

Multimodal neuroimaging and early neurobehavioral and developmental correlates of alcohol-exposed infants in Cape Town

Kirsten Ann Mary Donald

MBChB, MPhil (UCT), MRCPCH (UK), DCH (SA), FCPaed (SA),
Cert. Paed Neuro. (SA)

Thesis Presented for the Degree of DOCTOR OF PHILOSOPHY
in the Division of Developmental Paediatrics, Department of Paediatrics and Child Health, Faculty of Health Sciences,
UNIVERSITY OF CAPE TOWN August 2015

Supervisors

Prof Dan J Stein, University of Cape Town

Prof Katherine L Narr, University of California, Los Angeles

Prof Edward P Riley, San Diego State University

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

Declaration

I, Kirsten Ann Mary Donald, hereby declare that this thesis is my own work, both in concept and execution, apart from the normal guidance received from my supervisors and contributions from others as outlined in the acknowledgements. The assistance I received with study management, data collection, analysis and manuscript review from the co-authors of the publications that form part of this thesis is described for each relevant chapter.

Neither the substance nor any part of the above thesis has been submitted in the past, or is being, or is to be submitted for a degree at this University or at any other university.

I grant the University of Cape Town free licence to reproduce the above thesis in whole or in part, for the purpose of research.

I present this thesis for examination for the degree of PhD.

Signed:

Signed by candidate

Dated: August 2015

Contents

List of Tables	6
List of Figures	7
Abstract	8
Acknowledgements	11
Abbreviations	13
Chapter 1: Introduction.....	15
Chapter 2: Neuroimaging effects of prenatal alcohol-exposure on the developing human brain: A magnetic resonance imaging review.....	30
Chapter 3: Study outline and methods.....	75
Chapter 4: Alcohol exposure in utero is associated with decreased posterior cingulate cortex and inferior temporal gyrus volume in neonates	91
Chapter 5: A study of the effects of prenatal alcohol exposure on white matter microstructural integrity at birth.....	110
Chapter 6: Reduced glutamate in white matter of male neonates exposed to alcohol <i>in utero</i> : a ¹ H-magnetic resonance spectroscopy study	131
Chapter 7: Inter-hemispheric functional brain connectivity in neonates with prenatal alcohol exposure.....	148
Supplementary material (chapter 7)	171
Chapter 8: Discussion	173
Appendix A: Informed consent 525/2012.....	186
Appendix B: ASSIST questionnaire	192
Appendix C: Dubowitz neurobehavior assessment	196
Appendix D: MRI imaging protocol	200
Appendix E: Ethical approval HREC 525/2012.....	201

List of Tables

Chapter 2

Table 1: Structural magnetic resonance imaging (MRI) in prenatally alcohol-exposed children	36
Table 2: Diffusion tensor imaging (DTI) in prenatally alcohol-exposed children	45
Table 3: Proton magnetic resonance spectroscopy (1H-MRS) in prenatally alcohol-exposed children	49
Table 4: Functional MRI (fMRI) in prenatally alcohol-exposed children	52

Chapter 4

Table 1: Demographic, anthropometric and Dubowitz data of infants	100
Table 2: Frequency and quantity of maternal alcohol use by trimester	100
Table 3; Regions with significant differences in gray matter volumes between healthy and alcohol-exposed infants	101
Table 4: Significant associations between BSID III scores and gray matter volumes in alcohol-exposed infants (n = 19)	102
Table 5: Significant associations between Dubowitz optimality scores and gray matter volume in alcohol-exposed neonates (n=27)	102

Chapter 5

Table 1: Demographic, anthropometric and Dubowitz data of infants	119
Table 2: Alcohol use of mothers by trimester	119
Table 3: Results of group differences in diffusion parameters by regions of interest. Axial diffusivity was significantly lower in the right superior longitudinal fasciculus of alcohol-exposed infants compared to controls.	121

Chapter 6

Table 1: Physical attributes, Dubowitz neurological exam scores, and maternal smoking status	139
Table 2: Maternal drinking during pregnancy	140
Table 3: ¹ H-Magnetic resonance spectroscopy absolute metabolites, with FWHM and SNR	141

Chapter 7

Table 1: Comparison of alcohol exposed and un-exposed neonates on demographic and developmental variables	158
Table 2: Alcohol use of PAE mothers by trimester	159

List of Figures

Chapter 1

Figure 1: South Africa with nine provinces labelled. 16

Chapter 3

Figure 1: DCHS: Study Timeline Overview..... 76

Figure 2: DCHS: Infant Imaging Sub-Study Timeline 76

Chapter 4

Figure 1: Mean volume images showing regions of decreased gray matter
volume in alcohol-exposed infants compared to healthy controls 101

Chapter 5

Figure 1: Association tracts superimposed on a 3D brain template 122

Chapter 6

Figure 1: Positioning of parietal white matter proton magnetic resonance
spectroscopy (^1H -MRS) voxel and example spectra, left hemisphere. . 136

Figure 2: Positioning of parietal gray matter proton magnetic resonance
spectroscopy (^1H -MRS) voxel and example spectra, midline..... 137

Chapter 7

Figure 1: Axial, sagittal and coronal slices of ICA derived networks..... 160

Abstract

Alcohol use and alcohol use disorders contribute a significant proportion of the burden of disease in low, middle, and high-income countries. As a result, fetal alcohol spectrum disorders (FASD) represent one of the most common preventable causes of intellectual disability globally. Understanding the core brain areas of susceptibility to prenatal alcohol as they manifest in early life is key to developing strategies for early focused identification and intervention.

This thesis explored the relative impact of prenatal alcohol exposure on the brain in infants as measured by multimodal brain imaging and the relationship of these findings to early neurobehavioral and developmental status. The **specific aims** the thesis addressed included leveraging structural magnetic resonance imaging (MRI), diffusion tensor imaging (DTI), proton magnetic resonance spectroscopy (^1H -MRS) and resting state functional MRI (rs-fMRI) scans in approximately 100 infants (50 alcohol exposed and a matched number of control, unexposed babies) at 2-4 weeks of age, to assess group differences in early brain development. Correlations between multimodal neuroimaging measures and neonatal neurobehavioral assessments and associations between early structural imaging findings and later infant developmental, as measured by the Bayley III assessment at 6 months, were further explored in the same group of infants. *These studies addressed the hypothesis that maternal alcohol use in pregnancy would result in quantitative MRI abnormalities demonstrable at 2-4 weeks of age and that these changes would correlate with early indicators of neurobehavior and development.*

Chapter 1 presents the rationale and outline of the thesis. The burden of fetal alcohol spectrum disorders (FASD) is described in the context of different resource settings around the world with detailed reference to South Africa. Chapter 2 presents a published systematic literature review of published studies of MRI in children and adolescents with prenatal alcohol exposure. Chapter 3 provides an overall description of the methods and context for this study. Although the results chapters each include a focused methods section, the word restrictions of journal articles did not allow for adequate contextual detail for the project as a whole. Subsequently the thesis includes four results chapters,

incorporating related aspects of this work presented as separate journal publications. In chapter 4 and 5 evidence for the structural and microstructural effects of prenatal alcohol exposure on gray matter (chapter 4) and white matter (chapter 5) of the neonatal brain are presented using structural MRI and DTI data respectively. In the following two chapters, I subsequently describe how functional aspects of brain development may be affected by alcohol exposure in utero. In chapter 6 I report the results of an analysis of ¹H-MRS imaging of parietal white matter of neonates exposed to alcohol in utero. In chapter 7 the resting state connectivity networks in infants exposed to prenatal alcohol is explored. In chapter 8 the significance of the findings from the previous four chapters are discussed and future directions explored.

The systematic review describes a huge variety of structural, microstructural, neurochemical and functional effects of prenatal alcohol exposure in the brains of exposed children. Identification of the gaps in the extant literature is presented, including the paucity of neuroimaging data in the crucial first weeks and months of life. The structural MRI paper describes reduced volume in posterior cingulate cortex and inferior temporal gyrus in infants with prenatal alcohol exposure. These findings persist even when controlling for sex, age at scanning and maternal smoking status. In addition early infant neurobehavior as measured by the Dubowitz neurobehavioral optimality scale as well as Bayley Scales of infant development III, correlate with volume findings across multiple brain areas. In the DTI paper we describe reduced white matter microstructural integrity in the superior longitudinal fasciculus in neonates with alcohol exposure in utero. In the functional chapters I report reduced levels of glutamate-glutamine concentrations in parietal white matter of alcohol exposed boy infants and in the final results chapter describe altered functional connectivity in six key networks in the brains of infants with prenatal alcohol exposure.

I conclude that this data provides preliminary evidence for the effect of alcohol on very early structure and organisation of the brain at a structural, microstructural, neurochemical as well as functional organisational level. Further evidence for this is the widespread correlations between developmental outcomes at 6 months and gray matter volume at birth. These data underscore the fact that the harmful effects of prenatal exposure to

alcohol on brain structure are already discernible in newborns, well before the age fetal alcohol syndrome (FAS) and FASD are typically diagnosed. Future areas for research priority in this area are discussed.

Acknowledgements

The work reported in this thesis was funded in part, by a research fellowship award from the Harry Crossley Foundation, a South African Medical Association PhD supplementary fellowship and a developmental grant from the Foundation for Alcohol Research (USA). In addition the umbrella Drakenstein Child Health Project was funded by the Bill and Melinda Gates Foundation [OPP 1017641].

I would like to acknowledge and thank the following people: Prof Colleen Adnams for encouraging me in the direction of this research and for her generous mentorship; Professor Roger Woods for his input and insightful comments throughout the project; Dr Clare Thompson for advice regarding the use of the Dubowitz optimality score; Dr Annerine Roos for assistance with patient care and data collection and analysis support, and comments on the manuscripts arising from this research; JP Fouche for tirelessly helping with technical aspects of this project and analysis. All my other co-authors for their well-timed input at various stages of the project. Nastassja Koen and Whitney Barnett for their support and insight around the parent project in which this sub-study is nested. The nurses and study staff at the research sites who assisted with the care and assessment of the mothers and infants; and the parents and newborns for participating in this research.

I would particularly like to thank my University of Cape Town supervisor, Prof Dan Stein, for his comments and guidance throughout the research process; and my supervisors, Professor Katherine Narr from University of California, Los Angeles, and Professor Edward Riley from San Diego State University, San Diego, for their tireless long-distance support.

I would finally like to thank my friends and family for their patience and support. Very special thanks go to my husband, Oliver for his support and patience; and to my children, Leo and Sophie for their unconditional love, energy and inspiration.

Abbreviations and style

A note on spelling and style convention: The papers that comprise the majority of the thesis have been submitted to or published by American journals. I have therefore used United States standard academic spelling conventions. Where the papers were published in European journals, I have changed only the spelling in the papers so as to maintain internal consistency through the thesis. I have also used Vancouver referencing throughout the thesis for the same reason. For the rest the papers are reproduced in the thesis as they were submitted/published with the exception of some particularly repetitious sections in the methodology made inevitable by the nature of how these papers fit into the parent project. Where I have deleted a paragraph for this reason I refer the reader to where the detail for that section can be found.

FAS:	Fetal alcohol syndrome
FASD:	Fetal alcohol spectrum disorders
DCHS:	Drakenstein Child Health Study
LMIC:	Low and middle-income countries
WHO:	World Health Organization
CNS:	central nervous system
MRI:	Magnetic resonance imaging
CUBIC:	Combined Universities brain imaging center
TR:	Relaxation time
TE:	Echo time
TI:	inversion time
DTI:	Diffusion Tensor Imaging
FA:	Fractional anisotropy
MD:	mean diffusivity
TBSS:	Tract based spatial statistics
¹ H-MRS:	Proton magnetic resonance spectroscopy

NAA: N-acetyl aspartate
Cho: Choline
Cr: Creatinine
Glu: Glutamate
RS-fMRI: Resting state functional magnetic resonance imaging
ICA: independent component analysis
CHESS: chemical shift selective pulses
PRESS: point resolved spectroscopy
EPI: echo planar imaging
BOLD: Blood oxygen level dependent
PAE: prenatal alcohol exposure

Chapter 1

Introduction

1.1 Rationale and problem statement

1.1.1 Context of alcohol-exposure effects

Alcohol is reported as the one of the top three risk factors for premature mortality, disability and loss of health worldwide. Globally, the World Health Organization (WHO) estimates the use of alcohol to result in approximately 3.3 million deaths annually (5.9% of deaths), and further that 5.1% of the global burden of disease may be attributed to alcohol consumption. (1-3) Global trends have shown that there has been an increase in both affordability as well as alcohol consumption in many low and middle income countries (LMICs) in recent years. (4,5)

In Southern regions of sub-Saharan Africa, alcohol ranks as the leading risk factor for mortality and morbidity. (6) In absolute terms, the proportion of South Africans who drink alcohol is fairly low (in the 2013 South African National Health and Nutrition Survey 39 % of men and 16 % of women reported drinking alcohol in the previous year). (7) However, amongst those individuals who do drink, South Africa has one of the highest per-capita rates of alcohol consumption in the world, (1,2,8) as well as a pattern of alcohol consumption associated with greater detrimental effects. (8,9)

The physical and neurodevelopmental effects of prenatal alcohol exposure have been formally documented in the medical literature since the seminal reports published by Jones *et al* 40 years ago. (10,11) Since then there has been growing recognition that the full-blown presentation of Fetal Alcohol Syndrome (FAS), defined by three criteria (central nervous system dysfunction, pre- and postnatal growth deficits and specific craniofacial features) represents the extreme end of the spectrum of effects caused by alcohol exposure on the developing infant. This recognition has resulted in a number of terms representing “milder” forms of the syndrome (including fetal alcohol effects, partial FAS and alcohol-related neurocognitive disorder). Current literature most commonly uses the term

Fetal Alcohol Spectrum Disorders (FASD) to incorporate all these terms and refers to the effects of prenatal alcohol exposure, especially those not part of the criteria for FAS. (12)

Figure 1: South Africa with nine provinces labelled.



Estimates of prevalence for FAS internationally are reported most recently at between 2-7/1000 and between 20-50/1000 for FASD. (13) In South Africa (**Figure 1**), though

no national data are available, prevalence has been reported to be extremely high in specific communities (as high as 63-91/1000 for FAS and 135-207/1000 for FASD in a periurban Western Cape community). (14) Infants born to these mothers frequently face the cumulative effects of multiple exposures in utero as well as postnatal exposure to a deprived environment, in which poverty and a relative lack of developmental stimulation may be seen. (15-17)

1.1.2 Mechanisms for damaging effects of alcohol

Alcohol abuse may affect the fetus via direct and indirect mechanisms. Direct effects include teratogenicity (effects on primary structural development) on the developing brain in utero as well as later effects, which can be subtler. These may involve brain growth, maturation, neurotransmitter concentration and dynamics as well as neural pathway development. Indirect effects may be due to placental insufficiency as a result of altered delivery of substrate or through maternal behaviour related to alcohol abuse: poor nutrition, poor health-seeking behaviour, increased exposure to violence, risk of mental illness and infection. (18-21) Alcohol passes freely across both placental and blood brain barrier and animal models have demonstrated that maternal levels are reflected significantly in the foetal brain. (22-26)

The current evidence from animal studies suggests that the primary mechanisms for the damaging effects of alcohol on the developing brain include influencing the regulation of key developmental processes, interfering with the early development of midline serotonergic neurons and disrupting their regulatory signalling function for other target brain structures, interfering with both growth regulation as well as pruning mechanisms. (27) Pruning improves the efficiency of impulse transmission (across nerve synapses) by reducing the number of redundant connections and constitutes a crucial aspect of early brain maturation processes. (28-30)

1.1.3 Neuroimaging in Alcohol-exposure

Advances in neuroimaging methods over the last decade have allowed researchers to study structural, metabolic, and functional abnormalities resulting from prenatal exposure to drugs of abuse. Publications to date investigating the structural effects of alcohol

on the developing brain have primarily focused on older children and adolescents. Neuroimaging studies of prenatal alcohol exposure have reported differences in the structure and metabolism of many brain circuits, including those in frontal, parietal, and temporal regions, in the cerebellum and basal ganglia, as well as white matter tracts that connect these brain regions with the most consistent findings being in the corpus callosum. (31)

There are limited data on children < 8 years of age and there are currently no reports on the effects of alcohol in the critical period covering early infancy. Brain imaging in young infants is technically difficult and has traditionally required sedation, which has limited its use in this age group for research purposes. In addition, the neonatal brain is physically much smaller, with a higher water content making differentiation of specific tissues and structures much more challenging both in analysis and interpretation of data. However, more recently studies have been successful in acquiring imaging data on unsedated infants during quiet sleep. (32-34)

Brain development is incomplete at birth and continues throughout childhood into early adulthood. However, growth and maturation is most rapid during the first 2 years of life. (28-30) There is limited data on the effect of teratogens on brain structure and function at this important age. I hoped to establish whether evidence of the effects of alcohol could be identified on neuroimaging data of young infants exposed to alcohol in the prenatal period. I aimed to describe their early neurobehavioral and developmental patterns and establish if there was a relationship between imaging findings and developmental profiles. These insights may ultimately help clinicians develop better diagnostic tools and devise appropriate therapeutic interventions to improve the condition of children with prenatal exposure to alcohol. From a public health perspective, a better understanding of the relevant mechanisms is key, as this may ultimately drive preventative/therapeutic approaches. Indeed, in the South African setting, where the bulk of the population is under 30, expectant mothers and their young infants are a particularly important focus group. Much more attention is needed to address maternal and infant health, in order to decrease early mortality and later morbidity in this vulnerable population.

1.2 Aims, objectives and overview

In this section, I present the aims and objectives, and an overview of the contents of the rest of the thesis.

The **general aim** of this project was to assess the relative impact of prenatal alcohol exposure on the brain in infants as measured by multimodal brain imaging and the relationship of these findings to early neurobehavioral and developmental status.

The **specific aims** were to:

1. Undertake structural Magnetic resonance imaging (MRI), Diffusion tensor imaging (DTI), proton magnetic resonance spectroscopy (¹H-MRS) and resting state blood oxygen level dependent (BOLD) functional MRI (rs-fMRI) scans in approximately 100 infants (50 alcohol exposed and a matched number of control, unexposed babies) at 2-4 weeks of age, in order to assess group differences in neuronal integrity.
2. Determine the correlation between the multimodal neuroimaging markers above and neonatal neurobehavioral assessment scores.
3. Determine the correlation between early structural imaging findings and later infant developmental assessment as measured by the Bayley III assessment at 6 months in the same group of infants

Hypothesis 1:

It is expected that there would be between-group differences in volume and/or morphology in particular regions, such as the parietal and temporal areas when comparing infants exposed to alcohol in utero with their unexposed age matched control counterparts at 2-4 weeks of age using T2 MRI scans. It is also expected that reduced volume would correlate with early neurobehavior and developmental function at 6 months of age.

Hypothesis 2:

It is expected that there would be between-group differences in the metrics of white matter microstructural integrity in the central white matter tracts when comparing infants

exposed to alcohol in utero with their unexposed age matched control counterparts at 2-4 weeks of age using diffusion tensor imaging and that reduced white matter integrity would correlate with early neurobehavior.

Hypothesis 3:

It is expected that there would be between-group differences in glutamate/glutamine levels in the parietal white matter when comparing infants exposed to alcohol in utero with their unexposed age matched control counterparts at 2-4 weeks of age using ¹H-MRS and that this would correlate with early neurobehavior.

Hypothesis 4:

It is expected that infants with prenatal alcohol exposure would show alteration in their functional connectivity metrics particularly in sensorimotor networks and that this would correlate with early neurobehavior.

This proposal to combine multimodal computational neuroimaging methods to pursue questions regarding the effects of prenatal exposure to alcohol on brain structure, function and neurochemistry in a very young human cohort is a novel one. In the following chapter the background literature regarding the neuroimaging findings across modalities in children exposed to alcohol in utero is described and discussed.

Overview:

Chapter 1 presents the rationale and outline of the thesis. The burden of FASD is described in the context of different resource settings around the world with detailed reference to South Africa. An outline of all chapters is given.

Chapter 2 presents a systematic literature review of published studies of magnetic resonance imaging (MRI) in children and adolescents with prenatal alcohol exposure. The published systematic review describes a huge variety of structural, microstructural, neurochemical and functional effects of prenatal alcohol exposure identified in the brains of exposed children. Identification of the gaps in the extant literature is presented, including the paucity of neuroimaging data in the crucial first weeks and months of life.

Chapter 3 provides an overall description of the methods and context for this study. Although the results chapters do individually include a focused methods section, the word restrictions of journal articles did not allow for adequate contextual detail for the project as a whole.

Subsequently, the thesis includes four results chapters, and includes imaging data of a group of prenatal alcohol exposed neonates compared to an unexposed control group from the same community. This cohort is a sub-study of the Drakenstein Child Health Study (DCHS). All these chapters are related aspects of this work and are presented as separate journal publications. In chapters 4 and 5 the evidence for the structural and microstructural effects of prenatal alcohol exposure on gray matter (chapter 4) and white matter (chapter 5) of the neonatal brain, using structural MRI and diffusion tensor imaging data respectively is cited. In the subsequent two chapters, I describe how functional aspects of brain development may be affected by alcohol exposure in utero. In chapter 6 we reported the results of the analysis of white matter 1H-MRS imaging of the parietal white matter of neonates exposed to alcohol in utero. In Chapter 7 the resting state connectivity networks in infants exposed to prenatal alcohol are explored.

Chapter 4

The structural MRI paper describes reduced volume in posterior cingulate cortex and inferior temporal gyrus in infants with prenatal alcohol exposure. These findings persist even when controlling for sex, age at scanning and maternal smoking status. In addition early infant neurobehavior as measured by the Dubowitz neurobehavioral optimality scale as well as Bayley Scales of infant development III, correlate with volume findings across multiple brain areas. This paper addresses aspects of all three specific aims.

Chapter 5

In the DTI manuscript I focus on the diffusion tensor imaging effects of prenatal alcohol exposure in neonates compared to an unexposed group. Reduced white matter microstructural integrity in the superior longitudinal fasciculus in neonates with alcohol exposure in utero in this cohort is described and correlation of cerebellar peduncle white matter microstructure with neonatal neurobehavior is presented. This paper addresses

aspects of both aims 1 and 2.

Chapter 6

This manuscript focuses on the evidence for the effects of prenatal exposure on glutamatergic function in the parietal region. This dynamic measure is a key aspect to investigate with respect to toxin exposure on the very young brain. In this chapter reduced levels of glutamate/glutamine complex concentrations in parietal white matter of alcohol exposed infants is discussed. This manuscript specifically addresses the functional aspects of aim 1 and 2.

Chapter 7

This manuscript is based on the resting state functional magnetic resonance imaging data of a group of prenatal alcohol exposed neonates compared to an unexposed control group from the same community. These data represent the activation of areas of the brain during rest and provides information about functional networks in the first few weeks of life and how these may be impacted by prenatal alcohol exposure. Here, I describe altered functional connectivity in six networks in the brains of infants with prenatal alcohol exposure. This manuscript further addresses the functional aims of the thesis, though failed to show correlation with infant neurobehavior.

Chapter 8 the significance of the findings from the above four chapters is discussed and future directions explored. We conclude that these data provide preliminary evidence for the effect of alcohol on very early structure and organisation of the brain at a structural, microstructural, neurochemical as well as functional organisational level. Further evidence for this is the widespread correlations between developmental outcomes at 6 months and gray matter volume at birth. These data underscore that the harmful effects of prenatal exposure to alcohol on brain structure are already discernible in newborns, well before the age FAS and FASD are typically diagnosed. Future areas for research priority in this area are discussed.

Author contributions to included manuscripts

The contributions to the manuscripts have been endorsed by the principal supervisor, Professor Dan Stein and all five papers have been approved by the University of Cape Town (UCT) doctoral degrees board and UCT Vice chancellor as being appropriate for inclusion in the thesis as per UCT policy.

1. Neuroimaging effects of prenatal alcohol-exposure on the developing human brain: A magnetic resonance imaging review, Donald, K.A., Eastman, E., Howells, F.M., Adnams, C., Riley, E.P., Woods, R.P., Narr, K.L. & Stein, D.J. *Acta Neuropsychiatrica*, 2015 DOI:<http://dx.doi.org/10.1017/neu.2015.12>.

In this review, I developed a search methodology with input from my supervisors, and then conducted the database and journal search. I extracted data into a spreadsheet (with EE). I then analysed and summarised all the data myself, and wrote the full first draft of the manuscript. My co-authors, DS, CA, KN, RW, ER and FH, reviewed the draft, made conceptual and intellectual contributions in specific areas of expertise. I integrated contributions and made all revisions prior to publication myself.

2. A study of the effects of alcohol exposure in utero on white matter microstructural integrity at birth Donald, K.A., Roos, A., Fouche, J., Koen, N., Howells, F.M., Woods, R.P., Zar, H.J., Narr, K.L. & Stein, D.J. 2015 *Acta neuropsychiatrica*, [Epub ahead of print], PMID: 26022619

I designed the neuroimaging project, finalized the imaging sequences and developed the method for imaging these infants safely without sedation in our context. I also led the design of the infant neurobehavioral assessment data, did a proportion of the neurobehavioral assessments myself and oversaw the rest. I was involved in post-processing of the neuroimaging data, analysis (with J Fouche and A Roos), final correlational analyses and interpretation of the data. I wrote the first draft of the paper and integrated contributions from the other authors. A Roos, F Howells were involved in the acquisition of the neuroimaging data and in discussions around analysis strategy. N Koen was involved in the design and roll-out of the psychosocial aspects of the umbrella DCHS, from whom

the infants were recruited. HZ is PI of the umbrella DCHS and is responsible for the overall design of the parent project. In addition to this critical role she provided input on the contextual factors of relevance to the study. ER, RW, KN and DS all provided expert advice both at the conceptual stages of the project and all authors provided contributions to the intellectual content of the publication.

3. Alcohol exposure in utero is associated with decreased posterior cingulate cortex and inferior temporal gyrus volume in neonates: Donald, Kirsten A; Fouche, JP; Roos, Annerine; Koen, N; Howells, Fleur M; Riley, Edward P; Woods, Roger P; Zar, Heather J; Narr, Katherine L; Stein, Dan J. *Under review: Metabolic Brain Disease*

I designed the neuroimaging project, finalized the imaging sequences and developed the method for imaging these infants safely without sedation in our context. I also led the design of the infant neurobehavioral and developmental assessment data. I was involved in post-processing of the neuroimaging data, analysis (with J Fouche), final correlational analyses and interpretation of the data. I wrote the first draft of the paper and integrated contributions from the other authors. A Roos, F Howells were involved in the acquisition of the neuroimaging data and in discussions around analysis strategy. N Koen was involved in the design and roll-out of the psychosocial aspects of the umbrella DCHS, from whom the infants were recruited. HZ is PI of the umbrella DCHS and is responsible for the overall design of the parent project. In addition to this critical role she provided input on the contextual factors of relevance to the study. ER, RW, KN and DS all provided expert advice both at the conceptual stages of the project as well as at the intellectual content of the publication.

4. Reduced glutamate in whitematter marks prenatal exposure to alcohol in neonates: A cross-sectional ¹H-MRS study. Howells, FM; Donald, KA; Roos, A; Woods, RP; Zar, HJ; Narr, KL; Stein, DJ. *Under Review, Progress in Neuro-Psychopharmacology & Biological Psychiatry.*

I included ¹H-MRS in the project from the conception, acquired the data on the neonates, and was involved in quality control of the data acquisition during the scan process (decision making around whether to repeat the sequence to get better data). FH took

full responsibility for the ¹H-MRS analysis, but I was involved throughout in discussions around strategies for what aspects to include with respect to covariates for the analysis and in the final interpretation of the data and presented the findings at Research Society on Alcoholism in 2013 as first author. I was involved in the original manuscript writing and have been involved throughout with providing input for how these results fit into the bigger picture of the exposed infant background and neurobehavioural outcomes. One of the strengths of this project is that it brings together a number of magnetic resonance imaging modalities which focus on different aspects of brain structure and function. Inclusion of this paper is directly relevant to the project as a whole. For this particular paper I have included a separate letter of motivation signed by FH as first author and DS as my primary supervisor confirming my contribution and support for inclusion of the paper in this thesis. AR provided support around data acquisition and contributed to the manuscript. HZ is PI of the umbrella DCHS and is responsible for the overall design of the parent project. In addition to this critical role she provided input on the contextual factors of relevance to the study. RW, KN and DS all provided expert advice both at the conceptual stages of the project and all authors provided contributions to the intellectual content of the publication.

5. Inter-hemispheric functional brain connectivity in neonates with prenatal alcohol exposure. Donald, KA; Roos, A; Fouche, J, Koen, N; Biswal, B; Howells, FM; Riley, EP; Woods, RP; Zar, HJ; Narr, KL; Stein, DJ. *Under review, Alcoholism: Clinical and Experimental Research*

As for the above papers, this aspect of the project formed part of my neuroimaging sub-study of the Drakenstein Child Health Study (DCHS). I designed the neuroimaging project, finalized the imaging sequences and developed the method for imaging these infants safely without sedation in our context. I also led the design of the infant neurobehavioral data. I was involved in post-processing of the neuroimaging data, analysis (with J Ipser), final correlational analyses and interpretation of the data. I wrote the first draft of the paper and integrated contributions from the other authors. A Roos, F Howells were involved in the acquisition of the neuroimaging data and in discussions around analysis strategy. N Koen was involved in the design and roll-out of the psychosocial aspects

of the umbrella DCHS, from whom the infants were recruited. HZ is PI of the umbrella DCHS and is responsible for the overall design of the parent project. In addition to this critical role she provided input on the contextual factors of relevance to the study. ER, RW, KN and DS all provided expert advice both at the conceptual stages of the project as well as at the intellectual content of the publication. All authors read and approved the manuscript for submission.

References

- (1) World Health Organisation. Global status report on alcohol and health – 2014 ed. WHO Press, World Health Organization 2014.
- (2) Rehm J, Rehn N, Room R, Monteiro M, Gmel G, Jernigan D, et al. The global distribution of average volume of alcohol consumption and patterns of drinking. *European Addiction Research* 2003;9:147-156.
- (3) Lim SS, Vos T, Flaxman AD, Danaei G, Shibuya K, Adair-Rohani H, et al. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. *The Lancet* ;380(9859):2224-2260.
- (4) Blecher E, editor. Alcohol affordability in South Africa. South African Community Epidemiology Network on Drug Use (SACENDU); 10 April 2014; : Medical Research Council; 2014.
- (5) Whiteford HA, Degenhardt L, Rehm J, Baxter AJ, Ferrari AJ, Erskine HE, et al. Global burden of disease attributable to mental and substance use disorders: findings from the Global Burden of Disease Study 2010. *The Lancet* 2013 11/9–15;382(9904):1575-1586.
- (6) Lim SS, Vos T, Flaxman AD, Danaei G, Shibuya K, Adair-Rohani H, et al. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. *The Lancet* ;380(9859):2224-2260.
- (7) Hoosain C, SANHANES-1 Team, Dwane N, Maluleke T, Reddy P, Jacobs, et al. South African National Health and Nutrition Examination Survey. 2013;1.
- (8) Rehm J, Room R, Graham K, Monteiro M, Gmel G, & Sempos CT. The relationship of average volume of alcohol consumption and patterns of drinking to burden of disease: An overview. *Addiction* 2003;98:1209-1228.
- (9) May PA, Blankenship J, Marais A, Gossage JP, Kalberg WO, Joubert B, et al. Maternal alcohol consumption producing fetal alcohol spectrum disorders (FASD): Quantity, frequency, and timing of drinking. *Drug Alcohol Depend* 2013 12/1;133(2):502-512.
- (10) Jones KL, Smith DW, Ulleland CN, Streissguth AP. Pattern of malformation in offspring of Chronic Alcoholic mothers. *Lancet* 1973;1(7815):1267-1271.
- (11) Jones KL, Smith DW, Hansen JW. The Fetal Alcohol Syndrome: clinical delineation. *Annals of the New York Academy of Sciences* 1976;273:130-139.
- (12) Riley EP, Alejandra Infante M, Warren K. Fetal Alcohol Spectrum Disorders: An Overview. *Neuropsychol Rev* 2011;21(2):73-80.

- (13) May PA, Gossage JP, Kalberg K, Robinson L, Buckley D, Manning Mea. Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies. *Developmental Disabilities Research Reviews* 2009;15(3):176-192.
- (14) May PA, Blankenship J, Marais A, Gossage JP, Kalberg WO, Barnard R, et al. Approaching the Prevalence of the Full Spectrum of Fetal Alcohol Spectrum Disorders in a South African Population-Based Study. *Alcoholism: Clinical and Experimental Research* 2013;37(5):818-830.
- (15) Grantham-McGregor S, Cheung YB, Cueto S, Glewwe P, Richter L, Strupp B. Developmental potential in the first 5 years for children in developing countries. *The Lancet* 2007 1/12;369(9555):60-70.
- (16) Gottlieb CA, Maenner MJ, Cappa C, Durkin MS. Child disability screening, nutrition, and early learning in 18 countries with low and middle incomes: data from the third round of UNICEF's Multiple Indicator Cluster Survey (2005–06). *The Lancet* ;374(9704):1831-1839.
- (17) Walker SP, Wachs TD, Grantham-McGregor S, Black MM, Nelson CA, Huffman SL, et al. Inequality in early childhood: risk and protective factors for early child development. *The Lancet* 2011 10/8–14;378(9799):1325-1338.
- (18) Jones H, Browne F, Myers B, Carney T, Ellerson RM, Kline T, et al. Pregnant and Non-pregnant women in Cape town, South Africa: Drug use, sexual Behaviour and the need for comprehensive services. *International Journal of Paediatrics* 2011 doi: 10.1155/2011/353410.
- (19) Lebel C, Sowell E. Diffusion Tensor Imaging Studies of Prenatal Drug Exposure: Challenges of Poly-Drug Use in Pregnant Women. *J Pediatr* 2011 11;159(5):709-710.
- (20) Behnke M, Smith VC, COMMITTEE ON SUBSTANCE ABUSE, COMMITTEE ON FETUS AND NEWBORN. Prenatal Substance Abuse: Short- and Long-term Effects on the Exposed Fetus. *Pediatrics* 2013 March 01;131(3):e1009-e1024.
- (21) Abar B, LaGasse LL, Derauf C, Newman E, Shah R, Smith LM, et al. Examining the relationships between prenatal methamphetamine exposure, early adversity, and child neuro-behavioral disinhibition. *Psychology of Addictive Behaviors* 2013 09;27(3):662-673.
- (22) Mitchell JJ, Paiva M, Blaine Moore D, Walker DW, Heaton MB. A comparative study of ethanol, hypoglycemia, hypoxia and neurotrophic factor interactions with fetal rat hippocampal neurons: a multi-factor in vitro model for developmental ethanol effects. *Dev Brain Res* 1998 2/10;105(2):241-250.
- (23) Dzierko M, Sifringer M, Klaus J, Endesfelder S, Brait D, Hansen HH, et al. Neurotoxic Ef-

fects of MDMA (Ecstasy) on the Developing Rodent Brain. *Dev Neurosci* 2010;32:197-197-207.

- (24) Jeng W, Wong AW, Ting AK, Wells PG. Methamphetamine-enhanced embryonic oxidative DNA damage and neurodevelopmental deficits. *Free Radic Biol Med* 2005;39(3):317-318-326.
- (25) Szeto HH. Kinetics of drug transfer to the fetus. *Clin Obstet Gynaecol* 1993;36(2):246-254.
- (26) Won L, Bubula N, McCoy H, Heller A. Methamphetamine concentrations in fetal and maternal brain following prenatal exposure. *Neurotoxicol Teratol* 2001;23(4):349-354.
- (27) Goodlett CR, Horn KH, F.C. Alcohol Teratogenesis: Mechanisms of Damage and Strategies for Intervention *Exp Biol Med* 2005;230:394-406.
- (28) Dubois J, Dehaene-Lambertz G, Kulikova S, Poupon C, Hüppi PS, Hertz-Pannier L. The early development of brain white matter: A review of imaging studies in fetuses, newborns and infants. *Neuroscience* 2014 <http://dx.doi.org/10.1016/j.neuroscience.2013.12.044>.
- (29) Geng X, Gouttard S, Sharma A, Gu H, Styner M, Lin W, et al. Quantitative tract-based white matter development from birth to age 2 years. *Neuroimage* 2012 7/2;61(3):542-557.
- (30) Gilmore JH, Shi F, Woolson SL, Knickmeyer RC, Short SJ, Lin W, et al. Longitudinal Development of Cortical and Subcortical Gray Matter from Birth to 2 Years. *Cerebral Cortex* 2012 November 01;22(11):2478-2485.
- (31) Norman AL, Crocker N, Mattson SN, Riley EP. Neuroimaging and fetal alcohol spectrum disorders. *Developmental Disabilities Research Reviews* 2009;15(3):209-217.
- (32) Gousias IS, Edwards AD, Rutherford MA, Counsell SJ, Hajnal JV, Rueckert D, et al. Magnetic resonance imaging of the newborn brain: Manual segmentation of labelled atlases in term-born and preterm infants. *Neuroimage* 2012 9;62(3):1499-1509.
- (33) Malamateniou C, Malik SJ, Counsell SJ, Allsop JM, McGuinness AK, Hayat T, et al. Motion-Compensation Techniques in Neonatal and Fetal MR Imaging. *American Journal of Neuroradiology* 2012 May 10.
- (34) Pannek K, Guzzetta A, Colditz P, Rose S. Diffusion MRI of the neonate brain: acquisition, processing and analysis techniques. *Pediatr Radiol* 2012 10/01;42(10):1169-1182.

Chapter 2

Neuroimaging effects of prenatal alcohol-exposure on the developing human brain: A magnetic resonance imaging review

Kirsten A. Donald¹ ; Emma Eastman¹; Fleur M. Howells²; Colleen Adnams²; Edward P. Riley³; Roger P. Woods⁴; Katherine L. Narr⁴; Dan J. Stein²

1 - Division of Developmental Paediatrics, Department of Paediatrics and Child Health, Red Cross War Memorial Children's Hospital, University of Cape Town, Cape Town, South Africa.

2 - Department of Psychiatry and Mental Health, University of Cape Town, Cape Town, South Africa.

3 - Department of Psychology, San Diego State University, San Diego, USA.

4 - Department of Neurology, David Geffen School of Medicine, University of California, Los Angeles, USA.

Running title: Neuroimaging in fetal alcohol spectrum disorders.

Corresponding author:

Kirsten A. Donald

Tel: +27 21 658 5535

Fax: +27 21 658 5530

Email: kirsty.donald@uct.ac.za

Postal address: Department of Paediatrics and Child Health, Red Cross War Memorial Children's Hospital, Klipfontein Road/Private Bag, Rondebosch, 7700/7701.

Abstract

Objective: This paper reviews the magnetic resonance imaging (MRI) literature on the effects of prenatal alcohol-exposure on the developing human brain.

Method: A literature search was conducted through the following databases: PubMed, PsycINFO and Google Scholar. Combinations of the following search terms and key-words were used to identify relevant studies: 'alcohol', 'fetal alcohol spectrum disorders', 'fetal alcohol syndrome', 'FAS', 'FASD', 'MRI', 'DTI', 'MRS', 'neuroimaging', 'children' and 'infants'.

Results: A total of 64 relevant articles were identified across all modalities. Overall, studies reported smaller total brain volume as well as smaller volume of both white and grey matter in specific cortical regions. The most consistent reported structural MRI findings were alterations in the shape and volume of the corpus callosum, and smaller volume in the basal ganglia and hippocampi. The most consistent findings in diffusion tensor imaging studies is lower fractional anisotropy in the corpus callosum. Proton magnetic resonance spectroscopy studies are few to date, but show altered neurometabolic profiles in the frontal and parietal cortex, thalamus and dentate nuclei. Resting state functional MRI studies report reduced functional connectivity between cortical and deep grey matter structures.

Discussion: There is a critical gap in the literature of MRI studies in alcohol-exposed children under 5 years of age across all MRI modalities. The dynamic nature of brain maturation and appreciation of the effects of alcohol exposure on the developing trajectory of structural and functional networks, argues for the prioritization of studies which include a longitudinal approach to understanding this spectrum of effects and potential therapeutic time-points.

Keywords: Neuroimaging, fetal alcohol spectrum disorders (FASD), fetal alcohol syndrome (FAS), magnetic resonance imaging (MRI)

Summations:

1. Prenatal alcohol exposure results in smaller total brain volume, specific gray and white matter, cortical regions and abnormalities in neurochemistry and functional connectivity.
2. There is a gap in the literature of neuroimaging studies in alcohol-exposed children under 5 years of age across all the modalities of neuroimaging.
3. The dynamic nature of brain maturation calls for the prioritization of studies which include a longitudinal approach to understanding the effects of alcohol exposure on the developing human brain.

Considerations:

1. Differing methodologies make inter-study comparisons difficult.
2. Reporting of structural or microstructural alterations in single regions of interest has resulted in the effects on specific regions being very well documented at the expense of a more exploratory approach.
3. Controlling for polysubstance abuse, environmental factors, and age is needed to assess consistency of results and inter-study comparisons.

Introduction

The term fetal alcohol syndrome (FAS) was only formalized in the 1970s when Jones and Smith (1973) described abnormalities and disabilities in children born to mothers with alcoholism. (1,2) The World Health Organization's (WHO) 2014 global status report on alcohol and health,(3) reports that harmful alcohol usage ranks in the top five contributors to disease, disability and mortality. FAS is further recognized as one of the major disease and disability categories within alcohol-related disorders. (4-6) Studies indicate the global prevalence of FAS, ranges between 2-7 per 1000 births, and fetal alcohol spectrum disorders (FASD) between 20-50 per 1000 births (7-9) with the highest rates in South Africa. (10) However, May and colleagues (11-14) argue that current prevalence estimates of FASD represent an underestimate, and FASD presents a much larger public health issue than previously recognized, especially in emerging economies such as South Africa.

FASD animal studies using MRI modalities demonstrate a spectrum of changes on the developing brain structure. (15) Findings in rodent and mouse FASD models include reports of enlarged ventricles, (16) diffuse reductions in brain volumes including: whole brain volume, (17-19) cortical gray matter volume, (19) caudate-putamen volume, (16) hippocampal volume, (20) cerebellar volume, (20) while pituitary and septal regions showed increased volumes. (20) Lipinski and colleagues (2012) have described the correlation between dysmorphology and brain abnormalities in mice models with prenatal alcohol exposure at different stages of gestation. (21) A single rat FASD diffusion tensor imaging study showed increased fractional anisotropy in cortical tissue. (22) In addition, a single spectroscopy FASD rat study has shown, post-natal day 4-9, neonatal alcohol exposure results in reduced NAA and taurine in the striatum and cerebellum, increased myo-inositol in the cerebellum. (23)

Early investigations into the effects of alcohol on the structure of the human brain have reported on autopsies of children who had died in infancy though likely to have been on the extreme severe end of the spectrum of alcohol exposure effects. Findings included microcephaly (most consistently), but also agenesis corpus callosum, ventriculomegaly,

a small cerebellum as well as a few further malformations due to neuronal and glial migration abnormalities. (24,25) Advances in neuroimaging methods over the last decade have allowed researchers to study structural, metabolic, and functional abnormalities resulting from prenatal exposure to drugs of abuse more closely.

Aims of the study

The aim of this paper is to review the current magnetic resonance imaging (MRI) literature on the effects of prenatal alcohol-exposure in children.

Methods

Search methods for identification of studies

An initial electronic search was conducted to identify studies. A search was conducted through the following databases: PubMed, PsycINFO and Google Scholar. Combinations of the following search terms and keywords were used to identify relevant studies: 'alcohol', 'fetal alcohol spectrum disorders', 'fetal alcohol syndrome', 'FAS', 'FASD', 'MRI', 'DTI', 'MRS', 'neuroimaging', 'children' and 'infants'. No starting date limits were enforced to restrict the search, however, the search extended to 15 August 2014 and papers were restricted to those that included human subjects <18 years of age as a proportion of their cohort and that were published in English language journals. Abstracts were manually examined in order to confirm relevance. Further studies were identified by searching the reference lists of studies identified in the initial database search. This was done to ensure any studies which were missed in the initial search were identified. A total of 64 relevant articles were identified and included in this review.

A qualitative approach was taken for this review instead of a quantitative comparison such as meta-analysis. Reasons for this decision included: data required to compute effect size was not always available; the methodological detail to define regions of interest in different studies varied considerably, preventing clear comparisons; there were important differences in secondary variables across different studies (in particular age, gender, extent and timing of alcohol exposure and polysubstance exposure); and finally different analytic methods were used to report the imaging findings (for example voxel-based versus regions-of-interest (ROI)-based) across studies. As a result, a qualitative approach

seemed more appropriate for this particular review, which covers over two decades of neuroimaging studies on the effects of prenatal alcohol exposure on the brain.

