Antenatal causes of cerebral palsy and adverse pregnancy outcomes: Investigating associations between inherited thrombophilia, cytokine polymorphisms and viral infections

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Thesis submitted for the degree of
Doctor of Philosophy

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Declaration

"This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text."

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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October 2005

Declaration ii
Index iii
Acknowledgements x
Publications arising from this Thesis xi
Contributions made by Co-authors xiii
Thesis Explanation xvii
Abbreviations xviii
Abstract 1
Chapter 1 Literature Review
Introduction 3
What is Cerebral Palsy? 4
Prevalence of Cerebral Palsy 4
Risk Factors for Cerebral Palsy 5
White Matter Damage 6
Infection, Inflammation and PVL 6
Inherited Thrombophilia 9
Factor V Leiden 10
Prothrombin Gene Mutation 11
Methylenetetrahydrofolate Reductase Gene Mutations 11
Inherited Thrombophilia and Cerebral Palsy 12
Cytokine Involvement in Cerebral Palsy 13
Tumour Necrosis Factor-α 13
TNF-α Polymorphism 15
Mannose Binding Lectin 16
MBL Polymorphisms and Susceptibility to Infection 17
Intrauterine Infection 19
Fetal Response to Infection 20
Viral Infection 22
Transplacental Transmission of Viruses 22
Obstetrical Syndromes and Adverse Pregnancy Outcomes 24
Conclusions 24
Hypothesis and Aims 25
Chapter 2: Patient selection and demographic data
Study Population – Cerebral Palsy 26
Clinical Characteristics 27

Study Population – Adverse Pregnancy Outcomes 35
Clinical Characteristics 36
Chapter 3: The prevalence of inherited thrombophilia in a Caucasian Australian population
Abstract 38
Introduction 39
Materials and Methods 40
Patient Selection 40
DNA Isolation and Amplification 40
Results 42
Discussion 44
Chapter 4: Associations between inherited thrombophilia, gestational age and cerebral palsy
Abstract 47
Introduction 48
Materials and Methods 49
Patient Selection 49
DNA Isolation and Amplification 49
Statistical Analysis 49
Power Calculations 49
Results 50
Cerebral Palsy All Gestational Ages 50
Cerebral Palsy Gestational Ages ≥37 weeks 50
Cerebral Palsy Gestational Ages 32-36 weeks 50
Cerebral Palsy Gestational Ages <32 weeks 50
Combination Thrombophilia at any Gestational Age 51
Analysis using Term Controls 51
Discussion 56
Methylenetetrahydrofolate Reductase Gene 56
MTHFR Polymorphism C677T 56
MTHFR Polymorphism A1298C 57
Factor V Leiden 57
Prothrombin Gene Mutation 58
Conclusions 59

Chapter 5 The association between inherited cytokine polymorphisms and cerebral palsy

Abstract 60

Introduction 61

The Fetal Inflammatory Response 61

Cytokine Responses 61

Materials and Methods 64

Patient Selection 64

DNA Extraction 64

MBL Genotyping 64

DNA Sequencing 65

TNF-α Genotyping 65

Statistical Analysis 65

Results 66

Prevalence of Cytokine Polymorphisms in Control Population 66

Cytokines and Cerebral Palsy 69

All Controls 69

Cerebral Palsy All Gestational Ages 69

Cerebral Palsy Gestational Ages ≥37 weeks 69

Cerebral Palsy Gestational Ages 32-36 weeks 69

Cerebral Palsy Gestational Ages <32 weeks 70

Term Controls 70

Cerebral Palsy All Gestational Ages 70

Cerebral Palsy Gestational Ages ≥37 weeks 70

Cerebral Palsy Gestational Ages 32-36 weeks 71

Cerebral Palsy Gestational Ages <32 weeks 71

Discussion 80

Tumour Necrosis Factor α 80

Mannose Binding Lectin 82

Chapter 6 Neurotropic viruses are associated with cerebral palsy

Abstract 84

Introduction 85

Materials and Methods 87

Patient Selection 87

Virus Detection 87

Virus sensitivity, limits of detection and positive control stocks 87
Extraction 88
DNA Viruses 88
RNA Viruses 88
Amplification 88
Sequencing Analysis 89
Internal Controls 89
Statistical Analysis 90
Results 91
Prevalence of viruses in control population 91
Viruses and Cerebral Palsy 95
All Gestational Ages 95
Gestational Ages ≥37 weeks 95
Gestational Ages <37 weeks 95
Combination viruses at any gestational age 99
Discussion 100
Chapter 7 Associations between inherited thrombophilia and adverse pregnancy outcomes
Abstract 104
Introduction 105
Materials and Methods 106
Patient Selection 106
Statistical Analysis 106
Results 107
Intrauterine Growth Restriction <10th percentile 107
Hypertension 107
Pregnancy-Induced Hypertensive Disorders 107
Pregnancy-Induced Hypertensive Disorders and IUGR <10th percentile 107
Pre-existing Hypertension 107
Antepartum Haemorrhage 108
Antepartum Haemorrhage and IUGR <10th percentile 108
Preterm Birth <37 weeks 108
Preterm Birth and IUGR <10th percentile 108
Discussion 112

Prothrombin Gene Mutation 112
Factor V Leiden 113
Methylenetetrahydrofolate Reductase Gene 113
Chapter 8 Associations between cytokine polymorphisms and adverse pregnancy outcomes
Abstract 116
Introduction 117
The Fetal Inflammatory Response 117
Cytokine Responses 117
Materials and Methods 119
Patient Selection 119
Statistical Analysis 119
Results 120
Intrauterine Growth Restriction <10th percentile 120
Hypertension 120
Pregnancy-Induced Hypertensive Disorders 120
Pregnancy-Induced Hypertensive Disorders and IUGR <10th percentile 120
Pre-existing Hypertension 121
Antepartum Haemorrhage 121
Antepartum Haemorrhage and IUGR <10th percentile 121
Preterm Birth <37 weeks 121
Preterm Birth and IUGR <10th percentile 122
Discussion 126
Tumour Necrosis Factor-α 126
TNF-α and Intrauterine Growth Restriction 126
TNF-α and Hypertension 127
TNF-α and Antepartum Haemorrhage 128
TNF-α and Preterm Birth 128
Mannose Binding Lectin 128
MBL and Intrauterine Growth Restriction 129
MBL and Hypertension 129
MBL and Antepartum Haemorrhage 129
MBL and Preterm Birth 130
Conclusions 130

Chapter 9 Neurotropic viruses are associated with adverse pregnancy outcomes

Abstract 131

Introduction 132

Materials and Methods 134

Patient Selection 134

Statistical Analysis 134

Results 135

Intrauterine Growth Restriction <10th percentile 135

Hypertension 135

Pregnancy-Induced Hypertensive Disorders 135

Pregnancy-Induced Hypertensive Disorders and IUGR <10th percentile 135

Pre-existing Hypertension 135

Antepartum Haemorrhage 136

Antepartum Haemorrhage and IUGR <10th percentile 136

Preterm Birth <37 weeks 136

Preterm Birth and IUGR <10th percentile 136

Discussion 140

Chapter 10 Do inherited thrombophilic polymorphisms, cytokine polymorphisms and perinatal viral exposure interact to further increase the risks of cerebral palsy?

Abstract 144

Introduction 145

Materials and Methods 146

Patient Selection 146

Genotyping for inherited thrombophilic polymorphisms 146

Genotyping for inherited cytokine polymorphisms 146

Detection of viral nucleic acids 146

Statistical Analysis 146

Results 147

Main Effects Model including prematurity 147

Interactions between inherited thrombophilia, cytokine polymorphisms and viral infections 147

Discussion 149

Chapter 11 General Discussion

Introduction 151

Inherited Thrombophilic Polymorphisms 151

Inherited Cytokine Polymorphisms 152

Viral Infection 154

Interactions between main outcome measures 155

Population Attributable Risk 156

Prevention 156

Study Caveats 157

Conclusions 160

Future Implications and Directions 160

Chapter 12 References 164

Appendices

Appendix 1: Published Literature Review 186

Appendix 2: Published Inherited Thrombophilia Prevalence 198

Appendix 3: Inherited Thrombophilia Detection Methods (Thesis Ch 3) 202

Appendix 4: Supplementary Results for Inherited Thrombophilia and Cerebral Palsy (Thesis Ch 4) 204

Appendix 5: Inherited Cytokine Polymorphism Detection Methods (Thesis Ch 5) 212

Appendix 6: Viral Infection Detection Methods (Thesis Ch 6) 215

Appendix 7: Supplementary Results for Inherited Thrombophilia and Adverse Pregnancy Outcomes (Thesis Ch 7) 218

Appendix 8: Supplementary Results for Inherited Cytokine Polymorphisms and Adverse Pregnancy Outcomes (Thesis Ch 8) 226

Appendix 9: Supplementary Results for Viral Infection and Adverse Pregnancy Outcomes (Thesis Ch 9) 234

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Publications Arising From This Thesis

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2. **Gibson CS**, MacLennan AH, Rudzki Z, Hague WM, Haan E, Sharpe P, Priest K, Chan A, Dekker GA. The prevalence of inherited thrombophilias in a Caucasian Australian population. *Pathology* 2005;37(2):160-163.

3. **Gibson CS**, MacLennan AH, Hague WM, Haan EA, Priest K, Chan A, Dekker GA. Associations between Inherited Thrombophilias, Gestational Age, and Cerebral Palsy. *Am J Obstet Gynecol* 2005;In Press, Accepted 22.02.05.

4. **Gibson CS,** MacLennan AH, Goldwater PN, Haan E, Priest K, Dekker GA. *Neurotropic viruses are associated with cerebral palsy.* Brit Med J; In Press, Accepted 02.09.05.

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Conference Papers

1. **CS Gibson**, AH MacLennan, WM Hague, Z Rudzki, P Sharpe, A Chan, GA Dekker, *Fetal Thrombophilic Polymorphisms are not a risk factor for Cerebral Palsy.* Medicine and Pregnancy Conference, Fremantle, Western Australia, p32 (2003).

2. **CS Gibson**, AH MacLennan, WM Hague, Z Rudzki, P Sharpe, A Chan, GA Dekker, *Fetal Thrombophilic Polymorphisms are not a risk factor for Cerebral Palsy.* Society for Maternal Fetal Medicine Conference, New Orleans, USA, 2004 (accepted for oral presentation).

3. **CS Gibson**, AH MacLennan, WM Hague, Z Rudzki, P Sharpe, A Chan, GA Dekker, *Fetal Thrombophilic Polymorphisms are not a risk factor for Cerebral Palsy.* Perinatal Society of Australia and New Zealand Conference, Sydney, Australia, 2004 (accepted for oral presentation).

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6. **CS Gibson,** AH MacLennan, PN Goldwater, EA Haan, K Priest, GA Dekker, *The association between inherited cytokine polymorphisms and cerebral palsy.* Society for Maternal Fetal Medicine Conference, Miami, USA, 2006 (submitted for presentation).

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8. **CS Gibson,** AH MacLennan, PN Goldwater, EA Haan, K Priest, GA Dekker, *The role of fetal viral infection in the development of adverse pregnancy outcomes.* Society for Maternal Fetal Medicine Conference, Miami, USA, 2006 (submitted for presentation).

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2. Gibson CS, **MacLennan AH**, Rudzki Z, Hague WM, Haan E, Sharpe P, Priest K, Chan A, Dekker GA. The prevalence of inherited thrombophilias in a Caucasian Australian population. *Pathology* 2005;37(2):160-163.

3. Gibson CS, **MacLennan AH**, Hague WM, Haan EA, Priest K, Chan A, Dekker GA. Associations between Inherited Thrombophilias, Gestational Age, and Cerebral Palsy. *Am J Obstet Gynecol* 2005;In Press, Accepted 22.02.05.

4. Gibson CS, **MacLennan AH**, Goldwater PN, Haan E, Priest K, Dekker GA. *Neurotropic viruses are associated with cerebral palsy.* Brit Med J; In Press, Accepted 02.09.05.

Professor MacLennan was my principal supervisor and therefore was listed as a co-author on all publications arising from this thesis. He participated in the design and interpretation of the study, and was Chief Investigator A on grants for this project. He is the Head of the South Australian Cerebral Palsy Research Group. In addition, he read multiple drafts of the papers.

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2. Gibson CS, MacLennan AH, Rudzki Z, Hague WM, Haan E, Sharpe P, Priest K, Chan A, **Dekker GA**. The prevalence of inherited thrombophilias in a Caucasian Australian population. *Pathology* 2005;37(2):160-163.

3. Gibson CS, MacLennan AH, Hague WM, Haan EA, Priest K, Chan A, **Dekker GA.** Associations between Inherited Thrombophilias, Gestational Age, and Cerebral Palsy. *Am J Obstet Gynecol* 2005;In Press, Accepted 22.02.05.

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2. Gibson CS, MacLennan AH, **Hague WM**, Haan EA, Priest K, Chan A, Dekker GA. Associations between Inherited Thrombophilias, Gestational Age, and Cerebral Palsy. *Am J Obstet Gynecol* 2005;In Press, Accepted 22.02.05.

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2. Gibson CS, MacLennan AH, Hague WM, **Haan EA**, Priest K, Chan A, Dekker GA. Associations between Inherited Thrombophilias, Gestational Age, and Cerebral Palsy. *Am J Obstet Gynecol* 2005;In Press, Accepted 22.02.05.

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2. Gibson CS, MacLennan AH, Hague WM, Haan EA, **Priest K**, Chan A, Dekker GA. Associations between Inherited Thrombophilias, Gestational Age, and Cerebral Palsy. *Am J Obstet Gynecol* 2005;In Press, Accepted 22.02.05.

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- Mr Graeme Casey, Division of Molecular Pathology, IMVS.
- Mr Graeme Tucker, Head, Health Statistics Unit, Epidemiology Branch, Department of Health.

Thesis Explanation

The format of this thesis is as follows: abstract, literature review, nine distinct research chapters, and general discussion. During the course of my candidature, the literature review and two research chapters were published, with a third chapter submitted for publication; therefore each chapter is written as a publication, complete with abstract, introduction, materials and methods, results and discussion. Repetitions of the introduction and materials and methods occur only as necessary for the format of the papers.

Abbreviations

APH	Antepartum haemorrhage
APO	Adverse pregnancy outcomes
CI	Confidence Intervals
CMV	Cytomegalovirus
CP	Cerebral palsy
DBS	Dried blood spots
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EBV	Epstein-Barr virus
FVL	Factor V Leiden
HHV-6	Human herpesvirus 6
HHV-7	Human herpesvirus 7
HHV-8	Human herpesvirus 8
HSV-1	Herpes simplex virus 1
HSV-2	Herpes simplex virus 2
IUGR	Intrauterine Growth Restriction
MBL	Mannose Binding Lectin
MTHFR	Methylenetetrahydrofolate Reductase Gene
OR	Odds Ratio
PCR	Polymerase chain reaction
PGM	Prothrombin Gene Mutation
PIHD	Pregnancy-Induced Hypertensive Disorders
PTB	Preterm Birth
PVL	Periventricular Leukomalacia
RNA	Ribonucleic acid
RT-PCR	Reverse-transcription polymerase chain reaction
TNF	Tumour necrosis factor
VZV	Varicella zoster virus
µl	Microlitres
µM	Micromolar
WT	Wild-type

Abstract

Objective: The objective of this thesis was to investigate three potential antenatal risk factors – inherited thrombophilic polymorphisms, cytokine polymorphisms and exposure to viral infections – and their possible association with the development of cerebral palsy (CP) and other adverse pregnancy outcomes (APO), including intrauterine growth restriction, pregnancy-induced hypertensive disorders, antepartum haemorrhage and preterm birth.

Methods: Newborn screening cards from 1,326 babies (443 CP cases and 883 non-CP controls for the CP study; 717 APO cases and 609 non-APO controls for the APO study) were tested for inherited thrombophilic polymorphisms, cytokine polymorphisms and exposure to viral infections using polymerase chain reaction technology in the largest study of its kind worldwide. The four inherited thrombophilic polymorphisms tested were: Factor V Leiden (FVL G1691A), Prothrombin gene mutation (PGM G20210A) and Methylenetetrahydrofolate reductase gene (MTHFR) C677T and MTHFR A1298C. Five cytokine polymorphisms were genotyped: Tumour necrosis factor alpha -308 (TNF-α -308), Mannose binding lectin -221, (MBL -221) and three polymorphisms in Exon 1 of the MBL gene at codons 52, 54 and 57. The newborn screening cards were also tested for viral nucleic acids from enteroviruses and herpesviruses.

Results: Inherited thrombophilic polymorphisms may play a role in the development of CP and adverse pregnancy outcomes, as suggested by previous small studies. This thesis determined that the MTHFR C677T thrombophilic polymorphism approximately doubled the risk of CP in preterm infants, and a combination of homozygous MTHFR C677T and heterozygous PGM increased the risk of quadriplegic CP five-fold at all gestational ages. The results also suggested that some fetal thrombophilia, in particular PGM, may be related to such adverse pregnancy outcomes as intrauterine growth restriction.

The role of the TNF-α -308 polymorphism and four polymorphisms within the MBL gene had not previously been described for the subsequent development of CP. Carriage of polymorphisms in the TNF-α and MBL genes were associated significantly with an increased risk of CP. The TNF-α -308 polymorphism was also found to be associated with intrauterine growth restriction, pregnancy-induced hypertensive disorders, antepartum haemorrhage and preterm birth, and the MBL polymorphisms were associated with antepartum haemorrhage, pregnancy-induced hypertensive disorders and preterm birth.

Viral nucleic acid sequences were detected from newborn screening cards in 46.1% of cases, compared with 39.8% of controls (OR 1.30, 95% CI 1.00-1.67). This study was the first to demonstrate that evidence of direct infection with herpesviruses is associated with CP. In particular, detection of herpes group B viruses were associated with the development of CP (OR 1.68, 95% CI 1.09-2.59). These viral nucleic acid sequences were also found to be associated with adverse pregnancy outcomes, in particular preterm birth and pregnancy-induced hypertensive disorders.

Multivariable analysis demonstrated no significant interactions between the three main outcome measures listed above and the development of CP. Bivariable analyses showed increased risks of CP. The combination of Herpes group B viruses and carriage of any cytokine polymorphism was associated with an increased risk of CP (OR 2.47, 95% CI 1.43-4.27). This relationship was linear and showed no significant synergistic relationship between the two outcome measures in the causation of CP.

Conclusions: This research has shown that thrombophilic polymorphisms, cytokine polymorphisms and viral infections are all independently associated with the subsequent development of CP. These three factors do not interact to further increase the risk of CP, and this may reflect different pathological pathways to the brain white matter damage and periventricular leukomalacia that ultimately leads to CP. Together, their potential attributable risk is 15% of cerebral palsy cases, but further studies of new polymorphisms and infections are likely to increase this attributable risk. This data set has also shown that these same inherited thrombophilic and cytokine polymorphisms and viral infections are associated with adverse pregnancy outcomes such as intrauterine growth restriction, pregnancy-induced hypertensive disorders, antepartum haemorrhage and prematurity. These associations suggest interaction between genes and environmental risk factors.

Implications: Future research should investigate interactions between genes and the environment. Possible preventative strategies should be explored, such as vaccination programmes against the neurotropic viruses identified in this thesis as being associated with CP. This research also has medico-legal and political implications. The possible causal pathways for most CP outcomes currently cannot be influenced by obstetric practice. Their detection in retrospect may lead to prospective testing and research into the antenatal causes of cerebral palsy and its eventual prevention, saving hundreds of millions of dollars annually.

Literature Review

Introduction

Cerebral palsy is a very common disorder, with increasing evidence suggesting antenatal origins are a major cause. Of major concern is that the incidence of cerebral palsy has not decreased over the last forty years (1), and because of its human suffering and resultant economic and social effects, there is the need for research into its causes. Notwithstanding major improvements of medicine, intrauterine growth restriction (IUGR), pregnancy-induced hypertensive disorders (PIHD), preterm birth and antepartum haemorrhage (APH) continue to be important causes of adverse pregnancy outcome (APO).

Intrauterine infection, inflammation and thrombophilia have recently been suggested to be important risk factors for cerebral palsy, and therefore warrant further investigation. The maternal and neonatal clinical records of babies that develop cerebral palsy often contain direct or indirect evidence of infection and/or inflammation during pregnancy; thus testing of neonatal blood for markers of infection and inflammation as well as thrombophilia, may reveal further evidence of intrauterine infection and inflammation. However, infection may be subclinical and investigations may not have been prompted at or soon after birth. Unfortunately, it is difficult to retrospectively test for evidence of infection and inflammation unless bacteriological or histological examination of the placenta and fetal membranes has been performed soon after birth, and it has recently been suggested that only about 25% of placentas are histopathologically examined in the case of suspected neonatal infection (2). Evidence of bacterial infection has been strongly associated with the development of cerebral palsy (3-6). Viruses also cause neuronal damage, and a number of viruses (eg cytomegalovirus, herpes simplex virus, varicella zoster virus etc.) have been shown to be neurotropic during fetal development and infancy (7), thus their possible association with cerebral palsy also deserves investigation. During fetal and neonatal development, the brain is particularly vulnerable to neurotropic influences, and it is now possible to detect viral nucleic acids from a variety of specimens, using molecular techniques such as polymerase chain reaction (PCR). PCR techniques also allow the identification of polymorphisms for thrombophilia and cytokines from genomic DNA (8) using extremely small volumes of blood. Therefore, the technology now exists to retrospectively test neonatal blood samples, using archived newborn screening card (Guthrie card) specimens, for viral nucleic acids and genetic polymorphisms. The finding of viral nucleic acid sequences present in neonatal blood at the time of birth would suggest

prenatal infection had occurred and that the virus(es) represented by the specific nucleic acid sequences could play a role in the causation of brain damage, either directly or indirectly mediated through immune system-mediated mechanisms triggered by the viral infection. The finding of genetic polymorphisms which predispose individuals to various forms of thrombophilia or which alter a neonate's responsiveness to infection and inflammation would also suggest a possible predisposition to neuronal pathology, and that the damage was not the result of human intervention at the time of birth. These findings would also provide a basis for possible preventative policies, eg immunisation or treatment and ultimately the prevention of this debilitating disorder. In addition, there are many medico-legal issues surrounding cerebral palsy, and the traditional view that sub-optimal intrapartum care leading to intrapartum asphyxia causes most cases of cerebral palsy needs to be addressed, as there is no longer factual basis for this view in the large majority of cases (9). This review sets out to appraise the published literature and describe cerebral palsy, its risk factors and suspected causes. It will investigate the link between intrauterine infection, inflammation and thrombophilia as possible causes of cerebral palsy.

What is Cerebral Palsy?

Cerebral Palsy (CP) is a complex, multifactorial disorder, with many different definitions (10). The one adopted for this review defines cerebral palsy as "a chronic disability characterised by aberrant control of movement or posture appearing early in life and not the result of recognised progressive disease" (11). This definition was chosen because of its widespread use throughout the literature, and its high regard by prominent researchers. Cerebral palsy is characterised by non-progressive, abnormal control of movement or posture, and is not always diagnosed until months, or even years after birth (12). It is not associated with damage to the spinal cord, but with damage to the upper motor neurons of the brain (10, 13), resulting in excessive muscular tonus, spasticity with increased stretch reflexes, and hyperactive tendon reflexes, are often present in cases of cerebral palsy (10).

Prevalence of Cerebral Palsy

Despite the increase in obstetric care over the last forty years, the frequency of cerebral palsy has remained relatively constant (12, 14-19), or may have increased slightly as a result of the increased survival rate of infants of very low birth weight (20). Approximately 2-2.5 in every 1000 children born are diagnosed with cerebral palsy, making it the most common physical disability in childhood (12, 17, 18, 21). In contrast, there have been substantial decreases in both perinatal and maternal mortality (12, 18), suggesting that poor obstetric care is not a major cause of cerebral palsy, as had been

previously thought (12, 15, 22-25). The risk of cerebral palsy is highly associated with premature infants, occurring 20-30 times more often in infants weighing less than 1500 grams at birth (26). Very preterm infants (<32 weeks) represent 2% of all births; however they also represent 25% of all children with CP (27). Despite this, term or near-term infants have been shown to account for at least half of all diagnosed cases of cerebral palsy (1, 28, 29).

Risk Factors for Cerebral Palsy

There are many risk factors for the occurrence of cerebral palsy, including maternal age, maternal infection, multiple births, shorter gestational age, and low birth weight (10, 19, 22, 24). The traditional view that cerebral palsy was mostly caused by intrapartum asphyxia and subsequent brain damage as a result of poor obstetric care and management has been refuted in a number of epidemiological studies (19, 30-33). These studies indicate that the large majority of cases are not associated with intrapartum asphyxia, but instead by maternal and antenatal factors such as prematurity, intrauterine growth restriction, intrauterine infection, fetal coagulation disorders, multiple pregnancy, antepartum haemorrhage, breech presentation, and chromosomal or congenital abnormalities (14, 15, 17, 18, 22, 34-38). Some of these studies also show that intrapartum hypoxia could not be the cause in approximately 90% of cerebral palsy cases (15, 38). In the remaining 10% of cases, it was shown that intrapartum indicators consistent with damaging hypoxia may have had either antenatal or intrapartum origins, and that neither could be ruled out (12, 30). This is consistent with other findings that show many infants with cerebral palsy have little or no evidence of asphyxia at birth (39). As a result of these studies, less than 10% of all cerebral palsy cases are now considered to have their origins in birth asphyxia (15). Unfortunately, in cerebral palsy no definitive cause can be identified in more than 75% of cases (40), and data indicates that most children diagnosed with cerebral palsy did not experience birth asphyxia. It is also important to note that most asphyxiated babies do not develop the disorder (40).

Low birth weight and prematurity are well-recognised risk factors for the development of cerebral palsy, with it being the most common severe disability in low birth weight infants (41). It has been demonstrated that the rate of cerebral palsy in infants less than 33 weeks gestation is up to 30 times greater than for infants born at or near term (42). Multiple births are also a major risk factor, with twins at a higher risk of developing cerebral palsy than singletons (43). The risks of having a child with cerebral palsy have also been shown to be 0.2%, 1.3% and 7.6% for singleton, twin, and triplet pregnancies respectively.

White Matter Damage

The central nervous system (CNS) comprises of grey matter, containing cell bodies, and white matter, which contains millions of axons (44). White matter damage is mostly described as periventricular leukomalacia (PVL), and can be observed by cranial ultrasonography (45). PVL usually occurs between 28 and 34 weeks gestation, and is caused by ischaemic processes in the watershed zone that exists in the periventricular white matter of the immature brain (46-48). It refers to necrosis of white matter adjacent to the lateral ventricles that results in the formation of cysts and subsequent gliosis (49). White matter damage appears to involve both axons and oligodendrocytes, with deficits of oligodendroglia, loss of axonal fibres, microgliosis, and astrogliosis all playing a role in the pathogenesis of PVL (50).

Cystic PVL is most likely a heterogeneous white matter lesion, inducible by a number of different insults, including both asphyxia and inflammatory processes (51, 52), and its presence has been shown to be a better predictor of CP than any other ultrasound appearance of the brain (53). Of preterm infants with PVL, 60-100% go on to develop CP, and approximately 90% of preterm infants who develop CP also have magnetic resonance imaging (MRI) of gliosis as a predictor of perinatal PVL (45, 54-56). Another study demonstrated that 96% of infants with PVL displayed EEG abnormalities during the neonatal period, and spastic diplegia developed in 91% of infants surviving with PVL (57). Therefore, the presence of PVL is a strong indicator of subsequent development of cerebral palsy. Figure 1 demonstrates possible pathways for the development of PVL.

Many differences are being observed between term and preterm infants in the development of CP. Antenatal, rather than intrapartum factors, are now emerging as the major determinants of CP among children born at term (14, 22). PVL found in infants born at term may be indicative of lesions occurring early in the third trimester, but that the brain insult responsible for these lesions was not severe enough to result in preterm birth and hence the pregnancy progressed to term (58).

Infection, Inflammation, and PVL

Oligodendroglia appear to be the major target in PVL (59), and Figure 1 illustrates the potential roles of infection and cytokine activity in the destruction of oligodendroglia, which leads to periventricular leukomalacia. Neonates with sepsis born to mothers with documented infection are at increased risk for developing PVL (60, 61), and antenatal steroids reduce the severity and effects of systemic inflammatory responses associated with

PVL, providing further evidence for an inflammatory response and subsequent cytokine cascade being involved in the development of PVL. Antenatal steroid treatment has been associated with a 56% reduction in the risk of PVL with intraventricular haemorrhage and a 58% reduction in the risk of PVL alone (62), and has also been shown to decrease the incidence of white matter lesions in very low birth weight infants (63, 64)

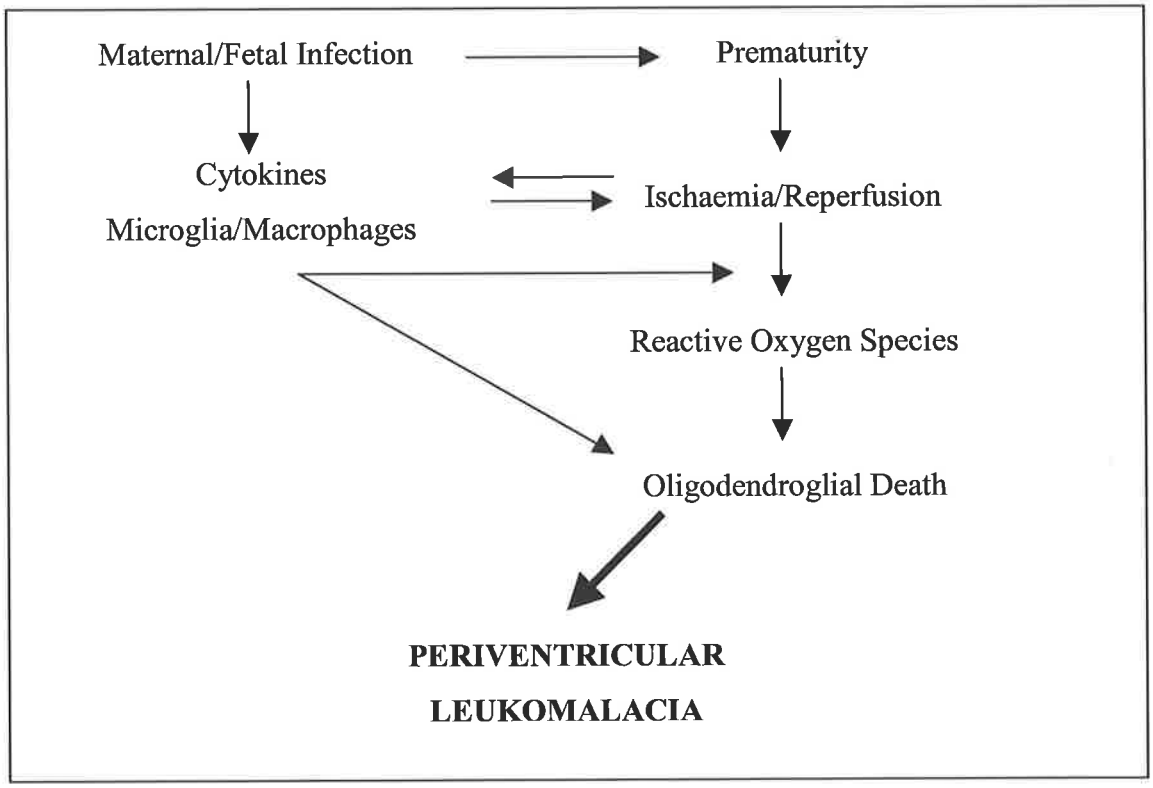

Figure 1: Possible pathways whereby infection, prematurity, and cytokine activity can combine to cause periventricular leukomalacia and subsequent cerebral palsy. Adapted from (59).

A number of studies have investigated the association between cytokine responses to infection and the development of periventricular leukomalacia and/or cerebral palsy (18, 25, 51, 65-83), as increased levels of cytokines in the amniotic fluid and fetal circulation appear to increase the risks for both neonatal brain injury and long term disability (78). A study by Urakubo and colleagues demonstrated that TNF-α levels were significantly increased in the placenta when pregnant rats were injected with lipopolysaccharide (LPS), suggesting that proinflammatory cytokine levels are increased in the fetal environment in response to maternal infection, which may significantly impact the developing brain (84). These studies suggest that cytokines are markers of infection and possible brain damage, and may even contribute to this brain damage in as yet unidentified pathways.

Inherited Thrombophilia

The coagulation cascade is the body's response to a breach in the vascular system, and is the body's most powerful haemostatic mechanism (85). Normally, the coagulation cascade is balanced, by both procoagulation and anticoagulation mechanisms. However, there are instances in which this balance is altered, to favour either procoagulation or anticoagulation. Thrombophilia favours procoagulation, and is an inherited or acquired condition, which predisposes individuals to thromboembolism, the obstruction of a blood vessel with thrombotic material carried by the blood stream from the site of origin. Common inherited thrombophilia include:

- heterozygosity or homozygosity for the factor V Leiden mutation causing activated protein C resistance (APCR).
- heterozygosity or homozygosity for the prothrombin gene mutation.
- homozygosity or compound heterozygosity for one or both of the common polymorphisms at positions 677 and 1298 in the gene for 5, 10-methylenetetrahydrofolate reductase (MTHFR), associated with hyperhomocysteinaemia.
- heterozygosity or homozygosity for the plasminogen activator inhibitor-1 (PAI-1) gene mutation.

There are also other, less common inherited thrombophilia, including deficiencies of proteins in the coagulation cascade, and also acquired thrombophilia, the most notable of which is the presence of lupus anticoagulant, and/or antibodies against cardiolipin. These inherited thrombophilic conditions predispose to the formation of thrombosis by impairing

the natural coagulation pathway, either by promoting excessive coagulation, or by impairing anticoagulation (86). In addition, pregnancy alone is an independent risk factor for thrombosis, as a result of major haemostatic changes involving decreasing anticoagulation and increasing coagulation such that with the progression of pregnancy the overall homeostatic balance of coagulation is altered towards hypercoagulability (87).

Endothelial cells line all blood vessels, and play an active role in the coagulation response. When injured, endothelial cells synthesise and release tissue factor, which goes on to initiate blood coagulation via the extrinsic pathway of the coagulation cascade. Endotoxin and TNF are among the substrates which can induce tissue factor activity (88-90). Chlamydia pneumoniae has also been postulated to induce infected monocytes to express tissue factor (91). TNF is also capable of attenuating the antithrombotic role of thrombomodulin, by promoting its endocytosis and degradation, and also inhibiting its transcription. In this way, coagulation is increased, whilst anticoagulation is decreased, leading to an overall shift towards a procoagulant state. Given that most thrombophilia require another risk factor to express the adverse phenotype, the presence of inflammatory cytokines (perhaps upregulated in response to infection) in conjunction with an inherited thrombophilia may provoke the development of thromboses.

Accumulating epidemiological evidence implicates thrombosis, as well as inflammation, as important factors in the development and causation of CP. Recently, inherited and acquired thrombophilic disorders have been associated with CP (92), and in particular, the Factor V Leiden mutation is now being implicated as a risk factor for the development of CP (18, 93-98).

Factor V Leiden Mutation

The factor V Leiden mutation is caused by a single base pair change (G→A) at nucleotide 1691 in the factor V gene, resulting in the arginine amino acid at position 506 being replaced by glutamine (99). Arg^{506} is one of three critical sites where protein C cleaves and so inactivates factor V and the incorporation of Gln^{506} alters the cleavage site such that factor V is now resistant to cleavage by protein C. The factor V Leiden mutation leads to excessive thrombin generation as factor V is now inactivated 10-20 times more slowly than the wild-type polymorphism (100). Inheritance of factor V Leiden (G1691A) is autosomal dominant, and compared to those with normal factor V, individuals homozygous for the factor V mutation have an 80 to 100 fold increased risk of developing thromboembolism

(101), and homozygosity for factor V Leiden has been associated with an increased risk of recurrent thromboembolism (102).

Prothrombin Gene Mutation

One of the more recently described heritable thrombophilic defects lies within the prothrombin gene. A G→A base pair substitution at nucleotide 20210 in the 3' untranslated region of the gene encoding prothrombin results in increased plasma prothrombin concentrations, and a 2.8 fold increase in the risk of thrombosis (87, 103).

Methylenetetrahydrofolate Reductase Gene Mutations

Homocysteine is a sulphur amino acid which undergoes metabolism via two different pathways – remethylation and transsulphuration. Normally, plasma levels are approximately 4-7μmol/L in women of childbearing age; in hyperhomocysteinaemia, plasma homocysteine concentrations are elevated, signalling a disturbance of homocysteine metabolism (104). Severe cases of hyperhomocysteinaemia often result from defects in the MTHFR gene (105).

Two mutations have been described in the MTHFR gene – C677T and A1298C. The C→T base pair substitution at position 677 is an autosomal recessive mutation, occurring in 6-12% of Caucasian populations in the homozygous form (106). This mutation results in a defective homocysteine metabolic pathway, leading to increased levels of homocysteine, and a state of hypercoagulability. It does this by altering the normal antithrombotic phenotype of the endothelium, enhancing the activities of factor XII and factor V, as well as depressing the activation of protein C (107, 108). Evidence suggests that the large amounts of homocysteine cause endothelial cell damage, which in turn causes recruitment and activation of platelets and leukocytes to repair the damage (109). A second mutation has also been described in the MTHFR gene, at position 1298 in the gene, where there is an A→C base pair substitution. This mutation also results in decreased enzyme activity, but not necessarily hyperhomocysteinaemia (110).

It has also been shown that co-inheritance of one or more of the inherited thrombophilia is associated with an increased risk of thromboembolism when compared with the risk of the inheritance of a single thrombophilia (111, 112).

Inherited Thrombophilia and Cerebral Palsy

The association between the tendency for thrombophilia and CP has been the focus of a number of recent studies (18, 92, 94, 113), with Factor V Leiden shown to be present in 26% of CP cases and only 1.5% of controls in the study by Nelson (18). In 1998, it was proposed that undiagnosed thrombophilia, both inherited and acquired, of the mother and/or fetus, may be responsible for thrombosis in the maternal and/or fetal circulation, subsequently resulting in adverse pregnancy outcomes, such as CP (114). There is evidence suggestive of a relationship between cerebral palsy and placental infarcts, often related to spiral artery thromboses (93, 114-116), with one study identifying thrombi in fetal vessels of the placenta in 11 of 15 infants with cerebral palsy (115). However, the literature is by no means conclusive. One study investigating the link between CP and thrombophilia suggested that, of patients with hemiplegic CP, thrombophilia in itself was not an important cause of CP (117). The authors of this study did, however, postulate that the pathogenesis of CP may be related to the interactions between thrombophilia and other risk factors for CP, including maternal and/or neonatal infections (118), and cytokines (71).

In both term and near-term infants, major causes of CP include intrauterine infection and fetal or neonatal stroke (24). A number of studies have investigated associations between CP and the factor V Leiden mutation, with the majority of cases classified as CP caused by fetal or neonatal stroke (18, 93, 94, 96, 119). It has been shown that 40% of CP can be clearly identified with vascular factors, such as infarction and haemorrhage (120), suggesting that thrombophilic polymorphisms, such as the factor V Leiden mutation, may play important aetiological roles in CP (92).

It has been proposed that one vascular mechanism of cerebral palsy causation is that dislodged thrombi from the placental circulation may travel to the fetus via the umbilical vein, ductus venosus, and inferior vena cava, before crossing the foramen ovale to reach the fetal cerebral circulation (114). Once here, there is the potential for widespread damage as the thrombus can lodge in blood vessels in the brain, effectively blocking the fetal circulation to the brain. This in turn can lead to white matter damage such as PVL, subsequently leading to the development of cerebral palsy. It is likely that this is only one of several complex interacting mechanisms that can cause neuronal damage. As will be discussed, cytokines may be increased by infection or infarction and these may have direct neurotropic actions.

Cytokine Involvement in Cerebral Palsy

Cytokines that are present in the amniotic fluid are risk markers of neonatal brain damage and subsequent long term disability (78). During the course of intrauterine infection, inflammatory cytokines play a pivotal role in the pathogenesis of brain white matter damage and the subsequent development of CP (71). An association has been demonstrated between cytokine concentrations from umbilical cord blood and the development of brain white matter lesions (71), and high expression of tumour necrosis factor alpha has been demonstrated in neonatal brains with PVL (121). However, it remains unclear whether the cytokines themselves mediate the damage, cause the damage, or whether the infection itself is responsible for the damage, and is probably a combination of all three. A cytokine hypothesis has been postulated, indicating that cytokines act as a final common pathway for injury to the central nervous system, and that they may be initiated by a number of different insults, including infection, hypoxic-ischaemic injury, reperfusion injury, and toxin-mediated injury (122, 123). Finally, the role of proinflammatory cytokines in preterm birth has been extensively investigated, demonstrating strong associations between preterm birth and the presence of proinflammatory cytokines (51, 77, 124-127). Preterm birth is a recognised risk factor for the development of cerebral palsy, and this is another mechanism by which proinflammatory cytokines may contribute to the development of cerebral palsy.

Tumour Necrosis Factor Alpha

Tumour necrosis factor alpha (TNF-α), a 17kDa protein made up of 157 amino acids, is a proinflammatory cytokine which is produced in response to infection (128). It mediates a variety of biologic processes, including growth, development, modulation, and immune responses of the brain (129), and is produced by a range of cells, including macrophages, haematogeneous and neural cells (130). Its main biological function, however, is its ability to recognise many pathogens and act quickly, promoting a broad range of immunological and inflammatory responses (128). Unfortunately, this ability to be rapidly produced may pose more of a risk than the infection through which it is elicited. If the production of TNF-α is excessive, and is released systemically in large quantities, fatal complications such as multiple organ failure may occur (128).

TNF-α is directly toxic to neurons and may cause white matter damage associated with PVL through its cytotoxic effect, damaging oligodendrocytes, cells responsible for deposition of myelin in white matter (72). TNF-α may also disturb developmental

transitions from the oligodendrocyte precursor to the mature oligodendrocyte (131). Cells positive for TNF-α have been identified in the white matter in 69% (9 of 13) of PVL cases when infant post-mortem tissues were stained with antibodies against TNF-α, suggestive of damage caused by cytokine production in the white matter regions of the brain (73). It has also been demonstrated that infants suffering PVL have increased TNF-α and IL-6 in brain tissue, as well as evidence of lymphocytes infiltrating the brain (72). As mentioned earlier, TNF-α exerts a powerful prothrombotic effect, one of the main mechanisms being the increased expression of tissue factor.

A number of studies have investigated the relationship between TNF-α and CP. One such study found that expression of TNF-α was more common with PVL (88% of cases) than without PVL (18% of cases) (72). It has also been shown that concentrations of TNF-α in amniotic fluid are higher in patients with intrauterine infection and subsequent preterm labour (68, 74). Proinflammatory cytokines in the brain are higher in infants who died with PVL than in infants with no evidence of white matter damage (71). Higher levels of proinflammatory cytokines were also measured in the amniotic fluid of preterm infants who later developed CP, and in term infants just days after birth, than those infants who did not develop CP (both preterm and term) (18, 132).

Fetal brain damage may also be initiated or mediated by abnormal cytokine production during infection in pregnancy (65). Elevated fetal plasma IL-6 has been identified as an independent risk factor for the occurrence of neonatal morbidity (51, 75, 133), further suggestive of the importance of cytokines in the aetiology of fetal brain damage and subsequent development of CP (51).

A number of animal studies demonstrate that inflammation and infection may directly damage the developing brain. Gilles and colleagues (1976) injected endotoxin into kittens, and observed the development of white matter lesions similar to the telencephalic leukoencephalopathy described in preterm infants (134). Another study showed that cerebral white matter lesions can be induced in fetal rabbits by experimental intrauterine infection with *E. coli* (72). Also, peritoneal administration of endotoxin to the immature rat results in rapid and prominent proinflammatory cytokine expression in the white and grey matter, and greatly sensitises the immature brain to injury. This suggests that the vulnerability of the immature brain following infection and hypoxia-ischaemia may involve the innate immune system (135). A dose-dependent cytokine increase in the fetal rat brain can also be observed after the administration of maternal endotoxin (136).

Another study has explained the association of inflammatory mediators and markers of autoimmune and coagulation disorders with CP (18). Using dried blood spots as their sample material, marked differences between CP and control cases in mean neonatal concentrations of cytokines were found, including many of the interleukin family, and TNF, as well as chemokines (18). This is also supportive of the importance of TNF-α in the development of CP. In direct contrast, however, a prospective clinical study found that TNF-α, although produced in relation to intrauterine infection, could not be directly associated with fetal cerebral white matter lesions (76). This study used amniocentesis to collect amniotic fluid for testing before birth, which could in part explain why no direct association could be identified. It is not known at what time the cytokine levels are at their highest, and this study could have missed the window of opportunity for maximal detection of TNF-α. Also, too much of a focus on damaging overproduction of cytokines could overlook the possibility that defective cytokine production, either because of immaturity or genetic basis, could leave the infant susceptible to infection and/or white matter damage.

Tumour Necrosis Factor Alpha Polymorphism

The gene encoding TNF is located on the short arm of chromosome 6, within the Major Histocompatibility Complex (MHC) (137). There are a number of different polymorphisms associated with the TNF-α gene, with the TNF-2 polymorphism being associated with high levels of TNF-α (138, 139). The TNF-2 polymorphism is a rare allele in the promoter region of the gene, and is represented as a single nucleotide G→A base pair substitution at nucleotide –308 relative to the transcriptional start site (128). It remains unclear whether this polymorphism in the promoter region of the TNF gene is able to upregulate TNF transcription (128), although it has been shown that this polymorphism alters the binding of nuclear factors to the promoter region of the TNF gene. The differential binding was found to be functional, with two-fold greater activity of the promoter of the TNF-2 allele compared to the TNF-1 allele (140). It has also been shown that human B cells transfected with the polymorphic promoter coupled with a reporter gene showed a 5-fold increase in reporter expression compared with the wild type promoter (141). Finally, a study by Westendorp and colleagues found that approximately 60% of the variation in the production of TNF is genetically determined (142), suggestive of the importance of polymorphisms in this gene. This polymorphism has been linked to the development of cerebral malaria, and it has been suggested that regulatory polymorphisms of cytokines such as TNF may be able to influence the outcomes of severe infection (143).

Studies investigating the effects of this particular polymorphism have found it was more common in pregnant women who delivered preterm babies after premature rupture of membranes (141), and premature rupture of membranes has been identified as a risk factor for CP (1). However, the sample population in this study were African American women, and the possibility exists that the distribution of this polymorphisms reflects a potential bias. Therefore further research is warranted in this area of genetic polymorphisms, to determine whether the association between the polymorphism and preterm rupture of membranes occurs in other racial groups, and whether this leads to a higher incidence of CP. It is also important to realise that there are other known reasons for premature rupture of membranes, including vaginal colonisation with certain bacteria (3).

In summary, the evidence suggests that increased levels of TNF-α can be detrimental to the fetus, and directly or indirectly cause brain damage. It also suggests that the -308 promoter polymorphism in the TNF-α gene results in higher circulating levels of TNF-α. Therefore, the combination of increased levels of TNF-α brought about by the promoter polymorphism at -308 and the normal physiological upregulation of TNF-α production as a result of infection may contribute to the pathogenesis of white matter damage, through its cytotoxic effect on brain oligodendrocytes and also its ability to increase permeability of the blood-brain barrier, allowing the passage of potentially toxic mediators into the incredibly sensitive developing brain.

Mannose Binding Lectin

The human immune system has two main types of immune response – the innate immune response and the adaptive immune response. The complement system is part of the innate immune system, and can be activated in three different ways: the classical pathway, the alternative pathway, and the mannose-binding lectin (MBL) pathway. The classical pathway is activated when specific antibodies bind to non-self determinants, whilst the alternative pathway is controlled by the action of inhibitory proteins present on membranes and in plasma (144). The MBL pathway of the complement system, however, is activated when MBL recognises and binds to carbohydrates, such as mannose- and N-acetyl-glucosamine-rich oligosaccharides, present on a wide range of bacteria, viruses, fungi and parasites (144, 145).

MBL is a serum protein of hepatic origin, and belongs to a family of calcium dependent collagenous lectins (146). The human MBL gene is found on chromosome 10, and has six known polymorphic sites (147), three of which are located within the promoter region of

exon 1. The polymorphism at -550 is a G to C base pair substitution (alleles H and L respectively). The polymorphism at -221 is also a G to C base pair substitution, giving rise to alleles X and Y respectively (148). Both of these mutations manifest as reduced levels of circulating MBL, by affecting the transcriptional activity of the basal-promoter complex (149). Finally, the polymorphism at +4 is a C to T base pair substitution, giving rise to alleles P and Q respectively (150).

The remaining three polymorphic sites are found in exon 1 of the MBL gene. The polymorphism at codon 52 is an Arginine to Cysteine amino acid substitution (CGT to TGT), whilst the polymorphism at codon 54 is a Glycine to Aspartic Acid amino acid substitution (GGC to GAC). Finally, the polymorphism at codon 57 is a Glycine to Glutamic Acid amino acid substitution (GGA to GAA) (151, 152). The amino acid substitutions at codons 54 and 57 result in disruption of the Gly-Xaa-Yaa structure of the collagenous backbone of MBL. This results in limited binding between the MBL subunits, causing significantly reduced levels of functional protein circulating in the serum (151). In contrast, the amino acid substitution at codon 52 does not interrupt the Gly-Xaa-Yaa sequence, but instead results in the formation of an extra disulphide bond, thus decreasing the stability of the molecule (152, 153).

These promoter and structural polymorphisms are found in various *cis* combinations, resulting in "haplotypes" associated with high, intermediate, and low levels of MBL (149). Currently, seven different haplotypes have been identified in a range of populations, and are HYPA, LYPA, LYQA, LXPA, LYPB, LYQC, and HYPD (149, 154). Additionally, another haplotype, HXPA, has been reported in three patients with systemic lupus erythematosus (155).

Mannose Binding Lectin Polymorphisms and Susceptibility to Infection

The first report of associations between recurrent infections and low levels of MBL was in 1989 (156). Since then, there have been many papers published investigating the associations between MBL efficiency and increased susceptibility of infection (144). It is important to note that 90% of MBL deficient individuals do not acquire repeated infections, possibly due to the redundancy of the complement system (144). It can therefore be postulated that the phenotypical manifestation of MBL deficiency is only apparent when combined with another immunodeficiency, either acquired or genetically determined. It has been shown that MBL deficient children presenting with recurrent infection also had concomitant or transient IgG subclass deficiency (157). MBL has also

been shown to bind to the human immunodeficiency virus (HIV), and prevent infection of cells (158). Therefore, low levels of MBL could have a deleterious effect on the fetus when exposed to infection, as the MBL pathway plays an important role in eliminating pathogens, especially in neonates and infants (159).

Low levels of maternal MBL have been associated with unexplained recurrent miscarriage (160, 161). and this is possibly associated with impaired resistance to infection at the feto-maternal interface. In one study, low MBL concentrations were observed in 16% of female partners and 14% of male partners experiencing recurrent miscarriage, in comparison with less than 5% of controls ($p<0.005$). Upon sub-group analysis for individuals presumed to be homozygous for MBL mutant alleles, this relationship was even stronger and more significant (9.5% compared to 1%, $p<0.002$). Based on these results, it was hypothesised that low concentrations of MBL within the feto-placental unit could increase susceptibility to fetal loss (160). In addition, relative MBL deficiency was not apparent in women whose recurrent miscarriages could be explained (162). This is consistent with the concept that pregnancy loss is predisposed to by a relative deficiency of innate immune factors, perhaps secondary to infection. A fetus deficient in MBL may be more susceptible to sub-clinical infections and/or inflammatory events *in utero* (163). Despite these findings, a study by Baxter et al., (2001) (164) found no association between recurrent miscarriage and variant alleles of the MBL, TNF, or LTA genes. These researchers were unable to explain the discrepancy between the earlier reports and their findings.

It has also been shown that mutations in the MBL gene are strongly associated with children presenting to hospital with infection, and that these mutations increase susceptibility to infection in children who are heterozygous or homozygous for the mutations (165). There is a highly significant association of mutant MBL genotypes with infection. In one particular study, the mutations were present in approximately twice as many children with infection as in control children. This is similar to an earlier study of children with an opsonic defect (166); however it contrasts another study which found no association between increased risk of infection and children heterozygous for mutant MBL gene alleles (167). This discrepancy may, however, be explained by the use of an adult control group rather than concurrent controls matched for age (167). This increased risk of infection may be of greatest importance when immune responses are either immature (such as in the neonate) or defective. Recent reports have described associations between low producing MBL alleles and disease severity in diseases where immunity is already

significantly impaired (168, 169). It has been suggested that low producing alleles of MBL will also influence the clinical phenotype of other immunodeficiency diseases (147). Therefore, the decreased levels of MBL as a result of polymorphisms in the gene may contribute to the pathogenesis of CP via a decreased response to infection, increasing susceptibility to infection and therefore the likelihood of adverse effects from infection.

