)
Mean Glx/Cr (SD)	1.95 (.21)	2.00 (.39)	9.99 (18.28)	1.90 (.21)

Mean and standard deviations of metabolite values as ratios to creatine are shown for both frontal and occipitoparietal voxels.
Frontal cortex data for one sibling control and two EOLD pre-Tx participants were excluded from analyses due to poor quality as a result of artefact induced by motion or non-neural tissues

Mean metabolite ratios are largely consistent with those reported previously in adults and children (Danielsen and Ross, 1999; Ozturk, Degaonkar et al., 2009). Distributional normality of spectroscopy data cannot be assumed. As data from Chapter 6 showed, the procedures used for data screening and the suitability of statistical analysis strategy may critically underpin the validity of the findings obtained from a given study, a point particularly important here as outlier data were deliberately retained in the analyses. As with data from Chapter 6, the unscreened data did not consistently satisfy distributional assumptions of normality (evaluated with the Shapiro-Wilk statistic at an α level of .05), and as a consequence were analysed using non-parametric statistical techniques.

7.4.2 ^{1}H-MRS metabolite differences between patients and controls

An Independent-samples Kruskall Wallis test showed no main effect of disease category on metabolite values. The significance values for each metabolite in both frontal and occipitoparietal cortex are shown in Table 7-3.

Table 7-3 Differences in neurometabolite values between sibling controls and liver disease groups

Metabolite	Kruskall Wallis p value (3 df)	Chi-square
Frontal voxel		
Cho/Cr	.563	2.05
NAA/Cr	.225	6.68
mI/Cr	.083	4.36
Glx/Cr	.838	.86
Occipitoparietal voxel		
Cho/Cr	.421	2.82
NAA/Cr	.094	2.12
mI/Cr	.549	6.39
Glx/Cr	.096	6.36

Non-parametric Kruskall-Walllis test was used to assess differences in neurometabolites in both frontal and occipitoparietal cortex as the unscreened data did not conform to a normal distribution.

Based on the mean values obtained in the sibling control cohort, none of the patients exceeded the diagnostic cut-offs established by Ross et al. (1994) in frontal cortex voxels (mI/Cr< .52 and Cho/Cr< .11, Glx> 2.16). The same is true of occipitoparietal, although frontal volumes were focused on due to their greater functional link to cognitive ability. Therefore, none of the patients in the present cohort could be diagnosed as having SHE using ^{1}H-MRS data alone.

Data illustrating the differences in neurometabolites according to disease category is shown for the frontal volume only (Figure 7-2 to Figure 7-5, pages 155 and 156) as this region is functionally related to the cognitive outcome measures of interest to a greater degree than the occipitoparietal cortex, a region which was used as a control.

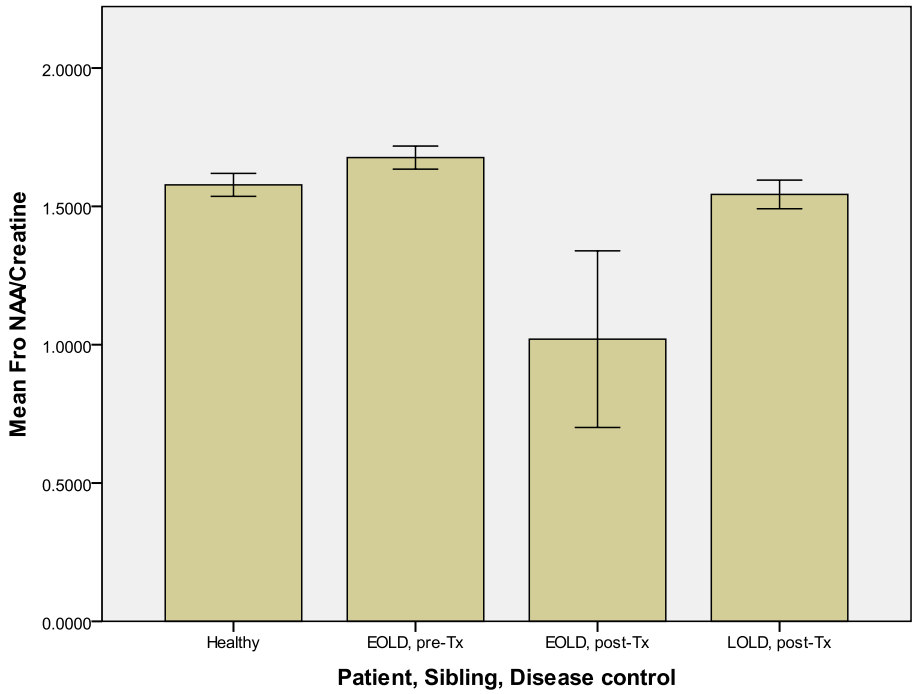

Figure 7-2 Comparison of mean frontal cortex NAA/Cr values between control and liver disease groups

Error bars represent ±1 standard error mean. EOLD post-Tx patients had lower NAA/Cr compared to the other three groups but this difference was not statistically significant.

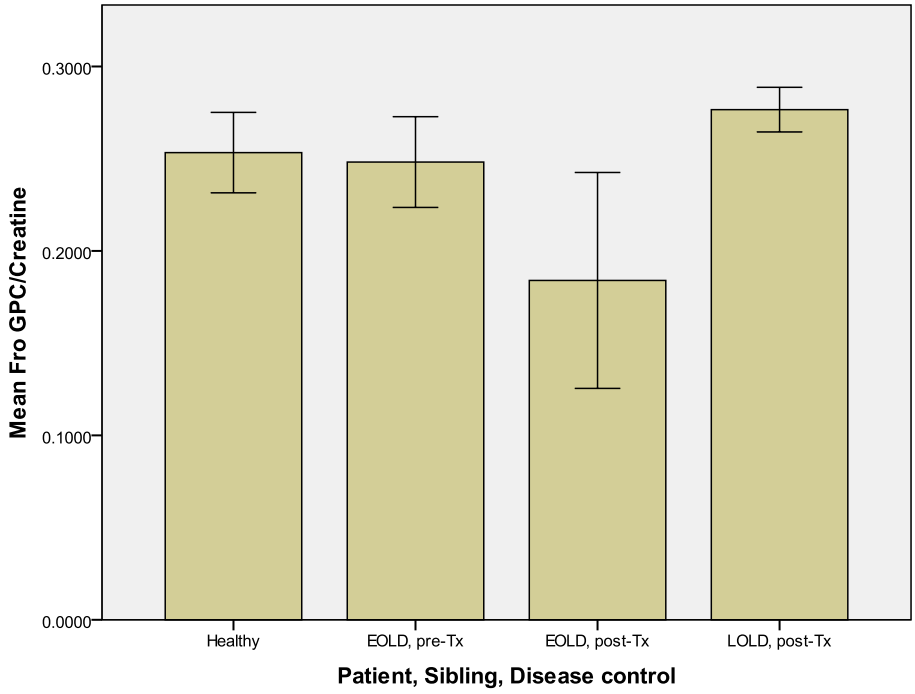

Figure 7-3 Comparison of mean frontal cortex Cho/Cr values between control and liver disease groups

Error bars represent ±1 standard error mean. EOLD post-Tx patients had lower Cho/Cr compared to the other three groups but this difference was not statistically significant.

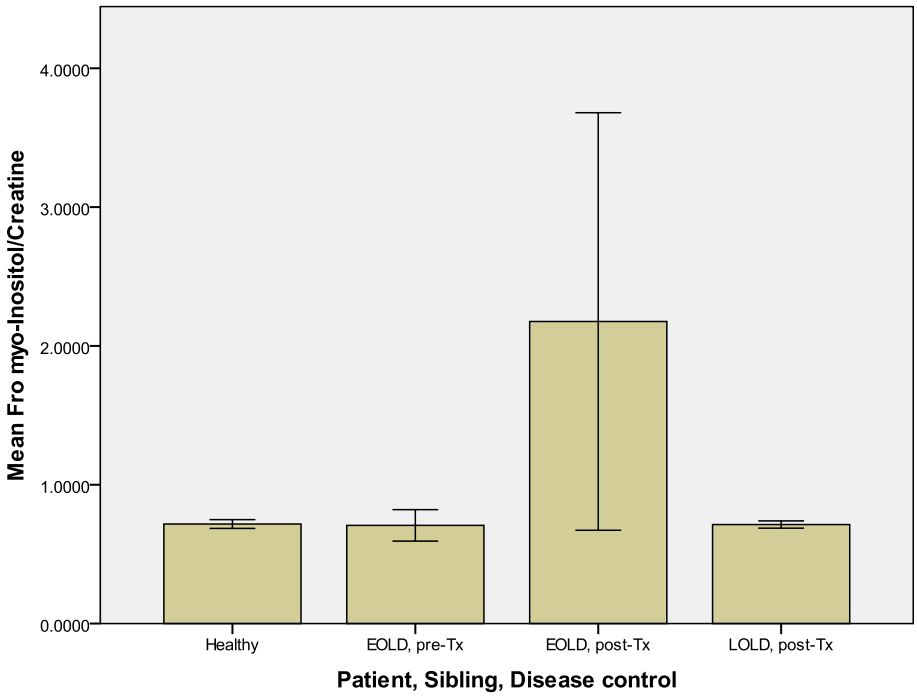

Figure 7-4 Comparison of mean frontal cortex mI/Cr values between control and liver disease groups

Error bars represent ±1 standard error mean. EOLD post-Tx patients had higher mI/Cr than the other three groups. The difference was not however statistically significant, an effect that can be attributed to the much greater variance in metabolite values in this patient group.

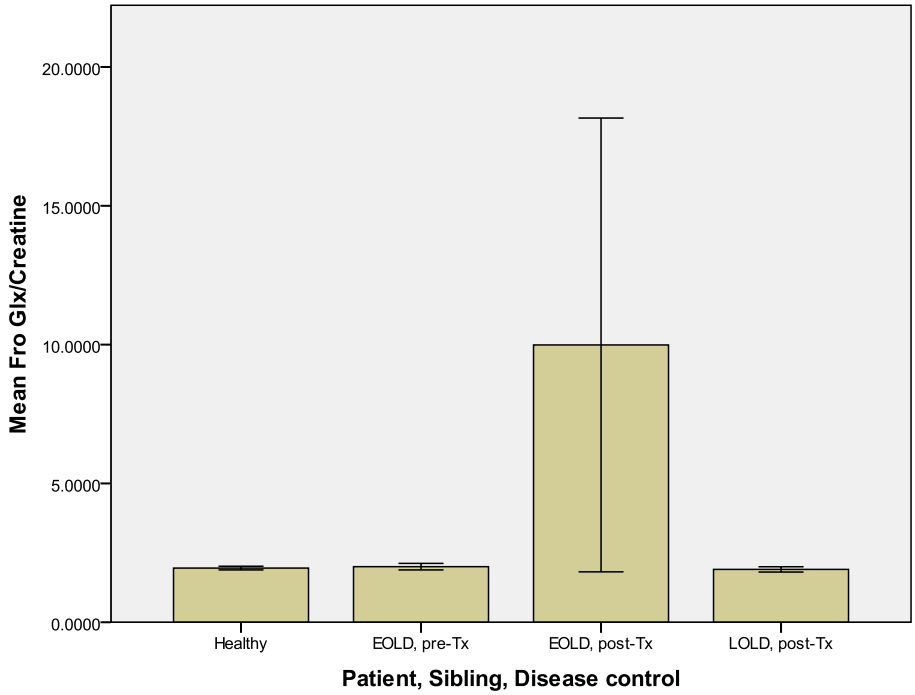

Figure 7-5 Comparison of mean frontal cortex Glx/Cr values between control and liver disease groups

Error bars represent ±1 standard error mean. EOLD post-transplant patients had higher Glx/Cr than the other three groups. The difference was not however statistically significant, an effect that can be attributed to the much greater variance in metabolite values in this patient group.