Results

Structural magnetic resonance imaging (MRI) in prenatally alcohol-exposed children

Since the early 1990's MRI technology has been used to report quantitative effects on the brains of children exposed to alcohol in the antenatal period. Structural neuroimaging studies of prenatal alcohol exposure have been most widely reported and 32 relevant studies were identified for this review and are listed alphabetically in **Table 1**.

Table 1: Structural magnetic resonance imaging (MRI) in prenatally alcohol-exposed children

Author	Journal	N = PAE	N = Control	Site	Age (years)	Findings		Comment
						Global	Focal	
(Archibald et al. 2001) (26)	Developmental Medicine & Child Neurology (DMCN)	26	41	California, San Diego, USA*	7-24	Smaller volumes in the cranial, cerebral, cerebellar vaults and cerebral and cerebellar gray (GM) and White matter (WM)	Smaller volume of the parietal lobe in WM & GM and caudate nucleus. Relative sparing of hippocampal volume and cerebellar hypoplasia greater than cerebral	Cerebellar hypoplasia greater than cerebral - approaching statistical significance p=0.069
(Astley et al. 2009) (27)	Alcoholism: Clinical and Experimental Research (ACER)	61	20	Washington, Seattle, USA**	8-15	Smaller total brain volume	Smaller volume of the frontal lobe, caudate, hippocampus, corpus callosum (CC).	Controlled for overall brainsize
(Autti-Rämö et al. 2002) (28)	DMCN	17	0	Helsinki, Finland	13-15	Smaller volume of cerebral area and skull surface area	Smaller volume in CC area, posterior fossa and mesencephalon areas and in length of splenium	No control subjects
(Bookstein et al. 2007) (29)	ACER	23	21	Washington, Seattle-Tacoma area, USA**	1-16 weeks	Ultrasound study only, showing a "hook" (obtuse angle) in the splenium of CC++		
(Bookstein et al. 2002a) (30)	NeuroImage	30	15	Washington, Seattle, USA**	18 – 36	MR study demonstrated variation in shape of the CC++		
(Bookstein et al. 2002b) (31)	The Anatomical Report	120	60	Washington, Seattle-area, USA**	14-37	MR study demonstrated variation in shape of the CC++		

Author	Journal	N = PAE	N = Control	Site	Age (years)	Findings		
						Global	Focal	Comment
(Chen et al. 2012) (32)	Human Brain Mapping	67	27	Atlanta, Georgia, USA#	Mean: 22	Smaller total brain volume	Smaller volume of the superior and inferior parietal lobule, precentral gyrus, pars opercularis (frontal lobe), superior temporal gyrus, pericalcarine cortex, lingual gyrus and isthmus of cingulate cortex	Controlled for overall brain size
(Cortese et al. 2006) (33)	Neurotoxicology and Teratology	11	4	Detroit, USA	9-12	Smaller total brain volume	Smaller volume of left caudate nucleus.	Controlled for overall brain size
(Johnson et al. 1996) (34)	American Journal of Medical Genetics	9	0	South Dakota, Vermillion, USA	4.5 months - 20 years	Total brain volume reduced	Agensis of the CC and cavum septi pellucidi, mild micrencephaly, hypoplastic CC, small cavum vergae, basal nasal meningocele, disproportionately small brain stem and hypoplasia of the inferior olivary eminences	No control subjects
(Joseph et al. 2014) (35)	Human Brain Mapping	12	19	Cape Town, South Africa	Mean: 11	Not indicated	Deformations in the hippocampus and caudate nucleus	Controlled for overall brain size
(Lebel et al. 2008) (36)	ACER	24	95	Alberta, Canada	5-13	Smaller GM, WM and total brain volume	Not applicable	Focus on diffusion imaging

Author	Journal	N = PAE	N = Control	Site	Age (years)	Findings		
						Global	Focal	Comment
(Lebel et al. 2012) (37)	Journal of Neuroscience	70	63	California, Los Angeles and San Diego, USA and Cape Town, South Africa*	5-16	Smaller total cerebral and lesser WM volume	Longitudinal changes in volume relative to controls across many parietal and temporal cortical regions	
(Li et al. 2009) (38)	Brain Imaging and Behaviour	7	7	Atlanta, USA#	18-24	Smaller total brain volume	Smaller volume of occipito-temporal area in both WM&GM	
(Mattson et al. 1992) (39)	ACER	2	9	California, San Diego, USA*	13-14	Smaller cerebral and cerebellar volume	Agensis of the CC and hypoplastic CC, enlarged lateral ventricles, temporal horns and caudate, volume reduction in thalamus, increased sub-arachnoid space and cerebral atrophy	
(Mattson et al. 1994) (16)	Neurotoxicology and Teratology	2	20	California, San Diego, USA*	16	Smaller volume in cranial and cerebellar vaults	Smaller volume of basal ganglia	
(Mattson et al. 1996) (40)	ACER	6	7	California, San Diego, USA*	8-19	Smaller cerebral volume	Smaller volume of the basal ganglia and caudate nucleus	Controlled for overall brain size
(Meintjes et al. 2014) (41)	NeuroImage	39	16	Cape Town, South Africa	9-11	Smaller total brain volume	Smaller volumes in particular brain regions were no longer significant after being controlled for overall brain size	

Author	Journal	N = PAE	N = Control	Site	Age (years)	Findings		
						Global	Focal	Comment
(Nardelli et al. 2011) (42)	ACER	28	56	Alberta, Canada	6-17	Smaller volumes of the intracranial vault, total WM and deep cortical GM	Bilaterally smaller volume in the hippocampus, thalamus, putamen, caudate, and globus pallidus	Controlled for overall brainsize
(O'Hare et al. 2005) (43)	Developmental Neuroscience	21	21	California, Los Angeles and San Diego, USA*	8-25	Not indicated	Displacement and smaller volume of the anterior vermal region++	Controlled for overall brain size
(Rajaprakash et al. 2014) (44)	Brain and Behavior	36	52	Toronto, Ontario, Canada	8-16	Smaller overall brain volume and GM volume	Narrower cortical thickness and smaller GM volume in frontal, L parietal and R temporal lobes and smaller cortical surface area in frontal, temporal and R occipital lobes	

Author	Journal	N = PAE	N = Control	Site	Age (years)	Findings		Comment
						Global	Focal	
(Riikonen et al. 1999) (45)	DMCN	11	6	Kymenlaakso, Kotka and Kupio Finland	3-13	Not indicated	Slightly dilated R ventricle (fronto-temporal), cortical (frontal) and cerebellar atrophy, delayed myelination, arnold-chiari type 1 malformation, atrophic cerebellum, slight reduction of white matter (occipital), large cortical and fronto-temporal post infarct damage (L), atrophic basal ganglion (L), hypoplasia of CC, and a larger left-right asymmetry of the hippocampus	
(Riikonen et al. 2005) (46)	Biological Psychiatry	12	10	Kupio, Finland	5-16	Smaller volume of the intracranial vault	Controlled for overall brain size. No significant differences between groups after normalization.	Left hippocampus was smaller than right in FASD groups
(Riley et al. 1995) (47)	ACER	13	12	California, San Diego, USA*	8-18	Smaller total brain volume	Agensis and smaller volume of the overall callosal area	When corrected for brain size, three of the five callosal regions were still smaller in the alcohol (ALC) group, although overall area of the CC was no longer significantly different

Author	Journal	N = PAE	N = Control	Site	Age (years)	Findings		
						Global	Focal	Comment
(Rousotte et al. 2012a) (48)	Human Brain Mapping	56	43	Los Angeles and San Diego, USA and Cape Town, South Africa*	8-16	Smaller total brain volume and lesser total cortical GM	Smaller volume of some regions of the basal ganglia, diencephalon, L putamen and R pallidum	Controlled for overall brain size
(Sowell et al. 1996) (49)	ACER	9	24	California, San Diego, USA*	8-24	Not indicated	Smaller volume of the anterior vermal regions (vermal lobules I-V)++	
(Sowell et al. 2001) (50)	Neuroreport	21	21	California, San Diego, USA*	8-25	Smaller total intracranial volume, and lesser total WM, GM and CSF volume	Left posterior temporo-parietal area shown relatively too much GM and too little WM	Controlled for overall brain size
(Sowell et al. 2002) (51)	NeuroImage	21	145	California, San Diego (and Yale) USA.*	7-25	Not applicable	Cortical surface and GM asymmetry in the posterior inferior temporal lobes (rightward-asymmetry)	
(Sowell et al. 2008) (52)	Cerebral Cortex	21	21	California, Los Angeles and San Diego, USA*	8-25	Smaller brain volume	Greater cortical thickness over lateral temporal, frontal and parietal lobes bilaterally. No significant change from controls over dorsal frontal and parietal lobes	Authors comment that this MRI measurement likely reflects decreased myelination of WM rather than actual increase of GM constituents (i.e. indicate difficulty of establishing the WM&GM boundary on imaging)

Author	Journal	N = PAE	N = Control	Site	Age (years)	Findings		Comment
						Global	Focal	
(Swayze et al. 1997) (53)	Pediatrics	10	119	Iowa City, Vermillion, USA	4-26	Not indicated	Microcephaly, midline developmental anomalies, agenesis of and a hypoplastic CC, thinning of posterior callosum body and inferior olivary eminences, cavum septa pellucidum, and cavum vergae.	
(Treit et al. 2013) (54)	Journal of Neuroscience	17	27	Alberta, Canada	5-15	Lesser total brain volume, WM and cortical GM	Smaller volumes of the basal ganglia (globus pallidus and putamen), thalamus and hippocampus	Corrected for overall brain size
(Willoughby et al. 2008) (55)	Journal of the International Neuropsychological Society	19	18	Toronto, Ontario, Canada	9-15	Smaller intracranial volume	Smaller L hippocampal volumes++	Corrected for overall brain size
(Yang et al. 2012a) (56)	ACER	82	71	Los Angeles and San Diego, USA and Cape Town, South Africa*	8-16	Not applicable	Lesser callosal thickness, anterior regions and the splenium++	
(Yang et al. 2012b) (57)	Cerebral Cortex	69	58	Los Angeles and San Diego, USA* and Cape Town, South Africa	8-16	Not applicable	Thicker cortices in several frontal, temporal and parietal regions	

Author	Journal	N = PAE	N = Control	Site	Age (years)	Findings		
						Global	Focal	Comment
(Zhou et al. 2011) (58)	NeuroImage	33	33 +66	Alberta, Canada	6-30	Smaller total brain volume	Lesser cortical thickness in the bilateral middle frontal lobe, bilateral pre- and post-central gyri, bilateral superior parietal lobe, L lateral temporal lobe, bilateral inferior temporal lobe, and bilateral occipital lobe.	Additional 66 controls recruited and matched for secondary analysis.
								Controlled for brain size.

Table 1 legend: GM: Gray Matter; WM: White matter; FASD: Fetal alcohol spectrum disorder; CC: Corpus callosum. L: left; R: right. The following codes denote sample overlap from specific regions * California/San Diego #Atlanta ^Minnesota **Washington ; The following codes denote report on a single ROI: ++

Diffusion tensor imaging (DTI) in prenatally alcohol-exposed children

White matter in the brain provides the connections that comprise the brain's structural neural networks. Its integrity is essential for the effective functioning of a wide spectrum of complex cognitive processes. In particular, white matter integrity has been demonstrated to play a critical role in normal executive function, attention and processing speed. (59-61)

White matter microstructure can be measured *in vivo* with diffusion tensor imaging (DTI), as well as other diffusion MRI approaches, and can estimate the overall directional diffusion of water molecules along fiber pathways. (62,63) Analysis of these data allows the degree of structural and organization of areas within the brain tissue to be determined. Traditional scalar metrics derived from DTI data include fractional anisotropy (FA), which quantifies the overall directionality of diffusion, and may represent variations in axon integrity and/or packing density. Mean diffusivity (MD), provides a measure of average diffusivity and may primarily reflect myelin breakdown, decreased cellular density or increased extra- or intracellular volumes although these relationships are less clearly defined in the developing brain. High FA and low MD values are typically associated with healthier neural microstructure in adults, whereas low FA and high MD values may indicate white matter pathology. However, it is also relevant to note that during brain maturation in healthy children and adolescents, axonal pruning and other biological processes may also lead to reduced FA. (64-66)

Studies reporting the effects of prenatal alcohol exposure on the white matter microstructure in children extend back 10 years . Seven studies have been reported using this modality and are detailed in **Table 2**.

Table 2: Diffusion tensor imaging (DTI) in prenatally alcohol-exposed children

Author	Journal	N = PAE	N = Control	Site	Age (years)	Findings		
						Mean Diffusivity (MD)	Fractional Anisotropy (FA)	Comment
(Fryer et al. 2009) (67)	ACER	15	12	California, San Diego, USA*	8-18	↑ (in ALC group) Left subcortex and superior frontal lobe	↓ (in ALC group) Bilateral occipital, inferior parietal lobe and superior frontal lobe, R temporo-parieto-occipital junction, parietal lobe and lateral frontal lobe and L superior frontal lobe, temporo-occipital junction and frontal lobe, and CC body	
						↓ (in ALC group) R inferior frontal lobe and temporo-occipito-parietal junction	↓ (in control group) R subcortex and cingulate gyrus	
(Lebel et al. 2008) (36)	ACER	24	95	Alberta, Canada	5-13	↑ (in ALC group) bilateral Inferior fronto-occipital fasciculus (IFO), L inferior longitudinal fasciculus (ILF), R corticospinal tracts (CST), globus pallidus, R putamen, and thalamus.	↓ (In ALC group) R cingulum, bilateral ILF and superior longitudinal fasciculus (SLF), splenium of CC and L thalamus	
						↓ (in ALC group) Genu of CC	↑ (In ALC group) Bilateral globus pallidus	

Author	Journal	N = PAE	N = Control	Site	Age (years)	Findings			Comment
						Mean Diffusivity (MD)	Fractional Anisotropy (FA)		
(Li et al. 2009) (68)	Human Brain Mapping	57	25	Atlanta, USA [#]	19-27	↑ (In ALC groups) Isthmus of CC ⁺⁺	↓ (In ALC groups) Isthmus and splenium of the CC to lateral callosal fibers		
(Ma et al. 2005) (69)	ACER	9	7	Atlanta, USA [#]	18-25	↑ (in FAS group) Genu and splenium of CC ⁺⁺	↓ (in FAS group) Genu and splenium of CC		
(Sowell et al. 2008b) (60)	Journal of Neuroscience	17	19	California, Los Angeles, USA [*]	7-15		↓ (in FASD group) Lateral splenium (medial superior parietal WM), posterior cingulate WM bilaterally, deep WM of right temporal lobe		MD was lower in the FASD group in some, but not all regions where FA was affected. Lower MD was observed in the lateral splenium of corpus callosum bilaterally and in R temporal association fibers
							↓ (in FASD group) R internal capsule and brainstem regions		
(Spottiswoode et al. 2011) (70)	ACER	13	12	Cape Town, South Africa	9-14		↓ (In FASD group) Middle cerebellar peduncle ⁺⁺		Association with poor eye-blink conditioning in prenatally alcohol exposed children

Author	Journal	N = PAE	N = Control	Site	Age (years)	Findings			Comment
						Mean Diffusivity (MD)	Fractional Anisotropy (FA)		
(Treit et al. 2013) (54)	Journal of Neuroscience	17	27	Alberta, Canada	5-15	↑ (In FASD group) Genu of CC	↓ (In FASD group) Superior Fronto-occipital Fasciculus (SFO)		Atypical developmental trajectories in superior and inferior fronto-occipital fasciculus and superior longitudinal fasciculus. Primarily MD findings (steeper decline in MD than controls)
(Wozniak et al. 2006) (59)	ACER	14	13	Minneapolis, Minnesota, USA [^]	10-13	↓ (in FASD group) Isthmus of CC ⁺⁺			
(Wozniak et al. 2009) (71)	ACER	33	19	Minneapolis, Minnesota, USA [^]	10-17	↑	↓ (in FASD group) Posterior midbody, isthmus, splenium of CC ⁺⁺		

Table 2 legend: GM: Gray Matter; WM: White matter; FASD: Fetal alcohol spectrum disorder; CC: Corpus callosum; FA: Fractional anisotropy; MD: Mean diffusivity; ALC: alcohol exposure; PAE: prenatal alcohol exposure; R: right; L: left. The following codes denote sample overlap from specific regions * California/San Diego #Atlanta ^Minnesota ***Washington ; The following codes denote report on a single ROI: ++

Proton magnetic resonance spectroscopy (¹H-MRS) in prenatally alcohol-exposed children

Both basic and clinical research has begun to implicate a number of neurometabolic processes that may underlie the association between maternal alcohol abuse and subsequent negative outcomes in offspring. (72,73) Proton magnetic resonance spectroscopy (¹H-MRS) is a non-invasive magnetic resonance technique which measures the concentration of several brain metabolites. Levels of individual brain metabolites may suggest abnormalities in neuronal microstructure and/or neurometabolism. The most commonly reported metabolites include N-acetyl aspartate (NAA) which is an indicator of neuronal integrity and or viability, choline metabolites (Cho), an indicator of neuronal-membrane turnover and myelination, creatine metabolites (Cr), energy metabolism and glutamate with its precursor glutamine (Glx), the brains major neuroexcitatory neurotransmitter. (74,75) Published ¹H-MRS studies only include 4 studies which were performed on children and adolescents, these data are present in **Table 3**.

Table 3: Proton magnetic resonance spectroscopy (1H-MRS) in prenatally alcohol-exposed children

Author	Journal	N = PAE	N = Control	Site	Age (years)	Neurometabolite	Site	Comment
(Astley et al. 2009a) (76)	Magnetic Resonance imaging	61	20	Washington, Seattle, USA	8-15	wCho	↓ R frontal-parietal WM voxel	Near significant p=0.07
						hCho	↓ Hippocampal voxel	Overall ANOVA not significant, but important to report from exploratory standpoint. WM p=0.058 and Hippocampal p=0.061.
						Cho/Cre ratio	↓ WM and hippocampus voxel	
(Cortese et al. 2006) (33)	Neurotoxicology & Teratology	11	4	Detroit, USA	9-12	wCho	↓ Volume of frontal lobe WM and midsagittal area of cerebellar vermis lobules 1-5	Fixed-size voxel will capture more surrounding tissue in a smaller brain than in a larger brain. Referred to as partial voluming.
						wCho	↓ Midsagittal area of CC	
						wNAA	↓ Midsagittal area of CC	
						hCho	↓ hippocampus, frontal lobe and CC Cerebellar vermis lobules 6-7, putamen and total brain	
						NAA/Cr	↑ L caudate nucleus	Associated with FAS facial dysmorphology
NAA	↑ L caudate							

Author	Journal	N = PAE	N = Control	Site	Age (years)	Neurometabolite	Site	Comment
(Fagerlund et al. 2006) (77)	ACER	10	10	Turku and Helsinki, Finland	14-21	NA/Choline & NAA/Cr	↓ Cerebral cortex (anterior cingulate and parietal cortex)	
						NAA/Cho	↓ Cerebral cortex (lateral frontal cortex)	
						NA/Cho & NAA/Cr	↓ Cerebral white matter (frontal white matter)	
						NAA/Cr	↓ Cerebral white matter (CC)	
						NAA/Cho	↓ Cerebral nuclei (thalamus)	
						NAA/Cho & NAA/Cr	↓ Cerebellum (Dentate nucleus)	
(Lindie du Plessis et al. 2014) (78)	ACER	37	17	Cape Town, South Africa	8-12	NAA	↓ Deep cerebellar nuclei	Associated with alcohol consumption at conception
						Cho	↓ Deep cerebellar nuclei	Associated with alcohol consumption throughout pregnancy
						Glx	↑ Deep cerebellar nuclei	Associated with alcohol consumption at conception and during pregnancy

Table 3 Legend: NAA – n-acetyl-aspartate; Cho – choline containing metabolites; Glx – glutamate with its precursor glutamine; Cr – creatine containing metabolites; R: right; L: left; PAE: prenatal alcohol exposure.

Functional MRI (fMRI) in prenatally alcohol-exposed children

Increasingly, over recent years, investigators have sought to document correlations between the functional deficits reported in children with FASD and the underlying neurobiology. Animal work (79,80) and human imaging studies in school-age children have demonstrated that *in utero* exposure to alcohol alters brain morphology and reduced white matter microstructural integrity. (15,26,36,37,59,67,81-85) However, there are few data on functional connectivity in these children. Functional connectivity in the imaging literature has been defined as dependencies among observed neurophysiological responses or “temporal correlation between spatially remote neurophysical events”. (86) Here, intrinsic brain activity is measured in the absence of an experimental task or “at rest” or during overt behavior. The ability of the brain to coordinate areas of activity in specific functional networks follows a developmental trajectory, reflected in increased functional network connectivity with age in childhood and early adulthood. (87) Two preliminary reports from a single cohort have described interhemispheric and global functional connectivity abnormalities in older children (10-17 years) with FASD.

Task-based fMRI is a directed method for investigating the function of the brain in humans. The technique measures the dynamic distribution of blood flow to specific regions of the brain during a defined motor or cognitive activity. The choice of tasks investigated has largely focussed on functional deficits previously described in children prenatally exposed to alcohol and include working memory,(88-95) sustained attention, (38) response inhibition , (96,97) verbal learning (98) and mathematical tasks.(99,100) Rousotte and colleagues have also reported connectivity in alcohol exposed and in polydrug exposed children aged 7-15 years, during a working memory task. **Table 4.**

Table 4: Functional MRI (fMRI) in prenatally alcohol-exposed children

Author	Journal	N = PAE	N = Control	Site	Age (years)	Brain function	Findings		Comment
							Brain activity	Site	
(Astley et al. 2009b) (94)	J Neurodev Disord	61	20	Washington, Seattle, USA**	8-15	Working memory	↓	(2-back tasks in FASD group) R inferior frontal gyrus, R posterior parietal lobe, R dorsolateral prefrontal cortex (DLPFC), and R middle frontal gyrus	Overall, performance on 1-back and 2-back tasks decreased in FASD groups, but activation levels only decreased on 2-back tasks and not on 1-back tasks
(Diwadkar et al. 2013) (93)	Human Brain Mapping	30	17	Cape Town, South Africa	8-10	Working memory	↑	(In syndromatic PAE group) R inferior parietal cortex and L cerebellum (Crus I/Lobule VI) and L inferior cerebellum (lobule VIIIB-VIIIA). (In non-syndromatic PAE group) Bilateral dorsal, prefrontal cortex, L caudate and L putamen	
(Fryer et al. 2007) (97)	ACER	13	9	California, San Diego, USA*	8-18	Go/No-Go	↑	(in FASD group) R middle frontal gyrus, L middle, medial and superior frontal gyri	
							↓	(In FASD group) R caudate nucleus	
(Li et al. 2008) (38)	Brain Imaging and behaviour	7	7	Atlanta, USA#	8-24	Sustained attention	↑	(In PAE groups) Occipital-temporal region, in the Z (inferior-superior) direction	Brain networks which were activated were more widespread in the PAE groups than in CON group
(Malisza et al. 2005) (90)	Pediatric research	24	25	Monitoba, Winnipeg, Canada	7-12 18-33	Spatial working memory	↑	(In FASD group) Inferior middle frontal gyrus	CON groups showed an overall increase in frontal activity with increasing task difficulty, while FASD group showed decreased activity

Author	Journal	N = PAE	N = Control	Site	Age (years)	Brain function	Findings			
							Brain activity	Site	Comment	
(Meintjes et al. 2010) (99)	ACER	15	18	Cape Town, South Africa	8-12	Number processing	↑	(In FAS groups) Angular gyrus, cuneus, posterior cingulate gyrus, anterior horizontal intraparietal sulcus (HIPS), superior frontal gyrus, R basal operculum, L sub callosal stratum, L deep precentral gyrus, L inferior temporal gyrus, R occipital gyrus, L anterior insula, R putamen, L thalamus, and red nucleus		
(Norman et al. 2013) (88)	ACER	18	17	California, San Diego, USA*	12-18	Spatial working memory	↑	(In PAE groups) Bilateral middle and superior frontal gyrus, lingual gyrus, cuneus, lentiform nucleus, insula and precuneus		PAE group showed fewer regions of activation, and limited frontal activation, in comparison to CON groups
(O'Brien et al. 2013) (96)	ACER	20	15	California, San Diego, USA*	8-18	Go/No-Go	↑	(In No-Go tasks, in PAE group) L precuneus, cingulate gyrus, anterior cingulate and R medial frontal gyrus		PAE group showed activation in more widespread areas, especially in the left hemisphere
							↓	(In No-go, cue-dependent tasks, in PAE group) L pre and post-central gyri		

Author	Journal	N = PAE	N = Control	Site	Age (years)	Brain function	Findings		
							Brain activity	Site	Comment
(O'Hare et al. 2009) (95)	Human Brain Mapping	20	20	California, Los Angeles, USA	7-15	Verbal working memory	↑	(In PAE group) L dorsal frontal, L inferior parietal, and bilateral posterior temporal regions	
							↓	(In PAE group) Bilateral anterior cingulate, R orbito-frontal, R frontal pole, R insula, R caudate and R putamen	
(Rousotte et al. 2011) (91)	Neuroimage	32	18	California, Los Angeles, USA	7-15	Working memory	↑	(In functional connectivity, in PAE group) Between caudate and frontal and prefrontal areas (only in lateral subregions: inferior frontal gyrus, in the R hemisphere) and R medial temporal lobe	
							↓	(In functional coupling, in PAE group) Between putamen and superior and inferior frontal subregions	
(Rousotte et al. 2012b) (92)	Developmental Neuroscience	32	18	California, Los Angeles, USA	7-15	Working memory	↑	(In functional connectivity, in PAE group) Between caudate and frontal and prefrontal areas (only in lateral subregions: inferior frontal gyrus, in the R hemisphere) and R medial temporal lobe	
(Santhanam et al. 2009) (100)	ACER	37	17	Atlanta, USA#	20-26	Number processing	↓	(In dysmorphic PAE group) L superior and R inferior parietal regions and medial frontal gyrus	

Author	Journal	N = PAE	N = Control	Site	Age (years)	Brain function	Findings		
							Brain activity	Site	Comment
(Sowell et al. 2007) (98)	Neuroreport	11	16	California, Los Angeles, USA	7-15	Verbal learning	↑	(In PAE group) L dorsal prefrontal cortices, R ventral and lateral frontal and superior parietal cortices	
							↓	(In PAE group) L medial temporal region	
(Spadoni et al. 2009) (89)	ACER	10	12	California, San Diego, USA*	10-18	Spatial working memory	↑	(In PAE group) Frontal, insular, superior, middle temporal, occipital, and subcortical regions	
(Wozniak et al. 2013) (101)	ACER	24	31	Minnesota, Minneapolis, USA^	10-17		↑	Characteristic Path Length	Global All measures are global. Study set out to specifically measure global connectivity rather than specific region to region connectivity.
							↓	Global Efficiency (GE)	Global GE positively correlated with cortical thickness in frontal, temporal and parietal regions
							↑	Local Efficiency	Global

Author	Journal	N = PAE	N = Control	Site	Age (years)	Brain function	Findings		
							Brain activity	Site	Comment
(Wozniak et al. 2011) (102)	ACER	21	23	Minnesota, Minneapolis, USA [^]	10-17		↓	(In functional group) Medial parietal (paracentral) regions	The author used the paracentral area as the region of interest as they had previously shown that children with FASD have microstructural abnormalities in these regions and the posterior CC which connects these regions

Table 4 legend: PAE: prenatal alcohol exposure; CON: control; R: right; L: left. The following codes denote sample overlap from specific regions * California/San Diego #Atlanta ^Minnesota **Washington

Discussion

The neuroimaging literature of the past 20 years has documented the deleterious effects of prenatal alcohol exposure on many important systems in the developing brain. These findings in children are consistent with animal neuroimaging literature, which has demonstrated the teratogenic effects of alcohol on the immature nervous system via a number of cellular and molecular mechanisms leading to structural, functional and metabolic abnormalities in a wide spectrum of brain regions.

Structural magnetic resonance Imaging (MRI)

Early reports on structural neuroimaging effects of prenatal alcohol exposure, focused largely on global effects and on the severe end of the FASD spectrum. Studies consistently reported smaller whole brain volume as well as more specifically in smaller volume of both white and grey matter across the whole brain. A small minority found proportionally greater volume differences in overall deep grey matter (42) or white matter (26) when controlling for overall brain volumes and though cortical thickness alterations have been reported, the direction of change remains mixed across studies. (37,44,48,54,56-58) However, these findings are not specific to alcohol-exposure and research focus moved to the identification of volume and shape differences of more defined regions.

The most consistent finding across studies of structural MRI in alcohol exposed children remains alterations in the volume, shape and area of the corpus callosum. Given that this structure represents the largest white matter tract in the brain, an important midline structure as well as the primary connection between the hemispheres, alterations in its shape and volume might be reasonably hypothesised in the context of alcohol exposure. Reported variations range from total absence of the structure or partial agenesis in individual cases (45) to more subtle between-group differences in volume, (47,48,56) area or length (27,28,47,103) and position (103) relative to unexposed children. These findings are likely to represent, in part, the well-described vulnerability of midline structures to alcohol-induced damage in exposed individuals, but also may represent downstream effects of alterations in other areas of the brain resulting in reduction in connections between these regions across the hemispheres. Finally, though these studies confirm that the corpus callosum is a heavily affected region in prenatal alcohol exposure, as can be

seen from Table 1, the region has frequently been singled out for discrete ROI analysis and so effects on the brains of these exposed children may in fact be more extensive than they would seem from these reports.

Differences in gray matter volumes and/or thickness have been reported for cortical regions as well as the deep gray matter. Frontal, parietal and temporal cortices have been implicated, though the direction of effect is not consistent between studies. (37,51,52,57,58) In particular the relationships between abnormalities of cortical structure have been related to cognitive functional outcomes as well as the differences in cortical development over time in alcohol exposed versus control groups have been reported by Sowell and colleagues. (37,52,54) This argues for the clinical importance of damage to these areas after prenatal alcohol exposure.

Other areas which have been reported as consistently affected include the deep gray matter structures. Most consistently reported is volume reduction in the hippocampi, even when corrected for total brain volume. (26,42,55) Additional studies found the hippocampi to have smaller volumes in proportion to overall brain volume reduction. (27,48) Basal ganglia volume reductions in alcohol-exposed children in comparison to their non-exposed peers have been reported by a number of authors. (16,26,33,40,42,46,48,76) Alcohol-exposure effects on the caudate have been most frequently cited, (16,26,33,40,42,46,48,76) but also discrete changes in the globus pallidus, (42,48,54) putamen, (42,46,48,54,76) or the lenticular nucleus as a whole. (40) The implication of alcohol targeting on these subcortical nuclei is not well established due, in part to the paucity of functional outcome data linked to these changes. However, their role in critical networks regulating behaviour and impulse control, amongst other functions, could be considered a consistent hypothesis in this group of affected children. (104)

Areas where findings have showed greater discrepancy include the thalamus which has been reported to have smaller volumes even when correcting for brain volume by some groups (39,42,54) and changes only in proportion to overall brain volume by others. (26,48) The diencephalon overall has also been reported as displaying reduced volume (40) or area (28) or no significant structural changes at all. (16)

The body of literature reporting structural brain changes associated with in utero alcohol exposure using cross-sectional study designs, described above, is now quite large. The studies have focused on a range of ages from 5 years through to young adulthood. However, the majority of participants were either in late childhood or adolescence and very few studies grouped participants in a narrow age-bracket. As a result, although there is reasonable consistency in the location of the PAE effects on the developing brain, there is very little clarity on when these changes have onset, the nature and functional importance of changes in specific areas at particular time points and the developmental trajectory of these changes across childhood or into later adulthood. There is established documentation of age-related changes in the developing brain, both at structural and microstructural level. (64,65,105,106) These changes do not necessarily follow linear trajectories and the different areas of the brain appear to follow different maturational patterns. (106) The CIFASD group, in one of the only reported longitudinal studies in alcohol-exposed children have reported altered trajectory of cortical development compared to non-exposed peers. (37,54) Particularly, parietal and some temporal regions demonstrated either inverted or flattened volume curves over time. Of interest are the different effects that were noted at different ages in specific areas. For example a cross sectional comparison of these groups at age 5-6 years may have demonstrated increased volume in the parietal cortex in prenatally alcohol exposed children whereas at age 11-12 years the volume relationship may have been reversed. This study supports the view that developmental trajectories may be a better indicator of atypical brain development, and emphasizes the conspicuous absence of data in children with prenatally alcohol exposed during the early years of life which represents the most rapid period of brain growth.

Diffusion tensor imaging (DTI)

DTI has proved to be a particularly useful tool in the investigation of the more subtle effects of the spectrum of alcohol and polysubstance exposure on white matter integrity of the developing brain. (59) The available data on DTI findings in children prenatally exposed to alcohol have consistently demonstrated reduced fractional anisotropy in the corpus callosum. Abnormalities in mean diffusivity have also been reported, though the nature

and specific location of these changes has been different across studies. (60,71,107) A recent review of seven DTI studies in older children and young adults exposed to alcohol prenatally reported white matter microstructural abnormalities (lower FA) in the corpus callosum, anterior-posterior fibre bundles and the cerebellum. (59,68,69,71,71,82,108) These abnormalities have also been reported in frontal, (36,67) temporal lobe regions (36,60) and subcortical structures (globus pallidus, thalamus and putamen). (36,37)

(Table 2)

Very few studies have addressed associations between white matter microstructural abnormalities and specific measures of cognitive and behavioral function. (60,84,84) Though negative findings exist for several functional domains, Sowell and colleagues (81) showed associations between reduced performance on a measure of visuospatial integration and reduced FA in the splenium of the corpus callosum and parietal white matter. Lebel *et al* reported significant associations between reduced FA in the left parietal lobe, cerebellum and brainstem with mathematical ability in 5-13 year old children. (108) These results suggest clinical significance of the DTI findings in these brain regions, at least in school-age children. However, existing studies have generally used relatively low angular resolution data (with between 6 and 35 gradient directions) and spatial resolution equal or greater than 2.5 mm. Thus more sensitive non-tensor based models have not been applied, which may have greater sensitivity. To date, almost no data exists regarding the impact of prenatal alcohol exposure in early infancy before higher-level brain networks have become established or the confounding post-natal environmental influences to which children from these backgrounds are exposed have come into play. Moreover, there are few data on when changes in white matter structural integrity have onset, regional specificity in early brain development, and whether there are any early neurobehavioral associations.

Proton magnetic resonance spectroscopy (¹H-MRS)

The first reported ¹H-MRS study in children exposed to alcohol in utero, reported increased NAA concentration in the caudate nucleus in a group of children with full-blown FAS. The group also reported relationships between NAA concentration and facial dysmorphometry. (33) A second study in slightly older children identified decreased NAA/Cr

and NAA/Cho ratios in multiple regions including cortical and subcortical regions. These changes were identified in regions including the parietal and frontal cortices, thalamus and cerebellar dentate nucleus as well as in the frontal white matter and corpus callosum. (77) These changes in neurometabolite ratios are suggested to reflect changes in glial proliferation (Cho and Cr) rather than decreased neuronal integrity/viability (NAA) in the children with FASD. A subsequent study in a larger cohort found reduced choline in frontal and parietal white matter of children with FASD compared to exposed children without the FAS facial phenotype or cognitive/behavioral dysfunction and unexposed children. Another group has also reported reduced Cho, but in the left striatal region in a group of children with a diagnosis of FASD compared to a control group. (109) Finally a well-characterised group of FASD children in South Africa were found to have lower NAA levels in the deep cerebellar nuclei associated with prenatal alcohol exposure around conception. Higher levels of alcohol consumption during pregnancy were related to reduced Cho and with increased concentrations of GLx in the deep cerebellar nuclei. (78) Despite discrepancies in site and type of neurometabolites reported in these studies, these individual studies indicate alterations in the neurochemistry across important areas in grey and white matter regions in children exposed to alcohol in utero. However, brain metabolism and associated neurochemistry is dynamic and site-specific. Certainly age specific changes in concentrations of these metabolites are documented (74) and the findings described above represent a broad age range as well as differing approaches to clinical classification for comparison groups. In addition to above, technical factors such as choice of the location of voxel placement make comparison across the studies largely unhelpful.

To date, no studies have reported the use of ^1H -MRS to explore the effects of prenatal alcohol exposure on the developing infant brain. This is an important period to investigate as the neurometabolic milieu at this early stage of development is likely to have a significant impact on subsequent brain development (74) and less likely to be contaminated with environmental factors. In particular Glutamate (Glu), an excitatory neurotransmitter, also plays a key role in early life in the regulation of cell proliferation, migration and pruning (110) and prenatal alcohol-exposure has been shown to disrupt this process in animal models. (23,72)

Functional magnetic resonance imaging (fMRI)

Functional changes in brain activity activation relating to specific tasks in children exposed to prenatal alcohol exposure across a number of cognitive domains. Working memory is the domain which has been most widely investigated (88-95) although other areas of cognitive function have been described. (38,96-100) All these studies reported differences in the distribution of activated brain areas during a working memory task in prenatal alcohol exposure compared to unexposed peers even in the absence of between group differences on the task itself. Alterations in blood flow regulation appear most consistently in the frontal regions in these studies and although the particular distribution of these changes is not consistent, there appears to be a more generalised pattern of activation in the prenatal alcohol exposed children possibly representing reduced efficiency in activation of specific neural pathways. Whether this relates to delayed functional maturation or more permanent impairment will be addressed by studies tracking these important cognitive functions over time and specifically into early adulthood.

Wozniak and colleagues in their initial study demonstrated that their children with prenatal alcohol exposure had abnormalities in white matter microstructural connectivity in the corpus callosum compared to healthy unexposed controls, as well as a disturbance of functional connectivity in the alcohol exposed group in this region. (71,102) A subsequent analysis, demonstrated further abnormalities in global measures of network connectivity using a graph theory approach. (101) The authors reported significantly higher characteristic path length and lower global efficiency in the brains of those children with prenatal alcohol exposure. These exploratory findings are an early indication that the dynamic activation of brain regions may provide key insights into the neuropathological basis of functional impairments demonstrated by children with FASD. (**Table 4**).

The central nervous system (CNS) development and maturation require a carefully patterned sequence of events and processes more complex and extending over a longer period than that of any other organ system. The brain is particularly vulnerable to prenatal environmental influences which may have long-term effects on its structure and function. (111,112) The complexity of the brain's structural and functional networks increase rapidly in the early months of life, representing a rapid acquisition of abilities across

motor, sensory and cognitive areas. (62,65,87,105) Although information is emerging on the effects of alcohol exposure on the longitudinal structural development of the brain in later childhood, (37) there are few human data on when changes have onset, where they are located at this initial stage, and how complex early behavioral milestones relate to functional and structural changes of the underlying neural substrate. More specifically, while preliminary studies have shown altered connectivity in the more mature brains of school-age children, the specific effects of alcohol exposure on the establishment of intrinsic connectivity in early infancy has not been explored. Characterizing the connectivity of regions in the brain that are key to early neurodevelopmental functional integration, including the thalamus and the motor cortex, may therefore serve as a sensitive indicator of the neuropathological effects of alcohol exposure in the human infant. (113)

Conclusions

The body of evidence documenting the neuroimaging changes associated with children and young adults exposed to alcohol during the prenatal period is now substantial. Limitations common to work in this field and which are likely to have had an impact on the consistency of the results include the issue of polysubstance abuse, even though alcohol exposure was in most cases the predominant reported exposure. Though expensive, the emerging use of biological measures of alcohol exposure such as biomarkers obtained from hair or nails are likely to improve the quantification of prenatal alcohol exposure in studies going forward. The exclusion or careful control of subjects with other substance misuse may also be improved with approaches such as urine identification of excreted drugs and cotinine measurements for tobacco exposure.

Differences in age and gender between subjects in neuroimaging studies were noted (**Tables 1-4**). These differences are unlikely to have affected within-study results as exposed and control groups were generally carefully matched. However, it is possible that age in particular, but also gender differences within studies may have affected the sensitivity for detecting changes between these two groups. As has been discussed in relation to the specific imaging modalities above, a particularly critical gap in the extant literature is the lack of neuroimaging studies in prenatally alcohol-exposed children under 5 years of age or those over 25 years of age when brain maturation has occurred

across all the modalities of imaging. In addition the dynamic nature of brain maturation and appreciation of the effects of a significant insult such as alcohol exposure on the developing trajectory of structural and functional network, argues for the prioritisation of studies which include a longitudinal approach to understanding this spectrum of effects. Hypothesis driven studies which include longitudinal time points as well as providing an integrated approach using a number of modalities at one age point, neuropsychological and behavioural outcomes and links to genetic vulnerabilities are likely to provide the most robust understanding of the neurobiological effects of prenatal alcohol-exposure on the developing brain. Refining our understanding at a neurobiological level is critical in developing not only earlier identification of the spectrum of prenatal alcohol-exposure effects, but also targeted interventions during this important window for early intervention.

In the next chapter we will discuss the detail of the methodology for this neuroimaging project overall.

References

- (1) Jones K, Smith D. Recognition of the fetal alcohol syndrome in early infancy. *Lancet* 1973;302(7836):999-1001.
- (2) May PA, Gossage JP, White-Country M, Goodhart K, Decoteau S, Trujillo PM, et al. Alcohol consumption and other maternal risk factors for fetal alcohol syndrome among three distinct samples of women before, during, and after pregnancy: the risk is relative. *Am J Med Genet* 2004;127(1):10-20.
- (3) World Health Organisation. Global status report on alcohol and health – 2014 ed. WHO Press, World Health Organization 2014.
- (4) Lupton C, Burd L, Harwood R. Cost of fetal alcohol spectrum disorders. *Am J Med Genet* 2004;127(1):42-50.
- (5) Popova S, Stade B, Bekmuradov D, Lange S, Rehm J. What do we know about the economic impact of fetal alcohol spectrum disorder? A systematic literature review. *Alcohol Alcohol* 2011;46(4):490-497.
- (6) Navarro HJ, Doran CM, Shakeshaft AP. Measuring costs of alcohol harm to others: A review of the literature. *Drug Alcohol Depen* 2011;114(2):87-99.
- (7) May PA, Blankenship J, Marais AS, Gossage JP, Kalberg WO, Joubert B, et al. Maternal alcohol consumption producing fetal alcohol spectrum disorders (FASD): Quantity, frequency, and timing of drinking. *Drug Alcohol Depen* 2013;133(2):502-512.
- (8) May PA, Fiorentino D, Gossage JP, Kalberg WO, Hoyme HE, Robinson LK, et al. Epidemiology of FASD in a province in Italy: prevalence and characteristics of children in a random sample of schools. *Alcohol Clin Exp Res* 2006(30):1562-1575.
- (9) May PA, Gossage JP, Kalberg WO, Robinson LK, Buckley DG, Manning M, et al. The prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on in-school studies. *Dev Disabil Res Revs* 2009(15):176-192.
- (10) Viljoen DL, Gossage JP, Brooke L, Adnams CM, Jones KL, Robinson LK, et al. Fetal alcohol syndrome epidemiology in a South African community: a second study of a very high prevalence area. *J Stud Alcohol* 2005;66(5):593.
- (11) May PA, Gossage JP, Marais AS, Adnams CM, Hoyme HE, Jones KL, et al. The epidemiology of fetal alcohol syndrome and partial FAS in a South African community. *Drug Alcohol Depen* 2007;88(2):259-271.

- (12) May PA, Fiorentino D, Coriale G, Kalberg WO, Hoyme HE, Aragón AS, et al. Prevalence of children with severe fetal alcohol spectrum disorders in communities near Rome, Italy: new estimated rates are higher than previous estimates. *Int. J. Environ. Res. Public Health* 2011;8(6):2331-2351.
- (13) May PA, Blankenship J, Marais A, Gossage JP, Kalberg WO, Barnard R, et al. Approaching the Prevalence of the Full Spectrum of Fetal Alcohol Spectrum Disorders in a South African Population-Based Study. *Alcohol Clin Exp Res* 2013;37(5):818-830.
- (14) May PA, Baete A, Russo J, Elliott AJ, Blankenship J, Kalberg WO, et al. Prevalence and characteristics of fetal alcohol spectrum disorders. *Pediatrics* 2014;134(5):855-866.
- (15) O'Leary-Moore S, Parnell S, Lipinski R. Magnetic Resonance-Based Imaging in Animal Models of Fetal Alcohol Spectrum Disorder. *Neuropsychol Rev* 2011;21:167-185.
- (16) Mattson SN, Riley EP, Jernigan TL, Garcia A, Kaneko WM, Ehlers CL, et al. A decrease in the size of the basal ganglia following prenatal alcohol exposure: a preliminary report. *Neurotoxicol Teratol* 1994;16(3):283-289.
- (17) O'Leary-Moore SK, Parnell SE, Godin EA, Dehart DB, Ament JJ, Khan AA, et al. Magnetic resonance microscopy-based analyses of the brains of normal and ethanol-exposed fetal mice. *Birth Defects Res., Part A* 2010;88(11):953-964.
- (18) Coleman Jr LG, Oguz I, Lee J, Styner M, Crews FT. Postnatal day 7 ethanol treatment causes persistent reductions in adult mouse brain volume and cortical neurons with sex specific effects on neurogenesis. *Alcohol* 2012;46(6):603-612.
- (19) Leigland LA, Ford MM, Lerch JP, Kroenke CD. The influence of fetal ethanol exposure on subsequent development of the cerebral cortex as revealed by magnetic resonance imaging. *Alcohol Clin Exp Res* 2013;37(6):924-932.
- (20) Parnell SE, O'Leary-Moore SK, Godin EA, Dehart DB, Johnson BW, Allan Johnson G, et al. Magnetic Resonance Microscopy Defines Ethanol-Induced Brain Abnormalities in Prenatal Mice: Effects of Acute Insult on Gestational Day 8. *Alcohol Clin Exp Res* 2009;33(6):1001-1011.
- (21) Lipinski RJ, Hammond P, O'Leary-Moore SK, Ament JJ, Pecevich SJ, Jiang Y, et al. Ethanol-induced face-brain dysmorphology patterns are correlative and exposure-stage dependent. *PloS one* 2012;7(8):e43067-doi: 10.1371/journal.pone.0043067.
- (22) Leigland LA, Budde MD, Cornea A, Kroenke CD. Diffusion MRI of the developing cerebral cortical gray matter can be used to detect abnormalities in tissue microstructure associated with fetal ethanol exposure. *NeuroImage* 2013;83:1081-1087.