Intrauterine Infection

Intrauterine infection is postulated to be an increasingly important contributor to the development of cerebral palsy, and as such is currently the focus of intensive research. To date, many studies have investigated the role of infection in the development of CP by using surrogate markers of infection, such as chorioamnionitis, and maternal pyrexia. Chorioamnionitis can be described as histopathological evidence of infection, characterised by an inflammatory leucocyte infiltration of the chorion and amnion, whilst pyrexia (fever) is a term given to body temperature greater than 37°C. Funisitis can be defined as inflammation of the umbilical cord detected by histologic examination of the placenta (133). Chorioamnionitis can make an infant of very low birth weight more vulnerable to neurologic damage (170), and increase the risk of cerebral palsy (29, 41, 83, 171-173). Also, a combination of maternal chorioamnionitis and neonatal seizures identifies infants of very low birth weight who are at increased risk of cerebral palsy (171, 174). Further evidence for the role of intrauterine infection in brain damage and cerebral palsy comes from an experiment which induced intrauterine infection in rabbits by injecting doses of *Escherichia coli* bacteria. This experiment showed that bacterial infection can lead to fetal white matter damage in the brain (175), providing more evidence for the role of intrauterine infection as a cause of CP. Another study has shown that the underlying process involved in the white matter damage is programmed cell death, or apoptosis (176). Debillon and colleagues (176) performed similar experiments to that of Yoon (175) by inoculating pregnant rabbits with *Escherichia coli*, and showed a causal association between intrauterine infection and programmed cell death in the periventricular white matter.

A recent meta-analysis investigated the potential association between chorioamnionitis and cerebral palsy in both full term and preterm infants (55). This meta-analysis has since been revisited and now includes studies published in 2000 (177). Clinical chorioamnionitis was found to be significantly associated with cerebral palsy (relative risk 1.9; 95% CI, 1.5-2.5), and also cystic periventricular leukomalacia (relative risk 2.6; 95% CI, 1.7-3.9),

demonstrating that chorioamnionitis is a risk factor for both cerebral palsy and cystic periventricular leukomalacia (177). Intrauterine exposure to maternal infection has also been associated with an increased risk of long term neurologic morbidity (22), although another study has suggested that abnormal labour, and not maternal infection, is causally related to neurologic abnormalities (178). A study by Yanowitz and colleagues in 2002 (179) found that chorioamnionitis was associated with increased cytokine concentrations in cord blood, and that infants with fetal vessel inflammation had higher levels of proinflammatory cytokines. This leads to speculation that in premature infants born after chorioamnionitis, the risk of brain injury is increased when cerebrovascular inflammation and systemic vasculitis are both present (179). It seems, therefore, that maternal infection is associated with white matter damage, periventricular leukomalacia (PVL), and long term neurological dysfunction or cerebral palsy. What is not yet clear, however, is how this pathway occurs, and if the brain damage is secondary to the proinflammatory cytokine response to the initial infection (25), although more recent evidence suggests that damage to the developing brain results from the host response to infection (180).

Infection remote from the brain causes the release of products of infection into the circulation. These products can cross the blood-brain-barrier (BBB), either as a result of immaturity of the BBB (181), or due to release of proinflammatory cytokines, which impair its integrity (70, 182, 183). Having penetrated the BBB, these products of infection, which include proinflammatory cytokines such as TNF-α, can cause damage to developing white matter, in the form of PVL. This white matter damage may result from a number of different mechanisms, including direct tissue damage, stimulation of fetal microglia to produce more TNF-α (125), and disruption of the endothelium and/or ependyma (70).

Fetal Response to Infection

The fetal inflammatory response is described as being a multisystem disorder that may result in preterm delivery and adverse neonatal outcome (184). One histopathologic representation of the fetal inflammatory response syndrome is funisitis, which has been associated with increased risks of both complications resulting from neonatal infection and the development of cerebral palsy (133, 185). It is now apparent that the fetal inflammatory response to intra-amniotic infection is biologically important, even more so than the maternal inflammatory response (186). A multicentre cohort of 1078 infants of birth weight <1500g showed that, in preterm births, fetal inflammatory responses contribute to cerebral white matter damage, and that maternal infection can damage the

fetal brain without the presence of fetal brain infection (186). Evidence is also increasing for the role of the fetus in chorioamnionitis. In the past, chorioamnionitis has been assumed to be a maternal infection; (187), however, it is now becoming apparent that involves primarily a fetal inflammatory response, with much data now supporting this view (26). It has been demonstrated that the onset of spontaneous preterm labour with preterm premature rupture of membranes, is preceded by a systemic proinflammatory cytokine response in the fetus, which is probably the fetal response to the presence of microbial products (188). There is also evidence suggesting that antenatal infection and brain white matter damage are linked by the fetal inflammatory response (78, 79). Therefore, where the role of intrauterine infection and its sequelae were once considered to be of maternal origin, the evidence is now suggestive of the role of the fetus in intrauterine infection. Finally, a recent study has demonstrated that umbilical vein plasma concentrations of interleukin-6 (IL-6), a cytokine produced by a wide range of cells and implicated in the regulation of the host response to infection, are increased in neonates born to mothers with clinical chorioamnionitis (189). Because plasma IL-6 concentrations have been used to define the fetal inflammatory response system, the results of this study suggest that the inflammatory process causing clinical chorioamnionitis is a fetal response.

Matrix metalloproteinases are a family of enzymes whose role is to hydrolyse specific components of the extracellular matrix (190). The chorioamnion cells of the fetal membranes synthesise some of these enzymes, and it is thought that rupture of these membranes is due to degradation by these enzymes (191), which are upregulated by proinflammatory cytokines. Using rhesus monkeys with bacterial and cytokine induced preterm labour and also spontaneous term labour, it was demonstrated that there were accompanying progressive increases in amniotic fluid matrix metalloproteinases, suggesting matrix metalloproteinases as markers of preterm labour in the presence of inflammation and/or infection (190). Matrix metalloproteinase-8 levels above the 90^{th} percentile have been identified as a marker for subsequent preterm premature rupture of membranes (192). An association between matrix metalloproteinase-9 and intra-amniotic infection has also been described, with increased sensitivity, specificity, and positive and negative predictive values in diagnosing intra-amniotic fluid when compared with interleukin-6 (193). This provides supportive evidence for the role of infection in preterm birth, which is also an independent risk factor for the development of cerebral palsy.

Viral Infection

During pregnancy, maternal resistance to some viral infections is decreased, as a result of the shift towards Th2 dominance associated with some suppression of Th1 cell-mediated immunity, and as such, the fetus may be at risk of transplacental virus transmission (194). There are many viruses capable of causing damage to the brain, in particular the developing brain, and these are collectively referred to as neurotropic viruses (7). Many of these neurotropic viruses are capable of causing brain damage in the human fetus, and are therefore classified as teratogens. Rubellavirus, cytomegalovirus, and varicella zoster virus are now accepted teratogens, whilst enteroviruses, adenoviruses, rotaviruses, and other members of the herpesvirus group are among a list of viruses suspected to be teratogenic (195). Many of these neurotropic viruses are included in a list of infections known as TORCH infections. These have the capability of causing childhood morbidity and mortality, and include infections caused by ***T***oxoplasma (a coccidian parasite), ***O***ther organisms (parvovirus, human immunodeficiency virus, Epstein-Barr virus, herpesviruses 6 & 8, varicella-zoster virus, Treponema pallidum (the bacterium of syphilis), enteroviruses, Listeria monocytogenes), ***R***ubella, ***C***ytomegalovirus, and ***H***erpes simplex viruses.

Transplacental Transmission of Viruses

For a pathogen to cause fetal damage *in utero*, it must first cross the placenta, which can be initiated during the viraemic, bacteraemic, or parasitaemic phase of maternal infection (196). The placenta acts as a potential barrier to maternal/fetal cross infection of viral infections. However, this barrier may be less effective during its development in early pregnancy, and potentially when the placenta is damaged by vascular disease, eg infarction. Placental dysfunction has been associated with thrombophilia, systemic lupus erythematosus (SLE), diabetes, chronic hypoxia, and severe preeclampsia. Disruption of the placental barrier may increase the vulnerability of the fetus to maternal infection. In addition, gestational age at the time of maternal infection plays an important role in the development of fetal infection. Although fetal transmission is much higher in infections contracted in the third trimester, exposure to fetal infection in the first or early second trimester is more likely to cause severe fetal damage (196). This gestational age effect may explain in part why not all patients with potentially damaging infections go on to develop severe neurological sequelae such as periventricular leukomalacia and cerebral

palsy as the brain may be more vulnerable during specific periods of fetal or neonatal brain development.

There are four potential histological outcomes following maternal infection via the bloodstream:

1. Absence of both fetal and placental infection
2. Fetal infection with no placental infection
3. Infection of both the placenta and fetus
4. Placental infection with no fetal infection

It is important to note that adverse consequences can result even in the absence of fetal and placental infection. In this case, it is believed that effects of maternal infection, such as fever or circulating toxins, such as cytokines, may cause indirect damage to the fetus (197).

Cytomegalovirus, herpes simplex viruses, varicella zoster virus, adenovirus, and enterovirus are all capable of crossing the placenta and setting up *in utero* infection (197-205), and as such are potential infectious agents responsible for the causation of cerebral palsy. The likelihood of maternal infection resulting in infection of the fetus varies according to the specific virus, whether the infection is primary or recurrent, and the gestational age of the fetus at the time of infection. For example, the overall rate of congenital cytomegalovirus infection is 1%, with only a small minority of infected mothers transmitting the virus *in* utero. Clinically apparent infection is even rarer, with only around 15% of infected newborns displaying signs of the infection (197). Once the infection has crossed the placenta into the fetal circulation, there is the potential for damage, both by the infectious agent directly, and also by the fetal inflammatory response to the infection.

As well as being capable of transplacental transmission and subsequent *in utero* infection, some viruses can persist for months or years after the initial infection (206-213). These viruses may have effects as long as thirty years after the original infection (212). In addition, it has been shown that adenovirus can induce TNF-α, a powerful proinflammatory cytokine capable of causing widespread damage (209).

Most infectious agents reach the CNS by hematogenous spread from extracranial foci, including infected circulating macrophages (monocytes) and other leucocytes, or by retrograde propagation of infected thrombi within emissary veins. Blood borne bacteria and viruses could then gain access to the CNS via vessels within the choroid plexuses,

meninges, and brain parenchyma (214). Therefore, it may be that babies with inherited thrombophilia are more susceptible to brain damage caused by infection, as a result of the infected thrombi providing entry into both the placenta and brain.

Obstetrical Syndromes and Adverse Pregnancy Outcome

Research over the past decades has shown that inflammation, thrombosis and infection are major players in the pathogenesis of the three great obstetrical syndromes, namely preeclampsia, preterm birth and intrauterine growth restriction (IUGR). Infectious and inflammatory processes have been investigated for these disorders, including inherited thrombophilia (215-222), cytokine polymorphisms (188, 223-232) and infections (126, 233-240). It should be noted that these studies are varied in their sample sizes and selections, ethnic groups, outcomes of interest, and have many methodological differences, making comparisons between these studies of adverse pregnancy outcomes difficult.

These three diseases are the major causes of adverse pregnancy outcome, and considering the overlap in underlying pathophysiological mechanisms, it comes as no surprise that these disorders, in particular preterm birth and IUGR are also known as major risk factors for developing CP.

Conclusions

In summary, there are many tests that can be performed to predict the risk of developing CP and other adverse pregnancy outcomes, including abnormalities in coagulation factors, chemokines, inflammatory factors, and autoimmunity (18). It has been suggested that most thrombophilia need another risk factor in order to express the adverse phenotype (causation of vascular thrombosis). It is likely that, in the fetus, viral or bacterial infection, inflammation and inflammatory cytokines may interact with the thrombophilic tendency to result in fetoplacental thrombosis and subsequent CP or other adverse pregnancy outcomes. Because of the overlap in underlying pathophysiological mechanisms and the epidemiological association between CP and the three major obstetrical disorders, the aim of this research program was to study common polymorphisms modulating the clotting and inflammation cascade and viral infections in the causation of CP, preeclampsia, IUGR and preterm birth. The formal hypothesis and aims of this study are delineated overleaf.

Hypothesis and Aims

The specific hypothesis of this research is that fetal thrombophilic polymorphisms, specific mediators for inflammation and infection, and evidence of fetal viral infection may all be associated with the future development of cerebral palsy. These factors may also be involved in the development of other adverse pregnancy outcomes, including IUGR, prematurity, APH and PIHD.

The specific aims of this study are to:

- Estimate the prevalence of four inherited thrombophilic polymorphisms in the Australian Caucasian population.
- Investigate any associations between inherited thrombophilia and cerebral palsy.
- Estimate the prevalence of five inherited cytokine polymorphisms in the Australian Caucasian population.
- Investigate any associations between inherited cytokine polymorphisms and cerebral palsy.
- Estimate the prevalence of specific viral infections in the Australian Caucasian population.
- Investigate any associations between specific viral infections and cerebral palsy.
- Investigate any associations between inherited thrombophilia and adverse pregnancy outcomes.
- Investigate any associations between inherited cytokine polymorphisms and adverse pregnancy outcomes.
- Investigate any associations between specific viral infections and adverse pregnancy outcomes.
- Investigate any interaction or synergism between the factors that affect the risk of a cerebral palsy outcome.

Patient Selection and Demographic Data

Study Population – Cerebral Palsy

The study population used for this research comprised all children with confirmed CP born in 1986-1999 in South Australia to Caucasian mothers (n=443), ascertained by the South Australian Cerebral Palsy Register from notifications by paediatricians, hospitals and child treatment centres. 883 babies born to Caucasian mothers between the years 1986-1999 were selected as controls for the CP cases. Newborn screening cards were identified by the South Australian Newborn Screening Program for each case. Potential controls were selected as the four babies whose screening cards were filed (by date of receipt) as closely as possible before (n=2) and after (n=2) the cards of cases. The dates of birth of the controls were within a few days of the case, the hospital from which the screening cards were taken was of the same category (metropolitan teaching, metropolitan private or country), and samples were taken on approximately the same day of life as the cases, to achieve similarity in condition of the cards. The control population included a higher proportion of preterm infants than the general population, as many of the cases of CP were born preterm and had been referred to metropolitan teaching hospitals. Linkage was attempted for all cases and potential controls to the South Australian Perinatal Data Collection of births, with its large number of sociodemographic and clinical variables, using Automatch Probabilistic Record Linkage Version 4.3 computer software (241). This was successful for all cases and 1691 controls. 268 (15.8%) of these 1691 controls were excluded as they were children of non-Caucasian mothers (n=102); children who had a birth defect as identified from the South Australian Birth Defects Register (n=161) or children who died in the first year of life (n=37) to ensure that they were not potentially cases of CP. Some controls were excluded for more than one reason. Two controls were then selected from the remaining controls in each group of four, using random numbers, to form the control population of 886. As three controls had inadvertently been selected more than once, the final number of controls was 883. The data from all cases and controls were de-identified before testing for polymorphisms and viral nucleic acids and statistical analysis. All testing was undertaken with blinding to case/control status. This research was approved by the Research Ethics Committee of the Women's and Children's Hospital (Approval number 1323/5/2005), and followed National Health and Medical Research Council guidelines. The ethics committee deemed that cases and controls must be

deidentified, and limited clinical data to that contained in the South Australian Cerebral Palsy Register and the South Australian Supplementary Birth Record.

Clinical Characteristics

The most common form of cerebral palsy for children in the case population was diplegia (31.2%), followed by hemiplegia (29.3%), quadriplegia (26.9%). The remaining 12.6% were classified otherwise (Figure 1).

Figure 2 illustrates the percentages of cases and controls by gestational age at birth. Although the risk of developing CP increases with decreasing gestation, the majority of CP babies are born at term. The control babies followed an expected pattern of gestational age, with the majority born at term. 54.9% of cases (243/443) were born at term (≥37 weeks), 14.2% (63/443) were born preterm (32-36 weeks), and 30.9% (137/443) were born very preterm (<32 weeks).

Figure 3 illustrates the birth weights of cases and controls. While the controls demonstrate the typical bell-curve of birth weights, 28% of the cases had birth weights less than 1500 grams at birth. 20.5% of the cases (91/443)were classified as being growth-restricted <10^{th} percentile for gestational age.

Demographic and clinical characteristics were compared by bivariable analysis for cerebral palsy cases and controls (Table 1). Gestation and birth weight were significantly lower for cases, as were 1 and 5 minute Apgar scores (Figure 4). 78.6% and 95.3% of CP cases had Apgar scores of 4 or greater at 1 and 5 minutes respectively, and 47.9% and 84% of CP cases had Apgar scores of 7 or greater at 1 and 5 minutes respectively. However, significantly more cerebral palsy cases had Apgar scores of less than 4 at 1 minute (OR 6.85, 95% CI 4.54-10.33) and 5 minutes (OR 14.70, 95% CI 4.36-49.58), compared with controls (Figure 5). Obstetric complications (including but not limited to threatened miscarriage, antepartum haemorrhage, pregnancy hypertension, suspected intrauterine growth restriction and gestational diabetes) were more prevalent among cases (60.9%) than controls (45.2%). Specifically, IUGR <5^{th} percentile was observed in significantly more CP cases than controls (12.9% vs. 8.9%). IUGR <10^{th} percentile was observed in 20.5% of CP cases compared with 17.0% of controls, a non-significant difference. Any recorded antepartum haemorrhage (defined as any bleeding after 20 weeks gestation, including abruption, placenta praevia, other and unknown causes) was also observed in significantly more CP cases than controls (CP 29.8% vs. controls 23.6%). The presence of any complications of labour, delivery and puerperium (including but not limited to postpartum

haemorrhage, fetal distress, retained placenta, prolonged labour >18 hours and cord prolapse) was significantly increased for cases, as was the need for resuscitation (not including aspiration). Cases were more likely to have a method of delivery other than normal spontaneous vaginal, and had more emergency caesarean section deliveries than controls. The presence of any medical conditions (including but not limited to anaemia, urinary tract infections, preexisting hypertension, preexisting diabetes, epilepsy and asthma) was not significantly different between cases and controls.

Despite associations between low Apgar scores and requirements for resuscitation for cerebral palsy, the percentage with very low Apgar scores (<4) at 5 minutes was 4.8%. This is one of several criteria required to indicate acute hypoxia at birth and its absence in over 95% or CP cases is in keeping with the recent literature. This study did not have access to cord or neonatal blood gases.

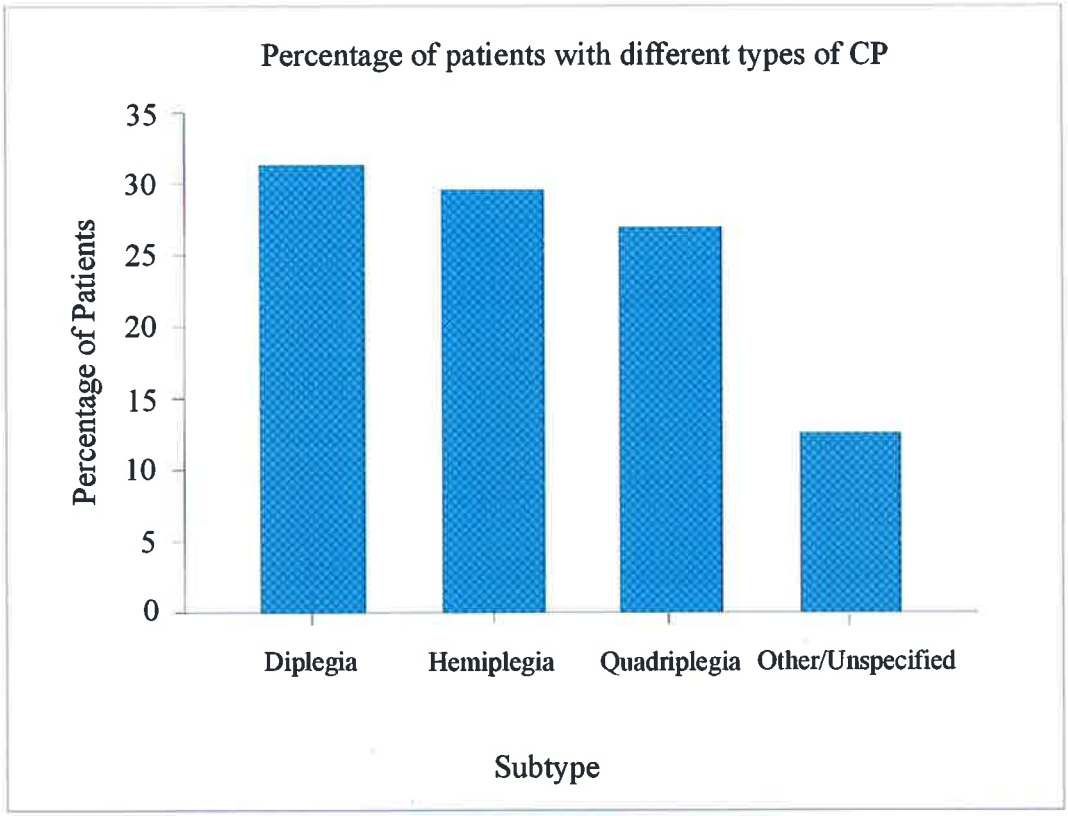

Figure 1: Cerebral palsy subtypes illustrated as a percentage of the total cerebral palsy cohort

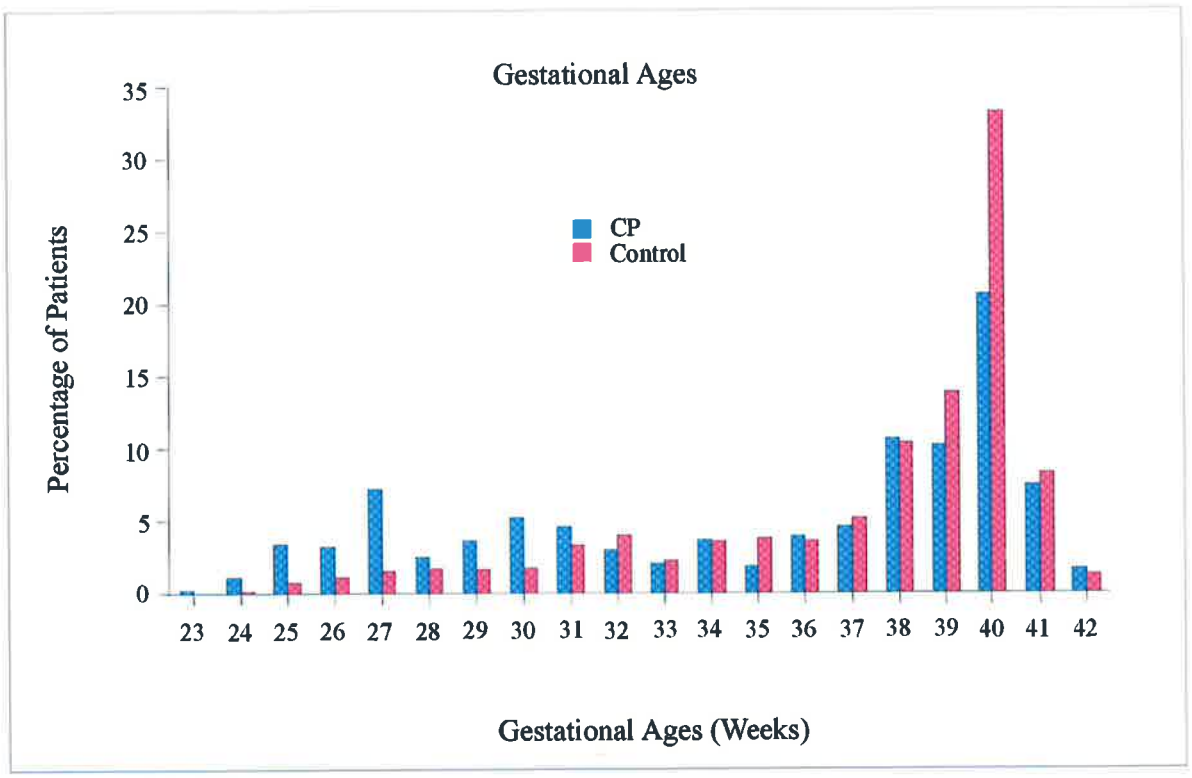

Figure 2: Percentage of cerebral palsy cases and controls by gestational age (weeks) at birth

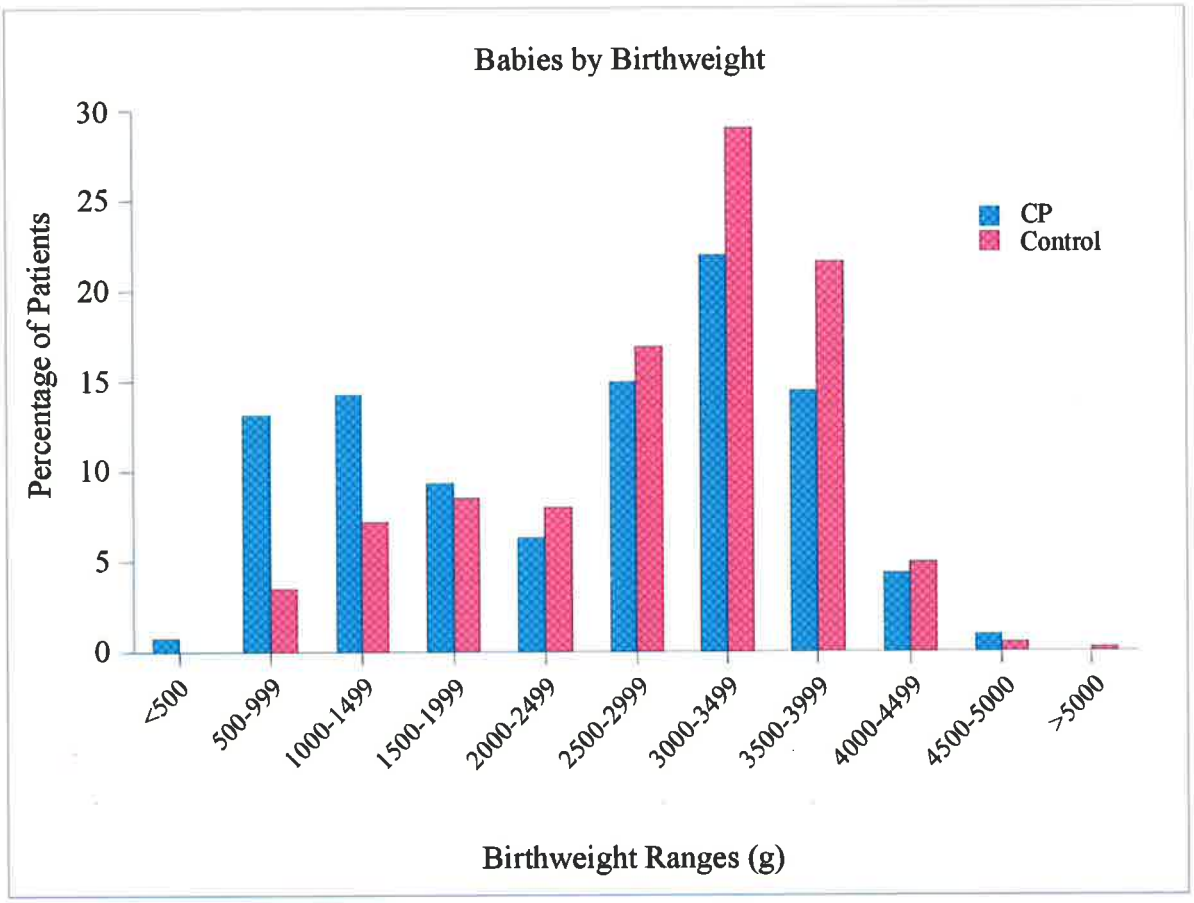

Figure 3: Percentage of cerebral palsy cases and controls by birthweight

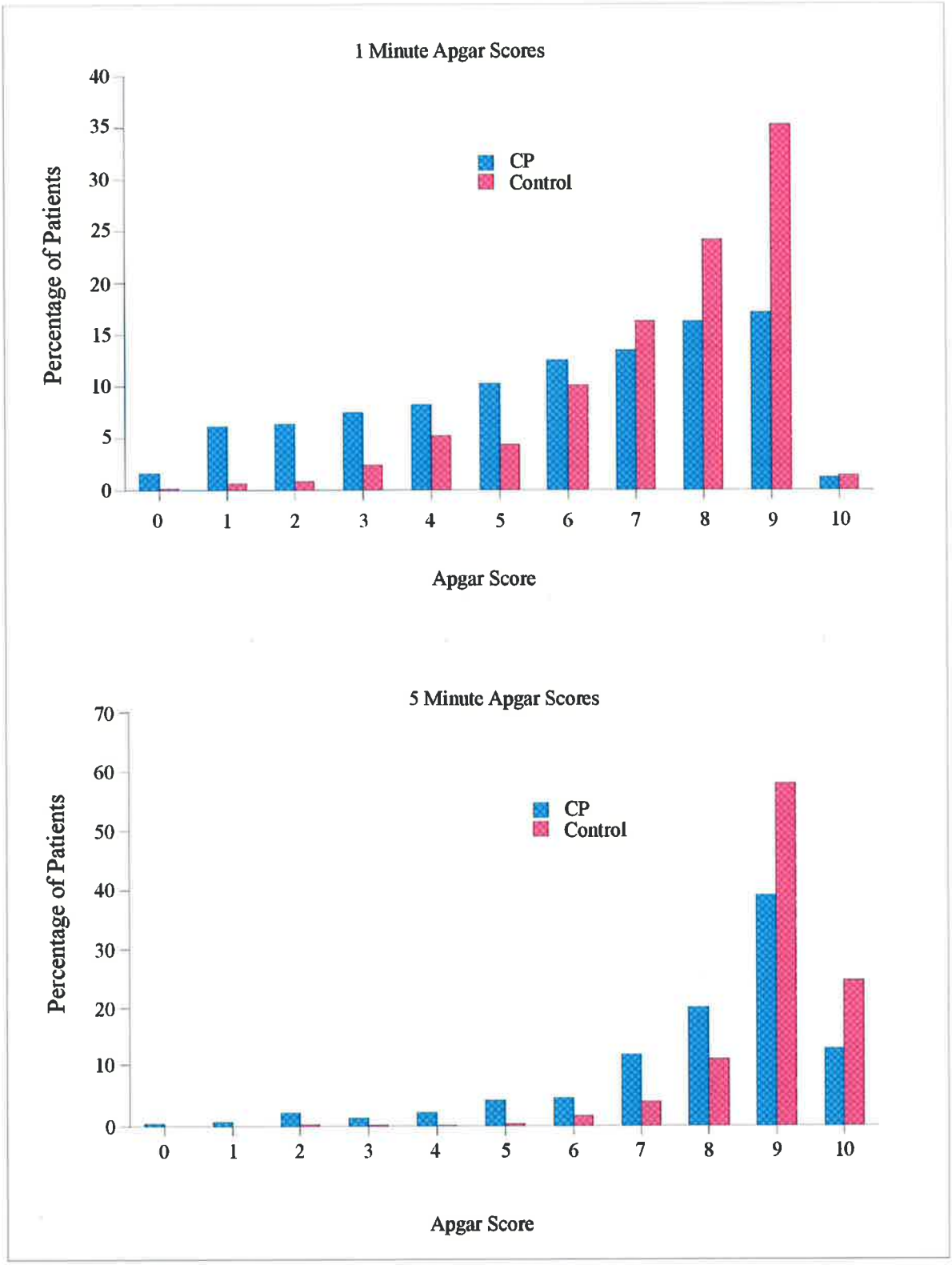

Figure 4: 1 and 5 minute Apgar Scores, expressed as a percentage of the total

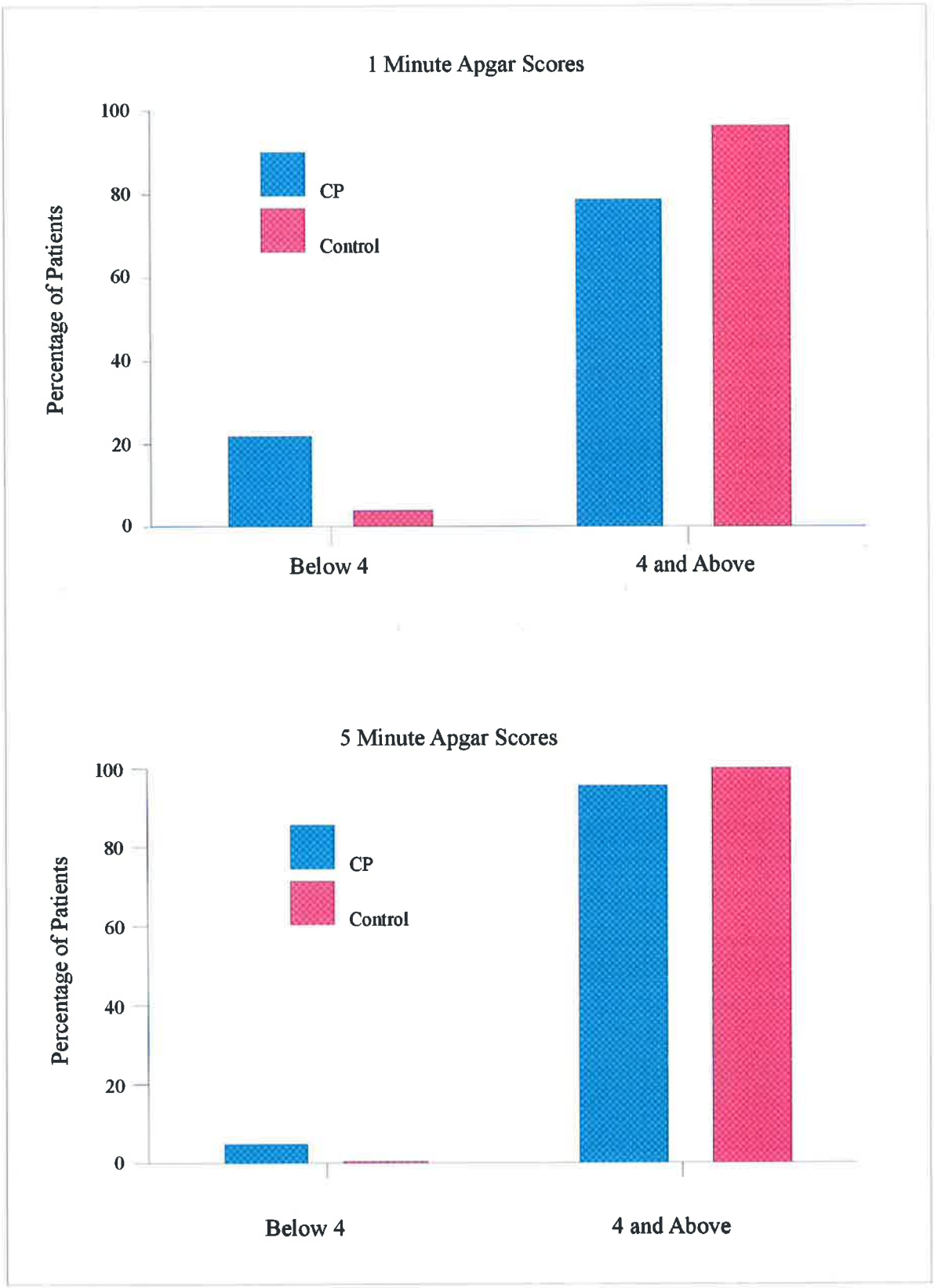

Figure 5: Percentage of cerebral palsy cases and controls with 1 and 5 minute Apgar scores of below 4 compared with 1 and 5 minute Apgar scores of 4 and above.

Table 1: Demographic and clinical variables for cerebral palsy cases and controls. NS = p>0.05. †Data are given as mean (95% CI). ‡Compared with normal spontaneous labour. §not including aspiration, compared with no resuscitation. ¥Compared with normal spontaneous delivery. LSCS = Lower segment caesarean section. IUGR = Intrauterine growth restriction. APH = Antepartum haemorrhage. PIHD = Pregnancy-induced hypertensive disorders. ** total number of cases and controls less than 443 and 883 respectively due to incomplete data collection in earlier years.

Variable	Cerebral Palsy Cases (n = 443)	Control (n = 883)	p-value
Maternal Age (y)	28.382 (27.873-28.890)	28.844 (28.486-29.202)	NS
Gestation (wk) †	35.09 (34.592-35.589)	37.254 (36.987-37.520)	<0.0001
Birthweight (g) †	2463.8 (2360.6-2823.5)	2884.6 (2823.5-2945.8)	<0.0001
1 minute Apgar †	5.866 (5.624-6.108)	7.435 (7.315-7.556)	<0.0001
1 minute Apgar <4 (n)	95 (21.5%)	34 (3.9%)	<0.0001
5 minute Apgar †	7.923 (7.742-8.104)	8.947 (8.882-9.012)	<0.0001
5 minute Apgar <4 (n)	21 (4.8%)	3 (0.3%)	<0.0001
Any anaesthesia (n) **	188 (70.9%)	364 (65.1%)	NS
Any analgesia (n) **	145 (54.7%)	351 (62.8%)	0.03
Any labour complications (n)	228 (51.5%)	332 (37.6%)	<0.0001
No labour (LSCS) (n)‡	115 (26%)	150 (17%)	0.0003
Any medical conditions (n)	124 (28%)	228 (25.8%)	NS
Any obstetric complications (n)	270 (60.9%)	399 (45.2%)	<0.0001
Resuscitation at delivery (n)§ **	118 (26.6%)	89 (10.1%)	<0.0001
Emergency LSCS (n)¥	163 (36.8%)	204 (23.1%)	<0.0001
IUGR <10th percentile (n)	91 (20.5%)	150 (17.0%)	NS
IUGR <5th percentile (n)	57 (12.9%)	79 (8.9%)	0.03
Any APH	132 (29.8%)	208 (23.6%)	0.02
PIHD	8 (1.8%)	15 (1.7%)	NS

Study Population – Adverse Pregnancy Outcomes

This thesis also examined the role of inherited thrombophilia, inherited cytokine polymorphisms and viral infection in the subsequent development of adverse pregnancy outcomes. For this analysis, we disregarded cerebral palsy as an outcome, and combined our cohort of 443 CP cases and 883 controls (total 1.326) before separating them on the basis of adverse pregnancy outcomes.

A total of 717 of the 1,326 babies (54.1%) met the following selection criteria for cases. Some cases had more than one condition:

1. Preterm birth <37 weeks gestation (451/717, 62.9%)
2. Intrauterine growth restriction (IUGR) less than the 10^{th} percentile, calculated from Roberts (242) (241/717, 33.6%).
3. Antepartum haemorrhage (any recorded bleeding at or after 20 weeks gestation, including placenta praevia, abruption, other and unknown causes), (340/717, 47.4%).
4. Pregnancy-induced hypertensive disorders (PIHD) (blood pressure ≥ 140/90 or higher on two occasions at least four hours apart, or ≥170/110 or higher on one occasion, first noted after 20 weeks gestation. The South Australian Pregnancy Outcome Database does not contain data on proteinuria, therefore in this study cases with PIHD include both gestational hypertension and preeclampsia, (23/717, 3.2%). The low frequency of PIHD in this study is most likely explained by the high incidence of preterm birth (62.9% of the overall study population).
5. Pre-existing hypertension (blood pressure ≥140/90 on two occasions at least 4 hours apart, or ≥170/110 on one occasion diagnosed before 20 weeks gestation), (20/717, 2.8%).

The remaining 609 babies (45.9%) had none of the above selection criteria, and were designated healthy, term controls.

Subanalysis was also performed, using the following selection criteria:

1. All pregnancy-induced hypertension + IUGR <10^{th} percentile (9/717)
2. All antepartum haemorrhage + IUGR <10^{th} percentile (108/717)
3. All preterm birth <37 weeks gestation + IUGR <10^{th} percentile (82/717)

Clinical Characteristics

Demographic and clinical characteristics were compared by bivariable analysis for the adverse pregnancy outcome cases and controls (Table 2). Babies with an adverse pregnancy outcome had significantly lower Apgar scores at both 1 minute and 5 minutes, and significantly more APO cases had an Apgar score less than 4 at 1 minute (OR 2.28, 95% CI 1.53-3.39). As expected, due to our case selection criteria, obstetric complications (including but not limited to threatened miscarriage, antepartum haemorrhage, pregnancy-induced hypertensive disorders, suspected intrauterine growth restriction and gestational diabetes) were more prevalent among cases (81.6%) than controls (13.8%). Significantly more cases had documented medical conditions (such as anaemia, urinary tract infections, preexisting hypertension or diabetes, epilepsy and asthma) during pregnancy (32.4% vs. 19.7%), and complications of labour, delivery and puerperium (such as postpartum haemorrhage, fetal distress, retained placenta, prolonged labour >18 hours and cord prolapse) (50.2% vs. 32.8%). APO cases were more likely to have a method of delivery other than normal spontaneous vaginal, and had more emergency caesarean section deliveries than controls. The need for resuscitation at delivery (not including aspiration) was also increased for cases compared with controls (56.6% vs. 20.3%). Interestingly, the mothers of significantly more controls required some form of analgesia than cases (59.6% vs. 51.9%). This is possibly due to the fact that the mothers of more controls had a normal spontaneous vaginal delivery than cases, with the mothers of many cases requiring caesarean section deliveries.

Table 2: Demographic and clinical variables for adverse pregnancy outcomes cases and controls. NS = p>0.05. †Data are given as mean (95% CI). ‡Compared with normal spontaneous labour. §not including aspiration, compared with no resuscitation. ¥Compared with normal spontaneous delivery. LSCS = Lower segment caesarean section. ** total number of cases and controls less than 717 and 609 respectively due to incomplete data collection in earlier years.

Variable	Adverse Pregnancy Outcome Cases (n = 717)	Control (n = 609)	p-value
Maternal Age (y)	28.596 (28.199-28.992)	28.800 (28.365-29.235)	NS
1 minute Apgar †	6.423 (6.254-6.592)	7.524 (7.370-7.677)	<0.0001
1 minute Apgar <4 (n)	92 (12.8%)	37 (6.1%)	<0.0001
5 minute Apgar †	8.396 (8.285-8.504)	8.867 (8.763-8.971)	<0.0001
5 minute Apgar <4 (n)	13 (1.8%)	11 (1.8%)	NS
Any anaesthesia (n) **	341 (72.6%)	252 (71.6%)	NS
Any analgesia (n) **	244 (51.9%)	211 (59.6%)	0.03
Any labour complications (n)	360 (50.2%)	200 (32.8%)	<0.0001
No labour (LSCS) (n)‡	327 (54.2%)	145 (28.3%)	<0.0001
Any medical conditions (n)	232 (32.4%)	120 (19.7%)	<0.0001
Any obstetric complications (n)	585 (81.6%)	84 (13.8%)	<0.0001
Resuscitation at delivery (n)§ **	162 (56.6%)	45 (20.3%)	<0.0001
Method of delivery (n)¥	441 (61.5%)	242 (39.7%)	<0.0001
Emergency LSCS (n)¥	293 (51.5%)	74 (16.8%)	<0.0001

The Prevalence of Inherited Thrombophilia in a Caucasian Australian Population

Abstract

Objective: To describe the prevalence of four inherited thrombophilic polymorphisms and their combinations for the first time in a large Caucasian Australian population.

Methods: 883 newborn screening cards of Caucasian babies born in South Australia in 1986-1999 were deidentified and tested for the following inherited thrombophilic polymorphisms: Factor V Leiden (G1691A), Prothrombin gene mutation (G20210A), Methylenetetrahydrofolate reductase gene (MTHFR) C677T and A1298C, as well as compound heterozygosity for the MTHFR polymorphisms.

Results: The birth prevalences of heterozygosity and homozygosity for the four thrombophilic polymorphisms were: Factor V Leiden 9.5% and 0.7%; Prothrombin gene 4.1% and 0.2%; MTHFR C677T 37.3% and 12.4% and MTHFR A1298C 38.3% and 11.8%. Compound heterozygosity for MTHFR C677T and A1298C was seen in 16.6% of the population. Overall, 64.2% and 24.5% of the population studied were heterozygous and homozygous respectively, for at least one of the four polymorphisms studied.

Conclusions: Inherited thrombophilic polymorphisms are common in the Caucasian Australian population. Knowledge of the background prevalence of these polymorphisms will allow further study of their associations in future disease research.

Introduction

Thrombophilia is an inherited or acquired condition which predisposes individuals to thrombosis. Most inherited thrombophilic conditions predispose to the formation of thrombosis by promoting excessive coagulation, or by impairing anticoagulation (86). Common inherited thrombophilia include: heterozygosity or homozygosity for the Factor V Leiden (FVL) mutation causing activated protein C resistance (APCR); heterozygosity or homozygosity for the Prothrombin gene mutation (PGM); and heterozygosity or homozygosity for polymorphisms at positions 677 and 1298 in the gene for 5, 10-methylenetetrahydrofolate reductase (MTHFR), associated with an increased tendency to hyperhomocysteinaemia; and compound heterozygosity for both MTHFR polymorphisms.

Inherited thrombophilic polymorphisms may influence a number of disease processes and, as part of ongoing studies investigating the possible role of inherited thrombophilia in the development of cerebral palsy, we have determined the prevalence of these four inherited thrombophilic polymorphisms in a Caucasian Australian population at birth.

Materials and Methods

Patient Selection

Please refer to Chapter 2 for information regarding patient selection.

DNA Isolation and Amplification

Four common polymorphisms associated with thrombophilia were screened for all subjects - FVL (G1691A), PGM (G20210A), MTHFR (C677T) and MTHFR (A1298C).

DNA was isolated from the newborn screening cards using the InstaGene Dried Blood Kit (BioRad, Hercules, California, USA) or the Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA). The dried blood spots (3mm diameter) were placed in 1 ml of water, and incubated twice for 10 minutes. The extraction reagents from the kit were then added to the spot, which was then incubated twice at 65°C for 10 minutes. The spot was washed three times with the provided wash reagent, and then washed with ethanol. Finally, the remaining ethanol was evaporated, and the spots were placed directly into tubes containing a polymerase chain reaction (PCR) mix to amplify the polymorphic regions of the four polymorphisms.

Please refer to Appendix 3 for detailed information regarding PCR reaction mixes, primer sequences, and cycling parameters. The polymorphic regions were amplified as four DNA fragments, ranging in size from 95bp to 120bp, in a single multiplex PCR reaction. Following PCR amplification, the polymorphic base in each gene was determined using the ABI Prism SNaPshot Multiplex Kit (Applied Biosystems, Inc, Foster City, California, USA). This kit, based on the primer extension method, uses fluorescently labelled dideoxy nucleotides to extend unlabelled oligonucleotide primers. Each primer was designed to hybridise to its PCR template so that the 3′ end of the primer was immediately upstream of the polymorphic site. The primer was then extended by one base at its 3' end using Taq polymerase, with the base incorporated determined by the sequence of the template. This system was successfully multiplexed by adding a mono-nucleotide tail of varying length to the 5′ end of each detection primer. A minimum size difference between detection primers of 5 bases was used to allow clear separation of each locus by the sequencer. The products were run on a DNA sequencer (ABI 3700) and results were analysed using Genotyper Version 3.7 software. In each case the locus was identified by primer length and the polymorphic base(s) determined by which fluorochrome(s) was incorporated into the primer.

These polymorphisms were reliably determined using newborn screening samples up to 15 years old. Prevalence proportions and exact 95% confidence intervals (CI) of the polymorphisms in the newborn screening cards tested were calculated using PEPI (243).

Results

Results were obtained from a total of 823 of the 883 cards. Not all polymorphisms gave a clear result for each newborn screening card, thus the total number of results for each polymorphism is less than 823. We investigated the effect of gestational age on the prevalence of these polymorphisms, because there was a higher rate of prematurity in the population from which many of the controls were selected, and found no significant difference between term and preterm deliveries. Table 1 shows the prevalence of hetero- and homozygosity of the four inherited thrombophilic polymorphisms in the newborn Caucasian population of South Australia.

We also investigated all combinations of thrombophilia, to determine the prevalence of multiple inherited thrombophilia in the sample population. Heterozygous FVL and heterozygous MTHFR A1298C was the most common combination with a prevalence of 4.1% (95% CI 2.9-5.7), followed by heterozygous FVL and heterozygous MTHFR C677T (3.5%, 95% CI 2.4-4.9). Heterozygous PGM and heterozygous MTHFR C677T had a prevalence of 2.2% (95% CI 1.4-3.5), whilst heterozygous FVL and compound heterozygous MTHFR had a prevalence of 2.0% (95% CI 1.2-3.2).

Genotype distributions were not in Hardy-Weinberg Equilibrium for Factor V Leiden, MTHFR C677T or MTHFR A1298C. In each of these polymorphisms, we observed a small excess of homozygotes.

Table 1: Prevalence of four inherited thrombophilic polymorphisms in a population of South Australian newborns, expressed as percentage positive of the total tested.

Mutation	Zygosity	Positive	Total	Prevalence (%) (95%CI)	
FVL (G1691A)	Heterozygous	78	823	9.5	7.6-11.6
	Homozygous	6		0.7	0.3-1.5
PGM (G20210A)	Heterozygous	34	820	4.1	2.9-5.7
	Homozygous	2		0.2	0.0-0.8
MTHFR (C677T)	Heterozygous	300	804	37.3	34.0-40.7
	Homozygous	100		12.4	10.3-14.9
MTHFR (A1298C)	Heterozygous	306	799	38.3	35.0-41.7
	Homozygous	94		11.8	9.7-14.1
MTHFR (C677T + A1298C)	Compound Heterozygous	131	787	16.6	14.2-19.4
Any of the above	Heterozygous	503	783	64.2	60.8-67.5
	Homozygous	192		24.5	21.6-27.6

Discussion

This is the first time that the prevalences of these four common thrombophilic polymorphisms and their combinations have been determined in a newborn Australian Caucasian population. A study in Brisbane (244) used 500 random blood donors (ethnicity undefined) to determine the prevalence of FVL, PGM, and MTHFR C677T in that population. They reported prevalences of 3.6% (heterozygous, FVL), 2.8% (heterozygous, PGM), and 10% (homozygous, MTHFR C677T). These lower prevalences than those reported here (9.5%, 4.1% and 12.4% respectively) could be explained by the possible inclusion of ethnic groups such as Asians in the Brisbane study. There have been no reports of FVL in any of the South East Asian countries, including China, Japan, and Indonesia (245). Rees (245) notes that the use of blood donors will underestimate the true prevalence of the polymorphisms. Our sample reflects the South Australian Caucasian population. Exclusion of babies with birth defects or death in the first year (a group of children in which the prevalence of thrombophilia could be higher than in the population as a whole) could potentially have resulted in a slightly lower observed prevalence than is actually the case. Our data, however, are more likely to reflect the prevalence of these polymorphisms in the normal adult population. A study by Wang and colleagues (246) reported FVL heterozygosity to be 4.9%, significantly less than our reported prevalence of 9.5%. Their study did not contain a control population, instead reporting prevalences from a selected population undergoing coronary angiography. Another study reported on the geographical and ethnic variation of the MTHFR C677T polymorphism (247), and reported Australian prevalences of 41% and 8% for heterozygous and homozygous MTHFR C677T respectively, compared with our reported values of 37.3% and 12.4%. Their population consisted of consecutive newborn screening cards, and it is possible that their population was not entirely Caucasian, which would influence their results, as demonstrated by their data showing geographical and ethnic variation of the MTHFR C677T polymorphism worldwide (247).

As reported, our population was not in Hardy-Weinberg equilibrium for FVL, MTHFR C677T or MTHFR A1298C. We observed small increases in the number of homozygotes for each of these polymorphisms. The departure from Hardy-Weinberg Equilibrium can occur for a number of reasons, such as the locus being under selection or where there is a null allele, both unlikely to be influencing factors in this population. Inbreeding or the presence of population substructures may also account for increases in homozygosity.

Increases in homozygosity as a result of population substructures is known as the Wahlund effect, and occurs when a large population contains sub-populations. Mathematically, a subdivided population will contain fewer heterozygotes than predicted, even if the individual sub-populations are in Hardy-Weinberg equilibrium. Inbreeding is very unlikely in a large, randomly selected population, and the Wahlund effect may reflect the recent multicultural origins of the Caucasian Australian population.

Inheritance of FVL is autosomal dominant, and compared with those with a normal factor V genotype, individuals homozygous for FVL have an 80 to 100 fold increased risk of developing thromboembolism (101). Homozygosity for FVL has also been associated with an increased risk of recurrent thromboembolism (102). The mean allele frequency of the FVL mutation throughout Europe is 2.7% (245), with the prevalence of this common mutation ranging from 2% to 15% in Caucasian populations (87, 248). Our values of 9.5% and 0.7%, for heterozygotes and homozygotes respectively, demonstrate a similar prevalence of this common polymorphism in the Australian Caucasian population (Table 1).

PGM results in increased plasma prothrombin concentrations, and a 2.8 fold increase in the risk of thrombosis (87, 103). PGM is found in the Caucasian population with a range between 1% and 8% (249). As with FVL, our values of 4.1% and 0.2%, for heterozygotes and homozygotes respectively, (Table 1) are consistent with other world populations.

Two common polymorphisms have been described in the MTHFR gene at positions 677 and 1298. The C677T polymorphism occurs in 6-12% of Caucasian populations in the homozygous form (106). It is important to realise that there is a low risk of thrombosis associated with homozygosity for the MTHFR C677T polymorphism. Data pooled from 15 studies shows that the risk of venous thromboembolism with the MTHFR 677TT genotype is 1.11 (95% CI 0.98-1.27) (250), suggesting that the phenotype of hyperhomocysteinaemia may be more important than the genotype with this polymorphism. The second polymorphism, A1298C, also results in decreased enzyme activity, but not necessarily hyperhomocysteinaemia (110). 37.3% and 12.4 %, respectively, of our population were heterozygous or homozygous for the MTHFR C677T polymorphism, whilst 38.3% and 11.8% of the population were heterozygous or homozygous for the MTHFR A1298C polymorphism. Compound heterozygosity for the MTHFR C677T and A1298C polymorphisms, a combination which decreases the enzyme activity of MTHFR and increases the risk of hyperhomocysteinaemia similarly to that

found in individuals homozygous for the C677T polymorphism (251), was observed in 16.6% of the study population, consistent with other Caucasian populations (252).