7.4.3 Inter-regional and gender differences in metabolite values

A Related-samples Wilcoxon Signed-rank test was used to assess inter-regional differences in metabolite values across all groups. No significant differences in median NAA/Cr, mI/Cr or Glx/Cr were observed between frontal and occipitoparietal voxels (p= .83 .37 .05 respectively). Cho/Cr was significantly higher in frontal cortex than occipital cortex (p= .02). An Independent-samples Kruskall Wallis test showed no main effect of gender on metabolite values (p> .05).

7.4.4 Relationships between ^{1}H-MRS-detectable metabolites and cognitive ability

The data from Chapter 4 showed that there were no significant differences between diagnostic groups for FSIQ. To supplement the findings in Chapter 6 for correlations (or lack of) between neurometabolites and cognitive variables in adults, the relationships between neurometabolites and cognitive ability were also assessed here. Analysis of the continuous relationships between MRS metabolites and cognitive variables across the entire cohort of 34 paediatric participants was justified as no statistically significant differences for metabolite values were observed between groups.

Non-parametric correlation coefficients for the unscreened data (n= 34) showed that the relationships between IPS or FSIQ and Cho/Cr, NAA/Cr, mI/Cr and Glx/Cr were neither strong nor statistically significant at a Bonferroni-corrected α value of .003 (.05/16 correlations).

Table 7-4 Correlations between neurometabolites in frontal cortex and IQ variables

		Correlation with cognitive ability; r (p value)			
	n	FSIQ	IPS	PIQ	VIQ
Cho/Cr	29	.33 (.84)	-.03 (.89)	.39* (.04)	.30 (.12)
NAA/Cr	29	.10 (.61)	-.06 (.77)	.03 (.86)	.14 (.46)
mI/Cr	29	-.03 (.87)	.19 (.34)	.01 (.96)	-.84 (.67)
Glx/Cr	29	-.04 (.85)	-.13 (.51)	-.02 (.92)	.12 (.54)

Non-parametric Spearman's Rho test was used to assess the relationship between neurometabolites and psychometric scores as the unscreened neurometabolite data did not conform to a normal distribution.
*p< .05, but not statistically significant at Bonferroni-corrected α level of .003

Screening of metabolite values to remove all univariate outliers (±2SD) results in the exclusion of data from six participants. Concentrations of the four metabolites of interest in the remaining sample of 28 were normally distributed when evaluated with the Shapiro-Wilk statistic at an α level of .05. However, no significant relationships between neurometabolite values and cognitive variables are observed when parametric (Pearson's r) statistics are applied (p> .05).

7.5 Discussion

7.5.1 ^{1}H-MRS-detectable metabolites as biomarkers in paediatric liver disease

^{1}H-MRS is a valuable addition to existing neuroimaging methods for the study of brain development in humans and is able to provide *in vivo* biochemical information with potential diagnostic value. ^{1}H-MRS was used in a cross-sectional study of a paediatric population to investigate whether children with liver disease have a neurometabolic pattern which differs from sibling controls in pattern association with subclinical hepatic encephalopathy. The most important finding of the present study was that there are no significant differences in metabolite concentrations, neither among the three groups of children with different stages of liver disease, nor between patients and age-matched sibling control children.

The findings of the present study are in contrast with previous studies of SHE in which two characteristic changes in ^{1}H-MRS spectra are described: (1) an elevation of glutamine (Glx) peak, and (2) the reduction of the mI and choline peaks. The area under

each peak in the ^{1}H-MRS spectra represents the relative concentration of nuclei detected for that particular molecule (see Chapter 3 for a detailed discussion), and is therefore reflective of the concentration of that metabolite in the tissue being assessed. The potential neuropathological changes associated with deviations in these particular metabolites in relation to liver disease has been almost exclusively observed in adults, and the mechanisms of action on cognitive outcomes in SHE are still largely unclear.

7.5.1.1 Glx in liver disease

The spectral peaks of glutamine and glutamate are often grouped together as Glx, because their spectral overlap makes it hard to resolve them adequately. Glutamine is easily detectable with ^{1}H-MRS, whilst glutamate is the most abundant amino acid in the brain and is released by approximately 90% of excitatory neurons (Magistretti, Pellerin et al., 1999). The role of Glx in liver disease is open to speculation (Zwingmann and Butterworth, 2005), but the increase in Glx peaks seen on MR spectroscopy may be a result of excess ammonia taken up by the astrocytes, which then convert glutamate to glutamine.

Ammonia metabolism is compromised by liver dysfunction and ammonia has consistently been shown to be important in the pathogenesis of HE (Lemberg and Fernández, 2009). Excessive ammonia is toxic to the CNS and can, for example, inhibit excitatory post-synaptic potentials, thereby producing a general depression of CNS function. In a small group of children with clinically suspected SHE, Foerster, Conklin et al. (2009) observed a positive significant correlation between grey matter Glx and ammonia levels (r= .66). Astrocytes are the site of ammonia detoxification in the brain and they eliminate ammonia by the synthesis of glutamine through amidation of glutamate. Accumulation of glutamine in astrocytes induced by hyperammonemia produces osmotic stress and astrocyte swelling (Häussinger, Kircheis et al., 2000). In the present study, however, elevated levels of Glx were not seen in the patient cohort compared to sibling controls, nor were ^{1}H-MRS values representative of these metabolites correlated with cognitive function.

7.5.1.2 mI in liver disease

A decrease in the concentration of brain organic osmolytes such as mI indicates the activation of the process of regulatory volume decrease of astrocytes. In response to elevated intracellular levels of glutamine resulting from excess ammonia, the astrocyte swelling hypothesis predicts that homeostatic mechanisms occur to limit the osmotic load in astrocytes, leading to the extracellular release of mI (Häussinger, Kircheis et al., 2000). MR studies in SHE have provided important *in vivo* data, which have contributed to the current theories on astrocyte swelling (Häussinger, Kircheis et al., 2000). Ross, Jacobson et al. (1994) report that decrease in mI levels may be the central derangement in chronic HE, with grey and white matter mI levels proving the best predictors of mild HE, but no significant difference between patients and controls were observed in the present study.

7.5.1.3 Choline in liver disease

The choline resonance contains contributions from phosphocholine and glycerophosphorylcholine, both of which are cell membrane precursor and degradation products (Vereb, Szollosi et al., 2003). The characteristic reduction of choline in SHE, which was not observed in this present study, is likely to reflect choline's importance in brain metabolism (Zeisel, 2000; Zeisel, 2004), specifically through alterations in phospholipid metabolism, membrane fluidity, or secondary changes in water content due to the osmolytic effects of choline.

7.5.1.4 NAA in liver disease

NAA provides a surrogate marker for the health and viability of neural tissue (Barker, 2001; Moffett et al., 2007). NAA concentrations in white matter reflect the metabolic function of axons as well as the extent and efficiency of their myelination (Bjartmar et al., 2002). In comparison to the ^{1}H-MRS detectable neurometabolites, it appears that neuronal loss and degeneration, as indexed by NAA, is largely unaffected in SHE, at least in adults (Binesh, Huda et al., 2005; Huda, Guze et al., 1998; Thomas, Huda et al., 1998). In a single, small study in children, Tkac, Hamernick et al. (2004) observed a reduction

of NAA/Cr in pre-transplant patients compared to sibling controls. However, to a large extent NAA has been overlooked as SHE does not seem to be associated with a loss of neurons or the reduced integrity of myelin.

In the present study, although no significant inter-group differences were observed, there was an interesting pattern of results for the early-onset post transplant group. Frontal cortex NAA/Cr and Cho/Cr showed a trend toward reduced values compared to sibling controls (see Table 7-2, page 153), (d)= 1.33 (see Figure 7-2, page 155) and 1.55 (see Figure 7-3, page 155, respectively. In addition, greater degree of variability in mI and Glx was observed in this group compared to the others (see Table 7-2 and, Figure 7-4 and Figure 7-5 for illustration). The range in metabolite values, particularly of Glx (see Figure 7-4, page 156), are important, because variation in metabolite values must be significantly higher than the precision of the MRS measurement (approximately 9%, see Chapter 3) before metabolite values can be confidently interpreted as a true reflection of biological variation in SHE.

In four paediatric post-transplant patients, Tkac, Hamernick et al. (2004) observed a regularisation in SHE markers that were previously abnormal. They noted, however, that the post-transplant concentration of glutamine specifically remained significantly higher compared to the normal adult brain, therefore indicating a slower than expected reversal of the effects of liver hypo-function on cerebral metabolic function, as detectable by ^{1}H-MRS. However, given the small sample size of both the patient and sibling control group and the lack of plausible explanation for reduced NAA/Cr in the context of liver disease, these findings must be interpreted with caution as the generality of the effects of liver disease are unknown.

7.5.2 The potential for age-related changes to mask disease-related effects

Foerster, Conklin et al. (2009) suggest that children with clinically suspected mild HE have changes in metabolic profiles of mI and Glx which are similar to adults. However, they make this claim without reference to age-matched control data for their cohort of 12 children, which they did not collect given ethical concerns about MR on small

children. The lack of a large database of normative age-matched data makes it extremely difficult to assess the true strength of disease-associated metabolite changes in childhood and is an important limitation for assessing the contribution ^{1}H-MRS as a diagnostic tool.

Two years of age marks a major landmark in brain development. The appearance of brain structures is similar to that of adults; the brain close to 80% of its adult weight, and all the major fibre tracts are identifiable by age three (Matsuzawa, Matsui et al., 2001). Levels of ^{1}H-MRS-detectable metabolites that may index various aspects of neurological development vary by anatomic region and have been shown to change nonlinearly with age. Metabolite changes, particularly for choline, mI and NAA, continue rapidly through the first year or so (Huppi, Fusch et al., 1995; Kimura, Fujii et al., 1995; Kreis, Ernst et al., 1993; Kreis, Hofmann et al., 2002; Pouwels, Brockmann et al., 1999; van der Knaap, van der Grond et al., 1990; Vigneron, Barkovich et al., 2001). Maturational changes of Glx are underreported in the literature due to their multiplet visibility and overlapping resonances of the two individual metabolites.