- (23) O'Leary-Moore SK, McMechan AP, Galloway MP, Hannigan JH. Neonatal Alcohol-Induced Region-Dependent Changes in Rat Brain Neurochemistry Measured by High-Resolution Magnetic Resonance Spectroscopy. *Alcohol Clin Exp Res* 2008;32(10):1697-1707.
- (24) Jones KL, Smith DW, Ulleland CN, Streissguth AP. Pattern of malformation in offspring of Chronic Alcoholic mothers. *Lancet* 1973;1(7815):1267-1271.
- (25) Coulter CL, Leech RW, Schaefer G, Scheithauer BW, Brumback RA. Midline cerebral dysgenesis, dysfunction of the hypothalamic-pituitary axis, and fetal alcohol effects. *Arch Neurol* 1993 July 1;50(7):771-775.
- (26) Archibald SL, Fennema-Notestine C, Gamst A, Riley EP, Mattson SN, Jernigan TL. Brain dysmorphology in individuals with severe prenatal alcohol exposure. *Dev Med Child Neurol* 2001;43(3):148-154.
- (27) Astley SJ, Aylward EH, Olson HC, Kerns K, Brooks A, Coggins TE, et al. Magnetic resonance imaging outcomes from a comprehensive magnetic resonance study of children with fetal alcohol spectrum disorders. *Alcohol Clin Exp Res* 2009;33(10):1671-1689.
- (28) Autti-Rämö I, Autti T, Korkman M, Kettunen S, Salonen O, Valanne L. MRI findings in children with school problems who had been exposed prenatally to alcohol. *Dev Med Child Neurol* 2002;44(2):98-106.
- (29) Bookstein FL, Connor PD, Huggins JE, Barr HM, Pimentel KD, Streissguth AP. Many infants prenatally exposed to high levels of alcohol show one particular anomaly of the corpus callosum. *Alcohol Clin Exp Res* 2007;31(5):868-879.
- (30) Bookstein FL, Streissguth AP, Sampson PD, Connor PD, Barr HM. Corpus callosum shape and neuropsychological deficits in adult males with heavy fetal alcohol exposure. *NeuroImage* 2002;15(1):233-251.
- (31) Bookstein FL, Sampson PD, Connor PD, Streissguth AP. Midline corpus callosum is a neuroanatomical focus of fetal alcohol damage. *Anat Rec* 2002;269(3):162-174.
- (32) Chen X, Coles CD, Lynch ME, Hu X. Understanding specific effects of prenatal alcohol exposure on brain structure in young adults. *Hum Brain Mapp* 2012;33(7):1663-1676.
- (33) Cortese BM, Moore GJ, Bailey BA, Jacobson SW, Delaney-Black V, Hannigan JH. Magnetic resonance and spectroscopic imaging in prenatal alcohol-exposed children: preliminary findings in the caudate nucleus. *Neurotoxicol Teratol* 2006;28(5):597-606.
- (34) Johnson VP, Swayze VW, Sato Y, Andreasen NC. Fetal alcohol syndrome: craniofacial and central nervous system manifestations. *Am J Med Genet* 1996;61(4):329-339.
- (35) Joseph J, Warton C, Jacobson SW, Jacobson JL, Molteno CD, Eicher A, et al. Three-dimen-

sional surface deformation-based shape analysis of hippocampus and caudate nucleus in children with fetal alcohol spectrum disorders. *Hum Brain Mapp* 2014;35(2):659-672.

- (36) Lebel C, Rasmussen C, Wyper K, Walker L, Andrew G, Yager Jea. Brain Diffusion abnormalities in children with fetal alcohol spectrum disorder. *Alcohol Clin Exp Res* 2008;32(10):1732-1740.
- (37) Lebel C, Mattson SN, Riley EP, Jones KL, Adnams CM, May PA, et al. A Longitudinal Study of the Long-Term Consequences of Drinking during Pregnancy: Heavy In Utero Alcohol Exposure Disrupts the Normal Processes of Brain Development. *J Neurosci* 2012 October 31;32(44):15243-15251.
- (38) Li Z, Coles CD, Lynch ME, Ma X, Peltier S, Hu X. Occipital-temporal reduction and sustained visual attention deficit in prenatal alcohol exposed adults. *Brain Imaging Behav* 2008;2(1):39-48.
- (39) Mattson SN, Riley EP, Jernigan TL, Ehlers CL, Delis DC, Jones KL, et al. Fetal alcohol syndrome: A case report of neuropsychological, MRI, and EEG assessment of two children. *Alcohol Clin Exp Res* 1992;16(5):1001-1003.
- (40) Mattson SN, Riley EP, Sowell ER, Jernigan TL, Sobel DF, Jones KL. A Decrease in the Size of the Basal Ganglia in Children with Fetal Alcohol Syndrome. *Alcohol Clin Exp Res* 1996;20(6):1088-1093.
- (41) Meintjes EM, Narr KL, der Kouwe AJWv, Molteno CD, Pirnia T, Gutman B, et al. A tensor-based morphometry analysis of regional differences in brain volume in relation to prenatal alcohol exposure. *NeuroImage: Clinical* 2014;5(0):152-160.
- (42) Nardelli A, Lebel C, Rasmussen C, Andrew G, Beaulieu C. Extensive deep gray matter volume reductions in children and adolescents with fetal alcohol spectrum disorders. *Alcohol Clin Exp Res* 2011;35(8):1404-1417.
- (43) O'Hare ED, Kan E, Yoshii J, Mattson SN, Riley EP, Thompson PM, et al. Mapping cerebellar vermal morphology and cognitive correlates in prenatal alcohol exposure. *Neuroreport* 2005;16(12):1285-1290.
- (44) Rajaprakash M, Chakravarty MM, Lerch JP, Rovet J. Cortical morphology in children with alcohol-related neurodevelopmental disorder. *Brain Imaging Behav* 2014;4(1):41-50.
- (45) Riikonen R, Salonen I, Partanen K, Verho S. Brain perfusion SPECT and MRI in foetal alcohol syndrome. *Dev Med Child Neurol* 1999;41(10):652-659.
- (46) Riikonen RS, Nokelainen P, Valkonen K, Kolehmainen AI, Kumpulainen KI, Könönen M, et al. Deep serotonergic and dopaminergic structures in fetal alcoholic syndrome: a study with

nor- β -CIT-single-photon emission computed tomography and magnetic resonance imaging volumetry. *Biol Psychiatry* 2005;57(12):1565-1572.

- (47) Riley EP, Mattson SN, Sowell ER, Jernigan TL, Sobel DF, Jones KL. Abnormalities of the corpus callosum in children prenatally exposed to alcohol. *Alcohol Clin Exp Res* 1995;19(5):1198-1202.
- (48) Roussotte FF, Sulik KK, Mattson SN, Riley EP, Jones KL, Adnams CM, et al. Regional brain volume reductions relate to facial dysmorphology and neurocognitive function in fetal alcohol spectrum disorders. *Hum Brain Mapp* 2012;33(4):920-937.
- (49) Sowell ER, Jernigan TL, Mattson SN, Riley EP, Sobel DF, Jones KL. Abnormal Development of the Cerebellar Vermis in Children Prenatally Exposed to Alcohol: Size Reduction in Lobules I-V. *Alcohol Clin Exp Res*, 1996;20(1):31-34.
- (50) Sowell ER, Thompson PM, Mattson SN, Tessner KD, Jernigan TL, Riley EP, et al. Voxel-based morphometric analyses of the brain in children and adolescents prenatally exposed to alcohol. *Neuroreport* 2001;12(3):515-523.
- (51) Sowell ER, Thompson PM, Peterson BS, Mattson SN, W, S.E., Henkenius AL, et al. Mapping cortical gray matter asymmetry patterns in adolescents with heavy prenatal alcohol exposure. *NeuroImage* 2002;17(4):1807-1819.
- (52) Sowell ER, Mattson SN, Kan E, Thompson PM, Riley EP, Toga AW. Abnormal Cortical Thickness and Brain–Behavior Correlation Patterns in Individuals with Heavy Prenatal Alcohol Exposure. *Cereb Cortex* 2008 January 01;18(1):136-144.
- (53) Swayze VW, Johnson VP, Hanson JW, Piven J, Sato Y, Giedd JN, et al. Magnetic resonance imaging of brain anomalies in fetal alcohol syndrome. *Pediatrics* 1997;99(2):232-240.
- (54) Treit S, Lebel C, Baugh L, Rasmussen C, Andrew G, Beaulieu C. Longitudinal MRI reveals altered trajectory of brain development during childhood and adolescence in fetal alcohol spectrum disorders. *J Neurosci* 2013;33(24):10098-10109.
- (55) Willoughby KA, Sheard ED, Nash K, Rovet J. Effects of prenatal alcohol exposure on hippocampal volume, verbal learning, and verbal and spatial recall in late childhood. *J Int Neuropsychol Soc* 2008;14(06):1022-1033.
- (56) Yang Y, Phillips OR, Kan,E., Sulik,K.K., Mattson SN, Riley EP, Jones KL, et al. Callosal Thickness Reductions Relate to Facial Dysmorphology in Fetal Alcohol Spectrum Disorders. *Alcohol Clin Exp Res* 2012;36(5):798-806.
- (57) Yang Y, Roussotte F, Kan E, Sulik KK, Mattson SN, Riley EP, et al. Abnormal cortical thick-

- ness alterations in fetal alcohol spectrum disorders and their relationships with facial dysmorphology. *Cereb Cortex* 2012;22(5):1170-1179.
- (58) Zhou D, Lebel C, Lepage C, Rasmussen C, Evans A, Wyper K, et al. Developmental cortical thinning in fetal alcohol spectrum disorders. *NeuroImage* 2011;58(1):16-25.
- (59) Wozniak JR, Mueller BA, Chang PN, Muetzel RL, Caros L, Lim KO. Diffusion Tensor Imaging in Children with Fetal Alcohol Spectrum Disorders. *Alcohol Clin Exp Res* 2006;30(10):1799-1806.
- (60) Sowell ER, Johnson A, Kan E, Lu LH, Van Horn JD, Toga AW, et al. Mapping White Matter Integrity and Neurobehavioral Correlates in Children with Fetal Alcohol Spectrum Disorders. *J Neurosci* 2008 February 06;28(6):1313-1319.
- (61) Hermoye L, Saint-Martin C, Cosnard G, Lee SK, Kim J, Nassogne MC, et al. Pediatric diffusion tensor imaging: normal database and observation of the white matter maturation in early childhood. *NeuroImage* 2006;29(2):493-494-504.
- (62) Pannek K, Guzzetta A, Colditz P, Rose S. Diffusion MRI of the neonate brain: acquisition, processing and analysis techniques. *Pediatr Radiol* 2012 10/01;42(10):1169-1182.
- (63) Beaulieu C. The basis of anisotropic water diffusion in the nervous system - a technical review. *NMR Biomed* 2002;15(7-8):435-436-455.
- (64) Dubois J, Dehaene-Lambertz G, Kulikova S, Poupon C, Hüppi PS, Hertz-Pannier L. The early development of brain white matter: A review of imaging studies in fetuses, newborns and infants. *Neuroscience* 2014 <http://dx.doi.org/10.1016/j.neuroscience.2013.12.044>.
- (65) Geng X, Gouttard S, Sharma A, Gu H, Styner M, Lin W, et al. Quantitative tract-based white matter development from birth to age 2 years. *NeuroImage* 2012 7/2;61(3):542-557.
- (66) Paus T, Pesaresi M, French L. White matter as a transport system. *Neuroscience* 2014 <http://dx.doi.org/10.1016/j.neuroscience.2014.01.055>.
- (67) Fryer S, Schweinsburg BC, Bjorkquist OA, Frank LK, Mattson SM, Spadoni ADea. Characterisation of white matter microstructure in fetal alcohol spectrum disorders. *Alcohol Clin Exp Res* 2009;33(3):514-521.
- (68) Li L, Coles CD, Lynch ME, Hu X. Voxelwise and Skeleton-Based Region of Interest Analysis of Fetal Alcohol Syndrome and Fetal Alcohol Spectrum Disorders in Young Adults. *Hum Brain Mapp* 2009;30(10):3265-3274.
- (69) Ma X, Coles CD, Lynch ME, LaConte SM, Zurkiyara O, Wang D, et al. Evaluation of Corpus Callosum Anisotropy in Young Adults With Fetal Alcohol Syndrome According to Diffusion Tensor Imaging. *Alcohol Clin Exp Res* 2005;29(7):1214-1222.

- (70) Spottiswoode BS, Meintjes EM, Anderson AW, Molteno CD, Stanton ME, Dodge NC, et al. Diffusion tensor imaging of the cerebellum and eyeblink conditioning in fetal alcohol spectrum disorder. *Alcohol Clin Exp Res* 2011;35(12):2174-2183.
- (71) Wozniak JR, Muetzel RL, Mueller BA, McGee CL, Freerks MA, Ward EE, et al. Microstructural Corpus Callosum Anomalies in Children With Prenatal Alcohol Exposure: An Extension of Previous Diffusion Tensor Imaging Findings. *Alcohol Clin Exp Res* 2009;33(10):1825-1835.
- (72) Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, et al. Ethanol induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science* 2000;287:1056-1060.
- (73) Roussotte FF, Soderberg L, Sowell ER. Structural, Metabolic and Functional Brain Abnormalities as a Result of Prenatal Exposure to Drugs of Abuse: Evidence from Neuroimaging. *Neuropsychol Rev* 2010;20:376-397.
- (74) Blüml S, Wisnowski JL, Nelson MD, Paquette L, Gilles FH, Kinney HC, et al. Metabolic Maturation of the Human Brain From Birth Through Adolescence: Insights From In Vivo Magnetic Resonance Spectroscopy. *Cereb Cortex* 2012 September 05.
- (75) Ross BD, Colletti P, Lin A. MR spectroscopy of the brain: Neurospectroscopy, In: Edelman, Hesselink, Zlatkin & Cruess, editor. *Clinical Magnetic Resonance Imaging*. 3rd ed. Philadelphia: Saunders-Elsevier, 2006. p. 1840-1910.
- (76) Astley SJ, Richards T, Aylward EH, Olson HC, Kerns K, Brooks A, et al. Magnetic resonance spectroscopy outcomes from a comprehensive magnetic resonance study of children with fetal alcohol spectrum disorders. *J Magn Reson* 2009;27(6):760-778.
- (77) Fagerlund Å, Heikkinen S, Autti-Rämö I, Korkman M, Timonen M, Kuusi T, et al. Brain metabolic alterations in adolescents and young adults with fetal alcohol spectrum disorders. *Alcohol Clin Exp Res* 2006;30(12):2097-2104.
- (78) du Plessis L, Jacobson JL, Jacobson SW, Hess AT, van der Kouwe A, Avison MJ, et al. An In Vivo 1H Magnetic Resonance Spectroscopy Study of the Deep Cerebellar Nuclei in Children with Fetal Alcohol Spectrum Disorders. *Alcohol Clin Exp Res* 2014;38(5):1330-1338.
- (79) Burke MW, Palmour RM, Ervin FR, Ptito M. Neuronal reduction in frontal cortex of primates after prenatal alcohol exposure. *Neuroreport* 2009;20(1).
- (80) An L, Zhang T. Spatial cognition and sexually dimorphic synaptic plasticity balance impairment in rats with chronic prenatal ethanol exposure. *Behav Brain Res* 2013 11/1;256(0):564-574.

- (81) Sowell ER, Johnson A, Kan E, Lu LH, Van Horn JD, Toga AW, et al. Mapping white matter integrity and neurobehavioral correlates in children with fetal alcohol spectrum disorders. *J Neurosci* 2008;28(6):1313-1319.
- (82) Lebel C, Roussotte F, Sowell ER. Imaging the Impact of Prenatal Alcohol Exposure on the Structure of the Developing Human Brain. *Neuropsychol Rev* 2011;21:102-118.
- (83) Wozniak JR, Muetzel RL. What does diffusion tensor imaging reveal about the brain and cognition in fetal alcohol spectrum disorders? *Neuropsychol Rev* 2011;21(2):133-147.
- (84) Lebel C, Rasmussen C, Wyper K, Andrew G, Beaulieu C. Brain Microstructure Is Related to Math Ability in Children With Fetal Alcohol Spectrum Disorder. *Alcohol Clin Exp Res* 2010;34(2):354-363.
- (85) Leigland LA, Ford MM, Lerch JP, Kroenke CD. The Influence of Fetal Ethanol Exposure on Subsequent Development of the Cerebral Cortex as Revealed by Magnetic Resonance Imaging. *Alcohol Clin Exp Res* 2013;37(6):924-932.
- (86) Biswal B, Zerrin Yetkin F, Haughton VM, Hyde JS. Functional connectivity in the motor cortex of resting human brain using echo-planar mri. *Magn Reson Med* 1995;34(4):537-541.
- (87) Hagmann P, Grant PE, Fair DA. MR Connectomics: A Conceptual Framework for Studying The Developing Brain. *Frontiers in Systems Neuroscience* 2012;6.
- (88) Norman AL, O'Brien JW, Spadoni AD, Tapert SF, Jones KL, Riley EP, et al. A functional magnetic resonance imaging study of spatial working memory in children with prenatal alcohol exposure: contribution of familial history of alcohol use disorders. *Alcohol Clin Exp Res* 2013;37(1):132-140.
- (89) Spadoni AD, Bazinet AD, Fryer SL, Tapert SF, Mattson SN, Riley EP. BOLD response during spatial working memory in youth with heavy prenatal alcohol exposure. *Alcohol Clin Exp Res* 2009;33(12):2067-2076.
- (90) Malisza KL, Allman AA, Shiloff D, Jakobson L, Longstaffe S, Chudley AE. Evaluation of spatial working memory function in children and adults with fetal alcohol spectrum disorders: a functional magnetic resonance imaging study. *Pediatric Research* 2005;58(6):1150-1157.
- (91) Roussotte FF, Bramen JE, Nunez SC, Quandt LC, Smith L, O'Connor MJ, et al. Abnormal brain activation during working memory in children with prenatal exposure to drugs of abuse: The effects of methamphetamine, alcohol, and polydrug exposure. *NeuroImage* 2011;54:3067-3075.
- (92) Roussotte FF, Rudie JD, Smith L, O'Connor MJ, Bookheimer SY, Narr KL, et al. Frontostriatal Connectivity in Children during Working Memory and the Effects of Prenatal Metham-

phetamine, Alcohol and Polydrug Exposure. *Dev Neurosci* 2012;34:43-57.

- (93) Diwadkar VA, Meintjes EM, Goradia D, Dodge NC, Warton C, Molteno CD, et al. Differences in cortico-striatal-cerebellar activation during working memory in syndromal and nonsyndromal children with prenatal alcohol exposure. *Hum Brain Mapp* 2013;34(8):1931-1945.
- (94) Astley SJ, Aylward EH, Olson HC, Kerns K, Brooks A, Coggins TE, et al. Functional magnetic resonance imaging outcomes from a comprehensive magnetic resonance study of children with fetal alcohol spectrum disorders. *J Neurodev Disord* 2009;1(1):61-80.
- (95) O'Hare ED, Lu LH, Houston SM, Bookheimer SY, Mattson SN, O'Connor MJ, et al. Altered frontal-parietal functioning during verbal working memory in children and adolescents with heavy prenatal alcohol exposure. *Hum Brain Mapp* 2009;30(10):3200-3208.
- (96) O'Brien JW, Norman AL, Fryer SL, Tapert SF, Paulus MP, Jones KL, et al. Effect of predictive cuing on response inhibition in children with heavy prenatal alcohol exposure. *Alcohol Clin Exp Res* 2013;37(4):644-654.
- (97) Fryer SL, Tapert SF, Mattson SN, Paulus MP, Spadoni AD, Riley EP. Prenatal alcohol exposure affects frontal-striatal BOLD response during inhibitory control. *Alcohol Clin Exp Res* 2007;31(8):1415-1424.
- (98) Sowell ER, Lu LH, O'Hare ED, McCourt ST, Mattson SN, O'Connor MJ, et al. Functional magnetic resonance imaging of verbal learning in children with heavy prenatal alcohol exposure. *Neuroreport* 2007;18(7):635-639.
- (99) Meintjes EM, Jacobson JL, Molteno CD, Gatenby JC, Warton C, Cannistraci CJ, et al. An FMRI study of number processing in children with fetal alcohol syndrome. *Alcohol Clin Exp Res* 2010;34(8):1450-1464.
- (100) Santhanam P, Coles CD, Li Z, Li L, Lynch ME, Hu X. Default mode network dysfunction in adults with prenatal alcohol exposure. *Psychiatry Res* 2011;194(3):354-362.
- (101) Wozniak JR, Mueller BA, Bell CJ, Muetzel RL, Hoecker HL, Boys CJ, et al. Global functional connectivity abnormalities in children with fetal alcohol spectrum disorders. *Alcohol Clin Exp Res* 2013;37(5):748-756.
- (102) Wozniak JR, Mueller BA, Muetzel RL, Bell CJ, Hoecker HL, Nelson ML, et al. Inter-Hemispheric Functional Connectivity Disruption in Children with Prenatal Alcohol Exposure. *Alcohol Clin Exp Res* 2011;35(5):849-861.
- (103) Sowell ER, Mattson SN, Thompson PM, Jernigan TL, Riley EP, & Toga AW. Mapping callosal morphology and cognitive correlates: effects of heavy prenatal alcohol exposure. *Neurology* 2001;57(2):235-244.

- (104) Fix JD. Basal Ganglia and the Striatal Motor System. In: ., editor. Neuroanatomy (Board Review Series). 4th ed ed. Baltimore: Wolters Kluwer; Lippincott Williams & Wilkins; 2008. p. 274-281.
- (105) Gilmore JH, Shi F, Woolson SL, Knickmeyer RC, Short SJ, Lin W, et al. Longitudinal Development of Cortical and Subcortical Gray Matter from Birth to 2 Years. *Cereb Cortex* 2012 November 01;22(11):2478-2485.
- (106) Shaw P, Kabani NJ, Lerch JP, Eckstrand K, Lenroot R, Gogtay N, et al. Neurodevelopmental Trajectories of the Human Cerebral Cortex. *J Neurosci* 2008 April 02;28(14):3586-3594.
- (107) Norman AL, Crocker N, Mattson SN, Riley EP. Neuroimaging and fetal alcohol spectrum disorders. *Dev Disabil Res Revs* 2009;15(3):209-217.
- (108) Wozniak JR, Muetzel RL. What Does Diffusion Tensor Imaging Reveal About the Brain and Cognition in Fetal Alcohol Spectrum Disorders. *Neuropsychol Rev* 2011;21(2):133-147.
- (109) Gonçalves R, de C., Vasconcelos MM, Faleiros LO, Cruz Jr, Luiz Celso, Hygino da, Domingues RC, Brito AR, et al. Proton magnetic resonance spectroscopy in children with fetal alcohol spectrum disorders. *Arq Neuropsiquiatr* 2009;67:254-261.
- (110) Karadottir R, Attwell D. Combining patch-clamping of cells in brain slices with immunocytochemical labeling to define cell type and developmental stage. *Nat Protoc* 2006;1:1977-1986.
- (111) Rodier PM. Vulnerable periods and processes during central nervous system development. *Environ Health Perspect* 1994;102(suppl. 2):121-124.
- (112) Rodier PM. Environmental causes of central nervous system maldevelopment. *Pediatrics* 2004;113:1076-1083.
- (113) Doria V, Beckmann CF, Arichi T, Merchant N, Groppo M, Turkheimer FE, et al. Emergence of resting state networks in the preterm human brain. *Proceedings of the National Academy of Sciences* 2010 November 16;107(46):20015-20020.

Chapter 3

Study outline and methods

In the previous chapter a critical review of the literature describing current knowledge of the effects of prenatal alcohol exposure on multimodal neuroimaging markers of the developing brain was presented. Here, an overview of the birth cohort in which this study is nested and specific methodological details of the this thesis project is described.

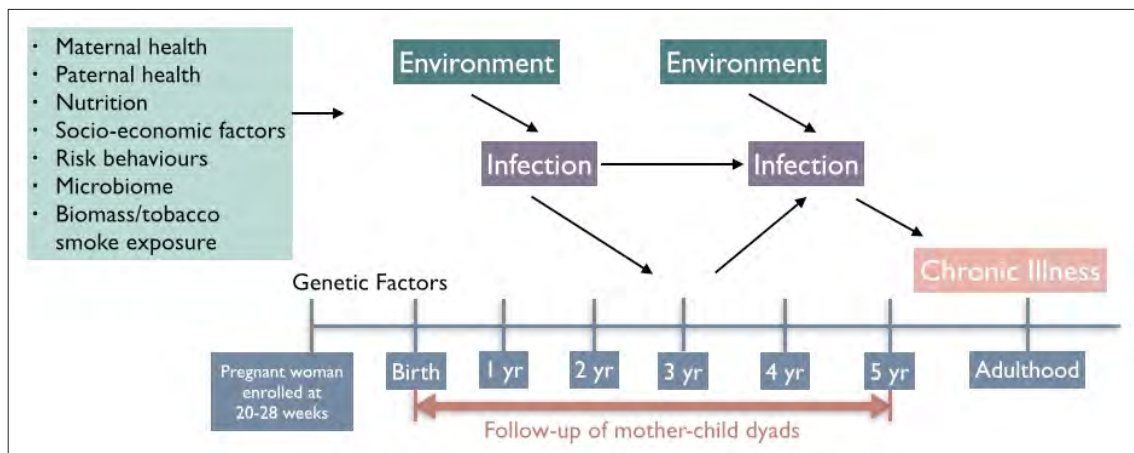
3.1 Study design and population

3.1.2 Background overview

Study participants are members of the established Drakenstein Child Health Study (DCHS), a birth cohort recruited from the Drakenstein sub-district, located near the Cape Winelands, Western Cape, South Africa (current N = 1100 mother-infant dyads) designed to study the etiology of childhood pneumonia and the determinants of child health in a peri-urban area in South Africa, funded by the Gates Foundation. The cohort consists of a South African sample of English, Afrikaans or isiXhosa mother-infant dyads (mothers will be aged 18+). The primary aim of the parent study is to investigate etiology, progression and risk factors for childhood pneumonia and the impact on child health. The study investigates the role and interaction of possible risk factors in seven areas (environmental, infectious, nutritional, genetic, psychosocial, maternal and immunological risk factors) that may impact child health.

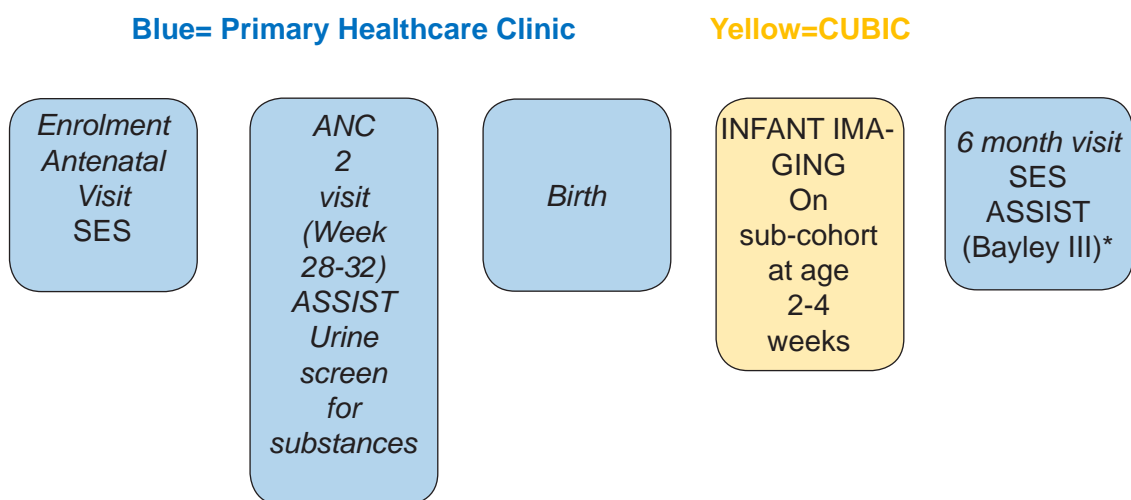
Figure 1 shows a conceptual framework for the study design. The birth cohort design recruits pregnant women attending one of two primary health care clinics serving different populations - TC Newman (serving a mixed race population) and Mbekweni (serving a black African population). Consenting women complete study questionnaires and specimen collection and are scheduled for follow-up study visits. Antenatal and postnatal follow-up visits occur at primary health care clinics, with the birth, 6 week and annual study visits taking place at Paarl hospital. Infants are followed at routine well baby visits according to the current national program, at 10 and 14 weeks and 6, 9, 18, 30, 42 and 54 months post delivery.

Figure 1: DCHS: Study Timeline Overview



In the nested sub-study used for this thesis project we assessed infants with significant alcohol exposure and infants with no significant history of substance abuse (Figure 2).

Figure 2: DCHS: Infant Imaging Sub-Study Timeline



The DCHS is located in the Drakenstein area in the town of Paarl, a peri-urban area, 60 km outside Cape Town, South Africa with a population of approximately 200,000. The community is stable, with low levels of immigration or emigration. The local economy is based around commercial agriculture and light industry. More than 90% of the population access health care in the public sector including antenatal and child health services. (1) This area has a well established, free primary health care system, with high coverage for childhood immunizations including Hemophilus B and 13-valent Pneumococcal conjugate vaccine. The public health system is comprised of 23 primary health care clinics

and one centralized hospital, Paarl Hospital, where all births and all hospital based paediatric care, including admissions occur. Similar to many low-middle income countries (LMICs), the area has a high burden of childhood diseases and pneumonia (2) and a high prevalence of risk factors associated with pneumonia or severe disease, such as alcohol exposure, tobacco smoke exposure, malnutrition and poverty. Mother-infant dyads are enrolled at 20-28 weeks' gestation and followed until children reach 5 years of age.

3.1.2 Recruitment and enrolment

Pregnant mothers were screened by the DCHS study staff at one of the two antenatal clinics in the community, TC Newman Hospital or Mbekweni clinic. Mothers were asked to provide informed consent to participate in the parent study and enrolled from 20-28 weeks' gestation during their routine antenatal visit for ultrasound scanning at Primary Health Care facilities or at Paarl Hospital. Exclusion criteria for the parent study were minimal in order to maximize generalizability: those who do not live in the region and thus cannot be readily followed-up or those who intended to move out of the district within the following 2 years. Infants were enrolled at birth and mother infant pairs followed up for 5 years on the parent study. This sub-study followed the infants to age 6 months.

Following written informed consent, women who wished to participate were requested to complete a selection of self-report questionnaires. Trained female fieldworkers administered the measures to study participants in the language of their choice (English, Afrikaans, or isiXhosa). Fieldworkers were fluent in English and either Afrikaans or isiXhosa, they had appropriate research experience as well as traits previously described as affecting women's willingness to divulge personal information. Prior to commencement of data collection, fieldworkers received in-service training on all aspects of Good Clinical Practice (GCP). Although most of these measures were designed to be self-administered, a study-wide decision was made that fieldworker-administration would improve data quality, and would minimise bias in data collection. This is standard practice in South African studies such as ours as a result of low literacy rates in our population. (3) Translation of questionnaires from English to Afrikaans and isiXhosa was done using standard forward- and back-translation processes.

The prenatal assessment included detailed sociodemographic enquiry. Alcohol and other drug use were measured by WHO's Alcohol, Smoking and Substance Involvement Screening Test (ASSIST), which has been validated for local pregnant populations. (4,5) Biological sampling included a blood draw and urine dipstick testing at clinic visits to serve as biomarkers of substance use. (6) This approach was taken in order to ensure that the problem of polysubstance abuse in the selected sample was minimized as far as possible. A cut-off score of moderate-severe alcohol use risk on the ASSIST tool was used for inclusion in the alcohol-exposure group.

A range of measures were used to assess medical, nutritional, psychological and sociological variables that may play a central role in impacting positively or negatively on maternal and child health in general. Several such measures have been well validated in local studies of maternal mental health, and in the South African Stress and Health Survey (4,5,7,8).

Inclusion/exclusion criteria for this **sub-study** varied depending on into which group the infant was being recruited. For inclusion into the alcohol exposed group, mothers reported a minimum score of 11 on the alcohol questions on the ASSIST. In addition to this initial screen, mothers were required to give a positive history of alcohol use in any of the 3 trimesters of pregnancy at levels consistent with WHO moderate-severe alcohol use (either drinking 2 or more times a week or 2 or more drinks per occasion). The healthy control group scored below above cut-off scores on the ASSIST and had a negative urine screen for any drugs of abuse. Exclusion criteria for both groups included a positive urine screen for other drugs of abuse (any group), prematurity (<34 weeks), low apgar scores (<7 at 5 minutes) and/or history of NICU admission for hypoxic ischemic encephalopathy or other significant neonatal complication (such as neonatal jaundice requiring phototherapy) and an identified genetic syndrome or congenital abnormalities (such as spina bifida or hydrocephalus).

Mothers were contacted telephonically in the weeks after birth based on above inclusion criteria. The mother had an opportunity to ask questions about the testing and an appointment was made then to attend the Combined Universities Brain Imaging Centre (CU-BIC), at Tygerberg Hospital campus when the infants were 2-4 weeks of age. Although

mothers had signed consent as part of the parent protocol consent for the imaging, they had the opportunity of withdrawing consent to participate with imaging sub-study without affecting their participation in the parent project when they were approached at this stage. Because this was an extra visit and not situated at the normal study sites, infants and mothers were collected from home and transported to CUBIC. After the scan and neurobehavioral assessment they were transported back to their homes.

At the imaging appointment the imaging team ensured the mother had received sufficient information about testing by going through a more detailed consent form and answering any questions. Mothers were asked to sign this separate consent form for infant brain imaging. (Appendix A)

3.2 Investigations and data collection

From the time of their first antenatal visit (week 20-28), mothers were assessed using scales as per DCHS protocol. This assessment included validated questionnaires addressing socioeconomic factors and substance use as detailed below.

The following psychosocial measures were included:

3.2.1 Maternal measures

Sociodemographic variables - A questionnaire to evaluate socioeconomic status (SES), modified from the version used in the *South African Stress and Health Study* (SASH), (8) assessed income and education; governmental financial assistance in the form of social grants; household composition; food security and amenities (including piped water electricity, electric stove and functional telephone).

Alcohol and Substance Misuse - Substance use was evaluated using the World Health Organisation's Alcohol, Smoking and Substance Involvement Screening Test (ASSIST) (5) (Appendix B) This tool was originally developed to detect substance use disorders in the primary care setting. It has good reported reliability, feasibility and validity across global, multisite studies. (4,5) The ASSIST comprises seven items assessing alcohol

and other drug use across ten categories (ie. tobacco products; alcoholic beverages; cannabis; cocaine; amphetamine-type stimulants; inhalants, sedatives or sleeping pills; hallucinogens; opioids; and a general category entitled “other”); as well as an eighth item enquiring about a history of intravenous drug use. Total scores are obtained for each substance by summing individual item responses - a higher score is indicative of greater substance-related risk. Scores of 0 – 10 for alcohol and 0 – 3 for illicit drugs were used to indicate that a participant was at low risk for substance-related health problems; 11–26 (alcohol) and 4–26 (illicit drugs) to indicate moderate risk; and scores >26 to indicate that a participant was at high risk of experiencing severe problems, with likely substance dependence. (5) Urine substance abuse screen was performed on all mothers at their antenatal visits. (6)

3.2.2 Infant measures

Infant anthropometry- Weight, length and head circumference were measured at the imaging visit. Trained clinical staff recorded infant weight, using a digital scale with a precision level of 10 g. Low weight-for-age Z (WAZ) score (standard deviation score) is defined as a score less than or equal to 2 standard deviations below the mean weight-for-age value (WHO 1995). Similarly, infants with low head circumference-for-age z score (HCAZ) were categorized as falling 2 or more standard deviations below the mean head circumference-for-age cut-off. The Z-score classification system is advantageous, as it is sex-independent, employs a linear scale, and enables a comparison of results across age groups and indicators.

Dubowitz neurobehavioral scale - This is a well-validated measure which has been used to study early neurological and behavioral changes. This measure was performed at the same visit as the neuroimaging in a quiet, warmed room on a comfortable surface in the Combined Universities Brain Imaging Centre. (Appendix C). (9)

Bayley III Scales of Infant Development at 6 months - This is a well-validated individually-administered developmental assessment tool used in infants from 0-42 months of age. (10) The primary purpose of this instrument is to identify children with developmental delay both at a point in time as well as longitudinally in order to provide informa-

tion regarding a child's developmental profile as well as changes over time. (11,12) The Bayley III assesses infant development across five scales - cognitive, language, motor, socio-emotional and adaptive behavior. These composite scales are further divided into subscales: the language scale into receptive and expressive communication subscales; the motor scale into gross and fine motor; and the adaptive behavior scale into ten subscales, seven of which are applicable to infants younger than one year – communication, health and safety, leisure, self-care, self-direction, social and motor. The cognitive, language and motor assessments are largely conducted using directly observed items administered to the infant. The socio-emotional and adaptive behavior domains are assessed using reported responses from a questionnaire completed by the primary caregiver. Items included in the Bayley III were informed by developmental theory and research identifying key behaviors typifying normal developmental in infants and toddlers. Psychometric data for this tool suggest a high degree of internal consistency (reliability) across all five scales, as well as construct validity within scales (ie. each item was found to have a higher correlation with the scale in which it is placed than with the other scales). (11,12) This assessment tool has been used widely in LMIC settings such as South Africa, (13) and remains a gold-standard measure of developmental milestones in infants and toddlers globally.

While no overall (total) developmental score is provided by the Bayley III, it does provide four types of norm-referenced scores across the subscales: scaled scores, composite scores, percentile ranks, and growth scores. For our purposes, scaled scores were calculated from captured total raw scores on each subtest using the specialised software *Bayley-III Scoring Assistant Update Version 2.0.2 with Bayley-III PDA conduit (BayleyIII_PDA_2_0_2.exe)*. Scaled scores represent a child's performance on a subtest relative to his or her same-age peers and are sensitive to subtle differences in developmental outcomes. They are scaled to a metric with a range of 1 to 19, a mean of 10 and a standard deviation of 3. While there is no universally accepted definition of developmental delay, criteria based on standard deviations (SDs) below the mean of a reference group is the most widely-used approach. (12)

Bayley III Scales of Infant Development was administered at 6 months of age at either

the TC Newman or Mbekweni clinic study site. The sites have a dedicated room in a quiet section of the clinic in close proximity to the well-baby clinic. The Bayley III was administered by one of a team of 3 trained professionals: Dr Kirsty Donald (Pediatric Neurologist and sub-study PI, KD), Sr. Robyn Kalan (professional nurse, RK) and Mrs Elizabeth Honeth (physiotherapist, EH). Dr Donald has trained as a pediatric neurologist at Red Cross War Memorial Children's Hospital, University of Cape Town. She has training and experience in a range of developmental assessment tools including the Bayley III Scales of Infant Development. RK received training in the Bayley III in 2010 during her involvement in another infant outcome study and is certified and experienced in the use of the tool. EH is an experienced physiotherapist with expertise in the assessment of young infants. She was trained in Cape Town by KD and RK. Quality control of the testing took place regularly in order to ensure fidelity to the verbatim instructions for test administration as well as detailed instructions for data scoring. KD visited sites regularly for the duration of the study to ensure compliance with the test manual and standardized data collection, and all completed protocols were checked independently to ensure accurate scoring procedures had been implemented. Individuals involved in Bayley III administration were periodically required to complete an assessment under observation to ensure overall accuracy in the assessment process.

Neuroimaging-Imaging was performed on a Siemens 3 Tesla Magnetom Allegra MRI scanner at CUBIC, situated on the Stellenbosch University Health Sciences Campus. This is a compact, small bore (22cm field of view), dedicated brain scanner. The Allegra system is equipped with a single channel head coil. Imaging children in this age group without sedation is a significant logistical challenge which is not under-estimated by our group. According to (14) Matthias, *et al.*, in their review of Pediatric Magnetic Resonance Research and the Minimal Risk Standard., the use of sedation in pediatric imaging constitutes greater than minimal risk. (1) (15) Natural sleep remains a good way of achieving MRI imaging in this young age group. In particular stage 3 sleep, with its higher arousal threshold and reduced spontaneous movements. This stage of sleep tends to occur early after sleep initiation, especially in young children.

Technical protocol (Appendix D)

3.2.3 Imaging summary:

Structural MRI:

MRI technology detects differences between various tissue-types based on the response of water molecules to a high intensity magnet. These data are reconstructed to produce visual images. Measures of structural MRI reported include qualitative descriptions as well as quantitative methods, which include morphometric properties of particular brain structures and volumetric analyses.(16)

Two T2 images, sagittal

3D T2-weighted MRI: repetition time (TR)=3500ms; TE (echo time)=354ms; slice thickness 1mm; 128 slices; voxel size 1.0×1.0×1.0mm. *Scan time*: 5min41s / scan.

Two T1-weighted images

3D T1-weighted multi-echo MRI: TR=2530ms; TE(1-4)=1.53, 3.21, 4.89, 6.57; flip angle=7°; slice thickness 1mm; 128 slices; voxel size 1.3×1.0×1.3 mm. *Scan time*: 6min45s / scan.

¹H-MRS voxels

Use of proton magnetic resonance spectroscopy (¹H-MRS) in brain research is the study of concentrations of specific metabolites important in neurotransmitter production and metabolism, measured in chosen regions of interest.

Sagittal T1-weighted images were captured using a five-echo 3D magnetization prepared rapid-gradient echo sequence [repetition time (TR) = 2530; echo time (TE) = 1.64, 3.5, 5.36, 7.22, 9.08; inversion time (TI) = 1200; flip Angle = 7°; field of view = 256 x 256 mm; matrix = 256 x 256, 1-mm thick slice, 192 slices; generalized autocalibrating partially parallel acquisitions (GRAPPA) acceleration factor = 2].

These images were used to define an ¹H-MRS voxel in the anterior cingulate. Spectroscopy data were acquired in the anterior cingulate using a motion-navigated point resolved

spectroscopy (PRESS) sequence with 2 mL voxels and 64 excitations. This sequence, which allows motion-correction, was developed through collaboration of UCT with Massachusetts General Hospital and uses an echoplanar imaging volumetric navigator to track motion and correct both voxel position and shim in real time for each TR. Chemical shift selective pulses (CHESS) water suppression was applied and an unsuppressed reference scan of 8 excitations was acquired for each voxel. Data were analyzed using LCModel to estimate metabolite ratios, in particular N-acetyl-aspartate (NAA)/Creatinine (Cr), Choline (Cho)/Cr and myo-Inositol (mI)/Cr. Absolute concentrations of glutamate, glutamine, NAA, Cho, Cr and mI were also calculated.

Brain area 1: Parietal white. Sequence: PRESS, TR=2000, TE=30 (128 averages); Voxel size 25x25x25 mm. *Scan time:* less than 6min total with water scaling.

Brain area 2: Parietal gray matter. Sequence: PRESS, TR=2000, TE=30 (128 averages); Voxel: mm, 25 x 25 x 25 mm. *Scan time:* less than 6min total with water scaling.

These areas provide reproducible ¹H-MRS data in neonates and allow a good comparison between the developing white and gray matter tissue.