The prevalence of individuals with multiple thrombophilic polymorphisms is also described here. Our results show that the combinations of these polymorphisms, as expected, are not as common as the individual polymorphisms, with the combination of heterozygous FVL and heterozygous MTHFR A1298C having the highest prevalence of 4.1% in the population. 24.5% of the population were homozygous and 64.2% of the population were heterozygous for at least one thrombophilic polymorphism.

In summary, we present here the birth prevalence of four common thrombophilic polymorphisms and their combinations in a Caucasian population of South Australia. These prevalences are comparable to those in Caucasian populations elsewhere in the world. The 64.2% and 24.5% prevalences of heterozygous and homozygous thrombophilic polymorphisms respectively gives a very high prevalence of these risk factors for thromboembolism in the Australian Caucasian population. It is likely that most of this population with such polymorphisms do not experience a clinically apparent thromboembolic episode during their lifetimes. Thus, other co-factors or pathological mechanisms are likely to be necessary to precipitate a thrombotic event. Until these co-factors are recognised, there is no clear advantage in widespread screening for these polymorphisms (87). Knowledge of the prevalence of these polymorphisms in appropriate control populations, eg in this study, the Caucasian population in Australia, allows clinicians and researchers to identify and study disease states or co-factors which may be associated with these polymorphisms.

Associations between Inherited Thrombophilia, Gestational Age, and Cerebral Palsy

Abstract

Objective: To investigate associations between inherited thrombophilic polymorphisms and cerebral palsy (CP) in a large case-control study.

Methods: This is a population-based case-control study. Genomic DNA from newborn screening cards of 443 Caucasian CP cases and 883 Caucasian controls was tested for Factor V Leiden (FVL, G1691A), Prothrombin gene mutation (PGM, G20210A), and Methylenetetrahydrofolate reductase (MTHFR) C677T and MTHFR A1298C.

Results: MTHFR C677T was associated with an increased risk of developing any CP (32-36 weeks gestation, homozygous OR 2.55 95% CI 1.12-5.74; heterozygous OR 1.91 95% CI 1.01-3.66). MTHFR C677T was also associated with diplegia at both <32 weeks gestation (Homozygous OR 2.76 95% CI 1.21-6.12) and all gestations (Heterozygous OR 1.58 95% CI 1.02-2.45). For children <32 weeks, FVL homozygosity may be associated with an increase in the risk of developing quadriplegia (OR 9.12 95% CI 0.86-53.71). MTHFR A1298C (heterozygous) was associated with a reduced risk of developing diplegia at 32-36 weeks gestation (OR 0.16 95% CI 0.02-0.70). There were no associations between any type of CP and thrombophilia for children born ≥37 weeks. Heterozygous PGM and homozygous MTHFR C677T combined were associated with quadriplegia at all gestational ages (OR 5.33 95% CI 1.06-23.25).

Conclusions: MTHFR C677T approximately doubles the risk of cerebral palsy in preterm infants. A combination of homozygous MTHFR C677T and heterozygous PGM increases the risk of quadriplegia fivefold at all gestational ages.

Introduction

Cerebral palsy (CP) is a disorder affecting 2-2.5 children in every 1000 births worldwide. Notwithstanding major improvements in perinatal medicine, the incidence of CP has not decreased over the last forty years (18). Research is needed into the causation of CP and the possibilities for its prevention.

A possible association between thrombophilia and CP has been the focus of several small studies. In 1998, it was proposed that both inherited and acquired thrombophilia of the mother and/or the fetus may be responsible for thrombosis in the maternal and/or fetal circulation, resulting in adverse pregnancy outcomes such as CP (114). There have been six small studies or case reports regarding inherited thrombophilic polymorphisms and the subsequent development of CP (18, 93, 94, 96, 117, 119). Of these six studies, only Smith and colleagues (117) did not find an association between thrombophilia and CP. The only case-control study demonstrated that 20 of 31 cases had surrogate indices for thrombophilia, compared with two of 65 controls (18). The studies by Thorarensen, Harum and Steiner (93, 94, 96) were case reports, with sample sizes of three, one, and one respectively. Finally, Halliday and colleagues found evidence of thrombophilia in five of 55 cases of CP (119).

This population-based study is the first large case-control study of CP and hereditary thrombophilia. The prevalence of thrombophilic polymorphisms, common in Caucasian populations, was compared in different types of CP cases at different gestational ages and in controls. The thrombophilic polymorphisms studied were: Factor V Leiden (FVL); prothrombin gene mutation G20210A (PGM); and methylenetetrahydrofolate reductase gene (MTHFR) polymorphisms C677T and MTHFR A1298C.

Materials and Methods

Patient Selection

Please refer to Chapter 2 for information regarding patient selection.

DNA Isolation and Amplification

Detailed information regarding DNA Isolation and Amplification can be found in Chapter 3 of this thesis.

Statistical Analysis

As controls were not matched for important covariates such as gestational age, analysis was undertaken using all controls without taking account of matching. Data analysis (EpiInfo Version 6) considered CP cases by gestational age range (<32 weeks, 32-36 weeks, ≥ 37 weeks, and all gestational ages), type of CP (diplegia, hemiplegia, quadriplegia and all types) and type of thrombophilia. Control data were considered in two groups: a) all controls irrespective of gestational age (Tables 1-4) and b) controls born ≥ 37 weeks (Appendix 4). This distinction was made because of the reported links between thrombophilia and a range of obstetric complications, such as pre-eclampsia and intrauterine growth restriction that may increase the risk of preterm birth (253-256). Results are expressed as odds ratios (OR) with 95% confidence intervals (CI), comparing homozygosity and heterozygosity for each polymorphism separately with homozygosity for the wild-type allele (Tables 1-4). Data for homozygosity and heterozygosity combined compared with the wild-type allele, and also homozygosity vs. heterozygosity are presented as supplementary material (Appendix 4). Some combinations of thrombophilic polymorphisms and CP types were not seen in some sub-groups: these odds ratios are not reported. P values less than 0.05 are highlighted in the tables. As other components of the CP study will examine other factors such as those relating to infection, multivariable analysis was not undertaken at this stage. No adjustments were made for multiple testing in this largely exploratory study.

Power Calculations

Two-sided power calculations for dichotomous data were performed using EpiInfo. They showed that a minimum prevalence of 4.5% of any thrombophilic polymorphism among the controls would be required for statistical significance (based upon 95% confidence, 80% power, case sample size of 444, 2:1 controls:cases and an odds ratio of 2).

Results

CP All Gestational Ages

No associations were found between CP and FVL or PGM (Table 1). For the MTHFR C677T polymorphism, an association for diplegia was found for heterozygotes (OR 1.58 95% CI 1.02-2.45) (Table 1), while the odds ratio for homozygotes was increased with borderline statistical significance (OR 1.81 95% CI 0.99-3.26). 55% of all CP cases were heterozygous or homozygous for the MTHFR C677T polymorphism, compared with 49.8% of all controls, giving an attributable risk of 5.2% for the presence of this polymorphism. In the group of infants with quadriplegia, there was a negative association with homozygosity in comparison with heterozygosity for MTHFR A1298C (OR 0.33 95% CI 0.1-0.87) (Appendix 4). The same trend appeared when homozygosity for MTHFR A1298C was compared with homozygosity for the wild-type allele (1298AA), but this did not reach statistical significance (Table 1). Almost all odds ratios for MTHFR A1298C were less than unity, unlike the odds ratios for the other thrombophilic polymorphisms.

CP Gestational Ages ≥ 37 weeks

225 of the 405 CP cases (55.6%) were born at gestational ages ≥ 37 weeks. The data presented in Table 2 shows no significant association between any of the tested thrombophilia and CP in children born at ≥ 37 weeks.

CP Gestational Ages 32-36 weeks

58 of the 405 CP cases (14.3%) were born between 32-36 weeks gestation. When all subtypes of CP were combined, MTHFR C677T was associated with CP (homozygous OR 2.55 95% CI 1.12-5.74; heterozygous OR 1.91 95% CI 1.01-3.66) (Table 3). MTHFR A1298C showed a negative association with diplegic CP (Table 3), which was statistically significant only for heterozygotes (OR 0.16 95% CI 0.02-0.70).

CP Gestational Ages < 32 weeks

122 of the 405 CP cases (30.1%) were born at gestational ages <32 weeks. MTHFR C677T (homozygous) was associated with diplegic CP (OR 2.76 95% CI 1.21-6.12) (Table 4). Homozygous FVL had an OR of 9.12 (95% CI 0.86-57.71) for quadriplegic CP (Table 4). The OR for homozygous FVL compared with heterozygous FVL was 26 (95% CI 1.09-1551.59) for all controls, and 29 (95% CI 1.11-1737.29) for term controls (Appendix 4).

Combination Thrombophilia at any Gestational Age

The combination of heterozygous PGM and homozygous MTHFR C677T was associated with quadriplegia at all gestational ages (OR 5.33, 95% CI 1.06-23.25). The OR was also elevated for babies born <32 weeks gestation (OR 9.33, 95% CI 0.77-70.09) when compared with all controls but the confidence intervals crossed unity. Comparisons with term controls showed similar trends. No other combination of thrombophilic polymorphisms was associated with any type of CP.

Analysis using Term Controls

All the associations were reanalysed using only term controls (babies born ≥37 weeks gestation), with very similar results. These data are presented in Appendix 4. Odds ratios for homozygosity or heterozygosity vs. wild-type normal and homozygosity vs. heterozygosity are also presented in Appendix 4, for both term controls and all controls.

Table 1: Odds ratios (95% CI) for CP for specified thrombophilia among babies born at all gestational ages. *p-values <0.05.

Type of CP	Zygosity	Odds Ratio (95% CI)					
		Any Thrombophilia	FVL	PGM	MTHFR C677T	MTHFR A1298C	MTHFR C677T+ A1298C
All Types	Homozygous	1.05 (0.68-1.60)	1.34 (0.28-5.68)	2.07 (0.15-28.62)	1.37 (0.93-2.01)	0.66 (0.42-1.04)	-
(n = 405)	Heterozygous	1.12 (0.78-1.62)	0.77 (0.49-1.22)	1.10 (0.59-2.03)	1.19 (0.91-1.56)	0.98 (0.75-1.28)	1.20 (0.77-1.86)
Diplegia	Homozygous	1.38 (0.69-2.85)	0.00 (0.00-4.01)	0.00 (0.00-12.54)	1.81 (0.99-3.26)	0.68 (0.32-1.38)	-
(n = 127)	Heterozygous	1.31 (0.71-2.47)	0.56 (0.21-1.24)	1.14 (0.38-2.83)	**1.58 (1.02-2.45)***	0.87 (0.57-1.32)	1.66 (0.81-3.50)
Hemiplegia	Homozygous	1.09 (0.50-2.45)	2.37 (0.23-13.45)	3.63 (0.06-70.11)	1.09 (0.54-2.18)	0.56 (0.24-1.26)	-
(n = 116)	Heterozygous	1.40 (0.73-2.75)	0.91 (0.43-1.88)	1.07 (0.32-2.83)	1.32 (0.84-2.07)	0.88 (0.56-1.36)	1.18 (0.54-2.57)
Quadriplegia	Homozygous	0.72 (0.35-1.50)	2.51 (0.24-14.29)	3.96 (0.07-76.51)	1.27 (0.68-2.37)	0.41 (0.12-1.06)	-
(n = 110)	Heterozygous	0.94 (0.53-1.75)	0.77 (0.33-1.72)	1.40 (0.47-3.48)	0.87 (0.54-1.40)	1.23 (0.78-1.90)	0.95 (0.46-1.95)

Table 2: Odds ratios (95% CI) for CP for specified thrombophilia among babies born at ≥ 37 weeks gestation

Type of CP	Zygosity	Odds Ratio (95% CI)					
		Any Thrombophilia	FVL	PGM	MTHFR C677T	MTHFR A1298C	MTHFR C677T + A1298C
All Types	Homozygous	0.90 (0.53-1.54)	1.21 (0.12-6.82)	3.72 (0.27-51.45)	1.02 (0.62-1.68)	0.73 (0.41-1.28)	-
(n = 225)	Heterozygous	1.05 (0.67-1.65)	0.84 (0.47-1.47)	1.09 (0.50-2.35)	1.03 (0.74-1.45)	1.05 (0.75-1.45)	0.99 (0.57-1.72)
Diplegia	Homozygous	1.29 (0.39-4.93)	0.00 (0.00-10.27)	0.00 (0.00-31.96)	0.96 (0.28-2.71)	0.92 (0.27-2.57)	-
(n = 49)	Heterozygous	1.58 (0.59-5.29)	0.40 (0.05-1.59)	0.48 (0.01-3.00)	1.41 (0.73-2.72)	1.13 (0.59-2.19)	2.03 (0.66-6.86)
Hemiplegia	Homozygous	0.90 (0.36-2.35)	3.47 (0.34-19.82)	5.23 (0.09-101.13)	0.76 (0.28-1.81)	0.52 (0.16-1.36)	-
(n = 80)	Heterozygous	1.33 (0.63-2.87)	0.93 (0.35-2.12)	1.23 (0.31-3.59)	1.13 (0.66-1.91)	1.02 (0.61-1.70)	1.02 (0.42-2.49)
Quadriplegia	Homozygous	0.70 (0.28-1.82)	0.00 (0.00-8.29)	6.88 (0.11-133.3)	1.26 (0.56-2.78)	0.57 (0.14-1.66)	-
(n = 64)	Heterozygous	0.90 (0.43-1.91)	0.82 (0.25-2.11)	1.62 (0.40-4.77)	0.84 (0.45-1.55)	1.22 (0.69-2.15)	0.74 (0.27-2.01)

Table 3: Odds ratios (95% CI) for CP for specified thrombophilia among babies born at 32-36 weeks gestation. *p-values <0.05

Type of CP	Zygosity	Odds Ratio (95% CI)					
		Any Thrombophilia	**FVL**	**PGM**	**MTHFR C677T**	**MTHFR A1298C**	**MTHFR C677T + A1298C**
All Types	Homozygous	1.21 (0.47-3.40)	0.00 (0.00-9.45)	0.00 (0.00-27.83)	**2.55 (1.12-5.74)***	0.50 (0.13-1.45)	-
(n = 58)	Heterozygous	1.14 (0.49-2.73)	1.11 (0.38-2.71)	1.26 (0.24-4.20)	**1.91 (1.01-3.66)***	0.77 (0.42-1.41)	1.60 (0.61-4.21)
Diplegia	Homozygous	0.65 (0.15-2.87)	0.00 (0.00-27.57)	0.00 (0.00-93.30)	1.73 (0.28-7.74)	0.53 (0.06-2.32)	-
(n = 20)	Heterozygous	0.59 (0.19-2.19)	1.05 (0.12-4.53)	4.07 (0.73-15.04)	1.92 (0.67-5.67)	**0.16 (0.02-0.70)***	0.44 (0.04-2.77)
Hemiplegia	Homozygous	Undefined	0.00 (0.00-51.19)	0.00 (0.00-148.43)	6.06 (0.68-73.09)	0.00 (0.00-2.36)	-
(n = 11)	Heterozygous	Undefined	0.95 (0.02-6.82)	0.00 (0.00-8.22)	4.04 (0.71-41.12)	0.75 (0.16-2.96)	Undefined
Quadriplegia	Homozygous	0.65 (0.08-4.91)	0.00 (0.00-38.78)	0.00 (0.00-99.45)	2.69 (0.55-11.58)	0.00 (0.00-1.83)	-
(n = 16)	Heterozygous	0.74 (0.18-4.31)	1.46 (0.16-6.62)	0.00 (0.00-5.63)	1.35 (0.36-5.09)	1.01 (0.34-3.01)	1.38 (0.29-7.12)

Table 4: Odds ratios (95% CI) for CP for specified thrombophilia among babies born at <32 weeks gestation. *p-values <0.05

Type of CP	Zygosity	Odds Ratio (95% CI)					
		Any Thrombophilia	FVL	PGM	MTHFR C677T	MTHFR A1298C	MTHFR C677T + A1298C
All Types	Homozygous	1.29 (0.62-2.70)	2.18 (0.21-12.37)	0.00 (0.00-13.43)	1.68 (0.90-3.04)	0.64 (0.27-1.35)	-
(n = 122)	Heterozygous	1.27 (0.67-2.42)	0.50 (0.18-1.18)	1.02 (0.30-2.70)	1.26 (0.80-1.99)	0.98 (0.63-1.51)	1.48 (0.69-3.23)
Diplegia	Homozygous	2.20 (0.75-7.79)	0.00 (0.00-8.92)	0.00 (0.00-27.32)	**2.76 (1.21-6.12)***	0.57 (0.14-1.66)	-
(n = 58)	Heterozygous	1.77 (0.67-5.91)	0.53 (0.10-1.69)	0.82 (0.09-3.36)	1.63 (0.83-3.23)	1.04 (0.57-1.89)	2.44 (0.75-9.16)
Hemiplegia	Homozygous	1.08 (0.21-7.05)	0.00 (0.00-21.38)	0.00 (0.00-71.25)	1.35 (0.23-5.52)	0.98 (0.18-3.67)	-
(n = 25)	Heterozygous	0.99 (0.26-5.53)	0.82 (0.09-3.44)	1.05 (0.02-6.89)	1.50 (0.54-4.22)	0.50 (0.14-1.52)	1.11 (0.15-8.41)
Quadriplegia	Homozygous	0.81 (0.17-4.15)	**9.12 (0.86-53.71)***	0.00 (0.00-59.92)	0.76 (0.14-2.72)	0.33 (0.01-2.23)	-
(n = 30)	Heterozygous	1.23 (0.40-5.05)	0.35 (0.01-2.19)	1.77(0.20-7.59)	0.76 (0.29-1.85)	1.40 (0.60-3.29)	1.11 (0.25-4.92)

Discussion

This study is the largest reported study of CP and inherited thrombophilia. Previous small studies varied in the specific thrombophilia investigated, and whether they tested for the polymorphisms directly or used surrogate indices for thrombophilia. The results of this large case control study suggest the possibility of a complex and heterogeneous relationship between inherited thrombophilia and subtypes of CP at different gestational ages.

672 separate analyses were performed on the individual polymorphisms, and further analyses involved combinations of these polymorphisms. Such multiple analyses increase the likelihood of identifying chance statistical associations. On the contrary, because of small numbers in some of the subanalyses, associations cannot be confidently excluded. Where associations are seen in one gestational age range, similar trends are seen in the same subgroup of CP with the same thrombophilic polymorphism in other gestational age ranges. This suggests that these may not be spurious associations and are therefore worthy of further study.

Methylenetetrahydrofolate Reductase Gene

Two common polymorphisms have been described in the MTHFR gene: C677T and A1298C. Homozygosity for the 677 C→T base pair substitution occurs in 6-12% of Caucasian populations (106), and in 12.4% of the control population described here. This polymorphism can result in hyperhomocysteinaemia, particularly in conditions of folate or vitamin B12 deficiency. Hyperhomocysteinaemia may exert thrombophilic effects by altering the normal antithrombotic phenotype of the endothelium, enhancing the activities of factors XII and V, as well as depressing the activation of protein C, and also via recruiting leukocytes and augmenting leukocyte-induced endothelial cell activation (107, 109). A second common polymorphism has been described at position 1298 in the MTHFR gene, where there is an A→C base pair substitution (110). This polymorphism also results in decreased enzyme activity, but not necessarily in hyperhomocysteinaemia (110).

MTHFR Polymorphism C677T

This is the first study investigating the association between MTHFR C677T and CP. The results presented in Tables 1-4 suggest an approximate doubling of the risk for CP for infants born at 32-36 weeks gestation and for diplegia in infants born at gestational ages

<32 weeks. Mild to moderate hyperhomocysteinaemia, with and without the C677T polymorphism, has been linked to arterial disease and venous thromboembolism (257, 258). The thrombophilic tendency associated with hyperhomocysteinaemia could in itself lead to neonatal stroke, but it appears unlikely that this mechanism could explain an increased risk of diplegic CP in very preterm babies (259). Preterm birth is a final common pathway of a variety of pathophysiological conditions. The association between infection and spontaneous preterm labour is now well established and is thought to be responsible for up to 40% of cases, with an especially strong association with very preterm birth (260, 261). Recent studies have shown that any inflammatory damage to the vascular lining could be augmented in an hyperhomocysteinaemic environment by recruiting more leukocytes and aggravating the leukocyte-induced endothelial damage (258, 262, 263). The significant positive association between the polymorphic variant at MTHFR C677T and very preterm infants with CP makes an additive adverse interaction between infection and mild to moderate increases in circulating homocysteine an attractive hypothesis deserving further study. It should be noted that the effect of the MTHFR genetic variant might be affected by the supplementation of folate in the diet. There was no mandatory fortification of food with folic acid in Australia during the study period; however if in the future fortification is implemented, it would be interesting to investigate its effects.

MTHFR Polymorphism A1298C

This is also the first study investigating the association between MTHFR A1298C and CP. Homozygosity for MTHFR A1298C is known to "protect" against having the MTHFR C677T polymorphism, probably by increasing the early embryonic loss rates (264). The presence of MTHFR A1298C heterozygosity appears to be "protective" for diplegia among babies born at 32-36 weeks gestation. The possibility of this finding being due to chance as a result of the many statistical analyses performed cannot be excluded, and further studies with larger sample sizes will be required to confirm or refute this finding.

Factor V Leiden

FVL is a dominantly inherited thrombophilia, with individuals homozygous for FVL having an 80-100 fold increased risk of developing thromboembolism (101). Factor V is usually associated with venous thrombosis. In the fetal circulation, however, the usually patent foramen ovale may allow venous thromboses to embolise to the brain and cause infarctions. Thus, cerebral arterial strokes in the fetus may have venous origins. FVL has been associated with hemiplegic (93, 119) and diplegic (94) CP, though none of these were

case-control studies. The only case-control study investigating the role of thrombophilia in the development of CP showed an association between abnormalities of coagulation factors and CP (18). This large case-controlled series also suggests that homozygous FVL may increase the risk for quadriplegia in very preterm infants. There was, however, a non-significant trend for heterozygous FVL to be negatively associated with quadriplegia in very preterm infants. Carrying one abnormal FVL allele may protect the vulnerable fetal brain from the more major degrees of intraventricular haemorrhage by increasing the clotting ability of the baby, and/or convey some protection in severe infectious processes (265).

Prothrombin Gene Mutation

PGM is a dominantly inherited thrombophilia which results in increased plasma prothrombin concentrations and a 2.8 fold increase in the risk of thrombosis (87, 103). Two uncontrolled studies have investigated the role of PGM in the development of CP (117, 119). Halliday and colleagues found that of 52 babies screened, only one was heterozygous for PGM, and none were homozygous (119). Similarly, Smith and colleagues found that none of 27 study participants carried PGM, in either heterozygous or homozygous form (117). The data presented here showed a trend towards a positive association between heterozygous PGM and diplegia in infants born between 32-36 weeks gestation (OR 4.07 95% CI 0.73-15.04). There was also an interaction between PGM and MTHFR C677T resulting in a 5-fold increase in the risk of quadriplegia at all gestations (OR 5.33 95% CI 1.06-23.25). The numbers are small for this analysis, and these results should be interpreted with caution. The prevalence of PGM is lower than that of the other thrombophilic polymorphisms in control populations, and therefore greater numbers would be required to investigate fully the role of PGM in CP subtypes.

Conclusions

In summary, an apparent doubling of the risk of developing CP among infants born preterm, and especially of diplegia for infants delivered <32 weeks gestation who are homozygous for MTHFR C677T was observed. A synergistic effect was seen between MTHFR C677T and PGM, increasing the risk of quadriplegia at all gestations. A possible association between FVL and an increased risk of CP was seen in the small group of preterm infants <32 weeks gestation with quadriplegia. Associations between some polymorphisms and CP subtypes in preterm infants may, however, suggest interaction with other factors, such as infections and responses to infection. Such possible interactions are currently being investigated by this group and will be the focus of multivariable analysis in the future.

The association between inherited cytokine polymorphisms and cerebral palsy

Abstract

Objective: To investigate associations between inherited cytokine polymorphisms and cerebral palsy (CP) in a large population-based case-control study.

Methods: Genomic DNA from the newborn screening cards of 443 Caucasian CP cases and 883 Caucasian controls was tested for five cytokine polymorphisms: Tumour Necrosis Factor alpha –308 (TNF-α -308), Mannose Binding Lectin –221 (MBL –221), and three polymorphisms in Exon 1 of the MBL gene at codons 52, 54 and 57.

Results: At all gestational ages MBL codon 52 was associated with an increased risk of developing quadriplegia (homozygous or heterozygous OR 2.74, 95% CI 0.95-6.96), and MBL codon 54 increased the risk of developing diplegia (homozygous or heterozygous OR 1.55, 95% CI 1.03-2.32). For babies born at term, the risk of developing quadriplegia was associated with heterozygous TNF-α (OR 1.82, 95% CI 1.04-3.15), and MBL codon 52 (homozygous or heterozygous OR 3.24, 95% CI 0.91-9.42). MBL codon 54 was associated with diplegia (homozygous or heterozygous OR 2.12, 95% CI 1.10-4.05). The presence of any polymorphism in MBL exon 1 at term approximately doubled the risk of developing diplegia (OR 1.94, 95% CI 1.05-3.62). Homozygous or heterozygous TNF-α was associated with hemiplegia for babies born <32 weeks gestation (OR 2.38, 95% CI 1.02-5.58). Overall, the presence of any cytokine polymorphism was associated with CP (OR 1.37, 95% CI 1.02-1.84).

Conclusions: Carriage of polymorphisms in the TNF-α and MBL genes are associated with an increased risk of cerebral palsy.

Introduction

The Fetal Inflammatory Response

The fetal inflammatory response is described as being a multisystem disorder, which may result in preterm delivery and adverse neonatal outcome (184). One histopathologic representation of the fetal inflammatory response syndrome is funisitis, which has been associated with increased risks of complications resulting from both neonatal infection and the development of cerebral palsy (83, 133, 185, 266, 267). It is becoming increasingly apparent that the fetal inflammatory response to intra-amniotic infection is biologically important, even more so than the maternal inflammatory response (186). A multicentre cohort of 1078 infants of birth weight <1500g showed that, in preterm births, fetal inflammatory responses contribute to cerebral white matter damage, and that maternal infection can damage the fetal brain without the presence of fetal brain infection (186). Evidence is also increasing for the role of the fetus in chorioamnionitis, with research indicating that chorioamnionitis primarily involves a fetal inflammatory response (26, 187). The onset of spontaneous preterm labour with preterm premature rupture of membranes, is preceded by a systemic proinflammatory cytokine response in the fetus, which is likely to be the fetal response to the presence of microbial products (188). Evidence also suggests that antenatal infection and brain white matter damage are linked by the fetal inflammatory response (78).

Cytokine Responses

TNF-α is a proinflammatory cytokine that is produced in response to infection (128). Its main biological function is an ability to recognise a broad range of pathogens and act quickly, promoting a broad range of immunological and inflammatory responses (128). Unfortunately, this ability to be rapidly produced may pose more of a risk than the infection through which it is elicited. If the production of TNF-α is excessive, and is released systemically in large quantities, fatal complications such as multiple organ failure may occur (128).

The TNF-2 polymorphism, located in the promoter region of the gene, is represented as a single nucleotide G→A base pair substitution at nucleotide -308 relative to the transcriptional start site (128). This polymorphism has been associated with high levels of TNF-α (138, 139), and can alter the binding of nuclear factors to the promoter region of the TNF gene, resulting in two-fold greater activity of the promoter of the TNF-2 allele

compared to the TNF-1 allele (140). It has also been shown that human B cells transfected with the polymorphic promoter coupled with a reporter gene demonstrated a five-fold increase in reported expression compared with the wild-type promoter (141). Finally, a study by Westendorp and colleagues found that approximately 60% of the variation in the production of TNF-α is genetically determined (142), suggestive of the importance of polymorphisms in this gene. This polymorphism has been linked to the development of cerebral malaria, and it has been suggested that regulatory polymorphisms of cytokines such as TNF-α may be able to influence the outcomes of severe infection (143).

Mannose-binding lectin (MBL) plays an anti-infectious role, identifying and removing potentially infectious pathogens from the body (144, 145). The human MBL gene is found on chromosome 10, and has a number of known polymorphic sites, capable of altering circulating levels of MBL in the body. Two of these polymorphisms are located within the promoter region of exon 1 (147), at positions -550 and -221. Both are G→C base pair substitutions, and give rise to alleles H and L or Y and X respectively (148). These promoter mutations manifest as reduced levels of circulating MBL by affecting the transcriptional activity of the basal-promoter complex (149); the -221 polymorphism in particular is associated with low MBL serum concentration compared with the wild-type allele (268). Three polymorphic sites are found in exon 1 of the MBL gene. The polymorphism at codon 52 is an Arginine to Cysteine amino acid substitution (CTG→TGT), whilst the polymorphism at codon 54 is a Glycine to Aspartic Acid amino acid substitution (GGC→GAC). Finally, the polymorphism at codon 57 is a Glycine to Glutamic Acid amino acid substitution (GGA→GAA) (151, 152). The amino acid substitutions at codons 54 and 57 result in disruption of the Gly-Xaa-Yaa structure of the collagenous backbone of MBL, resulting in limited binding between the MBL subunits, and significantly reduced levels of circulating functional protein (151). In contrast, the amino acid substitution at codon 52 does not interrupt the Gly-Xaa-Yaa sequence, but instead results in the formation of an extra disulphide bond, thus decreasing the stability of the molecule (152, Wallis, 1999 #500). Decreased levels of circulating MBL, as a result of polymorphisms in the gene, results in impaired ability to defend against infectious pathogens, reducing the body's immune response to such infections, and increasing the susceptibility to infection.

It remains unclear whether the cytokines themselves mediate or cause white matter damage indicative of cerebral palsy, or whether the infection which initiated the fetal inflammatory

response is responsible for the damage. A combination of factors is possibly involved. A cytokine hypothesis has been postulated, suggesting that cytokines act as a final common pathway for injury to the central nervous system, and that they may be initiated by a number of different insults, including infection, hypoxic-ischaemic injury, reperfusion injury, and toxin-mediated injury (122, 123). Finally, the role of proinflammatory cytokines in preterm birth has been extensively investigated, demonstrating strong associations between preterm birth and the presence of proinflammatory cytokines (51, 77, 124-126). Preterm birth is a recognised risk factor for the development of cerebral palsy, and proinflammatory cytokines may damage the immature (preterm) fetal brain, leading to the development of cerebral palsy.

The aim of this study was to investigate cytokine polymorphisms capable of altering the host response to infection or inflammation by either up or down-regulating cytokine production, and to assess their role in the development of cerebral palsy. In particular, the TNF-α -308 polymorphism and polymorphisms within the MBL gene may influence a number of disease processes and contribute to the pathogenesis of cerebral palsy. This study is the first large case-control study of cerebral palsy and cytokine polymorphisms, investigating the associations between cytokine polymorphisms in the TNF-α and MBL genes and the future development of cerebral palsy.

Materials and Methods

Patient Selection

Please refer to chapter 2 for information regarding case and control selection, and population demographics.

DNA Extraction

MBL and TNF-α polymorphisms were genotyped to determine the polymorphic base at the sites of interest. DNA was isolated from the newborn screening cards using a methanol fixation technique (269), slightly modified. Briefly, 1.2mm blood spots were placed in 1.5ml sterile microfuge tubes, and 100µl of methanol was added to each sample. These samples were air-dried (no lid) at ambient room temperature overnight in a fumehood. The following day, all tubes were boiled for 10 minutes to elute the DNA from the filter paper. 1xTE buffer was added in 25µl aliquots to each tube, and the lids replaced. Tubes were sealed with parafilm to minimise the risk of evaporation and contamination, and placed in a boiling water bath for 10 minutes, after which time they were removed and allowed to cool to room temperature and centrifuged for 3 minutes at 2,000g. The eluted DNA was stored at -20°C until use.

All amplification conditions were optimised using reference samples prior to adapting the method to amplification from dried blood spots (refer to Appendix 5 for details regarding primers, PCR mixes and cycling parameters). The reference samples for MBL genotyping were kindly provided by Professor Malcolm Turner, Immunobiology Unit, Institute of Child Health, University College London, UK. The reference samples for TNF genotyping were EBV Transformed B-cell lines from the Ninth/Tenth Histocompatibility Workshop, previously genotyped for the -308 polymorphism.

MBL Genotyping

Five µl of extracted patient DNA was amplified for the promoter region of the MBL gene using primers Shorty L and Shorty R, and for the exon 1 region of the MBL gene using primers Exon 1-L and Exon 1-R.

The samples for polymorphisms in the promoter and exon-1 regions of the MBL gene were genotyped using heteroduplexing methods. This method used synthetic DNA molecules based on the sequence of interest, but containing base insertions and deletions close to the sites of the mutations. The PCR product from sample DNA was mixed with the UHG and

heated before being allowed to anneal slowly. Heteroduplexes unique for the allele present in the sample DNA were formed, and could be visualised using non-denaturing polyacrylamide gel electrophoresis (270). Two different UHG molecules were used; one for the promoter polymorphism (271), and another for the 3 polymorphisms in exon 1 (270).

DNA Sequencing

Samples which did not yield a clear banding pattern were sequenced to determine genotypes. These samples were subjected to PCR amplification, using primers Shorty-R and Exon 1-R at concentrations of 0.5μM. These primers amplified a 550bp product which was cleaned up and amplified using Big Dye version 3. Primer Shorty-R was used for the sequencing reactions. The products were run on a DNA sequencer (ABI 3700) and results were analysed using Genotyper Version 3.7 software.

TNF-α Genotyping

TNF genotyping was performed by previously published methods (272, 273).

Statistical Analysis

These polymorphisms were reliably determined using newborn screening samples up to 15 years old. Prevalence proportions and exact 95% confidence intervals (CI) of the polymorphisms in the newborn screening cards tested were calculated using PEPI (243). As controls were not matched for important covariates such as gestational age, analysis was undertaken using all controls without taking account of matching. Data analysis (EpiInfo Version 6) considered cerebral palsy cases by gestational age range (<32 weeks, 32-36 weeks, ≥ 37 weeks, and all gestational ages), type of cerebral palsy (diplegia, hemiplegia, quadriplegia and all types) and cytokine polymorphism. Control data were considered in two groups: a) all controls irrespective of gestational age (Tables 3-6) and b) controls born ≥ 37 weeks (Tables 7-10). This distinction was made because of the reported links between TNF and MBL polymorphisms and a range of obstetric complications, such as infection, that may increase the risk of preterm birth (141, 156, 274). Results are expressed as odds ratios (OR) with 95% confidence intervals (CI), comparing homozygosity and heterozygosity for each polymorphism separately with homozygosity for the wild-type allele. Data for homozygosity and heterozygosity combined compared with the wild-type allele are also presented (Tables 3-10). P values less than 0.05 are highlighted in the tables.

Results

All researchers were blinded to case/control status of the newborn screening cards prior to commencement of the study. A unique barcode identification number was randomly assigned to each sample, with information regarding case/control status and other clinical data stored separately by the Epidemiology Branch, Department of Health, South Australia.

Results were obtained from a total of 414 of the 443 cerebral palsy samples (93.5%) and 856 of the 883 control samples (96.9%), a significant difference (OR 2.22, 95% CI 1.26-3.93 for not obtaining a result). This may cause a slight underestimation of the prevalence of cytokine polymorphisms in the cerebral palsy cases. Not all polymorphisms gave a clear result for each newborn screening card, thus the total number of results for each polymorphism was less than the maximum total of 414 or 856 for cases and controls respectively. Of the 414 cerebral palsy cases, 131 had diplegia, 119 had hemiplegia and 112 had quadriplegia; the remaining 52 cases had other or unspecified cerebral palsy.

Prevalence of Cytokine Polymorphisms in Control Population

The prevalence of 5 cytokine polymorphisms in the non-cerebral palsy newborn Caucasian population at birth was determined (Table 1). The polymorphisms investigated were in the TNF-α gene (-308) and in the MBL gene (-221 and codons 52, 54, and 57). Overall, 76.1% of our non-cerebral palsy population tested positive for at least one of the polymorphisms. MBL -221 was the most prevalent polymorphism in our non-cerebral palsy newborn Caucasian population, with 36.3% of babies being heterozygous. Of the babies with multiple cytokine polymorphisms, the most common combination observed was heterozygosity for both MBL -221 and codon 54 (9.5%), followed by heterozygosity for MBL -221 and TNF-α (9.2%) (Table 2).

The effect of gestational age on the prevalence of these polymorphisms was also investigated, as there was a higher rate of prematurity in the population from which many of the controls were selected. No significant differences were identified between the term and preterm populations.

Table 1: Prevalence of inherited cytokine polymorphisms in a population of non-cerebral palsy Caucasian South Australian newborns, expressed as percentage positive of the total tested with results.

Polymorphism	Zygosity	Positive	Total	Prevalence (%) (95%CI)	
TNF-α -308	Heterozygous	229	854	26.8	23.9-29.9
	Homozygous	37		4.3	3.1-5.9
MBL -221	Heterozygous	310	854	36.3	33.1-39.6
	Homozygous	40		4.7	3.4-6.3
MBL Exon 1 Codon 52	Heterozygous	19	845	2.3	1.4-3.5
	Homozygous	3		0.4	0.07-1.0
MBL Exon 1 Codon 54	Heterozygous	225	845	26.6	23.7-29.8
	Homozygous	20		2.4	1.5-3.6
MBL Exon 1 Codon 57	Heterozygous	19	845	2.2	1.4-3.5
	Homozygous	0		0	0.0-0.4
MBL Exon 1 Codon 52 and Codon 54	Compound Heterozygous	5	845	0.6	0.2-1.4
MBL Exon 1 Codon 52 and Codon 57	Compound Heterozygous	1	845	0.1	0.0-0.7
MBL Exon 1 Codon 54 and Codon 57	Compound Heterozygous	11	845	1.3	0.7-2.3
Any Cytokine Polymorphism	Homozygous or heterozygous	645	848	76.1	73.0-78.9

Table 2: Prevalence of inherited cytokine polymorphisms in more than one of the genes studied in a population of non-cerebral palsy Caucasian South Australian newborns, expressed as percentage positive of the total tested.

Mutation Combinations	Positive	Total	Prevalence (%)	(95%CI)
Heterozygous MBL -221, heterozygous TNF	78		9.2	7.3-11.3
Heterozygous MBL -221, homozygous TNF	14	852	1.6	0.9-2.7
Homozygous MBL -221, heterozygous TNF	10		1.2	0.6-2.1
Heterozygous MBL -221, heterozygous MBL 52	7		0.8	0.3-1.7
Heterozygous MBL -221, heterozygous MBL 54	80	843	9.5	7.6-11.8
Heterozygous MBL -221, heterozygous MBL 57	4		0.5	0.1-1.2
Heterozygous TNF, heterozygous MBL 52	4		0.5	0.1-1.2
Homozygous TNF, heterozygous MBL 52	1		0.1	0.0-0.7
Heterozygous TNF, heterozygous MBL 54	65		7.7	6.0-9.7
Heterozygous TNF, homozygous MBL 54	6		0.7	0.3-1.5
Homozygous TNF, heterozygous MBL 54	13		1.5	0.8-2.6
Heterozygous TNF, heterozygous MBL 57	5	844	0.6	0.2-1.4
Heterozygous TNF, heterozygous MBL 5254	1		0.1	0.0-0.7
Heterozygous TNF, heterozygous MBL 5257	1		0.1	0.0-0.7
Heterozygous TNF, heterozygous MBL 5457	2		0.2	0.0-0.8
Homozygous TNF, heterozygous MBL 5254	1		0.1	0.0-0.7
Homozygous TNF, heterozygous MBL 5457	1		0.1	0.0-0.7
Heterozygous MBL -221, heterozygous TNF, heterozygous MBL 52	2		0.2	0.0-0.9
Heterozygous MBL -221, heterozygous TNF, heterozygous MBL 54	16	842	1.9	1.1-3.1
Heterozygous MBL -221, homozygous TNF, heterozygous MBL 54	4		0.5	0.1-1.2

Cytokines and Cerebral Palsy

Associations between cytokine polymorphisms and cerebral palsy were investigated, taking into consideration gestational age and cerebral palsy subtypes.

All Controls

Cerebral Palsy All Gestational Ages

There was an increased risk of developing quadriplegia in the presence of heterozygous codon 52 (OR 2.72, 95% CI 0.85-7.40) and also in the presence of any abnormal allele (OR 2.74, 95% CI 0.95-6.96), despite the confidence intervals crossing unity (Table 3). For the exon 1 codon 54 polymorphism, an association was found for diplegia for heterozygotes (OR 1.62, 95% CI 1.07-2.44) as well as for the presence of any abnormal allele (OR 1.55, 95% CI 1.03-2.32) (Table 3). No associations were observed for TNF-α -308, MBL -221 or MBL Exon 1 codon 57, or when comparing any abnormal allele with all wild-type alleles in exon 1 (Table 3). Overall, the presence of any cytokine polymorphism increased the risk of CP, with an odds ratio of 1.37 (95% CI 1.02-1.84), with 335 (81.3%) CP cases carrying at least one cytokine polymorphism compared with 645 (76.1%) controls.

Cerebral Palsy Gestational Ages ≥37 weeks

227 of the 414 cerebral palsy cases (54.8%) were born at gestational ages ≥37 weeks. Heterozygous TNF-α was associated with quadriplegia (OR 1.82, 95% CI 1.04-3.15) (Table 4). Heterozygous codon 52 was also associated with quadriplegia (OR 3.75, 95% CI 1.03-11.13), as was any abnormal allele (OR 3.24, 95% CI 0.91-9.42). Codon 54 was associated with diplegia for those heterozygous for the polymorphism (OR 2.20, 95% CI 1.13-4.24) or possessing any abnormal allele (OR 2.12, 95% CI 1.10-4.05) (Table 4). Finally, the presence of any abnormal allele in exon 1 of the MBL gene approximately doubled the risk of developing diplegia, with an odds ratio of 1.94 (95% CI 1.05-3.62) (Table 4). No associations were observed for MBL -221 or Exon 1 codon 57.

Cerebral Palsy Gestational Ages 32-36 weeks

59 of the 414 cerebral palsy cases (14.3%) were born between 32-36 weeks gestation. The data presented in Table 5 shows no significant association between any of the tested cytokine polymorphisms and cerebral palsy in children born between 32 and 36 weeks gestation.

Cerebral Palsy Gestational Ages <32 weeks

128 of the 414 cerebral palsy cases (30.9%) were born at gestational ages <32 weeks. Heterozygous or homozygous TNF-α was associated with an increased risk of developing hemiplegia, with an odds ratio of 2.38 (95% CI 1.02-5.58) (Table 6). Heterozygous MBL -221 was also associated with an increased risk of hemiplegia (OR 3.88, 95% CI 0.88-13.28). There was a trend towards a protective effect for hemiplegia in the presence of any abnormal exon 1 allele (OR 0.33, 95% CI 0.08-0.97), however this did not reach statistical significance ($p < 0.05$) (Table 6). The presence of any cytokine polymorphism increased the risk of all types of CP (OR 2.20, 95% CI 1.28-3.81) and quadriplegia (OR 9.13, 95% CI 1.24-67.45). No associations were observed for any of the individual exon 1 codons (Table 6).

Term Controls

Cerebral Palsy All Gestational Ages

When compared with term controls, a number of associations were found for TNF-α. There was an increased risk of developing cerebral palsy, when all cerebral palsy cases were considered together, for heterozygous TNF-α (OR 1.36, 95% CI 1.02-1.82). Heterozygous TNF-α also increased the risk of developing hemiplegia (OR 1.59, 95% CI 1.01-2.48), as did the presence of any abnormal allele (OR 1.56, 95% CI 1.01-2.39) (Table 7). The presence of any abnormal allele in codon 52 was associated with the subsequent development of quadriplegia (OR 2.81, 95% CI 0.93-7.67), despite the confidence intervals crossing unity due to small sample sizes (Table 7). No associations were observed when comparing any abnormal allele with all wild-type alleles in exon 1 (Table 7). The risk of developing CP increased in the presence of any cytokine polymorphism (OR 1.39, 95% CI 1.02-1.90), with 335 (81.3%) CP cases carrying at least one cytokine polymorphism compared with 456 (75.7%) term-born controls. No associations were observed for MBL -221, exon 1 codon 54 or codon 57.

Cerebral Palsy Gestational Ages ≥37 weeks

Heterozygous TNF-α was associated with an approximate doubling of the risk of quadriplegia, with an odds ratio of 1.99 (95% CI 1.12-3.47) (Table 8). Heterozygosity for codon 52 in the MBL gene was associated with an increased risk of diplegia (OR 3.57, 95% CI 0.95-11.19), as was the presence of any abnormal allele (OR 3.33, 95% CI 0.89-10.31), although the confidence intervals cross unity due to the small numbers involved.

There was an approximate doubling in the risk of developing diplegia in the presence of heterozygous codon 54 (OR 2.07, 95% CI 1.05-4.01), and also in the presence of any abnormal allele (OR 2.02, 95% CI 1.04-3.89) (Table 8). No associations were observed for MBL -221 or exon 1 codon 57, and the presence of any abnormal allele in the exon 1 gene was not associated with CP.

Cerebral Palsy Gestational Ages 32-36 weeks

As with the all control data above, the data presented in Table 9 shows no significant association between any of the tested cytokine polymorphisms and cerebral palsy in children born between 32 and 36 weeks gestation.

Cerebral Palsy Gestational Ages <32 weeks

Heterozygous TNF-α was associated with an increased risk of developing any type of cerebral palsy (OR 1.56, 95% CI 1.01-2.39). The presence of any abnormal TNF-α allele was associated with an increased risk of developing hemiplegia (OR 2.57, 95% CI 1.10-6.07) (Table 10). Heterozygous MBL -221 was associated with an increased risk of developing hemiplegia (OR 4.20, 95% CI 0.93-14.84). The presence of any exon 1 codon 54 allele was associated with a decreased risk of hemiplegia (OR 0.29, 95% CI 0.05-0.98). This protective effect was also seen when the presence of any abnormal allele in exon 1 of the MBL gene was compared with the wild-type gene, with a decreased risk of developing hemiplegia (OR 0.31, 95% CI 0.08-0.94). The risk of developing CP increased in the presence of any cytokine polymorphism (OR 2.24, 95% CI 1.29-3.91). No associations were observed for MBL exon 1 codon 52 or codon 57 (Table 10).

The wide confidence intervals for some of the above observations reflect small numbers in these subgroups, especially where the prevalence of the individual polymorphisms are uncommon in the control population.

Table 3: Odds ratios (95% CI) for cerebral palsy for specified cytokine polymorphisms among cerebral palsy babies born at all gestational ages compared with controls of all gestational ages. GA = Gestational Age. *p-values <0.05.

Type of Cerebral Palsy	Zygosity	GA	Odds Ratio (95% CI)					
			TNF-α -308	MBL -221	MBL Exon 1 Codon 52	MBL Exon 1 Codon 54	MBL Exon 1 Codon 57	MBL Exon 1 Abnormal
All Types (n = 414)	Homozygous	All	0.65 (0.29-1.33)	0.91 (0.48-1.66)	1.46 (0.12-12.85)	1.10 (0.45-2.50)	-	
	Heterozygous	All	1.25 (0.96-1.63)	0.95 (0.73-1.23)	1.27 (0.54-2.86)	1.22 (0.93-1.60)	1.04 (0.41-2.45)	
	Homo or Hetero	All	1.17 (0.90-1.51)	0.94 (0.74-1.21)	1.30 (0.59-2.74)	1.21 (0.93-1.58)	-	1.18 (0.92-1.52)
Diplegia (n = 131)	Homozygous	All	0.55 (0.11-1.81)	1.16 (0.42-2.74)	2.47 (0.05-31.22)	0.74 (0.08-3.16)	-	
	Heterozygous	All	1.19 (0.78-1.82)	1.03 (0.68-1.54)	0.00 (0.00-1.51)	**1.62 (1.07-2.44)***	1.17 (0.22-4.13)	
	Homo or Hetero	All	1.11 (0.73-1.67)	1.04 (0.70-1.54)	0.34 (0.01-2.15)	**1.55 (1.03-2.32)***	-	1.37 (0.93-2.03)
Hemiplegia (n = 119)	Homozygous	All	1.32 (0.44-3.31)	1.31 (0.51-2.97)	0.00 (0.00-8.91)	1.72 (0.49-4.88)	-	
	Heterozygous	All	1.46 (0.94-2.25)	0.72 (0.45-1.12)	1.44 (0.35-4.50)	0.76 (0.45-1.25)	1.08 (0.20-3.80)	
	Homo or Hetero	All	1.44 (0.95-2.18)	0.79 (0.51-1.19)	1.25 (0.30-3.81)	0.84 (0.52-1.33)	-	0.88 (0.57-1.35)
Quadriplegia (n = 112)	Homozygous	All	0.45 (0.05-1.80)	0.19 (0.01-1.19)	2.87 (0.05-36.24)	1.29 (0.24-4.53)	-	
	Heterozygous	All	1.41 (0.90-2.18)	1.15 (0.75-1.75)	**2.72 (0.85-7.40)***	1.26 (0.78-2.01)	1.36 (0.25-4.80)	
	Homo or Hetero	All	1.28 (0.82-1.96)	1.04 (0.68-1.58)	**2.74 (0.95-6.96)***	1.26 (0.79-1.99)	-	1.36 (0.90-2.07)

Table 4: Odds ratios (95% CI) for cerebral palsy for specified cytokine polymorphisms among cerebral palsy babies born at ≥ 37 weeks gestation compared with controls of all gestational ages. GA = Gestational Age. *p-values <0.05.

Type of Cerebral Palsy	Zygosity	GA	Odds Ratio (95% CI)					
			TNF-α -308	MBL -221	MBL Exon 1 Codon 52	MBL Exon 1 Codon 54	MBL Exon 1 Codon 57	MBL Exon 1 Abnormal
All Types (n = 227)	Homozygous	All	0.43 (0.11-1.22)	0.52 (0.18-1.26)	1.35 (0.03-16.93)	1.62 (0.60-3.93)		
	Heterozygous	All	1.28 (0.92-1.78)	0.84 (0.60-1.15)	1.70 (0.63-4.18)	1.19 (0.84-1.67)	1.49 (0.52-3.79)	
	Homo or Hetero	All	1.17 (0.84-1.60)	0.80 (0.58-1.09)	1.65 (0.65-3.84)	1.22 (0.87-1.70)		1.23 (0.90-1.68)
Diplegia (n = 49)	Homozygous	All	0.96 (0.11-4.03)	0.79 (0.09-3.29)	7.86 (0.14-101.31)	1.18 (0.03-8.05)		
	Heterozygous	All	1.09 (0.53-2.14)	0.76 (0.38-1.48)	0.00 (0.00-4.86)	**2.20 (1.13-4.24)***	2.48 (0.26-11.32)	
	Homo or Hetero	All	1.07 (0.54-2.05)	0.77 (0.39-1.45)	1.07 (0.02-7.25)	**2.12 (1.10-4.05)***		**1.94 (1.05-3.62)***
Hemiplegia (n = 81)	Homozygous	All	0.62 (0.07-2.54)	0.68 (0.13-2.23)	0.00 (0.00-13.89)	2.66 (0.75-7.69)		
	Heterozygous	All	1.41 (0.83-2.34)	0.64 (0.36-1.09)	1.12 (0.12-4.85)	0.90 (0.49-1.59)	1.68 (0.31-5.98)	
	Homo or Hetero	All	1.30 (0.78-2.13)	0.64 (0.38-1.07)	0.97 (0.11-4.12)	1.04 (0.60-1.77)		1.05 (0.64-1.73)
Quadriplegia (n = 65)	Homozygous	All	0.00 (0.00-1.62)	0.00 (0.00-1.22)	0.00 (0.00-18.75)	1.43 (0.16-6.24)		
	Heterozygous	All	**1.82 (1.04-3.15)***	1.02 (0.58-1.76)	**3.75 (1.03-11.13)***	1.08 (0.56-2.00)	1.50 (0.16-6.61)	
	Homo or Hetero	All	1.57 (0.90-2.70)	0.90 (0.51-1.55)	**3.24 (0.91-9.42)***	1.11 (0.59-2.01)		1.27 (0.74-2.18)

Table 5: Odds ratios (95% CI) for cerebral palsy for specified cytokine polymorphisms among cerebral palsy babies born at 32-36 weeks gestation compared with controls of all gestational ages. GA = Gestational Age. All p-values NS.