An age-dependent increase of the total concentration of NAA in cerebral grey and white matter with normal brain development has been found predominantly during the first three years of life (Huppi, Fusch et al., 1995; Huppi, Posse et al., 1991; Pouwels, Brockmann et al., 1999). Since there is no major increase in the number of neurons after birth, this increase in NAA concentration must be related to other processes of neurological maturation, such as the proliferation of dendritic arborisations, synaptic connectivity and axonal myelination (Kato, Nishina et al., 1997).

Several of the studies investigating newborns (Cady, Penrice et al., 1996; Huppi, Posse et al., 1991; Kreis, Ernst et al., 1993) have demonstrated decreases in choline with age, particularly in the first year. During later childhood and adolescence, choline remains at a constant adult level and therefore exhibits the same regional distribution as described for adults (Pouwels and Frahm, 1998). mI has been observed as the dominant peak in the ^{1}H-MRS spectra at birth (Kreis, Ernst et al., 1993).

A few of the limited number of studies measuring maturational changes in mI have described subsequent decreases of mI during the first year of life and a continuing marked reduction of the metabolite up to the age of two (Huppi, Fusch et al., 1995; Kreis, Ernst et al., 1993). Whilst the majority of metabolite changes are apparent in neonates and infancy, they continue, but to a lesser degree, through adolescence (Horska, Kaufmann et al., 2002). Whilst explanations of the observed changes in ^{1}H-MRS-detectable neurometabolites are still largely a matter of debate, it is clear that they are related to maturational processes in the brain.

A Spearman's Rho tests for the relationship between non-normally distributed spectroscopy data and age in the healthy cohort of 11 showed no significant correlations for NAA, Cho or Glx in either frontal or occipitoparietal regions. Occipitoparietal mI was, however, strongly negatively correlated with age ($r= -.68$, $p= .02$). Pouwels et al. found that mI concentrations across the thalamus, basal ganglia and grey matter regions remain largely constant with age, but that mI in the cerebellum decreases from its highest value during early development by approximately 30% toward adolescence and adulthood (Pouwels, Brockmann et al., 1999).

However, as other work has found no age-dependant change of mI in the cerebellum or any other region (Huppi, Posse et al., 1991), the interpretation of the non-parametric correlation between age and occipitoparietal mI/Cr values in this small healthy cohort requires cautious interpretation. This caution is further justified by further analysis, which showed that the non-parametric correlation between occipitoparietal mI and age became non-significant when assessed across the entire cohort of 34 (which is theoretically justified as no group differences were observed); $r= -.31$, $p= .84$. All other correlations between metabolites and age also remained non-significant ($p> .05$). It is also important to recognise simple linear regression analyses may mask non-linear relationships between age and neurometabolite values, but much greater numbers of participants are required in order to adequately investigate these potentially subtle associations.

A deeper understanding of the changes in normal brain development is crucial prior to the application of ^{1}H-MRS to the study of pathological conditions. Given the immaturity

of the brain at birth and its rapid postnatal development to approximate adult structures, such normative data needs to be age-matched and referenced. More studies of large populations, in well-defined clinical settings, are required in order to confirm and extend current findings from existing longitudinal and cross-sectional studies and determine if *in vivo* ^{1}H-MRS can provide independent diagnostic and prognostic indices of liver disease.

7.5.3 ^{1}H-MRS-detectable metabolites and cognitive ability in paediatric liver disease

Non-parametric correlation coefficients for the unscreened data (n=34) showed that the relationships between IPS or FSIQ and Cho/Cr, NAA/Cr, mI/Cr and Glx/Cr were neither strong, nor statistically significant at a Bonferroni-corrected α level of .003 (see Table 7-4, page 1588). Analysis of the FSIQ sub-scores shows a moderate statistically significant correlation between frontal Cho/Cr and Performance IQ scores at an uncorrected α level of .05 (r= . 39, p= .037; see Table 7-4)). Taken as a whole however, and when using strictly appropriate statistical analyses, the results of the present study indicate that there is no correlation between the concentrations of brain metabolites and the developmental achievement of the child.

Others have observed relationships between SHE markers and cognitive performance. In a group of 17 patients diagnosed with SHE, Thomas, Huda et al. (1998) observed that performance on the Frontal Index (measured by performance on the assessments including the Colour Trails task, Rey-Osterreith and Digit Symbol subtests) was strongly correlated to baseline mI/Cr levels; patients with the lowest levels of mI/Cr were the most seriously impaired (r= .67, n= 17, p< 01). mI also correlated with Motor and Memory Indexes (r= .24), but the results were not statistically significant in their relatively small sample. This correlation with Frontal Index has also been observed by Huda, Guze et al. (1998), with Binesh, Huda et al. (2005) also finding a positive correlation between mI and motor speed and dexterity (r= .54).

The role of ammonia in cognitive deficits associated with SHE also appears to be important. Both Thomas, Huda et al. (1998) and Huda, Guze et al. (1998) found that

baseline blood ammonia levels are associated with lower performance on cognitive measures. However, both studies also found that neither Glx, nor Cho/Cr, were correlated with cognitive measures. In a group of 30 post-liver transplant children, Gilmour, Adkins et al. (2009) observed a strong correlations between PIQ measures and pre-transplant growth retardation and elevated serum ammonia (r= .45).

In support of ammonia as the potential link between cognitive ability and brain metabolism in SHE, Foerster, Conklin et al. (2009) found that that changes in choline and Glx within their patient group were proportional to biochemical markers of HE, including plasma ammonia level and the ratio of branched-chain to aromatic amino acids. However, as discussed earlier, no sibling controls were studied for comparison. Foerster et al.'s recent study is one of the few, along with Tkac, Hamernick et al. (2004), that employed ^{1}H-MRS in children with liver disease, an indication of the relative paucity of ^{1}H-MRS applications.

The results of the present study also show that the putative neuronal viability marker, NAA, commonly overlooked in studies of SHE, does not provide a biomarker of intellectual performance per se. In the evaluation of a child with liver disease, one might encounter normal, low or even elevated concentrations of NAA in the brain.

Pre and post-transplant data from children (Tkac, Hamernick et al., 2004) and adults (Thomas, Huda et al., 1998) and the limited studies investigating cognitive ability (Binesh, Huda et al., 2005; Huda, Guze et al., 1998; Thomas, Huda et al., 1998), suggest that the metabolite changes are not insignificant correlates of SHE and may reflect metabolism related to cerebral dysfunction. The relative reductions of NAA and choline, and greater variation in mI and Glx in the early-onset post transplant patients in the present study (see Figures 7-2 to 7-5), in parallel with specific deficits in IPS in this group (Chapter 3), is worthy of note and certainly motivates further investigations of MRS is paediatric liver disease patients.

However, the concerns raised by Moss, Tarter et al. (1992), in relation to cognitive function and SHE assessed with serum liver function tests, apply just as well to spectroscopic studies: the heterogeneity of the subjects studied with respect to age,

gender, socioeconomic status, hepatic diagnosis and disease severity, as well as other intra- or extra-hepatic pathologic mechanisms (e.g. fatty acid deprivation, alterations in membrane fluidity or aberrant amino acid metabolism), are likely to have contributed to the substantial variability in the relationships between metabolites and cognitive ability observed by others, or lack of relationships in the case of the present cohort. In terms of MRS specifically, many of the issues that trouble the work in sibling controls, which are discussed in Chapter 6, apply here. Issues of data analysis, metabolite quantification, voxel placement and the like, are compounded further in clinical studies, where studies are necessarily fewer and with smaller cohorts.

7.6 Conclusion

The consistency of the metabolites observed between the patient and sibling control suggests that neurodevelopment, as assessed by selective ^{1}H-MRS-detectable neurometabolite biomarkers, is ostensibly normal in this clinical cohort.

Early detection of alterations in brain metabolites may be helpful in mitigating the neurocognitive declines seen in some children with liver disease. Whilst the present findings do not confirm the specific pattern of metabolite changes typically seen in healthy adults in other studies of this kind, they add to the small, but important and growing, body of studies neuroimaging neurometabolism with MRS children.

MRS holds promise as a screening tool for biomarkers in liver disease because of the increased precision gained through using continuous measures rather than ordinal or categorical ones. It has the potential to become an alternative to neuropsychological test batteries for the assessment of the clinical manifestations of liver disease, and potentially allow tracking of the patient's response to clinical interventions designed to minimise the progression of their disease. However, the use of ^{1}H-MRS is still largely exploratory and what is currently missing is careful quantification, age-specific, reproducible, studies in newborns, infants, children and adolescents to provide context for the clinical data.

8 Discussion and conclusions

8.1 Introduction

Using a multi-modal approach, the present set of studies aimed to develop and refine analytic tools for biochemical assays, for use with both adult and paediatric populations. The main aim was to investigate some of the potential biochemical underpinnings of cognition, relating neural, systemic and behavioural levels of analysis.

Chapter 1 introduced the approaches employed to studying the biological bases of individual variation in cognitive ability and the utility of the converging methods approach to clarify biological mechanisms which account for differences in psychometric test performance. Chapter 2 described the nature of EFA metabolites and their importance and function in neural tissue, highlighting that the effects of fatty acids are multifactorial and are specific to individual classes of fatty acid. EFAs and their PUFA metabolites have important roles not only at the membrane level, primarily through their influence in phospholipid cell membrane fluidity, but also through their involvement in inflammatory processes. Both of these roles may be important in understanding the modulatory role of fatty acids on cognition in health and in disease.

Animal dietary deprivation models have demonstrated that chronic deficiency in EFAs, particularly in early neurodevelopment, is associated with significant cognitive impairment including deficits in memory and learning. Observational studies of breastfeeding versus formula feeding and randomised controlled trials comparing children fed formulas either supplemented or unsupplemented with EFAs and/or PUFAs are the common methods for investigating the cognitive effects of EFAs in human children. Whilst the data is not unequivocal, infants who receive formula milk, as opposed to breast milk which is naturally high in EFAs, suffer cognitive impairments including slower processing speed and lower general IQ. Supplementation with high levels of EFAs has been found to ameliorate some of these cognitive deficits, which are commonly assessed using the Mental and Psychomotor Development Indices of the Bayley Scales of Infant Development.

Disease models provide a useful paradigm for studying the cognitive deficits associated with suboptimal EFA levels in children. Intervention studies where EFA deficiency is induced are precluded by ethical considerations, such as the potential effects of long-term dietary restriction. In the present study, a paediatric liver disease model was used to answer questions of whether:

1. Sub-optimal concentrations of EFAs, as a result of fat malabsorption or dependence on inadequate dietary sources, is associated with deficits in cognitive ability.
2. ^{1}H-MRS-detectable metabolites can provide surrogate markers of sub-clinical changes in neuronal viability.

This investigation entailed four main bodies of work, three with the paediatric liver disease clinical population and one with a set of healthy adult controls. The specific convergent methods employed were described in Chapter 3.