Diffusion Tensor Imaging

Diffusion tensor imaging (DTI) is an MRI technique that determines the diffusion direction of water molecules. (17,18) Analysis of this data allows the degree of structural organization of a particular tissue to be determined. DTI measures are generally reported in terms of:

1. Fractional Anisotropy (FA): this represents the magnitude of the fraction tensor due to anisotropic diffusion (ie. The degree to which the diffusion direction of molecules is differentially restricted by, for example, cell membranes or myelin)
2. Mean diffusivity (MD): this represents the average orientation and magnitude of molecular diffusion in three dimensions.

Typically, higher FA and lower MD values are found in intact as compared to damaged/abnormal white matter. (18,19) However, DTI measures do not identify isolated aspects of white matter integrity or maturation, but are likely to represent integrity of axonal structure as well as organization at different levels

Spin echo EPI sequence: Two diffusion-weighted images per phase direction anterior-posterior (AP) and posterior-anterior (PA) respectively, each with the following parameters: 45 diffusion directions; $b=0\text{sec/mm}^2$; repetition time 7900 ms; echo time 90 ms; b-value of 1000sec/mm^2 ; slice thickness of 1.6 mm; voxel size: $1.3\times 1.3\times 1.6\text{ mm}$. Scan time: 6min27s / scan.

Ep2d_bold_resting

BOLD EPI: TR 2000 ms; TE 30 ms; flip angle= 77° slice thickness 4 mm; 25 slices; voxel size: $2.5\times 2.5\times 4.0\text{ mm}$. Scan time: 6min04s.

Total scan time: 55 minutes

Statistical Analysis

A preliminary power analysis based on existing neonatal imaging data indicated that our sample size was appropriate. Analysis of variance was undertaken to compare imaging variables in the subject groups. An n of 50 in each group was calculated to enable detection of an effect of Cohen's d of .5, with alpha at .05 and power at .80 for cross sectional analysis. For within subjects analysis, there is more power and detection of an effect size of .36 is possible. Correlational analyses were undertaken to explore the relationship between structural MRI volumes, DTI variables, resting blood oxygen level dependent (BOLD) variables, prenatal exposure to alcohol, and early behavioral and developmental measures. The analyses were designed to provide a key understanding of functional and structural brain abnormalities in infants after moderate to heavy prenatal alcohol exposure.

Structural MRI: Images were brain extracted with FSL 5.0 brain extraction tool (BET) (20) and exported to SPM8 (www.fil.ion.ucl.ac.uk/spm/software/spm8) for further processing. Within SPM8, the T2 images were co-registered to a custom infant T2 template in Montreal Neurological Institute (MNI) standard space created by a group at the University of North Carolina (UNC). (21) After co-registration, images were inspected for proper alignment before non-linear registration to the same infant template. After non-linear registration, images that displayed excessive warping or other problems with registration were discarded (28 alcohol-exposed and 45 control infants were included for the final analysis). T2 images were then segmented into gray and white matter maps by utilizing infant priors designed by UNC. (21) To preserve the regional volumetric information, the T2 images were modulated during the transformation to the gray matter priors.

Volumetric information was extracted from the gray matter segmented images for 90 anatomical regions as defined by the automated anatomical labeling (AAL) atlas. (22) The volumes were then exported to SPSS 22.0 for further statistical analysis.

Evaluation for correlations between imaging data as above and the functional measures of development and behaviour were made.

DTI: Traditional scalar metrics derived from DTI data were analyzed and included fractional anisotropy (FA), which represents axon integrity and/or packing density, and mean diffusivity (MD), which represents the mean water mobility within the white matter. Diffusion tensor images were analyzed using the FMRIB's Diffusion Toolbox (FDT) and Tract-Based Spatial Statistics (TBSS) (FMRIB Software Library - FSL v5.0). (23) Each subject's diffusion weighted image was registered to the corresponding b=0 image to correct for motion and eddy current distortion. The susceptibility-induced off-resonance field was estimated for the pair of images using the FSL topup tool after which the two images were combined into a single corrected image. (24) Images were then brain-extracted with Brain Extraction Tool and diffusion tensors were calculated with a weighted least squares fit of the tensor model to the diffusion data. Fractional anisotropy (FA) and mean diffusivity (MD) maps were extracted. Evaluation for associations between measures of white matter microstructural integrity and the functional measures of development and behaviour were made.

Resting State fMRI

BOLD data were despiked, motion-corrected and registered into UNC 2 year-old atlas space (Shi 2011) in a single step, spatially smoothed with a 6mm full width at half maximum (FWHM) gaussian kernel, and temporally filtered to exclude frequencies outside of the 0.01 to 0.1 Hz frequency band, in accordance with best practice guidelines and as implemented in the Analysis of Functional Images (AFNI) `afni_proc` script (afni.nimh.nih.gov/afni). Placing the echo-planar images (EPIs) into a common space was achieved via displacement parameters obtained through registering brain extracted T2 anatomical images to the UNC atlas Rigid-body motion estimates and their first order derivatives were regressed from the BOLD using `3dDeconvolve`, prior to bandpass filtering.

In order to identify spatially orthogonal intrinsic functional networks, a group probabilistic independent components analysis (ICA) was conducted using the melodic package provided as part of the FSL imaging software. Group networks were obtained by applying ICA to the temporally concatenated preprocessed residual time-series from each participant. This data-driven approach to identifying intrinsic connectivity networks has the advantage that connectivity patterns resulting from motion and physiological artifact are

identified as separate networks that can be excluded from the analysis.

Regions of interest (ROIs) for subsequent intra and inter motor network comparisons were subsequently defined through using AFNI's 3dExtrema to identify the most representative voxels on a slice-by-slice basis in components corresponding to the somatosensory motor network. In order to be included in the motor network masks, seeds were required to fall within masks of the pre-central and post-central gyrus extracted from the UNC AAL atlas, with any voxels within the contralateral hemisphere omitted. Surviving seeds were dilated by a single voxel, and constrained to fall within an 80% thresholded probabilistic gray matter segmentation mask for the UNC atlas.

3.3 Ethical considerations

Informed consent was obtained from the parents, caregivers or legal guardians of the infants. The informed consent form was written in a language that is easy to understand and its contents were explained fully and translated if needed. As much as possible steps and methods of the study were explained to the participants (mothers) and their verbal and written consent obtained.

The study was approved by the Faculty of Health Sciences, Human Research Ethics Committee, University of Cape Town (401/2009) and by the Western Cape Provincial Health Research committee. Written informed parental consent was obtained from for all participants at the time of enrolment. This sub-study protocol was independently reviewed and approved in 2012 (HREC REF 525/2012). (Appendix E)

Only the most recent ethics approval document has been included in the attachments. This is dated October 2014. The reason Professor Zar is addressed is that she is the PI of the Drakenstein Child Health study in which this sub-study is nested. Original University of Cape Town Human Research Ethics Committee approval was received in February 2012, well before we started recruiting to the study.

Following this chapter on the study design and methodology, I now describe the specific effects of prenatal alcohol exposure on the neonatal brain using structural MRI and correlations with infant neurobehavior and early developmental outcomes.

References

- (1) Western Cape Government. Regional Development Profile Cape Winelands District, 2013. . Accessed: 21 April 2014 Available from: <http://www.westerncape.gov.za/>; Provincial Treasury, Cape Town.
- (2) Massyn N, Day C, Dombo M, Barron P, English R, Padarath A. District Health Barometer 2012/13.
. Health Systems Trust, Durban Accessed: 20 April 2014. <http://www.hst.org.za/publications/district-health-barometer-201213>.
- (3) Stein DJ, Koen N, Donald KA, Adnams CM, Koopowitz S, Lund C, et al. Investigating the psychosocial determinants of child health in Africa: the Drakenstein Child Health Study. J Neuroscience methods 2015;doi:10.1016/j.jneumeth.2015.03.016.
- (4) Jackson PB, Williams DR, Stein DJ, Herman A, Williams SL, Redmond DL. Race and psychological distress: the South african stress and health study. J Health Soc Behav 2010 Dec;51(4):458-477.
- (5) Humeniuk R, Ali R, Babor TF, Farrell M, Formigoni ML, Jittiwutikarn J, et al. Validation of the Alcohol, Smoking And Substance Involvement Screening Test (ASSIST). Addiction 2008 Jun;103(6):1039-1047.
- (6) Lozarno J, Garcia-Algar O, Vall O, de la Torre R, Scaravelli G, Pichini S. Biological matrices for the evaluation of in utero exposure to drugs of abuse. There Drug Monit 2007;29(6):711-734.
- (7) Scholte WF, Verduin F, van Lammeren A, Rutayisire T, Kamperman AM. Psychometric properties and longitudinal validation of the self-reporting questionnaire (SRQ-20) in a Rwandan community setting: a validation study. BMC Med Res Methodol 2011 Aug 16;11:116.
- (8) Myer L, Stein DJ, Grimsrud A, Seedat S, Williams DR. Social determinants of psychological distress in a nationally-representative sample of South African adults. Soc Sci Med 2008 Apr;66(8):1828-1840.
- (9) Noble Y, Boyd R. Neonatal assessments for the preterm infant up to 4 months corrected age: a systematic review. Developmental Medicine & Child Neurology 2012;54(2):129-139.
- (10) Lung F, Shu B, Chiang T, Chen P, Lin L. Predictive validity of Bayley scale in language development of children at 6?36 months. Pediatrics International 2009;51(5):666-669.
- (11) Albers CA, Grieve AJ. Review of Bayley Scales of Infant and Toddler Development Third Edition. Journal of Psychoeducational Assessment 2007;25(2):180-190.
- (12) Bayley N. Bayley Scales of Infant and Toddler Development, Third Edition: Technical Man-

ual. 3rd ed ed. Bloomington: NCS Pearson Inc; 2006.

- (13) Ballot DE, Potterton J, Chirwa T, Hilburn N, Cooper PA. Developmental outcome of very low birth weight infants in a developing country. *BMC pediatrics* 2012;12(1):11.
- (14) Schmidt MH, Marshall J, Downie J, Hadskis MR. Pediatric Magnetic Resonance Research and the Minimal-Risk Standard. (Cover story). *IRB: Ethics & Human Research* 2011 Sep;33(5):1-6.
- (15) Malviya S, Voepel-Lewis T, Tait AR, Reynolds PI, Gujar SK, Gebarski SS, et al. Pentobarbital vs chloral hydrate for sedation of children undergoing MRI: efficacy and recovery characteristics. *Pediatric Anesthesia* 2004;14(7):589-595.
- (16) Eberhard-Gran M, Eskild A, Tambs K, Opjordsmoen S, Samuelsen SO. Review of validation studies of the Edinburgh Postnatal Depression Scale. *Acta Psychiatr Scand* 2001 Oct;104(4):243-249.
- (17) Pannek K, Guzzetta A, Colditz P, Rose S. Diffusion MRI of the neonate brain: acquisition, processing and analysis techniques. *Pediatr Radiol* 2012 10/01;42(10):1169-1182.
- (18) Beaulieu C. The basis of anisotropic water diffusion in the nervous system - a technical review. *NMR Biomed* 2002;15(7-8):435-436-455.
- (19) Wozniak JR, Mueller BA, Chang PN, Muetzel RL, Caros L, Lim KO. Diffusion Tensor Imaging in Children with Fetal Alcohol Spectrum Disorders. *Alcoholism: Clinical and Experimental Research* 2006;30(10):1799-1806.
- (20) Smith SM. Fast, robust human brain extraction. *Human Brain Mapping* 2002;17(3):143-144-155.
- (21) Shi F, Yap P, Wu G, Jia H, Gilmore JH, Lin W, et al. Infant Brain Atlases from Neonates to 1- and 2-year-olds. *PLoS ONE*, 2011; 6(4):e18746.
- (22) Tzourio-Mazoyer N, Landeau B, Papathanassiou D, Crivello F, Etard O, Delcroix N, et al. Automated anatomical labeling of activations in SPM using a macroscopic anatomical parcellation of the MNI MRI single-subject brain. *Neuroimage* 2002;15(1):273-289.
- (23) Smith SM, Jenkinson M, Johansen-Berg H, Rueckert D, Nichols T, Mackay CE, et al. Tract-based spatial statistics: Voxel-wise analysis of multi-subject diffusion data. *Neuroimage* 2006;31(4):1487-1488-1505.
- (24) Andersson JLR, Jenkinson M, Smith S. Non-linear registration, aka Spatial normalisation. FMRIB technical report TR07JA2. www.fmrib.ox.ac.uk/analysis/techrep 2007.

Chapter 4

Alcohol exposure in utero is associated with decreased posterior cingulate cortex and inferior temporal gyrus volume in neonates

*Donald, Kirsten A, MPhil¹; Fouche, JP, MSc^{2,3}; Roos, Annerine, PhD⁵; Koen, N, MBChB⁴; Howells, Fleur M, PhD⁴; Riley, Edward P, PhD⁶; Woods, Roger P, PhD⁷; Zar, Heather J, PhD⁸; Narr, Katherine L, PhD⁷; Stein, Dan J, PhD⁹.

Affiliations:

- 1 - *Division of Developmental Paediatrics, Department of Paediatrics and Child Health, Red Cross War Memorial Children's Hospital and University of Cape Town, South Africa*
 - 2 - *Department of Human Biology, University of Cape Town and*
 - 3 - *Department of Psychiatry, Stellenbosch University, Cape Town, South Africa*
 - 4 - *Department of Psychiatry and Mental Health, University of Cape Town, Cape Town, South Africa*
 - 5 - *MRC Unit on Anxiety & Stress Disorders, UCT/Stellenbosch University, Cape Town, South Africa.*
 - 6 - *Department of Psychology, San Diego State University, San Diego, United States of America*
 - 7 - *Department of Neurology, University of California, Los Angeles, United States of America*
 - 8 - *Department of Paediatrics and Child Health and MRC Unit on Child & Adolescent Health, Red Cross War Memorial Children's Hospital and University of Cape Town, Cape Town, South Africa*
 - 9 - *Department of Psychiatry and Mental Health and MRC Unit on Anxiety & Stress Disorders, University of Cape Town, Cape Town, South Africa*
- *corresponding author*

Corresponding author:

Kirsten A. Donald

Tel: +27 21 658 5535

Fax: +27 21 658 5530

Email: kirsty.donald@uct.ac.za

Postal address: Department of Paediatrics and Child Health, Red Cross War Memorial Children's Hospital, Klipfontein Road/Private Bag, Rondebosch, 7700/7701.

Keywords: alcohol; FASD; MRI, infant, neuroimaging, Dubowitz

Abstract

Background

Neuroimaging studies have indicated that prenatal alcohol exposure is associated with alterations in the structure of specific brain regions. However, the temporal and regional specificity of such changes and their behavioral consequences are less known. Here we explore the brain structure of infants with *in utero* exposure to alcohol shortly after birth.

Methods

T2 structural MRI images were acquired from 28 alcohol-exposed infants and 45 demographically matched healthy controls at 2-4 weeks of age on a 3T Siemens Allegra system as part of large birth cohort study, the Drakenstein Child Health Study (DCHS). Neonatal neurobehavior was assessed at this visit; early developmental outcome assessed on the Bayley Scales of Infant Development III at 6 months of age. Volumes of cortical regions were estimated based on the segmentations of the University of North Carolina neonate atlas.

Results

Significantly smaller gray matter volume was demonstrated for the alcohol-exposed cohort compared to healthy control infants in the right posterior cingulate and the right inferior temporal gyrus. These findings persisted even when correcting for infant age and gender, and maternal smoking status. Both early neurobehavioral and developmental adverse outcomes at six months across multiple domains were significantly associated with smaller regional volumes primarily in the parietal and frontal lobes in infants with prenatal alcohol exposure.

Conclusion

Alcohol exposure during the prenatal period has potentially enduring neurobiological consequences for exposed children. The effects of prenatal alcohol exposure on brain growth is present very early in the first year of life, a period during which the most rapid growth and maturation occurs.

Introduction

Four decades have passed since the physical and neurodevelopmental effects of prenatal alcohol exposure were first formally documented in the seminal reports of Jones and colleagues. (1,2) Documentation of the adverse effects of alcohol on the structure of the brains of children exposed in the prenatal period has grown exponentially since the introduction of magnetic resonance imaging (MRI) technology in the early 1990's. Smaller whole brain volumes were reported in exposed children in addition to reduced global volumes of both white and gray matter. (3,4) In subsequent studies, frontal, parietal and temporal alterations in cortical volume and thickness have been reported. (5-9) Associations reported between gray matter alterations and functional outcomes (both cognitive and behavioral), particularly in temporal and parietal areas, (5,7,10,11) underscores the functional and clinical consequences of damage to these areas after prenatal alcohol-exposure.

Though the effects of prenatal exposure to alcohol on central nervous system (CNS) development are now well-documented, prior studies leave several questions unanswered. First, reports to date have focused largely on ages 5 years through to young adulthood with the majority in late childhood or adolescence. (5,10) There is a paucity of data in children with prenatally alcohol-exposure during the first years of life, a period during which the most rapid brain growth occurs. (4,12) Second, questions remain regarding the impact of alcohol doses and timing of exposure. That is, the effects of alcohol exposure beyond the first trimester has been the period for which there has traditionally been the focus in determining CNS effects of alcohol exposure in previous studies. Finally, interactions of prenatal alcohol exposure with other specific contextual factors such as smoking have not been well studied.

South Africa is one of the regions in the world where FASD is highly prevalent. (13) We used a South African birth cohort sample to investigate whether the impact of prenatal alcohol exposure on early brain structure may be discernible in neonates and whether associations with neurobehavior and early development are present. We assessed alcohol doses and timing of exposure, as well as interactions with smoking. Based on the

literature in children and adolescents with FASD, we predicted that volume alterations would occur and be most prominent in temporal and parietal regions in the neonatal period even before formal diagnosis of FASD. (3,14)

Materials and Methods

Study design, population and procedures

This is a nested sub-study that included infants enrolled in a larger population-based birth cohort study, the Drakenstein Child Health Study (DCHS). The details of the umbrella study structure and methodology are detailed in chapter 3. (15) In this nested sub-study, 73 infants were selected and assessed; 28 infants with significant alcohol exposure and 45 infants with no significant history or biological evidence of substance exposure.

Mothers were recruited at 20-28 weeks gestation, written informed consent obtained, and background data collected for the DCHS. (16) For the group with alcohol exposure, mothers were identified based on a minimum score of 11 (moderate to high risk of experiencing severe problems as a result of their current pattern of use) on the alcohol questions on the ASSIST questionnaire – a widely validated World Health Organization scale to assess comorbid substance use. (17,18) Mothers were further required to give a positive history of alcohol use in any of the 3 trimesters of pregnancy at levels consistent with WHO moderate-severe alcohol use (either drinking 2 or more times a week or 2 or more drinks per occasion; **Table 2**). After birth, infants from mothers identified through this approach were included for study unless the mothers had a positive urine screen for any other drug abuse, (19) the infant was premature (<36 weeks) or had a low apgar score (<7 at 5 minutes) and/or admission for hypoxic ischemic encephalopathy or other significant neonatal complication (such as neonatal jaundice requiring phototherapy). Infants were also excluded if they had an identified genetic syndrome or congenital abnormality.

Two to four week old infants underwent brain magnetic resonance imaging. They were wrapped, fed and then imaged in quiet, natural (unsedated) sleep. Earplugs and mini-muffs were used for double ear protection; a pulse oximeter was used to monitor pulse and oxygenation, and a qualified neonatal nurse or pediatrician was present with the infant in the scanner room for the duration of the imaging session. At the time of scanning, basic anthropometry was acquired including weight, occipito-frontal head circumference and length. The Dubowitz neurobehavioral scale, a well-validated measure of neonatal neuromotor and neurobehavioral status, was used to study early neurological and be-

havioral state. This tool includes an optimality score allowing it to be used for quantitative analysis of potential associations with neuroimaging findings. The score is based on the distribution of the scores for each item in a population of low-risk term infants. The total optimality score is the sum of the optimality scores of individual items. For this study, specific item clusters were chosen as being of particular interest in this population. As defined by the Dubowitz scale authors, the “behavior” cluster includes items scoring irritability, cry, consolability, alertness, visual and auditory orientation and eye movements. The “abnormal signs” cluster has focus on posture, tremor and startle items. (20,21) Infant developmental function was assessed using the Bayley Scales of Infant and Toddler Development, Third Edition (BSID-III). (22) Either a pediatric physiotherapist or a registered neonatal nurse administered this assessment independently, at the age of 6 months. Administrators had background experience in pediatric clinical and research environments and were blinded to the exposure status of the infants. Assessors were trained and piloting took place prior to formal data collection to ensure fidelity to specific instructions for test administration and data scoring. A pediatric neurologist (KD) performed regular site visits to ensure compliance with test administration, and completed protocols were checked systematically to ensure scoring accuracy.

The BSID III assesses infant development across five scales - cognitive, language, motor, socio-emotional and adaptive behavior. (22,23) The language scale is further subdivided into receptive and expressive communication subtests; the motor scale into gross and fine motor subtests; and the adaptive behavior scale into ten subtests, seven of which are applicable to infants younger than one year – communication, health and safety, leisure, self-care, self-direction, social and motor. The cognitive, language and motor assessments are conducted using items administered directly to the infant, while the socio-emotional and adaptive behavior domains are assessed via a questionnaire completed by the primary caregiver. This tool has been used widely across low and middle income country settings such as South Africa, (24) and is considered a gold-standard measure of developmental milestones in infants and toddlers.

Ethical approval for human subjects research was obtained from the Research Ethics Committee of the Faculty of Health Sciences of University of Cape Town (HREC UCT

REF 401/2009) for the Drakenstein Child Health Study. This sub-study protocol was independently reviewed and approved by the same institutional ethics committee (HREC UCT REF 525/2012). Informed consent was signed by the mothers on behalf of herself and her infant for participation in this study.

Image acquisition

Magnetic resonance imaging (MRI) was acquired at the Cape Universities Brain Imaging Centre (CUBIC), Tygerberg, Cape Town. Images were acquired on a Siemens Magnetom 3T Allegra MRI system with a one-channel RF transmit/receive head coil. To overcome limitations with scanning smaller volumes of tissue, voltage was reduced to optimize signal, and the head coil was loaded with a wet clay inlay (40 x 40 cm with a thickness of 2 cm, standard sculpting clay commercially bought – white stoneware clay with grog). A 3D T2 image was acquired on the 3T Siemens Allegra in the sagittal direction with the following parameters: FOV = 160 x 160mm, TR = 3500ms, TE = 354ms, 128 slices, in-plane resolution = 1.3mm x 1.3mm and a slice thickness of 1.0mm.

Image processing

Images were brain extracted with FSL 5.0 brain extraction tool (BET) (25) and exported to SPM8 (www.fil.ion.ucl.ac.uk/spm/software/spm8) for further processing. Within SPM8, the T2 images were co-registered to a custom infant T2 template in MNI standard space created by a group at the University of North Carolina (UNC). (26) After co-registration, images were inspected for proper alignment before nonlinear registration to the same infant template. After non-linear registration, images that displayed excessive warping or other problems with registration were discarded (28 alcohol-exposed and 45 control infants were included for the final analysis). T2 images were then segmented into gray and white matter maps by utilizing infant priors designed by UNC. (26) To preserve the regional volumetric information, the T2 images were modulated during the transformation to the gray matter priors.

Volumetric information was extracted from the gray matter segmented images for 90 anatomical regions as defined by the automated anatomical labeling (AAL) atlas. (27) The volumes were then exported to SPSS 22.0 for further statistical analysis.

Statistical analysis

Group effects and age-by-group interaction effects were investigated for gray matter volumes of the 45 AAL regions of interest per hemisphere in SPSS 22.0 by utilizing a general linear model that included total gray matter volume, age, gender and maternal smoking status as covariates of no interest. Bonferroni correction was considered overly conservative, particularly in view of the available literature documenting volume changes predominantly in frontal, temporal and parietal regions in older children and our a priori hypotheses. Hence, a two-tailed p-value of .05 was used at the threshold of statistical significance. To investigate associations of gray matter volume with behavior, partial correlational analyses were performed between the neonate gray matter volumes and BSID III scores acquired from the same infants at 6 months of age.

Results

The final sample for analysis included 28 alcohol-exposed and 45 healthy control infants (**Table 1**). There was no significant difference in length of neonates, gestation, age, gender or weight of the infants between groups. However alcohol-exposed infants showed a significantly smaller head circumference compared to controls. In addition maternal smoking status was significantly different between the groups, where the ratio of smokers to non-smokers was higher in the alcohol-exposed group compared to the control group. For the Dubowitz scores, no significant differences were apparent between the two groups. Distribution of alcohol exposure by trimester in terms of frequency and intensity is presented in **Table 2** and highlights that, though the majority of the infants in this study were exposed to alcohol in the 1st trimester of pregnancy, a significant proportion were also exposed during the later trimesters.

Table 1: Demographic, anthropometric and Dubowitz data of infants

	Alcohol-exposed (n=28)	Controls (n=45)	Statistics
Gestation (weeks, SD)	38.75 (1.78)	38.80 (1.83)	t=0.12, p=0.91
Maternal smoking (yes/no)	12/16	8/37	$\chi^2=5.46$, p=0.02*
Age (days, SD)	20.54(5.98)	22.24(6.11)	t=1.18, p=0.24
Gender (male/female)	11/17	28/17	$\chi^2=3.65$, p=0.06
Weight (kg, SD)	3.80(0.68)	4.09(0.66)	t=1.76, p=0.08
Head circumference (cm, SD)	35.55(1.37)	36.71(1.62)	t=3.25, p=0.002*
Length (cm, SD)	51.27(5.03)	50.92(3.97)	t=-0.31, p=0.76
Dubowitz optimality scores (mean,(SD))			
Tone (/10)	7.93(2.11)	8.44(1.37)	t=1.25, p=0.22
3 Reflex (/6)	4.69(0.56)	4.77(0.56)	t=0.60, p=0.55
Spontaneous movement (/2)	1.93(0.30)	1.95(0.18)	t=0.42, p=0.68
Behavior (/7)	4.54(1.32)	4.22(1.26)	t=-1.02, p=0.31
Abnormal signs (/3)	2.86(0.36)	2.60(0.93)	t=-1.37, p=0.18

*p<0.05

Table 2: Frequency and quantity of maternal alcohol use by trimester

	Trimester 1	Trimester 2	Trimester 3
Alcohol usage (n, %)	22, 78.6%	11, 39.3%	7, 25%
Once per week or less	15	6	3
2 to 3 times per week	7	5	4
Number of drinks per occasion			
< 2	2	0	0
2 to 3	20	11	7

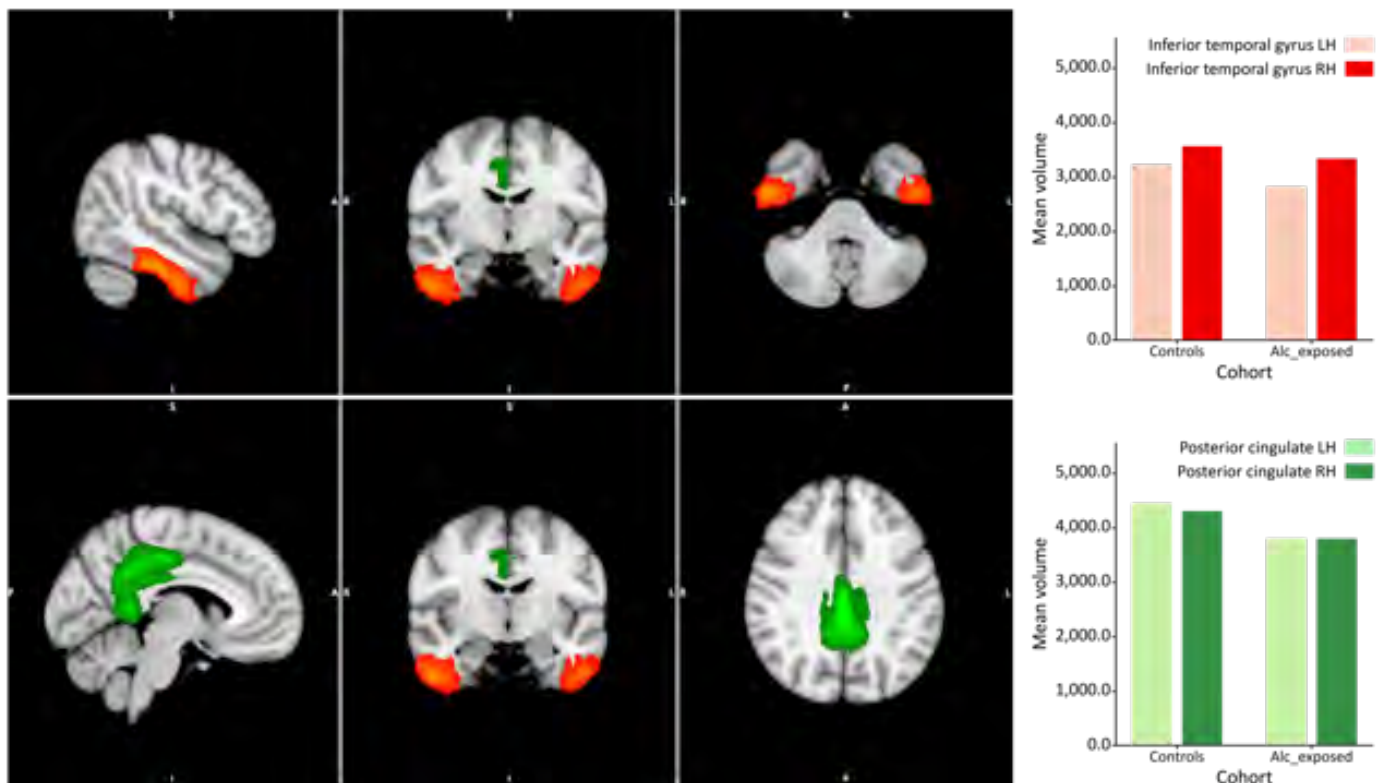
Volumetric analyses controlled for age, gender, maternal smoking status and total gray matter volume. There was no significant interaction of age and group for any region. Significantly lower gray matter volume was demonstrated for the prenatal alcohol-exposed cohort compared to healthy control infants ($p < 0.001$). Prior to correction for total gray matter volume all regions (with the exception of the limbic subcortical regions) were significantly different between infants with prenatal alcohol exposure and unexposed control infants with p values ranging from $p=0.05$ to $p=0.001$. Regions that displayed lower volume (after correction for overall gray matter volume) were the right posterior cingulate and inferior temporal gyrus (Table 3, Figure 1)

Table 3: Regions with significant differences in gray matter volumes between healthy and alcohol-exposed infants

AAL region	Hemisphere	Mean gray matter volume (mm ³)		F-value	p-value
		Controls (SD)	Alcohol-exposed (SD)		
Controls > Alcohol-exposed					
Posterior cingulate gyrus	Right	423.9 (93.9)	394.7 (98.0)	5.657	0.020
Inferior temporal gyrus	Right	3557.1 (903.4)	3332.5 (972.2)	7.231	0.009

AAL - Automated anatomic labeling. Analyses were controlled for age, gender, maternal smoking status and total gray matter volume at $p < 0.05$. There was no significant interaction of age and group within these regions

Figure 1 Mean volume images showing regions of decreased gray matter volume in alcohol-exposed infants compared to healthy controls.



Areas showing decreases in posterior cingulate cortex volume are presented in green and inferior temporal gyrus volume are presented in red ($p < 0.05$). Results are presented in the form of bar graphs where mean volume is shown in mm³, as well as superimposed onto a representative infant atlas image in mid-sagittal, coronal and axial slices (from left to right).

The significant associations between the early neurobehavioral outcomes and specific brain regions are presented in **Table 4** and between the BSID III at 6 months and specific brain regions in **Table 5**. Regions with which there were widespread associations included areas in the frontal and parietal lobe.

Table 4: 7 Significant associations between BSID III scores and gray matter volumes in alcohol-exposed infants (n = 19)

AAL region	Hemisphere	R-value	p-value
CS General Adaptive Behavior			
Medial orbitofrontal gyrus	Right	0.763	0.004
Middle frontal gyrus	Right	0.627	0.029
CS Socio-Emotional			
Inferior frontal gyrus	Left	-0.625	0.030
Precentral gyrus	Right	-0.643	0.024
Amygdala	Left	0.651	0.022
Superior parietal gyrus	Left	-0.650	0.022
CS Motor			
Inferior frontal gyrus	Left	-0.627	0.029
Middle occipital gyrus	Left	-0.617	0.033
Inferior occipital gyrus	Right	-0.631	0.028
Superior orbitofrontal gyrus	Right	0.607	0.036
Precuneus	Right	0.798	0.002
CS Language			
Inferior parietal lobule	Left	0.711	0.010
	Right	0.674	0.016
Supramarginal gyrus	Left	0.676	0.016
CS Cognitive			
Superior frontal (dorsal) gyrus	Left	-0.616	0.033
Superior occipital gyrus	Right	-0.623	0.030
Angular gyrus	Right	0.639	0.025
Rolandic operculum	Left	0.753	0.005

Results shown at $p < 0.05$. The model included total gray matter volume, maternal smoking status, age and gender as covariates of no interest. AAL - Automated anatomic labeling. CS – Composite score, AB – Adaptive Behavior

Table 5: Significant associations between Dubowitz optimality scores and gray matter volume in alcohol-exposed neonates (n=27)

AAL region	Hemisphere	R-value	P-value
Dubowitz abnormal signs			
Calcarine cortex	Right	0.538	0.008
Dubowitz behavior			
Inferior orbitofrontal gyrus	Right	-0.463	0.026

Medial orbitofrontal gyrus	Right	-0.492	0.017
Rectus gyrus	Right	-0.460	0.027
Dubowitz spontaneous movement			
Rolandic operculum	Left	0.718	<0.001
	Right	0.803	<0.001
Olfactory gyrus	Left	-0.461	0.027
Medial orbitofrontal gyrus	Left	-0.511	0.013
Insula	Left	0.544	0.007
	Right	0.674	<0.001
Anterior cingulate gyrus	Right	0.602	0.002
Posterior cingulate gyrus	Right	-0.427	0.042
Parahippocampal gyrus	Left	-0.527	0.010
Amygdala	Left	-0.448	0.032
Calcarine gyrus	Left	-0.550	0.007
	Right	-0.472	0.023
Cuneus	Left	0.520	0.011
Lingual gyrus	Left	-0.518	0.011
	Right	-0.441	0.035
Superior occipital gyrus	Left	0.471	0.023
Middle occipital gyrus	Right	0.572	0.004
Inferior occipital gyrus	Left	-0.555	0.006
Supramarginal gyrus	Left	0.451	0.031
Caudate	Right	-0.511	0.013
Pallidum	Left	-0.435	0.038
Thalamus	Left	-0.553	0.006
	Right	-0.431	0.040
Heschl gyrus	Right	0.509	0.013
Superior temporal gyrus	Left	0.590	0.003
	Right	0.658	0.001
Middle temporal pole	Right	-0.523	0.011
Dubowitz reflex			
Precuneus	Left	0.480	0.021
Dubowitz tone			
Supramarginal gyrus	Left	0.437	0.037

Results shown at $p < 0.05$. The model included total gray matter volume, maternal smoking status, age and gender as covariates of no interest. AAL - Automated anatomic labeling

Discussion

To our knowledge this is the first report of the effect of prenatal alcohol exposure on the volume of brain regions in the first weeks of life. Neonates in this study were found to have reduced volumes in the right posterior cingulate cortex and right inferior temporal gyrus corrected for overall brain volume. These findings persisted even when correcting for infant age, gender and maternal smoking status. We also report correlations between early infant neurobehavior and 6-month developmental outcomes and smaller gray matter volumes in the neonatal period.

In this analysis in addition to controlling for key factors such as age, sex and maternal smoking, we additionally controlled for overall gray matter volume. The rationale for this was for us to be able to highlight the areas that are proportionally the most affected in our cohort with prenatal alcohol exposure compared to unexposed controls. Nevertheless, absolute smaller volume of individual regions and structures may still be important for these children in functional terms and should still be viewed as important.

The finding of decreased posterior cingulate cortex is consistent with previous literature on FASD in older children. This region is situated in the medial aspect of the inferior parietal lobe. (28) It is a region, which is highly connected with many brain networks. Adult imaging studies suggest a role for the posterior cingulate cortex for processing pain as well as episodic memory retrieval. (29) Of particular interest in the context of prenatal alcohol exposure is the role of the posterior cingulate cortex as a key node in the default mode network. Dysfunctional activation of the default mode network has been documented to result in deficits in cognitive function, visual attention and executive motor control. (28) These domains are all highly implicated in prior literature documenting the multidimensional cognitive effects of prenatal alcohol exposure in older children. (30,31) Further and most importantly, Lebel and colleagues (5) have implicated this region as being affected in their longitudinal study of prenatal alcohol exposure in the brains of school-age children over time.

The inferior temporal gyrus is situated in the anterior aspect of the temporal lobe. This region of the brain is reported to play a central role in visual object recognition (faces, patterns and objects) as well as in more general involvement in visual perception and

spatial processing and receives processed visual information from the occipital visual cortex. (32,33) The neuroimaging literature on the structural effects of prenatal alcohol exposure has repeatedly implicated the temporal lobe and in particular the right side in children with a diagnosis of alcohol related neurocognitive disorder (no facial features) (34) and those more generally on the FASD spectrum. (5) The consistency with which this area has been reported in this group implicates it as particularly susceptible to prenatal alcohol exposure.

Adverse neonatal neurobehavioral measures shortly after birth as well as multiple domains in both developmental and adaptive behavioral outcomes at 6 months of age on the BSID III were associated with decreased volumes in particularly parietal and frontal regions in the prenatal alcohol exposed infants. Associations between gray matter volumes, and motor, language and cognitive outcomes, indicate the global nature of the effects of prenatal alcohol exposure on the developing brain. This effect of prenatal alcohol exposure on multiple developmental domains in early life is consistent with a meta-analysis of nine studies of infants with any reported prenatal alcohol exposure. (35) The authors reported significantly lower mental development index at 12–13 months of age on the BSID for the exposed group. To our knowledge ours is the first report of structural abnormalities associated with developmental deficits at this early age.

Our study has a number of limitations. First, our findings were based on a relatively small sample size. Although the sample is adequately powered for the primary outcome analysis, secondary analyses with breakdown of prenatal alcohol exposure severity and timing with respect to gray matter volume outcomes was not possible. Second, although our study employed a cross-sectional design where infants were matched for age, gender, and maternal smoking during pregnancy, longitudinal study data on this group is going to be key in establishing the trajectory of developing brains exposed to alcohol during prenatal life and relationship with facial characteristics of FASD.

Despite these limitations, this is a very well characterized group of infants recruited as part of a population-based prospective study design, and the selection of the infant age-group is an additional strength. For this study, two to four week old infants were chosen

for imaging because cerebral changes are particularly intense during the last weeks of gestation and the first postnatal months; neuroanatomical abnormalities may be present and identified at this early age that may lead to motor and cognitive dysfunction in the later years. Secondly, imaging during the early postnatal period may more accurately reflect the effects of prenatal alcohol exposure on brain structure before postnatal risk factors known to be highly prevalent in our cohort can compromise brain development.

In summary, this paper addresses a critical gap in the literature. Given progressive as well as regressive developmental processes of gray and white matter, including myelination, synaptogenesis, pruning and synaptic modification, (36) it is important to understand the early impact of prenatal exposure. The findings here are consistent with work in older children, and correlation with neurodevelopmental measures suggests clinical relevance. Here we have shown that the effects of prenatal alcohol exposure manifest in structural brain changes very early in life and that changes in brain structure in the neonatal period correlate with poorer neurodevelopmental outcomes at 6 months of age.

Following this description of the structural effects of prenatal alcohol exposure on the neonatal brain, in the next chapter I now move on to discuss the effects at a microstructural level as measured by diffusion tensor imaging.

References

- (1) Jones KL, Smith DW, Hansen JW. The Fetal Alcohol Syndrome: clinical delineation. *Annals of the New York Academy of Sciences* 1976;273:130-139.
- (2) Jones KL, Smith DW, Ulleland CN, Streissguth AP. Pattern of malformation in offspring of Chronic Alcoholic mothers. *Lancet* 1973;1(7815):1267-1271.
- (3) Donald KA, Eastman E, Howells FM, Adnams C, Riley EP, Woods RP, et al. Neuroimaging effects of prenatal alcohol-exposure on the developing human brain: A magnetic resonance imaging review . *Acta Neuropsychiatrica* 2015;DOI:<http://dx.doi.org/10.1017/neu.2015.12>.
- (4) Riley EP, Alejandra Infante M, Warren K. Fetal Alcohol Spectrum Disorders: An Overview. *Neuropsychol Rev* 2011;21(2):73-80.
- (5) Lebel C, Mattson SN, Riley EP, Jones KL, Adnams CM, May PA, et al. A Longitudinal Study of the Long-Term Consequences of Drinking during Pregnancy: Heavy In Utero Alcohol Exposure Disrupts the Normal Processes of Brain Development. *The Journal of Neuroscience* 2012 October 31;32(44):15243-15251.
- (6) Sowell ER, Thompson PM, Peterson BS, Mattson SN, W, S.E., Henkenius AL, et al. Mapping cortical gray matter asymmetry patterns in adolescents with heavy prenatal alcohol exposure. *Neuroimage* 2002;17(4):1807-1819.
- (7) Sowell ER, Mattson SN, Kan E, Thompson PM, Riley EP, Toga AW. Abnormal Cortical Thickness and Brain–Behavior Correlation Patterns in Individuals with Heavy Prenatal Alcohol Exposure. *Cerebral Cortex* 2008 January 01;18(1):136-144.
- (8) Yang Y, Roussotte F, Kan E, Sulik KK, Mattson SN, Riley EP, et al. Abnormal cortical thickness alterations in fetal alcohol spectrum disorders and their relationships with facial dysmorphology. *Cerebral cortex* 2012;22(5):1170-1179.
- (9) Zhou D, Lebel C, Lepage C, Rasmussen C, Evans A, Wyper K, et al. Developmental cortical thinning in fetal alcohol spectrum disorders. *Neuroimage* 2011;58(1):16-25.
- (10) Treit S, Lebel C, Baugh L, Rasmussen C, Andrew G, Beaulieu C. Longitudinal MRI reveals altered trajectory of brain development during childhood and adolescence in fetal alcohol spectrum disorders. *The Journal of Neuroscience* 2013;33(24):10098-10109.
- (11) Gautam P, Lebel C, Narr KL, Mattson SN, May PA, Adnams CM, et al. Volume changes and brain–behavior relationships in white matter and subcortical gray matter in children with prenatal alcohol exposure . *Human brain mapping* 2015;36(6):2318-2329.
- (12) Moore EM, Migliorini R, Infante MA, Riley EP. Fetal Alcohol Spectrum Disorders: Recent

Neuroimaging Findings. *Current developmental disorders reports* 2014;1(3):161-172.

- (13) May PA, Baete A, Russo J, Elliott AJ, Blankenship J, Kalberg WO, et al. Prevalence and characteristics of fetal alcohol spectrum disorders. *Pediatrics* 2014;134(5):855-866.
- (14) Donald KA, Roos A, Fouche J, Koen N, Howells FM, Woods RP, et al. A pilot study of the effects of alcohol exposure in utero on white matter microstructural integrity at birth. *Acta neuropsychiatrica* 2015;[Epub ahead of print](PMID: 26022619).
- (15) Zar HJ, Barnett W, Myer L, Stein DJ, Nicol MP. Investigating the early-life determinants of illness in Africa: the Drakenstein Child Health Study. *Thorax* 2014;doi: 10.1136/thorax-jnl-2014-206242.
- (16) Stein DJ, Koen N, Donald KA, Adnams CM, Koopowitz S, Lund C, et al. Investigating the psychosocial determinants of child health in Africa: the Drakenstein Child Health Study. *J Neuroscience methods* 2015;doi:10.1016/j.jneumeth.2015.03.016.
- (17) Humeniuk R, Ali R, Babor TF, Farrell M, Formigoni ML, Jittiwutikarn J, et al. Validation of the Alcohol, Smoking And Substance Involvement Screening Test (ASSIST). *Addiction* 2008 Jun;103(6):1039-1047.
- (18) Jackson PB, Williams DR, Stein DJ, Herman A, Williams SL, Redmond DL. Race and psychological distress: the South african stress and health study. *J Health Soc Behav* 2010 Dec;51(4):458-477.
- (19) Lozarno J, Garcia-Algar O, Vall O, de la Torre R, Scaravelli G, Pichini S. Biological matrices for the evaluation of in utero exposure to drugs of abuse. *There Drug Monit* 2007;29(6):711-734.
- (20) Noble Y, Boyd R. Neonatal assessments for the preterm infant up to 4 months corrected age: a systematic review. *Developmental Medicine & Child Neurology* 2012;54(2):129-139.
- (21) Dubowitz L, Mercurio E, Dubowitz V. An Optimality Score for the Neurological Examination of The Term Newborn. *The Journal of pediatrics* 1998;133(3):406-416.
- (22) Bayley N. Bayley Scales of Infant and Toddler Development, Third Edition: Technical Manual. 3rd ed ed. Bloomington: NCS Pearson Inc; 2006.
- (23) Albers CA, Grieve AJ. Review of Bayley Scales of Infant and Toddler Development Third Edition. *Journal of Psychoeducational Assessment* 2007;25(2):180-190.
- (24) Ballot DE, Potterton J, Chirwa T, Hilburn N, Cooper PA. Developmental outcome of very low birth weight infants in a developing country. *BMC pediatrics* 2012;12(1):11.
- (25) Smith SM, Jenkinson M, Johansen-Berg H, Ruerket D, Nichols T, Mackay CE, et al. Tract-

based spatial statistics: Voxel-wise analysis of multi-subject diffusion data. *Neuroimage* 2006;31(4):1487-1488-1505.