Type of Cerebral Palsy	Zygosity	GA	Odds Ratio (95% CI)					
			TNF-α -308	MBL -221	MBL Exon 1 Codon 52	MBL Exon 1 Codon 54	MBL Exon 1 Codon 57	MBL Exon 1 Abnormal
All Types (n = 227)	Homozygous	All	1.14 (0.22-3.83)	1.05 (0.20-3.56)	0.00 (0.00-21.02)	0.00 (0.00-3.11)		
	Heterozygous	All	0.79 (0.38-1.54)	0.90 (0.49-1.64)	0.84 (0.02-5.61)	1.56 (0.85-2.81)	0.00 (0.00-3.27)	
	Homo or Hetero	All	0.84 (0.43-1.56)	0.92 (0.51-1.63)	0.72 (0.02-4.77)	1.43 (0.78-2.58)		1.32 (0.74-2.32)
Diplegia (n = 49)	Homozygous	All	0.00 (0.00-5.18)	1.15 (0.03-8.25)	0.00 (0.00-62.57)	0.00 (0.00-8.95)		
	Heterozygous	All	1.50 (0.49-4.18)	1.18 (0.41-3.27)	0.00 (0.00-9.43)	1.41 (0.46-3.93)	0.00 (0.00-9.43)	
	Homo or Hetero	All	1.29 (0.42-3.60)	1.18 (0.43-3.16)	0.00 (0.00-8.12)	1.29 (0.42-3.60)		1.19 (0.44-3.17)
Hemiplegia (n = 81)	Homozygous	All	1.99 (0.04-15.48)	1.58 (0.03-12.24)	0.00 (0.00-134.19)	0.00 (0.00-18.13)		
	Heterozygous	All	0.64 (0.07-3.25)	0.41 (0.04-2.06)	4.75 (0.10-42.11)	1.20 (0.19-5.70)	0.00 (0.00-19.12)	
	Homo or Hetero	All	0.83 (0.14-3.49)	0.54 (0.09-2.27)	4.10 (0.09-36.02)	1.10 (0.18-5.23)		1.49 (0.36-5.91)
Quadriplegia (n = 65)	Homozygous	All	2.44 (0.26-11.41)	1.40 (0.03-10.55)	0.00 (0.00-76.19)	0.00 (0.00-10.78)		
	Heterozygous	All	0.40 (0.04-1.77)	1.26 (0.40-3.86)	0.00 (0.00-11.37)	1.69 (0.54-4.97)	0.00 (0.00-11.37)	
	Homo or Hetero	All	0.68 (0.16-2.23)	1.28 (0.43-3.78)	0.00 (0.00-9.77)	1.55 (0.49-4.57)		1.25 (0.43-3.61)

Table 6: Odds ratios (95% CI) for cerebral palsy for specified cytokine polymorphisms among cerebral palsy babies born at <32 weeks gestation compared with controls of all gestational ages. GA = Gestational Age. *p-values <0.05.

Type of Cerebral Palsy	Zygosity	GA	Odds Ratio (95% CI)					
			TNF-α -308	MBL -221	MBL Exon 1 Codon 52	MBL Exon 1 Codon 54	MBL Exon 1 Codon 57	MBL Exon 1 Abnormal
All Types (n = 227)	Homozygous	All	0.80 (0.20-2.33)	1.67 (0.68-3.69)	2.29 (0.04-28.83)	0.69 (0.08-2.92)		
	Heterozygous	All	1.43 (0.94-2.17)	1.22 (0.81-1.83)	0.72 (0.08-3.09)	1.13 (0.72-1.75)	0.72 (0.08-3.09)	
	Homo or Hetero	All	1.34 (0.89-2.00)	1.27 (0.86-1.88)	0.94 (0.18-3.22)	1.09 (0.70-1.68)		1.04 (0.69-1.56)
Diplegia (n = 49)	Homozygous	All	0.39 (0.01-2.43)	1.53 (0.37-4.61)	0.00 (0.00-18.75)	0.71 (0.02-4.71)		
	Heterozygous	All	1.19 (0.64-2.15)	1.23 (0.69-2.18)	0.00 (0.00-2.92)	1.33 (0.72-2.39)	0.75 (0.02-4.99)	
	Homo or Hetero	All	1.08 (0.59-1.93)	1.27 (0.73-2.19)	0.00 (0.00-2.52)	1.28 (0.71-2.28)		1.08 (0.61-1.91)
Hemiplegia (n = 81)	Homozygous	All	3.67 (0.64-14.14)	**3.88 (0.88-13.28)***	0.00 (0.00-32.98)	0.00 (0.00-4.83)		
	Heterozygous	All	2.17 (0.87-5.34)	1.25 (0.48-3.13)	1.30 (0.03-8.94)	0.33 (0.06-1.11)	0.00 (0.00-5.09)	
	Homo or Hetero	All	**2.38 (1.02-5.58)***	1.55 (0.67-3.63)	1.12 (0.03-7.61)	0.30 (0.06-1.02)		**0.33 (0.08-0.97)***
Quadriplegia (n = 65)	Homozygous	All	0.00 (0.00-3.09)	0.00 (0.00-3.07)	12.04 (0.21-158.12)	1.81 (0.04-12.92)		
	Heterozygous	All	1.28 (0.53-2.93)	1.42 (0.63-3.16)	1.90 (0.01-13.67)	1.45 (0.55-3.58)	1.90 (0.04-13.67)	
	Homo or Hetero	All	1.11 (0.46-2.52)	1.26 (0.56-2.79)	3.28 (0.34-15.53)	1.47 (0.58-3.56)		1.67 (0.75-3.71)

Table 7: Odds ratios (95% CI) for cerebral palsy for specified cytokine polymorphisms among cerebral palsy babies born at all gestational ages compared with controls of term gestational ages. GA = Gestational Age. *p-values <0.05.

Type of Cerebral Palsy	Zygosity	GA	Odds Ratio (95% CI)					
			TNF-α -308	MBL -221	MBL Exon 1 Codon 52	MBL Exon 1 Codon 54	MBL Exon 1 Codon 57	MBL Exon 1 Abnormal
All Types (n = 414)	Homozygous	≥ 37	0.67 (0.30-1.44)	0.98 (0.50-1.91)	3.08 (0.16-181.98)	1.28 (0.49-3.29)	-	
	Heterozygous	≥ 37	**1.36 (1.02-1.82)***	0.92 (0.70-1.20)	1.21 (0.49-2.92)	1.14 (0.85-1.53)	0.99 (0.37-2.50)	
	Homo or Hetero	≥ 37	1.26 (0.96-1.66)	0.92 (0.71-1.20)	1.33 (0.57-3.06)	1.15 (0.88-1.53)	-	1.13 (0.87-1.48)
Diplegia (n = 131)	Homozygous	≥ 37	0.57 (0.11-1.94)	1.26 (0.44-3.11)	5.21 (0.07-409.63)	0.87 (0.09-4.02)	-	
	Heterozygous	≥ 37	1.30 (0.83-2.01)	0.99 (0.65-1.50)	0.00 (0.00-1.44)	1.52 (0.99-2.32)	1.12 (0.20-4.14)	
	Homo or Hetero	≥ 37	1.20 (0.78-1.82)	1.02 (0.68-1.52)	0.35 (0.01-2.33)	1.47 (0.97-2.24)	-	1.32 (0.88-1.97)
Hemiplegia (n = 119)	Homozygous	≥ 37	1.37 (0.45-3.56)	1.42 (0.53-3.38)	0.00 (0.00-188.09)	2.00 (0.54-6.32)	-	
	Heterozygous	≥ 37	**1.59 (1.01-2.48)***	0.69 (0.43-1.09)	1.37 (0.32-4.53)	0.72 (0.42-1.18)	1.03 (0.19-3.82)	
	Homo or Hetero	≥ 37	**1.56 (1.01-2.39)***	0.77 (0.50-1.18)	1.28 (0.30-4.17)	0.80 (0.49-1.29)	-	0.85 (0.55-1.31)
Quadriplegia (n = 112)	Homozygous	≥ 37	0.46 (0.05-1.92)	0.21 (0.01-1.33)	6.03 (0.08-474.65)	1.51 (0.27-5.80)	-	
	Heterozygous	≥ 37	1.54 (0.97-2.41)	1.11 (0.72-1.71)	2.59 (0.78-7.47)	1.18 (0.72-1.91)	1.30 (0.23-4.82)	
	Homo or Hetero	≥ 37	1.38 (0.88-2.14)	1.02 (0.66-1.56)	**2.81 (0.93-7.67)***	1.21 (0.75-1.92)	-	1.31 (0.85-2.01)

Table 8: Odds ratios (95% CI) for cerebral palsy for specified cytokine polymorphisms among cerebral palsy babies born at ≥ 37 weeks gestation compared with controls of term gestational ages. GA = Gestational Age. *p-values <0.05.

Type of Cerebral Palsy	Zygosity	GA	Odds Ratio (95% CI)					
			TNF-α -308	MBL -221	MBL Exon 1 Codon 52	MBL Exon 1 Codon 54	MBL Exon 1 Codon 57	MBL Exon 1 Abnormal
All Types (n = 227)	Homozygous	≥ 37	0.44 (0.11-1.31)	0.56 (0.19-1.43)	2.84 (0.04-223.17)	1.89 (0.65-5.15)		
	Heterozygous	≥ 37	1.40 (0.98-1.98)	0.81 (0.57-1.13)	1.62 (0.57-4.24)	1.11 (0.77-1.59)	1.42 (0.47-3.85)	
	Homo or Hetero	≥ 37	1.26 (0.90-1.76)	0.78 (0.56-1.08)	1.70 (0.64-4.26)	1.17 (0.82-1.65)		1.18 (0.85-1.63)
Diplegia (n = 49)	Homozygous	≥ 37	1.10 (0.11-4.29)	0.85 (0.09-3.68)	16.52 (0.20-1299.92)	1.38 (0.03-10.09)		
	Heterozygous	≥ 37	1.19 (0.57-2.35)	0.74 (0.36-1.44)	0.00 (0.00-4.65)	**2.07 (1.05-4.01)***	2.36 (0.25-11.26)	
	Homo or Hetero	≥ 37	1.16 (0.58-2.23)	0.75 (0.38-1.43)	1.10 (0.03-7.80)	**2.02 (1.04-3.89)***		1.87 (1.00-3.51)
Hemiplegia (n = 81)	Homozygous	≥ 37	0.65 (0.07-2.71)	0.73 (0.14-2.51)	0.00 (0.00-291.35)	3.10 (0.82-9.92)		
	Heterozygous	≥ 37	1.54 (0.90-2.58)	0.62 (0.35-1.06)	1.06 (0.11-4.85)	0.84 (0.46-1.50)	1.60 (0.28-6.00)	
	Homo or Hetero	≥ 37	1.41 (0.84-2.33)	0.63 (0.37-1.06)	0.99 (0.11-4.47)	0.99 (0.57-1.70)		1.01 (0.61-1.68)
Quadriplegia (n = 65)	Homozygous	≥ 37	0.00 (0.00-1.68)	0.00 (0.00-1.33)	0.00 (0.00-391.03)	1.67 (0.17-7.91)		
	Heterozygous	≥ 37	**1.99 (1.12-3.47)***	0.98 (0.55-1.71)	**3.57 (0.95-11.19)***	1.01 (0.52-1.90)	1.43 (0.15-6.59)	
	Homo or Hetero	≥ 37	1.70 (0.97-2.95)	0.88 (0.50-1.53)	**3.33 (0.89-10.31)***	1.06 (0.56-1.94)		1.22 (0.70-2.12)

Table 9: Odds ratios (95% CI) for cerebral palsy for specified cytokine polymorphisms among cerebral palsy babies born at 32-36 weeks gestation compared with controls of term gestational ages. GA = Gestational Age. All p-values NS.

Type of Cerebral Palsy	Zygosity	GA	Odds Ratio (95% CI)					
			TNF-α -308	MBL -221	MBL Exon 1 Codon 52	MBL Exon 1 Codon 54	MBL Exon 1 Codon 57	MBL Exon 1 Abnormal
All Types (n = 227)	Homozygous	≥ 37	1.18 (0.22-4.09)	1.14 (0.21-4.00)	0.00 (0.00-437.03)	0.00 (0.00-3.65)		
	Heterozygous	≥ 37	0.87 (0.41-1.70)	0.87 (0.47-1.59)	0.80 (0.02-5.57)	1.46 (0.79-2.66)	0.00 (0.00-3.13)	
	Homo or Hetero	≥ 37	0.91 (0.47-1.71)	0.90 (0.50-1.60)	0.75 (0.02-5.14)	1.37 (0.74-2.48)		1.26 (0.71-2.25)
Diplegia (n = 49)	Homozygous	≥ 37	0.00 (0.00-5.41)	1.24 (0.03-9.17)	0.00 (0.00-1238.25)	0.00 (0.00-10.62)		
	Heterozygous	≥ 37	1.63 (0.53-4.59)	1.14 (0.39-3.17)	0.00 (0.00-9.06)	1.32 (0.43-3.71)	0.00 (0.00-9.06)	
	Homo or Hetero	≥ 37	1.39 (0.46-3.91)	1.15 (0.42-3.11)	0.00 (0.00-8.44)	1.23 (0.40-3.46)		1.15 (0.42-3.06)
Hemiplegia (n = 81)	Homozygous	≥ 37	2.06 (0.04-16.33)	1.71 (0.04-13.56)	0.00 (0.00-2476.50)	0.00 (0.00-21.72)		
	Heterozygous	≥ 37	0.70 (0.07-3.56)	0.39 (0.04-1.99)	4.52 (0.09-41.18)	1.13 (0.18-5.37)	0.00 (0.00-18.46)	
	Homo or Hetero	≥ 37	0.90 (0.15-3.79)	0.53 (0.09-2.23)	4.22 (0.09-38.18)	1.06 (0.17-5.01)		1.43 (0.34-5.70)
Quadriplegia (n = 65)	Homozygous	≥ 37	2.53 (0.26-12.10)	1.52 (0.03-11.70)	0.00 (0.00-1485.90)	0.00 (0.00-12.82)		
	Heterozygous	≥ 37	0.43 (0.05-1.94)	1.22 (0.38-3.74)	0.00 (0.00-10.93)	1.58 (0.50-4.69)	0.00 (0.00-10.93)	
	Homo or Hetero	≥ 37	0.74 (0.17-2.42)	1.25 (0.41-3.71)	0.00 (0.00-10.18)	1.48 (0.47-4.38)		1.20 (0.41-3.49)

Table 10: Odds ratios (95% CI) for cerebral palsy for specified cytokine polymorphisms among cerebral palsy babies born at <32 weeks gestation compared with controls of term gestational ages. GA = Gestational Age. *p-values <0.05.

Type of Cerebral Palsy	Zygosity	GA	Odds Ratio (95% CI)					
			TNF-α -308	MBL -221	MBL Exon 1 Codon 52	MBL Exon 1 Codon 54	MBL Exon 1 Codon 57	MBL Exon 1 Abnormal
All Types (n = 227)	Homozygous	≥ 37	0.83 (0.21-2.50)	1.80 (0.71-4.19)	4.81 (0.06-378.52)	0.80 (0.09-3.71)		
	Heterozygous	≥ 37	**1.56 (1.01-2.39)***	1.18 (0.77-1.79)	0.69 (0.07-3.09)	1.06 (0.67-1.66)	0.69 (0.07-3.09)	
	Homo or Hetero	≥ 37	1.45 (0.95-2.20)	1.24 (0.83-1.86)	0.96 (0.17-3.52)	1.04 (0.66-1.62)		1.00 (0.66-1.52)
Diplegia (n = 49)	Homozygous	≥ 37	0.40 (0.01-2.58)	1.66 (0.40-5.19)	0.00 (0.00-391.03)	0.83 (0.02-5.93)		
	Heterozygous	≥ 37	1.30 (0.69-2.37)	1.19 (0.66-2.12)	0.00 (0.00-2.79)	1.25 (0.67-2.26)	0.71 (0.02-4.95)	
	Homo or Hetero	≥ 37	1.17 (0.63-2.10)	1.24 (0.71-2.16)	0.00 (0.00-2.60)	1.22 (0.67-2.19)		1.04 (0.58-1.85)
Hemiplegia (n = 81)	Homozygous	≥ 37	3.80 (0.65-15.02)	**4.20 (0.93-14.84)***	0.00 (0.00-675.41)	0.00 (0.00-5.70)		
	Heterozygous	≥ 37	2.37 (0.94-5.85)	1.21 (0.47-3.04)	1.23 (0.03-8.86)	0.31 (0.06-1.05)	0.00 (0.00-4.87)	
	Homo or Hetero	≥ 37	**2.57 (1.10-6.07)***	1.52 (0.65-3.57)	1.15 (0.03-8.19)	**0.29 (0.05-0.98)***		**0.31 (0.08-0.94)***
Quadriplegia (n = 65)	Homozygous	≥ 37	0.00 (0.00-3.22)	0.00 (0.00-3.35)	25.33 (0.30-1992.95)	2.11 (0.05-16.12)		
	Heterozygous	≥ 37	1.40 (0.57-3.22)	1.37 (0.61-3.07)	1.81 (0.04-13.51)	1.36 (0.51-3.38)	1.81 (0.04-13.51)	
	Homo or Hetero	≥ 37	1.20 (0.49-2.74)	1.23 (0.55-2.75)	3.38 (0.34-16.71)	1.40 (0.55-3.42)		1.60 (0.72-3.59)

Discussion

This is the first reported study examining the associations between CP and polymorphisms in the TNF-α and MBL genes. Previous research has focused on the measurement of cytokine levels rather than genotyping of polymorphic sites capable of altering circulating cytokine concentrations. The present study has demonstrated positive associations with cerebral palsy, in particular the hemiplegic and quadriplegic subtypes, for both the TNF-α and MBL polymorphisms, with increases in the risk of the three main cerebral palsy subtypes observed for babies of differing gestational ages. The involvement of these polymorphisms is in line with previous findings showing that infection and white matter damage can account for up to 14.7% of hemiplegia and 44.2% of quadriplegia (259). The prevalence of abnormal cytokine polymorphisms was 81.3% for CP cases and 76.1% for controls, giving a potential attributable risk of 5.2% of all CP cases if there is a causal relationship.

448 separate analyses were performed on the individual polymorphisms, with further analyses involving combinations of these polymorphisms. Such multiple analyses increase the likelihood of identifying chance statistical associations. Furthermore, because of small numbers in some of the subanalyses where the prevalence of some polymorphisms is uncommon in the control group, other associations cannot be confidently excluded. Where associations are seen in one gestational age range, similar trends are seen in the same subgroup of cerebral palsy with the same polymorphism in other gestational age ranges. This suggests that these may indicate true causal relationships and may not be attributed to chance. Nevertheless, these associations require further study.

Tumour Necrosis Factor α

TNF-α is a proinflammatory cytokine with a promoter polymorphism capable of altering circulating concentrations of TNF-α. The observed prevalences of 26.8% and 4.3% for heterozygous and homozygous carriers respectively are consistent with other documented Australian and world prevalences (232, 275, 276).

The role of proinflammatory cytokines, particularly TNF-α, in the development of preterm birth has been extensively investigated, demonstrating strong associations between the two (51, 77, 124-126, 141, 232, 277). Children who subsequently develop cerebral palsy have been shown to have higher mean neonatal concentrations of circulating proinflammatory cytokines, including TNF-α (18). Concentrations of TNF-α in amniotic fluid have also

been shown to be higher in patients with intrauterine infection and subsequent preterm labour (68, 74). Preterm birth is itself a recognised risk factor for the development of cerebral palsy, and this may be one mechanism by which proinflammatory cytokines may contribute to the development of cerebral palsy.

TNF-α is directly toxic to neurons and may cause white matter damage with periventricular leukomalacia (PVL) through its cytotoxic effect, by damaging oligodendrocytes (72), and also by disturbing developmental transitions from the oligodendrocyte precursor to the mature oligodendrocyte (131). Finally, inflammatory cytokines such as TNF-α can activate endothelial cells, promoting a shift from an antithrombotic to prothrombotic state (278). TNF-α can downregulate the expression of thrombomodulin on endothelial cells. Thrombomodulin forms a complex with thrombin, which subsequently activates protein C, an important anticoagulant protein, and downregulation of thrombomodulin thus causes the endothelial cell surface to be procoagulant rather than anticoagulant (90). One of the major pathways for TNF to exert a prothrombotic effect is by increasing tissue factor and suppressing the tissue factor pathway inhibitor.

PVL is a heterogeneous white matter lesion, inducible by a number of different insults, including both ischaemic and inflammatory processes (51). The presence of PVL is a strong indicator of subsequent development of cerebral palsy, with 60-100% of preterm infants with PVL developing cerebral palsy. Approximately 90% of preterm infants who develop cerebral palsy also have magnetic resonance imaging (MRI) of gliosis as a predictor of perinatal PVL (45, 54-56, 177). TNF-α expression in brain cells has been associated with PVL, with TNF-α positive cells identified in the white matter of 9/13 PVL cases (69%) in one study (73), and in 15/17 (88%) of cases with PVL compared with 3/17 (18%) of cases without PVL (72). These results are highly suggestive of damage caused by cytokine production in the white matter regions of the brain. Infants with higher TNF-α concentrations in brain tissue also show evidence of lymphocytes infiltrating the brain (72).

The results from the present study suggest that the TNF-2 polymorphism, which has been previously associated with higher circulating levels of TNF-α, can be detrimental to the fetus, and directly or indirectly cause brain damage. The combination of increased levels of TNF-α as a result of the promoter polymorphism at -308, and the normal physiological upregulation of TNF-α production as a result of infection, may contribute to the pathogenesis of white matter damage. Its cytotoxic effect on brain oligodendrocytes and

also its ability to increase permeability of the blood-brain barrier (70, 182), may also allow the passage of potentially toxic mediators into the incredibly sensitive developing brain. TNF-α may also exert its effects through initiation of preterm delivery, an independent risk factor for the development of cerebral palsy. Because of the prothrombotic effects of TNF-α, the additional presence of a thrombophilic polymorphism may affect the clinical phenotype. An infection in a fetus carrying the TNF-α polymorphism may result in a more severe FIRS with exaggerated degree of intravascular thrombosis, subsequently resulting in cerebral palsy, in a fetus also carrying a thrombophilic polymorphism. Such interactions are currently being investigated by this research group, where associations between inherited thrombophilia and cerebral palsy have been documented (279).

Mannose Binding Lectin

MBL is a serum protein of hepatic origin, and belongs to a family of calcium dependent collagenous lectins (146). The current observed prevalences of 4 polymorphisms within the MBL gene are consistent with other documented Australian and world prevalences (232, 275, 276, 280).

The first report of associations between recurrent infections and low levels of MBL was in 1989 (156). Since then, there have been many papers published investigating the associations between MBL efficiency and increased susceptibility of infection (144). It is important to note that 90% of MBL deficient individuals do not acquire repeated infections, possibly due to the redundancy of the complement system (144). It can therefore be postulated that the phenotypical manifestation of MBL deficiency is only apparent when combined with another immunodeficiency, either acquired or genetically determined. MBL has also been shown to bind to the human immunodeficiency virus (HIV), and prevent infection of cells (158). Therefore, low levels of MBL could be deleterious to the fetus when exposed to infection, as the MBL pathway plays an important role in eliminating pathogens, especially in neonates and infants (159). A fetus deficient in MBL may be more susceptible to sub-clinical infections and/or inflammatory events *in utero* (163).

Mutations in the MBL gene are strongly associated with children presenting to hospital with infection, and these mutations increase susceptibility to infection in children who are heterozygous or homozygous for the mutations (281). There is a highly significant association of mutant MBL genotypes with infection. In one particular study, mutations were present in approximately twice as many children with infection as in control children.

This is similar to an earlier study of children with an opsonic defect; (166), however it contrasts another study which found no association between increased risk of infection and children heterozygous for mutant MBL gene alleles (167). This discrepancy may, however, be explained by the use of an adult control group rather than concurrent controls matched for age (167). This increased risk of infection may be of greatest importance when immune responses are either immature (such as in the neonate) or defective. Recent reports have described association between low-producing MBL alleles and disease severity in diseases where immunity is already significantly impaired (168, 169). It has been suggested that low-producing alleles of MBL will also influence the clinical phenotype of other immunodeficiency diseases (147).

Results from this study show that there are associations between polymorphisms in the MBL gene and the subsequent development of cerebral palsy. All of the significant associations with the exon 1 codon 52 polymorphism result in 2.5-3.5 times increased risk of developing quadriplegia, consistent with the common etiologies of development of quadriplegia being PVL and infection (259). Positive associations seen in babies born at term were associated with diplegia and quadriplegia, whereas associations in very preterm babies (<32 weeks) were associated with hemiplegia. It is hypothesised that decreased levels of MBL as a result of polymorphisms in the gene may contribute to the pathogenesis of cerebral palsy via a decreased innate immune response to infection, thus increasing susceptibility to infection and therefore the likelihood of adverse effects from infection. This theory will be further examined using multivariable analysis.

In summary, cytokines are produced in response to infection, and are able to mediate intravascular cell adhesion, coagulation and/or thrombosis, and vasoconstriction (70). In the presence of an existing thrombophilia or a cytokine polymorphism resulting in either increased response or susceptibility to infection, the actions of these cytokines in the fetal brain may be enough of an insult to cause cerebral palsy. Associations between fetal thrombophilia and cerebral palsy, and fetal viral infection and cerebral palsy have also been found in this same cohort (279, 282). In this chapter it has been demonstrated that carriage of polymorphisms in cytokines capable of altering circulating levels of those cytokines are associated with the subsequent development of cerebral palsy, either directly through cytokine-mediated brain damage, or indirectly via infection or thrombosis and the subsequent altered immune response to that infection.

Neurotropic viruses are associated with cerebral palsy

Abstract

Objective: Signs of possible intrauterine infection are strongly associated with the development of cerebral palsy. Direct evidence for perinatal exposure to neurotropic viruses and cerebral palsy (CP) outcome was sought.

Methods: A population-based case-control study tested newborn screening cards of 443 Caucasian CP cases and 883 Caucasian controls for viral RNA and DNA from enteroviruses and herpesviruses using polymerase chain reaction.

Results: The prevalence of the viral nucleic acids in the control population was high, with 39.8% of controls testing positive. The detection of VZV, HHV-6 or HHV-7 viral nucleic acids increased the risk of developing CP when all CP cases were considered as a group (OR 1.68, 95% CI 1.09-2.59) and hemiplegia (OR 2.07, 95% CI 1.10-3.88) at all gestational ages. For babies born ≥37 weeks gestation, these viral nucleic acids were also associated with the development of diplegia (OR 2.45, 95% CI 1.02-5.89) and hemiplegia (OR 2.38, 95% CI 1.15-4.92); for babies born preterm these viral nucleic acids were associated with the development of quadriplegia (OR 2.87, 95% CI 1.09-7.59). The risk of CP also increased in the presence of any herpesvirus (OR 1.52, 95% CI 1.09-2.13) or any virus (OR 1.64, 95% CI 1.17-2.28). The presence of more than one virus did not add to the risk of CP.

Conclusions: This is the first direct evidence that perinatal viral exposure is associated with CP, and reinforces the possibility of a complex and heterogeneous relationship between exposure to viral infections and subtypes of CP at different gestational ages.

Introduction

Intrauterine infection is postulated to be an important contributor to the development of cerebral palsy (6, 22, 283). To date, many studies have investigated the role of infection in the development of CP by using surrogate markers of infection, such as chorioamnionitis (histopathological evidence of infection, characterised by an inflammatory leucocyte infiltration of the chorion and amnion), funisitis (inflammation of the umbilical cord detected by histologic examination of the placenta), maternal pyrexia (body temperature greater than 37°C), raised C-reactive protein (CRP) and interleukin-6 concentrations (133). The research described below has used molecular techniques to determine the presence of viral nucleic acids in the blood of newborn babies, to investigate the role of infection in the development of CP.

The herpesviruses (including cytomegalovirus, herpes simplex viruses 1 and 2, varicella zoster virus, Epstein-Barr virus, and human herpesvirus 6, 7 and 8), and enteroviruses are all capable of crossing the placenta and setting up *in utero* infection (197-204). These viruses are potentially neurotropic and could contribute directly or indirectly to the causation of cerebral palsy. The likelihood of maternal infection resulting in infection of the fetus varies according to the specific virus, whether the infection is primary or recurrent, and the gestational age of the fetus at the time of infection. For example, the overall rate of congenital cytomegalovirus infection is approximately 1%, and of these, a 50% risk of transmission to the fetus occurs in mothers with primary infection but only a minority (<1%) of mothers with reactivation (or reinfection) transmit the virus *in utero*. Approximately 10-15% of CMV-infected newborns of mothers with primary infection and <1% with infection arising from maternal CMV reactivation display signs of the infection (197). Once the infection has crossed the placenta into the fetal circulation, there is the potential for neuronal damage, both by the infectious agent directly, and also by the fetal inflammatory response to the infection where pro-inflammatory induced cytokines may adversely effect the developing brain. This combination of factors may determine who develops CP.

As well as being capable of transplacental transmission and subsequent *in utero* infection, some viruses can persist for months or years after the initial infection (206-213). These viruses may have effects as long as thirty years after the original infection (212).

The aim of this research was to investigate associations between potentially neurotropic viruses and cerebral palsy in a Caucasian Australian population. The hypothesis was that evidence of perinatal viral infection may be associated with the future development of cerebral palsy.

Materials and Methods

Patient Selection

Please refer to chapter 2 for information regarding case and control selection, and population demographics.

Virus Detection

The viruses of interest are all known to be neurotropic and were categorised into DNA viruses and RNA viruses. The DNA viruses included: Herpes Simplex Virus 1 (HSV-1), Herpes Simplex Virus 2 (HSV-2), Varicella Zoster Virus (VZV), Epstein-Barr Virus (EBV), Cytomegalovirus (CMV), Human Herpesvirus 6 (HHV-6), Human Herpesvirus 7 (HHV-7), and Human Herpesvirus 8 (HHV-8). The RNA viruses of interest included members of the Enterovirus family. The DNA viruses were divided into two PCRs, one detecting nucleic acids from HSV-1, HSV-2, EBV, CMV and HHV-8, hereafter designated Herpes group A viruses, and the other detecting nucleic acids from VZV, HHV-6 and HHV-7, hereafter designated Herpes group B viruses. Within the Herpes group A PCR, differentiation between CMV and the remaining viruses (HSV-1, HSV-2, EBV and HHV-8) was possible because of differences in PCR product band size visualised by agarose gel electrophoresis.

Virus Sensitivity and Limits of Detection and Positive Control Stocks

Positive control enterovirus material was obtained from the poliovirus vaccine (Poliomyelitis vaccine (oral) Sabin, SmithKline Beecham Biological SA, Rixensart, Belgium). Positive control material for the Herpes group A and B PCRs was obtained from Advanced Biotechnologies Inc. (Maryland, USA) (HSV-1 and VZV quantitated viral DNA). CMV positive control material (lysed CMV strain Towne infected human foreskin fibroblasts) was kindly supplied by Dr Barry Slobedman (Westmead Millennium Institute, NSW Australia). Initial tests were performed to determine the sensitivity of detection of the viral nucleic acids by spiking known amounts of virus in blood and spotting the blood onto newborn screening card filter paper. These spiked cards were extracted and amplified in the same manner as the sample cards, detailed below. The minimum number of detectable viral nucleic acid copies per 1.2mm bloodspot was determined for each PCR, and then extrapolated back to a minimum number of detectable viral nucleic acid copies per millilitre of blood. The minimum number of detectable viral nucleic acid copies was 2.8 per bloodspot ($5.6x10^3$ nucleic acid copies per ml of blood) for enterovirus, 1.6 per

bloodspot ($3.2x10^3$ nucleic acid copies per ml of blood) for herpes group A viruses, and 15 per microlitre ($3.2x10^3$ nucleic acid copies per ml of blood) for herpes group B viruses.

Extraction

DNA Viruses

For the detection of the DNA viruses, 1.2mm diameter punches of dried blood on newborn screening cards (collected by heel-prick at approximately 3 to 5 days of life) were extracted using the commercially available NucleoSpin® Tissue Extraction Kit (Macherey-Nagel, Germany). Following this extraction, the eluted DNA was stored at -20°C until amplified.

RNA Viruses

For the detection of enterovirus, 1.2mm diameter punches of dried blood on newborn screening cards were extracted using a phenolic wash method (284). Briefly, 2 x 5 minute washes were performed with 200µl phenolic wash solution (50g of phenol, 120mg of 8-hydroxyquinoline, 10ml of 1M Tris/acetate, pH 8, 1ml of β-mercaptoethanol), followed by 2 x 5 minute rinses with 250µl 95% Isopropanol/5% Tris solution. At the completion of these washes, the samples were dried in a block heater at 50°C for approximately 10 minutes or until dry. The spots were then subjected to nucleic acid amplification.

Amplification

All amplification conditions were optimised using reference samples prior to adapting the method to amplification from dried blood spots. The reference samples were RNA and DNA extracted from viral stocks for viral amplification. These reference samples were included as positive controls for all subsequent amplifications using dried blood spots in addition to negative (no template) controls. Amplifications were performed in 0.3ml 96-well microtitre trays in a Hybaid OmnE Thermocycler using previously published primer sequences. For details regarding primer sequences, reaction mixtures and amplification conditions please refer to Appendix 6.

RNA amplification required an initial reverse transcription (RT) step prior to PCR. This was performed using a one-step method (SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase, Invitrogen, California, USA).

Amplified PCR products (10µl) were mixed with 3µl of loading buffer (285) and electrophoresed on 2% agarose gels for 30-35 minutes at 180 volts using 1xTBE buffer

with ethidium bromide. The gels were then visualised under ultraviolet light using a Gel Doc camera system (BioRad, Hercules, California, USA). pUC19/HpaII molecular markers (Geneworks, Adelaide, Australia) were used to determine fragment sizes.

Sequencing Analysis

Sequencing analysis was performed on selected positive viral samples to confirm the amplification of the specific sequences of nucleic acid. The chosen PCR product was visualised using agarose gel electrophoresis to confirm the presence of product, and the remaining product purified to remove excess primers and dNTPs using the commercially available UltraClean™ PCR Clean-up™ Kit (Mo Bio Laboratories, California, USA) and electrophoresed on a 2% agarose gel. Upon visualisation of a band, the remaining PCR product was amplified using Sequencing Big Dye™ version 3.1 (Applied Biosystems, New Jersey, USA). The PCR products were then further purified to precipitate the Sequencing Big Dye™ PCR product. (Automated DNA Sequencing Chemistry Guide, Applied Biosystems, New Jersey, USA). The sequencing analysis was performed by either the Molecular Pathology Group at the IMVS or the Cytogenetics Group at the WCH.

Internal Controls

Internal controls in PCR are constitutively expressed nucleic acid sequences that are present in all cells, and are used to assess integrity of samples. The constitutively expressed K-ras oncogene was selected as the internal control for our samples. A separate PCR was performed using the same extracted DNA samples as the virus PCRs, to determine the presence of intact DNA in the samples. Samples not amplifying for this constitutively expressed oncogene were excluded from further analysis, owing to uncertainty of the presence of amplifiable DNA.

During validation of the Herpes group B PCR, an apparently "spurious" band of smaller size than the expected size for the viruses was observed. This band was sequenced and subsequently identified as being from a human transcription factor family (Gli), constitutively expressed in cells to regulate nucleobase, nucleoside, nucleotide and nucleic acid metabolism, and are critical components in many important processes, including development and neurogenesis (286). This gene was capable of acting as an internal control for the PCR, and was used within the Herpes group B PCR as a measure of template quality. Samples not testing positive for this internal control were excluded from Herpes group B analysis, as it could not be certain that the Herpes group B PCR result for the sample was true, and not PCR failure due to poor template quality.

Statistical Analysis

These viruses could be detected using newborn screening samples that had been stored for up to 18 years. Prevalence proportions and exact 95% confidence intervals (CI) of the viruses in the newborn screening cards tested were calculated using PEPI (243). As controls were not matched for important covariates such as gestational age, analysis was undertaken using all controls without taking account of matching. Data analysis (GraphPad Instat version 3.06) then considered CP cases and controls by gestational age range (<37 weeks, ≥ 37 weeks, and all gestational ages), type of CP (diplegia, hemiplegia, quadriplegia and all types) and virus. Results are expressed as odds ratios (OR) with 95% confidence intervals (CI), comparing positive with negative virus detection. P values less than 0.05 are highlighted in the tables.

Results

Results were obtained (blinded to case/control status) from a total of 414 of the 443 cerebral palsy samples (93.5%) and 856 of the 883 control samples (96.9%), a significant difference which may cause a slight underestimation of the prevalence of viral exposure in the CP cases (OR 2.22, 95% CI 1.26-3.93). Not all viruses gave a clear result for each newborn screening card, thus the total number of results for each virus was less than the maximum total of 414 or 856 for cases and controls respectively. The results are expressed as total readable results.

Prevalence of Viruses in Control Population

The prevalence of the viruses in the non-cerebral palsy newborn Caucasian population of South Australia was determined (Table 1). Cytomegalovirus (CMV) was the most prevalent virus in the study population, with 26.7% of babies testing positive for CMV. In addition, 42 babies (4.9%) were positive for more than one virus. Of the babies positive for multiple viruses, the most common combination observed was Herpes group B and CMV, with a prevalence of 3.1% (95% CI 1.9-4.6), followed by CMV and Herpes group A, with a prevalence of 1.1% (0.5-2.1). The remainder of the virus combinations were seen in very small numbers, with prevalences of less than 1%.

The effect of gestational age on the prevalence of these viruses was investigated, because there was a higher rate of prematurity in the population from which many of the controls were selected. Cytomegalovirus was significantly more prevalent in preterm than term babies (33.2% and 24.0% respectively), with an odds ratio of 1.57 (95% CI 1.14-2.17) (Table 2 and Figure 1). The presence of Herpes group A viruses was also significantly more prevalent in preterm than term babies (36.0% and 27.5% respectively) (OR 1.49, 95% CI 1.09-2.04). The same trend was observed for the presence of any herpesvirus, with 44.3% of preterm babies testing positive for any herpesvirus, compared with 35.7% of term babies (OR 1.43, 95% CI 1.04-1.97) (Table 2 and Figure 1). No other virus was significantly different when investigated for gestational age. There were no significant differences in the prevalences of any of the viruses between very preterm babies <32 weeks gestation and preterm babies 32-36 weeks gestation.

Table 1: Prevalence of four neurotropic viruses, individually and in combination, in a population of South Australian newborns, expressed as percentage positive of the total tested. HSV refers to HSV-1, HSV-2, EBV, HHV-8. Herpes group A refers to HSV-1, HSV-2, EBV, CMV, HHV-8. Herpes group B refers to VZV, HHV-6, HHV-7. Any Herpesvirus refers to HSV-1, HSV-2, EBV, CMV, HHV-8, VZV, HHV-6, HHV-7. Any virus refers to all herpesviruses and enteroviruses.

Virus	Positive	Total†	Prevalence (%) 95% CI	
Enterovirus	22	855	2.6	1.6-3.9
Cytomegalovirus	228	855	26.7	23.7-29.8
HSV	37	855	4.3	3.1-5.9
Herpes group A viruses	256	855	29.9	26.9-33.1
Herpes group B viruses	54	715	7.6	5.7-9.7
Any Herpesvirus	286	747	38.3	34.8-41.9
Any virus	298	749	39.8	36.3-43.4
Multiple Viruses (≥2)	42	856	4.9	3.6-6.6

† The number of samples with a valid test result varied between the different PCR tests. This accounts for the slightly different total number of babies tested for each virus.

Table 2: Prevalence of four neurotropic viruses in term and preterm South Australian newborns, expressed as percentage positive of the total tested. Odds Ratios (95% Confidence Intervals) for <37 weeks gestation compared with ≥37 weeks gestation. HSV refers to HSV-1, HSV-2. EBV, HHV-8. Herpes group A refers to HSV and CMV. Herpes B refers to VZV, HHV-6, HHV-7. Any Herpesvirus refers to Herpes group A and Herpes group B. Any virus refers to any herpesvirus and enterovirus.

Virus	Gestation (weeks)	Positive	Total†	Prevalence (%) 95% CI		OR 95% CI
Enterovirus	≥37	17	608	2.8	(1.6-4.4)	Reference
	<37	5	247	2.0	(0.7-4.7)	0.72 (0.26-1.97)
Cytomegalovirus	≥37	146	608	24.0	(20.7-27.6)	Reference
	<37	82	247	33.2	(27.4-39.4)	1.57 (1.14-2.17)**
HSV	≥37	29	608	4.8	(3.2-6.8)	Reference
	<37	8	247	3.2	(1.4-6.3)	0.67 (0.30-1.48)
Herpes group A	≥37	167	608	27.5	(24.0-31.2)	Reference
	<37	89	247	36.0	(30.0-42.4)	1.49 (1.09-2.04)*
Herpes group B	≥37	39	503	7.8	(5.6-10.4)	Reference
	<37	15	212	7.1	(4.0-11.4)	0.91 (0.49-1.68)
Any Herpesvirus	≥37	188	526	35.7	(31.6-40.0)	Reference
	<37	98	221	44.3	(37.7-51.2)	1.43 (1.04-1.97)*
Any Virus	≥37	199	528	37.7	(33.5-42.0)	Reference
	<37	99	221	44.8	(38.1-51.6)	1.34 (0.98-1.84)

† The number of samples with a valid test result varied between the different PCR tests. This accounts for the slightly different total number of babies tested for each virus.

*$p<0.05$ compared to term prevalence.

** $p<0.01$ compared to term prevalence.

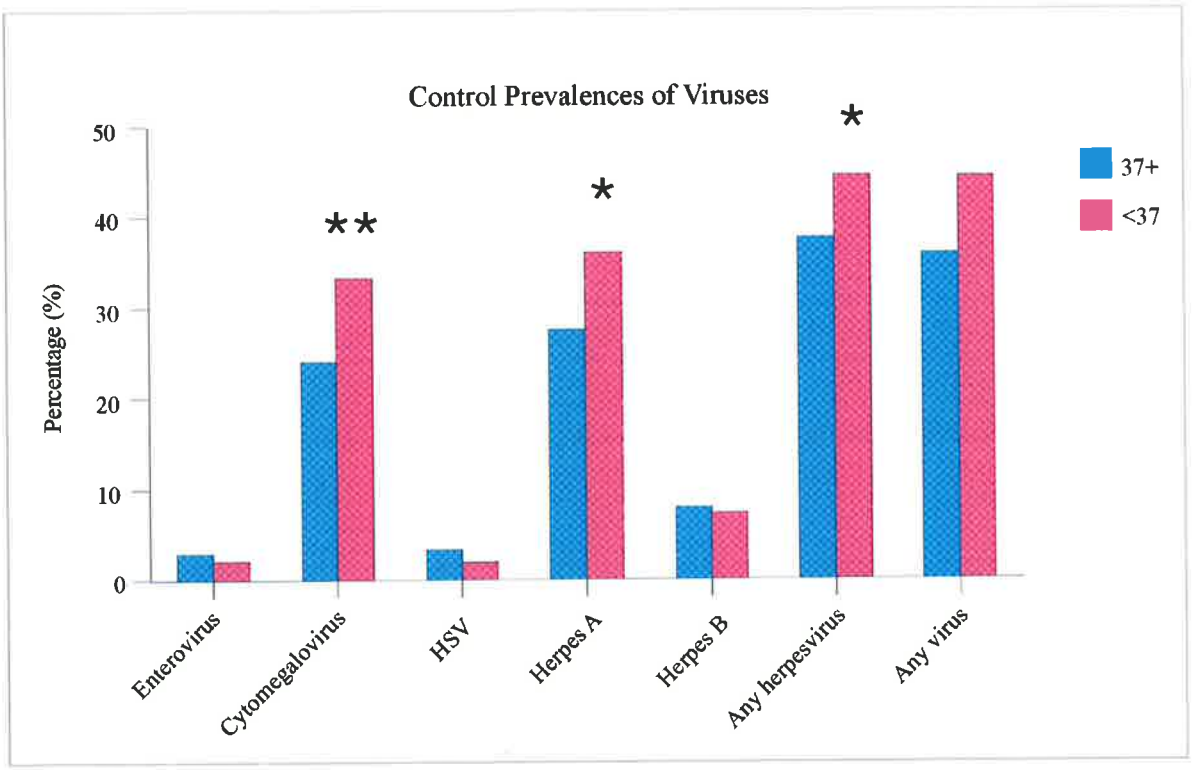

Figure 1: Prevalence of four neurotropic viruses in term and preterm South Australian newborns, expressed as percentage positive of the total tested. HSV refers to HSV-1, HSV-2, EBV, HHV-8. Herpes A refers to HSV and CMV. Herpes B refers to VZV, HHV-6, HHV-7. Any Herpesvirus refers to Herpes A and Herpes B. Any virus refers to any herpesvirus and enterovirus. *P<0.05 compared to term-born prevalence. **P<0.01 compared to term-born prevalence.

Viruses and Cerebral Palsy

Associations between neurotropic viruses and cerebral palsy were investigated, taking into consideration gestational age and cerebral palsy subtypes.

All Gestational Ages

The detection of Herpes group B viral nucleic acids increased the risk of developing all types of CP, with an odds ratio of 1.68 (95% CI 1.09-2.59) (Table 3). Herpes group B viruses were detected in a total of 40 (12.1%) CP cases with an obtainable result, compared with 54 (7.6%) of controls. This increased risk was also observed for the diplegic (OR 1.93, 95% CI 1.03-3.61) and hemiplegic (OR 2.07, 95% CI 1.10-3.88) subtypes of CP and Herpes group B. The detection of any viral nucleic acids was associated with the development of CP (OR 1.30, 95% CI 1.00-1.67). There were no significant associations for any of the other viruses (Table 3).

Gestational Ages ≥37 weeks

Overall the presence of any of the Herpes group B viruses was not associated with the development of CP at ≥37 weeks gestational age, although the trend towards such an association was observed (OR 1.69, 95% CI 0.98-2.92) (Table 4). Herpes group B viruses were associated with the development of diplegia (OR 2.45, 95% CI 1.02-5.89) and hemiplegia (OR 2.38, 95% CI 1.15-4.92). An association with quadriplegia could not be assessed, as no cases of quadriplegia tested positive for Herpes group B (OR 0.00, 95% CI 0.00-0.89). The risk of developing CP increased with the detection of any herpesvirus, with an odds ratio of 1.52 (95% CI 1.09-2.13), and also with the detection of any virus (OR 1.64, 95% CI 1.17-2.28). There were no significant associations for any of the other viruses (Table 4).

Gestational Ages <37 weeks

The detection of Herpes group A viral nucleic acids decreased the risk of developing all types of CP, with an odds ratio of 0.65 (95% CI 0.43-0.99) (CP n=50, controls n=89) (Table 5). Detection of Herpes group B increased the risk for quadriplegia (OR 2.87, 95% CI 1.09-7.59). There were no other significant associations for any other viruses (Table 5). In contrast to the findings in tables 3 and 4, where the majority of results tended towards an increased risk of CP, the findings in Table 5 demonstrate that the majority of the results tend towards a decreased risk of CP.

Table 3: Odds Ratios (95% CI) for cerebral palsy for specified viruses at all gestational ages compared with controls of all gestational ages. HSV refers to HSV-1, HSV-2, EBV, HHV-8. Herpes group B refers to VZV, HHV-6, HHV-7. Herpes group A refers to HSV-1, HSV-2, EBV, CMV, HHV-8. Any herpes refers to HSV-1, HSV-2, VZV, EBV, CMV, HHV-6, HHV-7, HHV-8. Any virus refers to any herpesvirus or enterovirus. Numbers of positive test results for each virus for CP cases are listed in parentheses. Significant results are indicated in bold text.

Virus	Odds Ratio (95% CI)			
	All Types of CP	Diplegia	Hemiplegia	Quadriplegia
HSV (13)	0.72 (0.38-1.37)	0.34 (0.08-1.44)	0.77 (0.27-2.20)	0.61 (0.18-2.01)
CMV (115)	1.06 (0.82-1.38)	1.08 (0.72-1.63)	0.93 (0.60-1.44)	1.15 (0.74-1.77)
Herpes group B (40)	**1.68 (1.09-2.59)**	**1.93 (1.03-3.61)**	**2.07 (1.10-3.88)**	1.00 (0.44-2.26)
Herpes group A (123)	0.99 (0.77-1.28)	0.96 (0.64-1.43)	0.94 (0.61-1.43)	1.02 (0.66-1.57)
Any Herpes (149)	1.22 (0.94-1.58)	1.27 (0.85-1.90)	1.10 (0.72-1.68)	1.10 (0.72-1.70)
Enterovirus (13)	1.24 (0.62-2.49)	0.60 (0.14-2.57)	1.68 (0.62-4.51)	1.42 (0.48-4.19)
Any Virus (160)	**1.30 (1.00-1.67)**	1.28 (0.86-1.92)	1.17 (0.77-1.78)	1.20 (0.79-1.85)

Table 4: Odds Ratios (95% CI) for cerebral palsy for specified viruses ≥37 weeks gestation compared with controls ≥37 weeks gestation. HSV refers to HSV-1, HSV-2, EBV, HHV-8. Herpes group B refers to VZV, HHV-6, HHV-7. Herpes group A refers to HSV-1, HSV-2, EBV, CMV, HHV-8. Any herpes refers to HSV-1, HSV-2, VZV, EBV, CMV, HHV-6, HHV-7, HHV-8. Any virus refers to any herpesvirus or enterovirus. Numbers of positive test results for each virus for CP cases are listed in parentheses. Significant results are indicated in bold text.

Virus	Odds Ratio (95% CI)			
	All Types of CP	Diplegia	Hemiplegia	Quadriplegia
HSV (10)	0.92 (0.44-1.92)	0.85 (0.20-3.67)	1.04 (0.35-3.03)	0.31 (0.04-2.33)
CMV (66)	1.30 (0.92-1.83)	1.03 (0.52-2.02)	1.04 (0.61-1.78)	1.62 (0.94-2.80)
Herpes group B (23)	1.69 (0.98-2.92)	**2.45 (1.02-5.89)**	**2.38 (1.15-4.92)**	**0.00 (0.00-0.89)**
Herpes group A (73)	1.25 (0.90-1.74)	0.95 (0.49-1.84)	1.11 (0.67-1.85)	1.45 (0.84-2.48)
Any Herpes (88)	**1.52 (1.09-2.13)**	1.41 (0.74-2.67)	1.38 (0.83-2.30)	1.25 (0.71-2.20)
Enterovirus (10)	1.63 (0.74-3.62)	1.55 (0.35-6.90)	1.83 (0.60-5.58)	1.12 (0.25-4.97)
Any Virus (96)	**1.64 (1.17-2.28)**	1.58 (0.83-2.98)	1.43 (0.86-2.37)	1.29 (0.74-2.24)

Table 5: Odds Ratios (95% CI) for cerebral palsy for specified viruses <37 weeks gestation compared with controls <37 weeks gestation. HSV refers to HSV-1, HSV-2, EBV, HHV-8. Herpes group B refers to VZV, HHV-6, HHV-7. Herpes group A refers to HSV-1, HSV-2, EBV, CMV, HHV-8. Any herpes refers to HSV-1, HSV-2, VZV, EBV, CMV, HHV-6, HHV-7, HHV-8. Any virus refers to any herpesvirus or enterovirus. Numbers of positive test results for each virus for CP cases are listed in parentheses. Significant results are indicated in bold text.

Virus	Odds Ratio (95% CI)			
	All Types of CP	Diplegia	Hemiplegia	Quadriplegia
HSV (3)	0.49 (0.13-1.87)	0.17 (0.00-1.75)	0.37 (0.00-3.85)	1.33 (0.27-6.46)
CMV (49)	0.72 (0.47-1.10)	0.88 (0.51-1.51)	0.72 (0.33-1.55)	0.61 (0.30-1.27)
Herpes group B (17)	1.73 (0.83-3.59)	1.67 (0.65-4.30)	1.41 (0.38-5.17)	**2.87 (1.09-7.59)**
Herpes group A (50)	**0.65 (0.43-0.99)**	0.78 (0.46-1.33)	0.63 (0.29-1.37)	0.54 (0.26-1.12)
Any Herpes (61)	0.82 (0.54-1.25)	0.99 (0.57-1.71)	0.66 (0.30-1.43)	0.84 (0.42-1.66)
Enterovirus (3)	0.79 (0.19-3.35)	0.27 (0.01-4.89)	1.31 (0.15-11.52)	2.15 (0.40-11.44)
Any Virus (64)	0.88 (0.58-1.33)	0.97 (0.56-1.68)	0.74 (0.34-1.59)	1.01 (0.51-1.99)

Combination Viruses at any Gestational Age

The presence of more than one virus was not associated with the risk of developing CP when compared with all controls (OR 1.13, 95% CI 0.65-1.98). There was also no association when comparing term CP with term controls (OR 1.29, 95% CI 0.64-2.61), or preterm CP with preterm controls (OR 0.98, 95% CI 0.38-2.53).

Discussion

This study is the largest reported study examining the association between CP and perinatal exposure to neurotropic viruses. The findings were based on the detection of viral nucleic acids from newborn screening card blood spots collected within a few days of birth and where detected, the findings indicate *in utero* or early neonatal viral exposure/infection. To date, research has focused on subjective, indirect measures of infection (mostly bacterial) and inflammation, such as chorioamnionitis, funisitis or maternal pyrexia (133). These data provide the first direct evidence that perinatal viral exposure and infection are associated with cerebral palsy, and reinforce the possibility of a complex and heterogeneous relationship between exposure to viral infections and subtypes of CP at different gestational ages. Other co-factors may trigger neurotropic damage.

84 separate analyses were performed on the individual viruses, and further analyses involved combinations of these viruses. Such multiple analyses increase the likelihood of identifying chance statistical associations. Due to small numbers in some of the sub-analyses, other associations cannot be confidently excluded. Where associations are seen in one gestational age range, similar trends are seen in the same subgroup of CP with the same virus in other gestational age ranges. This suggests that these may indicate true causal relationships and may not be attributed to chance. Nevertheless, these associations require further study.

This is the first study to document the prevalence of these viruses in a Caucasian control population, and has shown that exposure to viral nucleic acids is very common in the study population, with a prevalence of 39.8% (95% CI 36.3-43.4) for exposure to any of the viruses tested. Exposure to any of the herpesviruses tested was also common, with a prevalence of 38.3% (95% CI 34.8-41.9). Nearly 5% of the control population tested positive for more than one virus (95% CI 3.6-6.6). The prevalence of cytomegalovirus was significantly higher in those controls born before 37 weeks gestation, compared with those born at term (33.2% vs. 24.0%), suggesting that the presence of viral infectious agents *in utero* are also associated with subsequent preterm delivery (233, 261, 287, 288).