- Chapter 4 investigated cognitive outcomes in a group of paediatric patients with early-onset liver disease or acute liver failure compared to sibling controls using psychometric tests to assess the neuropsychological impact of the disease.
- Chapter 5 assessed the range of EFA concentrations and deficiency biomarkers in erythrocytes in the patient and control groups and examined the relationship between current EFA status and cognitive outcomes.
- Chapter 6 investigated the relationships between natural variations in neurometabolites assayed by ^{1}H-MRS and cognitive ability in a healthy adult population.
- Chapter 7 evaluated the potential of ^{1}H-MRS to add information to the study of children with liver disease by revealing abnormalities in cerebral metabolism.

The conclusions that can be drawn from the studies described in this thesis fall into two categories; those relating to the empirical results and their implications for the understanding of EFAs and ^{1}H-MRS-detectable metabolites in cognition in general and paediatric liver disease in particular, and those concerned with the experimental techniques employed and the theoretical framework in which they are currently used.

The following discusses the issues and limitations of ^{1}H-MRS and GC-MS methods, followed by examples of directions of research motivated the present studies and concluding remarks.

8.2 Key findings of the present studies

In the following discussion, mutually informative studies have been grouped together. Cognitive outcomes (Chapter 4) will be discussed in the context of EFA status (Chapter 5), followed by discussion of the ^{1}H-MRS studies in sibling controls and the paediatric liver disease patients (Chapters 6 and 7).

8.2.1 Studies 1 and 2: Cognitive outcomes and EFA status in paediatric liver disease

In the first set of analyses the effects of chronic liver disease on cognitive outcomes as assessed with age-appropriate, standardised assessments of verbal and nonverbal cognitive skills were investigated. Liver disease appears to have significant negative effects on specific aspects of cognitive development, with age at the onset of disease an important moderator of these effects. Whilst no significant deficit was observed in FSIQ between disease groups, significant differences were observed between the early onset, post-transplant group and age-matched sibling controls on a measure of IPS, with 31% of the variance in IPS explained by the effects of onset of liver disease coupled with transplantation.

The dissociation in results the between pre and post-transplant early-onset patients may be explained by the fact that the transplanted patients were those that were suffering the most severe effect of liver disease, which would therefore precipitate the most significant disturbances to normal development. This finding is in accordance with the literature, which has shown that earlier onset of liver disease is associated with a greater degree of cognitive deficits (Stewart, Campbell et al., 1992; Stewart, Uauy et al., 1988; Stewart, Uauy et al., 1989), and is in keeping with the hypothesis that potential disturbances in the early stages of neurodevelopment as a result of liver disease precipitate demonstrable deficits in cognitive ability later in life.

Disturbances in EFA status, due to dietary deficiency resulting from dependence on artificial nutritional feeds and/or malabsorption due to the specific deficits in liver function and lipid metabolism, was hypothesised to be one the mechanisms that contributed to cognitive deficits observed in patients with early-onset liver disease. Compared to sibling controls, no signs of fatty acid deficiency, indexed by EFA status biomarkers in erythrocyte membranes, were observed in any of the cohorts of patients with liver disease. If the acquired biomarkers are accurate, this suggests that: (1) these patients were not deficient in their dietary intake of the EFAs, LA and ALA at least in the three months prior to the participation in the study; and (2) these patients are able to sufficiently metabolise these precursor lipids to synthesise the LCPUFAs, DHA and EPA, to levels comparable to sibling controls.

Whilst the biomarkers indicate that the patients were not deficient in their current dietary intake, measures of actual dietary intake would provide useful context for the biomarkers detected in red cell membranes actual and help clarify potential dissociations between intake and metabolism. Dietary intake data was available for a limited number of patients recruited into the study (Appendix A, Table A, page 2076), but analysis of this incomplete set of data may not have been particularly informative.

The findings of these two studies have important implications in the treatment of patients with liver disease. With respect to clinical outcomes, there is increasing support for the use of omega-3 fatty acids in the treatment of liver disease, particularly parenteral nutrition associated liver disease (PNALD) (Diamond, Sterescu et al., 2008; Koletzko and Goulet, 2010). Fish-oil based PN feeds have, for example, been associated with the reversal of cholestasis and fatal liver disease compared to soya-based feeds, which lack appreciable levels of PUFAs (Gura, Lee et al., 2008).

The results of the present study suggest that early-onset patients, particularly those who are most ill and therefore require transplant, are those that may benefit from PUFA supplementation in early infancy. Phenylketonuria (PKU) is an inborn error of amino acid metabolism that precludes natural protein in a diet that is also typically DHA deficient and provides an analogous model to the one adopted in the present study. Whilst severe neurological damage is completely prevented in PKU-affected individuals

by adequate dietary management, in patients aged from 1 to 11, Koletzko, Beblo et al., (2009) observed that PKU patients showed slower visual evoked potentials, a measure of processing speed, compared to controls. These effects were ameliorated with 3 months of high-dose omega-3 supplementation.

The results from studies of PKU and PNALD patients provides rationale and motivation for better dietary management and prospective supplementation interventions in early-onset patients with the aim of normalising the deficits in processing speed potentiated by suboptimal EFA status in early development. The acute liver failure patients, whose liver disease developed after the critical perinatal stage, showed no signs of cognitive deficit as a result of EFA deficiency, and may not therefore require specific EFA supplementation to achieve normal developmental outcomes.

However, support for the use of PUFA supplementation must be tempered by the fact that the data on EFAs and cognitive outcomes is still largely exploratory. Studies of the effects of EFA supplementation on cognitive outcomes in children older than two years of age are severely limited in number (Eilander, Hundscheid et al., 2007), and have not consistently found positive ameliorative effects of supplementation (Gadoth, 2008). McCann and Ames (2005) stressed that the effects of omega-3s such as DHA may be overstated, because even in randomised controlled trials formulas are typically supplemented with other LCPUFAs, particularly AA, in addition to DHA. This confers an inherent lack of specificity and means that these studies are not therefore able to attribute significant effects to the presence of omega-3 fatty acids only.

In the present study the hypothesis that current levels of omega-3 fatty acids would correlate strongly with cognitive performance was not confirmed. The biomarker for omega-3 status (comprised of the sum of EPA and DHA, putatively the most important LCPUFAs), did not correlate significantly with FSIQ or IPS performance ($r = .03$ and $.20$; $p > .05$). The omega-6 index (comprised of the percentage total of LA and ALA) was significantly negatively correlated with FSIQ ($r = -.62$; $p < .001$; see Figure 5-2 , page 96) and showed a trend correlation with IPS ($r = -.39$; see Figure 5-3, page 96).

EFAs are known to modify several important physiological features of neurons such as: membrane fluidity; the action of membrane bound enzymes, receptors and ion channels; production and activity of neurotransmitters; and signal transduction, which controls the activity of neurotransmitters and neuronal growth factors. There are two mechanisms of these effects: (1) a long-term action on the composition and functioning of the membranes; and (2) a short-term action that would involve the metabolism of phospholipids (with subsequent modulation of signal transduction) and the action of EFA-derived metabolites such as eicosanoids.

The biomarkers of EFA status assessed in this study reflect intake and metabolism of EFAs over the preceding 3 months. That such a relatively transient measure is highly correlated with FSIQ (see Figure 5-2, page 96), which is ostensibly extremely stable over time, is worthy of note and implicates to a long-term moderator of EFA status. One such mechanism could be the recently identified genetic component of fatty acid metabolism, which is discussed in further detail as a future direction of research in section 8.5.2.

8.2.2 Studies 3 and 4: Cognitive spectroscopy and the utility of ^{1}H-MRS biomarkers of paediatric liver disease

The use of ^{1}H-MRS in cognitive research is still largely exploratory, so before applying these measures in a clinical paediatric cohort, a study was undertaken to establish the strength of relationships between neurometabolites and cognitive variables in a cohort of 38 healthy young adults. Whilst the correlations observed between ^{1}H-MRS detectable neurometabolites and IQ in the present study were within the range reported in the literature, the magnitude of these effects were dependent upon the extent to which outlying values were accounted for in statistical analyses (Chapter 6).

Coupled with the range of effect sizes reported in the literature, is substantial methodological variability between studies. Differences such as the age of participants, the cortical regions investigated, the ^{1}H-MRS acquisition parameters used and the cognitive skills targeted, pose a significant challenge for drawing inferences about the

strength of the relationship between neurometabolites obtained with proton spectroscopy and IQ variables at the population level.

The final study (Chapter 7) evaluated the extent to which ^{1}H-MRS can add information to the study of children with liver disease by revealing abnormalities in cerebral metabolism. A specific pattern of neurometabolite changes, specifically a decrease in choline and mI and increase in Glx, has been previously observed in adult and paediatric samples (Atkison, Ross et al., 2002; Kreis, Ross et al., 1992; Taylor-Robinson, Sargentoni et al., 1994b; Tkac, Hamernick et al., 2004). However, in this study, neither significant differences were observed in metabolite concentrations among the three groups of children with liver disease (early onset, pre-transplant, early onset, post-transplant and acute liver failure, post transplant), nor between these patients and sibling control children matched for age.

The consistency of the metabolite values observed in the patients and control groups (Table 6-2, page 130) suggests that neurodevelopment, assayed by surrogate neurometabolite markers, is normal in this cohort, and that: (1) deviant neurometabolism, indexed by the particular set of ^{1}H-MRS metabolites studied, is not the mechanism which explains deficits in processing speed seen in early-onset patients; (2) relations between neurometabolites and cognitive outcomes may be masked by other effects. The heterogeneity of the subjects studied with respect to age, gender, socioeconomic status, hepatic diagnosis and disease severity, as well as other intra- or extra-hepatic pathologic mechanisms (e.g. fatty acid deprivation, alterations in membrane fluidity or aberrant amino acid metabolism), are likely to have contributed to the substantial variability in the relationships between metabolites and cognitive ability observed by others, or lack of relationships in the case of the present cohort; (3) the cohort studied were not sufficiently compromised with respect to hepatic function for the previously reported pattern of neurometabolite changes to be precipitated. Participation in the current study required approval from the patient's consultant. For five patients hospitalised while awaiting transplantation it was judged that they would not to be able to tolerate an MRS scan, which means that the most severe cases of liver failure were not included in this study for ethical and practical reasons.

8.3 Recommendations for future studies

Based on the findings from the present set of studies and examination of the current state of the literature, three sets of specific recommendations are suggested to move the field forward.

8.3.1 Implementation and reporting of data screening

The first set of recommendations pertains to the reporting of data screening procedures and appropriate use of statistical analysis, particularly in ^{1}H-MRS studies where there is considerable variability between studies. Neurometabolite data obtained from normal populations cannot be assumed to be normally distributed and given that inappropriate use of parametric statistics on non-normal data can lead to errors in data interpretation, it is recommended that a concerted effort should be made to make explicit the extent of data screening employed in future studies.

8.3.2 Hypothesis-driven enquiry

The second set of recommendations concerns the framework in which studies are performed. To date, the substantial number of variables (EFA biomarkers or ^{1}H-MRS-detectable neurometabolites) under investigation, coupled with a lack of specifically identified *a priori* research hypotheses, renders the typical study susceptible to inflated Type 1 error resulting from the large number of statistical comparisons employed. The methodological approach to data analytic strategy in future studies should be directly informed by the *a priori* hypotheses adopted as this will govern the choice and range of psychometric, EFA and spectroscopy measures and how these are treated in the analyses, minimising over-extrapolation of the data.