- (26) Shi F, Yap P, Wu G, Jia H, Gilmore JH, Lin W, et al. Infant Brain Atlases from Neonates to 1- and 2-year-olds. *PLoS ONE*, 6(4): , 2011 2011;6(4):e18746.
- (27) Tzourio-Mazoyer N, Landeau B, Papathanassiou D, Crivello F, Etard O, Delcroix N, et al. Automated anatomical labeling of activations in SPM using a macroscopic anatomical parcellation of the MNI MRI single-subject brain. *Neuroimage* 2002;15(1):273-289.
- (28) Leech R, Sharp DJ. The role of the posterior cingulate cortex in cognition and disease. *Brain* 2014;137(1):12-32.
- (29) Nielsen FÅ, Balslev D, Hansen LK. Mining the posterior cingulate: segregation between memory and pain components. *Neuroimage* 2005;27(3):520-532.
- (30) Mattson S, Crocker N, Nguyen T. Fetal Alcohol Spectrum Disorders: Neuropsychological and Behavioral Features. *Neuropsychol Rev* 2011;21(2):81-101.
- (31) Mattson SN, Roesch SC, Glass L, Deweese BN, Coles CD, Kable JA, et al. Further Development of a Neurobehavioral Profile of Fetal Alcohol Spectrum Disorders. *Alcoholism: Clinical and Experimental Research* 2013;37(3):517-528.
- (32) Creem SH, Proffitt DR. Defining the cortical visual systems: “what”, “where”, and “how”. *Acta psychologica* 2001;107(1):43-68.
- (33) Kolb B, Whishaw IQ. *An introduction to brain and behavior*. Chicago: Worth Publishers; 2014.
- (34) Rajaprakash M, Chakravarty MM, Lerch JP, Rovet J. Cortical morphology in children with alcohol-related neurodevelopmental disorder . *Brain and behavior* 2014;4(1):41-50.
- (35) Testa M, Quigley BM, Eiden RD. The effects of prenatal alcohol exposure on infant mental development: a meta-analytical review. *Alcohol and Alcoholism* 2003;38(4):295-304.
- (36) T au GZ, Peterson BS. Normal development of brain circuits . *Neuropsychopharmacology* 2010;35(1):147-168.

Chapter 5

A study of the effects of prenatal alcohol exposure on white matter microstructural integrity at birth

*Donald, KA¹; Roos, A²; Fouche, J^{3,4}; Koen, N⁴; Howells, FM⁴; Woods, RP⁵; Zar, HJ⁶; Narr, KL⁵; Stein, DJ^{2,4}.

Affiliations:

- 1 - *Division of Developmental Paediatrics, Department of Paediatrics and Child Health, Red Cross War Memorial Children's Hospital and University of Cape Town, South Africa*
- 2 - *MRC Unit on Anxiety & Stress Disorders, Stellenbosch University, Cape Town, South Africa.*
- 3 - *Department of Human Biology, University of Cape Town and Department of Psychiatry, Stellenbosch University, Cape Town, South Africa*
- 4 - *Department of Psychiatry and Mental Health, University of Cape Town, Cape Town, South Africa*
- 5 - *Department of Neurology, University of California, Los Angeles, United States of America*
- 6 - *Department of Paediatrics and Child Health, Red Cross War Memorial Children's Hospital and University of Cape Town, Cape Town, South Africa*

**corresponding author*

Keywords: alcohol; fetal alcohol spectrum disorders; MRI, infant, neuroimaging, DTI, Dubowitz

Abstract

Background

Neuroimaging studies have indicated that prenatal alcohol exposure is associated with alterations in the structure of specific brain regions in children. However, the temporal and regional specificity of such changes and their behavioral consequences are less known. Here we explore the integrity of regional white matter microstructure in infants with *in utero* exposure to alcohol, shortly after birth.

Methods

Twenty-eight alcohol-exposed and 28 healthy unexposed infants were imaged using diffusion tensor imaging sequences to evaluate white matter integrity using validated tract-based spatial statistics analysis methods. Secondly, diffusion values were extracted for group comparisons by regions of interest. Differences in fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD) and radial diffusivity (RD) were compared between groups and associations with measures from the Dubowitz neonatal neurobehavioral assessment were examined.

Results

Lower AD values ($p < 0.05$) were observed in alcohol exposed infants in the right superior longitudinal fasciculus compared to non-exposed infants. Altered FA and MD values in alcohol-exposed neonates in the right inferior cerebellar peduncle were associated with abnormal neonatal neurobehavior.

Conclusion

These exploratory data suggest that prenatal alcohol exposure is associated with reduced white matter microstructural integrity even early in the neonatal period. The association with clinical measures reinforces the likely clinical significance of this finding. The location of the findings is remarkably consistent with previously reported studies of white matter structural deficits in older children with a diagnosis of fetal alcohol spectrum disorder.

Introduction

The current literature most commonly uses the term Fetal Alcohol Spectrum Disorders (FASD) to incorporate a range of conditions relating to maternal alcohol abuse, including a defined range of effects of prenatal alcohol exposure in individuals not meeting full criteria for fetal alcohol syndrome (FAS). (1-3) Central nervous system (CNS) development and maturation require a carefully patterned sequence of events. These developmental processes are more complex and extend over a longer period than those of any other organ system. Thus, the CNS is particularly vulnerable to prenatal environmental influences. (4,5) Animal work and human imaging studies have demonstrated that *in utero* exposure to alcohol alters brain morphology and neurocircuitry across multiple brain regions. (6,7)

White matter microstructure can be measured *in vivo* with diffusion tensor imaging (DTI) and can estimate the overall directional diffusion of water molecules along fiber pathways. (8,9) Analysis of this data allows the degree of microstructural integrity and organization of areas within the brain tissue to be determined. Traditional scalar metrics derived from DTI data include fractional anisotropy (FA), which quantifies the overall directionality of diffusion, and may represent variations in axon integrity and/or packing density. In contrast, mean diffusivity (MD), which provides a measure of average diffusivity, may reflect myelin degradation, decreased cellular density or increased extra- or intracellular volumes. High FA and low MD values are typically associated with healthier neural microstructure and improved behavioral function, whereas low FA and high MD values may indicate white matter pathology. (10,11) Radial diffusivity (RD) specifically reflects perpendicular diffusion towards membranes; RD is higher when myelination is reduced or there is myelin damage. Axial diffusivity (AD) specifically measures diffusion along axons. This is believed to be increased when neurofilaments are damaged (12) or decreased when axonal damage is present. (13,14) However, it is also relevant to note that during brain maturation in healthy children and adolescents, axonal pruning and other biological processes may also lead to reduced FA. (15,16)

A recent review of DTI studies in older children and young adults exposed to alcohol prenatally reported white matter microstructural abnormalities (lower FA) in the corpus

callosum, anterior-posterior fiber bundles and the cerebellum. (7,17-19,19-22) These abnormalities have also been reported in frontal (23,24) and temporal lobe regions, (24,25) and within subcortical structures (globus pallidus, thalamus and putamen). (24,26) Relatively few studies have addressed associations between white matter microstructural abnormalities and specific measures of cognitive and behavioral function. (25,27,27) Although negative findings have been reported for several functional domains, Sowell and colleagues (2008) showed associations between reduced performance on a measure of visuomotor integration and reduced FA in the splenium of the corpus callosum and parietal white matter. (25) Lebel *et al* reported significant associations between reduced FA in the left parietal lobe, cerebellum and brainstem with mathematical ability in 5-13 year old children. (17) Correlations between white matter microstructural integrity and eye-blink conditioning have been reported by Fan *et al*. (28) and oculomotor control by Green *et al*, (29) in a group of school age children with prenatal alcohol exposure. These results suggest the clinical significance of the DTI findings in these brain regions, at least in children of school-going age. To date, very little data exists regarding the impact of prenatal alcohol exposure in early infancy before higher-level brain networks have become established or the confounding post-natal environmental influences to which children from these backgrounds are exposed have come into play. Taylor and colleagues have reported altered axial diffusivity (AD) in 11 alcohol exposed neonates in six major network areas, including the association networks (superior longitudinal fasciculus, inferior longitudinal fasciculus and uncinate fasciculus). (30) Beyond this recent report, there remains few other data on white matter microstructure at this early period or an association with functional measures such as early neurobehavior.

To address gaps in the current literature concerning the presence, timing and regional specificity of altered white matter structural integrity in association with prenatal alcohol exposure, we conducted this study in South Africa, where estimates of prevalence for FAS are extremely high in certain communities. That is, globally quoted prevalence is between 2-7 per 1000 for FAS and between 20-50 per 1000 for FASD. (31) In contrast, though no national data are available, in South Africa prevalence has been reported to be as high as 63/1000 for FAS and 155/1000 for FASD in a periurban Western Cape community. (32) Using a cohort from this community, the current study thus investigated

whether the impact of prenatal alcohol exposure on early white matter microstructural integrity may be discernible in neonates and whether associations with neurobehavioral and physical measures of neonatal neurological health such as head circumference are present. Based on the literature in children and adolescents with FASD, we predicted that disruptions in white matter integrity would occur in association pathways connecting frontal and temporal regions and in the cerebellum. (33)

Methods

Study design, population and procedures

The current investigation is a nested sub-study that included infants enrolled in a larger population-based birth cohort study, the Drakenstein Child Health Study (DCHS) described in chapter 3. In this nested sub-study, 116 infants were assessed; 54 infants with significant alcohol exposure and 62 infants with no significant history or biological evidence of substance abuse.

Mothers were recruited at 20-28 weeks gestation, written informed consent obtained, and background data collected for the umbrella study as described by Stein and colleagues. (34) For the group with alcohol exposure, mothers were screened based on a minimum score of 11 (indicating that a participant is at moderate to high risk of experiencing severe problems as a result of their current pattern of use) on the alcohol questions on the ASSIST questionnaire – a widely validated World Health Organization (WHO) scale to assess comorbid substance use. (35,36) In addition to this initial screen, mothers were required to give a positive history of alcohol use in any of the 3 trimesters of pregnancy at levels consistent with WHO moderate-severe alcohol use (either drinking 2 or more times a week or 2 or more drinks per occasion), **Table 2**. After birth, infants from mothers identified through this approach were included for study unless the mothers also had a positive urine screen for other drugs of abuse (any group), (37) the infants were premature (<36 weeks) or had low apgar scores (<7 at 5 minutes) and/or history of neonatal ICU admission for hypoxic ischemic encephalopathy or other significant neonatal complication (such as neonatal jaundice requiring phototherapy). Infants were also excluded if they had an identified genetic syndrome or congenital abnormalities.

Two to four week old infants underwent brain imaging, wrapped and fed, in quiet natural (unsedated) sleep. Earplugs and mini-muffs were used for double ear protection; a pulse oximeter was used to monitor pulse and oxygenation, and a qualified neonatal nurse or pediatrician was present with the infant in the scanner room for the duration of the imaging session. At the time of scanning, basic anthropometry was acquired including weight, occipito-frontal head circumference and length. The Dubowitz neurobehavioral scale, a well-validated measure of neonatal neuromotor and neurobehavioral status, was used to study early neurological and behavioral state. This tool includes an optimality score allowing it to be used for quantitative analysis of potential associations with neuroimaging findings. The score is based on the distribution of the scores for each item in a population of low-risk term infants. The total optimality score is the sum of the optimality scores of individual items. However, for this study, specific item clusters were chosen as being of particular interest in this population. As defined by the Dubowitz scale authors, the “behavior” cluster includes items scoring irritability, cry, consolability, alertness, visual and auditory orientation and eye movements. The “abnormal signs” cluster has focus on posture, tremor and startle items. (38,39)

Ethical approval for human subjects research was obtained from the Research Ethics Committee of the Faculty of Health Sciences of University of Cape Town (HREC UCT REF 401/2009) for the Drakenstein Child Health Study. This sub-study protocol was independently reviewed and approved by the same institutional ethics committee (HREC UCT REF 525/2012).

DTI acquisition

Diffusion weighted images were acquired on a Siemens Magnetom 3T Allegra MRI system with a RF transmit/receive head coil using a spin-echo, echo-planar sequence and including 45 non-collinear directions. To overcome limitations with scanning smaller volumes of tissue, voltage was reduced to optimize signal, and the head coil was loaded with a wet clay inlay (40 x 40 cm with a thickness of 2 cm, standard sculpting clay commercially bought – white stoneware clay with grog). Images were obtained in the transverse plane with both anterior-posterior and posterior-anterior phase encoding to control for anatomic distortions and increase signal-to-noise. Parameters were as fol-

lows: repetition time (TR) 7900ms; echo time (TE) 90ms; slice thickness 1.6mm; field of view (FOV) 160mm; voxel size 1.3x1.3x1.6mm³; b-values 0 and 1000s/mm². Scan time per phase encoding acquisition was 6:27 minutes with a total time of 12:54 minutes for both acquisitions.

Data processing

Data were analyzed using a whole-brain approach followed by selected regions of interest. Firstly, the FMRIB's Diffusion Toolbox and Tract-Based Spatial Statistics (TBSS) processing streams (FSL v5.0) (40) were used to investigate diffusion of the whole brain, selecting a representative anatomical template from the sample as is described below. Secondly, diffusion values were extracted after preprocessing with FSL and data exported to Statistica 12 (Statsoft Inc.) for group comparisons by regions of interest that include white matter tracts that connect temporal and cerebellar regions with cortical areas. Analyses were controlled for gender and age, due to the rapidly evolving white matter maturation that occurs early in infant life. (16,41)

Preprocessing using FSL

Acquiring images in such small infants poses technical and logistical challenges. To ensure good quality data, strict criteria (images with at least 12 acquisition volumes without artifact) were deemed as the cut-off for inclusion for data pre-processing and statistical analyses. The Diffusion Toolbox was applied following manual quality control of data from each subject. Each subject's diffusion weighted image was registered to the corresponding b=0 image to correct for motion and eddy current distortion. Three b0 images were acquired. The susceptibility-induced off-resonance field was estimated for the pair of images using the FSL top-up tool after which the two images were combined into a single corrected image. (42) Images were then brain-extracted with the Brain Extraction Tool and diffusion tensors were calculated at each voxel with a weighted least squares fit of the tensor model to the diffusion data. FA, MD, AD and RD images were subsequently attained for each subject.

Whole-brain tract-based spatial statistics

The standard TBSS pipeline was applied. (40) However, the FMRIB adult FA template

is not appropriate for neonatal DTI analysis. Standard practice in FSL allows the user to identify an anatomical target after preprocessing that included stringent eddy correction and outlier rejection. Thus, every subject was registered to a representative target that was pre-selected from the control cohort. Each subject was registered to every other subject to find the most representative target, that is, the target with the lowest mean warp coefficient.

Individual FA images were aligned into the target image space and upsampled to $1 \times 1 \times 1 \text{ mm}^3$ voxel size taking into account previous estimated transformations. An average FA map was created and thinned to generate a mean FA skeleton. This skeleton represents the centre of all white matter tracts common to the study group. The skeleton was thresholded at an FA value of 0.2. As this study was explorative, we opted for a more stringent threshold compared to that of some previous infant studies that used a threshold of 0.15. FA and MD data were projected onto this skeleton prior to statistical analysis.

Variations in DTI metrics were examined voxelwise using FSL's Randomise tool. Specifically, t-tests and correlational analyses (5000 permutations per test) were used to investigate group differences. Analyses were corrected for multiple comparisons using threshold-free cluster enhancement (TFCE).

Analysis by regions of interest

Group main effects were investigated using extracted diffusion data by region of interest after FSL preprocessing. Individual brains were registered to the standard FMRIB58_FA template using affine registration. Mean diffusion values were then extracted by subject for regions of interest using the Johns Hopkins University white-matter atlas. (43) Regions of interest were major white matter tracts that connect temporal and cerebellar regions to the cortex. These included association fibers (superior longitudinal fasciculus, superior fronto-occipital fasciculus and uncinate fasciculus), tracts of the brain stem and cerebellum (cerebellar peduncles, corticospinal tract and cerebral peduncle), projection fibers (posterior thalamic radiation, fornix and cingulum) and commissural fibers (corpus callosum). Separate general linear models were used with infant age (in days) and gender as covariates. Results were Bonferroni corrected. Partial correlational analysis was

used to investigate associations between diffusion parameters and behavior, controlled for age and gender. Raw scores on the Dubowitz behavior and abnormal signs subscales, were converted to optimality scores as described by Dubowitz *et al* (1998) (39) for comparison. Tests were two-tailed and considered significant at $p < 0.05$.

Results

From the cohort of infants enrolled in the study, 13 scans from the initial alcohol-exposed group ($n=54$) and seven scans from the control group ($n=62$) were excluded due to movement or other artifacts and DTI imaging data were not acquired (infants awoke during the sequence so it could not be completed) for an additional 13 alcohol-exposed infants and 27 controls. Thus the final sample for the present analysis included 28 alcohol exposed infants and 28 healthy infants (**Table 1**). There were no significant differences in the mean values for gestational age, postnatal age at scanning, weight, length and head circumference for alcohol-exposed infants compared to controls. Although maternal smoking in pregnancy was prevalent in this cohort, there was no difference in this exposure between alcohol exposed and control infants. There was a significant difference between the scores on the behavior subscale of the Dubowitz for the infants exposed to alcohol prenatally compared to control infants [$t=2.13$, $p=0.04$].

Table 1: Demographic, anthropometric and Dubowitz data of infants

	Alcohol-exposed (n=28)	Controls (n=28)	Statistics
Gestation (weeks, SD)	38.36(1.81)	38.50(1.80)	t=-0.30, p=0.77
Maternal smoking (yes/no)	13/15	19/9	-x2=2.63,p=0.11
Age (days, SD)	20.71(5.29)	19.96(4.92)	t=0.55, p=0.58
Sex (male/female)	13/15	19/9	χ2=2.63, p=0.11
Weight (kg, SD)	3.69(0.56)	3.99(0.71)	t=-1.77, p=0.08
Head circumference (cm, SD)	35.63(1.28)	36.18(1.75)	t=-1.34, p=0.18
Length (cm, SD)	49.81(3.40)	50.56(4.59)	t=-0.69, p=0.49
Dubowitz optimality scores (mean,(SD))			
Tone (/10)	8.09(1.83)	8.32(1.35)	t=0.54, p=0.59
Reflex (/6)	4.83(0.46)	4.79(0.37)	t=0.42, p=0.67
Spontaneous movement (/2)	1.98(0.09)	2.00(0.00)	t=1.00, p=0.32
Behavior (/7)	4.39(1.13)	3.74(1.14)	t=2.13, p=0.04*
Abnormal signs (/3)	2.79(0.42)	2.61(0.92)	t=0.94, p=0.35

*p<0.05

Alcohol exposure was clustered to the first trimester, with 86% of mothers reporting alcohol use in this trimester. However, a third of mothers in the exposed group reported continued drinking through the second and third trimesters and those who continued to drink throughout demonstrated a pattern of heavier alcohol use (**Table 2**).

Table 2: Alcohol use of mothers by trimester

	Trimester 1	Trimester 2	Trimester 3
Alcohol usage (n,%)	24(86)	12(43)	9(32)
Once per week or less	18	7	3
2 to 3 times per week	5	5	6
4 to 5 times per week	1	0	0
Daily	0	0	0
Number of drinks per occasion			
<2	2	1	0
2 to 3	6	3	1
4 or more	16	8	8

Whole-brain group comparison of diffusion parameters

There were no significant group differences in any diffusion parameter. Head circumference was significantly positively correlated with FA, and significantly negatively correlated with MD, AD and RD across groups essentially in all white matter tracts.

Using general linear models by region of interest, there was evidence for a significant difference in diffusion by group in major white matter fibers that interconnect temporal, frontal and parietal regions. There was significantly lower AD in the right superior longitudinal fasciculus of alcohol-exposed infants compared to controls [$F(3,52)=3.46$, $p=0.023$] after correction for age and gender (**Table 3, Figure 1**). *Behavioral measures* Scores on the behavioral subscale were positively correlated with FA ($r=0.42$, $p=0.033$) and negatively correlated with MD ($r= -0.46$, $p=0.018$) in the right inferior cerebellar peduncle of alcohol-exposed infants.

Table 3: Results of group differences in diffusion parameters by regions of interest. Axial diffusivity was significantly lower in the right superior longitudinal fasciculus of alcohol-exposed infants compared to controls.

	Hemisphere	FA		MD		AD		RD	
		F	p	F	p	F	p	F	p
Superior longitudinal fasciculus	R	1.25	0.301	1.35	0.269	3.46	0.023**	0.76	0.520
	L	1.49	0.228	0.56	0.643	2.25	0.093	0.15	0.932
Superior fronto-occipital fasciculus	R	0.60	0.615	0.66	0.581	0.95	0.424	0.41	0.748
	L	0.05	0.987	0.27	0.844	0.27	0.847	0.26	0.856
Uncinate fasciculus	R	2.20	0.099	0.13	0.943	0.35	0.786	0.25	0.863
	L	1.61	0.197	1.26	0.296	1.35	0.268	1.21	0.317
Cerebellar peduncle									
– inferior	R	1.26	0.299	2.94	0.042*	3.31	0.027*	2.74	0.053
	L	1.11	0.352	0.85	0.473	0.12	0.945	1.11	0.353
– middle	R	1.02	0.390	2.40	0.078	2.89	0.044*	2.12	0.109
	L	1.28	0.292	0.38	0.770	0.90	0.449	0.41	0.746
– superior	R	2.57	0.064	1.17	0.330	2.51	0.068	0.31	0.820
	L	2.43	0.076	3.05	0.037*	1.98	0.129	3.56	0.020*
Corticospinal tract	R	0.70	0.557	0.36	0.786	0.63	0.600	0.37	0.772
	L	0.60	0.615	0.93	0.431	1.46	0.237	0.73	0.539
Cerebral peduncle	R	0.38	0.769	0.73	0.539	1.23	0.309	0.56	0.647
	L	0.84	0.478	0.63	0.599	1.00	0.402	0.48	0.699
Posterior thalamic radiation	R	1.31	0.282	2.32	0.086	2.30	0.088	2.19	0.100
	L	3.32	0.027*	2.02	0.122	1.82	0.154	2.21	0.097
Fornix	R	0.49	0.689	0.88	0.458	1.23	0.310	0.69	0.562
	L	0.05	0.985	0.95	0.423	1.25	0.301	0.80	0.497
Corpus callosum									
– genu	R	1.32	0.278	1.29	0.289	1.34	0.273	1.25	0.302
	L	0.83	0.482	2.05	0.119	2.01	0.125	2.01	0.125
– body	R	0.78	0.511	1.79	0.160	1.98	0.129	1.61	0.198
	L								

*Age contributed significantly to the model. **Significant group difference at $p < 0.05$ after controlling for age and gender.

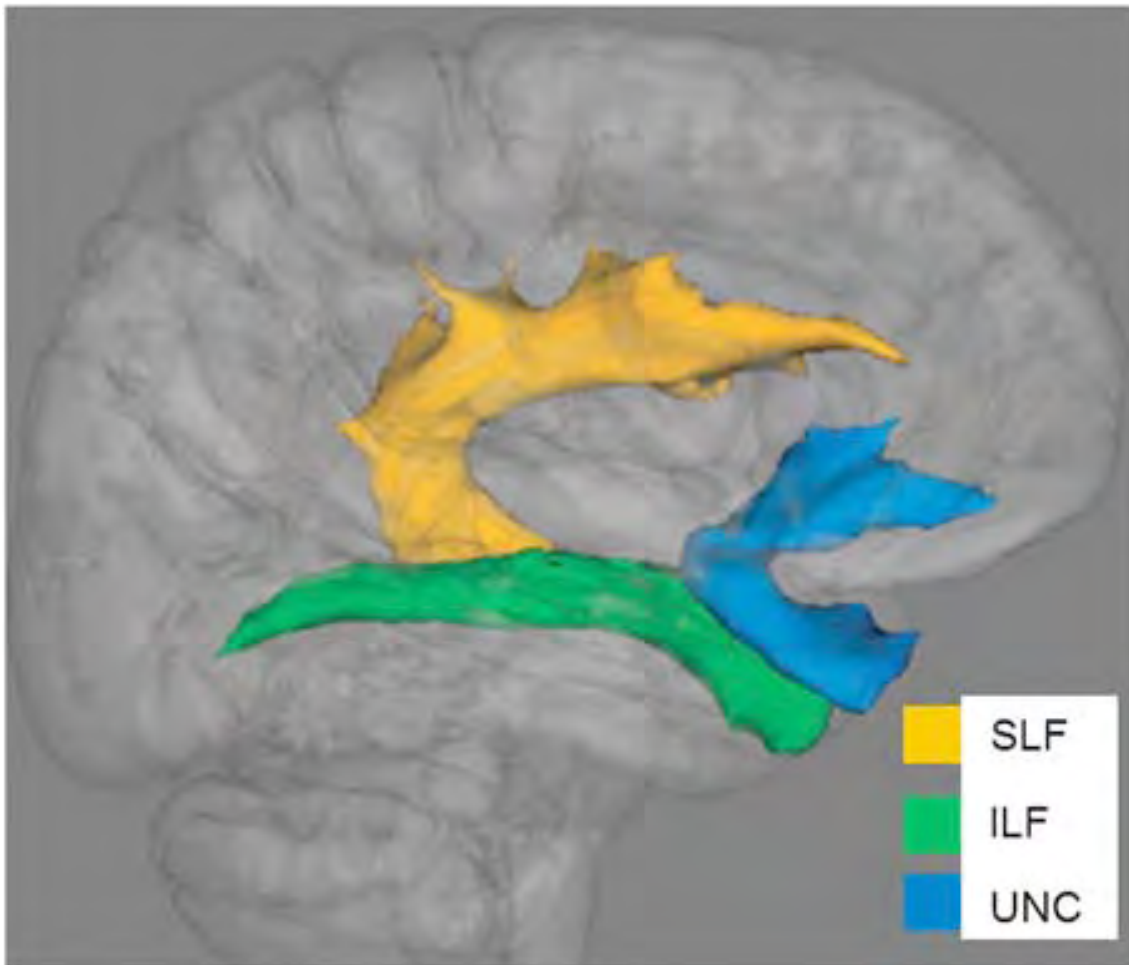
AD, axial diffusivity; FA, fractional anisotropy; MD, mean diffusivity; RD, radial diffusivity.

Axial diffusivity was significantly lower in the right superior longitudinal fasciculus of alcohol-exposed infants compared to controls.

* Post-hoc investigation of univariate tests of significance, revealed that age contributed significantly to the model.

† Significant group difference at $p < 0.05$ after controlling for age and gender.

Figure 1: Association tracts superimposed on a 3D brain template.



Yellow (SLF): superior longitudinal fasciculus, green (ILF) inferior longitudinal fasciculus, blue (UNC): uncinate fasciculus. Axial diffusivity was significantly lower in the right superior longitudinal fasciculus of alcohol-exposed infants compared to controls.

Discussion

Here, we report the effects of prenatal alcohol exposure on the white matter integrity of exposed infants as compared to a well-matched unexposed control group. Prenatal alcohol exposed infants were found to have lower AD in the superior longitudinal fasciculus compared to controls. Lower FA and higher MD in the inferior cerebellar peduncle were both correlated significantly with neonatal neurobehavioral scores in infants with prenatal alcohol exposure.

White matter microstructural abnormalities have been described in the anterior-posterior association pathways in prenatal alcohol exposed children, (33) specifically in the superior longitudinal fasciculus (22) and more recently even in the neonatal age group in

these areas. (30) Our findings in a larger, independent group of infants exposed to alcohol during prenatal life, replicating the report of reduced AD in the superior longitudinal fasciculus, reinforces the significance of these findings. The superior longitudinal fasciculus is one of the major fiber bundles linking frontal, temporal and parietal association cortices. Functions of these networks are believed to include associative tasks, higher level motor tasks, visual perception and attention. While these are not functions that can be teased out in the neonatal age group, they are functional areas which have been identified as being affected in children with prenatal alcohol exposure. (44,45) Following up these infants with developmental and behavioral assessments as they mature may help further define the functional significance of the abnormal white matter microstructure in these tracts.

Cerebellar structure has been found to be affected by prenatal alcohol exposure in children even taking into account differences in overall brain volumes. (7,46) In addition, O'Hare and colleagues demonstrated impaired verbal learning as a cognitive correlate of abnormal cerebellar vermis morphology. (47) Further, Fan *et al.*, (28) have reported lower FA and higher MD associated with eye-blink conditioning and Green *et al.*, (29) a correlation of FA with oculomotor control in a group of school age children with prenatal alcohol exposure. In our alcohol exposed infants, the presence of an association between infant neurobehavior scores with bilateral reduced FA and higher MD in the cerebellar peduncles, which link the cerebellum to the important cerebral motor and language networks, reinforces the role of these pathways in the primary effects of alcohol exposure on early brain development.

There was a significant association between reduced FA and smaller head size in these infants across both groups. This was a robust and widespread finding across multiple white matter areas and suggests that infants who have poorer overall brain growth in the antenatal and immediate post-natal period demonstrate poorer white matter organization. Although there was a reduced mean occipito-frontal circumference in the alcohol-exposed group compared to the unexposed controls, this finding did not reach statistical significance. This is not entirely surprising as the infants in the alcohol-exposed group have not been categorized by anything other than their exposure status and many of them may represent milder forms of the FASD spectrum. Follow up of this group of

prenatal alcohol-exposed children will be critical. Clinical outcomes may well vary in later childhood where other factors such as social, nutritional and other environmental factors may have impact.

The rate of brain development and in particular the rapidly maturing white matter tracts in the first weeks and months of life, as well as the paucity of imaging literature in this age group mean that the dynamics of exactly how DTI metrics relate to tissue microstructural integrity and organization is still incompletely understood. What is known is that the rate of white matter maturation is most rapid in the first 3 months of life (as compared to any other period) and that deep white matter structures (such as those we have reported here), already achieve approximately one half of the mean adult FA at this age. However, peripheral white matter remains barely discernible at the thresholds of detection. (8,48) In this context one has to assume that abnormal results especially in the large midline tracts should be considered as potentially relevant.

Limitations of the current study relate to the reduction of sample size due to movement artefact, this also led to an unbalanced ratio of female to male infant data used in the current analyses. In addition, although information was collected on the timing and severity of alcohol exposure, the number of infants for whom we acquired usable DTI data was insufficient to make meaningful comparisons between these exposure categories in order to help define particular windows of vulnerability to the effects of alcohol during the prenatal period. It is important to note that analyses were conducted in infants before any formal diagnosis could be made and thus effect sizes are expected to be smaller. Further, given the strong influence of age on DTI parameters (reflecting the rapid changes in myelination and maturation), although this was controlled for in the analysis, may have masked group differences resulting from prenatal alcohol exposure. The paucity of data in this age cohort it is an important knowledge gap and it remains highly relevant to provide observations of differences in white matter integrity to inform future studies and aid with the interpretation of the available data in older children. These data, representing congruent trends in an age-group which has not been previously described, is a meaningful addition to the literature in this important area.

In conclusion, the results reported here indicate that the neurobiological effects of prenatal alcohol exposure are observable in newborns, with reduced white matter integrity in major midline white matter tracts. The location of the findings is consistent with previously reported studies of white matter tracts in older children with FASD. Future work with larger cohorts may be able to identify specific windows of vulnerability to the effects of alcohol on the developing brain. In addition, the presence of findings such as these in such a young cohort presents the possibility of exploring the effectiveness of early therapeutic approaches using non-invasive neuroimaging techniques.

Following chapter 4 and 5, which discuss structural and microstructural effects of alcohol exposure in utero, the following chapter introduces data on a functional measure of brain status as measured by ¹H-MRS findings in this group of neonates.

References

- (1) Jones KL, Smith DW, Ulleland CN, Streissguth AP. Pattern of malformation in offspring of Chronic Alcoholic mothers. *Lancet* 1973;1(7815):1267-1271.
- (2) Jones KL, Smith DW, Hansen JW. The Fetal Alcohol Syndrome: clinical delineation. *Annals of the New York Academy of Sciences* 1976;273:130-139.
- (3) Riley EP, Alejandra Infante M, Warren K. Fetal Alcohol Spectrum Disorders: An Overview. *Neuropsychol Rev* 2011;21(2):73-80.
- (4) Rodier PM. Vulnerable periods and processes during central nervous system development. *Environ Health Perspective* 1994;102(suppl. 2):121-124.
- (5) Rodier PM. Environmental causes of central nervous system maldevelopment. *Pediatrics* 2004;113:1076-1083.
- (6) O'Leary-Moore S, Parnell S, Lipinski R. Magnetic Resonance-Based Imaging in Animal Models of Fetal Alcohol Spectrum Disorder. *Neuropsychol Rev* 2011;21:167-185.
- (7) Lebel C, Roussotte F, Sowell ER. Imaging the Impact of Prenatal Alcohol Exposure on the Structure of the Developing Human Brain. *Neuropsychol Rev* 2011;21:102-118.
- (8) Pannek K, Guzzetta A, Colditz P, Rose S. Diffusion MRI of the neonate brain: acquisition, processing and analysis techniques. *Pediatr Radiol* 2012 10/01;42(10):1169-1182.
- (9) Beaulieu C. The basis of anisotropic water diffusion in the nervous system - a technical review. *NMR Biomed* 2002;15(7-8):435-436-455.
- (10) Alexander AL, Lee JE, Lazar M, Field AS. Diffusion Tensor Imaging of the Brain. *Neurotherapeutics* 2007 7;4(3):316-329.
- (11) Assaf Y, Pasternak O. Diffusion Tensor Imaging (DTI)-based White Matter Mapping in Brain Research: A Review. *Journal of Molecular Neuroscience* 2008 01/01;34(1):51-61.
- (12) Song SK, Sun SW, Ramsbottom MJ, Chang C, Russell J, Cross AH. Dysmyelination revealed through MRI as increased radial (but unchanged axial) diffusion of water. *Neuroimage* 2002;17(3):1429-1436.
- (13) Kinoshita Y, Ohnishi A, Kohshi K, Yokota A. Apparent Diffusion Coefficient on Rat Brain and Nerves Intoxicated with Methylmercury. *Environ Res* 1999 5;80(4):348-354.
- (14) Harsan LA, Poulet P, Guignard B, Steibel J, Parizel N, De Sousa PL, et al. Brain dysmyelination and recovery assessment by noninvasive in vivo diffusion tensor magnetic resonance imaging. *J Neurosci Res* 2006;83(3):392-402.

- (15) Paus T, Pesaresi M, French L. White matter as a transport system. *Neuroscience* 2014 <http://dx.doi.org/10.1016/j.neuroscience.2014.01.055>.
- (16) Dubois J, Dehaene-Lambertz G, Kulikova S, Poupon C, Hüppi PS, Hertz-Pannier L. The early development of brain white matter: A review of imaging studies in fetuses, newborns and infants. *Neuroscience* 2014 <http://dx.doi.org/10.1016/j.neuroscience.2013.12.044>.
- (17) Wozniak JR, Muetzel RL. What Does Diffusion Tensor Imaging Reveal About the Brain and Cognition in Fetal Alcohol Spectrum Disorders. *Neuropsychology review* 2011;21(2):133-147.
- (18) Wozniak JR, Mueller BA, Chang PN, Muetzel RL, Caros L, Lim KO. Diffusion Tensor Imaging in Children with Fetal Alcohol Spectrum Disorders. *Alcoholism: Clinical and Experimental Research* 2006;30(10):1799-1806.
- (19) Wozniak JR, Muetzel RL, Mueller BA, McGee CL, Freerks MA, Ward EE, et al. Microstructural Corpus Callosum Anomalies in Children With Prenatal Alcohol Exposure: An Extension of Previous Diffusion Tensor Imaging Findings. *Alcoholism: Clinical and Experimental Research* 2009;33(10):1825-1835.
- (20) Ma X, Coles CD, Lynch ME, LaConte SM, Zurkiyara O, Wang D, et al. Evaluation of Corpus Callosum Anisotropy in Young Adults With Fetal Alcohol Syndrome According to Diffusion Tensor Imaging. *Alcoholism, Clinical and Experimental Research* 2005;29(7):1214-1222.
- (21) Li L, Coles CD, Lynch ME, Hu X. Voxelwise and Skeleton-Based Region of Interest Analysis of Fetal Alcohol Syndrome and Fetal Alcohol Spectrum Disorders in Young Adults. *Human Brain Mapping* 2009;30(10):3265-3274.
- (22) Treit S, Lebel C, Baugh L, Rasmussen C, Andrew G, Beaulieu C. Longitudinal MRI reveals altered trajectory of brain development during childhood and adolescence in fetal alcohol spectrum disorders. *The Journal of Neuroscience* 2013;33(24):10098-10109.
- (23) Fryer S, Schweinsburg BC, Bjorkquist OA, Frank LK, Mattson SM, Spadoni ADea. Characterisation of white matter microstructure in fetal alcohol spectrum disorders. *Alcohol Clinical and Experimental research* 2009;33(3):514-521.
- (24) Lebel C, Rasmussen C, Wyper K, Walker L, Andrew G, Yager Jea. Brain Diffusion abnormalities in children with fetal alcohol spectrum disorder. *Alcoholism, Clinical and Experimental Research* 2008;32(10):1732-1740.
- (25) Sowell ER, Johnson A, Kan E, Lu LH, Van Horn JD, Toga AW, et al. Mapping White Matter Integrity and Neurobehavioral Correlates in Children with Fetal Alcohol Spectrum Disorders. *The Journal of Neuroscience* 2008 February 06;28(6):1313-1319.

- (26) OLebel C, Mattson SN, Riley EP, Jones KL, Adnams CM, May PA, et al. A Longitudinal Study of the Long-Term Consequences of Drinking during Pregnancy: Heavy In Utero Alcohol Exposure Disrupts the Normal Processes of Brain Development. *The Journal of Neuroscience* 2012 October 31;32(44):15243-15251.
- (27) Lebel C, Rasmussen C, Wyper K, Andrew G, Beaulieu C. Brain Microstructure Is Related to Math Ability in Children With Fetal Alcohol Spectrum Disorder. *Alcoholism: Clinical and Experimental Research* 2010;34(2):354-363.
- (28) Fan J, Meintjes EM, Molteno CD, Spottiswoode BS, Dodge NC, Alhamud AA, et al. White matter integrity of the cerebellar peduncles as a mediator of effects of prenatal alcohol exposure on eyeblink conditioning. *Hum Brain Mapp* 2015:n/a-n/a.
- (29) Green CR, Lebel C, Rasmussen C, Beaulieu C, Reynolds JN. Diffusion Tensor Imaging Correlates of Saccadic Reaction Time in Children with Fetal Alcohol Spectrum Disorder. *Alcoholism: Clinical and Experimental Research* 2013;37(9):1499-1507.
- (30) Taylor PA, Jacobson SW, van der Kouwe A, Molteno CD, Chen G, Wintermark P, et al. A DTI-based tractography study of effects on brain structure associated with prenatal alcohol exposure in newborns. *Human Brain Mapping* 2015 DOI: 10.1002/hbm.22620;36(1):170-186.
- (31) May PA, Gossage JP, Kalberg K, Robinson L, Buckley D, Manning Mea. Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies. *Developmental Disabilities Research Reviews* 2009;15(3):176-192.
- (32) May PA, Blankenship J, Marais A, Gossage JP, Kalberg WO, Barnard R, et al. Approaching the Prevalence of the Full Spectrum of Fetal Alcohol Spectrum Disorders in a South African Population-Based Study. *Alcoholism: Clinical and Experimental Research* 2013;37(5):818-830.
- (33) Donald KA, Eastman E, Howells FM, Adnams C, Riley EP, Woods RP, et al. Neuroimaging effects of prenatal alcohol-exposure on the developing human brain: A magnetic resonance imaging review . *Acta Neuropsychiatrica* 2015;DOI:<http://dx.doi.org/10.1017/neu.2015.12>.
- (34) Stein DJ, Koen N, Donald KA, Adnams CM, Koopowitz S, Lund C, et al. Investigating the psychosocial determinants of child health in Africa: the Drakenstein Child Health Study. *J Neuroscience methods* 2015;doi:10.1016/j.jneumeth.2015.03.016.
- (35) Humeniuk R, Ali R, Babor TF, Farrell M, Formigoni ML, Jittiwutikarn J, et al. Validation of the Alcohol, Smoking And Substance Involvement Screening Test (ASSIST). *Addiction* 2008 Jun;103(6):1039-1047.

- (36) Jackson PB, Williams DR, Stein DJ, Herman A, Williams SL, Redmond DL. Race and psychological distress: the South african stress and health study. *J Health Soc Behav* 2010 Dec;51(4):458-477.
- (37) Lozarno J, Garcia-Algar O, Vall O, de la Torre R, Scaravelli G, Pichini S. Biological matrices for the evaluation of in utero exposure to drugs of abuse. *There Drug Monit* 2007;29(6):711-734.
- (38) Noble Y, Boyd R. Neonatal assessments for the preterm infant up to 4 months corrected age: a systematic review. *Developmental Medicine & Child Neurology* 2012;54(2):129-139.
- (39) Dubowitz L, Mercurio E, Dubowitz V. An Optimality Score for the Neurological Examination of The Term Newborn. *The Journal of pediatrics* 1998;133(3):406-416.
- (40) Smith SM, Jenkinson M, Johansen-Berg H, Rueker D, Nichols T, Mackay CE, et al. Tract-based spatial statistics: Voxel-wise analysis of multi-subject diffusion data. *Neuroimage* 2006;31(4):1487-1488-1505.
- (41) Dubois J, Hertz-Pannier L, Dehaene-Lambertz G, Cointepas Y, Le Bihan D. Assessment of the early organization and maturation of infants' cerebral white matter fiber bundles: a feasibility study using quantitative diffusion tensor imaging and tractography. *Neuroimage* 2006;30(4):1121-1132.
- (42) Andersson JLR, Jenkinson M, Smith S. Non-linear registration, aka Spatial normalisation. FMRIB technical report TR07JA2. www.fmrib.ox.ac.uk/analysis/techrep 2007.
- (43) Mori S, Wakana S, van Zijl PCM, Nagae-Poetscher LM. *MRI Atlas of Human White Matter*. Amsterdam: Elsevier; 2005.
- (44) Mattson S, Crocker N, Nguyen T. Fetal Alcohol Spectrum Disorders: Neuropsychological and Behavioral Features. *Neuropsychol Rev* 2011;21(2):81-101.
- (45) Mattson SN, Roesch SC, Fagerlund Å, Autti-Rämö I, Jones KL, May PA, et al. Toward a neurobehavioral profile of fetal alcohol spectrum disorders . *Alcoholism: Clinical and Experimental Research* 2010;34(9):1640-1650.
- (46) Mattson SN, Riley EP, Jernigan TL, Garcia A, Kaneko WM, Ehlers CL, et al. A decrease in the size of the basal ganglia following prenatal alcohol exposure: a preliminary report. *Neurotoxicology and teratology* 1994;16(3):283-289.
- (47) O'Hare ED, Kan E, Yoshii J, Mattson SN, Riley EP, Thompson PM, et al. Mapping cerebellar vermal morphology and cognitive correlates in prenatal alcohol exposure. *Neuroreport* 2005;16(12):1285-1290.

- (48) Provenzale JM, Liang L, DeLong D, White LE. Diffusion Tensor Imaging Assessment of Brain White Matter Maturation in the First Postnatal Year. *American Journal of Radiology* 2007;189:476-486.

Chapter 6

Reduced glutamate in white matter of male neonates exposed to alcohol *in utero*: a ¹H-magnetic resonance spectroscopy study

¹Howells FM, ²Donald KA, ³Roos A, ⁴Woods RP, ²Zar HJ, ⁴Narr KL, ¹Stein DJ

1 - *Department of Psychiatry and Mental Health, University of Cape Town, South Africa*

2 - *Department of Paediatrics and Child Health, Red Cross War Memorial Children's Hospital and University of Cape Town, South Africa*

3 - *Medical Research Council Anxiety and Stress Disorders Unit, University of Stellenbosch, South Africa*

4 - *Departments of Neurology and of Psychiatry and Biobehavioral Sciences, University of California Los Angeles, United States of America*

*Corresponding author: Dr Fleur M Howells and Dr K Donald. Physical & Postal address: Office 105, J-2 Block Old Groote Schuur Hospital, Anzio Road, Observatory, 7925. Telephone: +27(0)21 404 5480. Email address: howellsfleur@gmail.com; kirsty.donald@uct.ac.za

Abstract

Background

In utero exposure to alcohol leads to a spectrum of fetal alcohol related disorders (FASD). However, few studies used have used proton magnetic resonance spectroscopy (¹H-MRS) to understand how neurochemical disturbances relate to the pathophysiology of FASD. Further, no studies to date have assessed brain metabolites in infants exposed to alcohol *in utero*. We hypothesize that neonates exposed to alcohol *in utero* will show decreased glutamatergic activity, pre-emptive of their clinical diagnosis or behavioral phenotype.