This is also the first study to investigate the direct links between perinatal exposure to viral infection and the subsequent development of cerebral palsy. Using this large cohort of cerebral palsy cases and controls, specific viral DNA and RNA nucleic acid sequences have been identified, and show that exposure to viral nucleic acids (and presumably

infectious virus), in particular Herpes group B (VZV, HHV-6 or HHV-7), is associated with cerebral palsy. The results presented in tables 3 and 4 suggest that there is a 1.5-2.0 times increased risk of developing cerebral palsy when there has been perinatal exposure to Herpes group B (VZV, HHV-6 or HHV-7). The prevalence of Herpes group B viruses was 7.6% for controls and 12.1% for CP cases, giving a potential attributable risk of 4.5% of all CP cases if there is a causal relationship. These results also demonstrate an increased risk of developing cerebral palsy for those babies born at or after 37 weeks gestation when there has been perinatal exposure to any virus. In contrast, the results from Table 5 suggest negative associations between perinatal exposure to viral nucleic acids and subsequent cerebral palsy for babies born preterm. These conflicting results may be due to the higher prevalence of viral infection in control babies born preterm, thus diluting any positive association between viral infection and cerebral palsy for preterm babies. The varying results at different gestational ages may also reflect different timing of exposure to the virus. Exposure late in gestation may not result in preterm birth, instead having direct effects on the brain, whereas exposure early in gestation may result in preterm birth but increase the risk of neuropathology associated with prematurity. In this manner, exposure to perinatal viral infection may be indirectly associated with cerebral palsy.

This study used non-quantitative PCR methodology, and there remains the possibility of the results reflecting differing viral loads. It is possible that low viral loads are not sufficient to precipitate preterm birth, but continuing development *in utero* in the presence of infection, allows the infection to cause damage to vulnerable brain tissue. On the other hand, high viral loads may initiate or result in preterm birth and increase the risk of prematurity-associated neurological damage as well as infection-associated brain damage. Prospective studies designed to quantify viral loads to test this theory are planned. Also, this study was unable to test every potential virus. Other candidate viruses (for example adenoviruses, rotaviruses, human coronaviruses, parvovirus, paramyxoviruses (pneumovirus and human metapneumovirus) and lymphocytic choriomeningitis virus) also deserve investigation.

The results do not necessarily indicate active congenital or neonatal infection. Newborn screening cards were tested for the presence of viral DNA and RNA. While detection of viral nucleic acids in the blood of neonates indicates exposure to the respective virus or viruses, this study was not designed to detect evidence of an accompanying inflammatory response. Given the small sample volume and the limit of detection of 1-10 viral nucleic acid copies, it could be inferred that true viraemic infection was occurring. On the same

basis, it would seem unlikely that presence of viral nucleic acid merely reflected maternal-fetal cell trafficking or maternal blood contamination. These questions remain unanswered, and prospective investigations are required to follow women through pregnancy, quantitatively testing antenatal blood samples for viral nucleic acids, and determining if there is active infection in the fetus/neonate by examining leukocytes and sera for the presence of viral antigens associated with active viral replication. These findings may indicate that the Th2 cytokine shift of pregnancy could allow reactivation of herpesviruses at a rate higher than previously suspected. The advent of widespread childhood vaccination against VZV, which has been available here for approximately 10 years, will, in itself, provide an opportunity to investigate the potential role of maternal VZV infection and/or reactivation in the development of CP. It will be of interest to observe if there is a decline in CP rates in the next generation, following the vaccination of their mothers, in a way similar to that of the rubellavirus vaccine reducing the teratogenic effects of rubella during pregnancy.

It is unclear exactly how perinatal exposure to viral infection causes subsequent brain damage and cerebral palsy. Such damage can be caused either directly or indirectly. If the virus is able to cross the blood-brain-barrier, it is capable of setting up infection within the brain, and directly damage the vulnerable neuronal tissue. Herpesviruses are capable of establishing themselves within the brain, where they can remain latent indefinitely, or reactivate and cause damage. Indirectly, products of infection can be released locally and into the circulation. These products can cross the blood-brain-barrier, either as a result of immaturity of the blood-brain-barrier (181), or due to release of pro-inflammatory cytokines that impair its integrity (70, 182). Having penetrated the blood-brain-barrier, these pro-inflammatory cytokines such as TNF-α, can cause damage to developing white matter, resulting in periventricular leukomalacia (PVL). This white matter damage may result from a number of different mechanisms, including direct tissue damage, stimulation of fetal microglia to produce more TNF-α (125), and disruption of the endothelium and/or ependyma (70).

The high prevalence of exposure to viral infection in our control population suggests that "triggers" or co-factors are needed before brain damage can occur. Such triggers may include genetic susceptibility to infections, for example cytokine polymorphisms that increase or decrease the host immune response to the infection. The breakdown of vital barriers such as the placenta and blood-brain-barrier as a result of fetal blood clot-induced

infarctions secondary to inherited thrombophilia may also play a role in the transmission and establishment of infections that are capable of causing damage later resulting in cerebral palsy, and a role for infection acting as a co-factor in triggering thrombosis in the fetus with thrombophilia could also be postulated. An association between fetal thrombophilia and CP has also been found in this same cohort (279).

In summary, in this chapter it has been demonstrated that exposure to viral nucleic acids is common in the studied neonatal population, and cerebral palsy was increased nearly two-fold with exposure to Herpes group B viruses. Other factors, such as genetic susceptibility to infection and inherited thrombophilia or involvement of other clinical events such as growth restriction or prematurity, may be required for brain damage and subsequent cerebral palsy to occur.

Associations between Inherited Thrombophilia and Adverse Pregnancy Outcomes

Abstract

Objective: To investigate the role of fetal inherited thrombophilia in the development of a range of adverse pregnancy outcomes, including pregnancy-induced hypertensive disorders (PIHD), antepartum haemorrhage (APH), intrauterine growth restriction $<10^{th}$ percentile (IUGR) and preterm birth (PTB).

Methods: 717 cases and 609 controls were genotyped for Factor V Leiden (FVL, G1691A), Prothrombin gene mutation (PGM, G20210A), and Methylenetetrahydrofolate reductase (MTHFR) C677T and MTHFR A1298C using genomic DNA extracted from newborn screening cards.

Results: For babies born <28 weeks gestation, PGM was associated with an increased risk of IUGR (OR 6.40, 1.66-24.71) and APH with IUGR (OR 6.35, 1.63-24.75). Homozygous PGM also increased the risk of PIHD with IUGR for term babies (OR 50.81, 1.75-1476.90). Homozygosity for MTHFR A1298C was associated with an increased risk of IUGR for babies born 28-31 weeks gestation (OR 4.00, 1.04-15.37), and with APH and IUGR for babies born <32 weeks gestation (OR 3.57, 1.09-11.66). MTHFR C677T was associated with a reduced risk of PTB and IUGR (OR 0.52, 0.28-0.96) for babies born 32-36 weeks gestation. Homozygous FVL was associated with an increased risk of PIHD with IUGR for term babies (OR 37.15, 1.33-1041.30), but decreased the risk of PTB <32 weeks gestation (OR 0.55, 0.31-0.98). There were no associations with any thrombophilic polymorphism and APH alone.

Conclusions: These results suggest that some fetal thrombophilic polymorphisms may be related to adverse pregnancy outcomes, in particular IUGR, but that this may not be the only association. Further studies matching maternal and fetal genotypes are required to investigate if both are needed for the adverse pregnancy outcome phenotype to be expressed.

Introduction

Serious obstetric complications leading to adverse pregnancy outcomes, such as severe preeclampsia, severe intrauterine growth restriction (IUGR) and early preterm delivery (<32 weeks gestation) occur in up to 5% of pregnant women (289). Maternal inherited thrombophilic disorders, which predispose individuals to thrombosis, have been shown to be associated with such adverse outcomes (289). Most inherited thrombophilic conditions predispose to the formation of thrombosis by promoting excessive coagulation, or by impairing anticoagulation (86). Common inherited thrombophilia include: heterozygosity or homozygosity for the Factor V Leiden (FVL) mutation causing activated protein C resistance (APCR); heterozygosity or homozygosity for the Prothrombin gene mutation (PGM); and heterozygosity or homozygosity for polymorphisms at positions 677 and 1298 in the gene for 5, 10-methylenetetrahydrofolate reductase (MTHFR), associated with an increased tendency to hyperhomocysteinaemia; and compound heterozygosity for both MTHFR polymorphisms.

So far, researchers have focused on the role of maternal inherited thrombophilia, with only limited research investigating the role of fetal inherited thrombophilia in the development of adverse pregnancy outcomes (215-222). These few studies have demonstrated conflicting results, emphasising the need for further research in this area. Studies by Infante-Rivard, Glanville and McCowan (218-220) showed no effect of fetal thrombophilia on pregnancy outcome, whereas studies by Dizon-Townson, von Kries, Anteby and Dekker (215, 217, 221, 222) showed positive associations. All these studies differed widely in selected pregnancy outcomes, sample sizes and selected inherited thrombophilia, which may explain the discordant results.

The aim of this study was to investigate the role of fetal thrombophilia in a wide range of adverse pregnancy outcomes, including IUGR, pregnancy-induced hypertensive disorders (PIHD), antepartum haemorrhage, and preterm birth. To date, this is the largest case-control study investigating fetal inherited thrombophilia.

Materials and Methods

Please refer to chapter 5 for information regarding methods to detect the factor V Leiden polymorphism (FVL, G1691A), prothrombin gene mutation (PGM, G20210A), and two polymorphisms in the methylenetetrahydrofolate reductase gene (MTHFR C677T and A1298C).

Patient Selection

For detailed information on the selection of cases and controls, please refer to Chapter 2.

Statistical Analysis

These inherited thrombophilic polymorphisms were genotyped from newborn screening samples that had been stored for up to 18 years. As controls were not matched for important covariates such as gestational age, analysis was undertaken using all controls without taking account of matching. Data analysis (GraphPad Instat version 3.06) then considered cases by gestational age range (<28 weeks, 28-31 weeks, <32 weeks, 32-36 weeks, <37 weeks, ≥ 37 weeks, and all gestational ages). Results are expressed as odds ratios (OR) with 95% confidence intervals (CI). Homozygosity and heterozygosity were compared for each polymorphism separately with homozygosity for the wild-type allele. Data for homozygosity and heterozygosity combined compared with the wild-type allele are also presented. Please refer to Appendix 7 for tables detailing all calculated odds ratios and confidence intervals; only the significant results are presented in the main text. Some combinations of thrombophilic polymorphisms and adverse pregnancy outcomes types were not seen in some sub-groups: these odds ratios are not reported. p-values less than 0.05 are highlighted in the tables. No adjustments were made for multiple testing in this largely exploratory study.

Results

Intrauterine Growth Restriction <10th percentile

241 babies in this cohort (33.6%) were growth-restricted to less than the 10th percentile (inclusion criterion 2). Heterozygosity for PGM was associated with an increased risk of IUGR <10th percentile for babies born <28 weeks gestation (OR 6.40, 95% CI 1.66-24.71). Carriage of the variant allele (homozygosity or heterozygosity) for PGM was also associated with an increased risk of IUGR <10th percentile for babies born <28 weeks gestation (OR 5.71, 95% CI 1.49-21.93) (Table 1). Babies homozygous for MTHFR A1298C at gestational ages 28-31 weeks were four times more likely to be growth-restricted <10th percentile (OR 4.00, 95% CI 1.04-15.37) (Table 2).

Hypertension

The mothers of 42 babies in this cohort (5.9%) suffered from hypertension, either pregnancy-induced, (23) or pre-existing (20). One mother suffered from both.

Pregnancy-Induced Hypertensive Disorders

23 (54.8%) of the 42 mothers displaying signs of hypertension in this cohort developed pregnancy-induced hypertensive disorders (PIHD) (inclusion criterion 5). No significant associations were observed for any of the inherited thrombophilia at any gestational age.

Pregnancy-Induced Hypertensive Disorders and IUGR <10th percentile

9 (39.1%) of the 23 mothers who developed PIHD also gave birth to a growth-restricted baby (<10th percentile). For babies born at or after 37 weeks gestation, homozygosity for FVL (OR 37.15, 95% CI 1.33-1041.30) or PGM (OR 50.81, 95% CI 1.75-1476.90) was associated with an increased risk of PIHD and growth-restriction. (Table 3).

Pre-existing Hypertension

Of the 42 mothers displaying signs of hypertension in our cohort, 20 (47.6%) were classified as having pre-existing hypertension (inclusion criterion 4, please refer to Chapter 2 for full details). In this cohort, the majority of analyses were non-significant. Significantly more mothers with pre-existing hypertension had babies homozygous for PGM at <28 and 28-31 weeks gestation (both ORs 30.49, 95% CI 1.23-758.39) (Table 1).

Antepartum Haemorrhage

The mothers of 340 of 717 babies in this case cohort (47.4%) suffered from antepartum haemorrhage (APH) (inclusion criterion 3, please refer to Chapter 2 for full details). There were no associations between carriage of thrombophilic polymorphisms and APH at any gestational age.

Antepartum Haemorrhage and IUGR <10th percentile

Of the 340 mothers diagnosed with APH, 108 (31.8%) gave birth to a growth-restricted baby <10th percentile. For babies born <28 weeks gestation, PGM was associated with APH and growth-restriction (heterozygous OR 7.11, 95% CI 1.81-27.89; homozygous or heterozygous OR 6.35, 95% CI 1.63-24.75) (Table 1). Homozygosity for MTHFR A1298C was associated with APH and growth-restriction at both 28-31 weeks gestation (OR 5.00, 95% CI 1.21-20.60) and <32 weeks gestation (OR 3.57, 95% CI 1.09-11.66) (Table 2).

Preterm Birth <37 weeks

451 (62.9%) of this case cohort were born prematurely at a gestation age of less than 37 weeks (inclusion criterion 1, please refer to Chapter 2 for full details). FVL was associated with a decreased risk of preterm birth <32 weeks (heterozygous OR 0.48, 95% CI 0.26-0.89; homozygous or heterozygous OR 0.55, 95% CI 0.31-0.98) (Table 3). There were no increased risks of preterm birth for any of the thrombophilic polymorphisms genotyped.

Preterm Birth and IUGR <10th percentile

82 (18.2%) of the 451 preterm babies were growth-restricted <10th percentile. PGM was associated with prematurity and growth-restriction (heterozygous OR 6.40, 95% CI 1.66-24.71; homozygous or heterozygous OR 5.71, 95% CI 1.49-21.93) (Table 1). MTHFR C677T was associated with a decreased risk of preterm birth at 32-36 weeks gestation with IUGR (homozygous or heterozygous OR 0.52, 95% CI 0.28-0.96) (Table 3).

Table 1: Significant odds ratios (95% CI) for adverse pregnancy outcomes for Prothrombin gene mutation. WT = wild-type.

Adverse Pregnancy Outcome	Gestation (weeks)	Zygosity	Cases +ve/WT	Controls +ve/WT	Odds Ratio (95% CI)	p-value
IUGR <10th percentile	<28	Heterozygous	3/10	25/533	6.40 (1.66-24.71)	<0.05
	<28	Abnormal	3/10	28/533	5.71 (1.49-21.93)	<0.05
Preexisting hypertension	<28	Homozygous	0/2	3/533	30.49 (1.23-758.39)	>0.05
	28-31	Homozygous	0/2	3/533	30.49 (1.23-758.39)	>0.05
All PIHD and IUGR <10th percentile	≥ 37	Homozygous	0/1	3/533	50.81 (1.75-1476.90)	>0.05
All APH and IUGR <10th percentile	<28	Heterozygous	3/9	25/533	7.11 (1.81-27.89)	<0.05
	<28	Abnormal	3/9	28/533	6.35 (1.63-24.75)	<0.05
All Preterm Birth and IUGR <10th percentile	<28	Heterozygous	3/10	25/533	6.40 (1.66-24.71)	<0.05
	<28	Abnormal	3/10	28/533	5.71 (1.49-21.93)	<0.05

Table 2: Significant odds ratios (95% CI) for adverse pregnancy outcomes for Methylenetetrahydrofolate reductase gene mutations. WT = wild-type.

Adverse Pregnancy Outcome	Gestation (weeks)	Zygosity	Cases +ve/WT	Controls +ve/WT	Odds Ratio (95% CI)	p-value
IUGR <10th percentile	28-31	Homozygous 1298	4/5	56/280	4.00 (1.04-15.37)	0.05
All APH and IUGR <10th percentile	28-31	Homozygous 1298	4/4	56/280	5.00 (1.21-20.60)	<0.05
	<32	Homozygous 1298	5/7	56/280	3.57 (1.09-11.66)	<0.05
All Preterm Birth and IUGR <10th percentile	32-36	Abnormal 677	17/30	286/262	0.52 (0.28-0.96)	<0.05

Table 3: Significant odds ratios (95% CI) for adverse pregnancy outcomes for Factor V Leiden. WT = wild-type.

Adverse Pregnancy Outcome	Gestation (weeks)	Zygosity	Cases +ve/WT	Controls +ve/WT	Odds Ratio (95% CI)	p-value
All PIHD and IUGR <10th percentile	≥ 37	Homozygous	0/1	4/501	37.15 (1.33-1041.30)	>0.05
All Preterm Birth	<32	Heterozygous	13/238	57/507	0.48 (0.26-0.89)	<0.05
	<32	Abnormal	16/238	61/501	0.55 (0.31-0.98)	<0.05

Discussion

This is the first study to investigate fetal inherited thrombophilia for a wide range of adverse pregnancy outcomes, including intrauterine growth restriction (IUGR), pregnancy-induced hypertensive disorders (PIHD), antepartum haemorrhage, and prematurity. One major difference between the present study and other studies is that a wide range of adverse pregnancy outcomes were investigated, not limiting the focus to only one adverse outcome.

Within the case cohort of 717 babies, the mothers of 340 (47.4%) were diagnosed with some form of APH. The classification of APH within the South Australian Perinatal Data Collection of births includes diagnosis of placenta praevia and placental abruption, as well as other and unknown causes of APH. It is important to note that this is a pathology-enriched cohort of babies, with much higher incidences of these adverse pregnancy outcomes than would normally be expected in the general population.

642 separate analyses were performed on the individual polymorphisms, with 16 (2.5%) yielding significant associations. Such multiple analyses increase the likelihood of identifying chance statistical associations. On the contrary, because of small numbers in some of the subanalyses, associations cannot be confidently excluded. Any findings must be interpreted with caution, and further large-scale studies are necessary to definitively determine if there are associations between fetal inherited thrombophilia and adverse pregnancy outcomes.

Prothrombin Gene Mutation

PGM is a dominantly inherited thrombophilia which results in increased plasma prothrombin concentrations and a 2.8 fold increase in the risk of thrombosis (87, 103). The majority of analyses demonstrated no associations between adverse pregnancy outcomes and PGM. Any positive associations were mainly seen in the extremely preterm infants born <28 weeks gestation. The positive associations were seen for babies who were growth-restricted below the 10^{th} percentile for gestational age. A number of associations were seen with hypertension and growth-restriction for the PGM mutation; these associations demonstrated confidence intervals that did not cross unity, but were above the statistically significant p-value of 0.05. This phenomenon is most likely seen because of the small numbers involved in the analysis, and further studies are required with large sample sizes to investigate these possible associations. These results are consistent with

those of Anteby (221), but differ from those of Infante-Rivard and McCowan (218, 220), who both demonstrated a lack of association between PGM and an increased risk of IUGR or small for gestational age babies. Both of these studies had mixed ethnic groups, whereas this cohort was entirely Caucasian. The present study only demonstrated associations with IUGR for babies born <28 weeks gestation, and babies whose mothers were hypertensive were not excluded, which may explain the variation in results between the studies.

Factor V Leiden

FVL is a dominantly inherited thrombophilia, with individuals homozygous for FVL having an 80-100 fold increased risk of developing thromboembolism (101). Results demonstrated that for the majority of analyses, FVL was not associated with any of the adverse pregnancy outcomes studied, consistent with previous research demonstrating a lack of association between FVL and an increased risk of IUGR or small for gestational age babies (218, 220). These results also suggested that FVL decreases the risk of preterm birth <32 weeks gestation. FVL, a fairly common polymorphism in many populations worldwide, may confer a selective advantage to carriers, possibly by decreasing the risk of antepartum haemorrhage and subsequent PTB. Finally, a potentially very important association between PIHD with IUGR and FVL was observed. PIHD with IUGR is an important clinical entity, and the role of FVL in the development of these adverse outcomes needs to be further investigated with a larger cohort of cases. Any association between FVL and increased risks of adverse pregnancy outcomes is likely to be at least in part driven by the maternal genotype, with the fetal genotype playing a minor role.

Methylenetetrahydrofolate Reductase Gene

Two common polymorphisms have been described in the MTHFR gene: C677T and A1298C. Homozygosity for the 677 C→T base pair substitution occurs in 6-12% of Caucasian populations (106). This polymorphism can result in hyperhomocysteinaemia, particularly in conditions of folate or vitamin B12 deficiency. Hyperhomocysteinaemia may exert thrombophilic effects by altering the normal antithrombotic phenotype of the endothelium, enhancing the activities of factors XII and V, as well as depressing the activation of protein C, and also via recruiting leukocytes and augmenting leukocyte-induced endothelial cell activation (107, 109). A second common polymorphism has been described at position 1298 in the MTHFR gene, where there is an A→C base pair

substitution (110). This polymorphism also results in decreased enzyme activity, but not necessarily in hyperhomocysteinaemia (110).

For the majority of results, the present study is consistent with the few papers published in this area (218). MTHFR C677T was found to decrease the risk of PTB and IUGR <10th percentile, consistent with previous reports of MTHFR reducing the risk of IUGR (218). It has been proposed that this reduced risk is due to the increased availability of methylenetetrahydrofolate, which in turn reduces the likelihood that DNA synthesis will be reduced as a result of reduced substrate availability (218), however, further investigations are required to confirm or refute this theory. MTHFR A1298C was associated with APH in the presence of growth-restriction for babies born prematurely. Maternal thrombophilia are strongly associated with APH and placental abruption (290), via spiral artery thrombosis followed by retroplacental haematoma. Whether or not fetal MTHFR 1298AC works via that mechanism remains unclear, and needs further investigation.

One important limitation of this study is the absence of the maternal genotype. The effect that maternal genotypes have on the fetal circulation were unable to be assessed. Babies homozygous for an inherited thrombophilia must have mothers who are at least heterozygous for that thrombophilia, and this combination of polymorphic alleles in both the mother and fetus may be required for the development of adverse pregnancy outcomes. Both the fetal and maternal circulations should be considered when investigating the role of inherited thrombophilia in the development of adverse pregnancy outcomes, and future studies should investigate the role played by the maternal circulation in conjunction with the fetal circulation.

The role of fetal thrombophilia in the development of preeclampsia was unable to be investigated because data relating to proteinuria and oedema, which form part of the diagnostic criteria for preeclampsia, are not routinely collected in South Australia. Instead, investigations into pregnancy-induced hypertensive disorders, a milder pathological form of preeclampsia, were undertaken. It is also important to realise that a much lower than expected incidence of hypertension in this cohort of 5.9% was observed – similar studies based on data from the same Pregnancy Outcome Unit in South Australia have documented hypertension in 9.2% of pregnancies (291). The most likely explanation for the low incidence of pregnancy induced hypertensive disorders is the large percentage of preterm babies in this case cohort (62.9%). Most cases of pregnancy-induced hypertension

occur at or near term, and a high rate of prematurity would therefore predispose to a lower rate of pregnancy-induced hypertension.

All of these significant results arise from adverse pregnancy outcomes in the presence of growth-restriction. This is supportive of the hypothesis that fetoplacental thrombosis may be the common denominator, involved in the pathogenesis of such adverse pregnancy outcomes. Larger studies are required to further elucidate such associations.

This study has demonstrated that fetal inherited thrombophilic polymorphisms are associated with and may be involved in the pathogenesis of adverse pregnancy outcomes. The fetal clotting system may be a contributing factor in the development of adverse pregnancy outcomes, most likely in combination with other factors, such as maternal inherited thrombophilia, viral infection or cytokine imbalance. Our research group is currently working to address these questions.

In summary, the evidence presented here suggests that fetal thrombophilia are related to adverse pregnancy outcomes in the presence of growth-restriction. This may not be the only clinical association. Maternal thrombophilia may also be required to produce the adverse pregnancy outcome phenotype to be displayed. There is still much debate about the importance of fetal thrombophilia in the development of adverse pregnancy outcomes. This study has shown that any effect of thrombophilia on adverse pregnancy outcomes is unlikely to be the result of fetal inherited thrombophilia alone, and future studies should investigate the contribution of the combination of maternal and fetal inherited thrombophilia.

Associations between Cytokine Polymorphisms and Adverse Pregnancy Outcomes

Abstract

Objective: To investigate the role of fetal inherited cytokine polymorphisms in the development of a range of adverse pregnancy outcomes, including pregnancy-induced hypertensive disorders, antepartum haemorrhage (APH), intrauterine growth restriction (IUGR) and preterm birth (PTB).

Methods: 717 cases (babies with an adverse pregnancy outcome listed above) and 609 controls were genotyped for Tumour Necrosis Factor alpha (TNF-α) and four polymorphisms in the Mannose Binding Lectin Gene (promoter polymorphism at position –221 and three coding polymorphisms in Exon 1 at codons 52, 54 and 57) using genomic DNA extracted from newborn screening cards.

Results: TNF-α -308 was associated with a number of adverse pregnancy outcomes, including IUGR <10th percentile (OR 1.74, 95% CI 1.07-2.83), PIHD (OR 2.54, 95% CI 1.10-5.86), APH with IUGR (OR 1.78, 95% CI 1.04-3.05) and PTB <32 weeks (OR 1.42, 95% CI 1.03-1.96). For babies born <28 weeks gestation, MBL -221 was associated with APH (OR 2.86, 95% CI 1.01-8.12) and PTB (OR 2.29, 95% CI 1.02-5.15). Polymorphisms in exon 1 of the MBL gene were associated with preexisting hypertension (Codon 54 OR 6.00, 95% CI 1.17-30.74), PIHD (Codon 57 OR 7.55, 95% CI 1.88-30.37), and APH (Codon 52 OR 16.62, 95% CI 1.70-162.30).

Conclusions: The -308 polymorphism in the proinflammatory TNF-α gene and polymorphisms in the anti-infectious MBL gene are associated with IUGR, PIHD, APH and PTB.

Introduction

The Fetal Inflammatory Response

The fetal inflammatory response is described as being a multisystem disorder that may result in preterm delivery and adverse neonatal outcome (184). It is now becoming apparent that the fetal inflammatory response to intra-amniotic infection is biologically important, even more so than the maternal inflammatory response (186). The onset of spontaneous preterm labour with preterm premature rupture of membranes, is preceded by a systemic proinflammatory cytokine response in the fetus, which is probably the fetal response to the presence of microbial products (188). There is also evidence suggesting that antenatal infection and brain white matter damage are linked by the fetal inflammatory response (78).

Cytokine Responses

TNF-α is a proinflammatory cytokine that is produced in response to infection (128). Its biological functions include the beneficial effects for the host in inflammation and in protective immune responses against a variety of infectious pathogens. It is produced rapidly and acts quickly, promoting a broad range of immunological and inflammatory responses (128). Unfortunately, this ability to be rapidly produced may pose more of a risk than the infection through which it is elicited. If the production of TNF-α is excessive, and is released systemically in large quantities, fatal complications such as multiple organ failure or circulatory collapse may occur (128).

The TNF-2 polymorphism, located in the promoter region of the gene, is represented as a single nucleotide G$\rightarrow$A base pair substitution at nucleotide -308 relative to the transcriptional start site (128). This polymorphism has been associated with high levels of TNF-α (138, 139), and can alter the binding of nuclear factors to the promoter region of the TNF gene, resulting in two-fold greater activity of the promoter of the TNF-2 allele compared to the TNF-1 allele (140). It has also been shown that human B cells transfected with the polymorphic promoter coupled with a reporter gene showed a five-fold increase in reported expression compared with the wild-type promoter (141). Finally, a study by Westendorp and colleagues found that approximately 60% of the variation in the production of TNF-α is genetically determined (142), suggestive of the importance of polymorphisms in this gene. This polymorphism has been linked to the development of

cerebral malaria, and it has been suggested that regulatory polymorphisms of cytokines such as TNF-α may be able to influence the outcomes of severe infection (143).

Mannose-binding lectin (MBL) plays more of an anti-infectious role, identifying and removing potentially infectious pathogens from the body (144, 145). The human MBL gene is found on chromosome 10, and has a number of known polymorphic sites, capable of altering circulating levels of MBL in the body. Two of these polymorphisms are located within the promoter region of exon 1 (147), at positions -550 and -221. They are both G→C base pair substitutions, and give rise to alleles H and L or Y and X respectively (148). These promoter mutations manifest as reduced levels of circulating MBL by affecting the transcriptional activity of the basal-promoter complex (149), however the -221 polymorphism in particular is associated with low MBL serum concentration compared with the wild-type allele (268). Three polymorphic sites are found in exon 1 of the MBL gene. The polymorphism at codon 52 is an Arginine to Cysteine amino acid substitution (CTG→TGT), whilst the polymorphism at codon 54 is a Glycine to Aspartic Acid amino acid substitution (GGC→GAC). Finally, the polymorphism at codon 57 is a Glycine to Glutamic Acid amino acid substitution (GGA→GAA) (151, 152). The amino acid substitutions at codons 54 and 57 result in disruption of the Gly-Xaa-Yaa structure of the collagenous backbone of MBL, resulting in limited binding between the MBL subunits, and significantly reduced levels of circulating functional protein (151). In contrast, the amino acid substitution at codon 52 does not interrupt the Gly-Xaa-Yaa sequence, but instead results in the formation of an extra disulphide bond, thus decreasing the stability of the molecule (152, Wallis, 1999 #500). Decreased levels of circulating MBL, resulting from polymorphisms in the gene, culminates in an impaired ability to defend against infectious pathogens, reducing the body's immune response to such infections, and increasing the susceptibility to infection.

The aim of this study was to investigate cytokine polymorphisms capable of altering the host response to infection or inflammation by either up or downregulating cytokine production, and assess their role in the development of adverse pregnancy outcomes. This study is the first large case-control study of adverse pregnancy outcomes and cytokine polymorphisms, investigating the associations between cytokine polymorphisms in the TNF-α and MBL genes and the development of adverse pregnancy outcomes.

Materials and Methods

Please refer to chapter 5 for information regarding methods to detect cytokine polymorphisms in the TNF-α and MBL genes.

Patient Selection

Please refer to Chapter 2 for detailed information regarding patient selection.

Statistical Analysis

Cytokine polymorphisms were genotyped from newborn screening samples that had been stored for up to 18 years. As controls were not matched for important covariates such as gestational age, analysis was undertaken using all controls without taking account of matching. Data analysis (GraphPad Instat version 3.06) then considered cases by gestational age range (<28 weeks, 28-31 weeks, <32 weeks, 32-36 weeks, <37 weeks, ≥ 37 weeks, and all gestational ages). Results are expressed as odds ratios (OR) with 95% confidence intervals (CI), comparing homozygosity and heterozygosity for each polymorphism separately with homozygosity for the wild-type allele. Data for homozygosity and heterozygosity combined compared with the wild-type allele are also presented. Please refer to Appendix 8 for tables detailing all calculated odds ratios and confidence intervals; only the significant results are presented in the main text. Some combinations of cytokine polymorphisms and adverse pregnancy outcomes types were not seen in some sub-groups: these odds ratios are not reported. P values less than 0.05 are highlighted in the tables. No adjustments were made for multiple testing in this largely exploratory study.

Results

Intrauterine Growth Restriction <10th percentile

241 babies in this cohort (33.6%) were growth-restricted to less than the 10th percentile. The TNF-α -308 polymorphism was associated with growth-restriction for babies born <37 weeks gestation (heterozygous OR 1.74, 95% CI 1.05-2.88; homozygous or heterozygous OR 1.74, 95% CI 1.07-2.83), <32 weeks gestation (heterozygous OR 2.64, 95% CI 1.20-5.82; homozygous or heterozygous OR 2.68, 95% CI 1.25-5.75) and <28 weeks gestation (heterozygous OR 4.22, 95% CI 1.36-13.11; homozygous or heterozygous OR 4.18, 95% CI 1.38-12.66) (Table 1). None of the polymorphisms in the MBL gene were associated with IUGR (Tables 2 and 3).

Hypertension

The mothers of 42 babies in this cohort (5.9%) suffered from hypertension, either pregnancy-induced, (23) or pre-existing (20). One mother suffered from both.

Pregnancy-Induced Hypertensive Disorders

23 (54.8%) of the 42 mothers displaying symptoms of hypertension in this cohort developed pregnancy-induced hypertensive disorders (PIHD). TNF-α -308 was associated with an increased risk of PIHD at all gestational ages (heterozygous OR 2.40, 95% CI 1.00-5.76; homozygous or heterozygous OR 2.54, 95% CI 1.10-5.86) (Table 1). Homozygous codon 52 was associated with PIHD for term babies (OR 48.07, 95% CI 1.55-1491.10), 32-36 weeks gestational age babies (OR 34.33, 95% CI 1.18-998.49), and babies born 28-31 weeks gestation (OR 48.07, 95% CI 1.55-1491.10). Heterozygous codon 57 increased the risk of PIHD at all gestational ages (OR 7.55, 95% CI 1.88-30.37), <37 weeks gestation (OR 9.23, 95% CI 2.23-38.16), <32 weeks gestation (OR 9.23, 95% CI 1.70-50.21), and 28-31 weeks gestation (OR 13.85, 95% CI 1.18-162.72) (Table 3).

Pregnancy-Induced Hypertensive Disorders and IUGR <10th percentile

9 (39.1%) of the 23 mothers who developed PIHD also gave birth to a growth-restricted baby <10th percentile. No babies with PIHD and IUGR <10th percentile were born between 28-31 weeks gestation. In this cohort, homozygous MBL codon 52 was associated with PIHD and IUGR <10th percentile for preterm babies (32-36 GA OR 48.07, 95% CI 1.55-1491.10; <32 GA and <28 GA OR 34.33, 95% CI 1.18-998.49). No other polymorphisms were significantly increased in cases compared with controls (Table 3).

Pre-existing Hypertension

Of the 42 mothers displaying symptoms of hypertension in this cohort, 20 (47.6%) were classified as having pre-existing hypertension. No associations were observed for TNF-α or MBL –221. Homozygosity for MBL exon 1 codon 52 was associated with pre-existing hypertension for babies born at 32-36 weeks gestation (OR 34.33, 95% CI 1.18-998.49), and also at <32 and <28 weeks gestation (OR 80.11, 95% CI 2.23-2876.10). An association was observed for homozygous codon 54 at all gestational ages, with an odds ratio of 6.00 (95% CI 1.17-30.74) (Table 3).

Antepartum Haemorrhage

The mothers of 340 of 717 babies in the case cohort (47.4%) suffered from some form of antepartum haemorrhage (APH). This includes diagnoses of placenta praevia and abruption, as well as other and unknown causes. Homozygous MBL -221 was associated with APH for babies born <28 weeks gestation (OR 2.86, 95% CI 1.01-8.12) (Table 2). Homozygosity for MBL codon 52 was also significantly associated with APH, with odds ratios of 16.62 (95% CI 1.70-162.30) and 18.46 (95% CI 1.64-208.38) for gestational ages <32 weeks and 28-31 weeks respectively (Table 3).

Antepartum Haemorrhage and IUGR <10th percentile

108 of the 340 mothers diagnosed with APH (31.8%) gave birth to a growth-restricted baby <10th percentile. For this cohort, TNF-α -308 increased the risk of APH and growth-restriction <10th percentile for babies born <37 weeks gestation (heterozygous OR 1.79, 95% CI 1.02-3.13; homozygous or heterozygous OR 1.78, 95% CI 1.04-3.05) and <32 weeks gestation (heterozygous OR 2.42, 95% CI 1.05-5.60; homozygous or heterozygous OR 2.52, 95% CI 1.13-5.63) (Table 1). No other associations were observed for this cohort.

Preterm Birth <37 weeks

451 (62.9%) of the case cohort were born prematurely at a gestational age of less than 37 weeks. TNF-α -308 was associated with preterm birth for babies <32 weeks gestation (homozygous or heterozygous OR 1.42, 95% CI 1.03-1.96), 28-31 weeks gestation (homozygous OR 2.52, 95% CI 1.17-5.42), and <28 weeks gestation (heterozygous OR 1.62, 95% CI 1.02-2.57) (Table 1). Homozygous MBL -221 was also associated with preterm birth <28 weeks gestation (OR 2.29, 95% CI 1.02-5.15) (Table 2).

Preterm Birth and IUGR <10th percentile

82 (18.2%) of the 451 preterm babies were growth-restricted <10th percentile. TNF-α -308 was associated with PTB and IUGR <10th percentile for all preterm babies (heterozygous OR 1.74, 95% CI 1.05-2.88; homozygous or heterozygous OR 1.74, 95% CI 1.07-2.83), for babies <32 weeks gestation (heterozygous OR 2.64, 95% CI 1.20-5.82; homozygous or heterozygous OR 2.68, 95% CI 1.25-5.75). and for babies <28 weeks gestation (heterozygous OR 4.22, 95% CI 1.36-13.11; homozygous or heterozygous OR 4.18, 95% CI 1.38-12.66) (Table 1). No polymorphisms in the MBL gene were associated with PTB and IUGR <10th percentile.

Table 1: Significant odds ratios (95% CI) for adverse pregnancy outcomes for the tumour necrosis factor –α -308 polymorphism. WT = wild-type.

Adverse Pregnancy Outcome	Gestation (weeks)	Zygosity	Cases +ve/WT	Controls +ve/WT	Odds Ratio (95% CI)	p-value
IUGR <10th percentile	<28	Heterozygous	8/5	155/409	4.22 (1.36-13.11)	<0.05
	<28	Abnormal	9/5	176/409	4.18 (1.38-12.66)	<0.05
	<32	Heterozygous	13/13	155/409	2.64 (1.20-5.28)	<0.05
	<32	Abnormal	15/13	176//409	2.68 (1.25-5.75)	<0.05
	<37	Heterozygous	29/44	155/409	1.74 (1.05-2.88)	<0.05
	<37	Abnormal	33/44	176/409	1.74 (1.07-2.83)	<0.05
Any Pregnancy-Induced Hypertension	All	Heterozygous	10/11	155/409	2.40 (1.00-5.76)	0.05
	All	Abnormal	12/11	176/409	2.54 (1.10-5.86)	<0.05
Any APH and IUGR <10th percentile	<32	Heterozygous	11/12	155/409	2.42 (1.05-5.60)	0.06
	<32	Abnormal	13/12	176/409	2.52 (1.13-5.63)	<0.05
	<37	Heterozygous	23/34	155/409	1.79 (1.02-3.15)	<0.05
	<37	Abnormal	26/34	176/409	1.78 (1.04-3.05)	<0.05
All Preterm Birth	<28	Heterozygous	35/57	155/409	1.62 (1.02-2.57)	<0.05
	28-31	Homozygous	11/85	21/409	2.52 (1.17-5.42)	<0.05
	<32	Abnormal	87/142	176/409	1.42 (1.03-1.96)	<0.05
All Preterm Birth & IUGR <10th percentile	<28	Heterozygous	8/5	155/409	4.22 (1.36-13.11)	<0.05
	<28	Abnormal	9/5	176/409	4.18 (1.38-12.66)	<0.05
	<32	Heterozygous	13/13	155/409	2.64 (1.20-5.82)	<0.05
	<32	Abnormal	15/13	176/409	2.68 (1.25-5.75)	<0.05
	<37	Heterozygous	29/44	155/409	1.74 (1.05-2.88)	<0.05
	<37	Abnormal	33/44	176/409	1.74 (1.07-2.83)	<0.05

Table 2: Significant odds ratios (95% CI) for adverse pregnancy outcomes for the mannose binding lectin -221 polymorphism. WT = wild-type.

Adverse Pregnancy Outcome	Gestation (weeks)	Zygosity	Cases +ve/WT	Controls +ve/WT	Odds Ratio (95% CI)	p-value
Any APH	<28	Homozygous	5/24	26/357	2.86 (1.01-8.12)	0.06
All Preterm Birth	<28	Homozygous	9/54	26/357	2.29 (1.02-5.15)	0.07

Table 3: Significant odds ratios (95% CI) for adverse pregnancy outcomes for the mannose binding lectin exon 1 coding region polymorphisms. WT = wild-type.

Adverse Pregnancy Outcome	Gestation (weeks)	Zygosity	Cases +ve/WT	Controls +ve/WT	Odds Ratio (95% CI)	p-value
Any Preexisting Hypertension	<28, <32	Homozygous 52	0/1	1/360	80.11 (2.23-2876.10)	>0.05
	32-36	Homozygous 52	0/3	1/360	34.33 (1.18-998.49)	>0.05
	All	Homozygous 54	2/8	15/360	6.00 (1.17-30.74)	>0.05
Any PIHD	28-31	Homozygous 52	0/2	1/360	48.07 (1.55-1491.10)	>0.05
	28-31	Heterozygous 57	1/2	13/360	13.85 (1.18-162.72)	>0.05
	<32	Heterozygous 57	2/6	13/360	9.23 (1.70-50.21)	<0.05
	32-36	Homozygous 52	0/3	1/360	34.33 (1.18-998.49)	>0.05
	<37	Heterozygous 57	3/9	13/360	9.23 (2.23-38.16)	<0.05
	≥ 37	Homozygous 52	0/2	1/360	48.07 (1.55-1491.10)	>0.05
	All	Heterozygous 57	3/11	13/360	7.55 (1.88-30.37)	<0.05
Any PIHD & IUGR <10th percentile	<28, <32	Homozygous 52	0/3	1/360	34.33 (1.18-998.49)	>0.05
	32-36	Homozygous 52	0/2	1/360	48.07 (1.55-1491.10)	>0.05
Any APH	28-31	Homozygous 52	2/39	1/360	18.46 (1.64-208.38)	<0.05
	<32	Homozygous 52	3/65	1/3660	16.62 (1.70-162.30)	<0.05

Discussion

This is the largest study to investigate the associations between fetal cytokine polymorphisms and a wide range of adverse pregnancy outcomes, including intrauterine growth restriction (IUGR), pregnancy-induced hypertensive disorders, antepartum haemorrhage, and prematurity. The link between cytokines and prematurity is quite well established (188, 223, 224), as is the link between intrauterine growth restriction and cytokines (225). Using this large cohort of pathology-enriched cases and healthy term controls, polymorphisms within the TNF-α and MBL genes have been genotyped, and show associations with all the adverse pregnancy outcomes studied, including PIHD, IUGR, APH and preterm birth. Unfortunately, no information on the maternal genotypes for these polymorphisms was available. Maternal polymorphisms in these genes could affect uteroplacental circulation and/or the maternal systemic response to infection. Future studies would be strengthened by comparing maternal and fetal genotypes for these cytokine polymorphisms, and investigating interactions between the two.

787 separate analyses were performed on the individual cytokine polymorphisms, with 42 (5.3%) yielding significant associations. Such multiple analyses increase the likelihood of identifying chance statistical associations (Type I error). Equally, because of small numbers in some of the subanalyses, associations cannot be confidently excluded (Type II error). Any findings must be interpreted with caution, and further large-scale studies are necessary to definitively determine if there are associations between these and other cytokine polymorphisms and adverse pregnancy outcomes.

Tumour Necrosis Factor α

The TNF-α -308 polymorphism is associated with higher circulating TNF-α levels than the wild-type allele. When the fetal inflammatory response syndrome is activated, in response to infection or inflammation, circulating TNF-α levels would be increased even further, which can result in potentially fatal complications (128).

TNF-α and Intrauterine Growth Restriction

These results suggest that the TNF-α -308 polymorphism is associated with IUGR below the 10th percentile for gestational age, in premature babies born <37 weeks gestation. TNF-α may act to cause IUGR by enhancing vasoconstriction of the fetal placental vascular bed. Significantly higher levels of TNF-α have been observed in the perfusate of IUGR placentae, compared with normal placentae (225). Higher circulating levels of

TNF-α have also been observed in women who gave birth to an IUGR baby and who also had Doppler-defined placental insufficiency, compared with women who gave birth to an IUGR baby with no placental insufficiency, suggesting that higher TNF-α levels in these mothers could be specific to certain subsets of IUGR, identifying cases with placental dysfunction (226).

TNF-α and Hypertension

A weakness of this study is that it could not distinguish between gestational hypertension and preeclampsia. It should be noted, however, that the diagnosis of PIHD in the SA Pregnancy Outcome Database has previously been shown to be highly reliable (291-293). There are conflicting reports on the role of TNF-α in the development of preeclampsia. Increased concentrations of circulating TNF-α has been observed in placentas from preeclamptic patients (227), however another study found no associations with maternal or fetal concentrations of TNF-α and preeclampsia (228). Studies investigating the maternal genotype of TNF-α in the development of preeclampsia found no significant associations (229). Lachmeijer and colleagues did find that more preeclamptic patients carried the TNF-α -308 polymorphism, but showed no linkage in the affected sibling pair study (230). The only study investigating the fetal TNF-α -308 polymorphism did not find any associations for preeclampsia, however this study had a mixed ethnic group, with over 50% of the women in both the case and control groups being African-American (231). The TNF-α -308 polymorphism is highly ethnic-specific, and the pooling of ethnic groups in analysis may explain the lack of significant results (294). This study investigated the role of the fetal TNF-α -308 polymorphism in the development of hypertension, both preexisting and pregnancy-induced, in a Caucasian Australian population and found significant positive associations. This link may possibly be explained by the actions of TNF-α as part of the fetal inflammatory response to infection.

TNF-α is thought to play an important role in placentation and other early pregnancy events (229). Major features in the pathogenesis of preeclampsia include shallow endovascular cytotrophoblast invasion in the spiral arteries, an exaggerated inflammatory response, and inappropriate endothelial-cell activation (295, 296). The -308 polymorphism may be partly responsible for the exaggerated inflammatory response seen in preeclampsia. TNF-α can also induce thrombosis by increasing apoptosis and the expression of tissue factor; one of the major pathways for TNF-α to exert a prothrombotic effect is by increasing tissue factor and suppressing the tissue factor pathway inhibitor (297-300).

TNF-α and Antepartum Haemorrhage

Within the case cohort, 47.4% (340) of mothers were diagnosed with some form of APH. The classification of APH within the South Australian Perinatal Data Collection of births includes diagnosis of placenta praevia and placental abruption, as well as other and unknown causes of APH. It is important to note that this is a pathology-enriched cohort of babies, with much higher incidences of these adverse pregnancy outcomes than would normally be expected in the general population.

No studies to date have investigated the role of TNF-α -308 polymorphism in antepartum haemorrhage. The present study found significant associations between TNF-α -308 for APH when there was also growth-restriction $<10^{th}$ percentile. These associations were observed for premature babies, and the possibility remains that it is not a true association for APH alone, but instead these results may be due to the IUGR and prematurity. Unfortunately, differentiations between partial placental abruption, unexplained APH or bleeding due to placenta previa could not be made. Larger studies with better power are required to fully elucidate the role of TNF-α in the development of APH and its specific subcategories. One possible hypothesis for this association is that TNF may increase APH by increasing apoptosis in endovascular trophoblasts and trigger spiral artery thrombosis, which is associated with placental abruption.

TNF-α and Preterm Birth

There are many studies detailing associations between high circulating levels of TNF-α and preterm birth (66, 70, 124, 141, 224, 277, 301-305). Studies have also shown that the -308 promoter polymorphism is associated with preterm premature rupture of membranes and subsequent preterm delivery (141). These findings support these associations, and show that the TNF-α polymorphism is associated with preterm delivery, particularly very preterm deliveries <32 weeks gestation. This association remained for those babies who were premature and also growth-restricted $<10^{th}$ percentile. Again, it is unclear if it is the actions of prematurity or growth-restriction that "cause" these associations.

Mannose Binding Lectin

Polymorphisms in the MBL gene are associated with decreased circulating levels of MBL. In the event of *in utero* infection, lower circulating levels of MBL could decrease the immune response to the infection, and increase the risk of adverse pregnancy outcomes.

MBL and Intrauterine Growth Restriction

To date, no research has been conducted into the possible roles played by MBL in the development of IUGR. These results show very few associations, although larger studies are required to confirm or refute these findings. One possible reason for the lack of associations could be that these polymorphisms alone are not sufficient to cause adverse pregnancy outcomes. The adverse pregnancy outcomes investigated in this study are most likely multifactorial, and more than one abnormality may be needed for the development of adverse outcomes. Infection is a likely trigger, due to the increased susceptibility to infection as a result of the polymorphisms causing lower circulating levels of the anti-infectious MBL molecules, thus increasing the risk of infection.

MBL and Hypertension

This research demonstrated that polymorphisms in the MBL gene, particularly in the exon 1 coding region of the gene, are associated with PIHD. It remains unclear if this is a direct or indirect relationship. The polymorphisms in the MBL gene result in lower circulating levels of MBL (149, 151-153), leaving affected individuals more vulnerable to infection (156, 159, 165). Infections have been shown to cause vessel inflammation and artery wall thickening, contributing to the increased resistance of blood vessels and thus promoting a hypertensive state (236). Some viruses, particularly herpesviruses, are capable of causing thrombogenic changes to host cells and initiating the clotting cascade via the generation of thrombin (306), thus promoting vascular disease. Another study found that the onset of hypertension was often associated with HSV, CMV and EBV infections (307). It is possible that polymorphisms in the MBL gene are associated with hypertension because of the increased susceptibility to infections capable of causing vascular changes consistent with hypertension. The MBL polymorphisms were also found to have an association with pre-existing hypertension. There may be a potential interaction between carriage of MBL polymorphisms and an individual's response to infections such as *Chlamydia pneumoniae,* enterovirus and cytomegalovirus that have been associated with atherosclerosis (308)

MBL and Antepartum Haemorrhage

No studies to date have investigated the associations between polymorphisms in the MBL gene and APH. This study has shown positive associations between the two, in babies born before 32 weeks gestation. It is known that prematurity is associated with infection, and the polymorphisms in the MBL gene increase susceptibility to infection, therefore it is

logical to suggest that the MBL polymorphisms are associated with infection-related premature birth. Further studies are needed to investigate these postulated associations.

MBL and Preterm Birth

No associations were found between preterm birth and polymorphisms in the MBL gene, despite other researchers demonstrating such associations (232). However, there are many causes of preterm birth, and infection is only one postulated cause (69). The MBL polymorphisms may only be involved in infection-related preterm births; to demonstrate such associations, analysis would need to be conducted for infection-related prematurity separately to other causes of prematurity in prospective studies. As a whole, polymorphisms in the MBL gene are not associated with preterm birth, however, these results need to be interpreted with caution.

Conclusions

In summary, this research has shown that the -308 polymorphism in the proinflammatory TNF-α gene and polymorphisms in the anti-infection MBL gene are associated with a number of adverse pregnancy outcomes. Such associations need to be confirmed in larger case-control studies, and should be interpreted with caution at this stage. It remains unclear whether the cytokines themselves mediate or cause adverse pregnancy outcomes, or whether the infection which initiated the fetal inflammatory response is responsible for the damage. A combination of factors is likely.

Neurotropic Viruses are associated with Adverse Pregnancy Outcomes

Abstract

Objective: To investigate the role of fetal viral infection in the development of a range of adverse pregnancy outcomes, including pregnancy-induced hypertensive disorders (PIHD), antepartum haemorrhage (APH), intrauterine growth restriction <10th percentile (IUGR) and preterm birth (PTB).

Methods: The newborn screening cards of 717 cases and 609 controls were tested for viral RNA and DNA from enteroviruses and herpesviruses using polymerase chain reaction (PCR). The herpesviruses were detected using two PCRs, one detecting nucleic acids from HSV-1, HSV-2, EBV, CMV and HHV-8, hereafter designated Herpes group A viruses, and the other detecting nucleic acids from VZV, HHV-6 and HHV-7, hereafter designated Herpes group B viruses.

Results: Detection of cytomegalovirus (CMV) DNA was significantly associated with preterm birth <28 weeks gestation (OR 1.62, 95% CI 1.02-2.57). The risk of developing PIHD was increased in the presence of Herpes group B viruses (OR 3.57, 95% CI 1.10-11.70), CMV (OR 3.89, 95% CI 1.67-9.06), any herpesvirus (OR 5.70, 95% CI 1.85-17.57) and any virus (OR 5.17, 95% CI 1.68-15.94). Viral infections were not associated with IUGR or APH without hypertension; the combined presence of pre-existing hypertension and any herpesvirus was significantly associated with growth-restriction (OR 5.70, 95% CI 1.17-27.73).

Conclusions: Exposure to *in utero* viral infection, is significantly associated with very preterm birth <28 weeks gestation, and also with pregnancy-induced hypertensive disorders, but not with IUGR or APH.

Introduction

During pregnancy, a decrease in resistance to some viral infections is observed as a consequence of diminished cell-mediated immunity as part of the so-called Th1 shift. If this results in maternal viraemia, the fetus is at risk of infection via transplacental transmission (194). More recently it has been postulated that viral infection *in utero* may increase the risk of a wide range of adverse pregnancy outcomes, such as hypertension, intrauterine growth restriction (IUGR), and prematurity (236, 238, 239).

The placenta acts as a potential barrier to maternal/fetal viral infection. For a pathogen to cause fetal damage *in utero*, it must first cross the placenta, which can be initiated during the viraemic, bacteraemic, or parasitaemic phase of maternal infection (196). This placental barrier may be less effective in early pregnancy, and potentially when the placenta is damaged, for example by vascular disease, manifesting as infarction. Placental dysfunction has been associated with acquired and genetic thrombophilia, systemic lupus erythematosus (SLE), and pre-existing diabetes. Clinical syndromes associated with (utero)placental insufficiency and/or placental vasculopathy include preeclampsia, IUGR, preterm labour, fetal demise and preterm labour.