8.3.3 The need for normative data

In placing the clinical findings of the present study in context, it was stressed in Chapter 7 that the lack of a large database of normative age-matched data makes it difficult to assess the true strength of disease-associated metabolite changes in childhood and

is an important limitation for assessing the contribution ^{1}H-MRS as a diagnostic tool.
An understanding of the changes that take place in normal brain development is crucial prior to the application of ^{1}H-MRS in the study of pathological conditions, particularly given the demonstration of regionally specific, non-linear changes in metabolite concentrations, particularly in the first two years of life (Kreis, Ernst et al., 1993; Pouwels, Brockmann et al., 1999).

The diagnostic criteria for neurometabolite values in SHE, suggested by Ross, Jacobson et al. (1994), which were based on normative data derived from an ostensibly normal group of 12 adults, were used in the present study. Whilst these data provide useful guidelines, the value of the data is limited by the lack of sufficient sample sizes and at various ages, which would provide wider context for abnormal changes, particularly in paediatric populations.

Within the MRS community, there needs to be a concerted effort to follow the precedent set by the National Institute of Mental Health's Pediatric Brain Imaging Project (Lenroot and Giedd, 2007) to create a database of developmental MRS data across the lifespan, comparable to that created by the project for anatomical MRI data.

8.4 Methodological considerations and limitations in the present study

8.4.1 Fatty acid analytic methods

The application of conventional GCMS procedures to analysis of biological samples is disadvantaged by the high risk of contamination and recovery losses in multi-step procedures. Furthermore, these methods are impractical for analysing large series of samples, especially when the quantities of biological samples are limited. To overcome these disadvantages and owing to the time-intensive nature of GC, other studies have used alternative techniques such as High Performance Liquid Chromatography (HPLC) or Liquid Chromatography-Mass Spectrometry (LC-MS) (Peterson and Cummings, 2006). Additionally, Thin Layer Chromatography (TLC), described in detail by Christie (1982), allows for quantification of fatty acids in individual phospholipids, for example

phosphatidylcholine or phosphatidylinositol, providing not only a quantitative measure of total fatty acids, but also where in the lipid membrane these fatty acids are located, further helping clarify their functional roles.

Methods that combine extraction and derivitisation in a single step have been developed to provide higher resolving power and enable less abundant fatty acids to be detected. These include fast gas chromatography (Bondia-Pons, Castellote et al., 2004; Mondello, Casilli et al., 2004) and the use of a direct thermal desorption interface to profile the fatty acid composition of human plasma and whole human blood (Akoto, Vreuls et al., 2008). New optimised methods have been also devised that can profile lipids small quantity (i.e. fingertip (50μl) blood samples), with the ability to detect 100 fatty acids and related compounds (Bicalho, David et al., 2008). These methods provide the opportunity to develop an efficient and readily achievable database for large fatty acid methyl esters database from small, easily obtainable samples. Application of the analytic methods described above were beyond the scope and remit of the present study, but are worth considering in the broader scheme of its aims, particularly in the context of being able to easily create a databank of normative erythrocyte EFA data.

8.4.2 Paediatric ^{1}H-MRS

A persistent and inherent problem in paediatric MRI is the difficulty involved with determining the histological correlates of the various tissue classes that are assigned as cortical 'grey,' 'unmyelinated white,' 'myelinated white' and 'cerebrospinal fluid'. The ability to discriminate and measure different brain tissues is provided by the image contrast: the degree of the 'white' appearance of myelinated tissue, compared with the 'grey' of the adjacent cortical grey matter. Tissue contrast in infant MRI scans differs from scans obtained in later childhood, primarily because of the higher water content and lower myelin deposition in infant brains (Barkovich, Kjos et al., 1988; Paus, Collins et al., 2001). Accurate localisation of a voxel to a specific anatomic location without spectral contamination from adjacent tissues is especially difficult in infant populations as their small brain size makes it difficult to sample homogenous regions of cortical tissue.

The extent to which MRS samples grey or white matter can modulate the metabolite values obtained (Wiedermann, Schuff et al., 2001). In the present study, frontal and occipitoparietal white matter was assessed by positioning the voxel so as to maximise sampling of white matter and minimise grey matter content. Whilst tissue compartmentation can be performed *post hoc*, the MRS spectra is principally an average over all tissue types that occur within the volume at the time of sampling, which for neural tissues includes glial and neuronal cells, and with different extracellular spacing depending on the amount of white matter, grey matter or cerebrospinal fluid the volume of interest contains.

Smaller voxel sizes may aid in reducing the inhomogeniety of the sampled tissue, but decreasing voxel size leads to a corresponding decrease in the SNR of the MR signal (Freeman, 2003). Issues of tissue inhomogeniety and SNR can, in part, be overcome by multi-voxel techniques like chemical shift imaging (CSI), which acquires multiple spectra simultaneously from slices or volumes of the brain to form metabolite-specific images from the resulting peak-intensities.

Discussion of the applications of MRS and the need for more normative data must also consider the associated limitations and hazards of MR techniques in order to ensure the method is safe (McKinley, Bouffler et al., 2008; Shellock and Kanal, 1994), particularly when considering paediatric populations (Peterson and Ment, 2001). The practical difficulties of functional MRI have been discussed previously (Logan, 1999); many, if not all, of the same issues apply to MRS. The obvious limitations for the general use of MRS are that it is still, in relative terms, very expensive and reliant on specialised medical physics support, and is therefore not commonly available outside principally clinical settings.

MR methods also require a considerable degree of participant cooperation. This can be difficult to achieve with paediatric populations, where the unfamiliar environment of the scanner may induce anxiety. Immobility is also essential in order to minimise motion artefact and therefore sedation may be necessary for younger children. General anaesthesia undoubtedly allows MRI to be carried out in anxious or uncooperative children, but its use continues to be controversial and fraught with practical and ethical

complications (Lawson, 2000), and it may not be appropriate outside exclusively clinical settings.

Foerster, Conklin et al. (2009) did not collect data from healthy controls in their study of paediatric HE over concerns of the ethics and practicalities of MR on small children. ^{1}H-MRS is entirely safe if strict standard operating procedures are followed (Kanal, 2004; Shellock and Kanal, 1994). Whilst MR of young populations is undoubtedly difficult, in the present work, MRS data was collected in 34 of 40 children (including 11 sibling controls, one as young as 2 years old). Only three patients were unable to tolerate the scanner procedure (with claustrophobia once in scanner bore cited as the principal factor). Data from a further three participants were removed because of poor quality spectra as a result of motion artefact or encroachment in the voxel of non-neural tissue.

Successful scanning of young children in research settings is attributable to preparation with the child and guardian beforehand, and flexibility and patience with regards to scan sequences. As a part of the present study, an introductory video was developed that was aimed directly at the children and their guardians. The video provided a child-centred guide to the MR process and environment in order to familiarise participants with the research prior to consent and participation in the study.

In addition to issues of patient compliance, demands on scanner availability may preclude prolonged scan sessions. However, advances in MR technology are leading to progressively shortened scan times and allow acquisition of multiple spectra in a single occasion without the need for sedation. Compared with 1.5T, the improved SNR of 3T field strength adopted in the present studies shorted the total data acquisition time by a factor of 4 while maintaining a comparable SNR (Lin, An et al., 2003).

Applications of MR technologies still tend to be technology-led and improvements in technical methodologies are central to further developments in achieving better control of artefacts, greater spatial resolution and allowing ^{1}H-MRS to become progressively more quantitative within time-frames feasible for paediatric populations. With a large body of information on software, equipment, techniques, and activation results being

rapidly accumulated, cognitive-MRS studies should be easier to perform and the interpretation of the results more instructive in the future.

8.5 Future directions

A number of refinements to the methods and procedures employed and potential avenues of research motivated by the findings of the present work proposed.

8.5.1 A neurophysiological measure of processing speed

An alternative to behavioural paper-pencil or even computerised measures of processing speed is magnetoencephalography (MEG), a non-invasive neuroimaging tool that can tap processing speed at a neurophysiological level. By localising and characterising activity of the central nervous system through the measurement of the associated magnetic fields emanating from the brain, MEG is able to measure neural activity with millisecond precision.

The temporal resolution of the auditory system is exquisite, with neural systems capable of sub-millisecond resolution in decoding features in the acoustic signal (Eggermont, 2001). Psychophysical measures of gap detection, where a silent gap is inserted in a tone or noise burst and the minimum detectable gap is measured, are in wide use as an objective method with which to evaluate auditory temporal acuity in both healthy and clinical populations (Eggermont, 2000; Eggermont, 2001) and may provide a useful endophenotype for developmental delay.

As an adjunct to the present study, a small pilot investigation was conducted, with two sibling controls and two patients from the early onset, pre-transplant liver disease cohort, for the assessment of auditory-evoked field (AEF) responses in a gap detection task, in order to provide an efficient and objective index of auditory information processing in the human brain, with a view to use with young clinical populations.

The findings from the pilot study were presented at the 2010 BIOMAG meeting (see Appendix B, page 215). Whilst a full discussion of the MEG data is beyond the scope of

this thesis, the findings of this pilot work is promising, as it is suggests a deficit in auditory temporal processing in patients compared to controls. Event-related fields (ERFs) afford the opportunity to acquire neural measures of auditory processing speed within a passive experimental paradigm in which children are not required to attend or respond to stimuli. The absence of confounds related to task compliance makes it possible to asses perceptual-level processing and makes this paradigm a particularly promising approach in the study of neurocognitive development and deficits in young, clinical populations. This paradigm offers a potential functional measure of processing speed at the neural level to complement biochemical data from ^{1}H-MRS and the behavioural measures of psychometric tasks.

8.5.2 The influence of genetic variation in fatty acid metabolism

The Δ-5 and Δ-6 desaturase are the most important enzymes in the elongation and desaturation of EFA precursors (LA and ALA) to their long-chain PUFA metabolites (discussed in section 2.2, page 26; see Figure 2-4 and Figure 2-5, pages 29 and 30). Recent work suggests that there may be a strong genetic component to the fatty acid metabolism, with evidence of associations between single nucleotide polymorphisms (SNPs) in the two desaturase encoding genes (FADS1 and FADS2) and the concentration of omega-6 and omega-3 fatty acids (Lattka, Illig et al., 2010; Simopoulos, 2010).

The action of the FADS gene variants influence the levels of both serum (Tanaka, Shen et al., 2009) and RBC membrane phospholipid levels (Rzehak, Heinrich et al., 2008) of EFAs. Variants in the FADS1 and FADS2 have a frequency of 26% and the minor alleles associated with lower AA and higher LA account for 28% of the variation in serum phospholipid AA and up to 12% of its precursor fatty acids (Simopoulos, 2010).