Methods

Single voxel ¹H-MRS data, sampled in parietal white and gray matter, were acquired from 36 neonates exposed to alcohol *in utero*, and 31 control unexposed neonates, in their 2nd-4th week of life.

Results

Absolute glutamate with glutamine (Glx) concentrations of parietal white matter (PWM) was decreased in male infants exposed to alcohol *in utero* (n=8), compared with male controls (n=10; p=0.008). Absolute glutamate concentrations of PWM was decreased in male infants exposed to alcohol *in utero* (n=8), compared with male controls (n=10; p=0.02) and female infants exposed to alcohol *in utero* (n=9; p=0.02).

Conclusions

We found absolute concentrations of Glx and Glu were decreased in male neonates exposed to alcohol in utero. We suggest that thyroid hormone activity may underlie the decreased Glu in white matter of male neonates exposed to alcohol *in utero*, and further study is required to elucidate the relationship between glutamate, thyroid hormone activity, and oligodendrocyte maturation.

Keywords

MRS, alcohol exposure, gray matter, oligodendrocytes, thyroid hormone activity

Introduction

Proton magnetic resonance spectroscopy ($^1\text{H-MRS}$) may be particularly useful insofar as it can be used to infer information about cellular microstructure *in vivo* and to point to abnormalities in neurotransmission and cellular metabolism in fetal alcohol spectrum disorder (FASD). However, to date very few studies have investigated brain metabolite concentrations in FASD using $^1\text{H-MRS}$ in children and adolescence, while there are no studies in neonates exposed to alcohol *in utero*.

One study including 11 children with fetal alcohol syndrome (FAS) and four controls found *increased* n-acetyl-aspartate (NAA), a marker of neuronal integrity, in the caudate nucleus in FAS and reported associations between NAA and facial dysmorphometry measures (11). In contrast, a second study in adolescents and young adults with FASD (n=10 with FASD and n=10 controls) found *decreased* NAA in parietal and frontal cortices, frontal white matter, and the corpus callosum, thalamus, and cerebellar dentate nucleus (12). A third $^1\text{H-MRS}$ study reported *decreased* choline, a marker of phospholipid membrane turnover and myelination, in frontal/parietal white matter of preadolescent children with FASD or partial FASD (n=20) compared to children without the FAS facial phenotype or cognitive/behavioral dysfunction and controls (n=61) (13). A second study reported a *decreased* choline in the left striatum in eight male children with FASD compared to eight controls (14). Finally, a more recent study which assessed 37 children with heavy alcohol exposure and 17 non- or minimally exposed controls, has shown several $^1\text{H-MRS}$ relationships within the cerebellum and alcohol consumption; *decreased* NAA was related to increased maternal use of alcohol around time of conception, *decreased* choline was related to higher levels of alcohol consumption during pregnancy, and alcohol consumption around time of conception and during pregnancy were related to *increased* glutamate with glutamine (Glx) (15).

These studies demonstrate the utility of $^1\text{H-MRS}$ for targeting neurochemical abnormalities associated with symptoms of FASD during childhood. However, differences in the age range and clinical profiles of the developmental samples studied differences in the anatomic locations of $^1\text{H-MRS}$ voxel placement and variations in imaging acquisition and

analysis approaches have made it difficult to compare results across studies. Thus further empirical investigation at different stages of development is required.

It is important to characterize the permanent effects or outcome of *in utero* exposure to alcohol in children and adolescence, however is it feasible to intervene and improve the clinical and behavioral outcome of these children at a younger age – as neonates. The teratogenic effects of alcohol have not been fully elucidated; due to the complex cellular influences it exerts (16). The severity of FASD has been associated with the ability of the mother to metabolize alcohol, and the production of acetaldehyde – a highly toxic metabolite (17). Alcohol has been shown to decrease DNA synthesis, inhibit growth factors and protein synthesis, and reduce available glucose – all of which are necessary for healthy development (18). Studies in neonates exposed to alcohol *in utero* primarily assess the severity of FASD, investigating clinical phenotype through dysmorphometry and biomarkers obtained from meconium (19). As yet no studies have been able to suggest strategies for intervention to improve the neurobiological effects of *in utero* alcohol exposure.

Glutamate, the brain's major excitatory neurotransmitter is required to signal processes of myelination; achieved by glutamate's activation of **α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid** (AMPA), kainate and NMDA receptors on immature oligodendrocytes (20). The expression of these glutamate receptors on oligodendrocytes is achieved by the release of neuregulin and brain derived neurotrophic factor (BDNF) required for natural brain maturation (21), which is reportedly lacking in FASD (22). Oligodendrocytes sheath axons to ensure fast and efficient communication between brain regions, which is again lacking in children and adolescents with FASD (2,3,10). In healthy neonates glutamate concentration increases exponentially in white matter, and gray matter, during the first three months of life, then tapers to a steady level in the second year of life (23). Could this be a window of opportunity to develop an intervention, instead of simply characterizing metabolite profiles in childhood and adolescence.

We hypothesize that neonates exposed to alcohol *in utero* will show decreased glutamatergic activity, pre-emptive of their clinical diagnosis or behavioral phenotype. To address

these important gaps in the literature, the current investigation used single voxel $^1\text{H-MRS}$ to compare metabolite concentrations in infants exposed to alcohol *in utero* (**Table 2**), who have not yet been diagnosed with FASD, during their second to fourth week of life, hypothesizing that neonates exposed to alcohol *in utero* would show decreased Glu concentrations.

Materials and Methods

Participants and recruitment

We report data from 36 infants (19 female, 17 male) who were exposed to alcohol *in utero* and 31 infants (10 female, 21 male) who were not exposed to alcohol *in utero*, i.e. no history or biological evidence of substance abuse by the mother. Ethical approval for this study was obtained from the Faculty of Health Sciences Research Ethics Committee at the University of Cape Town (HREC REF 525/2012). The study was conducted in accordance with the Declaration of Helsinki .(24)

Infants and their mothers were recruited from a larger population-based birth cohort study, the Drakenstein Child Health Study. The details of the broader study and recruitment to this sub-study are described in chapter 3.

The infants were scanned in the second to fourth week after birth. On the day of scanning physical attributes were recorded, including: length (cm), weight (kg), and head circumference (cm). The Dubowitz neurological exam, a validated measure of infant neuromotor and neurobehavioral status (28) was performed by a qualified neonatal nurse (**Table 1**). Drug use history was also taken.

MRI scanning of infants and $^1\text{H-MRS}$ data collection

All infants were scanned without anaesthesia during natural sleep after they had been fed. During scanning, infants were wrapped in cotton swaddling with earplugs and mini-muffs for ear protection. A pulse oximeter was used to monitor oxygen saturations and heart rate, and a qualified neonate nurse or pediatrician was present with the infant in the scanner room for the duration of the imaging session. Imaging data were acquired on a 3T Allegra Siemens head-only system using a transmit-receive head coil. To overcome

limitations with scanning smaller volumes of tissue, voltage was reduced to optimize signal, and the head coil was loaded with a wet clay inlay (40 x 40 cm with a thickness of 2 cm, standard sculpting clay commercially bought – white stoneware clay with grog).

Anatomical images were obtained for ¹H-MRS voxel placement using a T2-weighted sequence (TR = 3000 msec, TE 355 msec, FOV = 220 mm, slice thickness = 1.0 mm, 128 slices, scan time 6:29 min). The T2-weighted images were reconstructed in 3D to facilitate accurate placement of voxels in left parietal white matter, which was positioned dorsolateral to the trigone of the lateral ventricle (PWM; standard placement **Figure 1**) and bilateral parietal gray matter, the center of this voxel was the divide of the two hemispheres and included bilateral precuneus and posterior cingulate (PGM; standard placement **Figure 2**). ¹H-MRS acquisition included a standard point resolved spectroscopy (PRESS) sequence (TE = 30 msec, TR = 2000 msec, 128 averages, delta = -2.6 ppm delta frequency, volume of interest (VOI) 25 x 25 x 25 mm, scan time 4:24 min, and water reference (2 averages, scan time 0:12 min).

Figure 1: Positioning of parietal white matter proton magnetic resonance spectroscopy (¹H-MRS) voxel and example spectra, left hemisphere.

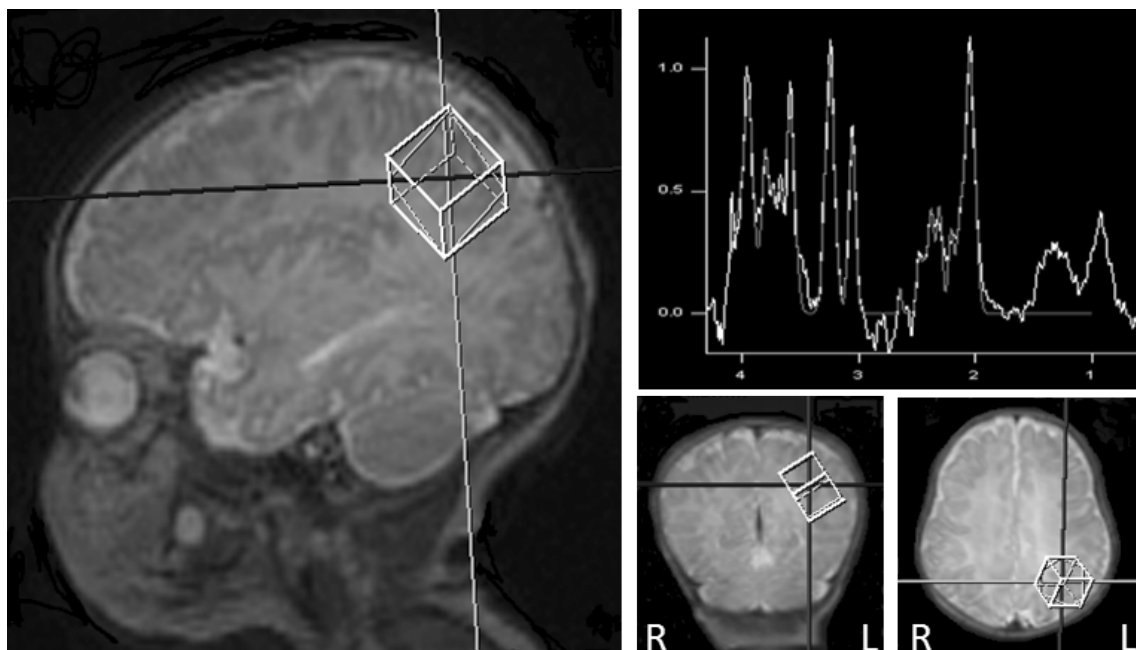
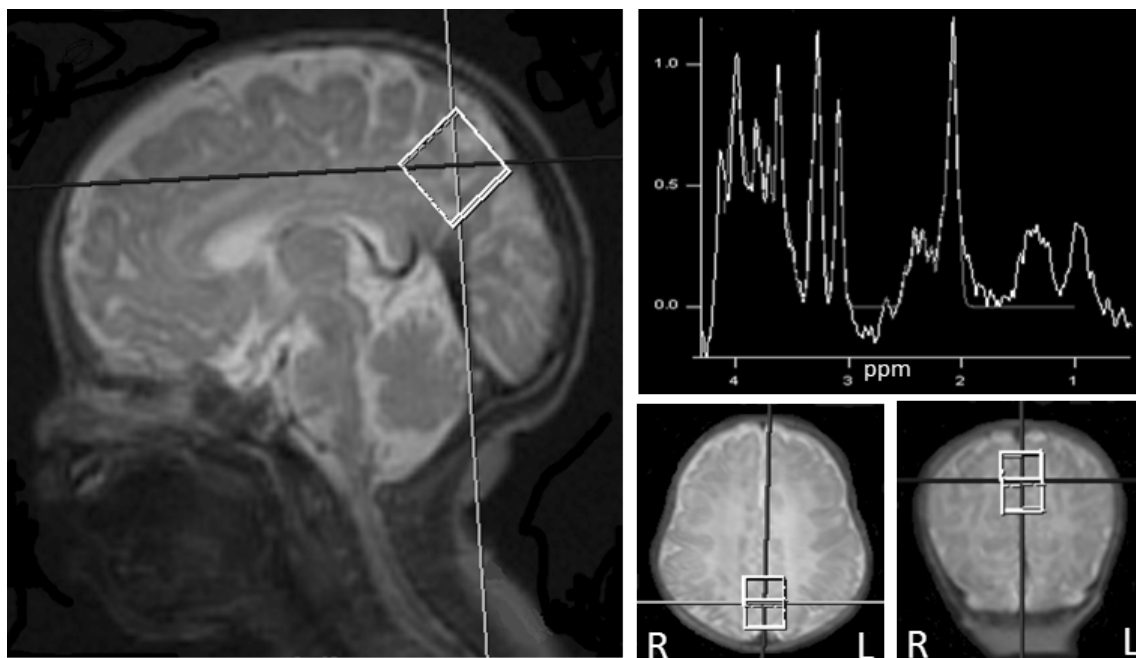


Figure 2: Positioning of parietal gray matter proton magnetic resonance spectroscopy (¹H-MRS) voxel and example spectra, midline.



¹H-MRS data processing

Processing of ¹H-MRS data were performed using the widely validated LCModel software package (29) and basis sets for PRESS sequences with a TE = 30 msec. Default parameters (ppmend = 0.2 and ppmst = 3.85) were used and a Cramér-Rao lower bound of < 20% was determined as the criterion for filtering out low quality spectra, we additionally report the full width at half maximum (FWHM) and the signal to noise ratio (SNR). From 36 infants exposed to alcohol in utero visually acceptable ¹H-MRS scans were obtain after the filtering these spectra – 31 PGM and 19 PWM spectra survived. From 31 control infants visually acceptable ¹H-MRS scans were obtained after filtering these spectra – 25 PGM and 21 PWM spectra survived. Absolute concentrations of glutamate (Glu) and glutamate with its precursor glutamine (Glx), n-acetyl-aspartate and n-acetyl-aspartyl-glutamate (NAA+NAAG), n-acetyl-aspartate (NAA), choline containing metabolites (GPc+PCh), *myo*-inositol (ml), creatine containing metabolites (PCr+Cr), and a robust macromolecule and lipid (MM09 & Lip09) are reported (Table 3). We additionally report and included FWHM and SNR for these voxels in our analysis.

Statistical analysis

Statistical analyses were performed using the Statistica software package (version 12) (30). Factorial analysis of variance (ANOVA) was used to model group differences for each brain area, PGM or PWM, and gender. We were unable to include brain area as a within variable due to the fall-out of either PWM or PGM creating a mismatch of data and the model were compromised. Significant findings were followed by Tukey's post-hoc tests, p-value < 0.05.

Correlation analyses were performed to determine whether there were significant relationships between clinical measures and metabolite concentrations, p-value < 0.01 were considered significant.

Results

No differences in physical properties (age, weight, length, head circumference) were found by group. No differences were found in outcome from the Dubowitz neurological exam or its subscales by group. No differences were found for number of active smokers (**Table 1**). Use of alcohol us by the mother during pregnancy is reported in **Table 2**.

Table 1: Physical attributes, Dubowitz neurological exam scores, and maternal smoking status

	Alcohol exposed infants (n=36)			Alcohol exposed female infants (n=17)			Alcohol exposed male infants (n=19)			Control infants (n=31)			Control female infants (n=10)			Control male infants (n=21)		
	ave.	std. dev	range	ave.	std.dev		ave.	std.dev		ave.	std.dev	range	ave.	std.dev		ave.	std.dev	
Physical attributes																		
Age (days old)	20.8	4.8	(12-35)	21.4	5.0		20.2	4.6		20.5	4.8	(11-31)	20.6	5.3		20.5	4.6	
Gestational age (weeks)	38.7	1.5	(36-42)	39.0	1.2		38.5	1.7		38.6	1.7	(36-41)	38.2	1.5		38.2	1.8	
Length (cm)	50.3	4.2	(45-65)	51.8	4.7		49.0	3.2		50.2	4.6	(38-62)	48.5	4.6		50.9	4.5	
Weight (kg)	3.79	0.56	(2.63-5.30)	3.73	0.57		3.84	0.56		3.95	###	(2.07-5.66)	3.65	0.49		4.10	0.87	
Head circumference (cm)	35.5	1.2	(33.0-37.5)	35.6	1.2		35.6	1.4		36.1	1.7	(31.8-39.0)	35.4	0.9		36.5	1.9	
Dubowitz neurological exam																		
Tone	8.4	1.8	(3.5-11.5)	8.5	1.7		8.4	1.9		8.7	1.3	(5.0-11.0)	8.0	1.5		9.0	1.1	
Reflexes	4.8	0.6	(3.5-6.5)	4.9	0.4		4.7	0.7		4.8	0.6	(3.5-6.5)	4.7	0.7		4.8	0.6	
Spontaneous movement	2.0	0.5	(0.5-2.0)	2.0	0.4		2.0	0.0		2.0	0.1	(1.5-2.0)	2.0	0.0		2.0	0.1	
Behavior	4.4	1.2	(1.0-7.0)	4.3	1.6		4.5	0.9		4.1	1.4	(2.0-7.0)	3.8	1.3		4.3	1.4	
Abnormal signs	2.8	0.4	(2.0-3.0)	2.8	0.4		2.8	0.4		2.6	0.9	(0.0-3.0)	2.5	0.8		2.6	1.0	
Optimality score/outcome	22.4	2.5	(15.0-26.5)	22.2	1.8		22.0	2.6		22.1	2.7	(15.5-26.0)	20.4	2.8		21.9	1.8	
Smoking of nicotine cigarettes (continine score > 500)																		
non-smoker/active-smoker	18/18									12/19								

No significant group differences were found for infants' physical attributes, Dubowitz neurological exam scores, or maternal smoking behaviour.

Table 2: Maternal drinking during pregnancy

	Trimester 1	Trimester 2	Trimester 3
Alcohol usage (n,%)	30(83)	15(42)	10(28)
Once per week or less	19	11	4
2 to 3 times per week	5	4	6
4 to 5 times per week	1	0	0
Daily	0	0	0
Number of drinks per occasion			
<2	3	1	0
2 to 3	10	5	2
4 or more	16	9	8

Use of alcohol during the three trimesters for alcohol exposed infants, consistent with WHO moderate-severe alcohol use (either drinking 2 or more times a week or 2 or more drinks per occasion).

¹H-magnetic resonance spectroscopy

Parietal white matter (PWM) showed a significant effect of alcohol exposure for group by gender ($F_{(10,20)} = 2.60$, $p = 0.03$). Glx and Glu were found to be significantly decreased in male infants prenatally exposed to alcohol ($n=8$). Glx was lower when compared with male control infants ($n=10$; $p=0.008$) and Glu was lower in male infants exposed to alcohol in utero when compared with male controls ($n=10$; $p=0.02$) and females exposed to alcohol in utero ($n=9$; $p = 0.02$) (**Table 3**). No further differences were found for PWM and no differences were found for PGM. In addition, no significant correlations were found to hold for either Glu or Glx when applying a p-value of 0.01.

Table 3: ¹H-Magnetic resonance spectroscopy absolute metabolites, with FWHM and SNR.

	Alcohol exposed infants		Alcohol exposed female infants		Alcohol exposed male infants		Control infants		Control female infants		Control male infants	
	ave.	std.dev	ave.	std.dev	ave.	std.dev	ave.	std.dev	ave.	std.dev	ave.	std.dev
1H Magnetic resonance spectroscopy - Parietal gray matter	(n=31)		(n=15)		(n=16)		(n=24)		(n=8)		(n=16)	
glutamate + glutamine (Glx)	6.49	1.16	6.55	1.20	6.45	1.17	6.23	1.01	6.16	0.99	6.26	1.05
glutamate (Glu)	5.12	0.69	5.12	0.67	5.12	0.73	5.14	0.71	5.04	0.88	5.19	0.65
n-acetyl-aspartate + n-acetyl-aspartyl-glutamate (NAA+NAAG)	3.45	0.35	3.43	0.24	3.47	0.45	3.40	0.67	2.99	0.75	3.58	0.57
n-acetyl-aspartate (NAA)	2.42	0.66	2.41	0.74	2.44	0.60	2.37	0.87	1.97	0.69	2.55	0.91
choline containing metabolites (GPC+PCh)	1.51	0.23	1.49	0.22	1.52	0.25	1.47	0.26	1.34	0.35	1.53	0.19
myo-inositol	7.28	0.85	7.10	0.90	7.44	0.79	2.24	0.94	7.09	1.16	7.30	0.86
creatine containing metabolites (PCr+Cr)	3.49	0.49	3.45	0.57	3.52	0.42	3.45	0.63	3.01	0.79	3.64	0.44
Macromolecule MM09 and Lipid 09	5.83	0.64	5.90	0.66	5.77	0.64	5.80	0.84	5.61	0.57	5.89	0.95
Full width at half maximum (FWHM)	0.07	0.03	0.07	0.03	0.07	0.03	0.07	0.03	0.05	0.02	0.07	0.03
Signal to noise ratio (SNR)	9.90	2.10	9.93	2.52	9.87	1.71	9.50	2.18	8.88	1.81	9.78	2.32
1H Magnetic resonance spectroscopy - Parietal white matter	(n=17)		(n=9)		(n=8)		(n=19)		(n=9)		(n=10)	
glutamate + glutamine (Glx)	5.03	0.93	5.38	0.89	4.63	0.85	5.77	0.75	5.52	0.66	5.94	0.78
glutamate (Glu)	3.79	0.61	4.10	0.67	3.45	0.30	4.21	0.66	4.00	0.66	4.34	0.64
n-acetyl-aspartate + n-acetyl-aspartyl-glutamate (NAA+NAAG)	2.76	1.10	2.60	1.23	2.93	1.00	3.17	0.68	3.24	0.81	3.12	0.61
n-acetyl-aspartate (NAA)	2.15	1.06	2.10	1.12	2.21	1.04	2.43	0.68	2.28	0.78	2.52	0.61
choline containing metabolites (GPC+PCh)	1.28	0.37	1.23	0.40	1.33	0.36	1.42	0.22	1.45	0.28	1.40	0.18
myo-inositol	5.00	1.71	4.68	1.91	5.37	1.50	5.98	1.00	6.03	1.91	5.95	1.50
creatine containing metabolites (PCr+Cr)	2.81	0.93	2.72	1.06	2.91	0.82	3.17	0.44	3.27	0.50	3.10	0.40
Macromolecule MM09 and Lipid 09	6.81	2.74	6.77	1.32	6.86	3.89	6.37	2.74	6.39	3.89	6.35	1.48
Full width at half maximum (FWHM)	0.07	0.03	0.06	0.02	0.08	0.04	0.08	0.04	0.08	0.04	0.07	0.05
Signal to noise ratio (SNR)	9.38	2.47	9.16	1.94	9.57	2.99	9.50	2.95	9.13	2.59	9.75	3.25

*Male infants exposed to alcohol in utero showed significantly decreased Glx in PWM when compared with healthy controls (p = 0.008). # Male infants exposed to alcohol in utero showed decreased Glu in PWM when compared with control males (p = 0.02) and females infants exposed to alcohol in utero (p = 0.02).

Discussion

This is the first study to examine variations in glutamate (Glu) and glutamate with glutamine (Glx) in neonates with and without prenatal exposure to alcohol. Our main findings indicate that parietal white matter (PWM) - Glx and Glu concentrations were decreased in male infants exposed to alcohol (**Table 3**). This study provides support of our developing hypothesis: decreased glutamate concentrations may account for the maturational lag in neonates exposed to alcohol *in utero* – i.e. decreased oligodendrocyte maturation in white matter. We would suggest to the following line of enquiry, which may serve as a potential route of intervention, in at least male neonates that have been exposed to alcohol *in utero*, improvement of thyroid hormone signalling.

As previously mentioned, glutamate, is required to signal processes of myelination; achieved by glutamates activation of AMPA/kainate and NMDA receptors on immature oligodendrocytes (20), so to is effective thyroid hormone signalling required for the development and maturation of oligodendrocytes (31-35). Oligodendrocytes sheath axons to ensure fast and efficient communication between brain regions, which is again lacking in children and adolescents with FASD (2,3,10) – i.e. oligodendrocytes are primarily found in white matter, the brain region we found affected in males exposed to alcohol *in utero*.

It has been shown that *in utero* alcohol affects thyroid hormone signalling – increased thyroid stimulating hormone and decreased thyroxine 4 (36). This may be related to decreased feedback from activated α -1 thyroid hormone receptors; as α -1 thyroid hormone receptor mRNA encoding is reduced in rodents exposed to alcohol *in utero* (Scott et al., 1998). A recent human and rabbit study has comprehensively shown that treatment with thyroxine restores myelination and clinical recovery after neonatal intraventricular haemorrhage, by induction of necessary oligodendrocyte transcription factors (37). These data suggest that the reduced Glu found in the current study may be a result of delayed maturation of oligodendrocytes.

Why did we find this decrease in Glu and Glx only in the male infants exposed to alcohol *in utero*? As mentioned earlier, thyroid hormone signalling is affected in neonates exposed to alcohol *in utero* – increased thyroid stimulating hormone and decreased

thyroxine 4, these levels are *further affected in male infants exposed to alcohol in utero* (36). This supports the proposed relationship between decreased glutamate, decreased thyroxine, and immature oligodendrocytes. We now need to determine the reason(s) why this relationship is limited to males exposed to alcohol in utero and whether females exert compensatory mechanisms.

Limitations of the present study include those associated with scanning neonates - including the loss of data due to movement artefact, with infants waking during the scan. Limitations of the ¹H-MRS protocol include the lack of partial volume correction, we also did not address the issue of chemical shift artefact related to the overlap of metabolite and water scans. Larger ¹H-MRS voxel sizes were also chosen to improve the signal-to-noise ratio, which may affect the homogeneity of tissue within selected voxels. Finally, though also a strength of this study, neonates were examined prior to diagnosis and thus were compared on the basis of known alcohol exposure only. While this suggests that Glx and Glu concentrations are an independent marker of prenatal alcohol exposure, we cannot rule out the possibility that some reports of maternal history of alcohol exposure may be less accurate.

In conclusion, we found absolute concentrations of Glx and Glu were decreased in male neonates exposed to alcohol in utero. We suggest that thyroid hormone activity may underlie the decreased Glu in white matter of male neonates exposed to alcohol *in utero*, and further study is required to elucidate the relationship between glutamate, thyroid hormone activity, and oligodendrocyte maturation.

Leading on from this manuscript which identified neurochemical changes observable using ¹H-MRS imaging, I now describe the final functional data acquired on this group of alcohol exposed neonates using resting state functional MRI.

References

- (1) Khalil A, O'Brien P. Alcohol and pregnancy. *Obstetrics, Gynaecology and Reproductive Medicine* 2010;20(10):311-313.
- (2) Treit S, Lebel C, Baugh L, Rasmussen C, Andrew G, Beaulieu C. Longitudinal MRI reveals altered trajectory of brain development during childhood and adolescence in fetal alcohol spectrum disorders. *J Neurosci* 2013 Jun 12;33(24):10098-10109.
- (3) Spottiswoode BS, Meintjes EM, Anderson AW, Molteno CD, Stanton ME, Dodge NC, et al. Diffusion tensor imaging of the cerebellum and eyeblink conditioning in fetal alcohol spectrum disorder. *Alcohol Clin Exp Res* 2011 Dec;35(12):2174-2183.
- (4) Lebel C, Mattson SN, Riley EP, Jones KL, Adnams CM, May PA, et al. A longitudinal study of the long-term consequences of drinking during pregnancy: heavy in utero alcohol exposure disrupts the normal processes of brain development. *J Neurosci* 2012 Oct 31;32(44):15243-15251.
- (5) Wozniak JR, Mueller BA, Muetzel RL, Bell CJ, Hoecker HL, Nelson ML, et al. Inter-hemispheric functional connectivity disruption in children with prenatal alcohol exposure. *Alcohol Clin Exp Res* 2011 May;35(5):849-861.
- (6) Wozniak JR, Muetzel RL, Mueller BA, McGee CL, Freerks MA, Ward EE, et al. Microstructural corpus callosum anomalies in children with prenatal alcohol exposure: an extension of previous diffusion tensor imaging findings. *Alcohol Clin Exp Res* 2009 Oct;33(10):1825-1835.
- (7) Wozniak JR, Mueller BA, Chang PN, Muetzel RL, Caros L, Lim KO. Diffusion tensor imaging in children with fetal alcohol spectrum disorders. *Alcohol Clin Exp Res* 2006 Oct;30(10):1799-1806.
- (8) Leigland LA, Budde MD, Cornea A, Kroenke CD. Diffusion MRI of the developing cerebral cortical gray matter can be used to detect abnormalities in tissue microstructure associated with fetal ethanol exposure. *Neuroimage* 2013 Dec;83:1081-1087.
- (9) Leigland LA, Ford MM, Lerch JP, Kroenke CD. The influence of fetal ethanol exposure on subsequent development of the cerebral cortex as revealed by magnetic resonance imaging. *Alcohol Clin Exp Res* 2013 Jun;37(6):924-932.
- (10) Wozniak JR, Mueller BA, Bell CJ, Muetzel RL, Hoecker HL, Boys CJ, et al. Global functional connectivity abnormalities in children with fetal alcohol spectrum disorders. *Alcohol Clin Exp Res* 2013 May;37(5):748-756.
- (11) Cortese BM, Moore GJ, Bailey BA, Jacobson SW, Delaney-Black V, Hannigan JH. Magnet-

- ic resonance and spectroscopic imaging in prenatal alcohol-exposed children: preliminary findings in the caudate nucleus. *Neurotoxicol Teratol* 2006 Sep-Oct;28(5):597-606.
- (12) Fagerlund A, Heikkinen S, Autti-Ramo I, Korkman M, Timonen M, Kuusi T, et al. Brain metabolic alterations in adolescents and young adults with fetal alcohol spectrum disorders. *Alcohol Clin Exp Res* 2006 Dec;30(12):2097-2104.
- (13) Astley SJ, Richards T, Aylward EH, Olson HC, Kerns K, Brooks A, et al. Magnetic resonance spectroscopy outcomes from a comprehensive magnetic resonance study of children with fetal alcohol spectrum disorders. *Magn Reson Imaging* 2009 Jul;27(6):760-778.
- (14) Goncalves Rde C, Vasconcelos MM, Faleiros LO, Cruz Jr LC, Domingues RC, Brito AR, et al. Proton magnetic resonance spectroscopy in children with fetal alcohol spectrum disorders. *Arq Neuropsiquiatr* 2009 Jun;67(2A):254-261.
- (15) du Plessis L, Jacobson JL, Jacobson SW, Hess AT, van der Kouwe A, Avison MJ, et al. An in vivo ¹H magnetic resonance spectroscopy study of the deep cerebellar nuclei in children with fetal alcohol spectrum disorders. *Alcohol Clin Exp Res* 2014 May;38(5):1330-1338.
- (16) Goodlett CR, Horn KH, Zhou FC. Alcohol teratogenesis: mechanisms of damage and strategies for intervention. *Exp Biol Med (Maywood)* 2005 Jun;230(6):394-406.
- (17) Gemma S, Vichi S, Testai E. Metabolic and genetic factors contributing to alcohol induced effects and fetal alcohol syndrome. *Neurosci Biobehav Rev* 2007;31(2):221-229.
- (18) Shibley IA, Jr, Pennington SN. Metabolic and mitotic changes associated with the fetal alcohol syndrome. *Alcohol Alcohol* 1997 Jul-Aug;32(4):423-434.
- (19) Memo L, Gnoato E, Caminiti S, Pichini S, Tarani L. Fetal alcohol spectrum disorders and fetal alcohol syndrome: the state of the art and new diagnostic tools. *Early Hum Dev* 2013 Jun;89 Suppl 1:S40-3.
- (20) Karadottir R, Attwell D. Combining patch-clamping of cells in brain slices with immunocytochemical labeling to define cell type and developmental stage. *Nat Protoc* 2006;1(4):1977-1986.
- (21) Lundgaard I, Luzhynskaya A, Stockley JH, Wang Z, Evans KA, Swire M, et al. Neuregulin and BDNF induce a switch to NMDA receptor-dependent myelination by oligodendrocytes. *PLoS Biol* 2013 Dec;11(12):e1001743.
- (22) Balaszczuk V, Bender C, Pereno G, Beltramino CA. Binge alcohol-induced alterations in BDNF and GDNF expression in central extended amygdala and pyriform cortex on infant rats. *Int J Dev Neurosci* 2013 Aug;31(5):287-296.
- (23) Bluml S, Wisnowski JL, Nelson MD, Jr, Paquette L, Gilles FH, Kinney HC, et al. Metabolic

maturation of the human brain from birth through adolescence: insights from in vivo magnetic resonance spectroscopy. *Cereb Cortex* 2013 Dec;23(12):2944-2955.

- (24) WMA General Assembly. World medical association declaration of Helsinki: Ethical principles for medical research involving human subjects. Adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, and amended by the 52nd WMA General Assembly, Edinburgh, Scotland, October 2000. 2000.
- (25) Stein DJ, Koen N, Donald KA, Adnams CM, Koopowitz S, Lund C, et al. Investigating the psychosocial determinants of child health in Africa: The Drakenstein Child Health Study. *J Neurosci Methods* 2015 Mar 19.
- (26) WHO ASSIST Working Group. The Alcohol, Smoking and Substance Involvement Screening Test (ASSIST): development, reliability and feasibility. *Addiction* 2002 Sep;97(9):1183-1194.
- (27) Gryczynski J, Kelly SM, Mitchell SG, Kirk A, O'Grady KE, Schwartz RP. Validation and performance of the Alcohol, Smoking and Substance Involvement Screening Test (ASSIST) among adolescent primary care patients. *Addiction* 2015 Feb;110(2):240-247.
- (28) Dubowitz L, Mercuri E, Dubowitz V. An optimality score for the neurologic examination of the term newborn. *J Pediatr* 1998 Sep;133(3):406-416.
- (29) Provencher SW. Estimation of metabolite concentrations from localized in vivo proton NMR spectra. *Magn Reson Med* 1993 Dec;30(6):672-679.
- (30) StatSoft Incorporated. STATISTICA data analysis software system. 2011;10.
- (31) Valcana T, Einstein ER, Csejtey J, Dalal KB, Timiras PS. Influence of thyroid hormones on myelin proteins in the developing rat brain. *J Neurol Sci* 1975 May;25(1):19-27.
- (32) Emery B. Regulation of oligodendrocyte differentiation and myelination. *Science* 2010 Nov 5;330(6005):779-782.
- (33) Fernandez M, Pirondi S, Manservigi M, Giardino L, Calza L. Thyroid hormone participates in the regulation of neural stem cells and oligodendrocyte precursor cells in the central nervous system of adult rat. *Eur J Neurosci* 2004 Oct;20(8):2059-2070.
- (34) Franco PG, Silvestroff L, Soto EF, Pasquini JM. Thyroid hormones promote differentiation of oligodendrocyte progenitor cells and improve remyelination after cuprizone-induced demyelination. *Exp Neurol* 2008 Aug;212(2):458-467.
- (35) Mohacsik P, Zeold A, Bianco AC, Gereben B. Thyroid hormone and the neuroglia: both source and target. *J Thyroid Res* 2011;2011:215718.

- (36) Herbstman J, Apelberg BJ, Witter FR, Panny S, Goldman LR. Maternal, infant, and delivery factors associated with neonatal thyroid hormone status. *Thyroid* 2008 Jan;18(1):67-76.
- (37) Vose LR, Vinukonda G, Jo S, Miry O, Diamond D, Korumilli R, et al. Treatment with thyroxine restores myelination and clinical recovery after intraventricular hemorrhage. *J Neurosci* 2013 Oct 30;33(44):17232-17246.

Chapter 7

Inter-hemispheric functional brain connectivity in neonates with prenatal alcohol exposure

Donald, KA¹; Ipser, JC²; Howells, FM²; Roos, A³; Fouche, JP^{2,4}; Riley, EP⁵; Koen, N², Woods, RP⁶; Biswal, B⁷; Zar, HJ⁸; Narr, KL⁶; Stein, DJ²

- 1 - *Division of Developmental Paediatrics, Department of Paediatrics and Child Health, Red Cross War Memorial Children's Hospital and University of Cape Town, South Africa*
- 2 - *Department of Psychiatry and Mental Health, University of Cape Town, Cape Town, South Africa*
- 3 - *MRC Unit on Anxiety & Stress Disorders, Stellenbosch University, Cape Town, South Africa*
- 4 - *Department of Psychiatry, Stellenbosch University, Cape Town, South Africa*
- 5 - *Department of Psychology, San Diego State University, San Diego, United States of America*
- 6 - *Department of Neurology, University of California, Los Angeles, United States of America*
- 7 - *Department of Biochemical Engineering, New Jersey Institute of Technology, New Jersey, United States of America*
- 8 - *Department of Paediatrics and Child Health & MRC unit on Child & Adolescent Health, Red Cross War Memorial Children's Hospital and University of Cape Town, Cape Town, South Africa*

Corresponding author:

Kirsten A. Donald

Tel: +27 21 658 5535

Fax: +27 21 658 5530

Email: kirsty.donald@uct.ac.za

Postal address: Department of Paediatrics and Child Health, Red Cross War Memorial
Children's Hospital, Klipfontein Road/Private Bag, Rondebosch, 7700/7701.

Abstract

Background

Children exposed to alcohol in utero demonstrate reduced white matter microstructural integrity. While early evidence suggests altered functional brain connectivity in the lateralization of motor networks in school-age children with prenatal alcohol exposure, the specific effects of alcohol exposure on the establishment of intrinsic connectivity in early infancy has not been explored.

Methods

Sixty subjects received functional imaging at 2-4 weeks of age for 6 to 8 minutes during quiet natural sleep. Thirteen alcohol-exposed (PAE) and 14 age-matched control (CTRL) participants with usable data were included in a multivariate model of connectivity between sensorimotor intrinsic functional connectivity networks. Post-hoc seed-based analyses of group differences in inter-hemispheric connectivity of intrinsic motor networks were also conducted. The Dubowitz neurological assessment was performed at the imaging visit.

Results

Prenatal alcohol exposure was associated with significant increases in connectivity between somatosensory, motor networks, brainstem/thalamic and striatal intrinsic networks. A reduction in inter-hemispheric connectivity of motor and somatosensory networks at trend level was also observed.

Conclusion

Though results are preliminary, findings suggest pre-natal alcohol exposure may disrupt the temporal coherence in blood oxygenation utilization in intrinsic networks underlying motor performance in new-born infants. Studies that employ longitudinal designs to investigate the effects of in utero alcohol exposure on the evolving resting state networks will be key in establishing the distribution and timing of connectivity disturbances already described in older children.

Keywords: Blood oxygen level-dependent, functional MRI, newborn, intrinsic brain activity, resting state MRI, alcohol exposure, FASD

Introduction

The complexity of the brain's structural and functional networks increase rapidly in the early months of life, representing a rapid acquisition of abilities across motor, sensory and cognitive areas. (1-4) The brain is likely to be particularly vulnerable during this developmental window to prenatal environmental influences, including maternal alcohol consumption during pregnancy, and these may have long-term effects on its structure and function. (5,6) Indeed, documentation of the physical and neurodevelopmental effects of prenatal alcohol exposure have a long tradition, extending back in the medical literature for the last 40 years. (7-12)

Investigators have increasingly sought to document correlations between the functional deficits reported in children who have been exposed to alcohol in-utero and underlying neurobiology. These endeavours have been given added impetus by the observation of functional deficits associated with prenatal alcohol exposure, even in cases where children do not exhibit the facial features required for a diagnosis of fetal alcohol syndrome (FAS). (12) The wide range of physical, behavioral and developmental abnormalities in children exposed to alcohol *in utero* is recognised by the inclusion of the Fetal Alcohol Spectrum Disorders (FASD) in extant classification systems. Indeed, animal work (13,14) and human imaging studies in school-age children with FASD have demonstrated that *in utero* exposure to alcohol alters brain morphology and reduces white matter microstructural integrity. (15-25)

However, few data exists regarding the impact of prenatal alcohol exposure in early infancy, before higher-level brain networks have become established, and before confounding postnatal environmental factors come into play. This is particularly striking with regards to the study of functional brain connectivity, described in the imaging literature as dependencies among observed neurophysiological responses or “temporal correlation between spatially remote neurophysical events” . (26) The ability of the brain to coordinate these areas of activity follows a developmental trajectory, reflected in increased functional network connectivity with age in childhood and early adulthood. (1)

Two preliminary reports have described interhemispheric and global functional connec-

tivity abnormalities in older children with FASD. Wozniak and colleagues in their initial study demonstrated that children with prenatal alcohol exposure (PAE) had abnormalities in white matter microstructural connectivity in the corpus callosum compared to healthy unexposed controls, as well as a disturbance of functional connectivity in the alcohol exposed group in this region. (27,28) A subsequent study demonstrated further abnormalities in global measures of network connectivity using a graph theory approach. The authors reported significantly higher characteristic path length and lower global efficiency in the brains of those children with PAE. (29) Roussotte and colleagues (2012) subsequently reported between - group abnormalities in frontostriatal connectivity in both alcohol and polydrug exposed children aged 7-15 years, during rest breaks between a working memory task. In both exposed groups, functional connectivity between the dorsal caudate and frontal executive network decreased while increases were noted in frontal cortical coupling with the posterior putamen, a brain region typically more strongly synchronised with the motor network. (30) These exploratory findings are an early indication that the dynamic activation of brain regions may provide key insights into the neuropathological basis of functional impairments demonstrated by children with FASD.

Nevertheless, despite this literature in older children, the effects of alcohol exposure on the longitudinal structural development of the brain in later childhood is still poorly characterized (23), with few human data on the onset of these effects, where they are located at this initial stage, and how the complex early behavioral milestones relate to functional and structural changes of the underlying neural substrate. More specifically, while preliminary studies have shown altered connectivity in the more mature brains of school-age children, the specific effects of alcohol exposure on the establishment of intrinsic connectivity in early infancy has not been explored. Based on the literature reviewed, characterizing the connectivity of regions in the brain that are key to early neurodevelopmental functional integration, including the thalamus and the motor cortex, would seem to be promising candidate markers of the neuropathological effects of alcohol exposure in the human infant. (31)

This study was conducted in an economically disadvantaged community in South Africa, in order to address gaps in the current literature concerning the presence, timing

and regional specificity of altered functional network integrity associated with prenatal alcohol exposure in a context in which FAS is rife. We hypothesized that there would be differences in functional brain networks in the first weeks of life as a result of gestational alcohol exposure. We also hypothesized that neonatal quantitative abnormalities associated with maternal alcohol use in pregnancy and detected using resting state functional magnetic resonance imaging (rs-fMRI) would correlate with early indicators of neurobehavioral health.

Materials and Methods

The current investigation is a nested sub-study that includes infants enrolled in a larger population-based birth cohort study, the Drakenstein Child Health Study (DCHS). (32,33) In this nested sub-study, rs-fMRI scans were acquired for 30 healthy unexposed infants and 30 alcohol-exposed infants at 2-4 weeks of age. Mothers and infants were recruited and screened using the approach described in chapter 3. (36,37)

Two to four week old infants underwent brain imaging. Basic anthropometry, including weight, occipito-frontal head circumference and length was acquired at the imaging visit. The Dubowitz neurobehavioral scale, used to study early neurological and behavioral changes, was also administered at this time. The Dubowitz is a well-validated measure of neonatal neuromotor and neurobehavioral status. The tool includes an optimality score which enables it to be used for quantitative analysis of potential associations with neuroimaging findings in this study. (39) The score is based on the distribution of the scores for each item in a population of low-risk term infants. The total optimality score was the sum of the optimality scores of individual items. However, for this study specific item clusters were chosen as being of particular interest. As defined by the authors of the tool, the “behavior” cluster includes items scoring irritability, cry, consolability, alertness, visual and auditory orientation and eye movements.