Herpesviruses (including cytomegalovirus, herpes simplex viruses 1 and 2, varicella zoster virus, Epstein-Barr virus, and human herpesvirus 6, 7 and 8), and enteroviruses are capable of crossing the placenta and setting up *in utero* infection (197-204). These viruses may contribute directly or indirectly to adverse pregnancy outcomes. The likelihood of maternal infection resulting in infection of the fetus varies according to the specific virus, whether the infection is primary or recurrent, and the gestational age of the fetus at the time of infection. For example, the overall rate of congenital cytomegalovirus infection is approximately 1%, and of these, a 50% risk of transmission to the fetus occurs in mothers with primary infection but only a minority (<1%) of mothers with reactivation (or reinfection) transmit the virus *in utero*. Approximately 10-15% of CMV-infected newborns of mothers with primary infection and <1% with infection arising from maternal CMV reactivation display signs of the infection (197). Once the infection has crossed the placenta into the fetal circulation, there is the potential for adverse outcomes. These can be caused by the infectious agent directly, or by the fetal inflammatory response to the infection where pro-inflammatory induced cytokines may adversely affect the developing brain and perhaps also placental function. This combination of factors plus genetic influences may determine fetal outcomes. Maternal infection through direct and indirect

effects of infection (eg cytokine responses) may also determine who develops adverse pregnancy outcomes such as hypertension, antepartum haemorrhage, and preterm birth.

The aim of this study was to investigate the role of fetal exposure to viral infection (detected through the presence of viral nucleic acids) in a wide range of adverse pregnancy outcomes, including IUGR, PIHD, antepartum haemorrhage, and preterm birth. To date, this is the largest case-control study investigating maternal/fetal infection as a cause of adverse pregnancy outcomes.

Materials and Methods

Please refer to chapter 6 for information regarding methods to detect viral nucleic acids from newborn screening cards. The viruses of interest are all known to be neurotropic and were categorised into DNA viruses and RNA viruses. The DNA viruses included: Herpes Simplex Virus 1 (HSV-1), Herpes Simplex Virus 2 (HSV-2), Varicella Zoster Virus (VZV), Epstein-Barr Virus (EBV), Cytomegalovirus (CMV), Human Herpesvirus 6 (HHV-6), Human Herpesvirus 7 (HHV-7), and Human Herpesvirus 8 (HHV-8). The RNA viruses of interest were members of the Enterovirus family. Detection of the DNA viruses was divided into two PCRs, one detecting nucleic acids from HSV-1, HSV-2, EBV, CMV and HHV-8, hereafter designated Herpes group A, and the other detecting nucleic acids from VZV, HHV-6 and HHV-7, hereafter designated Herpes group B. Within the Herpes group A PCR, differentiations were made between CMV and the remaining viruses (HSV-1, HSV-2, EBV and HHV-8) due to differences in PCR product band size visualised by agarose gel electrophoresis.

Patient Selection

Please refer to Chapter 2 for information regarding patient selection.

Statistical Analysis

Viral nucleic acids were detected from newborn screening samples that had been stored for up to 18 years. As controls were not matched for important covariates such as gestational age, analysis was undertaken using all controls without taking account of matching. Data analysis (GraphPad Instat version 3.06) then considered cases by gestational age range (<28 weeks, 28-31 weeks, <32 weeks, 32-36 weeks, <37 weeks, ≥ 37 weeks, and all gestational ages). Results are expressed as odds ratios (OR) with 95% confidence intervals (CI), comparing positive with negative virus detection. Please refer to Appendix 9 for tables detailing all calculated odds ratios and confidence intervals; only significant results are presented in the main text. Some combinations of viral nucleic acids and adverse pregnancy outcomes were not seen in some sub-groups: these odds ratios are not reported. P-values <0.05 are highlighted in the tables. No adjustments were made for multiple testing in this largely exploratory study.

Results

Intrauterine Growth Restriction <10th percentile

241 babies in the case cohort (33.6%) were growth restricted to less than the 10th percentile. There were no associations between any of the viruses tested and intrauterine growth restriction less than the 10th percentile (Table 1), or for the 136 babies below the 5th percentile.

Hypertension

The mothers of 42 babies in our cohort (5.9%) suffered from hypertension, either pregnancy-induced, (23) or pre-existing (20). One mother suffered from both.

Pregnancy Induced Hypertensive Disorders

Pregnancy-induced hypertension (PIH) developed in 23 (54.8%) of the 42 mothers displaying symptoms of hypertension in the cohort. In this cohort, the detection of Herpes group B viruses increased the risk of developing PIHD (OR 3.57, 95% CI 1.10-11.57) (Table 1). These significant associations were also observed in preterm babies. The detection of CMV was also significantly associated with PIHD (OR 3.89, 95% CI 1.67-9.06) (Table 2). The detection of any herpesvirus or any virus was also associated with PIHD, with ORs of 5.70 (95% CI 1.85-17.57) and 5.17 (95% CI 1.68-15.94), respectively (Table 3).

Pregnancy-induced Hypertensive Disorders and IUGR <10th percentile

Of the 23 mothers who developed PIHD, 9 (39.1%) also gave birth to a growth restricted baby (<10th percentile). No significant associations were observed for this cohort.

Pre-existing Hypertension

Of the 42 mothers displaying symptoms of hypertension in the case cohort, 20 (47.6%) were classified as having pre-existing hypertension. In this cohort, the majority of analyses were non-significant, with only one significant association found. Significantly more mothers with pre-existing hypertension had growth-restricted babies who tested positive for any herpesvirus (OR 5.70, 95% CI 1.17-27.73) (Table 3).

Antepartum Haemorrhage

The mothers of 340 of 717 babies in this case cohort (47.4% of cases) suffered from antepartum haemorrhage (APH). There were no associations between exposure to viral infection and APH.

Antepartum Haemorrhage and IUGR <10th percentile

Of the 340 mothers diagnosed with APH, 108 (31.8%) gave birth to a growth-restricted baby <10th percentile. The majority of associations investigated were non-significant. Babies born at 32-36 weeks gestation who tested positive for Herpes group B viral DNA were at greater risk of APH and growth-restriction, with an odds ratio of 2.79 (95% CI 1.08-7.25) (Table 1).

Preterm Birth <37 weeks

451 (62.9%) of this case cohort were born prematurely at a gestational age of less than 37 weeks. Detection of CMV DNA was significantly associated with preterm birth <28 weeks gestation (OR 1.62, 95% CI 1.02-2.57) (Table 2). In contrast, detection of HSV-1, HSV-2, EBV or HHV-8 DNA reduced the likelihood of delivering preterm <28 weeks (OR 0.12, 95% CI 0.01-1.94) (Table 2). This latter result may be due to the very small numbers involved in this particular analysis. No other viruses showed any significant associations.

Preterm Birth and IUGR <10th percentile

82 (18.2%) of the 451 preterm babies were growth-restricted <10th percentile. No significant associations were observed between prematurity with growth-restriction <10th percentile and exposure to viral infection.

Table 1: Significant odds ratios (95% CI) for adverse pregnancy outcomes for the presence of Herpes group B viruses.

Adverse Pregnancy Outcome	Gestation (weeks)	Cases +ve/-ve	Controls +ve/-ve	Odds Ratio (95% CI)	p-value
APH and IUGR <10th percentile	32-36	6/23	41/439	2.79 (1.08-7.25)	<0.05
Pregnancy induced hypertension	<28	2/3	41/439	7.14 (1.16-43.97)	0.06
	28-31	2/1	41/439	21.42 (1.90-241.38)	<0.05
	<32	4/4	41/439	10.71 (2.58-44.42)	<0.01
	<37	4/8	41/439	5.35 (1.55-18.55)	<0.05
	≥ 37	4/12	41/439	3.57 (1.10-11.57)	<0.05

Table 2: Significant odds ratios (95% CI) for adverse pregnancy outcomes for the presence of single and combined Herpes group A viruses.

Virus	Adverse Pregnancy Outcome	Gestation (weeks)	Cases +ve/-ve	Controls +ve/-ve	Odds Ratio (95% CI)	p-value
HSV	All Preterm Births	<28	0/94	25/265	0.12 (0.01-1.94)	<0.05
CMV	All Preterm Births	<28	33/61	147/440	1.62 (1.62-1.02-2.57)	<0.05
	Pregnancy-induced hypertensive disorders	≥ 37	5/1	147/440	14.97 (1.73-129.21)	<0.01
		All	13/10	147/440	3.89 (1.67-9.06)	<0.01
Herpes group A	Pregnancy-induced hypertensive disorders	≥ 37	5/1	167/420	12.58 (1.46-108.50)	<0.01
		All	13/10	167/420	3.27 (1.41-7.60)	<0.01

Table 3: Significant odds ratios (95% CI) for adverse pregnancy outcomes for the presence any herpesvirus or any virus.

Virus	Adverse Pregnancy Outcome	Gestation (weeks)	Cases +ve/-ve	Controls +ve/-ve	Odds Ratio (95% CI)	p-value
Any Herpes	Any Hypertension and IUGR <10th percentile	32-36	6/1	191/311	9.77 (1.17-81.82)	<0.05
		<37	8/3	191/311	4.34 (1.14-16.57)	<0.05
	Pre-existing hypertension	<37	7/2	191/311	5.70 (1.17-27.73)	<0.05
	Pregnancy induced hypertensive disorders	≥ 37	5/0	191/311	17.89 (0.98-325.63)	<0.01
		All	14/4	191/311	5.70 (1.85-17.57)	<0.01
Any Virus	Any Hypertension and IUGR <10th percentile	32-36	6/1	203/300	8.87 (1.06-74.24)	<0.05
		<37	8/3	203/300	3.94 (1.03-15.04)	0.06
	Pregnancy induced hypertensive disorders	<37	9/4	203/300	3.33 (1.01-10.95)	<0.05
		≥ 37	5/0	203/300	16.24 (0.89-295.57)	<0.05
		All	14/4	203/300	5.17 (1.68-15.94)	<0.01

Discussion

This is the largest study to investigate the associations between fetal exposure to viral infection detected by the presence of viral nucleic acids in blood collected within the first 2-3 days of neonatal life and a wide range of adverse pregnancy outcomes, including intrauterine growth restriction (IUGR), pregnancy-induced hypertensive disorders (PIHD), antepartum haemorrhage, and prematurity. The link between prematurity and infection is well established (126, 233, 234). The role of infection in other adverse pregnancy outcomes is not so well studied, although over the past decade the potential involvement of infection in the pathogenesis of preeclampsia has certainly received quite a bit of attention (235).

Using this large cohort of pathology-enriched cases and healthy term controls, specific viral DNA and RNA nucleic acid sequences have been identified, and show that exposure to viral infection is associated with adverse pregnancy outcomes, particularly pregnancy-induced hypertensive disorders.

394 separate analyses were performed on the individual viruses, with 5.3% (21) yielding significant associations. Such multiple analyses increase the likelihood of identifying chance statistical associations and because of small numbers in some of the subanalyses, associations cannot be confidently excluded. Any findings must be interpreted with caution, and further large-scale prospective studies are necessary to definitively determine if associations between *in utero* exposure to viral infection and adverse pregnancy outcomes exist.

This study has shown that *in utero* exposure to viral infection is associated with PIHD. These results demonstrate that detection of viral nucleic acids, in particular Herpes group B and CMV, are associated with PIHD in a wide range of gestational ages. One mechanism by which such associations could be explained is the inflammatory responses caused by these viruses. Vessel inflammation and artery wall thickening, as a result of viral infection, can contribute to the increased resistance of blood vessels (236), thus promoting an hypertensive state. HSV-1, HSV-2 and CMV, members of the herpesvirus family, are capable of causing thrombogenic changes to host cells and initiating the clotting cascade via the generation of thrombin (306), thus promoting vascular disease. Another study found that the onset of hypertension was often associated with HSV, CMV and EBV infections (307).

Pregnancy-induced hypertension is considered a precursor to preeclampsia, whose course can culminate in full-blown eclampsia and its life-threatening severe pathology. The findings from the present study are supported by the findings of others: von Dadelszen and colleagues (309) showed that women with early onset preeclampsia had higher levels of anti-CMV antibodies than women with late onset preeclampsia, women with normotensive IUGR, and women with normal pregnancies. Exposure to CMV and subsequent production of anti-CMV antibodies may generate pathogenic antiphospholipid antibodies which are capable of binding and activating endothelial cells (237). This in turn can enhance thrombus formation and increase inflammation, thus causing hypertension. A similar connection has also been identified for enteroviral infection and atherosclerosis (308), suggesting that viral infections in general are capable of increasing blood pressure and causing hypertension.

This study does not show any major associations between exposure to viral nucleic acids and IUGR or preterm birth, despite other research suggesting possible links between these adverse pregnancy outcomes (238, 239). Viral infection, in particular CMV, is one of the typical 'textbook' causes of IUGR, however no association was found in the present study.

No associations with APH were identified, an outcome not previously investigated for associations with viral infection. Within our case cohort of 717 babies, the mothers of 340 (47.4%) were diagnosed with some form of APH. The classification of APH within the South Australian Perinatal Data Collection of births includes diagnosis of placenta praevia and placental abruption, as well as other and unknown causes of APH. It is important to note that this is a pathology-enriched cohort of babies, with much higher incidences of these adverse pregnancy outcomes than would normally be expected in the general population. The results do, however, agree with van Dongen and colleagues (240), who found no associations between IUGR and adenoviruses or enteroviruses. These discrepancies may be explained by study design differences. This study used non-quantitative PCR methodology on archived newborn screening cards, and there remains the possibility of the results reflecting differing viral loads. Low viral loads may not invoke significant inflammatory/cytokine responses and therefore may not precipitate adverse pregnancy outcomes such as preterm birth or IUGR; alternatively, high viral loads, possibly associated with major inflammation and/or damaging cytokine production, could be necessary before such adverse outcomes are observed. Furthermore, these results may reflect different gestational ages at which exposure to the virus had first occurred. Infections that occur earlier in intrauterine life tend to be associated with more severe

clinical sequelae compared with those occurring later (310). One study found that positive amniotic fluid viral DNA PCR results were associated with an increased rate of fetal structural malformations, IUGR, hydrops and other fetal abnormalities (239). These tests were performed between 19-20 weeks gestation, compared with 3-5 days after birth in the current study, which may explain the differing results. Prospective studies designed to quantify viral loads at various gestational ages to further investigate this theory are planned. Also, this study was unable to test every potential virus. Other candidate viruses (for example adenoviruses, rotaviruses, human coronaviruses, parvovirus, paramyxoviruses (pneumovirus and human metapneumovirus) and lymphocytic choriomeningitis virus) also deserve investigation.

The results do not necessarily indicate active congenital or neonatal infection. Newborn screening cards were tested for the presence of viral DNA and RNA. While detection of viral nucleic acids in the blood of neonates indicates exposure to and replication of the respective virus or viruses, this study was not designed to detect evidence of an accompanying inflammatory response. Given the small sample volume of blood and the limit of detection of 1-10 viral nucleic acid copies, it could be inferred that true viraemic infection was occurring. On the same basis, it would seem unlikely that the presence of viral nucleic acid merely reflected maternal-fetal cell trafficking or maternal blood contamination. These questions remain unanswered, and prospective investigations are required to follow women through pregnancy, quantitatively testing antenatal blood samples for viral nucleic acids, and determining if there is active infection in the fetus/neonate by examining leukocytes and sera for the presence of viral antigens associated with active viral replication. PCR contamination was excluded by working in two designated separate laboratories for preparation of PCR samples (to eliminate the possibility of PCR product contamination) and by using appropriate controls in all assays. Furthermore, no other viral PCR work was being conducted by other users of the PCR laboratories.

When any adverse pregnancy outcome was investigated, a protective effect for HSV was observed for babies born <32 weeks gestation. This is most likely an artefactual result, due to only 1 baby testing positive for HSV at this gestational age. Further investigations using larger sample sizes are required to confirm or refute this finding.

In summary, in this chapter it has been demonstrated that exposure to viral infection (as demonstrated by the presence of viral nucleic acids) is associated with PIHD, but not with

other adverse pregnancy outcomes such as IUGR, preterm birth or antepartum haemorrhage. These findings support previously published indirect findings (such as antibody responses) of others that pointed to a relationship between CMV and hypertension of pregnancy. The implications of these findings suggest that specific preventive measures (for example vaccines or passive immunity via virus-specific immunoglobulin) could be undertaken to suppress viral reactivation or prevent primary infection in pregnancy. It is likely that adverse pregnancy outcomes are multifactorial, and may require other factors, such as genetic susceptibility to infection, genetically regulated proinflammatory cytokine responses and inherited thrombophilia for the adverse phenotypes to be expressed.

Do inherited thrombophilic polymorphisms, cytokine polymorphisms and perinatal viral exposure interact to further increase the risks of cerebral palsy?

Abstract

Objective: To investigate associations and interactions between the three main outcome measures: inherited thrombophilic polymorphisms, cytokine polymorphisms and viral infection, which may contribute to the future development of cerebral palsy.

Methods: Multivariable analysis was performed by stepwise backwards unconditional logistic regression using STATA, comparing all CP subtypes with all controls.

Results: Overall, preterm birth was the best predictor of CP, with the odds of developing CP increasing with decreasing gestational age (28-31 weeks OR 2.79, 95% CI 1.86-4.20; <28 weeks OR 5.35, 95% CI 3.30-8.69). No significant three-way interactions were observed for the three main outcome measures, with only main effects observed. The presence of Herpes group B viruses was associated with the development of CP (OR 1.69, 95% CI 1.09-2.59), as was carriage of any cytokine polymorphism (OR 1.36, 95% CI 1.01-1.82). The combined presence of Herpes group B viruses and carriage of any cytokine polymorphism was also significantly associated with CP (OR 2.47, 95% CI 1.43-4.27).

Conclusions: In this cohort of cerebral palsy cases and controls, no significant interactions were observed between the three main outcome measures and the development of CP. Prematurity was found to be the best predictor of CP. Herpes group B viruses and carriage of any cytokine polymorphism combined in a linear fashion to increase the risk of developing CP.

Introduction

Previous chapters have investigated individual associations between inherited thrombophilic polymorphisms, inherited cytokine polymorphisms and viral infections and cerebral palsy. The MTHFR C677T thrombophilic polymorphisms was found to approximately double the risk of developing CP in preterm infants, and the combination of homozygous MTHFR C677T and heterozygous PGM was shown to increase the risk of quadriplegia fivefold at all gestational ages. Carriage of any cytokine polymorphism was associated with an increased risk of cerebral palsy, with a significant odds ratio of 1.37 (95% CI 1.02-1.84). Specifically, the TNF-α -308 promoter polymorphism increased the risk of quadriplegia in babies born at term (OR 1.82, 95% CI 1.04-3.15) and hemiplegia in very preterm babies born <32 weeks gestation (OR 2.38, 95% CI 1.02-5.58). Polymorphisms in the exon 1 region of the MBL gene were also associated with cerebral palsy at various gestational ages. Finally, the detection of Herpes group B viral nucleic acids was associated with the development of CP (OR 1.68, 95% CI 1.09-2.59), providing the first direct evidence that perinatal viral exposure is associated with CP. Please refer to chapters 4, 5, and 6 for full thrombophilia, cytokine and viral results respectively.

The aim of this chapter was to perform multivariable analysis to investigate associations and interactions between the three main outcome measures which may contribute to the future development of cerebral palsy.

Materials and Methods

Patient Selection

Please refer to Chapter 2 for details regarding the selection of CP cases and controls.

Genotyping for Inherited Thrombophilic Polymorphisms

Please refer to Chapter 3 for details regarding the genotyping of four inherited thrombophilic polymorphisms (Factor V Leiden G1691A, Prothrombin Gene Mutation G20210A, and Methylenetetrahydrofolate Reductase Gene Polymorphisms C677T and A1298C).

Genotyping for Inherited Cytokine Polymorphisms

Please refer to Chapter 5 for details regarding the genotyping of 5 inherited cytokine polymorphisms in the TNF-α gene (-308) and the MBL gene (-221, Codon 52, Codon 54 and Codon 57).

Detection of Viral Nucleic Acids

Please refer to Chapter 6 for details regarding the detection of viral nucleic acids from members of the enterovirus family and the herpesvirus family. The herpesviruses were divided into two PCRs, one detecting nucleic acids from HSV-1, HSV-2, EBV, CMV and HHV-8, hereafter designated Herpes group A viruses, and the other detecting nucleic acids from VZV, HHV-6 and HHV-7, hereafter designated Herpes group B viruses. Within the Herpes group A PCR, differentiation between CMV and the remaining viruses (HSV-1, HSV-2, EBV and HHV-8) was possible because of differences in PCR product band size visualised by agarose gel electrophoresis.

Statistical Analysis

Multivariable analysis was performed by stepwise backwards unconditional logistic regression using STATA, comparing all CP subtypes with all controls. To ensure all cases and controls could be included in all analyses, an extra category was included to represent those cases and controls for which no data was recorded for a particular variable.

Results

Main Effects Model including prematurity

Significant results in this main effects model are summarised in Table 1. Overall, the best predictor of CP was preterm birth, with the odds of developing CP increasing with decreasing gestational age (28-31 weeks OR 2.79, 95% CI 1.86-4.20; <28 weeks OR 5.35, 95% CI 3.30-8.69). This model used gestation as a categorical variable; the same results were observed when gestation was treated as a continuous variable. Other predictors of CP in this model included intrauterine growth restriction $<5^{th}$ percentile, (OR 1.70 95% CI 1.17-2.49, p=0.006), the presence of chorioamnionitis (OR 5.01, 95% CI 1.93-13.00, p=0.001), and the presence of Herpes B nucleic acids (OR 1.67, 95% CI 1.06-2.62, p=0.026). Other factors considered in this analysis included the MTHFR polymorphisms, Exon 1 MBL polymorphisms, histological villitis, breech presentation and antepartum haemorrhage.

Interactions between Inherited Thrombophilia, Cytokine Polymorphisms and Viral Infections

The best predictor of CP was prematurity, which may have the effect of overshadowing other predictors of CP, therefore investigations were performed to assess potential interactions and associations between the three main outcome measures (thrombophilic polymorphisms, cytokine polymorphisms and viral infection) and the development of CP, irrespective of prematurity. No significant three-way interactions were found between these three outcome measures and CP, and only main effects were observed. In this model, the presence of any heterozygous cytokine polymorphism was significantly associated with the development of CP (OR 1.40, 95% CI 1.04-1.88), as was the carriage of any polymorphic cytokine allele (OR 1.37, 95% CI 1.02-1.83). The presence of Herpes group B viruses was also significantly associated with the development of CP (OR 1.69, 95% CI 1.09-2.59). The presence of both Herpes group B viruses (OR 1.69, 95% CI 1.10-2.60) and carriage of any cytokine polymorphism (OR 1.36, 95% CI 1.01-1.82) combined to significantly increase the odds of developing CP approximately 2.5 fold when compared with babies who did not possess any cytokine polymorphism or test positive for Herpes group B viruses (OR 2.47, 95% CI 1.43-4.27). Despite this significant finding, there was no significant interaction between the viral detection and presence of any cytokine polymorphism to dramatically increase the risk of developing CP in a synergistic manner.

Table 1: Main Effects Model 1: Multivariable analysis comparing all CP cases with all controls, best predictors of CP.

Factor		CP Cases (n=443)	Controls (n=883)	Bivariable OR (95% CI)	Adjusted OR (95% CI)	p-value
Gestation (weeks)	≥ 37	243	632	1.00 (Reference)	1.00 (Reference)	
	32-36	63	148	1.11 (0.80-1.54)	1.17 (0.82-1.67)	0.382
	28-31	70	73	2.49 (1.74-3.57)	2.79 (1.86-4.20)	0.000
	<28	67	30	5.81 (3.68-9.16)	5.35 (3.30-8.69)	0.000
IUGR for Gestation	≥ 5th percentile	385	804	1.00 (Reference)	1.00 (Reference)	
	<5th percentile	57	79	1.51 (1.05-2.16)	1.70 (1.17-2.49)	0.006
Chorioamnionitis*	Absent	60	102	1.00 (Reference)	1.00 (Reference)	
	Present	24	7	5.83 (2.37-14.34)	5.01 (1.93-13.00)	0.001
Herpes group B virus	Absent	291	661	1.00 (Reference)	1.00 (Reference)	
	Present	40	54	1.68 (1.09-2.59)	1.67 (1.06-2.62)	0.026

*Histological evidence of maternal and fetal inflammation (histological acute chorioamnionitis)

Discussion

This is the largest study to examine the effects of inherited thrombophilic polymorphisms, inherited cytokine polymorphisms and viral infection in the subsequent development of cerebral palsy. Bivariable analyses for each of these factors has shown significant associations (for full details please refer to Chapters 4, 5 and 6). Based on these analyses, multivariable analysis was undertaken in an attempt to gain a clearer understanding of the potential associations and interactions between these factors and the development of cerebral palsy, which is most likely a multifactorial disorder with genetic and environmental components.

Using stepwise backwards unconditional logistic regression using STATA, comparing all CP subtypes with all controls, this data has confirmed that prematurity is the most important predictor of cerebral palsy, and that the risk of developing CP increases with decreasing gestational age, with babies born <28 weeks gestation more than five times the risk of developing CP compared with babies born at term. This finding is not surprising; preterm birth has long been the main predictor of cerebral palsy (26, 27). This main effects model also demonstrated an increased risk of CP with IUGR <5th percentile. It has been suggested that IUGR is only a risk factor for CP in term babies (33), however the present data suggests that IUGR <5th percentile is a risk factor for CP, independent of gestation. The presence of histological chorioamnionitis was also a significant predictor of CP, a finding which supports that of other studies, including meta-analyses (29, 55, 177). Finally, the presence of Herpes group B viruses was found to be an important predictor of CP, increasing the risk of developing CP by two-thirds. This novel finding has not previously been demonstrated in a cohort of CP babies and unaffected controls.

Analysis was also performed on this case-control cohort to investigate other potential predictors and interacting factors in the development of CP without the influence of prematurity. These analyses demonstrated no significant interactions between the three main outcome measures (inherited thrombophilic polymorphisms, inherited cytokine polymorphisms and viral infection) and the subsequent development of cerebral palsy. These results suggest that there are a number of different pathways leading to the brain white matter damage that is ultimately responsible for the development of CP.

A significant association was observed for the combination of any cytokine polymorphism and the presence of Herpes group B viruses and the subsequent development of CP.

Individually, the odds ratios for these two outcome measures was 1.36 and 1.69 for the presence of any cytokine polymorphism and the detection of Herpes group B viruses respectively, and when combined increased the odds of developing CP in a linear fashion, with an increased odds ratio of 2.47. Although this was a significant finding, no significant interaction was observed between these two outcomes to suggest they act synergistically to increase the risk of developing CP. This suggests that these two outcomes may not act along the same causal pathway in the development of CP, and that they are instead contributing to the development of CP in different manners.

Despite there being no interactions between the three main outcome measures studied and CP, further research needs to be conducted in this area. One weakness of this study is the absence of maternal genotypes for the inherited thrombophilic and cytokine polymorphisms. A combination of maternal and fetal genotypes may be required for an interactive effect to occur. Furthermore, the list of polymorphisms investigated in this study is by no means exhaustive; there are still many candidate genes to be investigated.

Based on the findings discussed in this analysis and data reported in the literature, it is likely that interactions between genetic and environmental factors are needed to produce the required conditions for brain white matter damage to occur *in utero*. Future studies would benefit by quantifying viral loads present in both the mother and fetus. There may be threshold levels of viral infection, below which there is no significant association with CP. The timing of infection is also an important factor, one that this study was unable to investigate.

In conclusion, no significant interactions were observed between our three main outcomes of inherited thrombophilic polymorphisms, inherited cytokine polymorphisms and viral infection and the subsequent development of cerebral palsy. This suggests that these three factors may act along different causal pathways towards the development of cerebral palsy. Further research is required to fully investigate gene-environment interactions, which may provide more information on the development of CP.

General Discussion

Introduction

Cerebral Palsy is a complex and multifactorial disorder, with a prevalence of approximately 2-2.5 per 1000 children born (12, 17, 18, 21). Previously, it was believed that poor obstetric care culminating in intrapartum asphyxia was responsible for the subsequent development of cerebral palsy (12, 15, 22-25), however the frequency of cerebral palsy has remained relatively constant over the last forty years, despite significant improvements in obstetric care (12, 14-19). Research conducted in this study investigated the roles of antenatal factors, including the contribution of genetic factors, intrauterine infection and inflammation, in the subsequent development of cerebral palsy and adverse pregnancy outcomes, in particular preterm birth, PIHD and IUGR. This study is the largest of its kind and had a cohort of 1,326 babies available for study (443 CP cases and 883 non-CP controls in the CP study; 717 APO cases and 609 non-APO controls in the APO study). The newborn screening (Guthrie) cards of these cases and controls were used to genotype a number of thrombophilic and cytokine polymorphisms, and to detect the presence of viral nucleic acids. Basic clinical data was accessed through the South Australian Perinatal Births Collection, and through the Supplementary Birth Records for each baby. Ethics approval was granted for this project only if the cases and controls were deidentified; therefore access to detailed clinical and medical case notes was not possible.

Inherited Thrombophilic Polymorphisms

This study investigated four common inherited thrombophilic polymorphisms: Factor V Leiden (FVL, G1691A), Prothrombin Gene Mutation (PGM, G20210A), and two polymorphisms in the methylenetetrahydrofolate reductase gene (MTHFR) at positions 677 (C→T) and 1298 (A→C). These polymorphisms alter the balance of the coagulation cascade towards procoagulation. This predisposes individuals to thromboembolism, the obstruction of a blood vessel with thrombotic material carried by the bloodstream from the site of origin. A strength of this thesis was that it was the first large case-control study to investigate the associations between inherited thrombophilia and cerebral palsy. This builds on previous smaller studies (18, 93, 94, 96, 117, 119), which suggested a link between the two, with only one study failing to demonstrate any associations (117). Findings from the present study show a number of significant associations between the four inherited thrombophilic polymorphisms studied and CP at different gestational ages,

in keeping with the majority of previous research. Bivariable analysis for these polymorphisms showed an approximate doubling of the risk of developing CP among preterm infants, suggesting that changes to the fetal clotting cascade may enhance the risk of CP. Further investigations into the changes to the clotting cascades of both the fetus and the mother are now warranted, to extend our knowledge of interactions between the two.

This thesis also investigated four key adverse pregnancy outcomes: IUGR, PIHD, APH and preterm birth. Despite much interest in the roles of inherited thrombophilic polymorphisms in the development of these and other adverse pregnancy outcomes, most research to date has examined the roles of maternal inherited thrombophilia, with very limited research investigating the role of fetal inherited thrombophilia (215-222). The aim of this study was to further elucidate the role of fetal inherited thrombophilic polymorphisms. Key findings from this research demonstrated that there are significant associations, particularly in the presence of IUGR. How these polymorphisms lead to the development of IUGR is still unclear, however one proposed mechanism by which inherited thrombophilia may be associated with IUGR and other adverse outcomes is when thrombi lodge in the feto-placental (micro)circulation and therefore impair blood flow across the placenta. In addition, it should be noted that a fetus being heterozygous for a thrombophilia has at least a 50% chance of having a mother with a thrombophilia, with all the known potential risks for thrombotic processes affecting the spiral arteries and intervillous space. The effect of inherited thrombophilia on adverse pregnancy outcomes is unlikely to be the result of fetal inherited thrombophilia alone, and future studies should investigate the contribution of the combination of maternal and fetal inherited thrombophilia.

Inherited Cytokine Polymorphisms

Five cytokine polymorphisms from two different genes (TNF-α and MBL) were investigated in this thesis. Previous studies investigating associations for TNF-α have primarily focused on the measurement of cytokine levels rather than the genotyping of polymorphic sites capable of altering circulating cytokine concentrations. Findings from the present study demonstrated positive associations with CP, in particular the hemiplegic and quadriplegic subtypes, for both the TNF-α and MBL polymorphisms, with increases in the risk of the three main cerebral palsy subtypes being observed for babies of differing gestational ages. Whilst previous research demonstrated indirect associations for CP by investigating specific pathological conditions (such as PVL and prematurity), this study

was the first to show a direct relationship between the TNF-α -308 polymorphism and CP. The mechanism(s) behind this relationship remain unknown, and further research is required.

As with the inherited thrombophilia, this study also investigated the role played by the TNF-α -308 polymorphism in the development of the four adverse pregnancy outcomes studied. Significant associations were observed for all the adverse pregnancy outcomes studied, suggesting this polymorphism plays a key role in pathogenic outcomes. This extends previous research investigating IUGR which has shown higher levels of TNF-α in placentae affected by IUGR compared with normal placentae (225, 226). These results also extend research into PIHD and preeclampsia, with studies to date providing conflicting data on the role of TNF-α in the development of preeclampsia. This data differs from the only other study to investigate associations between fetal TNF-α -308 and PIHD (231), however that study used a group of mixed ethnicity, with over 50% of the women in both the case and control groups being African-American, which may explain the lack of concordant results. There is no previous research into the role of the TNF-α -308 polymorphism in the development of APH, and the significant findings presented in this thesis need to be confirmed in more large case-control studies. Finally, this thesis concurs with research into the role of TNF-α in preterm birth and the fetal inflammatory response syndrome (FIRS), although the mechanism(s) by which this occurs is yet to be fully determined (68, 69, 74, 75, 188, 226, 233).

Research in this thesis investigated polymorphisms in the MBL gene capable of decreasing circulating MBL levels. Significant associations for CP were observed for polymorphisms within the MBL gene, primarily the polymorphisms at codons 52 and 54 of the exon 1 region. Associations were also observed for babies with adverse pregnancy outcomes, for both the MBL -221 polymorphism and the three coding region polymorphisms. Although no significant interactions were observed between MBL polymorphisms and detection of viral particles, there is still the possibility that such interactions exist. Full haplotyping was not performed on the MBL polymorphisms, and quantitation of viral loads were not performed. The timing and nature of infection (primary vs. recurrent) during pregnancy may also be relevant, and need to be assessed in future studies utilising antenatal antibody testing.

Furthermore, this thesis studied these MBL polymorphisms, looking for associations with the adverse pregnancy outcomes. No associations were seen for IUGR, however larger

studies are required to confirm or refute these findings. Significant findings were observed for PIHD and APH with the polymorphisms in exon 1, and for APH and preterm birth with the promoter polymorphism. As with the CP results, it remains unclear whether these associations are direct, due to the lower circulating MBL levels brought about by the polymorphisms, or indirect, as a result of increased susceptibility to infection. Again, full haplotyping of the MBL gene is required, as well as further investigations into infection, both viral and bacterial, to fully understand the possible role of MBL polymorphisms in the development of adverse pregnancy outcomes.

The South Australian Cerebral Palsy Research Group is now involved in collaborative research with Dr Karin Nelson (NIH Bethesda, USA) investigating associations between cerebral palsy and 42 other cytokine and inflammatory polymorphisms using this set of 1,326 babies. It is hoped that this extended list of polymorphisms may show further associations with cerebral palsy and adverse pregnancy outcomes, and may provide more insights into the causal pathways of cerebral palsy, in addition to the development of new research directions in this area.

Viral Infection

Research indicates that viral infection may be involved in the development of a number of adverse pregnancy outcomes, including CP. This thesis was the first to look for direct evidence of viral infection as a potential causative agent in the development of adverse outcomes through the use of molecular techniques. The presence of viral nucleic acids in the newborn screening cards of significantly more babies with CP and adverse pregnancy outcomes compared with controls was a key finding. This suggests that these viruses are involved in the development of these disorders. This study focused on neurotropic viruses capable of crossing the placenta and causing damage to the developing fetal brain (197-204), and included members of the herpesvirus and enterovirus families. The novel approach taken in this thesis allowed determination of exposure to the specific infections studied during pregnancy for associations with the development of adverse pregnancy outcomes, including CP. This study could not comprehensively study exposure to all possible antenatal viral infections, bacterial infections or other infective agents.

Direct associations were observed between the presence of viral nucleic acids and CP, extending on from previous research which investigated surrogate markers of infection such as chorioamnionitis, funisitis, maternal pyrexia, raised C-reactive protein and interleukin-6 concentrations (29, 51, 76, 133, 185). Demonstrated links between direct

exposure to viral infection and preterm birth and PIHD was another significant finding of this study. Although the link between preterm birth and bacterial infection is well established, this study provides the first direct evidence of associations for specific viral nucleic acid sequences in the newborn screening cards of babies delivering preterm. Furthermore, this study also identified *in utero* infection as a potential contributing factor in the development of PIHD, potentially by affecting the magnitude of placental apoptosis and via this, the maternal inflammatory response associated with preeclampsia (296). The findings that specific neurotropic viruses may be involved in the development of these adverse outcomes allows for future research to target these and other potentially neurotropic viruses through early detection of infection during pregnancy. This may lead to potential vaccination programmes in the future. Planned future research will address such issues as the timing and nature of these infections, as this could influence fetal and neonatal outcomes.

Interactions between main outcome measures

An hypothesis of this thesis was that the three main outcome measures (inherited thrombophilia, inherited cytokine polymorphisms and viral infection) may interact to further increase the risk of CP. The combined presence of any cytokine polymorphism and Herpes group B viruses was significantly associated with the development of CP. This supports the bivariable results obtained in this study, which showed that the presence of any tested cytokine polymorphism and the detection of Herpes group B viral nucleic acids were both independently associated with the development of cerebral palsy. The combination of both viral infection and cytokine polymorphisms was also found to increase the risk of CP, although not in a synergistic fashion. No other significant interactions between any of these main outcome measures were observed, despite bivariable analysis demonstrating significant positive results for each of the three main outcome measures. This suggests that, contrary to the proposed hypothesis, these three factors are not acting synergistically to produce an *in utero* environment conducive to CP development. Importantly, this does not negate the significant bivariable findings which demonstrated associations between inherited thrombophilia and CP, cytokine polymorphisms and CP, and viral infection and CP. Instead, it is indicative that there are a number of different pathways by which CP can develop. It may be that different viral, or even bacterial infections, are required in the presence of inherited thrombophilia and cytokine polymorphisms for CP to result. This study has only investigated four inherited thrombophilia, five cytokine polymorphisms and nine viruses. There are many more

candidate genes, viral and bacterial infections, all of which may contribute, either individually or in combination, to the development of CP. The interactions between genes and the environment is most likely a key factor in determining the causes of CP; there is still much research to be conducted and the collection of clinical data during pregnancy, delivery and the puerperium is vital to the success of such research. CP is undoubtedly a multifactorial disorder, with many different causes and contributors leading to its development.

Population Attributable Risk

From data collected in this thesis, it is estimated that the population attributable fraction of three of the factors investigated for CP was 5.2% for the MTHFR polymorphism, 5.2% for the cytokine polymorphisms tested, and 4.5% for the Herpes group B viral infections. The total population attributable fraction of CP cases associated with these risk factors was therefore 14.9%. This suggests that specific antenatal causes of CP have been identified in approximately 15% of CP cases in this cohort, however further studies are needed to confirm the results presented in this thesis. It is likely that further studies of different thrombophilic polymorphisms, cytokine polymorphisms, neurotropic viruses and bacterial infections are likely to greatly increase the attributable risk of genetic polymorphisms and exposure to antenatal infections.

Prevention

With this information comes the potential for further understanding of the development of CP, and may lead to effective preventative strategies. Large-scale immunisation is very effective in reducing adverse effects of infection with viral and bacterial agents, as evidenced by the rubellavirus vaccine reducing the teratogenic effects of rubella during pregnancy. Children are already being immunised against VZV, and it will be of interest to observe if there is a decline in adverse pregnancy outcome rates, including CP, in the next generation, following the vaccination of their mothers. The introduction of vaccines for a wide range of viruses and bacteria would reduce the risk of women contracting infection during pregnancy, and therefore minimise the risk of transplacental transfer of infection to the fetus. Antiviral therapy may also play a role in reducing the risk of adverse outcomes, by providing an effective treatment option for pregnant women, also minimising the risk of transplacental transfer of infection to the fetus.

The reported associations between inherited thrombophilic and cytokine polymorphisms and adverse outcomes also need to be confirmed in further large-scale studies. There

remains many more candidate polymorphisms worthy of investigation, in order to fully understand any potential interactions between these genetic factors and the subsequent development of adverse pregnancy outcomes. If any of these polymorphisms are shown to be clinically relevant, intervention strategies can be implemented to reduce the risk of these polymorphisms contributing to adverse events. Although still controversial, antithrombotic therapy may be useful to women identified as having an increased risk of thrombosis. Fetal genetic screening *in utero* of these high-risk women may also be useful. It should be emphasised that current antithrombotic therapy using low-molecular weight heparins will potentially only "protect" the uteroplacental circulation and will not protect the fetus from (theoretical) adverse effects of fetoplacental thrombosis. Perhaps novel antithrombotic strategies such as ultra-low molecular weight heparins and other direct antithrombins may be able to prevent fetoplacental thrombosis without the terrible adverse consequences seen with the use of warfarin. Fetal genetic screening *in utero* for cytokine polymorphisms such as TNF-α -308 and polymorphisms in the MBL gene may also provide clinicians with more clinically relevant treatment options. Prophylactic antiviral or antibiotic treatment may be considered during pregnancy for women with cytokine polymorphisms capable of altering the immune response to infection.

Study Caveats

This study is the first of its kind in the world to investigate links between inherited thrombophilic polymorphisms, inherited cytokine polymorphisms and viral infection and their associations with the subsequent development of cerebral palsy and other adverse pregnancy outcomes, including IUGR, PIHD, APH and prematurity. Despite having the largest data set of its kind in the world, the available clinical data was limited to that contained within the Supplementary Birth Record, collected by the South Australian Pregnancy Outcomes Unit. This record contains only basic clinical information such as gestational age, birth weight, and basic obstetric histories. Medical records and case notes for both mother and child were inaccessible, as informed patient or parental consent was unable to be obtained retrospectively, and ethically the data obtained during this study had to be deidentified. This meant that information regarding such variables as infections during pregnancy, antibiotic use, and other relevant family histories, such as history of thrombosis or autoimmune disorders was therefore unable to be obtained. This information would have been beneficial for this study, as evidence of antibiotic use may suggest prior exposure to bacterial infection. A strong family history of venous thrombosis combined with the presence of an inherited thrombophilia may be relevant to the risk of a

prothrombotic event being involved in the development of CP or APO. All of this information has the potential to provide greater insights and understanding into the complex interactions involved in CP or APO causation and a prospective study with informed patient consent is planned.

Although this study only investigated inherited thrombophilic and cytokine polymorphisms it would be beneficial to correlate this data with functional clotting tests and measurements of cytokine levels. For example, the polymorphisms in the MTHFR gene can result in hyperhomocysteinaemia, which in turn may exert thrombophilic effects by altering the normal antithrombotic phenotype of the endothelium, enhancing the activities of factors XII and V. This may also depress the activation of protein C, and recruit leukocytes and augment leukocyte-induced endothelial cell activation (107, 109). However, it remains unclear whether the thrombophilic effects are due to the MTHFR genotypes, or the phenotype of hyperhomocysteinaemia, which may in turn be modulated by diet. Measurements of homocysteine levels in these mothers and babies could lead to an understanding of the mechanisms behind the associations between MTHFR polymorphisms and adverse pregnancy outcomes, including CP. In addition the maternal genotypes for the inherited thrombophilic and cytokine polymorphisms were unable to be determined, as this study focused only on fetal polymorphisms. A combined effect of both maternal and fetal genotypes may be required for adverse outcomes to occur; for example thrombotic processes may only occur when there is both a maternal and fetal inherited thrombophilia present. A similar situation may arise with the inherited cytokine polymorphisms. Abnormal alleles may be required in both the maternal and fetal genes before an adverse effect is observed. It is well documented that the TNF-α -308 polymorphism is associated with higher circulating TNF-α levels, and it is postulated that these higher circulating levels are involved in the development of adverse outcomes such as prematurity and CP. More damage may occur when both the maternal and fetal levels of TNF-α are raised, thus further increasing the risk of brain white matter damage. Polymorphisms in the MBL gene may also act in a similar manner as if the mother possesses polymorphisms in this gene, she may be more susceptible to infection, which can then transmit to the fetus. This transfer to the fetus may be more likely if the fetus also contains polymorphisms in this gene, thus creating an even bigger risk of infection-related white matter damage *in utero*. These questions can only be addressed if future studies investigate both the maternal and fetal genotypes. It is reasonable to assume that

polymorphisms in maternal genes may impact on the fetus, and vice versa, and therefore this is worthy of further study.

Some significant findings were found for polymorphisms in the MBL gene. There are currently six known polymorphic sites (147). This study investigated four of these polymorphisms; one promoter (position -221) and three exon 1 coding region (codons 52, 54 and 57) polymorphisms; these were deemed to be more likely to be relevant to the outcomes investigated. The remaining two known polymorphisms in the MBL gene are located at promoter positions -550 and +4. All six promoter and structural polymorphisms are found in various *cis* combinations, resulting in "haplotypes" associated with high, intermediate, and low levels of MBL (149). Currently, seven different haplotypes have been identified in a range of populations (149, 154). This study was unable to determine the complete MBL haplotypes for each CP case and control, because genotyping was not performed for the final two polymorphisms. Analysis of MBL haplotypes investigating associations with CP and APO would be advantageous, and, if correlated with viral and bacterial infection data, may provide more information regarding infectious and inflammatory processes in the development of such adverse outcomes. Despite not investigating all six polymorphisms, this study was the first to show that MBL polymorphisms are associated with CP and APO. Using this extensive database, collaborative research is now being undertaken with researchers at the National Institutes of Health in Bethesda, USA, to extend the number of cytokine and inflammatory polymorphisms studied for potential associations with CP. To date, a further 42 polymorphisms are being investigated, with results expected within the next six months.

This study has demonstrated for the first time links between both viral infection and CP, and viral infection and APO. The presence of viral nucleic acids is associated with CP, in particular viral nucleic acids represented by the herpes group B viruses (VZV, HHV-6 and HHV-7). This study used non-quantitative PCR methodology to detect viral nucleic acids, and data relating to viral load was not assessed. Future studies should utilise quantitative PCR methods to determine the viral load in positive samples in order to assess the importance of viral load in the development of CP. It is possible that there is a threshold level of viral load, above which the risk of CP is significantly increased, via the initiation of preterm birth, or directly via brain white matter damage initiated by the virus itself. The results of this study do not necessarily indicate active congenital or neonatal infection, only indicating exposure to the respective virus or viruses. This study was not designed to detect evidence of an inflammatory response, and these questions therefore remain

unanswered. Maternal blood samples taken at various timepoints during pregnancy would help to ascertain the timing of infection, as exposure to infection early in gestation may have different consequences than exposure late in gestation. Determination of active or latent infection in the neonatal period by examining leukocytes for the presence of viral antigens associated with active viral replication would also assist in gaining a clearer understanding of the role of viral infections in the development of CP. The role of bacterial infections was not addressed in this thesis, and remains an important avenue of study still to be performed. New methodologies to test for bacterial infection in proposed prospective studies are being tested.

Analyses for each of the three main outcome measures in this study involved multiple analyses, and therefore increased the risk of Type II error. This risk was partly reduced by having only a few specific *a priori* hypotheses and seeking consistencies in significant findings through subanalyses. However, there is still the potential for error, and all significant associations reported here require confirmation in future studies.

Lastly, this was a study of a Caucasian population only, and the incidence of the polymorphisms and infections detected in this study may vary in other populations. Further studies are required to determine if the incidence of the studied polymorphisms and infections are the same in populations of other ethnic backgrounds.

Conclusions

This research has shown that inherited thrombophilic polymorphisms, inherited cytokine polymorphisms, and viral infections are all independently associated with the subsequent development of CP. These three factors do not interact to further increase the risk of CP; this may reflect different pathological pathways to the brain white matter damage and periventricular leukomalacia that ultimately leads to CP. In addition, this data set has also shown that these same inherited thrombophilic polymorphisms, inherited cytokine polymorphisms and viral infections are associated with adverse pregnancy outcomes such as intrauterine growth restriction, pregnancy-induced hypertensive disorders, antepartum haemorrhage and prematurity. These associations with CP and APO suggest complex interactions between genes and the environment are occurring to create an *in utero* environment conducive to these adverse outcomes.

Future Implications and Directions

As well as scientific and medical implications, there are medico-legal and political implications arising from this research. Currently, cerebral palsy litigation is having a huge impact around the world, with many obstetricians choosing not to deliver babies for fear of litigation when they are blamed for cerebral palsy outcomes. Australia is facing an obstetrical crisis, with a survey of Australia's specialist obstetricians showing that only 24% of respondents expect to practice obstetrics in 10 years time (311). Two main reasons cited for ceasing obstetrics were fear of litigation and rising indemnity costs (311). The closure of obstetric hospitals, and difficulty in obtaining obstetric care in rural areas have been directly associated with the rising costs of medical indemnification and the threat of litigation (312). Cerebral palsy is currently not preventable, and the evidence suggests that the vast majority of CP cases are not the result of poor obstetric care in the intrapartum period. This thesis, by demonstrating antenatal associations, such as inherited thrombophilic and cytokine polymorphisms, and viral infection, with cerebral palsy, further suggests possible causative factors that cannot be influenced by obstetric care. It is hoped that research such as this, which demonstrates the presence of antenatal risk factors in the development of some CP, will reduce the risk of inappropriate litigation.

Currently, hundreds of millions of dollars are spent each year by the Government on cerebral palsy litigation, despite the fact that cerebral palsy is a disorder that obstetricians cannot yet prevent. Unfortunately, the majority of this money (up to 70%) is spent on the legal processes, with only a relatively small amount used for the benefit of the children with CP and their families. By conducting research to try and determine the causes and prevention of this debilitating disorder, the amount of money spent on litigation and medical needs may be reduced. Other solutions needed are to create a no-fault system for resolving disputes over birth outcomes (313, 314), which would compensate children with major neurological disabilities, thus removing the need for litigation and subsequent associated costs.

The research arising from this thesis has also raised many new research questions and future directions. Prospective studies investigating CP are made difficult because the time between birth and diagnosis can be up to five years, with approximately 85% of cases diagnosed by two years of age. Very large prospective studies would therefore be needed, with collection of samples from mothers and babies during pregnancy and immediately following birth. Because investigators would not have information regarding CP diagnosis

for a number of years, there would be great expense involved in the collection and correct storage of samples until testing. Ideally, maternal and cord blood samples and the placenta would be collected and stored from all deliveries in Australia, and be available for research into adverse pregnancy outcomes. Such a programme would allow the identification of risk factors and potential causal pathways for the development of adverse outcomes, and would have sufficient numbers of cases to properly investigate such outcomes. In the absence of such a biological sample bank, studies designed to investigate evidence of fetal inflammatory response syndrome and inflammation in a more select population of babies born preterm or with neurological injury may help understand the links between prematurity and CP, and thus help to decrease the risk of CP in preterm babies. These future studies need to assess the risk of infection during pregnancy, because this is the one factor that can be altered. Genetic makeup cannot be altered easily, although advances in gene therapy may make this possible in the future. Exposure to neurotropic viruses, however, can be limited by infection control measures and vaccinations. CP and other adverse pregnancy outcomes are likely to be complex disorders combining genetic and environmental factors, and any future research must take this into account.

The South Australian Cerebral Palsy Research Group is proposing a large prospective study which will extend the current retrospective research into the antenatal causes of cerebral palsy and other adverse pregnancy outcomes. The specific hypothesis of this planned prospective study is that an abnormal inflammatory response in fetuses with genetic polymorphisms affecting inflammation, coagulation and/or susceptibility to infection is a factor on the causal pathway to preterm delivery and neurological disability. This study plans to prospectively recruit preterm infants and term infants born with clinical signs of neonatal sepsis and/or neurological abnormality, such as neonatal encephalopathy (NE), as neurological abnormalities detected during the newborn period are the best available predictors of CP in tern and near-term infants. The two comparative groups will be healthy term babies with no neonatal complications and preterm babies without defined complications. This prospective approach, with informed parental consent, will allow the collection of clinical information recorded during pregnancy. This will include information such as folic acid supplementation, alcohol and cigarette intake, family history of birth defects, mental retardation, CP, neonatal seizures, prematurity, sexually transmitted diseases, numbers of miscarriages, completed pregnancies, duration and mode of delivery, number of live births, notable events in the current pregnancy, including febrile illness, exposure to possible infections, features of bacterial vaginosis, and drugs

taken, with special emphasis on antibiotic use and timing, preeclampsia, IUGR, preterm labour, and APH. The neonatal history will be recorded including neonatal encephalopathy, neonatal infection and results of investigations such as neuroimaging, which will help identify a subset of babies with fetal stroke. Based on the criteria to define an acute intrapartum hypoxic event, as described by the International Cerebral Palsy Task Force consensus statement about the causal relationship between acute intrapartum events and cerebral palsy (12), fetal, cord and neonatal blood gases also need to be collected and correlated, to better determine the presence of acute and/or chronic hypoxia at birth. This data then needs to be correlated with the antenatal risk factors identified in this study, and may provide a clearer picture of the pathways of development of neonatal brain damage leading to cerebral palsy. The SA Cerebral Palsy Research Group will investigate the possibility of multicentre collaborations, both here and overseas, in order to increase the number of babies studied and increase the power of the study. The concept of such a study was developed as a direct result of the research conducted during the course of this thesis candidature. The current study has increased our understanding and knowledge of the causes of adverse pregnancy outcomes, including cerebral palsy, and future research which is based on the current findings has the potential to have an enormous impact on society.

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Table I: Inherited Thrombophilic Polymorphism Amplification Primers.