The evidence gathered to date indicates that fats are systemically metabolised in different ways as a result of FADS gene variants, with a limited number of studies finding that this is associated with tangible changes in cognitive outcomes (Latta, Illig et al., 2010). Of particular relevance to the current study, Caspi, Williams et al. (2007) observed that IQ performance was higher in breastfed children compared with non-breastfed children, and that there was an interaction between a specific SNP in the

FADS2 gene (rs 174575), breastfeeding and cognitive performance. The suggestion is that those children carrying one particular genotype benefit more from breast milk than children with a different genotype who neither gained an advantage from breastfeeding nor suffered a disadvantage from not being breastfed.

Investigations into the influence of gene variants on EFA metabolism is an emerging field of research. Although this work is exploratory, if groups of fatty acids are differentially metabolised due to genetic variations, it is plausible that difference in EFA metabolism may lead to systemic changes in membrane EFA composition throughout the lifespan. Differences in EFA metabolism as a result of differences in FADS genes may, for example, explain the lack of association between breastfeeding and cognition observed by (Bakker, Ghys et al., 2003; Ghys, Bakker et al., 2002) in studies of early EFA status and cognitive outcomes.

It may also explain the findings from the present study of negative associations between omega-6 fatty acids and FSIQ and IPS, as current fatty acid levels may be representative of long-term metabolism and utilisation. A speculative explanation for this finding may be that the long-term preferential metabolism and accumulation of omega-6 fatty acids by particular FADS gene variants may lead to displacement of important long-chain omega-3 PUFAs such as DHA and EPA in phospholipid membranes, leading to saturated, less fluid membranes, and other functional alterations as discussed in section 2.3.

The incidence of allele variants of FADS1 and FADS2 and their impact on blood fatty acid levels and EFA utilisation may emerge as a productive area of research in contextualising the relationships between EFA status and cognitive outcomes. In terms of the wider study investigating the importance and potential deficiencies of EFAs in liver disease, identifying alleles associated with suboptimal EFA metabolism may be important, as these gene variants may exacerbate the potentially higher than normal requirements for these nutrients in patients with liver disease.

8.5.3 ^{31}Phosphorous spectroscopy

Protons are in most cases the default nuclei for cognitive spectroscopy studies as ^{1}H-MRS uses the same hardware, such as head coils, as standard MRI. Observing nuclei other than protons requires the development of radio-frequency coils and other specialised hardware tuned to their specific frequencies and this has limited investigations in spectroscopy studies to only those neurometabolites with a high proportion of proton constituents.

Phosphorous is an alternative nucleus that has particular relevance with the present study as ^{1}H-MRS may not be the ideal tool for investigating choline-related metabolic developments in neuronal maturation.
The normal phosphorous-MR spectrum of brain has seven major resonances (see Table 8-1, page 183) Five of these; one from each of the three phosphates of ATP, one from phosphocreatine (a high energy buffer compound); and one from inorganic phosphate (a product of ATP breakdown), can be used as a measure intracellular inorganic phosphate (pHi), a surrogate marker of bioenergetics and metabolism (Erecinska, Stubbs et al., 1977). The two remaining major peaks, phosphodiester (PDE) and monoesters (PME), are in a complex way related to the ^{1}H-MRS-detectable choline peak (Boulanger, Labelle et al., 2000). With proton nuclei, the biochemical interpretation of alterations in the MR-observed *in vivo* choline peak is complicated by the uncertainty in the metabolites contributing to the signal (see section 6.2.2, page 119). In neural tissue, choline is found primarily as membrane-bound phosphatidylcholine and also as a mixture of choline, phosphocholine and glycerophosphocholine in solution.

PME and PDE reveal important information about neuronal cell membranes. Specifically, the PME resonance in ^{31}P-MRS reflects membrane phospholipid anabolism as it includes the freely mobile precursors of membrane phospholipids such as phosphocholine and phosphoethanolamine. The PDE peak reflects membrane phospholipid catabolism as it contains contributions from breakdown products such as glycerophosphocholine and glycerophosphoethanolamine. It also contains contributions from less mobile phosphodiester-containing molecules, such as molecules involved in membrane structure (both cell membranes and intracellular organelle

membranes). As cell membranes are continually generated and broken down, expressing the PME and PDE levels as a ratio provides a measure of turnover equilibrium of membrane phospholipids.

Table 8-1 Metabolites detectable by ^{31}P MRS

Metabolites	Structure/function
Phosphomonoester (PME)	Phosphocholine, phosphoethanolamine and l-phosphoserine contribute to the PME peak
Phosphodiester (PDE)	Glycerophosphocholine and glycerophospho-ethanolamine contribute to the PDE peak
Adenosine triphosphate moieties	High energy phosphates and nucleotide and nucleoside phosphates
Phosphocreatine (PCr)	Energy storing moiety
Inorganic phosphate (pHi)	Intermediate in energy metabolism
Macromolecular signals (broad components)	Membrane phospholipids; phosphorylated proteins

The PME peak is known to be elevated in areas of rapidly growing tissue and in cases of rapid membrane synthesis, such as in growing brain. It is probable that the elevation is caused by the increased presence of compounds meant for the production of membrane phospholipids (Boulanger, Labelle et al., 2000). If the choline peak mainly reflects structural components of cell membranes, especially myelin sheaths, a change in the Cho/Cre ratio may be closely related to the process of myelination. Depending on the region studied, decreases in choline values generally appear to be most prominent only after the first 2 years of life, which corresponds with the completion of the majority myelination (Kreis, Ernst et al., 1993). Investigation with ^{31}P-MRS may aid in tapping more directly into the potentially disturbed processes of myelination in the early-onset patients with liver disease which may be precipitating deficits in IPS later in childhood.

^{31}P-MRS in cognitive research has largely focused on schizophrenia and schizo-affective disorders as a means of evaluating the role of the cell membrane in the aetiology of these disorders, specifically the membrane phospholipid theory of schizophrenia (Horrobin, 1998). These studies have yielded largely inconsistent results that none-the-less point to decreased PMEs and increased PDE (indicating decreased membrane stability) in patients with schizophrenia (Puri, 2006; Puri, Counsell et al., 2008; Reddy, Keshavan et al., 2004; Rzanny, Klemm et al., 2003).

In terms of applications to liver disease, reductions in the PME/βATP and PDE/βATP ratios, have been consistently observed in adult patients with chronic liver disease, and these correlate with the reduction in choline concentrations observed using ^{1}H-MRS (Patel, Forton et al., 2000; Taylor-Robinson, Sargentoni et al., 1994a). ^{31}P-MRS is, however, yet to be used with younger populations in this context.

There is also support for an association between peripheral and central measurements of membrane physiology. Richardson et al (2001) compared *in vivo* ^{31}P-MRS measurements averaged over 10 large voxels within the brain and erythrocyte PUFA composition in normal adult subjects, and demonstrated an negative correlation between DHA and EPA acid content and PDE levels (Richardson, Allen et al., 2001).

A correlation between RBC PUFAs and brain phospholipid metabolites does not indicate a direct causal relationship. If confirmed, however, such a correlation would provide a unique opportunity to simultaneously investigate convergent central and peripheral biochemistry with relation to EFA status and clinical and cognitive measures, in ways that are not possible with ^{1}H-MRS measures of neurometabolic status.

8.6 Conclusion

This is the first study to have employed parallel measures of brain and blood biochemistry in a paediatric liver disease population with the aim of investigating some of the potential biochemical underpinnings of cognition.

Multiple cellular functions and responses are affected as a consequence of neurometabolite and membrane lipid variations. Cognitive spectroscopy and studies of EFA and cognition in children are still largely exploratory, with a lack of consensus around the strength and significance of relationships between these biochemical markers and cognitive abilities. Inconsistencies and gaps in knowledge remain, making it difficult to draw general conclusions regarding the cognitive and functional changes in response to natural or induced variations in these neurometabolite and EFA substrates. The subtlety of these relationships motivates the development of an explanatory

framework that discriminates between statistical significance and statistical relevance, particularly in the studies of rare and difficult populations.

The evidence presented here suggests that the numerous activities of the liver that are disturbed as a consequence of liver disease may each contribute in some small degree to the observed deficits in cognitive functioning, specifically information processing speed, and particularly in patients whose early neurodevelopment was impacted.

Further cross-sectional longitudinal studies using ^{1}H-MRS and GC-MS, or their variant techniques, in larger subject populations, will be instrumental in confirming and extending current findings. Quantitative studies that seek correlation with subsequent behaviour and cognition are likely to provide valuable insight into the organisation of neural systems that underlie cognitive development in children, the differential contribution of neurometabolites and EFAs to cognitive functioning across the life span, and the mechanisms that are impacted in abnormal states like liver disease. Studies may need to be multicentre in order to achieve reliably large numbers of subjects, or if performed in a single centre, conducted in such a way as to be comparable with data from other sites in future analyses.

Converging methodologies offer a challenging, but promising and novel approach to explore brain-behaviour relationships from micro- to macro-scopic levels of analysis.

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Appendix A: Catalogue of measures

Below is a comprehensive list of the data and measures obtained in the present study. Inclusion and analysis of some portions of data collected was considered beyond the scope of this thesis, but will be informative in interpreting the data in future analyses.

Demographic data

- Date of birth
- Age
- Gender
- Postcode (for calculation of Deprivation Index and estimate of SES)
- Ethnicity.

Clinical measures

- Disease diagnosis
- Disease category (early onset, pre-transplant; early onset, post-transplant; acute liver failure , pre-transplant)
- Age of liver disease onset
- Date of liver transplant
- Length of hospitalisation
- Bilirubin level at time of study.

Anthropometry

- Height (cm) + height z-score
- Weight (kg) + weight z-score
- Mid-arm circumference (cm)
- Triceps skinfold (cm)
- Head circumference (cm).

Dietary data

- Estimate of frequency of current fish intake (per month)
- Total dietary intake (g/day)
- Percentage intake of average requirement
- Dietary fatty acid intake (oleic, linoleic, α-linolenic, arachidonic, DHA; see Table A).

Breastfeeding data

- Duration of breastfeeding (weeks)
- Estimate of frequency of maternal fish intake during pregnancy (per month).

Psychometric assessment

Wechsler Preschool and Primary Scale of Intelligence for Children — 3rd Edition (WPPSI-III) (Wechsler, 2002)
Wechsler Intelligence Scale for Children – 4th Edition, (WISC-IV) (Wechsler, 2003)
Wechsler Adult Intelligence Scale – 3rd Edition (WAIS-III) (Wechsler, 1997b)
Wechsler Abbreviated Scale of Intelligence (WASI) (Wechsler, 1997a)

- Full-scale IQ
- Verbal IQ
- Information Processing Speed Index
- Working Memory.