Intrinsic functional brain connectivity MRI scans were acquired on a 3T Siemens Allegra system at the Cape Universities Brain Imaging Centre, using a single channel head coil. Infants were in quiet natural (unsedated) sleep during the course of the scan, after being wrapped and fed. Earplugs and mini-muffs were used for double ear protection;

a pulse oximeter was used to monitor pulse and oxygenation, and a qualified neonatal nurse or pediatrician was present with the infant in the scanner room for the duration of the imaging session. Quality control procedures identified poor tissue specificity in T1-weighted MPRAGE images that were acquired to facilitate the anatomical localization of group differences in functional brain connectivity. Accordingly, these were substituted with a 5 minute T2-weighted image sequence (TR=3500ms; TE=354ms; slice thickness 1mm; 128 slices; voxel size 1.0x1.0x1.0mm). Whole-brain 3T gradient echo T2-weighted echoplanar images (EPI) were acquired for 6 minutes (TR 2000 ms; TE 30 ms; flip angle=77° slice thickness 4 mm;33 slices, voxel resolution = 2.5x2.5x4.0mm). The EPI sequence was extended to 8 minutes (238 volumes) towards the end of the study, whilst holding all other scanning parameters the same.

Image preprocessing was conducted using AFNI. (40) The first four volumes of the EPI sequence were removed, to allow for stabilization of the magnetic field, and outlier signal intensities in each voxel's time-series were truncated using 3dDespike. The blood oxygen level dependent data (BOLD) was subsequently motion-corrected (via rigid-body alignment of each of the EPI volumes to the 3rd volume), resampled to 2.5mm in the three spatial dimensions, and registered into University of North Carolina (UNC) neonatal atlas space (via displacement parameters obtained through the intermediary registration with the brain extracted T2 anatomical images, (41) all in a single step. As the final step, the data were spatially smoothed with a 5mm full width at half-maximum (FWHM) Gaussian kernel. Only participants for whom more than two-thirds of their BOLD data would have been retained after removing time-points with excessive movement, defined as 0.3mm relative to the preceding time-point, were included in the analysis. This corresponded to a minimum of 4 and 5.2 minutes of data for infants with the shorter and longer EPI sequences, respectively.

Motor networks, as well as networks that previous functional and resting-state connectivity analyses have implicated as being functionally integrated with the motor network, including the somatosensory, thalamic and striatal networks, were identified using probabilistic independent components analysis (ICA), as implemented in FSL's melodic tool. (42) ICA employs a model-free signal separation algorithm to identify spatially orthogonal

components based on voxel time-series, and allows investigators to identify connectivity patterns resulting from motion and physiological sources of no interest as separate networks that can be excluded from the subject's data prior to conducting between-group analyses. It was with this in mind that ICA was performed individually for each infant's rs-fMRI dataset in this study, in order to identify networks using the criteria of Kelly et al. (2010) (43) that corresponding to motion artefacts, as well as signals of no interest from white matter (WM) and cerebrospinal fluid (CSF) tissue (please see the supplementary material for the exact criteria employed). The time-series for these components were subsequently regressed from the preprocessed EPI data for that subject, using the `fsl_regfilt` tool.

Components corresponding to the motor, somatosensory, striatal and thalamic networks across the entire sample were obtained by applying ICA to the temporally concatenated denoised time-series from each participant, after truncating each individual dataset at 176 volumes and normalising the mean intensity of each volume. Voxels with a BOLD time-course that mapped onto the component's time-course, using a Z-score threshold of greater than 4 were designated as members of this component. Verification of candidate networks of interest were obtained through visual comparison with the published literature on neonate and adult ICA networks, (31,42,44) and through consulting the automated anatomical labeling (AAL) anatomical segmentation map provided with the University of North Carolina (UNC) infant atlas.

A multivariate general linear modelling (MVM) framework was employed to assess the effect of prenatal alcohol exposure on connectivity between the time-courses for six networks identified through ICA as being of interest (see results section). To prepare the data for this analysis, group component spatial maps were regressed from each infant's pre-processed 4D EPI dataset, yielding subject-specific separate time-series corresponding to each of the group components. Group differences in Fisher Z transformed Pearson's correlation connectivity estimates between the network time-courses were identified using a wrapper to AFNI's 3dMVM tool, provided as part of the FATCAT toolbox. (45) 3dMVM allows one to treat correlations between each pairwise combination of regions of interest as within subject repeated-measure factors, whilst, uniquely amongst

MRI imaging analysis software packages, simultaneously adjusting for the effect of continuous covariates. (46) A significant effect for an omnibus model of group differences in connectivity between any of the networks can be used to justify the subsequent inspection of the results of post-hoc models for each pair-wise comparison, to help determine specifically which of the networks are differentially connected as a result of prenatal alcohol exposure. Subject age in days and gender were included as covariates of no interest for both the omnibus and post-hoc models in this paper.

The clinical significance of differences in connectivity between the six networks was assessed by correlating the networks time-series for each infant with both the total and subscale scores from the Dubowitz neurological examination. In addition, the moderation by group of any association between development and inter-network connectivity was assessed by multivariate modelling of an interaction effect of group status and Dubowitz total score on Fisher Z transformed connectivity scores.

Ethical approval was obtained from the Research Ethics Committee of the Faculty of Health Sciences of UCT (HREC REF 401/2009) for the Drakenstein Child Lung Health Study. This sub-study protocol was independently reviewed and approved (HREC REF 525/2012).

Results:

Resting-state fMRI data were acquired from sixty infants, at 2-4 weeks of age (30 CTRL, 30 PAE). Thirteen alcohol-exposed (PAE) and 14 age-matched control (CTRL) participants were included in the analysis. A total of 33 participants were excluded (16 CTRL, 17 PAE). Reasons for excluding data included excessive subject motion (7 CTRL, 5 PAE), missing EPI or T2 anatomical data (8 CTRL, 3 PAE), pronounced EPI signal loss (1 CTRL, 7 PAE), and poor registration of the EPI into standard space (2 PAE). Five of the CTRL infant and two PAE children were scanned with the longer eight minute EPI sequences. Low rates of psychopathology were observed in this sample, with a single PAE mother screening positive for depression on a self-report measure.

Table 1 presents the comparison of the PAE and CTRL infants on demographic and developmental variables. The groups were comparable with respect to sex, with boys rep-

representing approximately half of the infants in the PAE (N = 7) and CTRL (N = 8) groups, as well as with respect to physical development ($\alpha > 0.2$ for all comparisons of weight, length, and head circumference). The average age at scan was 21 days (range: 11-32), and also did not differ by group. The proportion of mothers of PAE infant who drank, as well as the quantity of alcohol consumed per occasion was highest in the first trimester, with only a third (4/12) of the PAE mothers who drank during the first trimester still drinking during the third (**Table 2**). With respect to performance on the Dubowitz scale, no differences were observed between the PAE and CTRL participants for the tone, behavior and total scores. Very low variability in scores for spontaneous motion, reflex and abnormal movement prevented any meaningful comparison of group differences on these subscales (**Table 1**).

Table 1: Comparison of alcohol exposed and un-exposed neonates on demographic and developmental variables

	PAE (N = 13)	CTRLS (N = 14)	Test statistic
Gestation in weeks	38.55 2.07	38.11 1.54	Z = 0.691, p = 0.490
Age (in days)	21.38 (5.5)	20.71 (5.25)	Z = 0.244, p = 0.981
% Male	53.85	57.14	Chi-sq 0.030, p = 0.863
Weight^b (kilograms)	3.86 (0.69)	3.98 (0.66)	Z = -0.489, p = 0.625
Length^b (cm)	49.88 (4.11)	50.93 (4.39)	Z = -0.619, p = 0.536
Head circumference^b	35.66 (1.45)	36.16 (1.6)	Z = -0.876, p = 0.381
Dubowitz scale			
Tone subscale^c	8.27 (1.69)	8.54 (1.22)	Z = -0.104, p = 0.917
Behavior subscale	4.35 (1.3)	4.43 (1.34)	Z = 0.075, p = 0.941
Total score^b	22.23 (2.9)	22.42 (2.67)	Z = -0.283, p = 0.777

Table 1. All continuous variables reported as Means (SDs). Mann-Whitney tests conducted for group comparisons on all continuous variables.

^a Data missing for 3 PAE and 5 CTRL participants

^b Data missing for a single PAE participant

^c Data missing for a single CTRL participant

Table 2: Alcohol use of PAE mothers by trimester

	Trimester 1	Trimester 2	Trimester 3
Alcohol usage (n,%)	12 (92.3)	7 (53.8)	4 (30.8)
Once per week or less	10	5	2
2 to 3 times per week	2	2	2
4 to 5 times per week	0	0	0
Daily	0	0	0
Number of drinks per occasion			
<2	1	2	1
2 to 3	3	1	3
4 or more	8	4	0

The number of components from the individual-level ICA ranged from 27 to 103, with more than half (57.5%, mean = 34.61, SD = 14.16) identified as resulting from artefacts or from non-GM sources, and regressed from the infant's rs-fMRI EPI sequences. A total of 30 group-level components were subsequently extracted from the denoised data. Of the 30 ICA networks, six were selected as networks suited to test the hypothesis that intra-uterine alcohol exposure will affect motor connectivity (**Figure 1**).

Figure 1: Axial, sagittal and coronal slices of ICA derived networks.

Figure 1 (a)

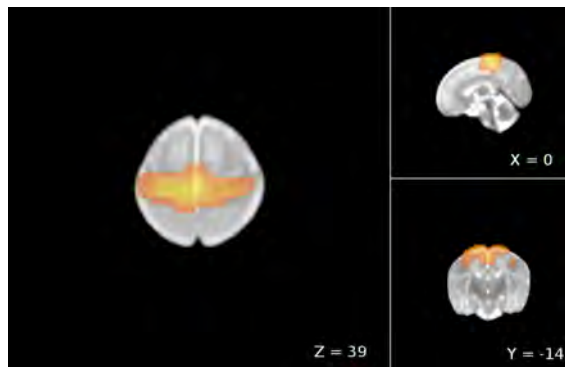


Figure 1(b)

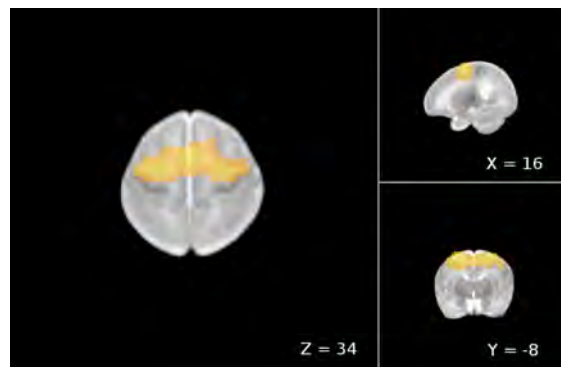


Figure 1(c)

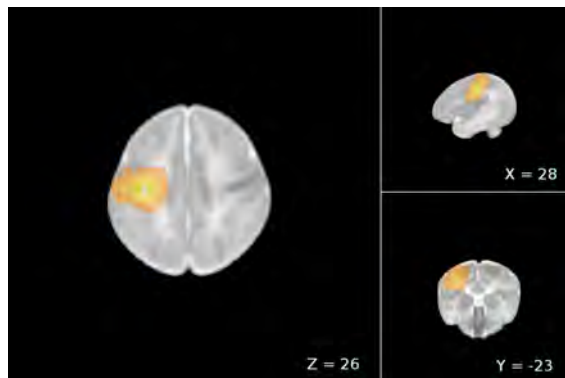


Figure 1(d)

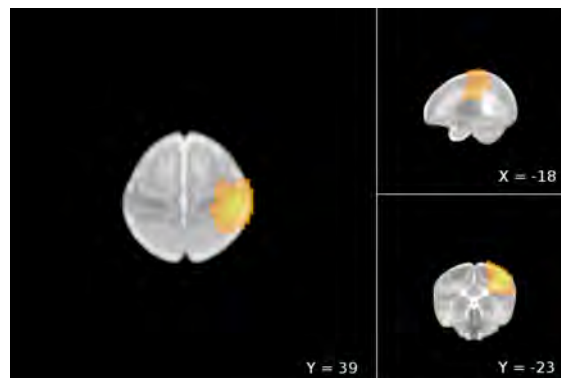


Figure 1(e)

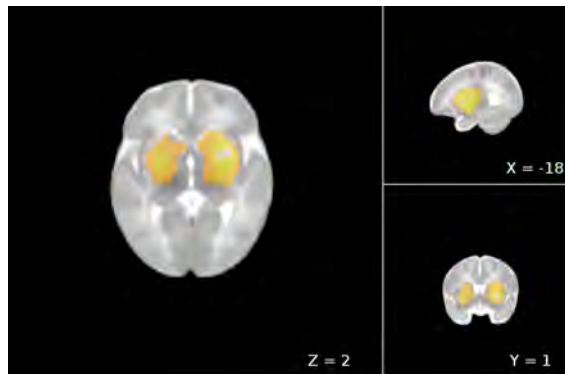


Figure 1(f)

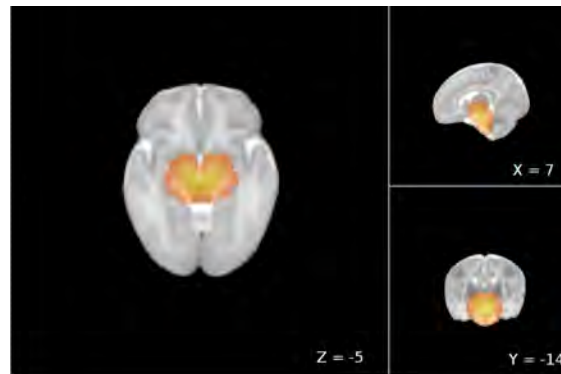


Figure 1. Axial, sagittal and coronal slices of ICA derived networks (shown in orange-yellow), thresholded at $Z = 4$ and superimposed on the UNC neonatal template (radiological convention: left of display equals right side of brain). Networks: (a) anterior motor, (b) posterior motor, (c) left somatosensory, (d) right somatosensory, (e) striatal and (f) brainstem/thalamic.

Of the six networks identified, two extended bilaterally within the precentral gyrus, and were designated as representing motor networks. The first was located more anteriorly, and included the supplementary motor area (SMA) and the superior frontal gyrus (**Figure 1a**). A more posterior network included the postcentral gyrus, the SMA and

the paracentral lobule (**Figure 1b**), as well as a separate small (10 voxel) cluster in the left thalamus. Two networks primarily situated within the postcentral gyrus, and largely mirroring one another in their more lateral position within the left and right hemispheres, were considered to represent somatosensory networks (**Figures 1c and 1d**). The right somatosensory network also included a relatively small (72 voxel) cluster in the left post-central gyrus. A bilateral network was evident in the striatum, with strongest activation in the left and right putamen, but also including the bilateral caudate and pallidum, as well as part of the left anterior insula (**Figure 1e**). Finally, a diffuse network was located in the brainstem, and included the bilateral thalamus, as well as parahippocampal gyrus, hippocampus, amygdala, and pallidum. (**Figure 1f**).

The MVM analysis revealed a significant effect of group in connectivity between all 6 networks (Chi-square = 11.080, $df = 1$, $p < 0.001$), after adjusting for age in days and gender (no significant effects on connectivity were observed for either of these covariates). Examination of the post-hoc comparisons of connectivity between particular networks indicated that alcohol exposure increased connectivity between the brainstem and the anterior motor network ($t = 2.426$, $df = 23$, $p = 0.024$), with a trend-level effect in the same direction observed between the brainstem and the left somatosensory network ($t = 2.0125$, $df = 23$, $p = 0.056$). Increased connectivity in the PAE infants was also observed between the striatum and both the anterior ($t = 3.045$, $df = 23$, $p = 0.006$) and posterior motor networks ($t = 3.257$, $df = 23$, $p = 0.004$). In the only instance of attenuated inter-network connectivity in the PAE relative to CTRL infants, the alcohol exposed infants displayed trend-level attenuation of inter-hemispheric connectivity between the left and right somatosensory networks ($t = -1.929$, $df = 23$, $p = 0.066$).

On the basis of the finding of a possible association between prenatal alcohol exposure and hemispheric asymmetry in the somatosensory network, a post-hoc seed-based analysis was conducted to further investigate the effect of prenatal alcohol exposure on connectivity between the hemispheres for both the anterior and posterior motor networks. A standard preprocessing pipeline was applied to the dataset described above, prior to denoising, and included regression of nuisance parameters, such as signal from a WM mask, mean, linear, quadratic and cubic temporal trends, as well as the 6 rigid-

body motion parameter estimates and their first order derivatives. Censoring of high motion volumes was also applied to the data. Fisher Z transformed Pearson's correlation coefficients were subsequently calculated between the time-series extracted separately from each hemisphere, per network, and compared using multiple linear regression procedures, controlling for infant gender and age in days (please refer to the supplementary documentation for additional information).

Although the hemispheric connectivity estimates were lower for the PAE than the CTRL infants in the anterior motor network (Mean; SD = 0.69; 0.1 and 0.75; 0.12), and marginally so for the posterior motor network (Mean; SD = 0.80; 0.07 and 0.82; 0.10) networks, these differences did not achieve statistical significance (Mann Whitney $Z = -1.359$, p -value = 0.174 and $Z = -0.679$, p -value = 0.497, respectively).

Finally, the MVM analysis of the clinical significance of inter-network differences failed to detect evidence for a moderating effect of group status on the association between inter-network connectivity and Dubowitz total, behavior and tone in this small cohort. Of the 15 post-hoc comparisons conducted, only connectivity between the striatum and anterior motor network achieved significance ($t = -2.2290$, $df = 22$, uncorrected $p = 0.0363$). Post-hoc non-parametric correlation tests revealed that greater connectivity between these networks predicted higher total clinical deficits on the Dubowitz scale in the CTRL infants (Spearman $Rho = 0.609$, $p = 0.027$), with no association detected amongst the PAE neonates ($Rho = -0.238$, $p > 0.1$). Replicating these analyses for the Dubowitz behavior and tone subscales produced no evidence of a moderating effect of group for behavior (chi-square = 0.165, $df = 1$, $p = 0.684$), but a possible effect for tone (chi-square = 3.288, $df = 1$, $p = 0.070$). Inspection of post-hoc connectivity between networks suggested that prior alcohol exposure had the greatest effect on the association between tone and striatal connectivity, with differences observed between this structure and both the anterior motor network ($t = -2.145$, $df = 22$, $p = 0.043$) and the left somatosensory network ($t = -2.249$, $df = 22$, $p = 0.035$). In line with the findings for the correlation tests using the total Dubowitz score, positive associations between striatal motor connectivity and tone for these networks ($Rho = 0.549$, $p = 0.052$ and $Rho = 0.529$, $p = 0.062$) were not evident in PAE infants ($Rho = -0.309$, $p = 0.305$ and $Rho = -0.307$, $p = 0.308$, respectively).

Discussion

This study reports preliminary data on functional network connectivity in alcohol exposed infants compared to controls. In this small sample, there were significant group differences in functional connectivity between networks underlying motor behavior, after correction for gender and age differences. Our results support the possibility that prenatal alcohol exposure may be associated with altered sensorimotor connectivity.

The only previous group to report resting state functional network abnormalities in alcohol exposed children reported robust asymmetry in inter-hemispheric connectivity of the sensorimotor cortex in exposed versus unexposed children. (28) Additional analyses by the authors revealed associations between hemispheric asymmetry in their cohort and the compromised microstructural integrity of the posterior corpus callosum, as well as impaired perceptual reasoning. We were able to replicate the findings of disrupted functional sensorimotor connectivity across brain hemispheres in a relatively immature sample, albeit at a trend level. In addition, our data suggests that intrinsic connectivity may be related differently to developmentally relevant behavioral measures.

Reductions in inter-network connectivity and increased connectivity within networks in adult intrinsic functional networks compared to healthy infants was recently argued as a hallmark of the functional compartmentalisation and segregation of brain networks. (47) The finding reported in this paper that alcohol exposure was associated with greater connectivity of the striatal and brainstem/thalamic networks to the rest of the sensorimotor system, but with possible reductions in inter-hemispheric motor and somatosensory connectivity is therefore consistent with the notion that exposure may play a role in delayed development of maturing brain networks. Apparently contradictory findings of increased inter-hemispheric connectivity of the motor and somatosensory networks as infants approach term, as well as greater thalamic connectivity to these networks (31) may reflect the dynamic nature of brain maturation, and underscores the importance of more finely grained longitudinal analyses of the maturation of functional brain networks in the first years of life.

Much literature in developmental neuroscience has focused on localizing and classifying the function of specific brain areas and how these regional specializations arise. The

sensorimotor cortex is a region that traditionally has been associated with tasks of motor planning and control. However, more recent functional models have explored broader involvement of this region in the integration of sensory stimuli, as well as the dynamic organization of the sensorimotor cortex during the generation of a wide range of complex cognitive and motor functions. (48,49) Although very little has been reported with respect to early neurobehavior in alcohol exposed neonates, studies that have investigated this outcome have identified poor habituation and low levels of arousal along with motor abnormalities in the infants of women who used alcohol heavily during pregnancy. (50,51) Sensitive functional outcomes in this age group remain difficult to define because structural and functional networks are still in their earliest stages. Published work on infants born prematurely suggests that the emerging connectivity of the thalamus at term with the visual, auditory and motor networks is indicative of its important developmental role in the formation of these networks. (31) Further, effective development of the structural core connecting the medial cortical regions, has been characterised as an integrated system that is critical to the coordination of left and right hemispheres. (1) It is therefore not surprising that previous work has revealed that this core network is particularly susceptible to the damaging effects of alcohol exposure during development. (52)

Imaging infants remains technically difficult both in terms of imaging acquisition as well as the inherent challenges in reliably analyzing data from brains that are small, have high water content and poor grey-white matter differentiation. (1,3,4) Subject motion has been identified as a potential source of artefactual group differences in rs-fMRI connectivity, particularly for pediatric samples. (53) Approximately half of the infants scanned for this study were excluded due to factors that included motion-induced imaging artefacts and signal attenuation. In addition, a series of steps were implemented to adjust for subject motion in the data that was included in this study. However, despite these efforts, it is possible that motion may have introduced confounding of the data. The results of this study should therefore be considered preliminary, and warrant replication in larger studies.

This study begins to address gaps in the current literature concerning the feasibility of functional connectivity studies in infants of this age and in particular of brain functional

connectivity studies in association with prenatal alcohol exposure. Studies that employ longitudinal designs to investigate the effects of in utero alcohol exposure on the evolving resting state networks will be key in establishing the distribution and timing of connectivity disturbances already described in older children. Preliminary study outcomes are starting to elucidate the early neurodevelopmental mechanisms leading to subsequent behavioral and neurological disturbances, which may allow opportunities for targeting interventions when brain plasticity is still relatively fluid.

References

- (1) Hagmann P, Grant PE, Fair DA. MR Connectomics: A Conceptual Framework for Studying The Developing Brain. *Frontiers in Systems Neuroscience* 2012;6.
- (2) Pannek K, Guzzetta A, Colditz P, Rose S. Diffusion MRI of the neonate brain: acquisition, processing and analysis techniques. *Pediatr Radiol* 2012 10/01;42(10):1169-1182.
- (3) Geng X, Gouttard S, Sharma A, Gu H, Styner M, Lin W, et al. Quantitative tract-based white matter development from birth to age 2 years. *Neuroimage* 2012 7/2;61(3):542-557.
- (4) Gilmore JH, Shi F, Woolson SL, Knickmeyer RC, Short SJ, Lin W, et al. Longitudinal Development of Cortical and Subcortical Gray Matter from Birth to 2 Years. *Cerebral Cortex* 2012 November 01;22(11):2478-2485.
- (5) Rodier PM. Vulnerable periods and processes during central nervous system development. *Environ Health Perspect* 1994;102(suppl. 2):121-124.
- (6) Rodier PM. Environmental causes of central nervous system maldevelopment. *Pediatrics* 2004;113:1076-1083.
- (7) Jones KL, Smith DW, Ulleland CN, Streissguth AP. Pattern of malformation in offspring of Chronic Alcoholic mothers. *Lancet* 1973;1(7815):1267-1271.
- (8) Jones KL, Smith DW, Hansen JW. The Fetal Alcohol Syndrome: clinical delineation. *Annals of the New York Academy of Sciences* 1976;273:130-139.
- (9) Jacobson SW, Jacobson JL, Stanton M. Biobehavioral Markers of Adverse Effect in Fetal Alcohol Spectrum Disorders. *Neuropsychol Rev* 2011;21:148-166.
- (10) Mattson S, Crocker N, Nguyen T. Fetal Alcohol Spectrum Disorders: Neuropsychological and Behavioral Features. *Neuropsychol Rev* 2011;21(2):81-101.
- (11) Mattson SN, Roesch SC, Glass L, Deweese BN, Coles CD, Kable JA, et al. Further Development of a Neurobehavioral Profile of Fetal Alcohol Spectrum Disorders. *Alcoholism: Clinical and Experimental Research* 2013;37(3):517-528.
- (12) Riley EP, Alejandra Infante M, Warren K. Fetal Alcohol Spectrum Disorders: An Overview. *Neuropsychol Rev* 2011;21(2):73-80.
- (13) An L, Zhang T. Spatial cognition and sexually dimorphic synaptic plasticity balance impairment in rats with chronic prenatal ethanol exposure. *Behav Brain Res* 2013 11/1;256(0):564-574.
- (14) Burke MW, Palmour RM, Ervin FR, Ptito M. Neuronal reduction in frontal cortex of primates after prenatal alcohol exposure. *Neuroreport* 2009;20(1).

- (15) O'Leary-Moore S, Parnell S, Lipinski R. Magnetic Resonance-Based Imaging in Animal Models of Fetal Alcohol Spectrum Disorder. *Neuropsychol Rev* 2011;21:167-185.
- (16) Lebel C, Roussotte F, Sowell ER. Imaging the Impact of Prenatal Alcohol Exposure on the Structure of the Developing Human Brain. *Neuropsychol Rev* 2011;21:102-118.
- (17) Wozniak JR, Mueller BA, Chang PN, Muetzel RL, Caros L, Lim KO. Diffusion Tensor Imaging in Children with Fetal Alcohol Spectrum Disorders. *Alcoholism: Clinical and Experimental Research* 2006;30(10):1799-1806.
- (18) Wozniak JR, Muetzel RL. What Does Diffusion Tensor Imaging Reveal About the Brain and Cognition in Fetal Alcohol Spectrum Disorders. *Neuropsychology review* 2011;21(2):133-147.
- (19) Sowell ER, Johnson A, Kan E, Lu LH, Van Horn JD, Toga AW, et al. Mapping White Matter Integrity and Neurobehavioral Correlates in Children with Fetal Alcohol Spectrum Disorders. *The Journal of Neuroscience* 2008 February 06;28(6):1313-1319.
- (20) Leigland LA, Ford MM, Lerch JP, Kroenke CD. The influence of fetal ethanol exposure on subsequent development of the cerebral cortex as revealed by magnetic resonance imaging . *Alcoholism: Clinical and Experimental Research* 2013;37(6):924-932.
- (21) Lebel C, Rasmussen C, Wyper K, Andrew G, Beaulieu C. Brain Microstructure Is Related to Math Ability in Children With Fetal Alcohol Spectrum Disorder. *Alcoholism: Clinical and Experimental Research* 2010;34(2):354-363.
- (22) Lebel C, Rasmussen C, Wyper K, Walker L, Andrew G, Yager Jea. Brain Diffusion abnormalities in children with fetal alcohol spectrum disorder. *Alcoholism, Clinical and Experimental Research* 2008;32(10):1732-1740.
- (23) Lebel C, Mattson SN, Riley EP, Jones KL, Adnams CM, May PA, et al. A Longitudinal Study of the Long-Term Consequences of Drinking during Pregnancy: Heavy In Utero Alcohol Exposure Disrupts the Normal Processes of Brain Development. *The Journal of Neuroscience* 2012 October 31;32(44):15243-15251.
- (24) Fryer S, Schweinsburg BC, Bjorkquist OA, Frank LK, Mattson SM, Spadoni ADea. Characterisation of white matter microstructure in fetal alcohol spectrum disorders. *Alcohol Clinical and Experimental research* 2009;33(3):514-521.
- (25) Archibald SL, Fennema-Notestine C, Gamst A, Riley EP, Mattson SN, Jernigan TL. Brain dysmorphology in individuals with severe prenatal alcohol exposure. *Developmental Medicine & Child Neurology* 2001;43(3):148-154.
- (26) Biswal B, Zerrin Yetkin F, Haughton VM, Hyde JS. Functional connectivity in the motor

cortex of resting human brain using echo-planar mri. *Magnetic Resonance in Medicine* 1995;34(4):537-541.

- (27) Wozniak JR, Muetzel RL, Mueller BA, McGee CL, Freerks MA, Ward EE, et al. Microstructural Corpus Callosum Anomalies in Children With Prenatal Alcohol Exposure: An Extension of Previous Diffusion Tensor Imaging Findings. *Alcoholism: Clinical and Experimental Research* 2009;33(10):1825-1835.
- (28) Wozniak JR, Mueller BA, Muetzel RL, Bell CJ, Hoecker HL, Nelson ML, et al. Inter-Hemispheric Functional Connectivity Disruption in Children with Prenatal Alcohol Exposure. *Alcoholism: Clinical and Experimental Research* 2011;35(5):849-861.
- (29) Wozniak JR, Mueller BA, Bell CJ, Muetzel RL, Hoecker HL, Boys CJ, et al. Global functional connectivity abnormalities in children with fetal alcohol spectrum disorders. *Alcoholism: Clinical and Experimental Research* 2013;37(5):748-756.
- (30) Roussotte FF, Rudie JD, Smith L, O'Connor MJ, Bookheimer SY, Narr KL, et al. Frontostriatal Connectivity in Children during Working Memory and the Effects of Prenatal Methamphetamine, Alcohol and Polydrug Exposure. *Developmental Neuroscience* 2012;34:43-57.
- (31) Doria V, Beckmann CF, Arichi T, Merchant N, Groppo M, Turkheimer FE, et al. Emergence of resting state networks in the preterm human brain. *Proceedings of the National Academy of Sciences* 2010 November 16;107(46):20015-20020.
- (32) Zar HJ, Barnett W, Myer L, Stein DJ, Nicol MP. Investigating the early-life determinants of illness in Africa: the Drakenstein Child Health Study. *Thorax* 2014;doi: 10.1136/thorax-jnl-2014-206242.
- (33) Stein DJ, Koen N, Donald KA, Adnams CM, Koopowitz S, Lund C, et al. Investigating the psychosocial determinants of child health in Africa: the Drakenstein Child Health Study. *J Neuroscience methods* 2015;doi:10.1016/j.jneumeth.2015.03.016.
- (34) May PA, Blankenship J, Marais A, Gossage JP, Kalberg WO, Barnard R, et al. Approaching the Prevalence of the Full Spectrum of Fetal Alcohol Spectrum Disorders in a South African Population-Based Study. *Alcoholism: Clinical and Experimental Research* 2013;37(5):818-830.
- (35) May PA, Gossage JP, Kalberg WO, Robinson LK, Buckley DG, Manning M, et al. The prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on in-school studies. *Developmental Disabilities Research Reviews* 2009(15):176-192.
- (36) Humeniuk R, Ali R, Babor TF, Farrell M, Formigoni ML, Jittiwutikarn J, et al. Validation of the

Alcohol, Smoking And Substance Involvement Screening Test (ASSIST). *Addiction* 2008 Jun;103(6):1039-1047.

- (37) Jackson PB, Williams DR, Stein DJ, Herman A, Williams SL, Redmond DL. Race and psychological distress: the South african stress and health study. *J Health Soc Behav* 2010 Dec;51(4):458-477.
- (38) Lozarno J, Garcia-Algar O, Vall O, de la Torre R, Scaravelli G, Pichini S. Biological matrices for the evaluation of in utero exposure to drugs of abuse. *There Drug Monit* 2007;29(6):711-734.
- (39) Dubowitz L, Mercurio E, Dubowitz V. An Optimality Score for the Neurological Examination of The Term Newborn. *The Journal of pediatrics* 1998;133(3):406-416.
- (40) Cox R. AFNI: Software for Analysis and Visualization of Functional Magnetic Resonance Neuroimages. *Computers and Biomedical Research* 1996;29:162-173.
- (41) Shi F, Yap P, Wu G, Jia H, Gilmore JH, Lin W, et al. Infant Brain Atlases from Neonates to 1- and 2-year-olds. *PLoS ONE*, 6(4): , 2011 2011;6(4):e18746.
- (42) Beckmann CF, DeLuca M, Devlin JT, Smith SM. Investigations into resting-state connectivity using independent component analysis. *Philosophical Transactions of The Royal Society Biological Sciences* 2005;360(1457):1001-1013.
- (43) Kelly Jr. RE, Alexopoulos GS, Wang Z, Gunning FM, Murphy CF, Morimoto SS, et al. Visual inspection of independent components: Defining a procedure for artifact removal from fMRI data. *J Neurosci Methods* 2010 6/15;189(2):233-245.
- (44) Smith SM, Fox PT, Miller KL, Glahn DC, Fox PM, Mackay CE, et al. Correspondence of the brain's functional architecture during activation and rest. *Proceedings of the National Academy of Sciences* 2009;106(31):13040-13045.
- (45) Taylor, PA & Saad, ZA. FATCAT: (An Efficient) Functional And Tractographic Connectivity Analysis Toolbox. *Brain Connectivity* 2013;3(5):523-535.
- (46) Chen G, Adleman NE, Saad ZS, Leibenluft E, Cox RW. Applications of multivariate modeling to neuroimaging group analysis: A comprehensive alternative to univariate general linear model. *Neuroimage* 2014 10/1;99(0):571-588.
- (47) Wylie KP, Rojas DC, Ross RG, Hunter SK, Maharajh K, Cornier M, et al. Reduced brain resting-stae network specificity in infants compared with adults. *Neuropsychiatric Disease and Treatment* 2014;10:1349-1359.
- (48) Bouchard KE, Mesgarani N, Johnson K, Chang EF. Functional organization of human sensorimotor cortex for speech articulation. *Nature* 2013 03/21;495(7441):327-332.

- (49) Crone NE, Miglioretti DL, Gordon B, Sieracki JM, Wilson MT, Uematsu S, et al. Functional Mapping of human sensorimotor cortex with electrocorticographic spectral analysis. *Brain* 1998;121:2271-2299.
- (50) Chiriboga CA. Fetal alcohol and drug effects. . *Neurologist* 2003;9(6):267-279.
- (51) Streissguth AP, Barr HM, Martin DC. Maternal alcohol use and neonatal habituation assessed with the Brazelton scale. *Child Dev* 1983;54(5):1109-1118.
- (52) Wozniak JR, Muetzel RL. What does diffusion tensor imaging reveal about the brain and cognition in fetal alcohol spectrum disorders? *Neuropsychology review* 2011;21(2):133-147.
- (53) Power JD, Barnes KA, Snyder AZ, Schlaggar BL, Petersen SE. Spurious but systematic correlations in functional connectivity MRI networks arise from subject motion. *Neuroimage* 2012;59(3):2142-2154.

Supplementary material (chapter 7)

Denoising of resting-state EPI datasets

Components of no interest were identified using the criteria of Kelly et al. (2010); (1) candidate components for removal included those (a) with minimal ($< 10\%$) overlap with gray matter (as determined using the GM mask from the UNC atlas), (b) with rimming effects identified as resulting from subject motion, (c) that corresponded to the spatial distribution of WM or CSF spatial compartments, (d) with greater than 10% gray matter coverage but with a blotchy or speckled appearance resulting from small clusters, and (e) those components containing large motion spikes, defined as exceeding a difference of five standard deviations relative to the preceding volume. A similar denoising procedure has been effectively implemented in other neonate rs-fMRI datasets. (2)

Preprocessing of EPI data for seed-based inter-hemispheric connectivity analyses

Prior to calculating connectivity measures, a series of preprocessing steps were applied to the EPI timeseries for each participant, using the AFNI imaging suite. (3) The first four volumes of the EPI sequence were removed, to allow for stabilization of the magnetic field, and outlier signal intensities in each voxel's time-series were truncated using 3dDespike. The blood oxygen level dependent data (BOLD) was subsequently motion-corrected (via rigid-body alignment of each of the EPI volumes to the 3rd volume), resampled to 2.5mm in the three spatial dimensions, and registered into University of North Carolina (UNC) neonatal atlas space (via displacement parameters obtained through the intermediary registration with the brain extracted T2 anatomical images, (4) all in a single step. Following spatial smoothing with a 5mm full width at half-maximum (FWHM) Gaussian kernel, nuisance and motion estimates were regressed from the EPI time-series using AFNI's 3dDeconvolve. These regressors included the mean, linear, quadratic and cubic temporal trends, as well as the 6 rigidbody motion parameter estimates and their first order derivatives. In addition the influence of physiological sources of artefact was addressed through regressing out the average timeseries from a WM mask constructed from a tissue-segmented UNC atlas. The time-series was averaged over voxels in the mask identified as having a greater than 99% probability of being WM. Finally, as

an additional control against motion, the set of regressors also included a vector identifying volumes that deviated by at least 0.3mm relative to the preceding volume. These volumes, and a single subsequent volume, were censored from the data, in an attempt to mitigate the propagation of the effects of motion down the time-series. (5)

Inter-hemispheric connectivity estimates were obtained by partitioning 50% gray matter thresholded masks for the anterior and posterior motor networks along the midline of the brain ($x = 0$ in MNI152 atlas space), calculating the mean time-series for the hemisphere-specific masks for each network, and correlating the mean times-series within each network for each subject. The Pearson's correlations coefficients were transformed using the Fisher Z algorithm, to improve the distributional qualities of the data, and subsequently compared between groups using linear multiple regression in the R statistical computing platform (version 3.1.3), controlling for infant gender and age in days.

References

- (1) Kelly Jr., R.E., Alexopoulos, G.S., Wang, Z., Gunning, F.M., Murphy, C.F., Morimoto, S.S., Kanellopoulos, D., Jia, Z., Lim, K.O. & Hoptman, M.J. 2010, "Visual inspection of independent components: Defining a procedure for artifact removal from fMRI data", *Journal of neuroscience methods*, vol. 189, no. 2, pp. 233-245.
- (2) Wylie, K.P., Rojas, D.C., Ross, R.G., Hunter, S.K., Maharajh, K., Cornier, M. & Tregellas, J.R. 2014, "Reduced brain resting-state network specificity in infants compared with adults", *Neuropsychiatric Disease and Treatment*, vol. 10, pp. 1349-1359.
- (3) Cox, R. 1996, "AFNI: Software for Analysis and Visualization of Functional Magnetic Resonance Neuroimages", *Computers and Biomedical Research*, vol. 29, pp. 162-173.
- (4) Shi, F., Yap, P., Wu, G., Jia, H., Gilmore, J.H., Lin, W. & Shen, D. 2011, "Infant Brain Atlases from Neonates to 1- and 2-year-olds", *PLoS ONE*, 6(4): , 2011, vol. 6, no. 4, pp. e18746.
- (5) Power, J. D.; Mitra, A.; Laumann, T. O.; Snyder, A. Z.; Schlaggar, B. L. & Petersen, S. E. (2013). Methods to detect, characterize, and remove motion artifact in resting state fMRI. *Neuroimage*, 84C, 320-341 R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org/>.

Chapter 8

Discussion

Alcohol use and alcohol use disorders contribute a significant proportion of the burden of disease in low, middle, and high-income countries. Taken together, substance use disorders comprise one of the largest public health problems globally. (1) Secondary neurological disorders are a significant threat to childhood health in the world, according to the World Health Organization (WHO), and may severely impact children for a lifetime. (1-3) For neurodevelopmental disorders, studies have consistently shown that early intervention leads to better long-term outcomes. But early intervention is predicated on early detection and targeted interventions. Understanding the core areas of susceptibility to prenatal alcohol effects as they manifest in early life is key to developing strategies for early focused identification and interventions.

The general aim of this thesis was to assess the impact of prenatal alcohol exposure on the brain in infants as measured by multimodal brain imaging and the relationship of these findings to early neurobehavioral and developmental status. The specific aims included undertaking structural MRI, DTI, 1H-MRS and rs-fMRI scans in approximately 100 infants (50 alcohol exposed and a matched number of control, unexposed babies) at 2-4 weeks of age, in order to assess group differences in both structural, microstructural, neurochemical and functional brain development. We also aimed to determine the correlation between multimodal neuroimaging extracted from the above imaging modalities and neonatal neurobehavioral assessment and the correlation between early structural imaging findings and later infant developmental assessment as measured by the Bayley III assessment at 6 months in the same group of infants. These studies addressed the hypothesis that maternal alcohol use in pregnancy would result in quantitative MRI abnormalities demonstrable at 2-4 weeks of age and that these changes would correlate with early indicators of neurobehavior and development.

8.1 Study endpoints and applicability

The five papers presented in the body of this thesis address these aims directly and are summarised separately here. Following this, a section on the limitations of this neuroimaging substudy data chapters are reported and general conclusions for the whole thesis are discussed.

8.1.1 Systematic review

The extant literature on neuroimaging (MRI) in the field of FASD are outlined in chapter 2 and describe the range of abnormalities in structure and function of both general and specific regions of the brain. A total of 64 relevant articles were identified across all modalities. Overall, studies reported smaller total brain volume as well as smaller volume of both white and gray matter in specific cortical regions. The most consistent reported structural MRI findings were alterations in the shape and volume of the corpus callosum, and smaller volume in the basal ganglia and hippocampi. The most consistent findings in diffusion tensor imaging studies was lower fractional anisotropy in the corpus callosum. Proton magnetic resonance spectroscopy studies are few to date, but showed altered neurometabolic profiles in the frontal and parietal cortex, thalamus and dentate nuclei. Resting state functional MRI studies reported reduced functional connectivity between cortical and deep gray matter structures. Important questions which remained to be answered after review of the literature included a relative paucity of data in the preschool age group, limited longitudinal data on the evolving trajectory of brain development in the context of children exposed to alcohol during the prenatal period as well as limited understanding of the windows of vulnerability during pregnancy for different types or degrees of effect.

Limitations to interpretation and generalisability of the findings in this systematic review included the following. Firstly, authors reported differing methodologies both in magnetic resonance acquisition as well as in post-processing and analysis approaches. This made inter-study comparisons difficult. Secondly, studies reporting on structural, microstructural or spectroscopy alterations in single regions of interest have resulted in the effects on specific regions being very well documented at the expense of a more exploratory

approach. Thirdly, there remains variation in how authors have chosen to control (or not) for a variety of important contextual factors which include polysubstance abuse, environmental factors, and age. Clear reporting on these aspects in future studies is needed in order to assess consistency of results and inter-study comparisons across the literature and argues the need for large multicentre collaborative efforts to pool data and employ consistent methods across data acquisition as well as analysis strategy.

8.1.2 Structural MRI

In chapter 4, we reported the results of a comparison between 28 prenatal alcohol exposed infants and 45 unexposed control infants at 2-4 weeks of age using the T2 structural MRI sequence. In the manuscript we reported reduced volume in posterior cingulate cortex and inferior temporal gyrus in the infants with prenatal alcohol exposure. These findings persisted even when controlling for overall gray matter volume, sex, age at scanning and maternal smoking status. In addition early infant neurobehavior as measured by the Dubowitz neurobehavioral optimality scale, correlated with volume findings across multiple brain areas. BSID-III scores at 6 months of age across motor, language and general cognitive subscales as well as parental report of adaptive behavior correlated with the volumes of brain regions which extended widely, but particularly in the frontal and parietal regions.

The vast majority of infants reported in this analysis (78%) had maternal history of moderate-severe alcohol use during the first trimester. Although the pattern of drinking appeared to follow that of “binge drinking” (ie mothers reported drinking heavily rather than primarily with high frequency). This is a pattern which has been previously documented in South African communities and appears to be associated with more severe effects on children exposed to alcohol in this pattern during antenatal life. (4)

Recent work from the NIAAA sponsored Collaborative Initiative on Fetal Alcohol Spectrum Disorders (CIFASD) (5,6) has established that prenatal alcohol exposure affects the volumes of specific brain regions in different ways at different time points in older children. At 5-6 years, alcohol exposed children demonstrated larger cortical volumes (particularly in the parietal cortex) compared to unexposed control subjects. Over time

these regions in alcohol-exposed children showed a linear drop-off in volume with ultimately lower volumes in adolescence than control children. This suggests reduced efficiency and plasticity in brain development. However, information on the critical infant years when the greatest changes in brain volumes occur has remained a largely missing piece in understanding the initial effects of prenatal alcohol exposures until now. Imaging neonates safely and successfully is challenging. This study represents one of the first and largest cohorts at this critical age investigating the neurobiological effects of prenatal alcohol exposure. Our findings in this paper are in regions consistent with those reported in these longitudinal cohorts in older children and reinforces the importance of these areas as particularly susceptible to prenatal alcohol exposure. This also highlights the focus of these areas as being primarily an alcohol effect rather than an effect from the multiple additional postnatal risk factors children in these environments experience and which may confound investigations in older children.