Gene	Direction	Primer Sequence 5'-3'	Primer Length
Factor V Leiden	Forward	GGC TAA TAG GAC TAC TTC TAA TCT GTA AGA GC	32
	Reverse	AAT TTC TGA AAG GTT ACT TCA AGG ACA	27
Prothrombin Gene Mutation	Forward	CCA ATC CCG TGA AAG AAT TAT TTT T	25
	Reverse	AGA GCT GCC CAT GAA TAG CAC	21
MTHFR C677T	Forward	TGA AGC ACT TGA AGG AGA AGG TG	23
	Reverse	GCA AGT GAT GCC CAT GTC G	19
MTHFR A1298C	Forward	TAC CTG AAG AGC AAG TCC CCC	21
	Reverse	TCA CTT TGT GAC CAT TCC GGT	21

Table II: Inherited Thrombophilic Polymorphism Detection Primers.

Gene	Primer Sequence 5'-3'	Primer Length
Factor V Leiden	**7C** + AAG GAC AAA ATA CCT GTA TTC CT	30
Prothrombin Gene Mutation	**17C** + TCC CAA TAA AAG TGA CTC TCA GC	40
MTHFR C677T	**26C** + CTG CGT GAT GAT GAA ATC G	45
MTHFR A1298C	**30C** + GGA GGA GCT GAC CAG TGA AG	50

Table III: Components of PCR Reaction Mixes.

PCR	Primers (μM)		$MgCl_2$ (mM)	AmpliTaq Gold DNA Polymerase (U)	dNTP with dUTP (μM)	AmpErase Uracil N-glycosylase (U)	Total Reaction Volume (μl)
Multiplex Amplification PCR	FVL	80	2	0.6	200	0.5	25
	PGM	240	2	0.6	200	0.5	25
	MTHFR 677	480	2	0.6	200	0.5	25
	MTHFR 1298	480	2	0.6	200	0.5	25

Table IV: Components of SnaPshot Multiplex Detection PCR Master Mix.

PCR	Primers (μM)		SNaPshot Reaction Buffer (μl)	PCR Product (μl)
SNaPshot	FVL	0.25	2	2
Multiplex	PGM	0.5	2	2
Detection	MTHFR 677	2	2	2
PCR	MTHFR 1298	1	2	2

Table V: PCR Cycling Conditions for Inherited Thrombophilic Polymorphism PCRs.

PCR Programme	Initial Temperature (°C)[1]	PCR Cycling Conditions				Final Temperature (°C)
		Denaturation Temperature (°C)	Annealing Temperature (°C)	Extension Temperature (°C)	Number of Cycles	
Amplification	95 (12 min)	95 (1 min)	65 (30 sec)[2]	72 (30 sec)	15	72 (8 min)
		95 (1 min)	50 (30 sec)	72 (30 sec)	25	
Detection	-	96 (10 sec)	50 (1 min)	60 (30 sec)	35	-

[1] = Required for activation of AmpliTaq Gold DNA Polymerase.
[2] = Annealing Temperature reducing by 1°C per cycle

Table I: Odds ratios (95% CI) for cerebral palsy for specified thrombophilia among babies born at all gestational ages compared with controls of all gestational ages. *p-values <0.05.

Type of CP	Zygosity	Control Gestation (weeks)	Odds Ratio (95% CI)				
			Any Thrombophilia	FVL	PGM	MTHFR C677T	MTHFR A1298C
All Types	Homo or Hetero	All	1.11 (0.77-1.58)	0.81 (0.52-1.26)	1.15 (0.63-2.08)	1.24 (0.96-1.59)	0.91 (0.71-1.16)
(n = 405)	Homo vs Hetero	All	0.93 (0.69-1.26)	1.73 (0.33-7.87)	1.89 (0.13-27.76)	1.15 (0.78-1.70)	0.68 (0.42-1.08)
Diplegia	Homo or Hetero	All	1.34 (0.73-2.49)	0.52 (0.20-1.15)	1.08 (0.36-2.66)	**1.63 (1.09-2.46)***	0.82 (0.56-1.22)
(n = 127)	Homo vs Hetero	All	1.05 (0.66-1.67)	0.00 (0.00-11.63)	0.00 (0.00-33.91)	1.15 (0.64-2.05)	0.78 (0.36-1.63)
Hemiplegia	Homo or Hetero	All	1.35 (0.71-2.61)	1.02 (0.51-1.99)	1.21 (0.41-2.99)	1.26 (0.83-1.93)	0.80 (0.53-1.22)
(n = 116)	Homo vs Hetero	All	0.78 (0.46-1.32)	2.60 (0.22-17.19)	3.40 (0.05-74.48)	0.83 (0.41-1.66)	0.64 (0.26-1.47)
Quadriplegia	Homo or Hetero	All	0.87 (0.49-1.55)	0.90 (0.42-1.85)	1.54 (0.56-3.63)	0.97 (0.64-1.49)	1.04 (0.68-1.58)
(n = 110)	Homo vs Hetero	All	0.76 (0.43-1.32)	3.25 (0.27-22.33)	2.83 (0.04-61.04)	1.46 (0.75-2.82)	**0.33 (0.1-0.87)***

Table II: Odds ratios (95% CI) for cerebral palsy for specified thrombophilia among babies born at ≥37 weeks gestation compared with controls of all gestational ages. All p-values NS.

Type of CP	Zygosity	Control Gestation (weeks)	Odds Ratio (95% CI)				
			Any Thrombophilia	FVL	PGM	MTHFR C677T	MTHFR A1298C
All Types	Homo or Hetero	All	1.02 (0.66-1.59)	0.86 (0.50-1.48)	1.24 (0.60-2.52)	1.03 (0.75-1.40)	0.97 (0.71-1.32)
(n = 225)	Homo vs Hetero	All	0.85 (0.58-1.26)	1.44 (0.13-8.95)	3.40 (0.21-50.94)	0.99 (0.59-1.66)	0.69 (0.39-1.24)
Diplegia	Homo or Hetero	All	1.58 (0.61-5.22)	0.37 (0.04-1.48)	0.45 (0.01-2.82)	1.30 (0.70-2.43)	1.08 (0.58-2.02)
(n = 49)	Homo vs Hetero	All	0.82 (0.37-1.77)	0.00 (0.00-76.04)	0.00 (0.00-682.5)	0.68 (0.20-1.92)	0.81 (0.23-2.32)
Hemiplegia	Homo or Hetero	All	1.22 (0.59-2.61)	1.12 (0.50-2.41)	1.45 (0.43-3.87)	1.04 (0.63-1.71)	0.90 (0.55-1.47)
(n = 80)	Homo vs Hetero	All	0.68 (0.35-1.29)	3.71 (0.3-26.27)	4.25 (0.06-95.58)	0.68-0.24-1.63)	0.51 (0.15-1.37)
Quadriplegia	Homo or Hetero	All	0.82 (0.40-1.73)	0.76 (0.23-1.95)	1.91 (0.56-5.15)	0.95 (0.55-1.64)	1.06 (0.62-1.84)
(n = 64)	Homo vs Hetero	All	0.79 (0.38-1.59)	0.00 (0.00-17.67)	4.25 (0.06-95.58)	1.50 (0.63-3.51)	0.47 (0.12-1.38)

Table III: Odds ratios (95% CI) for cerebral palsy for specified thrombophilia among babies born at 32-36 weeks gestation compared with controls of all gestational ages. *p-values <0.05.

Type of CP	Zygosity	Control Gestation (weeks)	Odds Ratio (95% CI)				
			Any Thrombophilia	FVL	PGM	MTHFR C677T	MTHFR A1298C
All Types	Homo or Hetero	All	1.15 (0.51-2.70)	1.04 (0.35-2.51)	1.19 (0.23-3.95)	**2.07 (1.14-3.79)***	0.70 (0.40-1.25)
(n = 58)	Homo vs Hetero	All	1.06 (0.54-2.05)	0.00 (0.00-14.03)	0.00 (0.00-74.27)	1.33 (0.61-2.87)	0.65 (0.16-2.01)
Diplegia	Homo or Hetero	All	0.56 (0.19-2.02)	0.98 (0.11-4.20)	3.84 (0.69-14.15)	1.88 (0.69-5.25)	**0.25 (0.06-0.78)***
(n = 20)	Homo vs Hetero	All	1.09 (0.30-3.38)	0.00 (0.00-76.04)	0.00 (0.00-74.27)	0.90 (0.16-3.59)	3.26 (0.23-45.31)
Hemiplegia	Homo or Hetero	All	Undefined	0.88 (0.02-6.32)	0.00 (0.00-9.06)	4.54 (0.93-43.42)	0.57 (0.12-2.26)
(n = 11)	Homo vs Hetero	All	0.98 (0.17-4.15)	0.00 (0.00-513.5)	-	1.50 (0.24-7.17)	0.00 (0.00-5.01)
Quadriplegia	Homo or Hetero	All	0.75 (0.20-4.22)	1.35 (0.15-6.13)	0.00 (0.00-5.92)	1.68 (0.55-5.69)	0.78 (0.26-2.30)
(n = 16)	Homo vs Hetero	All	0.87 (0.15-3.55)	0.00 (0.00-76.04)	-	2.00 (0.41-8.61)	0.00 (0.00-2.31)

Table IV: Odds ratios (95% CI) for cerebral palsy for specified thrombophilia among babies born at <32 weeks gestation compared with controls of all gestational ages. *p-values <0.05.

Type of CP	Zygosity	Control Gestation (weeks)	Odds Ratio (95% CI)				
			Any Thrombophilia	FVL	PGM	MTHFR C677T	MTHFR A1298C
All Types	Homo or Hetero	All	1.28 (0.68-2.42)	0.62 (0.27-1.37)	0.96 (0.29-2.54)	1.37 (0.90-2.08)	0.90 (0.59-1.35)
(n = 122)	Homo vs Hetero	All	1.02 (0.62-1.66)	4.33 (0.35-31.89)	0.00 (0.00-41.38)	1.33 (0.72-2.45)	0.65 (0.28-1.45)
Diplegia	Homo or Hetero	All	1.88 (0.73-6.16)	0.49 (0.10-1.56)	0.78 (0.09-3.16)	**1.91 (1.04-3.53)***	0.93 (0.53-1.64)
(n = 58)	Homo vs Hetero	All	1.24 (0.65-2.34)	0.00 (0.00-36.64)	0.00 (0.00-124.83)	1.70 (0.78-3.65)	0.54 (0.13-1.64)
Hemiplegia	Homo or Hetero	All	1.07 (0.30-5.76)	0.77 (0.09-3.19)	0.99 (0.02-6.49)	1.46 (0.58-3.74)	0.61 (0.23-1.60)
(n = 25)	Homo vs Hetero	All	1.09 (0.30-3.38)	0.00 (0.00-76.04)	0.00 (0.00-682.5)	0.90 (0.16-3.59)	1.95 (0.30-10.23)
Quadriplegia	Homo or Hetero	All	1.08 (0.36-4.38)	0.98 (0.19-3.28)	1.68 (0.19-7.14)	0.76 (0.33-1.72)	1.15 (0.51-2.60)
(n = 30)	Homo vs Hetero	All	0.65 (0.19-1.83)	**26 (1.09-1551.59)***	0.00 (0.00-124.83)	1.00 (0.17-4.11)	0.23 (0.01-1.57)

Table V: Odds ratios (95% CI) for CP for specified thrombophilia among babies born at all gestational ages compared with term-born controls. *p-values <0.05.

Type of CP	Zygosity	Control Gestation (weeks)	Odds Ratio (95% CI)					
			Any Thrombophilia	FVL	PGM	MTHFR C677T	MTHFR A1298C	MTHFR C677T+ A1298C
All Types (n = 405)	Homo	≥37	0.88 (0.56-1.39)	1.45 (0.27-7.81)	1.50 (0.11-20.71)	1.20 (0.80-1.80)	0.63 (0.39-1.01)	-
	Hetero	≥37	1.03 (0.69-1.52)	0.75 (0.46-1.21)	1.17 (0.60-2.30)	1.18 (0.88-1.57)	0.98 (0.74-1.30)	1.09 (0.68-1.75)
	Homo or Hetero	≥37	0.98 (0.67-1.45)	0.79 (0.50-1.26)	1.20 (0.63-2.27)	1.18 (0.91-1.54)	0.89 (0.68-1.16)	-
	Homo vs Hetero	≥37	0.86 (0.62-1.18)	1.93 (0.33-11.08)	1.28 (0.08-19.13)	1.02 (0.68-1.54)	0.64 (0.39-1.05)	-
Diplegia (n = 127)	Homo	≥37	1.16 (0.57-2.44)	0.00 (0.00-4.34)	0.00 (0.00-9.07)	1.58 (0.86-2.88)	0.64 (0.29-1.31)	-
	Hetero	≥37	1.20 (0.65-2.37)	0.54 (0.20-1.22)	1.22 (0.40-3.17)	1.55 (0.99-2.44)	0.87 (0.56-1.33)	1.52 (0.72-3.25)
	Homo or Hetero	≥37	1.20 (0.64-2.25)	0.50 (0.19-1.14)	1.12 (0.37-2.89)	**1.56 (1.03-2.37)***	0.81 (0.54-1.22)	-
	Homo vs Hetero	≥37	0.96 (0.60-1.55)	0.00 (0.00-14.93)	0.00 (0.00-23.37)	1.02 (0.56-1.85)	0.74 (0.34-1.58)	-
Hemiplegia (n = 116)	Homo	≥37	0.92 (0.42-2.10)	2.56 (0.23-18.08)	2.63 (0.04-50.74)	0.96 (0.47-1.94)	0.53 (0.22-1.21)	-
	Hetero	≥37	1.29 (0.67-2.65)	0.88 (0.41-1.85)	1.14 (0.33-3.16)	1.30 (0.82-2.07)	0.87 (0.55-1.38)	1.07 (0.48-2.41)
	Homo or Hetero	≥37	1.20 (0.62-2.36)	0.99 (0.49-1.97)	1.26 (0.41-3.24)	1.21 (0.78-1.86)	0.79 (0.51-1.21)	-
	Homo vs Hetero	≥37	0.71 (0.41-1.23)	2.90 (0.23-23.16)	2.30 (0.03-51.33)	0.74 (0.36-1.50)	0.60 (0.25-1.42)	-
Quadriplegia (n = 110)	Homo	≥37	0.60 (0.29-1.29)	2.71 (0.24-19.19)	2.86 (0.05-55.37)	1.12 (0.59-2.10)	0.39 (0.12-1.02)	-
	Hetero	≥37	0.87 (0.47-1.6)	0.75 (0.32-1.69)	1.49 (0.48-3.90)	0.86 (0.53-1.40)	1.23 (0.78-1.92)	0.87 (0.41-1.83)
	Homo or Hetero	≥37	0.77 (0.43-1.41)	0.88 (0.41-1.84)	1.60 (0.57-3.94)	0.93 (0.60-1.44)	1.02 (0.66-1.58)	-
	Homo vs Hetero	≥37	0.70 (0.39-1.22)	3.63 (0.28-29.79)	1.92 (0.03-42.07)	1.30 (0.65-2.54)	**0.32 (0.09-0.84)***	-

Table VI: Odds ratios (95% CI) for CP for specified thrombophilia among babies born at ≥ 37 weeks compared with term-born controls. All p-values NS.

Type of CP	Zygosity	Control Gestation (weeks)	Odds Ratio (95% CI)					
			Any Thrombophilia	FVL	PGM	MTHFR C677T	MTHFR A1298C	MTHFR C677T+ A1298C
All Types (n = 225)	Homo	≥37	0.75 (0.43-1.32)	1.30 (0.12-9.17)	2.69 (0.19-37.23)	0.89 (0.53-1.49)	0.69 (0.38-1.23)	-
	Hetero	≥37	0.97 (0.60-1.55)	0.81 (0.45-1.45)	1.17 (0.51-2.62)	1.02 (0.72-1.45)	1.04 (0.74-1.47)	0.90 (0.51-1.61)
	Homo or Hetero	≥37	0.91 (0.57-1.45)	0.84 (0.48-1.47)	1.29 (0.60-2.74)	0.98 (0.71-1.36)	0.96 (0.69-1.32)	-
	Homo vs Hetero	≥37	0.78 (0.52-1.17)	1.61 (0.13-12.27)	2.30 (0.14-35.11)	0.88 (0.51-1.50)	0.66 (0.36-1.20)	-
Diplegia (n = 49)	Homo	≥37	1.09 (0.32-4.19)	0.00 (0.00-11.11)	0.00 (0.00-23.12)	0.84 (0.24-2.40)	0.88 (0.25-2.47)	-
	Hetero	≥37	1.45 (0.54-4.04)	0.39 (0.05-1.56)	0.51 (0.01-3.31)	1.39 (0.72-2.71)	1.13 (0.58-2.20)	1.85 (0.60-6.34)
	Homo or Hetero	≥37	1.41 (0.53-4.69)	0.37 (0.04-1.46)	0.47 (0.01-3.02)	1.24 (0.66-2.34)	1.07 (0.57-2.00)	-
	Homo vs Hetero	≥37	0.75 (0.34-1.64)	0.00 (0.00-89.11)	0.00 (0.00-468.00)	0.61 (0.17-1.72)	0.77 (0.22-2.23)	-
Hemiplegia (n = 80)	Homo	≥37	0.76 (0.30-2.00)	3.75 (0.33-26.59)	3.78 (0.06-73.19)	0.67 (0.24-1.60)	0.49 (0.15-1.31)	-
	Hetero	≥37	1.22 (0.57-2.67)	0.90 (0.34-2.09)	1.31 (0.32-4.00)	1.11 (0.65-1.91)	1.01 (0.60-1.70)	0.93 (0.37-2.34)
	Homo or Hetero	≥37	1.09 (0.52-2.36)	1.09 (0.48-2.38)	1.51 (0.44-4.18)	0.99 (0.60-1.65)	0.89 (0.54-1.46)	-
	Homo vs Hetero	≥37	0.62 (0.32-1.20)	4.14 (0.31-34.78)	2.88 (0.04-65.87)	0.60 (0.22-1.47)	0.48 (0.14-1.32)	-
Quadriplegia (n = 64)	Homo	≥37	0.59 (0.23-1.56)	0.00 (0.00-8.97)	4.97 (0.08-96.47)	1.11 (0.49-2.47)	0.54 (0.13-1.60)	-
	Hetero	≥37	0.82 (0.39-1.85)	0.79 (0.24-2.07)	1.73 (0.42-5.31)	0.83 (0.44-1.55)	1.21 (0.68-2.17)	0.67 (0.23-1.89)
	Homo or Hetero	≥37	0.73 (0.35-1.56)	0.74 (0.22-1.93)	1.99 (0.57-5.57)	0.91 (0.52-1.58)	1.05 (0.60-1.83)	-
	Homo vs Hetero	≥37	0.72 (0.35-1.47)	0.00 (0.00-22.31)	2.88 (0.04-65.87)	1.33 (0.55-3.15)	0.44 (0.11-1.33)	-

Table VII: Odds ratios (95% CI) for CP for specified thrombophilia among babies born at 32-36 weeks gestation compared with term-born controls. *p-values <0.05.

Type of CP	Zygosity	Control GA (weeks)	Odds Ratio (95% CI)					
			Any Thrombophilia	FVL	PGM	MTHFR C677T	MTHFR A1298C	MTHFR C677T + A1298C
All Types (n = 58)	Homo	≥37	1.02 (0.39-2.90)	0.00 (0.00-10.22)	0.00 (0.00-20.13)	2.24 (0.97-5.09)	0.47 (0.12-1.40)	-
	Hetero	≥37	1.04 (0.45-2.53)	1.08 (0.36-2.67)	1.34 (0.25-4.67)	1.89 (0.98-3.69)	0.76 (0.41-1.41)	1.46 (0.55-3.91)
	Homo or Hetero	≥37	1.03 (0.45-2.44)	1.01 (0.34-2.49)	1.24 (0.23-4.25)	**1.98 (1.08-3.66)***	0.69 (0.39-1.24)	-
	Homo vs Hetero	≥37	0.97 (0.49-1.90)	0.00 (0.00-17.88)	0.00 (0.00-51.19)	1.19 (0.54-2.58)	0.62 (0.15-1.94)	-
Diplegia (n = 20)	Homo	≥37	0.54 (0.12-2.44)	0.00 (0.00-29.93)	0.00 (0.00-67.50)	1.52 (0.25-6.83)	0.50 (0.06-2.22)	-
	Hetero	≥37	0.54 (0.17-2.02)	1.02 (0.11-4.43)	**4.35 (0.76-16.61)***	1.90 (0.65-5.62)	**0.16 (0.02-0.70)***	0.40 (0.04-2.55)
	Homo or Hetero	≥37	0.50 (0.17-1.82)	0.95 (0.11-4.14)	4.00 (0.70-15.16)	1.79 (0.66-5.05)	**0.25 (0.06-0.78)***	-
	Homo vs Hetero	≥37	1.00 (0.27-3.12)	0.00 (0.00-89.11)	0.00 (0.00-51.19)	0.80 (0.14-3.21)	3.10 (0.22-43.26)	-
Hemiplegia (n = 11)	Homo	≥37	Undefined	0.00 (0.00-55.93)	0.00 (0.00-107.39)	5.31 (0.59-64.25)	0.00 (0.00-2.24)	-
	Hetero	≥37	Undefined	0.92 (0.02-6.66)	0.00 (0.00-8.87)	3.99 (0.7-40.65)	0.74 (0.16-2.97)	Undefined
	Homo or Hetero	≥37	Undefined	0.86 (0.02-6.22)	0.00 (0.00-9.62)	4.35 (0.89-41.61)	0.56 (0.12-2.24)	-
	Homo vs Hetero	≥37	0.90 (0.15-3.82)	0.00 (0.00-575.25)	-	1.33 (0.21-6.41)	0.00 (0.00-4.80)	-
Quadriplegia (n = 16)	Homo	≥37	0.54 (0.07-4.16)	0.00 (0.00-43.22)	0.00 (0.00-71.95)	2.36 (0.48-10.21)	0.00 (0.00-1.74)	-
	Hetero	≥37	0.68 (0.16-3.98)	1.41 (0.15-6.47)	0.00 (0.00-6.06)	1.33 (0.35-5.04)	1.01 (0.33-3.02)	1.26 (0.26-6.56)
	Homo or Hetero	≥37	0.67 (0.18-3.78)	1.32 (0.14-6.04)	0.00 (0.00-6.29)	1.61 (0.52-5.46)	0.76 (0.25-2.27)	-
	Homo vs Hetero	≥37	0.80 (0.14-3.27)	0.00 (0.00-89.11)	-	1.78 (0.36-7.70)	0.00 (0.00-2.21)	-

Table VIII: Odds ratios (95% CI) for CP for specified thrombophilia among babies born at <32 weeks gestation compared with term-born controls. *p-values <0.05.

Type of CP	Zygosity	Control GA (weeks)	Odds Ratio (95% CI)					
			Any Thrombophilia	FVL	PGM	MTHFR C677T	MTHFR A1298C	MTHFR C677T + A1298C
All Types (n = 122)	Homo	≥37	1.09 (0.52-2.31)	2.35 (0.21-16.62)	0.00 (0.00-9.72)	1.48 (0.78-2.70)	0.60 (0.25-1.30)	-
	Hetero	≥37	1.16 (0.60-2.34)	0.49 (0.17-1.16)	1.09 (0.32-3.02)	1.25 (0.78-1.99)	0.98 (0.62-1.52)	1.35 (0.61-3.01)
	Homo or Hetero	≥37	1.14 (0.60-2.19)	0.61 (0.26-1.36)	1.00 (0.29-2.75)	1.31 (0.85-2.01)	0.88 (0.58-1.35)	-
	Homo vs Hetero	≥37	0.93-0.56-1.54)	4.83 (0.35-41.80)	0.00 (0.00-28.52)	1.19 (0.63-2.21)	0.62 (0.27-1.40)	-
Diplegia (n = 58)	Homo	≥37	1.85 (0.62-6.62)	0.00 (0.00-9.65)	0.00 (0.00-19.77)	**2.42 (1.05-5.42)***	0.54 (0.13-1.60)	-
	Hetero	≥37	1.63 (0.61-5.47)	0.51 (0.10-1.65)	0.88 (0.10-3.72)	1.61 (0.81-3.21)	1.04 (0.56-1.90)	2.22 (0.68-8.45)
	Homo or Hetero	≥37	1.68 (0.64-5.54)	0.48 (0.09-1.54)	0.81 (0.09-3.40)	1.83 (0.99-3.40)	0.92 (0.52-1.63)	-
	Homo vs Hetero	≥37	1.14 (0.59-2.16)	0.00 (0.00-44.51)	0.00 (0.00-86.02)	1.51 (0.68-3.29)	0.52 (0.13-1.58)	-
Hemiplegia (n = 25)	Homo	≥37	0.91 (0.17-5.98)	0.00 (0.00-23.17)	0.00 (0.00-51.55)	1.18 (0.20-4.87)	0.93 (0.17-3.51)	-
	Hetero	≥37	0.90 (0.24-5.10)	0.80 (0.09-3.37)	1.12 (0.03-7.58)	1.48 (0.53-4.18)	0.50 (0.14-1.52)	1.01 (0.13-7.73)
	Homo or Hetero	≥37	0.92 (0.27-5.17)	0.75 (0.08-3.14)	1.03 (0.02-6.93)	1.40 (0.55-3.60)	0.60 (0.23-1.59)	-
	Homo vs Hetero	≥37	1.00 (0.27-3.12)	0.00 (0.00-89.11)	0.00 (0.00-468.00)	0.80 (0.14-3.21)	1.86 (0.28-9.81)	-
Quadriplegia (n = 30)	Homo	≥37	0.68 (0.14-3.52)	**9.85 (0.85-71.52)***	0.00 (0.00-43.35)	0.66 (0.12-2.41)	0.31 (0.01-2.13)	-
	Hetero	≥37	1.13 (0.36-4.66)	0.34 (0.01-2.15)	1.9 (0.21-8.38)	0.75 (0.29-1.84)	1.40 (0.60-3.30)	1.01 (0.22-4.54)
	Homo or Hetero	≥37	0.96 (0.32-3.93)	0.95 (0.18-3.24)	1.74 (0.19-7.65)	0.72 (0.32-1.65)	1.13 (0.50-2.58)	-
	Homo vs Hetero	≥37	0.60 (0.17-1.69)	**29 (1.11-1737.29)***	0.00 (0.00-86.02)	0.89 (0.15-3.68)	0.22 (0.01-1.51)	-

Table I: Primer Sequences used in the genotyping of polymorphisms in the mannose-binding lectin gene and the tumour necrosis factor alpha gene.

Cytokine Gene Region	Primer Name	Primer Direction	Primer Sequence 5'-3'	Primer Length
TNF-α Promoter	308G for	Forward	GGC AAT AGG TTT TGA GGG GCG TGG	24
	308A rev	Reverse	ACC CTG GSG GCT GAA CCC CGT CCT	24
	ForC	Forward	GCC CCT CCC AGT TCT AGT TCT ATC	24
	RevC	Reverse	AAG CGG TAG TGG GCC CTG CAC CTT	24
	TNF for	Forward	GAC CTG GTC CCC AAA AGA AAT GGA GGC AAT AGG TTT TGA GGG CCA T	46
MBL Promoter	Shorty-R	Forward	AGG CAT AAG CCA GCT GGC AAT	21
	Shorty-L	Reverse	CTA AGG AGG GGT TCA TCT G	19
	UHG Shorty Overlap 1	Forward	CAC TGC CAC CCA TGT TTA TAG TCT TCC AGC	30
	UHG Shorty Overlap 2	Reverse	AAA CAT GGG TGG CAG TGA GAA CAA ATG	27
MBL Exon 1	Exon-1 L	Forward	CTG TGA CCT GTG AGG ATG C	19
	Exon-1 R	Reverse	CCA ACA CGT ACC TGG TTC C	19
	Exon-1 UHG	Forward	CTG TGA CCT GTG AGG ATG CCC AAA AGA CCT GCC CTG	36
	Exon-1 UHG Overlap	Reverse	CCA ACA CGT ACC TGG TTC CCC CTT TTC TCT GGT GCA ACA TCA CGC C	46

Table II: PCR Reaction Mixes.

PCR		Primers (μM)		$MgCl_2$ (mM)	Taq Polymerase	DNTP (μM)	Total Reaction Volume (μl)
Shorty	UHG Overlap	Shorty L UHG Overlap 1	0.5	2	0.6U AmpliTaq Gold	200	25
		Shorty R UHG Overlap 2	0.5	2	0.6U AmpliTaq Gold	200	25
	UHG DNA	Shorty L Shorty R	1	2	0.6U AmpliTaq Gold	200	25
Exon 1	UHG	Exon 1 UHG Exon 1 UHG Overlap	0.5	2	1.25U AmpliTaq Gold	200	50
	DNA	Exon 1-R Exon 1-L	0.5	2	0.6U AmpliTaq Gold	200	25
TNF-α	TNF 1st PCR	For C Rev C	0.5	2	0.6U AmpliTaq Gold	200	25
	TNF Nested PCR	308 G For 308 A Rev For C Rev C	0.5 0.6 0.4 0.4	2	0.6U AmpliTaq Gold	200	25
	TNF Nco1 PCR	TNF For Rev C	1	1.5	1U Platinum Taq	200	25

Table III: PCR Cycling Conditions.

PCR Programmes	Initial Temperature (°C)[1]	PCR Cycling Conditions				Final Temperature (°C)
		Denaturation Temperature (°C)	Annealing Temperature (°C)	Extension Temperature (°C)	Number of Cycles	
MBL Promoter	95 (10 min)	95 (1 min)	48 (1.5 min)	72 (1 min)	35	72 (10 min)
MBL Exon-1	95 (10 min)	95 (1 min)	52 (1 min)	72 (30 sec)	35	72 (10 min)
		96 (1 min)	70 (1 min)	72 (1 min)	5	
TNF-α AmpliTaq Gold	96 (10 min)	96 (1 min)	65 (1 min)	72 (1 min)	26	72 (5 min)
		96 (1 min)	55 (1 min)	72 (2 min)	4	
TNF-α Platinum Taq	94 (2 min)	94 (1 min)	57 (1.5 min)	72 (1 min)	35	72 (5 min)

[1]= Required for activation of AmpliTaq Gold and Platinum Taq Polymerase.

Table I: Primer sequences for detection of viral infection.

Virus	Gene Region	Primer Name	Primer Direction	Primer Sequence 5'-3'	Primer Length	Reference
Enterovirus	5' Untranslated Region	En-1	Forward	TCC TCC GGC CCC TGA AT	17	(1)
		En-3	Reverse	CAC CGG ATG GCC AAT CCA	18	
Herpesvirus group A PCR	DNA Polymerase	HSV-P1	Forward	GTG GTG GAC TTT GCC AGC CTG TAC CC	26	(2)
		HSV-P2	Reverse	TAA ACA TGG AGT CCG TGT CGC CGT AGA TGA	30	
Herpesvirus group B PCR	DNA Polymerase	VZV-P1	Forward	GTC GTG TTT GAT TTT CAA AGT TTA TAT CC	29	(2)
		VZV-P2	Reverse	ATA AAC ACA CAA TCC GTA TCA CCA TAA ATA ACC T	34	
K-ras PCR	Codon 12	K-ras c	Forward	AAG TGC TCT ACT ATC CAC A	19	(1)
		K-ras d	Reverse	CAC TAA ATT CCC TGG TAA TC	20	

1. Van den Veyver, I, Ni, J, Bowles, N, Carpenter, R, Weiner, C, Yankowitz, J, Moise, K, Henderson, J and Towbin, J (1998). Detection of Intrauterine Viral Infection Using the Polymerase Chain Reaction. *Mol Genet Metab*, 63: 85-95.
2. Johnson, G, Nelson, S, Petric, M and Tellier, R (2000). Comprehensive PCR-based assay for detection and species identification of human herpesviruses. *J Clin Microbiol*, 38: 3274-9..

Table II: Components of PCR Reaction Mixes for detection of viral infection.

PCR	Primers (mM)		$MgCl_2$ (mM)	Platinum Taq (U)	dNTP (mM)	DMSO (%)	Total Reaction Volume (µl)
Enterovirus	En-1	0.4	1.6	-	0.2	-	25
	En-3	0.4					
Herpesvirus PCR A	HSV-P1	0.4	2	1	0.2	5	25
	HSV-P2	0.4					
Herpesvirus PCR B	VZV-P1	0.4	2	1	0.2	5	25
	VZV-P2	0.4					
K-ras	K-ras c	0.2	2	1	0.2	-	25
	K-ras d	0.2					

Table III: PCR Cycling Conditions for detection of viral infection.

PCR Programme	Initial Temperature (°C)[1]	PCR Cycling Conditions				Final Temperature (°C)
		Denaturation Temperature (°C)	Annealing Temperature (°C)	Extension Temperature (°C)	Number of Cycles	
Enterovirus[2]	94 (2 min)	94 (30 sec)	60 (30 sec)	68 (1 min)	35	68 (5 min)
Herpesvirus PCR A	94 (2 min)	94 (1 min)	60 (1 min)	72 (1 min)	40	72 (7 min)
Herpesvirus PCR B	94 (2 min)	94 (1 min)	47 (1 min)	72 (1 min)	40	72 (3 min)
K-ras	94 (2 min)	94 (1 min)	58 (1 min)	72 (1 min)	35	72 (7 min)
Sequencing Big Dye PCR	-	96 (30 sec)	50 (10 sec)	60 (4 min)	30	-

[1] = Required for activation of Platinum Taq Enzyme and deactivation of reverse tramscriptase enzyme.
[2] = RNA viruses underwent an initial reverse transcription step of 55°C for 30 minutes.

Table I: Odds Ratios (95% CI) for all IUGR <10th percentile for specified thrombophilic polymorphisms. *p-values <0.05.

GA (weeks)	Zygosity	Odds Ratio (95% CI)				
		FVL	PGM	C677T	A1298C	677 + 1298
<28	Homozygous	4.13 (0.21-80.62)	7.26 (0.35-149.67)	1.03 (0.21-5.04)	1.25 (0.14-11.40)	-
	Heterozygous	0.32 (0.02-5.51)	**6.40 (1.66-24.71)***	0.35 (0.07-1.71)	1.99 (0.55-7.15)	0.51 (0.05-5.74)
	Homo or Hetero	0.30 (0.02-5.15)	**5.71 (1.49-21.93)***	0.52 (0.15-1.81)	1.84 (0.53-6.34)	-
28-31	Homozygous	4.46 (0.23-87.40)	5.65 (0.28-114.85)	0.51 (0.06-4.24)	**4.00 (1.04-15.37)**	-
	Heterozygous	0.73 (0.09-5.74)	0.77 (0.04-13.41)	0.88 (0.27-2.81)	0.80 (0.19-3.37)	2.04 (0.18-22.94)
	Homo or Hetero	0.68 (0.09-5.36)	0.69 (0.04-11.97)	0.79 (0.26-2.37)	1.47 (0.46-4.68)	-
<32	Homozygous	2.19 (0.11-41.73)	3.24 (0.16-64.66)	0.77 (0.22-2.75)	2.78 (0.90-8.60)	-
	Heterozygous	0.35 (0.05-2.65)	2.78 (0.78-9.89)	0.62 (0.24-1.55)	1.33 (0.52-3.40)	1.02 (0.20-5.20)
	Homo or Hetero	0.33 (0.04-2.47)	2.48 (0.70-8.77)	0.65 (0.29-1.50)	1.63 (0.69-3.83)	-
32-36	Homozygous	1.34 (0.07-25.39)	1.64 (0.08-32.24)	1.25 (0.54-2.91)	0.50 (0.15-1.70)	-
	Heterozygous	1.29 (0.52-3.16)	0.46 (0.06-3.50)	0.80 (0.41-1.58)	0.62 (0.32-1.20)	0.44 (0.16-1.19)
	Homo or Hetero	1.20 (0.49-2.95)	0.41 (0.06-3.11)	0.92 (0.50-1.67)	0.59 (0.32-1.10)	-
<37	Homozygous	0.84 (0.04-15.75)	1.10 (0.06-21.47)	1.07 (0.52-2.20)	1.03 (0.45-2.31)	-
	Heterozygous	0.93 (0.41-2.13)	1.24 (0.42-3.66)	0.73 (0.42-1.28)	0.78 (0.45-1.35)	0.54 (0.23-1.28)
	Homo or Hetero	0.87 (0.38-1.99)	1.10 (0.38-3.24)	0.82 (0.50-1.35)	0.83 (0.51-1.38)	-
37+	Homozygous	1.01 (0.11-9.12)	0.58 (0.03-11.30)	0.77 (0.42-1.39)	1.45 (0.80-2.64)	-
	Heterozygous	0.71 (0.35-1.43)	0.49 (0.15-1.64)	0.72 (0.48-1.09)	1.13 (0.75-1.71)	0.72 (0.34-1.50)
	Homo or Hetero	0.73 (0.37-1.43)	0.44 (0.13-1.46)	0.73 (0.50-1.07)	1.20 (0.82-1.76)	-
All	Homozygous	0.66 (0.07-5.94)	0.38 (0.02-7.40)	0.87 (0.53-1.42)	1.29 (0.77-2.16)	-
	Heterozygous	0.79 (0.45-1.39)	0.75 (0.32-1.75)	0.72 (0.51-1.03)	1.00 (0.71-1.41)	0.64 (0.35-1.15)
	Homo or Hetero	0.78 (0.45-1.35)	0.67 (0.29-1.55)	0.76 (0.55-1.05)	1.06 (0.77-1.46)	-

Table II: Odds Ratios (95% CI) for pre-existing hypertension for specified thrombophilic polymorphisms. *p-values <0.05.

GA (weeks)	Zygosity	Odds Ratio (95% CI)				
		FVL	PGM	C677T	A1298C	677 + 1298
28-31	Homozygous	22.29 (0.93-533.74)	**30.49 (1.23-758.39)**	-	0.99 (0.05-20.98)	-
	Heterozygous	1.74 (0.08-36.81)	4.18 (0.20-89.51)	6.15 (0.29 (128.84)	0.27 (0.01-5.56)	-
	Homo or Hetero	1.63 (0.08-34.39)	3.74 (0.18-79.88)	4.58 (0.22-95.94)	0.21 (0.01-4.39)	-
<32	Homozygous	22.29 (0.93-533.74)	**30.49 (1.23-758.39)**	-	0.99 (0.05-20.98)	-
	Heterozygous	1.74 (0.08-36.81)	4.18 (0.20-89.51)	6.15 (0.29 (128.84)	0.27 (0.01-5.56)	-
	Homo or Hetero	1.63 (0.08-34.39)	3.74 (0.18-79.88)	4.58 (0.22-95.94)	0.21 (0.01-4.39)	-
32-36	Homozygous	7.43 (0.37-150.75)	10.16 (0.48-214.64)	0.90 (0.10-8.16)	0.45 (0.02-8.28)	-
	Heterozygous	0.58 (0.03-10.32)	1.40 (0.08-25.12)	0.62 (0.11-3.39)	0.53 (0.10-2.76)	0.34 (0.03-3.34)
	Homo or Hetero	0.54 (0.03-9.64)	1.25 (0.07-22.41)	0.69 (0.15-3.10)	0.42 (0.08-2.18)	-
<37	Homozygous	5.87 (0.29-116.89)	8.02 (0.39-166.48)	0.90 (0.10-8.16)	0.33 (0.02-5.88)	-
	Heterozygous	0.46 (0.03-8.00)	1.10 (0.06-19.46)	1.23 (0.30-4.98)	0.38 (0.08-1.84)	0.34 (0.03-3.34)
	Homo or Hetero	0.43 (0.02-7.47)	0.99 (0.06-17.37)	1.15 (0.30-4.31)	0.30 (0.06-1.46)	-
37+	Homozygous	7.43 (0.37-150.75)	10.16 (0.48-214.64)	0.32 (0.02-5.94)	0.99 (0.05-20.98)	-
	Heterozygous	0.58 (0.03-10.32)	1.40 (0.08-25.12)	0.49 (0.09-2.56)	2.65 (0.48-14.63)	1.02 (0.06-16.59)
	Homo or Hetero	0.54 (0.03-9.64)	1.25 (0.07-22.41)	0.37 (0.07-1.91)	2.10 (0.38-11.55)	-
All	Homozygous	3.38 (0.17-65.39)	4.62 (0.23-93.16)	0.40 (0.05-3.20)	0.26 (0.01-4.56)	-
	Heterozygous	0.26 (0.02-4.47)	0.63 (0.04-10.87)	0.82 (0.29-2.34)	0.88 (0.31-2.53)	0.51 (0.09-2.86)
	Homo or Hetero	0.25 (0.01-4.17)	0.58 (0.03-9.70)	0.71 (0.26-1.94)	0.70 (0.25-1.99)	-

Table III: Odds Ratios (95% CI) for all pregnancy-induced hypertensive disorders for specified thrombophilic polymorphisms. All p-values NS.

GA (weeks)	Zygosity	Odds Ratio (95% CI)				
		FVL	PGM	C677T	A1298C	677 + 1298
<28	Homozygous	8.57 (0.42-176.23)	13.86 (0.64-301.73)	0.51 (0.03-10.00)	0.71 (0.04-13.93)	-
	Heterozygous	0.67 (0.04-12.07)	1.90 (0.10-35.37)	0.41 (0.04-3.97)	0.88 (0.15-5.34)	0.51 (0.05-5.74)
	Homo or Hetero	0.63 (0.03-11.28)	1.70 (0.09-31.56)	0.31 (0.03-2.96)	0.70 (0.12-4.22)	-
28-31	Homozygous	15.92 (0.71-355.49)	21.78 (0.94-505.55)	1.19 (0.05-29.55)	-	-
	Heterozygous	2.93 (0.30-28.65)	2.99 (0.15-59.46)	3.69 (0.38-35.75)	11.94 (0.64-223.07)	7.15 (0.36-140.48)
	Homo or Hetero	2.74 (0.28-26.75)	6.35 (0.64-63.00)	2.75 (0.28-26.60)	9.44 (0.51-176.26)	-
<32	Homozygous	5.87 (0.29-116.89)	8.97 (0.43-187.52)	0.40 (0.02-7.46)	0.71 (0.04-13.93)	-
	Heterozygous	0.98 (0.12-7.85)	2.67 (0.32-22.15)	1.23 (0.30-4.98)	2.65 (0.66-10.74)	2.04 (0.37-11.43)
	Homo or Hetero	0.91 (0.11-7.33)	2.38 (0.29-19.70)	0.92 (0.23-3.70)	2.10 (0.52-8.47)	-
32-36	Homozygous	10.13 (0.48-211.99)	11.73 (0.55-250.88)	0.71 (0.03-15.05)	0.99 (0.05-20.98)	-
	Heterozygous	1.76 (0.20-15.32)	1.61 (0.09-29.38)	1.85 (0.31-11.15)	2.65 (0.48-14.63)	2.04 (0.18-22.94)
	Homo or Hetero	1.64 (0.19-14.30)	1.44 (0.08-26.22)	1.37 (0.23-8.29)	2.10 (0.38-11.55)	-
<37	Homozygous	3.84 (0.20-74.81)	5.26 (0.26-106.58)	0.27 (0.02-4.94)	0.45 (0.02-8.28)	-
	Heterozygous	1.26 (0.28-5.67)	1.52 (0.19-12.05)	1.44 (0.48-4.34)	2.65 (0.89-7.88)	2.04 (0.50-8.42)
	Homo or Hetero	1.17 (0.26-5.29)	1.36 (0.17-10.72)	1.07 (0.35-3.22)	2.10 (0.71-6.22)	-
37+	Homozygous	8.57 (0.42-176.23)	13.86 (0.64-301.73)	2.39 (0.39-14.60)	0.99 (0.05-20.98)	-
	Heterozygous	0.67 (0.04-12.07)	1.90 (0.10-35.37)	0.18 (0.10-3.42)	1.99 (0.33-12.02)	-
	Homo or Hetero	0.63 (0.03-11.28)	1.44 (0.08-26.22)	0.61 (0.10-3.69)	1.57 (0.26-9.49)	-
All	Homozygous	2.72 (0.14-52.23)	3.91 (0.19-78.36)	0.80 (0.17-3.77)	0.33 (0.02-5.88)	-
	Heterozygous	0.88 (0.20-3.86)	1.12 (0.14-8.73)	0.96 (0.35-2.61)	2.46 (0.97-6.29)	2.04 (0.50-8.42)
	Homo or Hetero	0.82 (0.19-3.60)	1.00 (0.13-7.76)	0.92 (0.36-2.34)	1.95 (0.77-4.96)	-

Table IV: Odds Ratios (95% CI) for pregnancy-induced hypertensive disorders and IUGR <10th percentile for specified thrombophilic polymorphisms. *p-values <0.05.

GA (weeks)	Zygosity	Odds Ratio (95% CI)				
		FVL	PGM	C677T	A1298C	677 + 1298
<28	Homozygous	15.92 (0.71-355.49)	21.78 (0.94-505.55)	0.71 (0.03-15.05)	0.71 (0.04-13.93)	-
	Heterozygous	1.25 (0.06-24.44)	2.99 (0.15-59.46)	0.25 (0.01-5.15)	0.19 (0.10-3.69)	0.20 (0.10-4.32)
	Homo or Hetero	1.17 (0.06-22.84)	2.67 (0.13-53.06)	0.18 (0.01-3.84)	0.15 (0.10-2.92)	-
<32	Homozygous	15.92 (0.71-355.49)	21.78 (0.94-505.55)	0.71 (0.03-15.05)	0.71 (0.04-13.93)	-
	Heterozygous	1.25 (0.06-24.44)	2.99 (0.15-59.46)	0.25 (0.01-5.15)	0.19 (0.10-3.69)	0.20 (0.10-4.32)
	Homo or Hetero	1.17 (0.06-22.84)	2.67 (0.13-53.06)	0.18 (0.01-3.84)	0.15 (0.10-2.92)	-
32-36	Homozygous	15.92 (0.71-355.49)	16.94 (0.76-378.15)	0.71 (0.03-15.05)	0.99 (0.05-20.98)	-
	Heterozygous	2.93 (0.30-28.65)	2.33 (0.12-44.39)	0.62 (0.06-6.83)	1.33 (0.19-9.50)	0.34 (0.01-8.47)
	Homo or Hetero	2.74 (0.28-26.75)	2.08 (0.11-39.61)	0.46 (0.04-5.08)	1.05 (0.15-7.50)	-
<37	Homozygous	8.57 (0.42-176.23)	21.78 (0.94-505.55)	0.40 (0.02-7.46)	0.45 (0.02-8.28)	-
	Heterozygous	1.47 (0.17-12.39)	1.40 (0.08-25.12)	0.31 (0.03-2.77)	0.53 (0.10-2.76)	0.15 (0.10-2.87)
	Homo or Hetero	1.37 (0.16-11.57)	1.25 (0.07-22.41)	0.23 (0.03-2.06)	0.42 (0.08-2.18)	-
37+	Homozygous	**37.15 (1.33-1041.30)**	**50.81 (1.75-1476.90)**	1.19 (0.05-29.55)	-	-
	Heterozygous	2.91 (0.12-72.26)	6.97 (0.28-175.59)	0.41 (0.02-10.12)	3.98 (0.16-98.23)	-
	Homo or Hetero	2.72 (0.11-67.51)	6.24 (0.25-156.72)	0.31 (0.01-7.54)	3.15 (0.13-77.62)	-
All	Homozygous	7.43 (0.37-150.75)	8.97 (0.43-187.52)	0.32 (0.02-5.94)	0.45 (0.02-8.28)	-
	Heterozygous	1.26 (0.15-10.40)	1.23 (0.07-21.93)	0.31 (0.03-2.77)	0.80 (0.19-3.37)	0.15 (0.10-2.87)
	Homo or Hetero	1.17 (0.14-9.70)	1.10 (0.06-19.57)	0.18 (0.02-1.58)	0.63 (0.15-2.66)	-

Table V: Odds Ratios (95% CI) for all antepartum haemorrhage for specified thrombophilic polymorphisms. All p-values NS.

GA (weeks)	Zygosity	Odds Ratio (95% CI)				
		FVL	PGM	C677T	A1298C	677 + 1298
<28	Homozygous	1.31 (0.07-24.78)	1.84 (0.09-36.18)	1.00 (0.36-2.78)	0.53 (0.12-2.33)	-
	Heterozygous	0.63 (0.19-2.09)	1.56 (0.45-5.39)	1.23 (0.62-2.42)	1.47 (0.77-2.80)	2.04 (0.59-7.02)
	Homo or Hetero	0.59 (0.18-1.95)	1.39 (0.41-4.78)	1.17 (0.62-2.22)	1.27 (0.68-2.39)	-
28-31	Homozygous	1.15 (0.06-21.67)	1.37 (0.07-26.95)	1.20 (0.54-2.66)	1.88 (0.83-4.25)	-
	Heterozygous	1.47 (0.66-3.25)	0.78 (0.18-3.36)	0.73 (0.38-1.39)	1.22 (0.66-2.23)	1.02 (0.34-3.03)
	Homo or Hetero	1.37 (0.62-3.03)	0.69 (0.16-2.99)	0.85 (0.48-1.50)	1.36 (0.77-2.37)	-
<32	Homozygous	0.62 (0.03-11.54)	0.79 (0.04-15.42)	1.12 (0.58-2.15)	1.28 (0.62-2.63)	-
	Heterozygous	1.07 (0.54-2.13)	1.11 (0.41-2.97)	0.93 (0.57-1.50)	1.33 (0.84-2.10)	1.39 (0.61-3.19)
	Homo or Hetero	1.00 (0.51-1.98)	0.99 (0.37-2.63)	0.98 (0.63-1.52)	1.32 (0.85-2.03)	-
32-36	Homozygous	0.84 (0.04-15.75)	1.07 (0.05-20.86)	1.26 (0.64-2.50)	0.95 (0.42-2.14)	-
	Heterozygous	1.20 (0.57-2.53)	1.20 (0.41-3.55)	0.83 (0.48-1.43)	0.79 (0.47-1.34)	0.61 (0.26-1.47)
	Homo or Hetero	1.12 (0.53-2.36)	1.07 (0.37-3.15)	0.94 (0.58-1.53)	0.82 (0.51-1.34)	-
<37	Homozygous	0.36 (0.02-6.66)	0.46 (0.02-8.86)	1.18 (0.71-1.96)	1.12 (0.63-1.99)	-
	Heterozygous	1.13 (0.66-1.93)	2.61 (0.12-55.41)	0.89 (0.61-1.30)	1.06 (0.74-1.53)	0.94 (0.50-1.76)
	Homo or Hetero	1.05 (0.62-1.80)	1.03 (0.47-2.22)	0.96 (0.68-1.36)	1.07 (0.76-1.51)	-
37+	Homozygous	1.12 (0.12-10.11)	0.64 (0.03-12.54)	0.94 (0.51-1.73)	1.44 (0.78-2.66)	-
	Heterozygous	0.86 (0.44-1.70)	0.90 (0.34-2.41)	0.89 (0.58-1.36)	1.01 (0.66-1.55)	0.89 (0.47-1.72)
	Homo or Hetero	0.88 (0.46-1.69)	0.81 (0.31-2.13)	0.90 (0.61-1.34)	1.10 (0.74-1.63)	-
All	Homozygous	0.47 (0.05-4.21)	0.27 (0.01-5.19)	1.08 (0.70-1.66)	1.25 (0.79-1.99)	-
	Heterozygous	1.02 (0.64-1.61)	1.05 (0.54-2.05)	0.89 (0.65-1.21)	1.04 (0.77-1.41	0.92 (0.56-1.51)
	Homo or Hetero	0.98 (0.62-1.54)	0.80 (0.40-1.60)	0.94 (0.70-1.24)	1.09 (0.82-1.44)	-

Table VI: Odds Ratios (95% CI) for all APH and IUGR <10th percentile for specified thrombophilic polymorphisms. *p-values <0.05.