Table A Examples of fatty acid dietary intake data for patients enrolled in the current study

Disease category	Subject	Total dietary fat (g/day)	% average requirement†	Oleic (18:1) (g)	Linolenic (18:2) (g)	Alpha-linoleic (18:3) (mg)	Arachidonic (20:4) (mg)	DHA (22:6) (mg)‡
Metabolic disease (Tyrosinaemia)	1	34.93	73	8.7	1.7	110	0	0
	2	33.95	133	14.4	1.7	260	20	0
	3	38.75	93	12.6	2.3	160	0	0
	4	84.5	183	13.9	36.85	230	0	0
Early-onset liver disease (EOLD), no transplant	5	71.9	80	24.1	7.44	320	0	0
	6	43.8	54	13.2	3.5	70	30	30
Early-post liver disease + transplant	7	43.27	51	17.8	4.61	50	0	20
Acute liver failure (ALF) + transplant	8	28.7	50	10	1.09	120	30	30
	9	81.8	104	2.11	1.18	60	280	70
Healthy sibling	10	44.03	66	10.3	1.32	220	50	10

† average of fat generally below recommended intake
‡ Recommended DHA intake is 120mg (see Table 2-2, page 34)

Gas chromatography-mass spectrometry

Table B Summary of fatty acids detected in erythrocyte membranes with standard GC-MS

Common name	Carbon number
Saturated fats	
myristic	14:0
palmitic	16:0
stearic	18:0
Monounsaturated fats	
oleic	18:1
Omega-9	
mead	20:3
Omega-6	
linoleic	18:2
dihomo-γ-linolenic	20:3
arachidonic	20:4
adrenic	22:4
osbond	22:5
Omega-3	
alpha-linolenic	18:3
eicosapentaenoic	20:5
docosahexaenoic	22:6

Table C Summary of erythrocyte biomarkers of essential fatty acid status

Index	Fatty acids
SFA	stearic + palmitic + myristic
MUFA	oleic
Omega-3 index	DHA + EPA
Omega-6 index	arachidonic + linoleic
Omega-3:Omega-6	Omega-3 index/Omega-6 index
EFA shortage marker	mead acid
Functional DHA shortage marker	DHA/osbond acid

Proton Magnetic Resonance Spectroscopy

- N acetyl aspartate/creatine
- Choline/creatine
- Myo-Inositol/creatine
- Glutamate-Glutamine(Glx)/Creatine.

Metabolite values were obtained in two principal regions: frontal and occipitoparietal cortex. Additional temporal cortex data was also collected in 11 participants.

Appendix B: Conference abstracts

The following is a list of abstracts of work from the present study presented at conferences. In addition to the investigations described in the main body of this thesis, some of the work presented included disease control patients with intestinal failure and metabolic disease who were recruited into the study and a number of clinical case studies.

Aston University Postgraduate Research Day (July 2009)
Birmingham, UK

Biochemical correlates of cognitive function
Abstract: Poster presentation

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School of Life & Health Sciences, Aston University, Birmingham, UK

Developments in psychological assessment and neuroimaging techniques are enabling the study of the neurophysiological basis of individual variation in cognitive ability with increasing refinement. Localized Proton Magnetic Resonance Spectroscopy (^{1}H-MRS) allows non-invasive, *in vivo* quantification of neurometabolites such as N-acetyl-aspartate (NAA), choline and myo-Inositol, which have been related to cognitive functions including information processing speed (IPS) (Ross and Sachdev. *Brain Res Rev*. 44, 83-102, 2004). This cross-sectional investigation of 35 healthy volunteers combined neuroimaging and biochemistry with cognitive testing (Wechsler Adult Intelligence Scales III), with the aim of linking brain-biochemistry to variability in behaviour. It was hypothesized that there would be a positive correlation between NAA levels, a marker of neuronal health, and cognitive performance, particularly IPS. Conversely, levels of choline and myo-Inositol, associated with membrane turnover and gliosis, would be expected to be negatively correlated with cognitive performance. A significant correlation was observed between frontal region NAA/creatine concentration and Matrix Reasoning score ($r = 0.386$, $p = 0.043$), with NAA/Creatine accounting for 14% of the variance in scores. No relationship was found between metabolite concentrations and IPS. No significant correlations were observed between cognition and metabolites in occipitoparietal region. ^{1}H-MRS is potentially a sensitive tool for assessing the biochemical correlates underlying cognitive function. Possible mechanisms underlying the association of neurometabolites with cognition are discussed and future directions of work, including the addition of a more extensive test battery and assessing and improving the reliability of ^{1}H-MRS measures, helping refine the use of MRS in behavioural neurosciences, are presented.

XIth International Symposium on Small Bowel Transplantation (September 2009)

Bologna, Italy

Tracking paediatric cognitive outcomes following small bowel transplantation: a case study
Abstract: Poster presentation

Patel T[1], Blyth J[2], Mears J[2], Sira J[2], Clarke S[2], Kelly DA[2], Gupte G[2], Griffiths G[1], Beath SV, Talcott JB[1]
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Introduction: Infants with intestinal failure (IFx) now survive and grow satisfactorily with parenteral nutrition (PN), but IQ may be affected. A potential reason for this may be dependency on a single source of lipid based on soya oil (Intralipid), which lacks essential polyunsaturated fatty-acids (PUFAs) normally found in breast milk and diet.

Methods: Psychometric data collected over ten years were used to assess developmental outcomes of an 11 year old boy who received SBTx at age 9 months because of liver failure secondary to PN and IFx caused by Hirchsprung's disease. Current PUFA status was measured and neurochemistry non-invasively assessed with Proton-Magnetic Resonance Spectroscopy (^{1}H-MRS).

Results: Pre-Tx, the patient demonstrated mild cognitive delay and normal motor development. Six months post-Tx the patient displayed significant motor and mental delay. Between three and five years post-Tx, IQ plateaued in the borderline/low average range (72 and 79 respectively). By ten years post-Tx, IQ had risen to 97, well within average range. His PUFA intake was negligible until commencing Nutrtini orally (662 mg/L PUFA), 6 months post-SBTx. He is now on a normal diet (300mg PUFA/day). Neurometabolite values, such as N-acteyl aspartate, which provide markers of neuronal health, showed a normal profile for a healthy 11 year old child.

Conclusion: In contrast to a cohort of children maintained on PN for 5 years or more (Hill et al *Arch Dis Child.* 2005;90:A16), this boy, who commenced normal diet from 1 year, has a normal IQ and distribution of neurometabolites, demonstrating that early SBTx is consistent with good long-term cognitive outcome.

British Society of Paediatric Gastroenterology, Hepatology and Nutrition (BSPGHAN) (January 2010)

Liverpool, UK

Tracking paediatric cognitive outcomes following combined liver small bowel transplantation: a case study

Abstract: Oral presentation

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Introduction: Infants with intestinal failure (IFx) now survive and grow satisfactorily with parenteral nutrition (PN), but IQ may be affected. A potential reason for this may be dependency on a single source of lipid based on soya oil (Intralipid), which lacks essential polyunsaturated fatty-acids (PUFAs) normally found in breast milk and diet.

Aim: Our aim was to assess the long-term cognitive and developmental effects of combined liver and small bowel transplantation and the potential detrimental effects of PN-induced PUFA deficiency.

Methods: Psychometric data collected over ten years were used to assess developmental outcomes of an 11 year old boy who received SBTx at age 9 months because of liver failure secondary to PN and IFx caused by Hirchsprung's disease. Current dietary intake was assessed with a comprehensive 5-day diet diary. PUFA status was assessed in red blood cell (RBC) membranes by conventional Gas chromatography-mass spectrometry. Neurochemistry was non-invasively assessed with proton magnetic resonance spectroscopy (^{1}H-MRS) in occipitoparietal and frontal cortex regions.

Results: Pre-Tx, the patient demonstrated mild cognitive delay and normal motor development. Six months post-Tx the patient displayed significant motor and mental delay. Between three and five years post-Tx, IQ plateaued in the borderline/low average range (72 and 79 respectively). By ten years post-SBTx, IQ had risen to 97, well within average range. His PUFA intake was negligible until commencing Nutrini orally (662 mg/L PUFA), 6 months post-SBTx. The patient is now on a normal diet (300mg PUFA/day). Neurometabolite values recorded by MRS, such as N-acteyl aspartate and choline, which provide markers of neuronal health, showed a normal profile for a healthy 11 year old child. At 10 years post-SBTx, the patient's essential PUFA levels, specifically docosahexaenoic acid and eicosapentaenoic acid, were no different to healthy age-matched controls (4% vs 4.23% of total fatty acid content), which consistent with his MRS and cognitive assessments.

Conclusion: In contrast to a cohort of children maintained on PN for 5 years or more (S. Hill et al *Arch Dis Child.* 2005;90:A16), this boy, who commenced normal diet from 1 year, has a normal IQ and distribution of neurometabolites and essential blood lipids, demonstrating that early SBTx is consistent with good long-term cognitive outcome.

17th International Conference on Biomagnetism (BIOMAG) (March 2010)

Dubrovnik, Croatia

Sensitivity to gaps in noise: Using MEG to assess auditory temporal resolution in paediatric populations

Abstract: Poster presentation*

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*Poster presented on my behalf by JB Talcott

Impaired neural timing has been demonstrated across a range of developmental disorders (e.g. dyslexia) and clinical conditions (e.g. hepatic encephalopathy). Neural timing can be operationally defined in different ways, but one core feature that underlies performance on many cognitive and behavioural tasks is the encoding of stimulus changes that occur within a millisecond timescale. Auditory gap detection provides a measure of temporal resolution on this scale, where psychophysical thresholds indicate the shortest silent gap in noise which can be detected. Although gap detection has been measured previously with MEG, this study focused on developing an optimised paradigm for use with young children and patient populations where full compliance and vigilance for a neuroimaging task may not be assured. MEG data was collected using a 252 channel CTF scanner, while participants were presented with a 420 second continuous diotic Gaussian noise stimulus. The noise was interrupted at jittered intervals around 500ms with pseudo-randomised gap durations of either 3, 6, 10 or 30ms. Subsequent to the removal of baseline trend, the data was subdivided into 500ms epochs centred around the gap and averaged for each gap length. Mean amplitudes of the evoked response for each gap duration was calculated for the sensor with the peak response and normalised to the response for the 30-ms gap. The gradient of the linear relationship between response amplitude and gap length therefore provided a metric of physiological sensitivity to gap duration. Data from both adult listeners and children with probable hepatic encephalopathy indicate that this MEG gap detection paradigm yields a reliable and valid index of auditory temporal resolution with potential clinical utility. MEG paradigms which minimise the length of the recording epochs are particularly beneficial for obtaining data with younger and other variably compliant populations.