8.1.3 Diffusion Tensor Imaging

In chapter 5 we reported the investigation of the effects of alcohol on the microstructural integrity of the major white matter tracts which constitute the structural connections between gray matter regions. In this manuscript we reported 28 alcohol-exposed and 28 healthy unexposed infants who were imaged using DTI sequences to evaluate white matter integrity using validated tract-based spatial statistics analysis methods. Secondly, diffusion values were extracted for group comparisons by regions of interest. Differences in fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD) and radial diffusivity (RD) were compared between groups and associations with measures from the Dubowitz neonatal neurobehavioural assessment were examined. Lower AD values ($p < 0.05$) were observed in alcohol exposed infants in the right superior longitudinal fasciculus compared to non-exposed infants. Altered FA and MD values in alcohol-exposed neonates in the right inferior cerebellar peduncle were associated with abnormal neonatal neurobehaviour.

The superior longitudinal fasciculus forms part of the major central association pathways of the brain and is one of the major fiber bundles linking frontal, temporal and parietal association cortices. (7) These networks are believed to include associative tasks, high-

er level motor tasks, visual perception and attention. While these are not functions that can be easily teased out in the neonatal age group, they are functional areas which have been identified as being affected in children with prenatal alcohol exposure. In addition, the location of this important connecting white matter tract between the parietal and temporal regions in which we have described gray matter volume changes in neonates exposed to alcohol in the prenatal period is further evidence for the coherence of the argument for this region being susceptible to alcohol exposure in utero.

Further, early neurobehavioral outcome correlated with reduced white matter microstructural integrity in the inferior cerebellar peduncles. Cerebellar structure has previously been described as being a key area affected by prenatal alcohol exposure in children both in structural and functional studies. (8-10) In addition, of all the areas reported to be associated with clinical or neuropsychological outcomes, the cerebellar peduncles seem to be a prominently implicated region. Fan and colleagues, (9) reported lower FA and higher MD associated with eye-blink conditioning and Green *et al*, (12) a correlation of FA with oculomotor control in a group of school age children with prenatal alcohol exposure. In our alcohol exposed infants, the presence of an association between infant neurobehavior scores with bilateral reduced FA and higher MD in the cerebellar peduncles, which link the cerebellum to the important cerebral motor and language networks, reinforces the role of these pathways in the primary effects of alcohol exposure on early brain development.

8.1.4 ¹H-MRS

In the previous two chapters I have described evidence for the structural and microstructural effects of prenatal alcohol exposure on the neonatal brain. In the following two chapters, I now describe how functional aspects of brain development may be affected by alcohol exposure in utero. In chapter 6 we reported the results of the analysis of white matter ¹H-MRS imaging of the parietal white matter of neonates exposed to alcohol *in utero*. Few studies used have used ¹H-MRS to investigate how neurochemical disturbances relate to the pathophysiology of FASD. Further, no studies to date have reported assessing brain metabolites in the infant age group with a history of prenatal alcohol exposure *in utero*.

We demonstrated that in this group, at this age, altered glutamate/glutamine complex concentrations were present in the parietal WM of the exposed infants. Absolute glutamate with glutamine (Glx) concentration of parietal white matter (PWM) was decreased in male infants exposed to alcohol *in utero* (n=8), compared with male controls (n=10; p=0.008). Absolute glutamate concentration of PWM was decreased in male infants exposed to alcohol *in utero* (n=8), compared with male controls (n=10; p=0.02) and female infants exposed to alcohol *in utero* (n=9; p=0.02).

This study provides possible support for the hypothesis that reduced concentrations of glutamate may reflect delay in the brain maturation in neonates exposed to alcohol *in utero*. However, neurochemical concentrations are a very dynamic measure of brain function which are highly likely to change not only with age and brain development, but also with length of time from the last exposure (birth). Although these findings are consistent in terms of region with the effects of prenatal alcohol exposure that we have described in the structural analyses at this time point, evaluating these measures at later ages will give us clearer understanding of their long-term significance.

8.1.5 Resting state fMRI

In the final paper of this thesis we explored the functional connectivity networks using resting state fMRI imaging in a small group of neonates with prenatal alcohol exposure compared to healthy control infants. Using an independent component analysis approach we demonstrated that there were indeed differences between the default mode networks of infants exposed to alcohol *in utero* and their unexposed counterparts. Prenatal alcohol exposure was associated with significant increases in connectivity between somatosensory, motor networks, brainstem/thalamic and striatal intrinsic networks. Trend-level evidence for reductions in inter-hemispheric connectivity of motor and somatosensory networks was also observed. Though again, results are preliminary, findings suggest pre-natal alcohol exposure may disrupt the temporal coherence in blood oxygenation utilization in intrinsic networks underlying motor performance in new-born infants.

8.2 Limitations of this study

Imaging infants remains technically difficult both in terms of imaging acquisition as well as the inherent challenges in reliably analysing data from brains that are small, have high water content and poor gray-white matter differentiation. (13-15) As with the other imaging studies in this age group, this project was limited by the reduction of sample size due to movement artefact across all the modalities. In certain modalities (such as the resting state fMRI sequence), data on nearly half of the original sample scanned with this sequence had to be excluded from the final analysis.

Information was collected on the timing and severity of alcohol exposure. However, the number of infants for whom we acquired usable MRI data was insufficient to make meaningful comparisons between these exposure categories in order to help define particular windows of vulnerability to the effects of alcohol during the prenatal period. Further, the influence of age on parameters across the modalities (reflecting the rapid changes in structural and functional maturation) is particularly strong in these early weeks of life. Although this was controlled for in the analysis, it may in fact have masked group differences resulting from prenatal alcohol exposure.

Specific technical limitations of the ^1H -MRS protocol included the lack of partial volume correction. In addition, the issue of chemical shift artefact related to the overlap of metabolite and water scans was not addressed in the analysis. Large ^1H -MRS voxel sizes were necessary to improve the signal-to-noise ratio in this age group. This may have had an effect on the homogeneity of tissue within selected voxels. Finally the selection of one specific area for investigation, though hypothesis driven, nevertheless means that other key areas which may have shown differences have not been identified in this study.

Although our study employed a cross-sectional design where infants were matched for age, gender, and active maternal smoking during pregnancy, longitudinal study data on this group is going to be key in establishing the trajectory of alterations in structure and function in developing brains exposed to alcohol during prenatal life. Despite these considerations, I believe this work to demonstrate preliminary evidence for the direct effects of alcohol exposure on the developing brain using complimentary, but different neuroim-

aging modalities in the first weeks of life. The results of this study warrant replication in larger studies in independent samples.

8.3 So what does this information tell us?

Putting the data together there is evidence for gray matter volume differences, differences in the integrity of white matter microstructure in white matter tracts which connect to both these areas, abnormal glutamate/glutamine concentrations in the parietal lobe as well as differences in the organisation of functional networks for a small group of these infants. Together this provides evidence for the effect of prenatal alcohol exposure on the brain at a structural, microstructural, neurochemical as well as functional organisational level in the *same group of infants*. This has not been previously reported. These alterations suggest prenatal alcohol exposure affects multiple biological processes that result in physical growth of the brain (overall as well as regional volume changes), quality and maturation of the connecting circuitry (white matter microstructural metrics), neurotransmitter concentrations in the first weeks of life (glutamatergic function) as well as functional connectivity, both within intrinsic networks as well as between hemispheres. Although some of these effects may represent delayed maturation, the coherence of the described regions with previously described findings in older children as well as in our understanding of the functional deficits we expect to see in children on the FASD spectrum argues against this as the only explanation. (17) The regions and connecting circuitry described here are likely to be at least part of the *primary core* effect of alcohol exposure on the developing brain.

It is important to note that analyses were conducted in infants before any formal diagnosis could be made and thus effect sizes are expected to be smaller. This is true of all the analyses described here, the changes described represent the group effects of infants with moderate-severe alcohol exposure at any stage in the prenatal period. This was a potentially risky strategy to adopt for defining the alcohol exposed group (against describing the severe end of the exposure spectrum). However, based on the previous literature in the imaging and developmental psychology fields, I believed that there was evidence for the harmful effects of *any* prenatal alcohol on the developing brain. The combined data which is described in this thesis provides support for this hypothesis.

Further evidence for this effect are the widespread correlations between developmental outcomes at 6 months and gray matter volume at birth. These data underscore the fact that the harmful effects of moderate-severe prenatal exposure to alcohol on brain development at neurobiological and clinical level are already discernible in the first months of life, well before the age FAS and FASD are typically diagnosed. Although the use of the newer quantitative MRI techniques are not yet easily translatable into use in individual identification (these reports represent between group effects), this is something that could be developed. Approaches such as identifying “cut-off” values/ranges for any of the different MRI modalities described at a specific age, outside of which exposed children could be considered at risk, especially if they had additional clinical concerns may be avenues for adding the huge bulk of imaging information the research community has acquired over the last 25 years, to pathways to care. However, to do this effectively, large collaborative analyses using data from multiple populations will be necessary and data collected across understudied time-points, especially the early years.

8.4 Future directions

Most of our infants were exposed to alcohol during the first trimester of pregnancy with a significant minority being exposed through all three trimesters. It is well documented that early pregnancy alcohol exposure puts infants at risk of developing significant central structural defects. (18,19) Our data were unable to answer the question of whether later exposure *only* also puts children at risk of neurobiological damage. As understanding of the mechanisms of brain maturation evolves, the importance of the secondary processes of radial migration and maturation of cortical neurons has become apparent. The process of cortical migration can be affected at a number of discrete steps and each step can be affected in different ways depending on the type, dose and timing of an insult. (20,21) It remains to be seen exactly how prenatal alcohol exposure affects these more subtle processes.

Our study was not designed to answer this question. However, what is known is that disruption of normal neuronal migration and plasticity mechanisms may result in subtle disorders of neuronal position resulting in behavioral symptoms ranging from mild to severe. Evidence is emerging that indicates that exposure to neurotoxins during critical

periods in development may impact not only the number and size of neurons in affected areas, but may also impact the highly sensitive processes of secondary neuronal migration and axonal guidance, network formation and selective pruning necessary for cerebral maturation. The specific role of prenatal alcohol exposure in the etiology of abnormalities of higher brain function are unknown. Large numbers and innovative in vivo techniques may be required to detect subtle changes in achieving proper neuronal position and connections caused by exposure to neurotoxins such as alcohol. Animal studies may be able to answer some of these questions or at least provide direction, but ultimately understanding how these processes generalize to human beings is necessary.

Expanding the understanding of how exposure to neurotoxic substances during fetal life and subsequent early development leads to permanent structural and plasticity deficits is clinically relevant. Critical future focus areas that are needed to begin to explain the variation in clinical phenotype of children with prenatal alcohol exposure and hence risk, include the following: the investigation of hypothesis driven candidate gene expression/alterations (with the SRC/Fyn gene being a promising current candidate in cohorts with European ancestry), epigenetic modification of genetic code in prenatal alcohol exposure both at an individual and intergenerational level which may explain the gene-environment interactions which we know exist. (20,21) Studies which explore these critical areas and relate them to both clinical, neuroimaging and facial morphological outcomes are likely to provide a deeper understanding of the mechanisms for vulnerability to the effects of prenatal alcohol exposure and in so doing providing potential directed targets for biological interventions to ameliorate the effects of prenatal alcohol on subsequent brain development and functional outcomes when prevention fails. There is a need for a better understanding of the underlying mechanisms of development if we want to promote optimal development, not only in terms of cognition, but also in reducing risk for later alcohol use disorders in these children as they emerge into adolescence.

The other key remaining question is that of the meaning of these findings in the context of these children's later brain development both at a neuroanatomical and functional level. Documenting the trajectory over time, may further address gaps in our understanding of which areas and networks have key functional importance for this group of children.

An independent study has reported volume changes in prenatal alcohol exposed infants using tensor-based morphometry analysis. (22) These findings were most prominent in cerebellum, striatal and frontal midline areas. The location of these results is remarkably consistent with an established theme in prenatal alcohol exposure studies of the importance of midline structures. We expect that the identification of alcohol effects on key midline structures in the infant brain may be an early marker for later functional cognitive and behavioral deficits. However, only longitudinal imaging, developmental and behavioral data will enable us to confirm the clinical significance of very early identification of these children. Further, our preliminary resting state functional MRI analysis demonstrated significant differences in the motor network in neonates with prenatal alcohol exposure and suggests that alcohol exposure disrupts the temporal coherence in blood oxygen utilisation across the brain at rest. This highly sensitive approach may prove to be valuable in identifying affected children early as well as monitoring the effectiveness of interventions. A longitudinal approach may consolidate these early findings by the addition of later time points and establish relationships between structural and functional imaging findings and clinical measures including not only formal cognitive or developmental and behavioral assessments, but also more integrative outcomes such as academic achievement in real-world settings.

This project is an early step towards forming an effective strategy to detect and identify targets for ameliorating interventions for the devastating long-term effects of alcohol exposure on brain development in young children. These data will form the basis for a future research to extend these findings to subsequent imaging time-points with a larger range of developmental and neurobehavioral assessments to further examine the longitudinal effects of prenatal alcohol exposure on the trajectory of the developing brain in these critical early years. In addition, the interaction between structural and functional connectivity and manifest developmental states will need to be analyzed to explore the long-term clinical significance of these neurobiological findings. The goal is to enable routine, early detection of childhood brain disorders from all causes, opening a window of opportunity for prevention or reduction of symptom severity.

References

- (1) World Health Organisation. Global status report on alcohol and health – 2014 ed. WHO Press, World Health Organization 2014.
- (2) Abel, E. & Sokol, R. Fetal alcohol syndrome is now leading cause of mental retardation. *The Lancet* 1986;328(8517):1222.
- (3) Maulik PK, Darmstadt GL. Childhood Disability in Low- and Middle-Income Countries: Overview of Screening, Prevention, Services, Legislation, and Epidemiology. *Pediatrics* July 2007 July 2007;120(Supplement 1):S1-S55.
- (4) Eckstrand KL, Ding Z, Dodge NC, Cowan RL, Jacobson JL, Jacobson SW, et al. Persistent Dose-Dependent Changes in Brain Structure in Young Adults with Low-to-Moderate Alcohol Exposure In Utero. *Alcoholism: Clinical and Experimental Research* 2012;36(11):1892-1902.
- (5) Gautam P, Lebel C, Narr KL, Mattson SN, May PA, Adnams CM, et al. Volume changes and brain-behavior relationships in white matter and subcortical gray matter in children with prenatal alcohol exposure . *Human brain mapping* 2015;36(6):2318-2329.
- (6) Lebel C, Mattson SN, Riley EP, Jones KL, Adnams CM, May PA, et al. A Longitudinal Study of the Long-Term Consequences of Drinking during Pregnancy: Heavy In Utero Alcohol Exposure Disrupts the Normal Processes of Brain Development. *The Journal of Neuroscience* 2012 October 31;32(44):15243-15251.
- (7) Mori S, Wakana S, van Zijl PCM, Nagae-Poetscher LM. *MRI Atlas of Human White Matter*. Amsterdam: Elsevier; 2005.
- (8) Diwadkar VA, Meintjes EM, Goradia D, Dodge NC, Warton C, Molteno CD, et al. Differences in cortico-striatal-cerebellar activation during working memory in syndromal and nonsyndromal children with prenatal alcohol exposure. *Human Brain Mapping* 2013;34(8):1931-1945.
- (9) Fan J, Meintjes EM, Molteno CD, Spottiswoode BS, Dodge NC, Alhamud AA, et al. White matter integrity of the cerebellar peduncles as a mediator of effects of prenatal alcohol exposure on eyeblink conditioning. *Hum Brain Mapp* 2015:n/a-n/a.
- (10) Lebel C, Roussotte F, Sowell ER. Imaging the Impact of Prenatal Alcohol Exposure on the Structure of the Developing Human Brain. *Neuropsychol Rev* 2011;21:102-118.
- (11) O'Hare ED, Kan E, Yoshii J, Mattson SN, Riley EP, Thompson PM, et al. Mapping cerebellar vermal morphology and cognitive correlates in prenatal alcohol exposure. *Neuroreport* 2005;16(12):1285-1290.

- (12) Green CR, Lebel C, Rasmussen C, Beaulieu C, Reynolds JN. Diffusion Tensor Imaging Correlates of Saccadic Reaction Time in Children with Fetal Alcohol Spectrum Disorder. *Alcoholism: Clinical and Experimental Research* 2013;37(9):1499-1507.
- (13) Hagmann P, Grant PE, Fair DA. MR Connectomics: A Conceptual Framework for Studying The Developing Brain. *Frontiers in Systems Neuroscience* 2012;6.
- (14) Geng X, Gouttard S, Sharma A, Gu H, Styner M, Lin W, et al. Quantitative tract-based white matter development from birth to age 2 years. *Neuroimage* 2012 7/2;61(3):542-557.
- (15) Gilmore JH, Shi F, Woolson SL, Knickmeyer RC, Short SJ, Lin W, et al. Longitudinal Development of Cortical and Subcortical Gray Matter from Birth to 2 Years. *Cerebral Cortex* 2012 November 01;22(11):2478-2485.
- (16) Power JD, Barnes KA, Snyder AZ, Schlaggar BL, Petersen SE. Spurious but systematic correlations in functional connectivity MRI networks arise from subject motion. *Neuroimage* 2012;59(3):2142-2154.
- (17) Mattson S, Crocker N, Nguyen T. Fetal Alcohol Spectrum Disorders: Neuropsychological and Behavioral Features. *Neuropsychol Rev* 2011;21(2):81-101.
- (18) Moore EM, Migliorini R, Infante MA, Riley EP. Fetal Alcohol Spectrum Disorders: Recent Neuroimaging Findings. *Current developmental disorders reports* 2014;1(3):161-172.
- (19) Sowell ER, Mattson SN, Thompson PM, Jernigan TL, Riley EP, & Toga AW. Mapping callosal morphology and cognitive correlates: effects of heavy prenatal alcohol exposure. *Neurology* 2001;57(2):235-244.
- (20) Rakic P. - Genetic Control of Cortical Convolution. - *Science* (- 5666):- 1983.
- (21) Rakic P. Genetic Control of Cortical Convolution. *Science* 2004 03/26;303(5666):1983-1984.
- (22) Meintjes EM, Jacobson JL, Moltano CD, Gatenby JC, Warton C, Cannistraci CJ, et al. An FMRI study of number processing in children with fetal alcohol syndrome. *Alcoholism: Clinical and Experimental Research* 2010;34(8):1450-1464.

Appendix A: Informed consent 525/2012

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM FOR USE BY PARENTS/LEGAL GUARDIANS

Infants Control Version

TITLE OF THE RESEARCH PROJECT: *Alcohol exposure effects in infants: MRI, DTI, MRS and neurobehavioural correlates*

REFERENCE NUMBER: HREC 525/2012

PRINCIPAL INVESTIGATOR: Dr Kirsty Donald

ADDRESS: School of Child and Adolescent Health, Red Cross Children's Hospital and the University of Cape Town

CONTACT NUMBER: (021) 6585322

Your child is being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how your child could be involved. Also, your child's participation is entirely voluntary and you are free to decline to participate. If you say no, this will not affect you or your child negatively in any way whatsoever. You are also free to withdraw him/her from the study at any point, even if you do initially agree to let him/her take part.

This study has been approved by the Committee for Human Research at the University of Cape Town and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

In the event of an injury or illness that is determined to be directly related to the use of the study medication or properly performed study procedures, the University of Cape Town will pay all reasonable and necessary medical expenses that treat such illness or injury through their no fault insurance policy which covers this trial, provided you have followed

the directions of the study doctor and his/her staff. Compensation will be in accordance with the Association of British Pharmaceutical Industry guidelines. Your doctor has a copy of the guidelines if you would like to review them.

What is this research study all about?

This study looks at the structure of your child's brain using a brain scan (Magnetic resonance imaging). By doing so we hope to get a better understanding of how the brain looks and also what goes wrong in certain disorders so (in the long term) we can identify problems and develop better treatments.

Why has your child been invited to participate?

We would like to look at the structure of his/her brain compared to other babies of the same age.

What will your responsibilities be?

You would be required to bring your baby to the unit (we will collect you from Paarl hospital and return you there) so we can get the images and wait with your child while we are scanning. The visit will take 1-2 hours in total.

Will your child benefit from taking part in this research?

Your baby's's development will be assessed and brain scanned. Possible problems may be picked up early and your child will be referred for treatment. We will provide you with a picture of your child's brain after the scan.

Are there any risks involved in your child taking part in this research?

No. We will wrap your baby in a blanket and let them sleep naturally, they will experience no pain. If at any time they become upset, the task will be stopped. There will be a neonatal nurse and/or medical doctor present at all times.

Who will have access to your child's medical records?

Only members of the research team will have access to the data gathered here. All information will remain confidential and if the results of this study are published no participant will be identified.

Will you or your child be paid to take part in this study and are there any costs involved?

You or your child will not be paid to take part in the study, but your/your child's transport and meal costs will be covered for each study visit. There will be no costs involved for you if your child does take part.

Is there any thing else that you should know or do?

- ▶ You should inform your family practitioner or usual doctor that your child is taking part in a research study.
- ▶ You can contact Dr Kirsty Donald at tel 021-6585322 if you have any further queries or encounter any problems.
- ▶ You can contact the Committee for Human Research if you have any concerns or complaints that have not been adequately addressed by your child's study doctor.
- ▶ You will receive a copy of this information and consent form for your own records.

Declaration by parent/legal guardian

By signing below, I (*name of parent/legal guardian*)
agree to allow my child (*name of child*) who is
years old, to take part in a research study entitled (*insert title of study*)

I declare that:

- I have read or had read to me this information and consent form and that it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to let my child take part.
- I may choose to withdraw my child from the study at any time and my child will not be penalised or prejudiced in any way.
- My child may be asked to leave the study before it has finished if the study doctor or researcher feels it is in my child's best interests, or if my child does not follow the study plan as agreed to.

Signed at (*place*) on (*date*) 2010.

Signature of parent/legal guardian

Signature of witness

Declaration by investigator

I (*name*) declare that:

- I explained the information in this document to
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understand all aspects of the research, as discussed above
- I did/did not use a translator (*if a translator is used, then the translator must sign the declaration below*).

Signed at (*place*) on (*date*) 2010.

Signature of parent/legal guardian

Signature of witness

Declaration by translator

I (*name*) declare that:

- I assisted the investigator (*name*) to explain the information in this document to (*name of parent/legal guardian*) using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the parent/legal guardian fully understands the content of this informed consent document and has had all his/her questions satisfactorily answered.

Signed at (*place*) on (*date*) 2010.

Signature of parent/legal guardian

Signature of witness

Appendix B: ASSIST

ASSIST QUESTIONNAIRE – WHO

These are some questions about your experience of using substances across your lifetime and in the past three months. These substances can be smoked, swallowed, snorted, inhaled, injected or taken in the form of pills. Some of the substances listed may be prescribed by a doctor (like amphetamines, sedatives, pain medications). For these questions, do not record medications that are used as prescribed by your doctor.

However, if you have taken such medications for reasons other than prescription, or taken them more frequently or at higher doses than prescribed, please record these. While we are also interested in knowing about your use of various illicit (illegal) drugs, please be assured that information on such use will be treated as confidential.

1	In your life, which of the following substances have you ever used? (NON-MEDICAL USE ONLY)	NO	YES
A	Tobacco products (cigarettes, chewing tobacco, cigars, etc.)	0	3
B	Alcoholic beverages (beer, wine, spirits, etc.)	0	3
C	Cannabis (marijuana, pot, grass, hash, dagga, etc.)	0	3
D	Cocaine (coke, crack, etc.)	0	3
E	Amphetamine-type stimulants (speed, diet pills, ecstasy, Tik, etc.)	0	3
F	Inhalants (nitrous, glue, petrol, paint thinner, etc.)	0	3
G	Sedatives or Sleeping Pills (Valium, Serepax, Rohypnol, etc.)	0	3
H	Hallucinogens (LSD, acid, mushrooms, PCP, Special K, etc.)	0	3
I	Opioids (heroin, morphine, methadone, codeine, etc.)	0	3
J	Other – specify:	0	3
If all answers are negative: “Not even when you were in school?”		If “No” to all items, go to Question 6. If “Yes” to any of these items, answer Question 2 for each substance ever used.	

2	In the past three months, how often have you used the substances you mentioned (FIRST DRUG, SECOND DRUG, ETC)?	Never	Once or Twice	Monthly	Weekly	Daily or Almost Daily
A	Tobacco products (cigarettes, chewing tobacco, cigars, etc.)	0	2	3	4	6
B	Alcoholic beverages (beer, wine, spirits, etc.)	0	2	3	4	6
C	Cannabis (marijuana, pot, grass, hash, dagga, etc.)	0	2	3	4	6
D	Cocaine (coke, crack, etc.)	0	2	3	4	6
E	Amphetamine- type stimulants (speed, diet pills, ecstasy, Tik, etc.)	0	2	3	4	6
F	Inhalants (nitrous, glue, petrol, paint thinner, etc.)	0	2	3	4	6
G	Sedatives or Sleeping Pills (Valium, Serepax, Rohypnol, etc.)	0	2	3	4	6
H	Hallucinogens (LSD, acid, mushrooms, PCP, Special K, etc.)	0	2	3	4	6
I	Opioids (heroin, morphine, methadone, codeine, etc.)	0	2	3	4	6
J	Other – specify:	0	2	3	4	6
If “Never” to all items in Question 2, skip to Question 6.						
If any substances in Question 2 were used in the previous three months, continue with Question 3, 4 & 5 for each substance used.						

3	During the <u>past three months</u> , how often have you had a strong desire or urge to use (<i>FIRST DRUG, SECOND DRUG, ETC</i>)?	Never	Once or Twice	Monthly	Weekly	Daily or Almost Daily
A	Tobacco products (cigarettes, chewing tobacco, cigars, etc.)	0	3	4	5	6
B	Alcoholic beverages (beer, wine, spirits, etc.)	0	3	4	5	6
C	Cannabis (marijuana, pot, grass, hash, dagga, etc.)	0	3	4	5	6
D	Cocaine (coke, crack, etc.)	0	3	4	5	6
E	Amphetamine-type stimulants (speed, diet pills, ecstasy, Tik, etc.)	0	3	4	5	6
F	Inhalants (nitrous, glue, petrol, paint thinner, etc.)	0	3	4	5	6
G	Sedatives or Sleeping Pills (Valium, Serepax, Rohypnol, etc.)	0	3	4	5	6
H	Hallucinogens (LSD, acid, mushrooms, PCP, Special K, etc.)	0	3	4	5	6
I	Opioids (heroin, morphine, methadone, codeine, etc.)	0	3	4	5	6
J	Other – specify:	0	3	4	5	6

4	During the <u>past three months</u> , how often has your use of (<i>FIRST DRUG, SECOND DRUG, ETC</i>) led to health, social, legal or financial problems?	Never	Once or Twice	Monthly	Weekly	Daily or Almost Daily
A	Tobacco products (cigarettes, chewing tobacco, cigars, etc.)	0	4	5	6	7
B	Alcoholic beverages (beer, wine, spirits, etc.)	0	4	5	6	7
C	Cannabis (marijuana, pot, grass, hash, dagga, etc.)	0	4	5	6	7
D	Cocaine (coke, crack, etc.)	0	4	5	6	7
E	Amphetamine-type stimulants (speed, diet pills, ecstasy, Tik, etc.)	0	4	5	6	7
F	Inhalants (nitrous, glue, petrol, paint thinner, etc.)	0	4	5	6	7
G	Sedatives or Sleeping Pills (Valium, Serepax, Rohypnol, etc.)	0	4	5	6	7
H	Hallucinogens (LSD, acid, mushrooms, PCP, Special K, etc.)	0	4	5	6	7
I	Opioids (heroin, morphine, methadone, codeine, etc.)	0	4	5	6	7
J	Other – specify:	0	4	5	6	7

5	During the <u>past three months</u> , how often have you failed to do what was normally expected of you because of your use of (<i>FIRST DRUG, SECOND DRUG, ETC</i>)?	Never	Once or Twice	Monthly	Weekly	Daily or Almost Daily
A	Tobacco products (cigarettes, chewing tobacco, cigars, etc.)	0	5	6	7	8
B	Alcoholic beverages (beer, wine, spirits, etc.)	0	5	6	7	8
C	Cannabis (marijuana, pot, grass, hash, dagga, etc.)	0	5	6	7	8
D	Cocaine (coke, crack, etc.)	0	5	6	7	8
E	Amphetamine-type stimulants (speed, diet pills, ecstasy, Tik, etc.)	0	5	6	7	8
F	Inhalants (nitrous, glue, petrol, paint thinner, etc.)	0	5	6	7	8
G	Sedatives or Sleeping Pills (Valium, Serepax, Rohypnol, etc.)	0	5	6	7	8
H	Hallucinogens (LSD, acid, mushrooms, PCP, Special K, etc.)	0	5	6	7	8
I	Opioids (heroin, morphine, methadone, codeine, etc.)	0	5	6	7	8
J	Other – specify:	0	5	6	7	8

Answer Questions 6 & 7 for all substances ever used (i.e. those endorsed in Question 1)

6	Has a friend or relative or anyone else ever expressed concern about your use of (FIRST DRUG, SECOND DRUG, ETC)?	No, Never	Yes, in the past 3 months	Yes, but not in the past 3 months
A	Tobacco products (cigarettes, chewing tobacco, cigars, etc.)	0	6	3
B	Alcoholic beverages (beer, wine, spirits, etc.)	0	6	3
C	Cannabis (marijuana, pot, grass, hash, dagga, etc.)	0	6	3
D	Cocaine (coke, crack, etc.)	0	6	3
E	Amphetamine-type stimulants (speed, diet pills, ecstasy, Tik, etc.)	0	6	3
F	Inhalants (nitrous, glue, petrol, paint thinner, etc.)	0	6	3
G	Sedatives or Sleeping Pills (Valium, Serepax, Rohypnol, etc.)	0	6	3
H	Hallucinogens (LSD, acid, mushrooms, PCP, Special K, etc.)	0	6	3
I	Opioids (heroin, morphine, methadone, codeine, etc.)	0	6	3
J	Other – specify:	0	6	3

7	Have you ever tried and failed to control, cut down or stop using (FIRST DRUG, SECOND DRUG, ETC)?	No, Never	Yes, in the past 3 months	Yes, but not in the past 3 months
A	Tobacco products (cigarettes, chewing tobacco, cigars, etc.)	0	6	3
B	Alcoholic beverages (beer, wine, spirits, etc.)	0	6	3
C	Cannabis (marijuana, pot, grass, hash, dagga, etc.)	0	6	3
D	Cocaine (coke, crack, etc.)	0	6	3
E	Amphetamine-type stimulants (speed, diet pills, ecstasy, Tik, etc.)	0	6	3
F	Inhalants (nitrous, glue, petrol, paint thinner, etc.)	0	6	3
G	Sedatives or Sleeping Pills (Valium, Serepax, Rohypnol, etc.)	0	6	3
H	Hallucinogens (LSD, acid, mushrooms, PCP, Special K, etc.)	0	6	3
I	Opioids (heroin, morphine, methadone, codeine, etc.)	0	6	3
J	Other – specify:	0	6	3

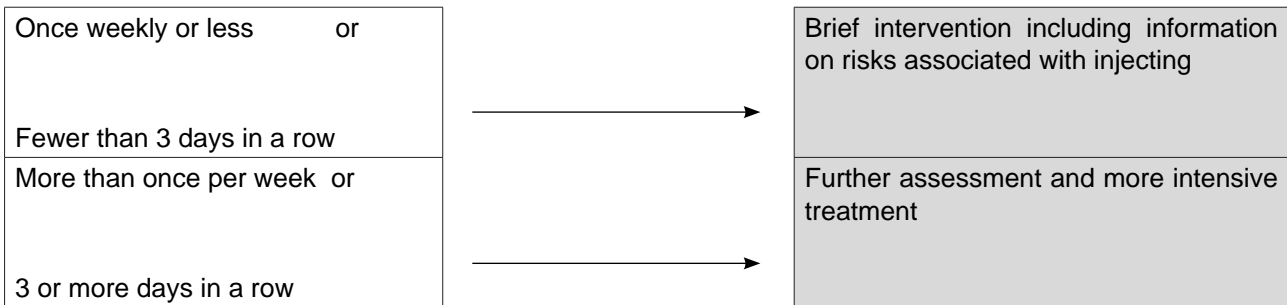
8	Have you ever used any drug by injection? (NON-MEDICAL USE ONLY)	<input type="checkbox"/> No, never <input type="checkbox"/> Yes, in the past 3 months <input type="checkbox"/> Yes, but not in the past 3 months		
---	--	--	--	--

IMPORTANT NOTE

If you have injected drugs in the last 3 months, please indicate your pattern of injecting during this period (below):

PATTERN OF INJECTING

INTERVENTION GUIDELINES



SCORING GUIDELINES

For each substance (labeled A to J) add up the scores received for questions 2 through 7 inclusive. Do not include the results from either Q1 or Q8 in this score. For example, a score for cannabis would be calculated as: Q2c + Q3c + Q4c + Q5c + Q6c + Q7c

Note that Q5 for tobacco is not coded, and is calculated as: Q2a + Q3a + Q4a + Q6a + Q7a

	Record specific substance score	No intervention	Receive brief intervention	More intensive treatment
Tobacco		0-3	4-26	27+
Alcohol		0-10	11-26	27+
Cannabis		0-3	4-26	27+
Cocaine		0-3	4-26	27+
Amphetamine		0-3	4-26	27+
Inhalants		0-3	4-26	27+
Sedatives		0-3	4-26	27+
Hallucinogens		0-3	4-26	27+
Opioids		0-3	4-26	27+
Other drugs		0-3	4-26	27+

Scoring: Count up questions 2-7

Total: Drug 1: _____ (name of drug) _____ (score)

Total Drug 2: _____ (name of drug) _____ (score)

Total Drug 3: _____ (name of drug) _____ (score)

Total Drug 4: _____ (name of drug) _____ (score)

Appendix C: Dubowitz neurobehavior assessment



CRF0X: Dubowitz Scale

Visit: Neonatal imaging

Mother Participant ID: ____/____/____/____

Date: ____/____/____

Child Participant ID: ____/____/____/____

DD / MMM / YYYY

ASSESSMENT CATEGORY	SCORING GUIDELINES				
POSTURE					
POSTURE Infant supine, look mainly at position of legs but also note arms. <i>score predominant posture</i>	arms & legs extended or very slightly flexed 	legs slightly flexed 	leg well-flexed but not adducted 	leg well flexed & adducted near abdomen 	abnormal posture: a) opisthotonus b)

LIMBS					
ARM RECOIL Take both hands, quickly extend arms parallel to the body, Count to three. Release. Repeat X 3	arms do not flex 	arms flex slowly, not always; not completely 	arms flex slowly; more complete 	arms flex quickly and completely 	arms difficult to extend; snap back forcefully
ARM TRACTION Hold wrist and pull arm upwards. Note flexion at elbow and resistance while shoulder lifts off table. <i>Test each side separately</i>	arms remain straight; no resistance felt 	arms flex slightly or some resistance felt 	arms flex well till shoulder lifts, then straighten 	arms flex at approx 100° & maintained as shoulder lifts 	flexion of arms <100°; maintained when body lifts up
LEG RECOIL Take both ankles in one hand, flex hips+knees. Quickly extend. Release. Repeat X3	No flexion 	incomplete or variable flexion 	complete but slow flexion 	complete fast flexion 	legs difficult to extend; snap back forcefully
LEG TRACTION Grasp ankle and slowly pull leg upwards. Note flexion at knees and resistance as buttocks lift. <i>Test each side separately</i>	legs straight - no resistance felt 	legs flex slightly or some resistance felt 	legs flex well till bottom lifts up 	knee flexes remains flexed when bottom up 	flexion stays when back+bottom up
POPLITEAL ANGLE Fix knee on abdomen, extend leg by gentle pressure with index finger behind the ankle. Note angle at knee. <i>Test each side separately</i>	180° R L	= 150° R L	≈ 110° R L	≈ 90° R L	< 90° R L



CRFOX: Dubowitz Scale

Visit: Neonatal imaging Mother Participant ID: ____/____/____ Date: ____/____/____
 Child Participant ID: ____/____/____ DD / MMM / YYYY

HEAD/TRUNK					
HEAD CONTROL (1) <i>(extensor tone)</i> Infant sitting upright; Encircle chest with both hands holding shoulders. Let head drop forward.	no attempt to raise head 	infant tries: effort better felt than seen 	raises head but drops forward or back 	raises head: remains vertical; it may wobble 	
HEAD CONTROL (2) <i>(flexor tone)</i> Infant sitting upright; Encircle chest with both hands holding shoulders. Let head drop backward.	no attempt to raise head 	infant tries: effort better felt than seen 	raises head but drops forward or back 	raises head: remains vertical; it may wobble 	head upright or extended; cannot be passively flexed
HEAD LAG Pull infant to towards sitting posture by traction on both wrists & support head slightly. Also note arm flexion	head drops & stays back 	tries to lift head but it drops back 	able to lift head slightly 	lifts head in line with body 	head in front of body
VENTRAL SUSPENSION Hold infant in ventral suspension; observe back, flexion of limbs and relation of head to trunk. If it looks different, DRAW	back curved, head & limbs hang straight 	back curved, head ↓, limbs slightly flexed 	back slightly curved, limbs flexed 	back straight, head in line, limbs flexed 	back straight, head above body



CRFOX: Dubowitz Scale






Visit: Neonatal imaging

Mother Participant ID: ____/____/____

Date: ____/____/____

Child Participant ID: ____/____/____

DD / MMM / YYYY

REFLEXES						
TENDON REFLEX test biceps, knee and ankle jerks.	absent	felt, not seen	seen	'exaggerated'	clonus	
SUCK / GAG Little finger into mouth with pulp of finger upwards.	no gag / no suck	weak irregular suck only: No stripping	weak regular suck Some stripping	strong suck: (a) irregular (b) regular Good stripping	no suck but strong clenching	
PALMAR GRASP Put index finger into the hand and gently press palmar surface. Do not touch dorsal surface.	no response R L	short, weak flexion of fingers R L	strong flexion of fingers R L	strong finger flexion, shoulder ↑ R L	very strong grasp; infant can be lifted off couch R L	
PLANTAR GRASP Press thumb on the sole below the toes.	no response R L	partial plantar flexion of toes R L	toes curve around the examiner's finger R L			
PLACING Lift infant in an upright position and stroke the dorsum of the foot against a protruding edge of a flat surface. Test each side separately	No response R L	dorsiflexion of ankle only R L	full placing response with flexion of hip, knee & placing sole on surface R L			
MORO One hand supports infant's head in midline, the other the back. Raise infant to 45° and when relaxed let his head fall through 10°. Note if jerky. Repeat 3 times	no response or opening of hands only	full abduction at shoulder and extension of the arms; no adduction 	full abduction but only delayed or partial adduction 	partial abduction at shoulder and extension of arms followed by smooth adduction 	<ul style="list-style-type: none"> • no abduction or adduction; • only forward extension of arms from the shoulders • marked adduction only  or 	

SPONTANEOUS MOVEMENT (quantity) Watch infant lying supine	no movement	sporadic and short isolated movements	frequent isolated movements	frequent generalised movements	continuous exaggerated movements
SPONTANEOUS MOVEMENT (quality) Watch infant lying supine	only stretches	stretches and random abrupt movements; some smooth movements	fluent movements but monotonous	fluent alternating movements of arms + legs; good variability	<ul style="list-style-type: none"> • cramped synchronised; • mouthing • jerky or other abn. mov.



CRFOX: Dubowitz Scale

Visit: Neonatal imaging

Mother Participant ID: ____/____/____

Date: ____/____/____

Child Participant ID: ____/____/____

DD / MMM / YYYY

OTHER NEUROLOGIC SIGNS					
EYE APPEARANCES	does not open eyes		full conjugated eye movements	<i>transient</i> • nystagmus • strabismus • roving eye movements • sunset sign	<i>persistent</i> • nystagmus • strabismus • roving eye movements abnormal pupils
AUDITORY ORIENTATION Infant awake. Wrap infant. Hold rattle 10 - 15 cms from ear.	no reaction	auditory startle; brightens and stills; no true orientation	shifting of eyes, head might turn towards source	prolonged head turn to stimulus; search with eyes; smooth	turns head and eyes towards noise every time; jerky abrupt
VISUAL ORIENTATION Wrap infant, wake up with rattle if needed or rock gently. Note if baby can see and follow red ball (B) or target (T)	does not follow or focus on stimuli B T	stills, focuses, follows briefly to the side but loses stimuli B T	follows horizontally and vertically; no head turn B T	follows horizontally and vertically; turns head B T	follows in a circle B T
ALERTNESS <i>Tested as response to visual stimuli (B or T)</i>	will not respond to stimuli	when awake, looks only briefly	when awake, looks at stimuli but loses them	keeps interest in stimuli	does not tire (hyper-reactive)
IRRITABILITY in response to stimuli	quiet all the time, not irritable to any stimuli	awakes, cries sometimes when handled	cries often when handled	cries always when handled	cries even when not handled
CONSOLABILITY Ease to quiet infant	not crying consoling not needed	cries briefly; consoling not needed	cries; becomes quiet when talked to	cries; needs picking up to console	cries cannot be consoled
CRY	no cry at all	whimpering cry only	cries to stimuli but normal pitch		High pitched cry; often continuous

MOVEMENTS					
ABNORMAL HAND OR TOE POSTURES		hands open, toes straight most of the time	intermittent fisting or thumb adduction	continuous fisting or thumb adduction; index finger flexion, thumb opposition	continuous big toe extension or flexion of all toes
TREMOR		no tremor or tremor only when crying	tremor only after Moro or occasionally when awake	frequent tremors when awake	continuous tremors
STARTLE	no startle even to sudden noise	no spontaneous startle but react to sudden noise	2-3 spontaneous startles	more than 3 spontaneous startles	continuous startles

CRF Completed by: _____

Date: ____/____/____
DD / MMM / YYYY

Appendix D: MRI imaging protocol

1. Structural:

Two T2 images, saggital

t2_tse3d_sag: repetition time (TR)=3500ms; TE (echo time)=354ms; slice thickness 1mm; 128 slices; voxel size 1.0×1.0×1.0mm. *Scan time*: 5min41s / scan.

Two T1-weighted images

tfl_mge_me: TR=2530ms; TE(1-4)=1.53, 3.21, 4.89, 6.57; flip angle=7°; slice thickness 1mm; 128 slices; voxel size 1.3×1.0×1.3 mm. *Scan time*: 6min45s / scan.

2. 1H-MRS voxels

Brain area 1: Parietal white. Sequence: PRESS, TR=2000, TE=30 (128 averages) with water scaling (30, 75, 100, 144, 500, 1000) and water reference (0 water suppression); voxel size 18x18x18 mm. *Scan time*: 6min.

Brain area 2: Parietal grey matter. Sequence: PRESS, TR=2000, TE=30 (128 averages) with water scaling (30, 75, 100, 144, 500, 1000) and water reference (0 water suppression); Voxel 18x18x18 mm. *Scan time*: 6min.

3. Diffusion Tensor Imaging

ep2d_diff_MGH: Two diffusion-weighted images per phase direction AP and PA respectively, each with the following parameters: 45 diffusion directions; 1 b=0sec/mm²; repetition time 7900 ms; echo time 90 ms; b-value of 1000sec/mm²; slice thickness of 1.6 mm; voxel size: 1.3×1.3×1.6 mm. *Scan time*: 6min27s / scan.

4. Resting state

ep2d_bold_resting: TR 2000 ms; TE 30 ms; flip angle=77° slice thickness 4 mm; 25 slices; voxel size: 2.5×2.5×4.0 mm. *Scan time*: 6min04s.

Total scan time: 55min49s

Appendix E: Ethical approval HREC 525/2012

000001 0000

HREC office use only (FWA00001637; IRB00001938)		
This serves as notification of annual approval. Including any documentation described below.		
<input checked="" type="checkbox"/> Approved	Annual progress report	Approved until/next renewal date 30/12/2015
<input type="checkbox"/> Not approved	See attached comments	
Signature Chairperson of the HREC		Date Signed 14/1/2015

Comments to PI from the HREC

1. Protocol information

Date (when submitting this form)	6 Feb 2015		
HREC REF Number	525/2012	Current Ethics Approval was granted until	30 Dec 2014
Protocol title	Multimodal imaging and neurobehavioural correlates of alcohol and methamphetamine exposed infants in Cape Town		
Protocol number (if applicable)			
Are there any sub-studies linked to this study?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> N		
If yes, could you please provide the HREC Ref's for all sub-studies? Note: A separate FHS016 must be submitted for each sub-study.	*This study is a sub-study of the Drakenstein Child Lung Health Study (401/2009)		
Principal Investigator	Dr. K.A Donald		
Department / Office Internal Mail Address	Health Drakenstein's Hospital, Klipfontein 7701 Kirsty.donald@uct.ac.za		

1.1 Does this protocol receive US Federal funding?	<input type="checkbox"/> Yes	<input checked="" type="checkbox"/> No
1.2 If the study receives US Federal Funding, does the annual report require full committee approval?	<input type="checkbox"/> Yes	<input type="checkbox"/> No
1.3 Has sponsorship of this study changed? If yes, please attach a revised summary of the budget	<input type="checkbox"/> Yes	<input checked="" type="checkbox"/> No