GA (weeks)	Zygosity	Odds Ratio (95% CI)				
		FVL	PGM	C677T	A1298C	677 + 1298
<28	Homozygous	4.46 (0.23-87.40)	8.02 (0.39-166.48)	1.03 (0.21-5.04)	1.67 (0.17-16.33)	-
	Heterozygous	0.35 (0.02-5.97)	**7.11 (1.81-27.89)***	0.35 (0.07-1.71)	2.65 (0.66-10.74)	0.51 (0.05-5.74)
	Homo or Hetero	0.33 (0.02-5.58)	**6.35 (1.63-24.75)***	0.52 (0.15-1.81)	2.45 (0.63-9.56)	-
28-31	Homozygous	4.85 (0.25-95.43)	6.10 (0.30-124.51)	0.60 (0.07-5.05)	**5.00 (1.21-20.60)***	-
	Heterozygous	0.80 (0.10-6.31)	0.84 (0.05-14.54)	1.03 (0.31-3.41)	1.00 (0.22-4.50)	5.11 (0.24-107.93)
	Homo or Hetero	0.75 (0.09-5.89)	0.75 (0.04-12.98)	0.92 (0.29-2.88)	1.84 (0.53-6.34)	-
<32	Homozygous	2.37 (0.12-45.38)	3.55 (0.18-70.85)	0.83 (0.23-2.99)	**3.57 (1.09-11.66)***	-
	Heterozygous	0.38 (0.05-2.88)	3.05 (0.85-10.90)	0.66 (0.26-1.69)	1.33 (0.46-3.84)	1.53 (0.25-9.39)
	Homo or Hetero	0.36 (0.05-2.69)	2.72 (0.77-9.67)	0.70 (0.30-1.64)	1.80 (0.70-4.64)	-
32-36	Homozygous	1.89 (0.10-35.94)	2.42 (0.12-47.90)	1.93 (0.74-5.02)	0.56 (0.13-2.46)	-
	Heterozygous	0.91 (0.27-3.08)	0.69 (0.09-5.25)	1.14 (0.51-2.54)	0.88 (0.42-1.88)	1.02 (0.32-3.29)
	Homo or Hetero	0.85 (0.25-2.87)	0.61 (0.08-4.67)	1.34 (0.65-2.77)	0.82 (0.40-1.67)	-
<37	Homozygous	1.06 (0.06-20.00)	1.45 (0.07-28.51)	1.38 (0.64-2.99)	1.40 (0.28-3.40)	-
	Heterozygous	0.68 (0.24-1.94)	1.64 (0.55-4.90)	1.80 (0.87-3.73)	1.12 (0.61-2.05)	1.15 (0.43-3.11)
	Homo or Hetero	0.63 (0.22-1.81)	1.46 (0.49-1.34)	1.02 (0.59-1.78)	1.18 (0.67-2.07)	-
37+	Homozygous	1.49 (0.08-28.14)	1.88 (0.10-37.09)	0.94 (0.37-2.39)	0.95 (0.31-2.88)	-
	Heterozygous	1.19 (0.45-3.14)	0.53 (0.07-4.04)	0.70 (0.34-1.41)	1.07 (0.55-2.09)	0.51 (0.15-1.76)
	Homo or Hetero	1.11 (0.42-2.93)	0.48 (0.06-3.59)	0.76 (0.40-1.42)	1.05 (0.56-1.97)	-
All	Homozygous	0.62 (0.03-11.67)	0.82 (0.40-16.09)	1.17 (0.63-2.18)	1.20 (0.58-2.45)	-
	Heterozygous	0.89 (0.42-1.86)	1.16 (0.43-3.11)	0.80 (0.50-1.30)	1.10 (0.69-1.75)	0.83 (0.38-1.82)
	Homo or Hetero	0.83 (0.40-1.73)	1.04 (0.39-2.75)	0.90 (0.58-1.38)	1.12 (0.72-1.73)	-

Table VII: Odds Ratios (95% CI) for preterm births (<37 weeks GA) for specified thrombophilic polymorphisms. *p-values <0.05.

GA (weeks)	Zygosity	Odds Ratio (95% CI)				
		FVL	PGM	C677T	A1298C	677 + 1298
<28	Homozygous	2.98 (0.54-16.55)	0.92 (0.05-18.06)	0.98 (0.45-2.14)	0.54 (0.21-1.43)	-
	Heterozygous	0.31 (0.10-1.03)	1.04 (0.35-3.07)	1.49 (0.91-2.48)	0.95 (0.59-1.54)	1.39 (0.61-3.19)
	Homo or Hetero	0.49 (0.19-1.25)	0.93 (0.32-2.72)	1.36 (0.85-2.18)	0.87 (0.55-1.37)	-
28-31	Homozygous	0.88 (0.10-7.96)	0.64 (0.03-12.54)	1.15 (0.64-2.08)	1.03 (0.52-2.05)	-
	Heterozygous	0.62 (0.31-1.24)	0.90 (0.34-2.41)	0.93 (0.59-1.43)	1.05 (0.69-1.61)	0.97 (0.49-1.91)
	Homo or Hetero	0.64 (0.33-1.24)	0.81 (0.31-2.13)	0.98 (0.66-1.47)	1.05 (0.70-1.57)	-
<32	Homozygous	1.58 (0.35-7.11)	0.38 (0.02-7.40)	1.09 (0.66-1.80)	0.82 (0.45-1.47)	-
	Heterozygous	**0.48 (0.26-0.89)***	0.96 (0.44-2.09)	1.13 (0.80-1.61)	1.01 (0.72-1.42)	1.12 (0.64-1.96)
	Homo or Hetero	**0.55 (0.31-0.98)***	0.86 (0.40-1.85)	1.12 (0.81-1.56)	0.97 (0.70-1.34)	-
32-36	Homozygous	0.74 (0.08-6.64)	0.42 (0.02-8.27)	0.95 (0.56-1.59)	0.89 (0.50-1.59)	-
	Heterozygous	0.93 (0.53-1.63)	1.19 (0.56-2.53)	0.97 (0.68-1.39)	0.91 (0.64-1.29)	0.83 (0.48-1.45)
	Homo or Hetero	0.92 (0.53-1.58)	1.06 (0.51-2.23)	0.97 (0.69-1.35)	0.90 (0.65-1.26)	-
<37	Homozygous	1.37 (0.34-5.51)	0.20 (0.01-3.90)	1.02 (0.68-1.53)	1.34 (0.89-2.03)	-
	Heterozygous	0.74 (0.47-1.18)	1.07 (0.58-1.97)	1.05 (0.79-1.39)	0.96 (0.73-1.26)	0.96 (0.62-1.50)
	Homo or Hetero	0.79 (0.51-1.22)	0.95 (0.53-1.73)	1.04 (0.80-1.36)	0.64 (0.72-1.22)	-

Table VIII: Odds Ratios (95% CI) for preterm births (<37 weeks GA) and IUGR <10th percentile for specified thrombophilic polymorphisms. *p-values <0.05.

GA (weeks)	Zygosity	Odds Ratio (95% CI)				
		FVL	PGM	C677T	A1298C	677 + 1298
<28	Homozygous	4.13 (0.21-80.62)	7.26 (0.35-149.67)	0.90 (0.10-8.16)	1.25 (0.14-11.40)	-
	Heterozygous	0.32 (0.02-5.51)	**6.40 (1.66-24.71)***	1.85 (0.51-6.63)	1.99 (0.55-7.15)	0.51 (0.05-5.74)
	Homo or Hetero	0.30 (0.02-5.15)	**5.71 (1.49-21.93)***	1.60 (0.46-5.54)	1.84 (0.53-6.34)	-
28-31	Homozygous	4.46 (0.23-87.40)	5.65 (0.28-114.85)	2.87 (0.75-10.97)	4.00 (1.04-15.37)	-
	Heterozygous	0.73 (0.09-5.74)	0.77 (0.04-13.41)	0.74 (0.17-3.13)	0.80 (0.19-3.37)	2.04 (0.18-22.94)
	Homo or Hetero	0.68 (0.09-5.36)	0.69 (0.04-11.97)	1.28 (0.40-4.09)	1.47 (0.46-4.68)	-
<32	Homozygous	2.19 (0.11-41.73)	3.24 (0.16-64.66)	1.99 (0.65-6.14)	2.78 (0.90-8.60)	-
	Heterozygous	0.35 (0.05-2.65)	2.78 (0.78-9.88)	1.23 (0.48-3.15)	1.33 (0.52-3.40)	1.02 (0.20-5.20)
	Homo or Hetero	0.33 (0.04-2.47)	2.48 (0.70-8.77)	1.43 (0.61-3.35)	1.63 (0.69-3.83)	-
32-36	Homozygous	1.34 (0.07-25.39)	1.64 (0.08-32.24)	0.36 (0.11-1.21)	0.50 (0.15-1.70)	-
	Heterozygous	1.29 (0.52-3.16)	0.46 (0.06-3.50)	0.57 (0.30-1.11)	0.62 (0.32-1.20)	0.44 (0.16-1.19)
	Homo or Hetero	1.20 (0.49-2.95)	0.41 (0.06-3.11)	**0.52 (0.28-0.96)***	0.59 (0.32-1.10)	-
<37	Homozygous	0.84 (0.04-15.75)	1.10 (0.06-21.47)	0.74 (0.33-1.65)	1.03 (0.45-2.31)	-
	Heterozygous	0.93 (0.41-2.13)	1.34 (0.42-3.66)	0.73 (0.42-1.25)	0.78 (0.45-1.35)	0.54 (0.23-1.28)
	Homo or Hetero	0.87 (0.38-1.99)	1.10 (0.38-3.24)	0.75 (0.46-1.24)	0.83 (0.51-1.38)	-

Table I: Odds Ratios (95% CI) for all IUGR <10th percentile for specified cytokine polymorphisms. *p-values <0.05.

GA (weeks)	Zygosity	TNF -308	MBL -221	MBL Exon 1			
				Codon 52	Codon 54	Codon 57	Any Abnormal
<28	Heterozygous	**4.22 (1.36-13.11)***	1.17 (0.41-3.33)	2.65 (0.31-22.40)	1.63 (0.56-4.76)	1.57 (0.09-28.67)	-
	Homozygous	3.90 (0.44-34.87)	0.71 (0.04-12.55)	14.14 (0.54-373.05)	1.37 (0.08-24.81)	-	-
	Hetero or Homo	**4.18 (1.38-12.66)***	1.04 (0.36-2.95)	2.50 (0.30-21.09)	1.49 (0.51-4.37)	-	1.41 (0.51-3.95)
28-31	Heterozygous	1.65 (0.53-5.12)	0.97 (0.32-2.94)	1.21 (0.07-21.87)	1.63 (0.56-4.76)	1.57 (0.09-28.67)	-
	Homozygous	2.44 (0.29-20.39)	0.71 (0.04-12.55)	14.14 (0.54-373.05)	1.37 (0.08-24.81)	-	-
	Hetero or Homo	1.74 (0.60-5.10)	0.86 (0.29-2.61)	1.15 (0.06-20.64)	1.49 (0.51-4.37)	-	1.21 (0.41-3.54)
<32	Heterozygous	**2.64 (1.20-5.82)***	1.07 (0.50-2.31)	1.32 (0.17-10.58)	1.63 (0.75-3.52)	0.81 (0.05-14.22)	-
	Homozygous	3.00 (0.63-14.15)	0.36 (0.02-6.22)	7.28 (0.29-185.81)	0.70 (0.04-12.30)	-	-
	Hetero or Homo	**2.68 (1.25-5.75)***	0.95 (0.44-2.05)	1.25 (0.16-9.96)	1.49 (0.69-3.22)	-	1.31 (0.62-2.78)
32-36	Heterozygous	1.36 (0.72-2.56)	1.31 (0.73-2.37)	0.76 (0.10-5.90)	1.39 (0.75-2.59)	0.99 (0.12-7.84)	-
	Homozygous	1.26 (0.28-5.61)	0.49 (0.06-3.75)	4.22 (0.17-105.96)	0.41 (0.02-7.00)	-	-
	Hetero or Homo	1.35 (0.74-2.48)	1.22 (0.68-2.18)	0.71 (0.09-5.55)	1.28 (0.69-2.37)	-	1.27 (0.71-2.27)
<37	Heterozygous	**1.74 (1.05-2.88)***	1.22 (0.75-1.97)	0.96 (0.22-4.31)	1.48 (0.90-2.44)	0.63 (0.08-4.93)	-
	Homozygous	1.77 (0.58-5.39)	0.30 (0.04-2.25)	2.70 (0.11-67.35)	0.26 (0.02-4.45)	-	-
	Hetero or Homo	**1.74 (1.07-2.83)***	1.11 (0.69-1.79)	0.91 (0.20-4.05)	1.36 (0.82-2.23)	-	1.28 (0.80-2.06)
37+	Heterozygous	0.92 (0.61-1.40)	1.22 (0.84-1.77)	0.22 (0.03-1.68)	0.88 (0.58-1.33)	1.44 (0.50-4.15)	-
	Homozygous	0.92 (0.34-2.49)	0.32 (0.07-1.37)	1.25 (0.05-30.83)	0.50 (0.11-2.23)	-	-
	Hetero or Homo	0.92 (0.62-1.37)	1.12 (0.78-1.61)	0.21 (0.03-1.58)	0.85 (0.57-1.28)	-	0.84 (0.57-1.23)
All	Heterozygous	1.16 (0.82-1.64)	1.22 (0.89-1.68)	0.45 (0.13-1.57)	1.07 (0.76-1.51)	1.19 (0.44-3.19)	-
	Homozygous	1.17 (0.52-2.61)	0.31 (0.09-1.05)	0.86 (0.03-21.14)	0.34 (0.08-1.52)	-	-
	Hetero or Homo	1.16 (0.84-1.61)	1.12 (0.82-1.53)	0.43 (0.12-1.48)	1.01 (0.72-1.41)	-	0.98 (0.71-1.35)

Table II: Odds Ratios (95% CI) for all pre-existing hypertension for specified cytokine polymorphisms. *p-values <0.05.

GA (weeks)	Zygosity	TNF -308	MBL -221	MBL Exon 1			
				Codon 52	Codon 54	Codon 57	Any Abnormal
<28	Heterozygous	0.88 (0.04-21.68)	0.58 (0.02-14.38)	6.87 (0.27-174.84)	0.72 (0.03-17.82)	8.90 (0.35-228.92)	-
	Homozygous	6.35 (0.25-160.60)	4.50 (0.18-113.18)	**80.11 (2.23-2876.10)**	7.75 (0.30-198.26)	-	-
	Hetero or Homo	0.77 (0.03-19.09)	0.52 (0.02-12.76)	6.50 (0.26-165.08)	0.66 (0.03-16.35)	-	0.54 (0.02-13.27)
28-31	Heterozygous	2.64 (0.16-42.48)	1.75 (0.11-28.15)	-	6.50 (0.26-160.42)	-	-
	Homozygous	6.35 (0.25-160.60)	4.50 (0.18-113.18)	-	-	-	-
	Hetero or Homo	2.32 (0.14-37.39)	1.55 (0.10-24.96)	-	5.96 (0.24-147.12)	-	4.84 (0.20-119.40)
<32	Heterozygous	1.32 (0.12-14.66)	0.88 (0.08-9.72)	6.87 (0.27-174.84)	2.17 (0.13-34.91)	8.90 (0.35-228.92)	-
	Homozygous	3.81 (0.18-81.89)	2.70 (0.13-57.70)	**80.11 (2.23-2876.10)**	7.75 (0.30-198.26)	-	-
	Hetero or Homo	1.16 (0.10-12.91)	0.78 (0.07-8.61)	6.50 (0.26-165.08)	1.99 (0.12-32.00)	-	1.61 (0.10-25.96)
32-36	Heterozygous	0.88 (0.18-4.41)	0.70 (0.13-3.64)	7.06 (0.70-71.50)	2.17 (0.43-10.86)	3.82 (0.19-77.65)	-
	Homozygous	1.47 (0.08-26.89)	2.75 (0.31-24.40)	**34.33 (1.18-998.49)**	8.00 (0.78-81.56)	-	-
	Hetero or Homo	0.77 (0.15-3.88)	0.93 (0.22-3.94)	6.67 (0.66-67.35)	2.65 (0.59-11.98)	-	2.69 (0.64-11.37)
<37	Heterozygous	0.99 (0.26-3.78)	0.75 (0.19-2.93)	5.29 (0.56-49.99)	2.17 (0.54-8.78)	2.97 (0.15-57.98)	-
	Homozygous	1.12 (0.06-20.07)	1.96 (0.23-16.56)	26.70 (0.95-748.84)	6.00 (0.63-57.04)	-	-
	Hetero or Homo	0.87 (0.23-3.33)	0.89 (0.26-3.07)	5.00 (0.53-47.08)	2.49 (0.66-9.37)	-	2.42 (0.68-8.68)
37+	Heterozygous	2.11 (0.56-7.97)	1.40 (0.37-5.27)	2.29 (0.12-44.24)	1.63 (0.36-7.35)	6.92 (0.72-66.39)	-
	Homozygous	1.73 (0.09-32.37)	1.23 (0.07-22.80)	26.70 (0.95-748.84)	6.00 (0.63-57.04)	-	-
	Hetero or Homo	1.86 (0.49-7.01)	1.24 (0.33-4.67)	2.17 (0.11-41.77)	1.99 (0.49-8.05)	-	2.02 (0.54-7.60)
All	Heterozygous	1.42 (0.56-3.63)	1.02 (0.40-2.64)	2.65 (0.31-22.40)	1.90 (0.68-5.32)	3.46 (0.40-29.77)	-
	Homozygous	0.71 (0.04-12.28)	1.14 (0.14-9.15)	14.14 (0.54-373.05)	**6.00 (1.17-30.74)**	-	-
	Hetero or Homo	1.25 (0.49-3.19)	1.04 (0.42-2.57)	2.50 (0.30-21.09)	2.24 (0.85-5.90)	-	2.22 (0.88-5.60)

Table III: Odds Ratios (95% CI) for all pregnancy-induced hypertensive disorders for specified cytokine polymorphisms. *p-values <0.05.

GA (weeks)	Zygosity	TNF -308	MBL -221	MBL Exon 1			
				Codon 52	Codon 54	Codon 57	Any Abnormal
<28	Heterozygous	3.52 (0.78-15.91)	1.75 (0.35-8.75)	2.29 (0.12-44.24)	0.54 (0.06-4.89)	6.92 (0.72-66.39)	-
	Homozygous	2.72 (0.14-54.41)	4.58 (0.46-45.58)	26.70 (0.95-748.84)	2.58 (0.13-50.19)	-	-
	Hetero or Homo	3.10 (0.69-13.99)	2.07 (0.46-9.34)	2.17 (0.11-41.78)	0.50 (0.06-4.48)	-	1.21 (0.27-5.46)
28-31	Heterozygous	0.88 (0.09-8.53)	0.19 (0.01-3.63)	10.59 (0.91-122.68)	0.43 (0.02-9.08)	**13.85 (1.18-162.72)**	-
	Homozygous	2.72 (0.14-54.41)	1.50 (0.08-28.61)	**48.07 (1.55-1491.10)**	4.65 (0.21-101.15)	-	-
	Hetero or Homo	2.32 (0.14-37.39)	0.17 (0.01-3.22)	10.00 (0.87-115.57)	0.40 (0.02-8.32)	-	1.61 (0.23-11.55)
<32	Heterozygous	2.20 (0.66-7.31)	0.75 (0.19-2.93)	3.53 (0.40-31.00)	0.36 (0.04-3.03)	**9.23 (1.70-50.21)***	-
	Homozygous	1.47 (0.08-26.89)	1.96 (0.23-16.56)	18.49 (0.69-498.27)	1.79 (0.10-33.23)	-	-
	Hetero or Homo	1.94 (0.58-6.43)	0.89 (0.26-3.07)	3.33 (0.38-29.19)	0.33 (0.04-2.78)	-	1.35 (0.41-4.46)
32-36	Heterozygous	1.76 (0.29-10.63)	0.35 (0.04-3.02)	2.94 (0.15-59.26)	1.45 (0.24-8.74)	9.23 (0.90-94.91)	-
	Homozygous	6.49 (0.65-65.13)	1.23 (0.07-22.80)	**34.33 (1.18-998.49)**	3.32 (0.16-67.22)	-	-
	Hetero or Homo	2.32 (0.46-11.63)	0.31 (0.04-2.68)	2.78 (0.14-55.94)	1.33 (0.22-8.01)	-	1.61 (0.32-8.07)
<37	Heterozygous	2.05 (0.75-5.61)	0.58 (0.19-1.83)	2.35 (0.28-19.66)	0.72 (0.19-2.71)	**9.23 (2.23-38.16)***	-
	Homozygous	2.16 (0.26-17.90)	1.14 (0.14-9.15)	12.65 (0.48-331.36)	1.22 (0.07-22.02)	-	-
	Hetero or Homo	2.07 (0.78-5.44)	0.65 (0.22-1.86)	2.22 (0.27-18.52)	0.66 (0.18-2.48)	-	1.44 (0.55-3.78)
37+	Heterozygous	3.96 (0.65-23.93)	0.88 (0.16-4.82)	10.59 (0.91-122.68)	3.25 (0.54-19.66)	5.34 (0.24-116.83)	-
	Homozygous	9.74 (0.85-111.81)	1.50 (0.08-28.61)	**48.07 (1.55-1491.10)**	4.65 (0.21-101.15)	-	-
	Hetero or Homo	4.65 (0.84-25.62)	0.78 (0.14-4.27)	10.00 (0.87-115.57)	2.98 (0.49-18.02)	-	3.23 (0.59-17.78)
All	Heterozygous	**2.40 (1.00-5.76)**	0.66 (0.25-1.70)	3.85 (0.79-18.76)	1.18 (0.43-3.25)	**7.55 (1.88-30.37)**	-
	Homozygous	3.54 (0.74-17.01)	0.86 (0.11-6.73)	10.45 (0.40-270.79)	1.01 (0.06-17.97)	-	-
	Hetero or Homo	**2.54 (1.10-5.86)***	0.68 (0.28-1.68)	3.64 (0.75-17.65)	1.09 (0.39-2.98)	-	1.76 (0.76-4.06)

Table IV: Odds Ratios (95% CI) for all pregnancy-induced hypertensive disorders and IUGR <10th percentile for specified cytokine polymorphisms.

GA (weeks)	Zygosity	TNF -308	MBL -221	MBL Exon 1			
				Codon 52	Codon 54	Codon 57	Any Abnormal
<28, <32	Heterozygous	7.92 (0.82-76.72)	1.75 (0.24-12.52)	2.94 (0.15-59.26)	0.72 (0.07-7.01)	3.82 (0.19-77.65)	-
	Homozygous	6.35 (0.25-160.60)	2.70 (0.13-57.70)	**34.33 (1.18-998.49)**	3.32 (0.16-67.22)	-	-
	Hetero or Homo	6.97 (0.72-67.52)	1.55 (0.22-11.10)	2.78 (0.14-55.94)	0.66 (0.07-6.42)	-	0.54 (0.06-5.21)
32-36	Heterozygous	0.38 (0.02-7.33)	0.58 (0.06-5.65)	4.12 (0.19-89.18)	2.17 (0.30-15.54)	5.34 (0.24-116.83)	-
	Homozygous	6.49 (0.65-65.13)	1.93 (0.10-38.33)	**48.07 (1.55-1491.10)**	4.65 (0.21-101.15)	-	-
	Hetero or Homo	0.77 (0.08-7.50)	0.52 (0.05-5.01)	3.90 (0.18-84.20)	1.99 (0.28-14.24)	-	1.61 (0.23-11.55)
<37	Heterozygous	1.98 (0.44-8.95)	1.05 (0.25-4.44)	1.87 (0.10-35.26)	1.30 (0.31-5.51)	2.43 (0.13-46.21)	-
	Homozygous	4.87 (0.52-45.52)	1.23 (0.07-22.80)	21.85 (0.80-598.53)	2.11 (0.11-40.00)	-	-
	Hetero or Homo	2.32 (0.57-9.40)	0.93 (0.22-3.94)	1.77 (0.09-33.28)	1.19 (0.28-5.05)	-	0.97 (0.23-4.09)
37+	Heterozygous	7.90 (0.32-195.12)	0.58 (0.02-14.38)	-	6.50 (0.26-160.42)	-	-
	Homozygous	-	4.50 (0.18-113.18)	-	-	-	-
	Hetero or Homo	6.96 (0.28-171.83)	0.52 (0.02-12.76)	-	5.96 (0.24-147.12)	-	4.84 (0.20-119.40)
All	Heterozygous	2.64 (0.65-10.69)	0.88 (0.22-3.54)	1.87 (0.10-35.26)	1.74 (0.46-6.55)	2.43 (0.13-46.21)	-
	Homozygous	4.87 (0.52-45.52)	1.04 (0.06-18.94)	21.85 (0.80-598.53)	2.11 (0.11-40.00)	-	-
	Hetero or Homo	2.91 (0.77-10.95)	0.78 (0.19-3.14)	1.77 (0.09-33.28)	1.59 (0.42-6.00)	-	1.29 (0.34-4.86)

Table V: Odds Ratios (95% CI) for all antepartum haemorrhage for specified cytokine polymorphisms. *p-values <0.05.

GA (weeks)	Zygosity	TNF -308	MBL -221	MBL Exon 1			
				Codon 52	Codon 54	Codon 57	Any Abnormal
<28	Heterozygous	1.66 (0.88-3.13)	1.31 (0.70-2.48)	0.81 (0.10-6.37)	1.08 (0.54-2.16)	1.07 (0.13-8.47)	-
	Homozygous	1.44 (0.32-6.48)	**2.86 (1.01-8.12)**	13.85 (0.84-227.91)	1.85 (0.40-8.51)	-	-
	Hetero or Homo	1.64 (0.89-3.02)	1.49 (0.82-2.70)	1.54 (0.34-7.00)	1.03 (0.53-1.99)	-	1.24 (0.68-2.28)
28-31	Heterozygous	1.15 (0.63-2.09)	1.04 (0.60-1.81)	1.63 (0.46-5.81)	0.78 (0.41-1.47)	0.34 (0.02-5.80)	-
	Homozygous	2.50 (0.89-6.99)	0.74 (0.17-3.25)	**18.46 (1.64-208.38)***	1.23 (0.27-5.58)	-	-
	Hetero or Homo	1.31 (0.76-2.28)	1.01 (0.59-1.73)	2.56 (0.90-7.29)	0.73 (0.40-1.34)	-	0.87 (0.50-1.52)
<32	Heterozygous	1.36 (0.86-2.14)	1.15 (0.74-1.77)	1.30 (0.42-4.00)	0.90 (0.55-1.46)	0.43 (0.05-3.31)	-
	Homozygous	2.07 (0.84-5.05)	1.58 (0.66-3.79)	**16.62 (1.70-162.30)***	1.48 (0.48-4.59)	-	-
	Hetero or Homo	1.44 (0.94-2.22)	1.20 (0.79-1.81)	2.15 (0.86-5.36)	0.85 (0.54-1.35)	-	1.02 (0.67-1.56)
32-36	Heterozygous	1.07 (0.63-1.79)	1.18 (0.72-1.92)	0.37 (0.05-2.80)	0.71 (0.41-1.23)	0.23 (0.01-3.89)	-
	Homozygous	0.68 (0.16-2.99)	1.49 (0.55-4.08)	2.05 (0.08-51.07)	0.83 (0.18-3.72)	-	-
	Hetero or Homo	1.02 (0.62-1.68)	1.22 (0.76-1.94)	0.34 (0.05-2.63)	0.65 (0.38-1.09)	-	0.67 (0.40-1.11)
<37	Heterozygous	1.22 (0.85-1.76)	1.16 (0.82-1.64)	0.86 (0.31-2.38)	0.81 (0.55-1.19)	0.23 (0.03-1.74)	-
	Homozygous	1.43 (0.64-3.19)	1.54 (0.75-3.16)	8.78 (0.90-85.24)	1.17 (0.44-3.09)	-	-
	Hetero or Homo	1.25 (0.88-1.77)	1.20 (0.86-1.70)	1.30 (0.55-3.07)	0.75 (0.52-1.09)	-	0.85 (0.60-1.20)
37+	Heterozygous	1.22 (0.80-1.34)	1.05 (0.71-1.56)	0.80 (0.23-2.81)	1.10 (0.72-1.68)	1.75 (0.61-5.06)	-
	Homozygous	0.88 (0.29-2.61)	0.86 (0.32-2.30)	4.56 (0.28-73.69)	0.91 (0.26-3.23)	-	-
	Hetero or Homo	1.18 (0.79-1.75)	1.03 (0.70-1.51)	1.01 (0.33-3.08)	0.97 (0.64-1.46)	-	1.08 (0.74-1.59)
All	Heterozygous	1.22 (0.90-1.65)	1.11 (0.84-1.48)	0.84 (0.36-1.98)	0.92 (0.68-1.26)	0.82 (0.31-2.20)	-
	Homozygous	1.19 (0.59-2.43)	1.25 (0.66-2.36)	7.13 (0.79-64.25)	1.07 (0.46-2.50)	-	-
	Hetero or Homo	1.22 (0.91-1.63)	1.13 (0.86-1.49)	1.19 (0.56-2.52)	0.94 (0.69-1.27)	-	0.94 (0.71-1.25)

Table VI: Odds Ratios (95% CI) for all antepartum haemorrhage and IUGR <10th percentile for specified cytokine polymorphisms. *p-values <0.05.

GA (weeks)	Zygosity	TNF -308	MBL -221	MBL Exon 1			
				Codon 52	Codon 54	Codon 57	Any Abnormal
<28	Heterozygous	3.17 (0.95-10.53)	1.09 (0.35-3.39)	3.53 (0.40-31.00)	2.17 (0.69-6.83)	2.05 (0.11-38.39)	-
	Homozygous	3.90 (0.44-34.87)	0.79 (0.04-14.14)	18.49 (0.69-498.27)	1.79 (0.10-33.23)	-	-
	Hetero or Homo	3.25 (1.02-10.39)	0.97 (0.31-3.00)	3.33 (0.38-29.19)	1.99 (0.63-6.26)	-	1.88 (0.62-5.68)
28-31	Heterozygous	1.89 (0.59-6.03)	1.09 (0.35-3.39)	1.37 (0.08-25.04)	1.86 (0.62-5.62)	1.78 (0.10-32.83)	-
	Homozygous	2.78 (0.33-23.68)	0.79 (0.04-14.14)	16.02 (0.60-426.69)	1.55 (0.08-28.41)	-	-
	Hetero or Homo	1.99 (0.66-6.01)	0.97 (0.31-3.00)	1.30 (0.07-23.64)	1.71 (0.56-5.15)	-	1.38 (0.46-4.17)
<32	Heterozygous	**2.42 (1.05-5.60)**	1.09 (0.49-2.46)	1.63 (0.20-13.19)	2.00 (0.89-4.48)	0.99 (0.06-17.53)	-
	Homozygous	3.25 (0.68-15.45)	0.41 (0.02-7.01)	8.90 (0.35-228.92)	0.86 (0.05-15.18)	-	-
	Hetero or Homo	**2.52 (1.13-5.63)***	0.97 (0.43-2.18)	1.54 (0.19-12.42)	1.84 (0.82-4.11)	-	1.61 (0.73-3.55)
32-36	Heterozygous	1.44 (0.70-2.98)	1.23 (0.61-2.48)	0.92 (0.12-7.23)	1.67 (0.72-3.88)	0.57 (0.03-9.86)	-
	Homozygous	0.89 (0.11-6.89)	0.69 (0.09-5.32)	5.11 (0.20-129.07)	0.49 (0.03-8.53)	-	-
	Hetero or Homo	1.37 (0.68-2.79)	1.16 (0.58-2.32)	0.87 (0.11-6.81)	0.86 (0.40-1.86)	-	0.84 (0.41-1.73)
<37	Heterozygous	**1.79 (1.02-3.13)***	1.17 (0.68-2.01)	1.18 (0.26-5.30)	1.33 (0.76-2.32)	0.37 (0.02-6.29)	-
	Homozygous	1.72 (0.49-6.06)	0.38 (0.05-2.90)	3.29 (0.13-82.35)	0.32 (0.02-5.44)	-	-
	Hetero or Homo	**1.78 (1.04-3.05)***	1.08 (0.63-1.84)	1.11 (0.25-4.98)	1.22 (0.69-2.13)	-	1.12 (0.66-1.92)
37+	Heterozygous	0.82 (0.40-1.72)	0.97 (0.51-1.87)	0.39 (0.02-6.65)	1.08 (0.54-2.16)	3.20 (0.86-11.93)	-
	Homozygous	0.61 (0.08-4.67)	0.51 (0.07-3.89)	4.54 (0.18-114.13)	0.44 (0.03-7.54)	-	-
	Hetero or Homo	0.80 (0.39-1.62)	0.92 (0.48-1.75)	0.37 (0.02-6.28)	0.99 (0.50-1.98)	-	1.06 (0.56-1.99)
All	Heterozygous	1.32 (0.84-2.08)	1.08 (0.70-1.67)	0.68 (0.15-3.03)	1.22 (0.78-1.93)	1.34 (0.37-4.84)	-
	Homozygous	1.18 (0.39-3.55)	0.44 (0.10-1.88)	1.92 (0.08-47.77)	0.19 (0.01-3.15)	-	-
	Hetero or Homo	1.30 (0.84-2.02)	1.01 (0.66-1.55)	0.65 (0.15-2.85)	1.12 (0.72-1.76)	-	1.09 (0.71-1.67)

Table VII: Odds Ratios (95% CI) for all preterm births (<37 weeks GA) for specified cytokine polymorphisms. *p-values <0.05.

GA (weeks)	Zygosity	TNF -308	MBL -221	MBL Exon 1			
				Codon 52	Codon 54	Codon 57	Any Abnormal
<28	Heterozygous	**1.62 (1.02-2.57)***	1.04 (0.65-1.66)	0.40 (0.05-3.07)	1.27 (0.79-2.05)	1.05 (0.23-4.76)	-
	Homozygous	0.68 (0.16-2.99)	**2.29 (1.02-5.15)**	6.79 (0.42-110.31)	1.36 (0.38-4.85)	-	-
	Hetero or Homo	1.51 (0.96-2.37)	1.18 (0.76-1.83)	0.75 (0.17-3.35)	1.28 (0.80-2.03)	-	1.25 (0.80-1.94)
28-31	Heterozygous	1.21 (0.79-1.85)	1.39 (0.95-2.04)	0.99 (0.32-3.00)	0.91 (0.59-1.40)	0.32 (0.04-2.50)	-
	Homozygous	**2.52 (1.17-5.42)***	0.75 (0.25-2.22)	8.37 (0.75-93.45)	0.56 (0.13-2.49)	-	-
	Hetero or Homo	1.37 (0.92-2.02)	1.32 (0.90-1.92)	1.40 (0.54-3.62)	0.88 (0.58-1.34)	-	0.88 (0.60-1.31)
<32	Heterozygous	1.38 (0.98-1.93)	1.24 (0.90-1.71)	0.76 (0.28-2.11)	1.05 (0.74-1.48)	0.60 (0.17-2.13)	-
	Homozygous	1.78 (0.87-3.66)	1.41 (0.70-2.82)	7.77 (0.80-75.37)	0.86 (0.31-2.42)	-	-
	Hetero or Homo	**1.42 (1.03-1.96)***	1.26 (0.92-1.71)	1.15 (0.49-2.71)	1.03 (0.74-1.44)	-	1.02 (0.75-1.40)
32-36	Heterozygous	1.12 (0.78-1.60)	0.90 (0.64-1.28)	0.47 (0.13-1.62)	0.78 (0.54-1.14)	0.81 (0.26-2.54)	-
	Homozygous	0.70 (0.26-1.89)	1.42 (0.71-2.84)	0.88 (0.04-21.76)	0.88 (0.31-2.48)	-	-
	Hetero or Homo	1.07 (0.76-1.51)	0.96 (0.69-1.33)	0.44 (0.13-1.52)	0.79 (0.55-1.14)	-	0.77 (0.55-1.08)
<37	Heterozygous	1.25 (0.95-1.65)	1.07 (0.82-1.40)	0.62 (0.26-1.45)	0.92 (0.69-1.22)	0.70 (0.28-1.79)	-
	Homozygous	1.25 (0.65-2.38)	1.41 (0.80-2.49)	3.93 (0.41-37.98)	0.87 (0.39-1.97)	-	-
	Hetero or Homo	1.25 (0.96-1.63)	1.11 (0.86-1.43)	0.80 (0.37-1.72)	0.91 (0.69-1.20)	-	0.90 (0.69-1.16)

Table VIII: Odds Ratios (95% CI) for all preterm births (<37 weeks GA) and IUGR <10th percentile for specified cytokine polymorphisms. *p-values <0.05.

GA (weeks)	Zygosity	TNF -308	MBL -221	MBL Exon 1			
				Codon 52	Codon 54	Codon 57	Any Abnormal
<28	Heterozygous	**4.22 (1.36-13.11)***	1.17 (0.41-3.33)	2.65 (0.31-22.40)	1.63 (0.56-4.76)	1.57 (0.09-28.67)	-
	Homozygous	3.90 (0.44-34.87)	0.71 (0.04-12.55)	14.14 (0.54-373.05)	1.37 (0.08-24.81)	-	-
	Hetero or Homo	**4.18 (1.38-12.66)***	1.04 (0.36-2.95)	2.50 (0.30-21.09)	1.49 (0.51-4.37)	-	1.41 (0.51-3.95)
28-31	Heterozygous	1.65 (0.53-5.12)	0.97 (0.32-2.94)	1.21 (0.07-21.87)	1.63 (0.56-4.76)	1.57 (0.09-28.67)	-
	Homozygous	2.44 (0.29-20.39)	0.71 (0.04-12.55)	14.14 (0.54-373.05)	1.37 (0.08-24.81)	-	-
	Hetero or Homo	1.74 (0.60-5.10)	0.86 (0.29-2.61)	1.15 (0.06-20.64)	1.49 (0.51-4.37)	-	1.21 (0.41-3.54)
<32	Heterozygous	**2.64 (1.20-5.82)***	1.07 (0.50-2.31)	1.32 (0.17-10.58)	1.63 (0.75-3.52)	0.81 (0.05-14.22)	-
	Homozygous	3.00 (0.63-14.15)	0.36 (0.02-6.22)	7.28 (0.29-185.81)	0.70 (0.04-12.30)	-	-
	Hetero or Homo	**2.68 (1.25-5.75)***	0.95 (0.44-2.05)	1.25 (0.16-9.96)	1.49 (0.69-3.22)	-	1.31 (0.62-2.78)
32-36	Heterozygous	1.36 (0.72-2.56)	1.31 (0.73-2.37)	0.76 (0.10-5.90)	1.39 (0.75-2.59)	0.99 (0.12-7.84)	-
	Homozygous	1.26 (0.28-5.61)	0.49 (0.06-3.75)	4.22 (0.17-105.96)	0.41 (0.02-7.00)	-	-
	Hetero or Homo	1.35 (0.74-2.48)	1.22 (0.68-2.18)	0.71 (0.09-5.55)	1.28 (0.69-2.37)	-	1.27 (0.71-2.27)
<37	Heterozygous	**1.74 (1.05-2.88)***	1.22 (0.75-1.97)	0.96 (0.22-4.31)	1.48 (0.90-2.44)	0.63 (0.08-4.93)	-
	Homozygous	1.77 (0.58-5.39)	0.30 (0.04-2.25)	2.70 (0.11-67.35)	0.26 (0.02-4.45)	-	-
	Hetero or Homo	**1.74 (1.07-2.83)***	1.11 (0.69-1.79)	0.91 (0.20-4.05)	1.36 (0.82-2.23)	-	1.28 (0.80-2.06)

Table I: Odds Ratios (95% CI) for all IUGR <10th percentile for specified viral nucleic acids. All p-values NS.

GA (weeks)	Herpes B (n=22/194)	CMV (n=57/226)	HSV (n=10/226)	Entero (n=5/227)	Herpes A (n=65/226)	Any Herpes (n=81/198)	Any Virus (n=86/199)
<28	0.97 (0.12-7.73)	1.20 (0.37-3.88)	0.76 (0.04-13.12)	0.94 (0.05-16.25)	1.01 (0.31-3.25)	1.16 (0.36-3.72)	1.06 (0.33-3.37)
28-31	0.46 (0.03-7.96)	0.82 (0.22-2.97)		2.29 (0.28-18.44)	0.69 (0.19-2.49)	0.54 (0.15-2.03)	0.74 (0.22-2.49)
<32	0.49 (0.06-3.71)	1.00 (0.42-2.40)	0.39 (0.02-6.52)	1.06 (0.04-8.24)	0.84 (0.35-2.01)	0.81 (0.34-1.94)	0.89 (0.38-2.07)
32-36	1.84 (0.73-4.62)	1.16 (0.61-2.22)	1.43 (0.42-4.93)	0.29 (0.02-4.84)	1.18 (0.64-2.20)	1.79 (0.95-3.37)	1.63 (0.86-3.06)
<37	1.32 (0.56-3.07)	1.10 (0.65-1.88)	0.90 (0.27-3.05)	0.38 (0.05-2.89)	1.05 (0.63-1.77)	1.36 (0.81-2.28)	1.31 (0.78-2.19)
37+	1.40 (0.75-2.61)	0.96 (0.63-1.46)	1.12 (0.47-2.63)	0.83 (0.28-2.47)	1.00 (0.67-1.49)	1.03 (0.69-1.52)	1.04 (0.71-1.54)
All	1.37 (0.79-2.37)	1.01 (0.71-1.44)	1.04 (0.49-2.20)	0.67 (0.25-1.82)	1.02 (0.73-1.43)	1.13 (0.81-1.58)	1.13 (0.81-1.57)

n = total number of cases testing positive for the virus/total number of cases yielding positive or negative results for the virus

Table II: Odds Ratios (95% CI) for all pre-existing hypertension for specified viral nucleic acids. *p-values <0.05.

GA (weeks)	Herpes B (n=2/14)	CMV (n=6/20)	HSV (n=1/20)	Entero (n=0/19)	Herpes A (n=7/20)	Any Herpes (n=9/18)	Any Virus (n=9/18)
<28	-	8.96 (0.36-221.30)	7.35 (0.29-185.12)	9.68 (0.38-245.56)	7.53 (0.31-185.95)	4.88 (0.20-120.49)	4.43 (0.18-109.37)
28-31	3.53 (0.14-88.10)	2.99 (0.19-48.19)	4.41 (0.21-94.37)	5.81 (0.27-125.23)	2.52 (0.16-40.47)		
<32		5.99 (0.54-66.54)	3.15 (0.16-62.69)	4.15 (0.21-83.20)	5.03 (0.45-55.88)	8.13 (0.39-170.44)	7.38 (0.35-154.71)
32-36	5.35 (0.95-30.13)	1.00 (0.20-5.00)	3.21 (0.38-27.13)	1.71 (0.10-30.70)	1.51 (0.36-6.39)	4.07 (0.78-21.20)	3.70 (0.71-19.24)
<37	4.28 (0.81-22.78)	1.71 (0.49-5.93)	2.25 (0.28-18.26)	1.26 (0.07-22.23)	2.10 (0.63-6.96)	**5.70 (1.17-27.73)***	**5.17 (1.06-25.16)**
37+	0.71 (0.04-12.59)	0.86 (0.18-4.16)	1.16 (0.07-20.52)	1.71 (0.10-30.70)	0.72 (0.15-3.50)	0.47 (0.10-2.26)	0.42 (0.09-2.05)
All	1.79 (0.39-8.25)	1.28 (0.48-3.40)	1.18 (0.15-9.20)	0.75 (0.04-12.80)	1.35 (0.53-3.45)	1.63 (0.64-4.18)	1.48 (0.58-3.79)

n = total number of cases testing positive for the virus/total number of cases yielding positive or negative results for the virus

Table III: Odds Ratios (95% CI) for all pregnancy-induced hypertensive disorders for specified viral nucleic acids. *p-values <0.05.

GA (weeks)	Herpes B (n=4/16)	CMV (n=13/23)	HSV (n=1/23)	Entero (n=1/23)	Herpes A (n=13/23)	Any Herpes (n=14/18)	Any Virus (n=14/18)
<28	7.14 (1.16-43.97)	1.20 (0.23-6.24)	1.47 (0.08-26.48)	1.94 (0.11-35.16)	1.01 (0.19-5.24)	1.63 (0.33-8.15)	1.48 (0.30-7.40)
28-31	**21.42 (1.90-241.38)***	8.98 (0.93-87.04)	2.45 (0.13-46.80)	3.23 (0.17-62.11)	7.55 (0.78-73.09)	11.39 (0.58-221.81)	10.34 (0.53-201.33)
<32	**10.71 (2.58-44.42)***	2.49 (0.75-8.30)	0.96 (0.05-16.74)	1.26 (0.07-22.23)	2.10 (0.63-6.96)	3.26 (0.80-13.18)	2.96 (0.73-11.96)
32-36	1.18 (0.06-22.25)	2.99 (0.60-15.00)	1.70 (0.09-30.98)	2.24 (0.12-41.12)	2.52 (0.50-12.59)	4.89 (0.50-47.32)	4.43 (0.46-42.95)
<37	**5.35 (1.55-18.55)***	2.66 (1.01-7.02)	0.63 (0.04-10.78)	0.83 (0.05-14.32)	2.24 (0.85-5.89)	3.66 (1.13-12.06)	**3.33 (1.01-10.95)***
37+	1.18 (0.06-22.25)	**14.97 (1.73-129.21)***	4.50 (0.51-39.96)	5.96 (0.66-53.54)	**12.58 (1.46-108.50)***	**17.89 (0.98-325.63)***	**16.24 (0.89-295.57)***
All	**3.57 (1.10-11.57)***	**3.89 (1.67-9.06)***	1.02 (0.13-7.89)	1.35 (0.17-10.58)	**3.27 (1.41-7.60)***	**5.70 (1.85-17.57)***	**5.17 (1.68-15.94)***

n = total number of cases testing positive for the virus/total number of cases yielding positive or negative results for the virus

Table IV: Odds Ratios (95% CI) for all Pregnancy-Induced Hypertensive Disorders and IUGR <10th percentile for specified viral nucleic acids. All p-values NS.

GA (weeks)	Herpes B (n=0/4)	CMV (n=3/9)	HSV (n=0/9)	Entero (n=0/9)	Herpes A (n=3/9)	Any Herpes (n=3/5)	Any Virus (n=3/5)
<28	2.12 (0.10-44.89)	1.00 (0.10-9.67)	2.45 (0.13-46.80)	3.23 (0.17-62.11)	0.84 (0.09-8.12)	0.81 (0.07-9.05)	0.74 (0.07-8.21)
28-31	-	-	-	-	-	-	-
<32	2.12 (0.10-44.89)	1.00 (0.10-9.67)	2.45 (0.13-46.80)	3.23 (0.17-62.11)	0.84 (0.09-8.12)	0.81 (0.07-9.05)	0.74 (0.07-8.21)
32-36		2.99 (0.42-21.45)			2.52 (0.35-18.01)	8.13 (0.39-170.44)	7.38 (0.35-154.71)
<37	1.18 (0.06-22.25)	1.80 (0.42-7.61)	1.30 (0.07-23.12)	1.71 (0.10-30.70)	1.51 (0.36-6.39)	2.44 (0.40-14.76)	2.22 (0.37-13.39)
37+	-	1.00 (0.04-24.59)	7.35 (0.29-185.12)	9.68 (0.38-245.56)	0.84 (0.03-20.66)	-	
All	1.18 (0.06-22.25)	1.50 (0.37-6.06)	1.16 (0.07-20.52)	1.53 (0.09-27.24)	1.26 (0.31-5.09)	2.44 (0.40-14.76)	2.22 (0.37-13.39)

n = total number of cases testing positive for the virus/total number of cases yielding positive or negative results for the virus

Table V: Odds Ratios (95% CI) for all antepartum haemorrhage for specified viral nucleic acids. All p-values NS.

GA (weeks)	Herpes B (n=27/259)	CMV (n=89/323)	HSV (n=10/323)	Entero (n=5/323)	Herpes A (n=95/323)	Any Herpes (n=113/271)	Any Virus (n=118/272)
<28	2.22 (0.87-5.65)	1.76 (0.94-3.29)	0.24 (0.01-3.96)	0.31 (0.02-5.15)	1.47 (0.79-2.76)	1.92 (0.98-3.75)	1.74 (0.89-3.40)
28-31	0.93 (0.32-2.72)	1.16 (0.64-2.09)	0.37 (0.05-2.82)	1.01 (0.23-4.44)	1.05 (0.59-1.88)	0.97 (0.53-1.75)	1.03 (0.58-1.86)
<32	1.43 (0.69-2.97)	1.39 (0.89-2.18)	0.21 (0.03-1.58)	0.56 (0.13-2.45)	1.22 (0.79-1.90)	1.30 (0.82-2.05)	1.29 (0.82-2.03)
32-36	1.23 (0.53-2.86)	1.24 (0.74-2.07)	0.85 (0.25-2.89)	0.18 (0.01-2.95)	1.24 (0.75-2.02)	1.50 (0.91-2.47)	1.36 (0.82-2.24)
<37	1.34 (0.74-2.43)	1.33 (0.92-1.90)	0.49 (0.17-1.42)	0.32 (0.07-1.37)	1.23 (0.86-1.75)	1.38 (0.96-1.98)	1.32 (0.92-1.89)
37+	1.12 (0.54-2.31)	0.90 (0.58-1.40)	1.05 (0.42-2.62)	0.69 (0.20-2.36)	0.82 (0.53-1.27)	0.90 (0.59-1.39)	0.91 (0.60-1.38)
All	1.25 (0.75-2.08)	1.14 (0.84-1.55)	0.72 (0.34-1.52)	0.47 (0.17-1.27)	1.05 (0.78-1.41)	1.17 (0.86-1.57)	1.13 (0.84-1.53)

n = total number of cases testing positive for the virus/total number of cases yielding positive or negative results for the virus

Table VI: Odds Ratios (95% CI) for all antepartum haemorrhage and IUGR <10th percentile for specified viral nucleic acids. *p-values <0.05.

GA (weeks)	Herpes B (n=11/87)	CMV (n=21/103)	HSV (n=2/103)	Entero (n=1/104)	Herpes A (n=23/103)	Any Herpes (n=34/89)	Any Virus (n=35/89)
<28	1.07 (0.14-8.58)	1.00 (0.27-3.74)	0.88 (0.05-15.33)	0.18 (0.06-18.77)	0.84 (0.22-3.14)	1.09 (0.30-3.90)	0.99 (0.27-3.54)
28-31	0.46 (0.03-7.96)	0.90 (0.24-3.31)	0.82 (0.05-14.14)	2.48 (0.31-20.09)	0.75 (0.21-2.78)	0.54 (0.15-2.03)	0.74 (0.22-2.49)
<32	0.51 (0.07-3.89)	0.95 (0.37-2.41)	0.43 (0.03-7.31)	1.19 (0.15-9.26)	0.79 (0.31-2.02)	0.76 (0.30-1.90)	0.84 (0.35-2.05)
32-36	**2.79 (1.08-7.25)***	0.62 (0.25-1.52)	1.36 (0.31-6.00)	0.41 (0.02-6.92)	0.75 (0.33-1.67)	1.43 (0.68-2.99)	1.29 (0.62-2.71)
<37	1.70 (0.72-4.02)	1.70 (0.72-4.02)	0.78 (0.18-3.36)	0.50 (0.07-3.78)	0.77 (0.41-1.43)	1.10 (0.62-1.98)	1.08 (0.61-1.93)
37+	1.34 (0.45-3.97)	0.79 (0.37-1.69)	0.25 (0.02-4.24)	0.33 (0.02-5.63)	0.67 (0.31-1.42)	0.88 (0.44-1.77)	0.81 (0.40-1.61)
All	1.55 (0.76-3.15)	0.77 (0.46-1.28)	0.45 (0.10-1.91)	0.29 (0.04-2.19)	0.72 (0.44-1.19)	1.01 (0.63-1.60)	0.96 (0.60-1.52)

n = total number of cases testing positive for the virus/total number of cases yielding positive or negative results for the virus

Table VII: Odds Ratios (95% CI) for all preterm births (<37 weeks GA) for specified viral nucleic acids. All p-values NS.

GA (weeks)	Herpes B (n=32/358)	CMV (n=131/433)	HSV (n=11/433)	Entero (n=8/434)	Herpes A (n=139/433)	Any Herpes (n=159/375)	Any Virus (n=163/375)
<28	1.90 (0.93-3.89)	**1.62 (1.02-2.57)***	**0.12 (0.01-1.94)***	0.32 (0.04-2.40)	1.36 (0.86-2.16)	1.55 (0.96-2.50)	1.40 (0.87-2.27)
28-31	0.73 (0.32-1.67)	1.09 (0.71-1.67)	0.17 (0.02-1.25)	1.15 (0.40-3.13)	0.95 (0.62-1.44)	0.84 (0.55-1.28)	0.88 (0.58-1.34)
<32	1.17 (0.65-2.09)	1.29 (0.92-1.81)	0.10 (0.01-0.73)	0.80 (0.31-2.02)	1.11 (0.79-1.55)	1.08 (0.77-1.52)	1.07 (0.76-1.50)
32-36	0.93 (0.49-1.75)	1.31 (0.92-1.86)	1.16 (0.55-2.46)	0.29 (0.07-1.28)	1.29 (0.91-1.81)	1.34 (0.95-1.88)	1.21 (0.86-1.71)
<37	1.05 (0.65-1.71)	1.30 (0.98-1.71)	0.59 (0.29-1.20)	0.56 (0.24-1.29)	1.19 (0.91-1.56)	1.20 (0.91-1.58)	1.14 (0.87-1.49)

n = total number of cases testing positive for the virus/total number of cases yielding positive or negative results for the virus

Table VIII: Odds Ratios (95% CI) for all preterm births (<37 weeks GA) and IUGR <10th percentile for specified viral nucleic acids. All p-values NS.

GA (weeks)	Herpes B (n=7/64)	CMV (n=21/78)	HSV (n=3/78)	Entero (n=1/79)	Herpes A (n=23/78)	Any Herpes (n=30/66)	Any Virus (n=31/66)
<28	0.97 (0.12-7.73)	1.20 (0.37-3.88)	0.76 (0.04-13.12)	0.94 (0.05-16.25)	1.01 (0.31-3.25)	1.16 (0.36-3.72)	1.06 (0.33-3.37)
28-31	0.46 (0.03-7.96)	0.82 (0.22-2.97)		2.29 (0.28-18.44)	0.69 (0.19-2.49)	0.54 (0.15-2.03)	0.74 (0.22-2.49)
<32	0.49 (0.06-3.71)	1.00 (0.42-2.40)	0.39 (0.02-6.52)	1.06 (0.14-8.24)	0.84 (0.35-2.01)	0.81 (0.34-1.94)	0.89 (0.38-2.07)
32-36	1.84 (0.73-4.62)	1.16 (0.61-2.22)	1.44 (0.42-4.93)	0.29 (0.02-4.84)	1.18 (0.64-2.20)	1.79 (0.95-3.37)	1.63 (0.86-3.06)
<37	1.32 (0.56-3.07)	1.10 (0.65-1.88)	0.90 (0.27-3.05)	0.38 (0.05-2.89)	1.05 (0.63-1.77)	1.36 (0.81-2.28)	1.31 (0.78-2.19)

n = total number of cases testing positive for the virus/total number of cases yielding positive or negative results for the virus