Aston Postgraduate Research Day (June 2010)
Birmingham, UK

Investigating biochemical correlates of cognitive function with Proton Magnetic Resonance Spectroscopy: refining the methodological framework
Abstract: Poster presentation

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Proton Magnetic Resonance Spectroscopy (^{1}H-MRS) is a non-invasive imaging technique that enables quantification of neurochemicals *in vivo* and thereby facilitates investigation of the biochemical underpinnings of human cognition. Studies have typically focused on relationships between measures of N-acetyl aspartate (NAA), a surrogate marker of neuronal health and function, and broad measures of cognitive performance, such as Full-scale IQ. In this cross-sectional study of 34 healthy individuals, we assessed NAA levels in occipitoparietal and frontal cortical white matter in parallel with IQ measures. We hypothesized a positive correlation between NAA and Full-scale IQ, and with Information Processing Speed in particular. In contrast to several previous studies, we found neither strong, nor significant, predictive relationships between NAA and cognitive ability. The range of relationships and effect sizes in the literature reveals the exploratory nature of current cognitive spectroscopy. Sources of variability between studies include methodological differences in spectroscopic protocols, the neuroanatomical location of voxels, the neuropsychological assessments employed and heterogeneity in population samples. Of particular concern is the multiple comparisons problem inherent to studies that have measured several neurometabolites over multiple brain regions and employed a battery of intelligence measures without detailing specific research hypotheses *a priori*, as these are particularly susceptible to inflated Type 1 error rates. Although ^{1}H-MRS offers a sensitive tool for assessing neurochemistry, the relationships between brain metabolites and broad aspects of human behaviour are subtle, and highlight the need to develop an explanatory framework that discriminates between statistical relevance and statistical significance in investigations of this kind.

American Association for the Study of Liver Disease (AASLD) Annual Meeting (November 2010)

Boston, USA

Polyunsaturated fatty acid status and cognitive outcomes in paediatric liver disease

Abstract: Poster presentation*

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Introduction: Essential Polyunsaturated Fatty Acids (PUFAs), omega-6 (n-6) and the omega-3 (n-3) are complex lipids found in high concentrations in the central nervous system, where they serve a multitude of structural and functional roles, which may modulate cognitive function. Fat malabsorption in liver disease, or abnormal fat intake caused by dependency on EFA-deficient intravenous nutrition, may lead to suboptimal concentrations of PUFAs in red blood cells, which may manifest as changes in cognitive ability. Our aim was to measure the range of PUFA concentrations in children with liver disease compared to sibling controls and assess the relationship between PUFA status and cognitive ability.

Method: PUFA status was assessed in red blood cell (RBC) membranes by conventional Gas Chromatography-Mass Spectrometry. Percentage total of EPA and DHA were taken as markers of omega-3 status and Linoleic and Arachidonic acid of omega-6. Full-scale IQ was assessed with an age-appropriate Weschler's psychometric test battery (Wechsler FSIQ).

Results: We observed no significant difference in IQ between the groups, but a significant negative correlation was found between pro-inflammatory omega-6 fatty acids and FSIQ across the cohort ($r = -0.525$, $p = 0.025$). The omega-3:omega-6 ratio was also found to be significantly lower the chronic liver disease group compared to the sibling control and post-transplant group ($F(2,15) = 4.88$, $p= 0.023$, effect size (d) = 1.66 and 1.23, respectively).

Group	n	Mean age	Mean n-3 (% total)	Mean n-6 (% total)	n-3:n6	FSIQ
Sibling control	5	15.4	15.0	16.2	0.92	105
Chronic liver disease (no Tx)	3	14	13.9	18.1	0.77	85
Post-Tx	10	14.1	15	16.8	0.90	95

Conclusion: Our findings suggest no significant deficiency of omega-3 fatty acids in liver disease patients, but the relationship between pro-inflammatory fatty acids and IQ requires further investigation. Liver transplantation in early childhood is consistent

with recovery of good long-term cognitive outcome. Longitudinal studies of transplant patients assessing dietary intake and PUFA and cognitive status will help clarify the role of EFAs in cognitive development in paediatric liver disease.

American Association for the Study of Liver Disease (AASLD) Annual Meeting (November 2010)

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Essential fatty acids (EFAs) and polyunsaturated fatty acids (PUFAs) in patients with intestinal failure and after small bowel transplantation (SBTx): relationship to cognitive outcomes

Abstract: Poster presentation*

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Aim: To measure the status of EFAs and PUFAs in patients before and after SBTx, because the exposure to these fatty acids in parenteral nutrition and after transplantation is known to be widely different from healthy children.

Methods: 4 children aged 11–15yrs and one infant aged 2 years were recruited: two had undergone SBTx 5 and 10 years previously and three were on the transplant list for small bowel transplantation. None of the children had been breast fed and all had received Intralipid, which lacks polyunsaturated fatty-acids (PUFAs), from birth. The 3 children awaiting SBTx had been converted to a new source of lipid containing fish oils 1–12 months prior to blood sampling (SMOFlipid Fresenius Kabi). We compared their exposure to EFAs and PUFAs at the same time as measuring their body PUFA stores in erythrocyte (RBC) membranes using Gas Chromatography Mass Spectrometry (GC-MS), and related these measures to their Full-scale IQ (Wechsler FSIQ).

Results: All the children on SMOFlipid had satisfactory levels of DHA, but they had high levels of the pro-inflammatory PUFA arachidonic acid. Subjects 3 and 4 were carrying mead acid in RBC membranes, suggesting that they had had insufficient EFAs in the weeks preceding the blood sampling. The diet of subjects who had been successfully transplanted were associated with the EFA deficiency marker mead acid (subject 1) and subject 2 had relatively low amounts of DHA, neither subject showed a pro-inflammatory bias.

Subject	Total cals and type of lipid	Fatty acid intake (mg/kg per day) 18:1, 18:2, 18:3, 20:4; 22:6	RBC 20:3 %	RBC 20:4 %	RBC 22:5 %	RBC 22:6 %	FSIQ
1 MK	Oral diet	Pending (10mg/ kg PUFA/day)	9.19	8.96	0.0	8.13	97
2 RS	Oral diet	Pending	7.52	7.83	6.34	6.56	90
3 DS	48cals/kg/d2g/kg SMOF/day	556, 374; 50; 10; 44;	10.76	11.40	0.0	10.72	100
4 JB	76cals/kg/d2g/kg SMOF/day	556; 374; 50; 10; 44	7.68	9.63	0.31	10.35	94
5 AF	80cals/kg/d2.5g/ kg SMOF/day	687; 460; 60; 12; 54	0.0	12.28	0.0	10.58	
Controls			8.1-8.6	8.1-9.2	6.7-7.6	7.1-8.3	100

18:1 oleic acid; 18:2 linoleic acid, 18:3 linolenic acid, 20:3 mead, 20:4 arachidonic acid, 22:5 osbond, 22:6 docosahexaenoic acid (DHA)

Conclusion: Children may require EFA supplements after SBTx. SMOFlipid is associated with above average DHA concentrations, but this is associated with a pro-inflammatory bias in patients awaiting SBTx.

British Association for Parenteral and Enteral Nutrition (BAPEN) (November 2010)

Sequential changes in polyunsaturated fatty acid composition of red cell membranes before and after small bowel transplant; a case report
Abstract: Poster presentation*

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*Poster presented on my behalf by SV Beath

Children with intestinal failure are dependent on intravenous lipid solutions to survive and historically the lipid source has been soya oil, which is rich in essential fatty acids. Recently there have been concerns that reliance on a single source of lipid, which contains a high proportion of so-called pro-inflammatory omega-6 fatty acids, may be a factor in the development of liver disease (Koletzko and Goulet. *Curr Opin Clin Nutr Metab Care.* 2010;13:321-6). The use of a multi-source lipid (SMOFresnius) consisting of soya oil, medium chain fatty acids, olive oil and fish oil became available in the UK in 2007 and is being increasingly used, although the long-term effects on the liver function and other tissues have not yet been determined. The aim of this study was to evaluate the polyunsaturated fatty acid status in an 11 year old boy prior to small bowel transplant, when he was receiving SMOF as part of his parenteral nutrition, and periodically until 12 months after transplant, when he was established on the enteral feed Peptamen. PUFA levels were measured in red blood cell (RBC) membranes by Gas chromatography-mass spectrometry. The dietary intake of fatty acids was calculated from the PN prescription and dietetic records.

The markers of both omega-6 (20:4) and omega-3 (22:6) PUFAs decreased significantly and came down to just above the range for healthy volunteers by 5 months post-transplant. This change in PUFAs was associated with reduced intake of long chain fatty acids when PN was stopped and Peptamen Junior commenced. We conclude that the PN fat source may have been over-providing essential fatty acids and DHA, and despite a reduction in EFA intake and lack of DHA in Peptamen Junior, this child was able synthesise DHA satisfactorily from his enteral food source.

	Pre-transplant	2 months after Tx	5 months after Tx	8 months after Tx	12 months after Tx
Intravenous FA intake (mg/kg/ day)					
18:1, 18:2;	556; 374;				
18:3n3, 20:4, 22:6	50;10; 44	0	0	0	0
n3: n6 ratio	-0.24				
Enteral FA* intake (mg/kg/ day)					
18:1, 18:2,		132: 320;	132: 320;	155; 374;	155; 374;
18:3n3, 20:4, 22:6	0	70; 0; 0	70; 0; 0	82; 0	82; 0
n3: n6 ratio		-0.22	-0.22	-0.22	-0.22
RBC PUFA (%)					
18:1; 18:2; 18:3	20; 10; 9;	10; 10; 11;	8; 10: 10;	10; 8; 11;	10;11;11;
20:4; 22:6	11.3; 12.1	11.9; 8.4;	10; 9.4	10; 9.9;	11.4; 9.9
n3:n6 ratio	-0.71	-0.79	-1.05	-0.92	-0.88

18:1: oleic acid; 18:2: linoleic acid; 18:3: α-linolenic acid; 20:4: arachidonic acid; 22:6: docosahexanaenoeic acid (DHA)

Society for Neurosciences Annual Meeting (November 2010)

Biochemical correlates of cognition: exploring the relationships between blood, brain and behaviour
Abstract: Poster presentation

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This multi-modal investigation utilises convergent biochemical assay techniques to explore the relationships between brain and blood biochemistry and cognitive ability. It is well-established that essential fatty acids (EFAs) are crucial for normal brain development and function, but their specific behavioural effects are yet to be elucidated. We employed conventional Gas chromatography-mass spectrometry of erythrocyte membranes in conjunction with standard batteries of psychometric assessment, such as the Wechsler's Adult Intelligence Scale, to assess the relationship between EFAs and their long-chain polyunsaturated metabolites, such as arachidonic acid and docosahexaenoic acid, and specific cognitive abilities. In our cohort of healthy individuals, significant negative correlations were observed between levels of pro-inflammatory omega-6 fatty acids in erythrocyte membranes and Full-scale IQ ($r = -0.401$, $p = 0.035$). Alongside the blood measures, we employed single-voxel Proton-Magnetic Resonance Spectroscopy (^{1}H-MRS) in frontal and occipitoparietal cortex to assess biochemistry at the neural level. In contrast with previous cognitive spectroscopy studies, we observed no significant correlations between measures of cognitive ability and surrogate markers of neuronal viability such as N-acetyl aspartate and choline.

Our results suggest that the relationships between brain and blood metabolites and broad aspects of human behaviour are subtle and complex, and highlight the need for both hypothesis-driven enquiry and for distinguishing between statistical relevance and statistical significance in studies of this kind. Multi-modal investigations offer a promising, novel approach to exploring brain-behaviour relationships from micro- to macroscopic levels of analysis, but the conceptual framework within which these techniques are employed requires refinement.